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EFFECTS OF 2,4,5-T ON MAN AND THE ENVIRONMENT

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Senator HART. Our next witness, as the Surgeon General has already indicated, is Dr. Jacqueline Verrett.

STATEMENT OF DR. JACQUELINE VERRETT, FOOD AND DRUG ADMINISTRATION, DEPARTMENT OF HEW

Dr. VERRETT. Thank you, Mr. Chairman, for this opportunity to discuss our investigations of the relationships between chlorinated phenoxy herbicides, chlorinated dibenzo-p-dioxins, and the chick edema factors.

Chick edema disease was first recognized in 1957, when large numbers of broiler flocks in the United States suffered what appeared to be an epidemic disease. The affected birds appeared droopy, with ruffled feathers, and had difficulty breathing. In many flocks, more than 50 percent of the birds died as a result of the disease. Of the millions of birds affected, those autopsied consistently displayed hydropericardium (accumulation of fluid in the pericardial sac), accumulation of fluid in the abdominal cavity, subcutaneous edema, and additionally liver and kidney damage.

In 1958 the investigations of a number of laboratories indicated that the causal agent was contained in fats, and specifically in the unsaponifiable fraction of fats in the commercial poultry rations. In laying hens the toxic fat caused a rapid drop in egg production. Pullets receiving toxic fat during the full growing period did not come into production, and mortality was very high. Hydropericardium, the most common lesion found in young birds, was not found in birds of laying age.

The chick edema factor found in the toxic fat in the 1957 outbreak was presumed to have arisen as a byproduct of industrial production of stearic and oleic acids, since the unsaponifiable materials from this process were the components of fat in the poultry ration. Subsequently, the toxic substance was found to be present in several different types of fats. It was demonstrated to be present in samples of commercially produced oleic acids and triolein, in acidulated vegetable oils, and in inedible animal tallows. The demonstration of the presence of the chick edema factor in commercial fats led to the ruling by the Food and Drug Administration in 1961 that higher fatty acids intended for food additive use must be free of the chick edema factors. The presence of the factor was to be ascertained by a chick bioassay based on the volume of pericardial fluid in birds fed the fat under investigation.

Beginning in 1958, fat that had first been proved to be toxic to chicks was used by various investigators in experiments with other species, and was demonstrated also to produce deleterious effects in rats, mice, turkeys, pigeons, guinea pigs, swine, dogs, and monkeys.

Early investigations of feeding toxic fats to rats indicated that they are more resistant than chicks in short term feedings, but when fed in sufficient dosage, extracts of the toxic fat produced definite deleterious effects as shown by growth depression, enlarged and fatty livers, marked involution of the thymus, and enlarged adrenals.

Guinea pigs fed 2½ percent toxic fat stopped growing at 6 weeks, and death losses occurred at 8 weeks. At a level of 4½ percent the weight losses occurred after 3 weeks, and death at 4 weeks. The pathology observed was congestion of the lungs and mottled livers.

Dogs fed 10 percent toxic fat in their rations lost hair on their backs and shoulders (alopecia), and there was poor reproduction and lactation performance. Whelped pups were either dead or weak, and the mothers seemed to have an insufficient milk supply. Pups removed before weaning and fed a normal ration showed an immediate and dramatic increase in growth. Other litters maintained on the toxic fat ration postweaning demonstrated inferior growth performance.

Monkeys have demonstrated considerable sensitivity to toxic fat materials. In one study nine monkeys received a toxic triolein at a level of 25 percent in their diets. One monkey died at 1 month, and four died at 3 months. At the 3-month period, corn oil was substituted for the toxic triolein, but the other four monkeys died from 3 weeks to 5 weeks later, in spite of this substitution. Of the nine monkeys fed the toxic triolein, eight were autopsied and showed signs of jaundice, pancreatic atrophy, and fibrosis, hemosiderosis, fatty liver with necrosis, bile duct proliferation, and gross hemorrhage in the intestinal tract. No such pathology was seen in the control monkeys in this study.

A second study with 36 monkeys given a toxic fat at levels from 0.125 to 10 percent of their diet, demonstrated an inverse relationship between the concentration of the toxic fat in the diet and their mean survival time. Those given the highest level (10 percent) had a mean survival time of only 91 days, while those given the lowest level (0.125 percent) had a mean survival time of 445 days. It has been estimated that the highest level provided approximately 728 micrograms total chick edema factors, while the lowest level diet provided approximately 100 micrograms total intake. The toxic fat was lethal at all levels studied, and the animals were sacrificed when possible just before death. During the last 30 days of life, all monkeys developed alopecia, generalized subcutaneous edema, accumulation of fluid in the abdominal and thoracic cavities, and hydropericardium. There were decreases in red and white blood cell counts, total serum protein values, and altered serum-protein ratios. There was also cardiac dilatation and myocardial hypertrophy and edema. Finally, the experimental monkeys had reduced hematopoiesis and spermatogenesis, degeneration of the blood vessels, focal necrosis of the liver and gastric ulcers.

Limited experimentation with mice, pigeons, and turkeys, indicated that toxic fat in the diet led to reduction in growth without hydropericardium or accumulation of abdominal fluid. Similarly, swine, fed toxic fat at a level of 9 percent of their ration, showed poor weight gain, but one pig sacrificed 6 weeks after the start of the study showed no gross or microscopic lesions attributable to the ration.

One important finding in the studies with chickens, is the apparent storage of the chick edema factors in chick tissues. The unsaponifiable fraction of carcasses (exclusive of intestines, head, and feet) of chickens fed the toxic fat was very potent in producing hydropericardium in other birds when incorporated in their rations. Other investigations of the distribution of the chick edema factors in the

chick tissues indicated significant levels in bone, heart, intestine, kidney, liver, and skin. The liver contained more than 80 percent of the total detected. A similar determination of the distribution in rats indicated the presence of chick edema factors only in liver and in the feces.

During the years that the previously described toxicity investigations were taking place, the toxic fats were undergoing intensive chemical analyses to concentrate, purify, and finally determine the nature of the compounds responsible for chick edema disease. At all steps of these procedures, the path of the toxic material was confirmed by assay in young chicks. This proved to be a time-consuming and difficult job because of the complexity of the fatty materials. A major breakthrough in this effort came when it was found that a highly purified crystalline material possessing the properties of chick edema factor contained chlorine. This indicated that it was not a natural component of the fat in which it occurred.

Work in several laboratories obtained similar results, and examination of the purified material by a variety of analytical techniques suggested that chick edema factors could be highly substituted (chlorinated) derivatives of naphthalene, biphenyl, anthracene, or even structures common to the chlorinated pesticides of the DDT family. These latter compounds were ruled out when tested in the chick feeding assay, but some derivatives of the former classes of compounds were tested and found in some instances to be toxic, and indeed produce similar lesions to those observed with authentic toxic fat. However, none of these compounds demonstrated the high order of toxicity, or the complete chick edema syndrome when so tested. Finally, by means of single crystal X-ray crystallography, it was demonstrated that a pure compound isolated from a toxic fat was a hexachloro-dibenzo-p-dioxin. This structure was verified by infrared, ultraviolet, and mass spectrometry data. Final confirmation came when this particular compound was synthesized and found to produce the same lesions in chicks as the compound isolated from the toxic fat.

The finding that a chlorinated dibenzo-p-dioxin was a chick edema factor explained why different investigators had isolated materials similar in their capacity to elicit chick edema disease, but yet in their purest forms, had slightly different chemical properties. The large number of isomers possible (more than 60) in this family ranging from mono- to octa-chloro-dibenzo-p-dioxins illustrates the complexity of the problem. It then became a problem of determining whether some or all of these compounds are in fact chick edema factors, and what their relative capacities in this regard might be.

The chlorinated dibenzo-p-dioxin structures have been known in organic chemistry many years, and became particularly noteworthy, when in manufacturing processes with chlorophenols, their formation as byproducts posed serious occupational hazards. The most potent in this regard seems to have been the symmetrical tetrachloro-p-dioxin which was formed in the manufacture of 2,4,5-trichlorophenol. These chlorinated compounds were found to cause a serious and persistent disease referred to as chloracne. This disease was first described in 1899. Associations of this disease with chlorinated di-benzo-p-dioxins were made by the Germans, who had several outbreaks of this disease in their factories. There have also been similar occurrences in the Netherlands and in this country, in factories manufacturing chlorophenol compounds. It should also be pointed out, that other compounds, such as the chlorinated naphthalenes, anthracenes, biphenyls and dibenzofurans, are known to be acnegenic, but as in the case of the toxic response in chicks, these materials are less potent than the chlorinated dibenzo-p-dioxins. In the case of the chloracne associated with dioxin, the human symptomatology extends to other mucous membrane irritation, porphyria cutanea tarda, hirsutism, hyperpigmentation, increased skin fragility, severe damage to the internal organs, particularly hepatotoxicity, and central nervous system disorders, as indicated by neuromuscular symptoms and psychologic alterations, and other systemic symptoms. Most of these occupational exposures in Germany occurred in the 1950's, and followup examination of these affected workers in recent years indicate that the recovery period is lengthy, with many workers still having demonstrable adverse effects from prior exposure. Similar observations have been made on exposed workers in the United States of America. The tetrachlorodibenzo-p-dioxin was demonstrated to produce the chloracne in humans after the application of only 20 micrograms. The rabbit ear is especially sensitive, with concentration of 0.001 percent to 0.005 percent producing severe reactions after local application. This assay using the rabbit ear is apparently used as an indicator in some plants of the content of this particular dioxin in the manufacturing process. Hence, the serious health significance of these compounds for humans has, inadvertently, been clearly documented.

Research in Germany and Japan indicated that the magnitude of this problem was indeed large, since the formation of the chlorinated di-benzo-p-dioxins would be facilitated in the saponification procedures used in various processes involving chlorophenols. A further complication is that a given chlorophenol preparation is generally contaminated with other isomers, increasing the possibility of formation of a wide spectrum of chlorinated di-benzo-p-dioxins beyond those to be expected from the predominant component. Evidence that this does occur will be discussed shortly in connection with the chicken embryo studies of these materials.

During the time the previously described investigations of the chick edema factors were underway, many of which were carried out by FDA investigators, methodology was developed for detecting the chick edema factors using sensitive gas-liquid-chromatographic (GLC) techniques. It became apparent that authentic toxic fats consistently gave peaks with specific retention times, and these peaks were used as an indication of chick edema factor in a suspect sample. Confirmation of this was obtained using the chick feeding assay. In the light of recent knowledge of the chlorodioxins as chick edema factors, it has been possible to establish that the materials being detected were hexa-, hepta-, and octa-chlorodibenzo-p-dioxins. Although toxic fat samples did indeed contain peaks corresponding to dioxins of lesser chlorine content, i.e., di-, tri-, tetra-, penta-, these are not detectable with this particular analytical procedure because their particular peaks are obscured by other components,

including pesticide residues present in the samples. Other GLC procedures are being developed to detect these latter dioxins.

In the early 1960's the chicken embryo was being used in toxicological evaluations of a wide variety of materials. It was hoped to develop a rapid and sensitive screening system to pinpoint compounds with significant toxic and teratogenic effects for further study. In view of the demonstrated sensitivity of the chicken to chick edema factors, the chicken embryo was used to assay toxic fat samples, and found to present the same syndrome as observed in the chick feeding assay. A high mortality was observed with toxic fat extracts, and additionally, hydropericardium, generalized and massive edema, eye, beak, and leg defects, and necrotic livers were apparent on gross observation. No microscopic studies have been conducted on embryos or hatched chicks in these investigations.

In parallel with other investigations, the chicken embryo was used to test the toxicity of the chick edema factors isolated from toxic fats. It was also found that the chlorinated biphenyls, naphthalenes, anthracenes, and other compounds did indeed elicit a toxic response, and in some instances, the chick edema syndrome was present. But in no case were any of these materials as potent as the toxic components isolated from toxic fats, and were generally less potent by a few orders of magnitude.

After the identification in 1966 of a hexachloro dibenzo-p-dioxin as a chick edema factor, studies were initiated in which various isomers of chlorinated dibenzo-p-dioxin were prepared and tested in the embryos. Although the investigation was not extensive or complete, it illustrated that isomers prepared by pyrolyzing selected chlorophenols did give chloro-dibenzo-p-dioxins with GLC retention times duplicating those in the authentic toxic fats, and likewise, produced the chick edema syndrome in the treated embryos. It was also apparent from this study that the various isomers (that is, those with different chlorine content, and those with identical chlorine content, but with chlorine atoms positioned differently on the molecule) varied in their toxicity, although in all cases only microgram or less quantities were required to elicit the toxic response. It is not possible to give exact figures for the toxicities obtained in this study, since most of the individual dioxins were contaminated with traces of others. Nevertheless, it was apparent that the symmetrical chlorodioxin prepared from 2,4,5-trichlorophenol (2,3,6,7-tetrachloro dibenzo-p-dioxin) was more potent than any of the others tested, even recognizing its lack of purity.

During this investigation, samples of the chlorophenols, both technical and reagent grades, were examined by GLC to determine if preformed chloro-dioxins were present. The presence of chlorodioxins was demonstrated by GLC, and these materials, which can be removed by appropriate techniques, were then tested in the chicken embryo system and did indeed produce chick edema. A current study of similarly contaminated chlorophenols, containing from 18 ppb to 95 ppm of chloro-dioxins with six or more chlorine atoms are currently under test.

This investigation was not pursued further in view of the fact that there had been no known occurrences of chick edema disease since the late 1950s and, hence, such research had a low order of priority.

However, in early 1969 there was another large outbreak of the disease in North Carolina and the toxic factor was traced to the fat component of the feed. The toxicity was confirmed by the chicken embryo test with fractions of the hexa-, hepta-, and octa-chloro-dibenzo-p-dioxins from the crude fat.

An investigation of the processing plant in which the toxic vegetable oil products were produced revealed a proximate operation for the manufacture of chlorophenol formulations. However, it is still not possible to conclude that this accounted solely for the presence of dioxins in the fat, or whether they were at least to some extent in crude oil from a prior contamination.

Since that time FDA has initiated an investigation of the oils of other manufacturers and processors and in a few cases GLC analysis has indicated the presence of chloro-dioxins. These have not as yet been confirmed by chicken embryo bioassay; however, it is noteworthy that in the case of these subject samples there is no known adjacent manufacturing operation that would give rise to direct chloro-dioxin or chlorophenol contamination, so that entry of the chloro-dioxins from other sources must be considered.

This 1969 outbreak of chick edema disease, coupled with the question of contamination of the herbicide 2,4,5-T by chlorinated dioxins led us to renew this investigation. In the past 6 months the herbicides 2,4-D and 2,4,5-T, as well as the particular tetrachlorodioxin purported to be the teratogenic agent responsible for the effects in the Bionetics study of 2,4,5-T have been under study.

In an effort to assess the edema-producing capacity, the teratogenic activity, and the acute toxicity of these materials, samples of the original Bionetics 2,4,5-T, from Diamond-Alkali, were obtained for comparison with a sample representative of the current manufacture of Dow Chemical Co.

The Bionetics 2,4,5-T is reported to contain 27 plus or minus 8 parts per million of the 2,3,6,7-tetrachloro dibenzo-p-dioxin, with the content of other dioxins unknown. The current production of Dow 2,4,5-T has 0.5 parts per million of this dioxin, with no analysis for higher chloro-dioxins reported, but does contain almost 5 percent of other impurities, mostly isomers of 2,4,-D 2,4,5-T, and chlorophenols and chlorophenoxy compounds of undetermined structure.

All investigations using the chicken embryo involved administration of the compounds by injection through the air cell of the egg, either preincubation or at the 4th day of incubation.

A comparison of the Bionetics 2,4,5-T with the Dow 2,4,5-T indicates that with respect to the ability of the materials to produce embryonic mortality, the Bionetics 2,4,5-T is more potent. The Bionetics 2,4,5-T has an LD_{50}—that is, kills 50 percent of the treated embryos—of approximately 25 micrograms per egg, 0.5 parts per million, while the Dow 2,4,5-T LD_{50} is approximately 100 micrograms per egg, 2 parts per million.

With respect to teratogenic effects, both samples produce chick edema syndrome in the nonviable embryos, and hatched chicks, including eye defects, beak defects—predominantly cleft palate—short and twisted feet—the result of tendon slippage—and diffuse and localized edema in various parts of the body.

With both of these samples of 2,4,5-T these teratogenic effects are observed at levels inducing no significant embryonic mortality.

The Dow 2,4,5-T still produces the chick edema syndrome at 50 micrograms, one part per million, a level where only 12 percent mortality is observed, while the Bionetics sample has similar effects as low as 6.25 micrograms per egg, 0.125 parts per million, a level inducing only 16 percent mortality. Both of these mortalities are close to that induced by the solvent alone.

It should also be emphasized that the chick edema syndrome is not observed in the embryos treated with the solvents only, at any level, or in the control flock.

A sample of 2,4,5-T from a chemical supply company was subjected to three recrystallizations before test. With the present GLC techniques no chlorodioxins are detectable in this purified sample. When tested in embryos it produced chick edema syndrome at 5, 10, and 25 parts per million, all levels which induced no more than 15 percent mortality in the embryos.

This same sample was subjected to an additional purification by seven extractions to remove dioxins that might have been present, but were below the current detection levels.

This repurified sample is still clearly teratogenic in the embryos since when tested at a level of 2.5 parts per million it produced 20 percent incidence of the malformations previously described, though no significant edema was seen grossly. The mortality induced was 24 percent, which is higher than that of the sample prior to the extensive extraction procedure.

It is also noteworthy that the embryonic mortality occurred soon after treatment, and the hatched chicks had bleached down, indicative of an aberration in the normal pigment formation.

With respect to the 2,3,6,7-tetrachlorodibenzo-p-dioxin, early investigations of this compound in a preparation containing some 2,3,7-trichlorodibenzo-p-dioxin indicate a high order of toxicity and teratogenicity.

Whether prepared by pyrolysis of 2,4,5-trichlorophenol, or direct chlorination of dibenzo-p-dioxin, the test preparations, which contained approximately 50 to 55 percent of the tetrachlorodioxin and 20 to 25 percent of the trichlorodioxin, produced significant mortality, that is greater than 20 percent, and chick edema syndrome in more than 40 percent of the treated embryos at levels of five ten-millionths of a milligram per egg, or 10 parts per trillion.

More recent investigations, with two samples of the tetrachlorodioxin, both of purity greater than 95 percent, indicate edema and terata at 20 parts per trillion. These samples have only become available within the past month and additional testing is underway at lower and higher levels.

It should also be mentioned that the herbicide 2,4-D as a commercially available sample, and a purified sample, a mixture of the N-butyl esters of 2,4-D and 2,4,5-T, and a sample of silvex, a related herbicide, have been tested.

Terata and chick edema syndrome have been observed with all of these materials at levels of 10 parts per million and above. Lower levels are under investigation and the levels of dioxins in these samples are also being determined.

Studies have recently been initiated in the FDA using pregnant golden hamsters, intubated on day 6 through 10 of organogenesis with the test compounds.

The Dow 2,4,5-T, 0.5 parts per million tetrachlorodioxin, tested at 100 m./k. yielded about 80 percent fetal deaths and those pups born alive had gastrointestinal hemorrhages.

The thrice-recrystallized 2,4,5-T sample referred to earlier, with no detectable chlorodioxins, when tested at a level of 100 m./k. produced an average fetal mortality of 55 percent.

Among 38 live pups, three abnormals were found: One with a deformed hind limb and two with inadequate fusion of the skull.

At lower doses the fetal mortality was less, but still higher than that observed in control hamsters.

When the extensively repurified sample of 2,4,5-T was tested in hamsters no gross terata were observed at 100 m./k., but the number of early fetal deaths was 70 percent, indicating a definite embryotoxic effect and corroborating the observation of increased mortality in the chick embryo studies. Additional tests with this compound are underway.

A dioxin preparation containing approximately 51 percent 2,3,6,7-tetrachloro- and 21 percent 2,3,6 trichlorodibenzo-p-dioxin yielded 98 percent fetal deaths at 9.1 micrograms per kilogram. Gastrointestinal hemorrhages and eye anomalies—absence of lid—were present in many of the pups.

Tests with the purer tetrachlorodibenzo-p-dioxins are underway.

The numbers of animals in the hamster tests are too small to be considered statistically valid, but there are definite indications that alterations in fetal viability and gastrointestinal hemorrhages do occur at the levels tested.

In summary, the chick embryo studies and additionally the preliminary hamster data indicate that the current production 2,4,5-T containing 0.5 parts per million of the 2,3,6,7-tetrachlorodibenzo-p-dioxin is teratogenic and embryotoxic in these test systems.

Further, an extensively purified 2,4,5-T sample, with no chlorodioxins detectable with the present techniques, has indicated significant embryotoxicity in the hamster and chick embryo, and additionally produced gross terata in the chicken embryos, making it impossible at this point in time to exonerate it of teratogenic or other adverse effects on the embryos that may have some health significance.

The data for 2,4-D in chick embryos likewise demonstrate these effects in current production materials.

These studies have in no way assessed another and perhaps more complicated aspect of this problem, and that is the interactions of the various chlorodioxin isomers with each other in the many combinations in which they are likely to occur, or the possible interactions, including potentiation or synergism, between the chlorodioxins and the chlorophenols, herbicides and other materials in which they are found.

At this point, with your permission, Senator Hart, I would like to insert in the record the documents containing the data from which this testimony was derived.

Thank you. That is the end of my prepared statement.

Senator HART. Yes, they will be received and placed in the record after your oral testimony

Dr. VERRETT. I realize I presented a rather lengthy and complicated piece. I will be happy to answer questions and elaborate if you wish.

I also have some samples of embryos, if you would care to see them, or chicks.

Senator HART. Yes, I heard about them.

The statement is not an easy one for one not trained in your discipline.

I take it, Doctor, these are some of the chicks that have the deformities that you are talking about?

Dr. VERRETT. That is right. These are chicks that have been treated with the tetrachlorodibenzo-dioxin. I would like to put them out on the table but it is not safe.

As you can see, they cannot stand up. These chicks hatched early this week. They have noticeable edema. This is really the ankle, if you wish, of the chicken and this is really a result of the malformation.

These are normal chicks; as you can see, they have no difficulty standing and walking.

These are chicks that have been treated with the ethanol, the solvent alone or have been untreated. We always run some at the same time that have had no treatment and I think the comparison between that one and this one, if you wish, is quite obvious.

Senator HART. Your last statement referred to a series of compounds.

Dr. VERRETT. Yes.

Senator HART. Exactly what are these?

Dr. VERRETT. It is a family of compounds which I may say very briefly is a series of phenols having a varying chlorine content. They go from dichlorophenol, which is the precursor or the compound from which 2,4-D is made, all the way up to pentachlorophenol. All of these materials are very widely used as herbicides. They also have a very broad use in industry and, in other words, are capable of being in the environment and if they are contaminated with dioxins, of also putting dioxins in the environment.

What I was trying to say is that we have looked at these chlorophenols, apart from just the herbicides which are derived from them, and we know from past study, and there is a paper in the material that I have included in the record which demonstrates, that these materials are already contaminated with dioxin. The amount of the contamination at this point is——

Senator HART. It is very difficult to hear the witness. I do not know what the distraction is, but please be patient.

Dr. VERRETT. We do know, at least by GLC (gas-liquid-chromatography) just chemical techniques—I will distinguish between chemical and biological techniques—by chemical analysis, we know these materials are contaminated with chlorodioxins.

In addition to that, we know there are many of these chlorinated dioxins that are involved and not simply the single one which has been the subject of the 2,4,5-T. There are approximately 60 or more.

We are aware from our previous work that these materials are already in chlorophenols as they are being used. This is the point I was trying to make.

Senator HART. The point I was trying to make by raising it is the necessity that there be no more delay with respect to establishing the dangers, the hazards, the potentials for harm in this whole variety, this whole family of products, given the kind of damage that you have so dramatically demonstrated here from one single element of the environment.

Dr. VERRETT. Yes. I might say—of course I could not bring very many—these are chicks treated with the tetradioxin in question, which is the most pertinent to the hearing. There are some here which have been treated with 2,4-D. Thes are 2,4,5-T treated chicks. We do have underway an investigation of all the other dioxins. It is a question of having to synthesize these materials and test them in pure form. But by inference at least, in our experience with the chick edema problem in the past, we know all of these dioxins are in fact toxic. We do know that. We have that information.

As I said, at least in chicks, we know there is a storage and possible transmission from chickens that have been fed these materials to the human. The possibility does exist.

Senator HART. By handling, as you just did?

Dr. VERRETT. No. I hope not. The tetradioxin is potent. I do not know whether handling this bird will harm you, but chloracne is a very serious disease and persistent disease and there is no known cure.

We are not aware that the other dioxins are as potent as that in this regard, but they have yet to be evaluated.

To our knowledge the herbicides we have been discussing have not actually been tested to see if any other of the dioxins are also present.

The concern, apparently, has revolved around the particular dioxin because it is extremely potent; even if the others are less potent, of course, they may still be important.

Senator HART. I understood your explanation with the rats and the monkeys to indicate that this—that the dioxin in 2,4,5-T has an accumulative effect.

Dr. VERRETT. The effects that are seen would indicate that. I would be unable to say that has been proven, but the fact that, for example, in the one monkey study, when they were fed for 3 months and then the animals that did not die during that course were put on a ration free of it, still died in subsequent months, and that would indicate that either the damage had been done by the initial exposure or there was storage such that it finally did have its effect.

But it was fatal in all cases. So the animal data would indicate—again we cannot apply that with certainty about the human—storage or persistence, if you wish to use that word.

Senator HART. Then your attitude, which you say is not conclusive and certainly does not relate directly to humans, points to the persistence, the cumulative character of the dioxin, rather than in the other direction, that it is not cumulative?

Dr. VERRETT. I would say it indicates it is probably cumulative.

Senator HART. Do you know any experiments that point in the direction that it is probably not cumulative?

Dr. VERRETT. I am not aware of any, no. The human data I cited with respect to occupational exposure would also indicate that it is probably cumulative in the human, of course.

Senator HART. You used a word here which I take it means burning.

Dr. VERRETT. Pyrolysis. Not exactly burning. In the sense I used it, it means reacting under conditions of elevated temperature, but not the actual burning of the material itself. It indicates a high temperature reaction, perhaps, heat applied to the material in order to make the reaction take place, but not in the sense of actually igniting it.

But it does indicate that heat, in other words, facilitates the formation of these compounds (dioxins), if that is what you are arriving at.

Senator HART. What if some of this material is just put in the city dump and burned? Could that burning inadvertently produce dioxin?

Dr. VERRETT. I could only say that the likelihood is there; yes.

Again, lack of actual experimentation does not give us any evidence or proof of this, but the fact is that these materials are formed when chlorophenols are subjected to heat and that would indicate to me that that is definitely a possibility.

Senator HART. What common products, or what common articles contain chlorophenol?

Dr. VERRETT. There are so many that I wouldn't be able to name all. But, every piece of newspaper or paper of any kind probably has chlorophenol used in the manufacture of paper. I am sorry I am not in an area which would enable me to give you total usage figures, but they are considerable.

This is washed out to some extent, but there probably are some chlorophenol residues, and paper would be one item and one which we can say is very widely used.

Another example—well, leather is cured by using chlorophenols in the tanning process. So leather materials contain it. There would be any number of other everyday items that would possibly have it.

Senator HART. Well, just as I am reluctant to have pictures taken, I am reluctant to make these contrary statement and yet this one is not inappropriate.

If the materials that we customarily—and for generations, centuries I guess—have been throwing into a fire contain chlorophenol, when you burn them, it is your opinion that dioxins can result——

Dr. VERRETT. Could possibly result; yes.

Senator HART. Is it possible that some of the birth defects for which there has been no medical explanation to date are the result of this kind of thing, where we have always done it and it has never seemed to hurt us?

Dr. VERRETT. I would say it is a possibility. It would be for others to assess this situation, but I think it is a distinct possibility because of the fact that these materials (chlorophenols) are ubiquitous, and if, in fact, chlorodioxins are formed in the environment, this is a possibility.

I should point out the studies done in mammals have been done by feeding, while the largest exposure may come from inhalation or dermal contact. That was the source of exposure occupationally to the tetrachlorodioxin. So here we have to be concerned not only about eating, but inhalation and perhaps contact exposure.

This brings up the subject of other materials. For example, we are also investigating——

Senator HART. I was just going to say that it is hard to visualize a substitute for paper or leather, but can one have leather and paper without this material?

Dr. VERRETT. I would think so. I should point out some of the materials (chlorophenols) are washed out in the processing. The total amount used in the processing does not always remain in the products. I did not mean to imply that. That brings up another question: Are they washed out into the rivers et cetera?

Nevertheless, I am not really sufficiently knowledgeable of the technology to say whether something else could be substituted or not.

Senator HART. Mr. Bickwit?

Mr. BICKWIT. I have heard several people criticize these chick embryo studies as being overly sensitive. I am not sure of this, but I believe the Secretary of HEW has criticized them.

Can you respond to that criticism?

Dr. VERRETT. Well, I have not as yet. As I mentioned in the testimony very briefly, this technique was started with just that idea, of finding a sensitive technique, if at all possible, for assessing toxic and also teratogenic response.

One of the great difficulties in all of the toxicology animal work, using mammals and primates, even, is the difficulty of relating, of course, to the human. There is perhaps another generation gap, if you can use that word, between the chicken or an avian species.

I would say without hesitation that these studies, for example, proved that this material is not for the birds, if I may phrase it that way. I would certainly not say that you can conclusively state that there is a human hazard from the results with the chick embryo.

However, inferentially we have evidence of that, and we have the inadvertent tie-in because of the chlorodioxin toxicity already known in primates and humans. I would certainly hope the human is less sensitive than the chick, but I feel what we really need, and I think that is pointed out very much by these hearings, is a very sensitive test, and then it remains to show this is not the case in the species more closely related to man.

I would rather demonstrate that something has an adverse effect in a sensitive system and then, by appropriate study, find out whether it will be relevant to man, than miss it altogether in an insensitive test. Although we do not try to make direct correlations, we feel certain anything seen in this system is worthy of further study, and I should also like to add we are trying to keep the study in the proper perspective.

That is, we use levels (doses) when possible that are relevant to the human exposure or other animal exposure and not simply try to produce effects with excessive amounts of material.

Mr. BICKWIT. You state on page 4 that the most potent dioxin in the production of serious occupational hazards, seems to have been the tetradioxin.

What actual experimental evidence is there that suggests or confirms that tetra is the most potent of the dioxins?

Dr. VERRETT. You mean in animal work?

Mr. BICKWIT. Yes.

Dr. VERRETT. Of course the tests referred to earlier by Dr. Steinfeld show in that system that the tetradioxin is extremely potent in rats. I am not aware that the other dioxins have ever been studied.

Mr. BICKWIT. Have the other dioxins ever been compared in potency in chick analysis?

Dr. VERRETT. Yes. As far as I know only in the chick embryos.

Mr. BICKWIT. So, unless we can rely on the chick studies, we may be in very serious trouble, even more serious than it now appears on the basis of available evidence.

Dr. VERRETT. That is right.

Mr. BICKWIT. Are there any data available, either in mammalian studies or chick studies on the relative toxicity of dioxins compared to thalidomide?

Dr. VERRETT. If you used the chick as an exhibit or even the mammalian studies with the tetradioxine, which is the only one being studied at the moment, you would have to say this material is some 100,000 to a million times more potent in these particular species.

Now, I should add that the abnormal effects (terata) that we are seeing are not the same as we see with thalidomide, but the potential for producing abnormalties that we do find, it is of that order.

Senator HART. Doctor, thank you very much.

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THE CHICK EDEMA FACTOR

A toxic factor in some feed grade fats and fatty acids produces hydropericardium and ascites in young chicks when 9 µg. per kilogram body weight are fed per day. There are a number of these toxic compounds, containing large amounts of chlorine. One has been characterized and synthesized.

In 1957, large numbers of chickens suffered from what appeared to be an epidemic disease. Losses attributable to this epidemic have been estimated in the millions of dollars. The affected birds appeared droopy, showed ruffled feathers, and had difficulty breathing (L. Friedman, *Feedstuffs, March 17, 1962*).

In some flocks, over 50 per cent of the birds died with typical symptoms of the disease (V. L. Sanger *et al., J. Am. Vet. Med. Assn.* **133,** *172 (1958)*). When autopsied, the birds had pale hearts with hydropericardium, and livers that were pale, mottled, and had an irregular granular surface. In the advanced stages, the abdomens were distended, and contained 100 to 500 ml. of clear, straw-colored fluid. The pericardial cavity is most susceptible to fluid accumulation, with the abdominal cavity next, and then subcutaneous tissue (D. F. Flick, C. D. Douglass, and L. Gallo, *Poultry Sci.* **42.** *855 (1963)*). The kidneys were pale and swollen: the fatty tissue of the gizzard was edematous: and the duodenum was swollen and soft (Sanger *et al., loc. cit.*). Hydropericardium was not as prominent in the older birds as in the broilers (Friedman, *loc. cit.*).

No acceptable explanation is available for the abnormalities associated with feeding the chick edema factor. Despite accumulation of fluid in the pericardial and abdominal cavities of the affected birds, the hematocrit, blood volume, and moisture content of the heart, skeletal muscle, skin, and kidney were normal. However, the total body water content of the birds was significantly increased. On the basis of these observations, it was suggested that the chick edema factor increased permeability of the cardiac vascular bed (Flick, Douglass, and Gallo, *loc. cit.*). Additional support for this hypothesis came from the finding that the toxic factor produced no appreciable change in the proportion or level of plasma proteins (Flick, D. Firestone, and J. P. Marliac, *Poultry Sci.* **44.** *1214 (1965)*).

The type of diet had a marked effect on the rate at which the chicks' bodies accumulated water. Chicks fed a natural grain ration containing 4 per cent toxic fat showed an increased body moisture content (78 versus 72 per cent for the controls) only at the end of the third week. Birds fed the same level of toxic fat in a semi-purified diet showed a marked increase in body water content after seven days (79 versus 73 per cent) (Flick, Douglass, and Gallo, *loc. cit.*).

During 1958, a number of laboratories traced the disorder to the presence of a toxic substance in the unsaponifiable fraction of fats added to commercial poultry

rations. This fraction contained a toxic substance(s) which presumably was present in a byproduct of industrial production of stearic and oleic acids.

The toxic substance was present in a variety of types of fats. These included a sample of triolein, which on the basis of chemical analyses was found to be of "excellent quality" (A. Yartzoff *et al., J. Am. Oil Chem. Soc.* **38.** *60 (1961)*). When this triolein was incorporated into a chick ration at a level of 15 per cent, the birds showed severe symptoms of hydropericardium. Similar symptoms were produced when chicks were fed rations containing distillates or residues secured in commercial production of fatty acids. The toxic substance(s) was also found in inedible animal tallows, acidulated vegetable oils, and several commercially produced oleic acids and triolein (Firestone, W. Horwitz, Friedman, and G. M. Shue, *J. Am. Oil Chem. Soc.* **38.** *418 (1961)*). A report from another laboratory confirmed the presence of the chick edema factor in various industrial fats (J. C. Alexander, R. J. Young, C. M. Burnett, and H. D. Hathaway, *Poultry Sci.* **41.** *22 (1962)*).

The presence of the chick edema factor in commercial fats led to the ruling by the Food and Drug Administration that methyl esters of higher fatty acids intended for use as a food additive must be free of the chick edema factor (*Federal Register Dec. 9, 1961. 26 F.R. 11828; 121.224*). The presence of the factor was to be ascertained by a chick bioassay based on the volume of pericardial fluid in birds fed the fat under investigation.

That salt was necessary in the chicks' ration for the development of hydropericardium when toxic fats were fed was suggested by Alexander and co-workers. They observed typical symptoms of the disturbance only when the ration contained sodium chloride; extra salt accentuated the condition. Although this syndrome had many of the earmarks of a vitamin E deficiency, neither antioxidants (including vitamin E) nor selenium had any curative effect.

Sensitivity of different species to the toxic factor in these fats varies considerably. When monkeys were fed a sample of triolein that produced hydropericardium in chicks, the five monkeys died within three months (Yartzoff *et al., loc. cit.*). The ration contained 25 per cent triolein. The monkeys showed signs of jaundice, pancreatic atrophy and fibrosis, hemosiderosis, fatty liver with necrosis, and gross hemorrhage in the intestinal tract.

Pigs fed a ration composed of equal parts of a highly toxic chick ration and a normal swine ration gained weight and appeared to show no abnormalities (Sanger *et al., loc. cit.*). However, when a toxic fat was incorporated into a swine ration at a level of 9 per cent, the five shoats gained 0.72 pounds per day while the controls gained 2 pounds (L. C. Scott, *J. Am. Vet. Med. Assn.* **137.** *258 (1960)*). Despite the poor weight gains, the one pig sacrificed about six weeks after the start of the study showed no gross or microscopic lesions attributable to the ration.

The fat from the latter pig was rendered and incorporated into a chick ration at a level of 4 per cent. None of the chicks fed the ration containing the rendered lard developed toxicity signs or symptoms. This finding suggested that the pig did not store appreciable amounts of the toxic substance in its adipose tissue.

The apparent absence of the toxic factor in pork fat is in contrast to chicks' fat, where storage appears to occur. The unsaponifiable fraction of carcasses of chickens fed the toxic fat was very potent in producing hydropericardium in other birds (Friedman *et al., J. Assn. Official Agr. Chem.* **42.** *129 (1959)*).

A more recent study of the response of different species to the chick edema factor utilized the unsaponifiable fraction of a toxic fat. This was fed to young chicks and rats (T. C. Campbell and Friedman, *Proc.*

Soc. Exp. Biol. Med. **121.** *1283 (1966)).* The weanling rats fed the unsaponifiable fraction at a level of 9 μg. per kilogram body weight per day of the chick edema factor showed a 3.7 per cent loss in weight over the six day feeding trial. No gross tissue alterations were apparent on autopsy. The livers, however, were 21 per cent heavier than those of the controls when expressed on a body weight basis, while the adrenals were 50 per cent heavier. The heart, kidney, and spleen were of normal size.

Chicks fed a comparable amount of concentrate developed hydropericardium in six days. The livers of these birds were 15 per cent heavier than those of the controls. The statement was made that "the increase in liver weight in the chicks was not due to moisture or fat." Such an observation should have been documented and checked by histological studies.

A few years ago, R. E. Harman and collaborators (*J. Am. Chem. Soc.* **82.** *2078 (1960)*) reported isolation of a crystalline substance from a feed-grade tallow. This compound produced hydropericardium in chicks when incorporated into a commercial ration at a level of 0.1 p.p.m. A report (Yartzoff *et al., loc. cit.*) indicated that the crystalline substance contained 47 per cent chlorine.

Apparently, there are a number of compounds that behave like the chick edema factor. These compounds move closely with the toxic factor during molecular distillation, on thin layer chromatography, and show similar peaks on gas chromatography. Not all these compounds are toxic, and toxicity appears to vary in those that are.

Part of these difficulties may be resolved with characterization and synthesis of one of the toxic substances. By means of single crystal x-ray crystallography, J. S. Cantrell, N. C. Webb, and A. J. Mabis reported (*Abstracts, Am. Crystallographic Assn. Meeting, p. 27 (1967)*) that the compound isolated in their laboratories was 1,2,3,7,8,9-hexachloro-dibenzo-p-dioxin. This work was supported by infra-red, ultra-violet, and mass spectrometry principally by J. C. Wootton and W. R. Courchene (*J. Agr. Food Chem.* **12,** *94 (1964)*). This structure was verified by synthesizing the compound. The latter produced the same lesions in chicks as the compound isolated from the toxic fat.

Significant progress has been made in indentifying one of the toxic substances present in some batches of fats intended for animal foods. There are still a number of unanswered questions, the most important of these being: What is the possible significance of these compounds to human health? Since substances with physiological properties similar to the chick edema factor can be stored in the adipose tissue of chicks, can they also accumulate in human tissue when minimal amounts are inadvertently ingested?

Another important question is: What is the source of these compounds? Apparently, the chick edema factor has been associated only with fats and fatty acids subjected to a considerable amount of heat during their processing. If the source of the chlorine in the toxic compound could be identified, it might be possible to devise methods for its removal or elimination before marketing the fat.

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Studies of the Chicken Edema Disease Factor*

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Introduction

When the problem of "X" or "Edema" in poultry was brought to our attention in December of 1957, a considerable amount of work had already been done. It was established that drugs added to the feed, as well as contamination by drugs or heavy metals such as lead or arsenic, was not responsible. The disease was not caused by bacterial, viral, or parasitic infection, and evidence was accumulating that the only feed ingredient associated with all the various outbreaks was the fat. The symptoms have been described in detail in the preliminary report of Schmittle, *et al.* (*J. Am. Vet. Med. Assoc.*, *132*, *216 (1958)*), which also summarized some of the work leading to the incrimination of the fat added to the ration.

The characteristic symptoms of this disease are the presence of excessive fluid in the pericardial sac, in the abdominal cavity (water belly), and less often subcutaneously, accompanied by high mortality beginning approximately in the third week. The striking resemblance of the "edema disease" symptoms to those recorded for the exudative diathesis syndrome of vitamin E deficiency in chicks made attractive the hypothesis that the disease outbreaks were the result of an induced vitamin E deficiency caused by the use of poor quality fats.

It was convenient, because of other work in progress in our laboratory at the time, to use the A.O.A.C. vitamin D_3 chick test ration and single comb white Leghorn chicks in an attempt to produce the condition.

Our first feeding trial with a sample of feed collected from one of the "disease areas" was a false start, since after six weeks on the ration the chicks were normal and showed no unusual symptoms upon postmortem examination. The first experiment in which we successfully produced the characteristic symptoms of the "chick edema disease" involved a sample of fat that had been collected by one of our Food and Drug inspectors at a feed manufacturing plant and which had been known to have produced the disease, and also a sample of a "tarry" by-product from the manufacture of oleic and stearic acid, which presumably had been mixed into this fat sample to the extent of about 40%. In this experiment the vitamin E hypothesis was tested. Also, the suitability of the A.O.A.C. ration for this investigation was checked against a feed resembling more closely a practical commercial ration.

Basal Ration and the Vitamin E Hypothesis.—Table 1 shows the composition of the A.O.A.C. basal ration and the special basal ration. The major differences are the substitution of Drackett (an isolated soybean protein) for the casein of the A.O.A.C. diet, and the inclusion of alfalfa leaf meal and linseed oil meals. From the results shown in Table 2, it is clear that: *(1)* the fatty acid (F.A.) by-product is much more effective in producing the disease than the sample of fat (INV. Fat) that had actually been used by feed manufacturers; *(2)* the special basal ration is to be preferred to the A.O.A.C. diet, since the severity of the edema symptoms is increased, growth performance is improved, and gizzard erosion is eliminated; *(3)* the feeding of vitamin E in large

Table 1. Basal chick rations

	A.O.A.C.	Special
Whole yellow corn, ground	58	15
Wheat flour middlings	25	19
Crude precipitated casein	12	—
Calcium phosphate (precipitated)	2	2.14
Iodized salt	2	2.04
Nonirradiated yeast	2	3.2
$MnSO_4 \cdot 4H_2O$	0.02	0.02
Cod liver oil	1	1
Dehydrated alfalfa leaf meal	—	1
Linseed oil meal	—	10.6
Drackett protein	—	16
	102.02	100.00

* Presented at the Seventy-second Annual Meeting of the Association of Official Agricultural Chemists, Oct. 13-15, 1958, at Washington, D.C.

Table 2. Effect of suspect fat and by-product fat on chicks

Group	Diet	Added Fat	Av. 20-Day Wt. (Grams)	Death	No. of Chicks Showing Edema Symptoms Marked	Mild	No. Normal
1	A.O.A.C.	6% lard	147	0	—	—	20
2	A.O.A.C.	6% INV. fat	133	0	—	7	12
3	Special	6% INV. fat	153	0	10	2	4
4	Special	3.6% lard + 2.4% F.A. by-product	147	(4) [b]	7	9	—
5	Special	3.6% lard + 2.4% F.A. by-product + vitamin E [a]	143	0	5	5	9

[a] 10 mg *dl*-alpha tocopherol per chick per day by mouth.
[b] Av. survival time, 29.2 days.

doses does not prevent the occurrence of the disease, but does seem to decrease the severity of the symptoms. However, in an independent study by P. C. Underwood and C. G. Durbin at the Beltsville Laboratories of our Veterinary Medical Branch, with Rhode Island Reds, the oral administration of 1 mg per day per chick of *dl*-alpha tocopherol acetate had no effect, the symptoms being slightly more pronounced in the supplemented group. The conclusion that some material associated with the "fatty acid by-product" is responsible for the disease, and not a simple vitamin E deficiency, was confirmed in a subsequent test, when the fatty by-product was fed at 7% of the diet and all chicks showed marked to severe symptoms at autopsy; only 2 of 19 chicks survived the twenty-third day, and the average survival of 17 chicks was 19.4 days. In a similar group fed 10 mg of *dl*-alpha tocopherol per chick per day, the average survival was 20.5 days for 15 chicks; 4 chicks survived to the twenty-third day. All showed marked to severe symptoms.

Chemical Findings.—Part of the chemical data on these samples is shown in Table 3. Findings of particular significance were that these "fats" (F.A. by-product) were actually free fatty acids containing an unusually large amount of unsaponifiable material. Furthermore, the unsaponifiable fraction is qualitatively different from that found in fresh animal fat such as lard, by virtue of the large percentage that reacts in the Liebermann-Burchard test, compared to the much smaller proportion that reacts with digitonin. In the lard the proportion is almost one to one.

Table 3. Comparison of suspect fats with lard

	% Unsap.	Liebermann-Burchard Positive % of Unsap.	Digitonin Precipitable % of Unsap.
Lard	0.35	23.0	21.7
Inv. fat	3.8	70.0	7.6
F. A. by-product	10.7	92.5	2.3

[a] Δ-5 [illegible] sterol.
[b] 3-β-OH Δ 5-6 [illegible] sterol.

Urea Fractionation.—The fatty acids from the fatty by-product, after saponification, were fractionated (Fig. 1) with urea into two portions: the normally occurring fatty acids that form urea adducts, and modified or abnormal fatty acids that do not form urea adducts. About 76% of the fatty acids form urea adducts; the remainder (24%) comprise the urea filtrate.

Effect of Urea Fractions on Rats and Chicks.—The urea filtrate fatty acid fraction was fatal to weanling rats (40–60 g) upon oral administration. Two successive daily doses of 0.4 ml resulted in marked loss of weight and death by the fourth day. Two doses of 0.2 ml caused a marked weight loss from which recovery began at the fourth day. This effect is similar to that observed in our laboratory with the fatty acids that do not form urea adducts which were derived from heated cottonseed oil, and to the observations recorded by Crampton, *et al.* (*J. Nutrition*, 44, 177 (1951)) with heated linseed oil. Chicks react similarly, although they seem to be more resistant to the lethal effects of direct feeding than the rats. However, not enough observations were made to establish the relative susceptibility of the two species. All polymers and hydrogenation products (see Fig. 1) were toxic.

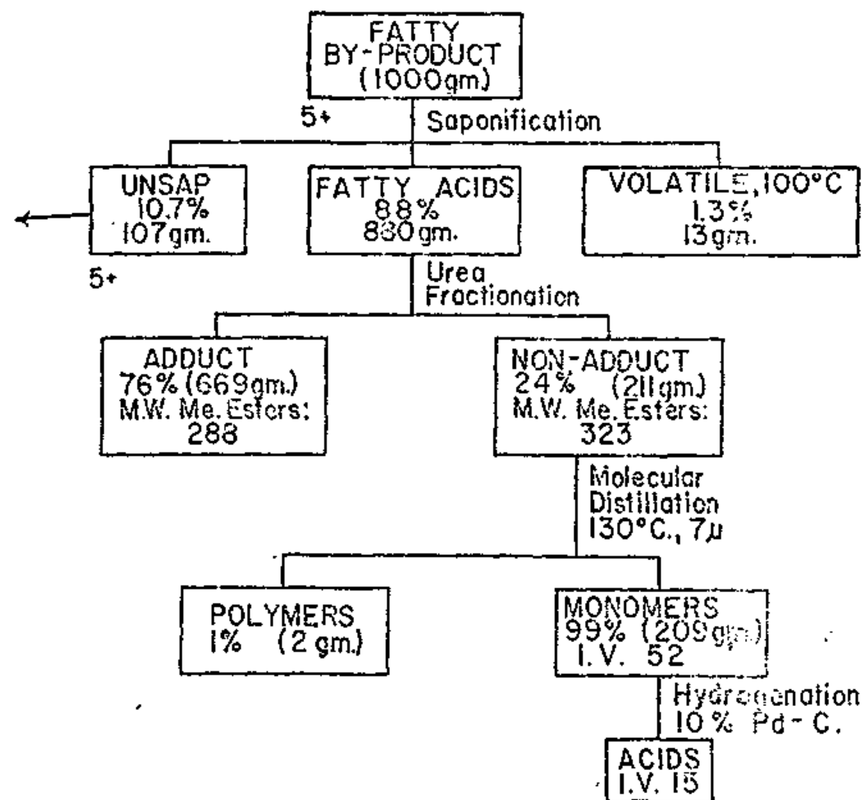

Fig. 1—Fractionation of fatty acid by-product. (Numbers followed by + sign are toxicity scores; see footnote c, Table 5.)

The urea adduct fraction of the fatty acids produced no unusual effects either in rats or in chicks.

Effect of Unsaponifiable Fraction on Rats.—The significant findings of feeding the unsaponifiable fraction of the fatty acid by-product to rats are seen in Table 4. One ml of the unsaponifiable fraction was fed to each of four rats every day for 22 days. Control animals received no supplement to the basal ration. The experimental group contained two males from different litters, with litter mates in the control group. The females of both the test and control groups were all from one litter. The growth rate was significantly depressed—females were more drastically affected than males; the size of the thymus decreased; and the size of the liver markedly increased, which was accounted for only in part by an increase in liver fat. No other significant differences in the relative weights of organs (kidneys, thyroids, hearts, adrenals, etc.) were noted, although small differences cannot be demonstrated by such small groups.

Although deleterious effects were produced in rats both by the urea filtrate and by the unsaponifiable portion, symptoms similar to those of the "edema disease" syndrome were not observed in this species.

Effect on Chicks.—When added to the diet of chicks at a level equivalent to 7% of the original fatty by-product, the urea filtrate produced a significant growth depression but, as Table 5 shows, very few of the disease symptoms. The small amount of activity

Table 4. Results of feeding unsaponifiables from "fatty acid by-product" to weanling rats by stomach tube (1.0 ml/rat/day)

	Males[a]		Females[b]	
	Control	Fed	Control	Fed
22-day wt gain, grams	138	91	114	49
	112	91	115	48
Liver wt, g/100 g body wt	6.00	9.65	5.65	10.00
	5.77	10.03	6.31	9.68
% Fat in dry liver	12.6	11.8	10.9	14.9
Thymus, g/100 g body wt	0.220	0.095	0.23?	0.084
	0.258	0.102	0.16?	0.073

[a] Males paired by litter; 2 litters.
[b] Females all from one litter.

indicated in the urea filtrate was shown in later experiments to reside in the unsaponifiable residue that had not been completely extracted. It is clear that the edema disease-producing material is associated with the unsaponifiable portion of the fatty by-product. In Table 5 are also shown the variations in severity of the disease produced by a series of graded doses. Although such a tabulation as shown here cannot give a complete picture of the range of observations encountered, it indicates how the pattern of symptoms seen in any one group depends upon the dose of active material fed. In these studies the dose was usually high enough to produce severe symptoms, and as we became more familiar with the disease we became more confident of the results obtainable with smaller groups. Whenever possible we now use five chicks per group; occasionally we have used as few as two, when the amount of sample was limited.

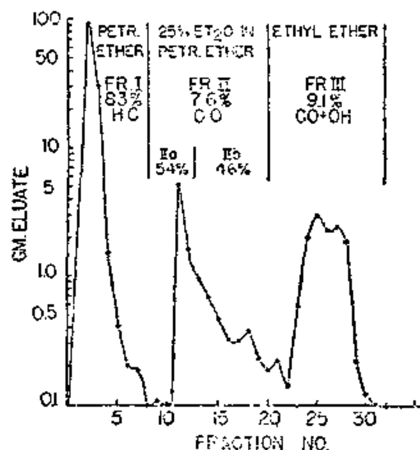

Fig. 2—Alumina chromatography of unsaponifiable fraction from toxic fatty by-product.

Concentration of Toxic Factor

Alumina Chromatography.—The first step in our attempts to isolate the factor that caused the "edema disease" from the unsaponifiable portion of the fatty by-product was chromatography on alumina (1:6). A chromatogram (Fig. 2) was obtained by plotting the weight of material eluted by increments of solvent. Petroleum ether was used until the elution curve seemed to have reached a minimum, then was followed by 25% ethyl ether in petroleum ether. The elution was completed with 100% ethyl ether to give three distinct fractions of increasing polarity. In general, Fraction I could be characterized as hydrocarbon (HC), Fraction II as containing carbonyl compounds, and Fraction III as containing sterols. The ultraviolet absorption spectra show that Fraction I has characteristic absorption peaks at 223, 227, 234, and 243 mμ, suggesting the presence of significant amounts of cholestadiene, either the 2,4 or 3,5 isomer or both.

In anticipation of the possibility that the toxicity would reside in Fraction I (83% of the unsaponifiable material) a sample of 3,5-cholestadiene was prepared by W. L. Hall in time to be included in the third feeding trial. The results of this experiment indicated clearly that there was no response to cholestadiene but that all the activity responsible for the chick edema disease was in Fraction II, which constituted approximately 8% of the unsaponifiable or about 0.55% of the original product.

Purification of Fraction II.—Three differ-

Table 5. Effect of by-product fat and its fractions on chicks

Expt.	Sample	% in Diet	Deaths[a]	No. Chicks Showing Edema Symptoms: Severe	Moderate	Mild	No. Normal	Tox. Score[c]
2	F.A. by-product	7	17 (19.4)	2	—	—	—	4.9
2	F.A. by-product + vitamin E[b]	7	15 (20.5)	2	2	—	—	4.7
2	Urea adduct	4.8	—	—	—	—	20	—
2	Urea filtrate	1.5	—	—	—	4	14	0.2
2	Unsap.	0.70	15 (20.2)	6	—	—	—	4.7
4	Unsap.	0.50	3 (20.3)	6	—	1	—	4.0
4	Unsap.	0.25	—	—	7	3	—	2.4
4	Unsap.	0.125	—	1	5	2	3	2.0
4	Unsap.	0.062	—	—	—	—	10	—

[a] Figures in parentheses indicate average survival time in days.
[b] 10 mg dl-alpha tocopherol per chick per day by mouth.
[c] The toxicity score is computed by assigning a score of 5 for each chick that died of the edema disease, 4 for each chick that showed one or more of the characteristic symptoms, i.e., hydropericardium, hydroperitoneum, or subcutaneous edema, in severe degree, a score of 3 for moderate symptoms, and a score of 1 or 2 for mild symptoms. The average of the individual scores is tabulated as the toxicity score.

ent approaches were made simultaneously to the problem of further purifying Fraction II: (*1*) counter current distribution (iso-octane: methanol solvent system with 500 transfers[1]), (*2*) chemical separation of carbonyl containing compounds by the Girard Reagent T, and (*3*) re-chromatography on alumina with a higher ratio of adsorbent to sample and more gradual elution.

For the counter-current distribution experiment 8.4 grams of Fraction II were loaded into the first 10 tubes of a 500 tube Craig all-glass counter-current apparatus. After 500 transfers, the contents of every 10 consecutive tubes were combined, and after evaporation of the solvents the weight of solids was determined in each of the 50 fractions obtained. The resulting distribution curve, i.e., solute *vs.* tube numbers, is shown in Fig. 3.

The 50 fractions were combined according to the peaks indicated by the distribution curve into 10 fractions for biological testing (Fig. 3). Each fraction was added to the test diet at a level equivalent to 7% of the original fatty by-product. Figure 4 shows the separation procedure diagrammatically, and indicates the amount of material recovered in each fraction. The activity seemed to be equally distributed between Fraction 5 (tubes 240-320 containing 2.239 grams) and Fraction 6 (tubes 321-343 containing 0.600 grams), as indicated by the toxicity scores. Fraction 6 was the most potent, showing as much activity at a level of 4.2 mg per 100 grams of feed as had been observed with 5 grams of original product. It is of interest that the toxic fractions 5 and 6 did not react with digitonin and did not show the L.B. reaction.

In preparing material for the chemical separation (Fig. 5) Fraction II was cut into two approximately equal portions; a characteristic yellow band was used as the landmark for the separation. Each of the fractions (IIA and IIB) was treated identically. First, after evaporation of the ether the material was heated with methanol, then cooled. When the hot methanol solution cooled, white crystals were obtained. The infrared spectrum for these crystals indicated a carbonyl compound with a long aliphatic chain. A synthetic compound, dipalmitone (prepared by Jonas Carol), had a very similar infrared spectrum.

Approximately 16% of Fraction IIA was insoluble in *methanol*. To the methanol-soluble portion was added twice the weight of Girard Reagent T and to the mixture was added 10% its volume of glacial acetic acid. The mixture was allowed to stand for two hours at room temperature before the water-soluble derivatives were separated from the unreacted material ("non-ketonic"). The "non-ketonic" portion was dissolved in hot acetic acid and heated with two times its weight of Girard Reagent T on the steam bath for 10 minutes, and the unreacted material was separated from the water-soluble derivatives. Approximately 23% of sample

[1] We gratefully acknowledge our debt to Procter & Gamble workers for suggesting the solvent system.

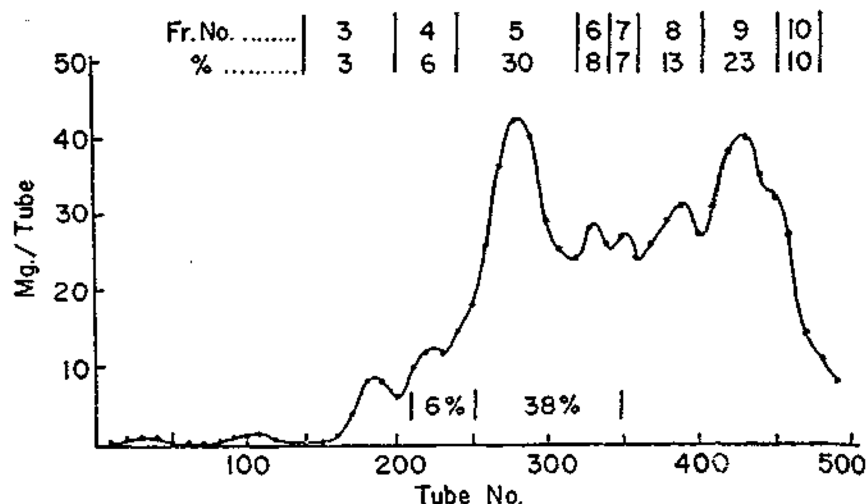

Fig. 3—Counter-current partition of Fraction II. Methyl alcohol:iso-octane system, 500 tubes, 22 hours.

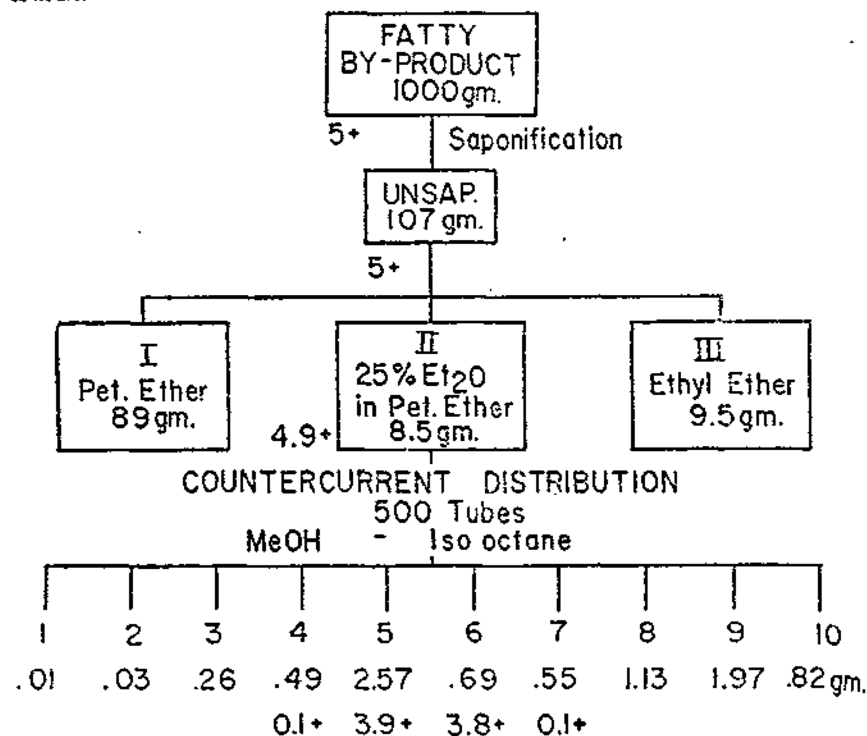

Fig. 4—Partition of Fraction II with methyl alcohol:iso-octane counter-current system. (Numbers followed by + sign are toxicity scores; see footnote c, Table 5.)

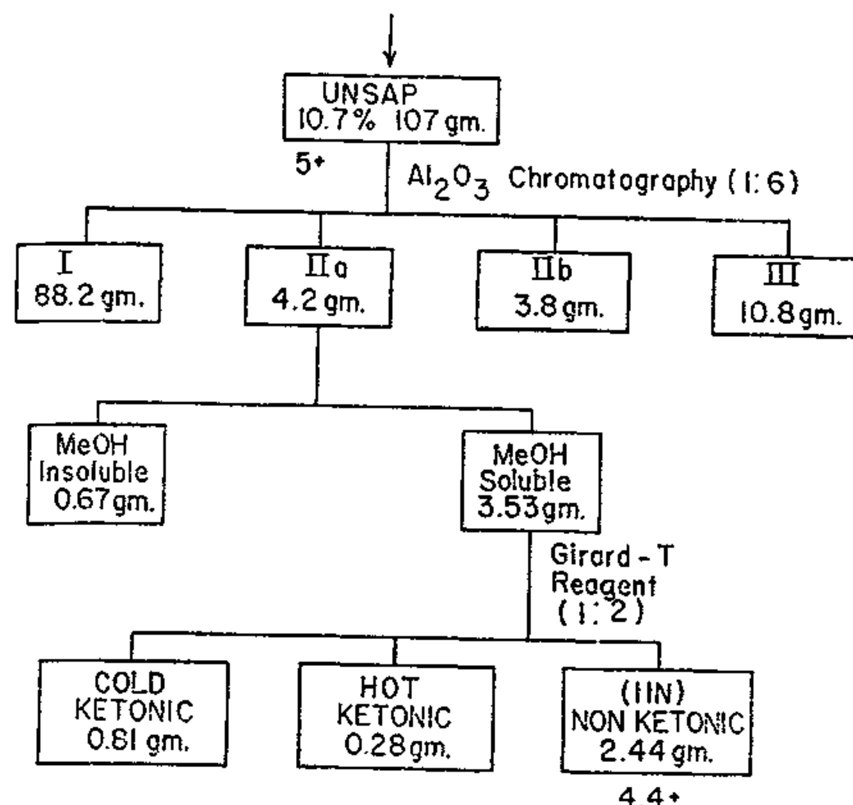

Fig. 5—Further fractionation of Fraction II.

had reacted in the cold and 8% more with heat; 69% did not react under these conditions and was classified as "non-ketonic" (IIN). The various fractions derived from Fraction IIA in Fig. 5, as well as a similar set derived from Fraction IIB, were also fed at a 7% equivalent level in the diet. The synthetic dipalmitone was fed at a level of 10 mg per 100 grams. As indicated, there was no disease-producing activity in any of the fractions obtained from IIB and only the "non-ketonic" Fraction IIN was very active. Surprisingly, however, the infrared spectrum of Fraction IIN definitely indicated the presence of carbonyl groups. This so-called "non-ketonic" material was again treated with Girard Reagent T, this time with five times the weight of reagent instead of two times, and was heated at 80° C for 15 minutes. After the water-soluble derivatives were separated from the unreacted non-ketonic material, two-thirds of the IIN fraction proved to have reacted. The non-ketonic residue was again treated with excess Girard reagent and heat, and this time 50% of the "non-ketonic" material reacted. When the original material from the Girard derivatives was regenerated and the reaction was repeated with excess Girard reagent, again only 67% reacted to form ketone-Girard derivatives. It was clear that our active "non-ketonic" Fraction IIN consisted largely of compounds containing "hindered" carbonyl groups that would react with Girard reagent with great reluctance. Of additional interest is the fact that these "hindered ketone" fractions had characteristic ultraviolet spectra with a major absorption max-

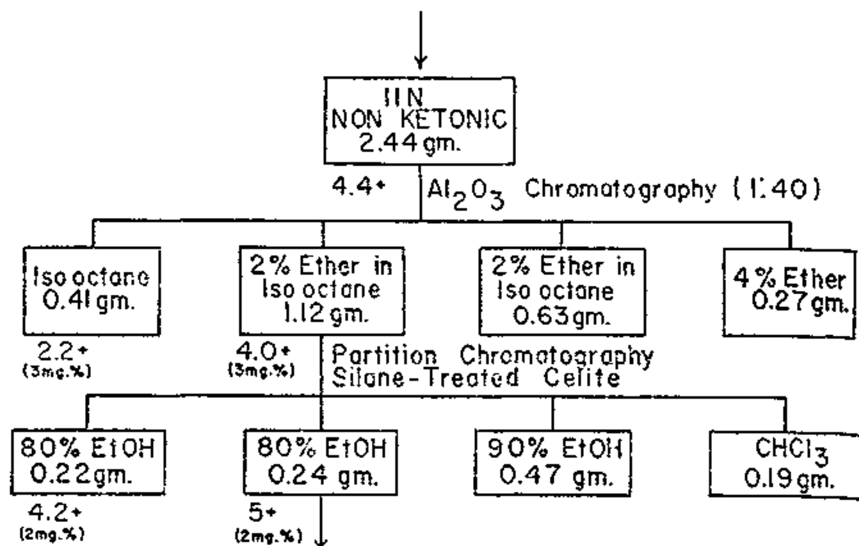

Fig. 6—Chromatography of Fraction 11N. (Toxicity scores at the levels fed appear below boxes.)

imum at 233 mμ and a secondary maximum at 260 mμ.

The disease-producing activity of Fraction 11N was further concentrated (Fig. 6) by chromatography, first on alumina. A ratio of one part sample to 40 parts alumina was used, and the column was eluted successively with iso-octane, 2% ethyl ether in iso-octane, and 4% ethyl ether in iso-octane. The fractions were monitored by ultraviolet spectrophotometry as they were eluted and then were combined for feeding on the basis of spectral characteristics. The first part of the material eluted with 2% ethyl ether in iso-octane had a definite phenanthrene type of ultraviolet spectrum with characteristic maxima at 259 mμ and 300 mμ, and with *no* peak at 234 mμ. The fraction immediately preceding, eluted with 100% iso-octane, did not show a phenanthrene type of spectrum but did have a major absorption peak at 234 mμ and a smaller one at 260 mμ. The

Next, this "phenanthrene" material was further concentrated by partition chromatography on a column consisting of 5 grams of silane-treated Celite plus 4 ml of iso-octane. Mobile solvents were 80% ethyl alcohol saturated with iso-octane, followed by 90% ethyl alcohol saturated with iso-octane. The first and second 80% alcohol fractions were both very toxic at a level of 2 mg per 100 grams of feed. These two steps (Fig. 6) represent approximately an 8-fold concentration of the toxic material of Fraction 11N. The first 80% alcohol cut had a typical phenanthrene ultraviolet spectrum and no peak at 234 mμ, whereas the second 80% alcohol fraction shows a major absorption maximum at 234 mμ, in addition to the characteristic phenanthrene peaks at 259 and 300 mμ.

Simultaneously with the work just discussed, another study of chromatography on

and that only the first cut contained edema disease-producing material (Fig. 2). This Fraction IIA was eluted from the second column with large volumes of petroleum ether (three arbitrary cuts), followed in succession by large volumes of 0.2%, 1%, 2%, and 5% and 25% ethyl ether in petroleum ether. The seven fractions indicated in Fig. 7 were tested at a level of 3 mg per 100 grams of feed. Only Fractions 2 and 3 showed activity. The methyl alcohol-insoluble portion from Fraction 3 showed activity. This finding did not agree with earlier experience, but may be explained by the larger quantities of material involved in these particular operations as compared to the earlier experiment. Apparently the active material has a more limited solubility in methanol than first supposed. In one of our most recent experiments, the methanol-soluble portion of Fraction 3 from the second alumina column was subjected to partition chromatography on silane-treated Celite as previously described (Fig. 8). This time only 80% ethanol was used as the mobile phase and the eluate was partitioned into forerun, tailings, and two major fractions, 82A and 82B, according to their ultraviolet spectra. Fraction 82A consisted mainly of material with a phenanthrene spectrum, and 82B showed the major peak at 234 mμ. Fraction 82A was rechromatographed on the silane column, using 75% ethanol as the mobile solvent, and again cut according to the ultraviolet spectra into a forerun, a fraction with a phenanthrene spectrum, an overlap fraction showing approximately equal absorption peaks at 259 and 234 mμ, and a fraction with a major peak at 234 mμ, approximately 6 times as intense as at 259 mμ. All of the fractions were fed, and each of the major fractions was found capable of producing the chick edema disease when fed at a level of 0.5 mg per 100 grams of diet with a severity approximately equal to that observed in chicks fed 5% of original fatty by-product. This is the first instance where fractions with distinctly different absorption characteristics had approximately the same biological activity.

Another recent experiment involves the reduction of one of our potent fractions with sodium borohydride. After the borohydride was separated, the treated material was

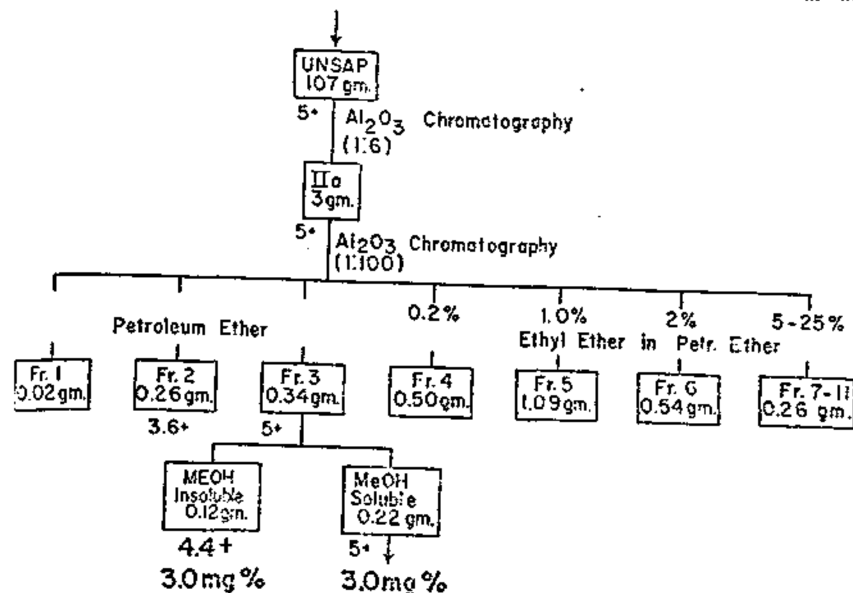

Fig. 7—Chromatography and elution of Fraction IIA (1:100 column).

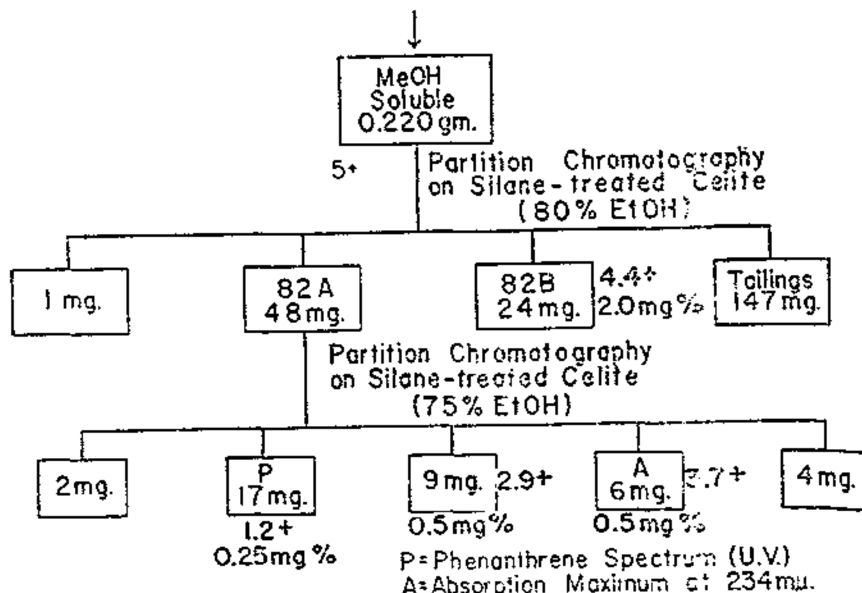

Fig. 8—Reverse phase partition chromatography of MeOH-soluble portion of Fraction 3.

chromatographed on alumina into three approximately equal (30:30:40) fractions of increasing polarity. The infrared spectra indicated that the first fraction was largely hydrocarbon, the second fraction had the carbonyl group present, indicating that borohydride also reacts reluctantly and incompletely with the hindered ketones, and the third fraction contained hydroxyl groups. There was very little disease-producing activity left, all of it limited to the first fraction. This may mean that either the activity is lost upon reduction but that not all of the material reacts under the conditions used, or that the reduced compound retains only part of the original activity. In either case, these data are additional evidence that the active material contains a carbonyl compound.

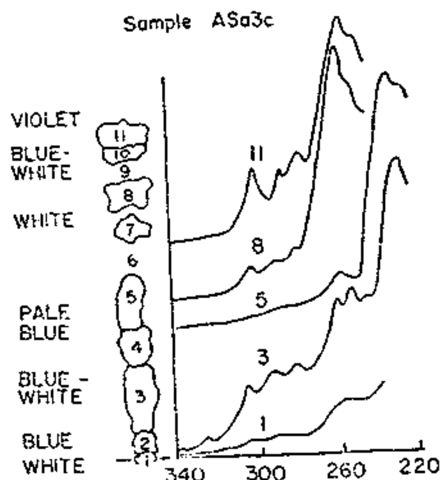

Fig. 9—Paper chromatography, and ultraviolet curves of fluorescent spots obtained from a highly toxic fraction.

Each of the fractions discussed here, and many others have been studied by ultraviolet and infrared spectrophotometry and the pertinent observations have been mentioned.

E. O. Haenni has been studying the fluorescence spectra, both activation and emission, of each fraction, and will report the results when the data have been analyzed.

In addition, as the potency of the disease producing factors has been concentrated, we have tested the purity of each fraction by paper chromatography. We are using 8 x

9½ Whatman No. 31 paper, washed with methanol and then coated with mineral oil that has been pre-washed with sulfuric acid and methanol. The papers are developed with methanol in an ascending system for periods varying from 2 to 20 hours. For the longer periods the solvent is permitted to evaporate from the edge of the paper through the slotted glass cover. After the papers are dried, they are examined under ultraviolet light and the fluorescent spots are traced. Figure 9 shows a typical paper chromatogram. The spots are cut out and eluted with methanol, and the ultraviolet spectra of these solutions are determined. Figure 9 represents a fraction of high purity that has been tested by a chick feeding experiment; it is of considerable interest to note that the ultraviolet spectra of these spots are of both types discussed earlier, namely, the phenanthrene type with an absorption maximum at 259 mμ and the type with a maximum at 234 mμ. The ultraviolet curve of the original solution was of the phenanthrene type.

If we were to speculate about the chemical nature of the disease-producing material from the evidence available at this point, we would visualize a pair or a series of closely related compounds, with a 2-ring naphthalene structure, or more likely a 3-ring nucleus, similar to phenanthrene; a carbonyl group in a long aliphatic side chain; and molecular weights of 500–600.

Appearance of Poison in Flesh of Chickens

Once it had been established that the chicken edema disease was caused by a toxic principle, the possibility that edible chicken flesh might contain this toxic material had to be considered. As soon as we discovered the unusual characteristics of the unsaponifiable material from the toxic fat and fatty by-product, we compared the unsaponifiable fraction of the carcasses of chickens that had been fed these products with the unsaponifiable fraction from the carcasses of normal control chickens. About 25% more unsaponifiable material per unit of fresh chicken weight was obtained from the test chickens than from the control group. When the unsaponifiable material was chromatographed on alumina, about 18 times more hydrocarbon material was obtained from the test group. The test group hydrocarbon fraction gave a strong positive reaction in the Liebermann-Burchard Test whereas comparable material from the control group was negative. Furthermore, upon comparing the hydrocarbon fractions by differential ultraviolet spectrophotometry, definite absorption peaks appeared which were similar to those observed from Fraction I of the unsaponifiable portion of the fatty by-product.

This evidence indicated beyond doubt that some of the material from the disease-producing fat was deposited in the flesh of chickens when they received it in their diet. This, of course, did not prove that the chicken meat contained the toxic material, especially after it was learned that the disease-producing activity was in Fraction II and not in Fraction I. In order to settle this point, the unsaponifiable portion obtained from freshly ground chicken carcasses, not including intestines, head, or feet, was fed to chicks at levels of 1.0, 0.5, 0.25, 0.125, 0.05, and 0.025% in the test ration. As can be seen in Table 6, symptoms of edema-disease were observed at all levels fed. The unsaponifiable material from normal control chickens fed at 0.5% of the diet produced no abnormal symptoms of any kind.

Table 6. Results of feeding unsaponifiable extract of chickens that had been fed fat or fatty acid by-product

Expt. No	% in Diet	Deaths*	No. Chicks Showing Edema Symptoms: Severe	Moderate	Mild	No. Normal
3	1.0	2 (15.5)	—	—	—	—
4	0.5	5 (16.6)	—	—	—	—
5	0.25	5 (19.0)	1	—	—	—
	0.125	4 (21.0)		—	—	—
6	0.050	—	—	3	2	1
	0.025	—	—	1	1	3

* Figures in parentheses show average survival time in days

We have examined samples of various types of fats and fatty acid products in a survey that is still continuing. The edema disease-producing product is a characteristic not confined to the production of a single manufacturer.

Summary

In summary, from the studies so far, it may be concluded that:

(1) The chicken edema disease is caused by a toxic factor in a fatty by-product of stearic and oleic acid manufacturing operations.

(2) The disease-producing factor has been concentrated approximately 10,000 times by saponification, chromatography of the unsaponifiable portion on alumina, and reverse phase partition chromatography on silane-treated Celite.

(3) The evidence presented indicates (a) the presence of compounds with a two-ring nucleus similar to naphthalene or a three-ring nucleus similar to phenanthrene, (b) the presence of a "hindered carbonyl" group, probably in a side chain. Based on the relatively low intensity of the carbonyl peak at 5.8 μ a compound of molecular weight 500–600 is suggested. However, the evidence on the presence of carbonyl in the toxic compounds is not conclusive and does not completely rule out the possibility of a toxic hydrocarbon.

(4) The fatty acid fraction from this fatty by-product contains significant quantities of non-urea adduct-forming compounds that are toxic, although they do not produce edema disease.

(5) The edema disease-producing compounds are present in significant amounts in the flesh of chickens on rations which contain toxic material.

(6) The edema disease-producing material is not confined to the production of a single manufacturer.

Note

Since this paper was presented, repeated chromatographic purifications of the toxic materials on alumina and on silane-treated Celite have now permitted the isolation of several distinctive fractions. One of these fractions, possessing the ultraviolet absorption spectrum of substituted naphthalenes ($\lambda_{max.}$ at 236, 286, 296 mμ; shoulder at 325 mμ; $\lambda_{min.}$ at 253 mμ; $A_{236} \sim 14 \times A_{286}$), proved highly toxic. The infrared spectrum of this material showed little absorption in the carbonyl region. It is concluded that the "naphthalene" fraction comprises a mixture of hydrocarbons. Assuming that alkyl-substituted naphthalene is the only chromophore present, the average molecular weight, calculated from the absorptivity at 236 mμ ($E^{1\%}_{1\,cm} \cong 3200$) is in the vicinity of 250.

Acknowledgment

The extensive work outlined in this paper was made possible by the active cooperation of many other individuals, both within and outside the Food and Drug Administration. We are indebted to W. C. Ault and D. H. Saunders of the Eastern Utilization Research and Development Division of the U.S. Department of Agriculture, who prepared 1,330 grams of unsaponifiable material from the fatty by-product, and to these members of the Food and Drug Administration: Stanley Nesheim, Division of Food, who prepared an equal amount of the unsaponifiable material; Jerome Eisner, Division of Food, who helped prepare the charts; J. H. Jones, John Winniger, and Meyer Dolinsky, Division of Cosmetics, who assisted in the countercurrent distribution study; Jonas Carol, Division of Pharmaceutical Chemistry, who helped to interpret the infrared curves and to synthesize a sample of dipalmitone; P. C. Underwood and C. G. Durbin, Bureau of Medicine, who provided market-size chickens that had been fed the toxic material, for use in the carcass residue studies; W. L. Hall, Division of Nutrition, who synthesized a sample of 3,5-cholestadiene; and the numerous inspectors of the Food and Drug Administration who provided essential information without which this work would not have progressed.

We are also indebted to the other individuals and laboratories, who are simultaneously investigating this problem, for discussions that provided information helpful in guiding us in our studies. Among these are Ralston Purina Company, the Procter & Gamble Company, and Hales and Hunter.

The Occurrence of the Chick Pericardial Edema Factor in Some Oleic Acids and Products Derived Therefrom [1]

Stanley R. Ames, William J. Swanson, Marion I. Ludwig, and George Y. Brokaw, Research Laboratories, Distillation Products Industries,

Division of Eastman Kodak Company, Rochester, New York

A material causing the chick edema syndrome has been reported (1, 2, 3, 4) to occur in specific lots of feed-grade animal fats. These fats were reported to contain the edema-producing factor as a trace impurity produced during certain fat-processing operations (2, 3). Birds fed diets containing this unidentified factor develop an edematous condition, characterized by pericardial edema (distention of the pericardial sac with fluid, also termed "hydropericardium") and in more severe cases ascites and gross liver and kidney damage. In the young chicks the initial gross symptoms are characterized by abdominal distention and labored breathing. Manifestations of the pericardial edema factor appear to be limited to poultry (1).

The active pericardial edema-producing factor has not yet been isolated, but certain characteristics of the pericardial edema factor have been reported by Brew *et al.* (2) as follows: a) presumably a hydrocarbon derivative, possibly of cholesterol, b) a molecular weight of about 360, c) associated with fractions which give the Liebermann-Burchard test for sterol residues, d) it can be concentrated by molecular distillation of fats containing the factor. The edema syndrome is not produced by fat *per se* but appears to be related to impurities in certain lots of fats subjected to special fat-processing operations.

F. W. Hill of Cornell University reported to us that high-level feeding to chicks of a sample of a glycerol ester of oleic acid resulted in some mortality with gross symptoms resembling pericardial edema. Our studies have confirmed his observations and furthermore have shown that the pericardial edema-producing factor is present in many samples of commercially-available oleic acid as well as in glycerol esters made therefrom. Molecular distillation of an active oleic acid or of a glycerol mono-ester of an active oleic acid was found to concentrate the pericardial edema-producing factor.

Technique

In this investigation a rapid, sensitive bioassay procedure, developed in our laboratories, was employed (5). The diet used is a modification of that described by Brew *et al.* (2) and consists principally of purified casein, gelatin, glucose, and 16% test fat. Day-old chicks are fed this diet *ad lib.* immediately on arrival. Subgroups are sacrificed at 10, 14, and 21 days. In general, a higher incidence of pericardial edema is observed in the subgroups sacrificed at 10 and 14 days than in the group sacrificed at 21 days. At autopsy, chicks are examined for the appearance of pericardial edema, ascites, and the appearance of complications, such as liver and kidney damage or labored breathing. Bioassay groups consist of 10 or 15 chicks for each sample of fat. Using a weighting procedure described in Table I, a pericardial edema-activity score is calculated. This makes possible a semi-quantitative comparison between the relative activities of various fat samples.

Occurrence

Oleic acid samples (U.S.P.) from four manufacturers were assayed biologically and chemically (acid value and unsaponifiable content). As indicated in Table I, several of the oleic acid samples were strongly active, but others showed little or no activity. The pericardial edema activity scores showed that no correspondence existed between the presence of the pericardial edema-producing factor and the acid value or the content of unsaponifiables.

Tests for the pericardial edema-producing factor in certain commercial samples of glycerol esters (monoglycerides and monodiglycerides) of oleic acid are summarized in Table II. Seven different manufacturers of glycerol esters are represented. The majority of these ester samples were active. Again, there is no correlation between unsaponifiable content and pericardial edema activity.

In four instances, samples of both the oleic acid and the glycerol ester pro-

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[1] Communication No. 265 from the Research Laboratories of Distillation Products Industries, Division of Eastman Kodak Company...

PERICARDIAL EDEMA ACTIVITY

Sample No.	Cut, percent	Analysis		Bioassay Results							
				Pericaridal edema						Activity ratio[c] pos./total	Activity score,[d] percent
		Acid value	Unsap. percent	Plus death	Plus complications[a]	Plus ascites	Uncomplicated	Other deaths[b]	Negative		
TABLE I.—OLEIC ACIDS AND THEIR PE (PERICARDIAL EDEMA) ACTIVITY											
1		201.0	0.45						21	0/21	0
2		202.5	0.68		2	1			7	3/21	110
3		201.2	0.77			1			8	1/10	33
4		202.8	0.52			2			8	2/10	60
5		203.0	0.18	1	1	1			6	3/10	133
6		202.5	0.28		4		3		4	7/11	200
7		201.3	0.51						10	0/10	0
8		204.9	0.50				1		8	1/9	22
9		204.4	0.77		3	4	3			10/10	300
10		203.9	0.52						10	0/10	0
11		202.0	0.20						10	0/10	0
TABLE II.—OLEIC ACID ESTERS AND THEIR PE (PERICARDIAL EDEMA) ACTIVITY											
1		2.2			3	1	2		0	6/6	320
2		1.6	0.12			1	5		4	6/10	130
3		1.7	0.13	3	4	4	13		6	24/30	230
4		2.2	0.26						11	0/11	0
5							1		13	1/14	14
6		0.5	0.32			2			9	2/11	55
7		0.3	0.63						9	0/9	0
8		2.8	0.5	1			4	3	2	5/10	160
9		4.7	0.49	2	1		3		4	6/10	200
10		7.1	0.35			1			8	1/9	33
11		1.2	0.46					3	6	0/9	33
12		5.5	1.2				2	3	9	2/14	50

TABLE IV.—MOLECULAR DISTILLATION OF AN OLEIC ACID *(SAMPLE 6, TABLE I)*

Sample No.	Cut, percent	Acid value	Unsap. percent	Plus death	Plus complications	Plus ascites	Uncomplicated	Other deaths	Negative	Activity ratio pos./total	Activity score, percent
Charge											
F-1	11.9				4		3		4	7/11	200
F-2	10.3								10	0/10	0
F-3	29.1 }				1				8	1/9	45
F-4	29.6 }										
F-5	9.7										
F-6	7.2										
r	1.2			2	1	5			2	8/10	290

TABLE V.—MOLECULAR DISTILLATION OF A GLYCEROL MONO-ESTER OF OLEIC ACID (SAMPLE 2, TABLE II)

Sample No.	Cut, percent	Acid value	Unsap. percent	Plus death	Plus complications	Plus ascites	Uncomplicated	Other deaths	Negative	Activity ratio pos./total	Activity score, percent
Charge											
F-1	10.4					1	5		4	6/10	130
F-2	9.4			3	2	4			1	9/10	350
F-3	30.6 }					1	1	1	6	2/9	67
F-4	31.5 }										
F-5	11.6										
F-6	2.8										
r	1.0				1	2	1		6	4/10	120

[a] Complications include gross liver and kidney damage or labored breathing.
[b] Early deaths in excess of control groups, PE not observed.
[c] Chicks with positive pericaridal edema/total chicks in test.
[d] The "PE activity score" is a numerical index of the severity of the activity. *The products of the number of chicks in each category times its weighting factor are summated and divided by the total number of chicks as follows:*

$$\text{PE activity score} = 100 \times \frac{5\times(\text{PE}+\text{death})+4\times(\text{PE}+\text{compl.})+3\times(\text{PE}+\text{ascites})+2\times(\text{PE})+1\times(\text{other deaths})}{\text{Total chicks in test group}}$$

duced therefrom were bioassayed as summarized in Table III. The glycerol esters showed activity only when the corresponding oleic acid was active. With inactive oleic acids or those with low activity the corresponding glycerol ester did not have increased activity. Thus the edema factor was not formed during the manufacture of the glycerol esters.

CONCENTRATION STUDIES

Molecular Distillation of an Oleic Acid. A sample of oleic acid known to contain a moderate amount of the pericardial edema-producing factor was fractionated in a 14-in. molecular still into six fractions as indicated in Table IV. The first and last fractions and a composite of the middle fractions were bioassayed as described above. The first 12% strip cut was entirely free of the pericardial edema-producing factor. The middle cuts, representing approximately 60% of the input fat, were only slightly active. The last 7% fraction showed a concentration of the edema factor with an increased activity over the input oleic acid. Thus the pericardial edema-producing factor was concentrated in the last fraction after the bulk of the oleic acid was removed by molecular distillation.

Distillation of a Glycerol Mono-ester of an Oleic Acid. A sample of a glycerol mono-ester of oleic acid, prepared from an oleic acid known to contain the pericardial edema-producing factor was fractionated into six fractions in a 14-in. molecular still, as described in Table V. The first 10% cut, the last 3% fraction, and an intermediate fraction representing approximately 60% of the input material were bioassayed as described above. An increased concentration of the pericardial edema-producing factor was observed in the first 10% cut. Some pericardial edema-producing factor was present in the other fractions examined.

DISCUSSION

The material which produces pericardial edema in chicks has been reported previously only in certain lots of fats subjected to special fat-processing operations (1, 2, 3). Therefore the finding that some U.S.P. oleic acids possessed a high degree of activity was unexpected. Feed-grade fats that contained the edema factor characteristically had high unsaponifiable levels ranging above 6% (2). However, in the present study, samples of the more active oleic acids had unsaponifiable levels ranging from 0.2 to 0.7%. This indicates a 10- to 30-fold concentration of the edema factor in the unsaponifiable fraction and suggests that the unsaponifiable fraction of an active oleic acid might be a starting material for isolation of the factor.

Molecular distillation of a fat which contains the factor concentrated this factor in the more volatile fractions (2). Molecular distillation of an active glycerol mono-ester of oleic acid also concentrated the edema factor in the more volatile fractions, but separation from the monoglyceride fraction was not as complete as it was from the higher-distilling triglycerides. Molecular distillation of an active oleic acid concentrated the edema factor in the less volatile fraction. Since fatty acids, such as oleic acid, distill at temperatures lower than the corresponding monoglycerides, the distillation range of the edema factor appears to lie above that of oleic acid and below that of the glycerol mono-ester of oleic acid.

TABLE III.—COMPARISON OF PE (PERICARDIAL EDEMA) ACTIVITY OF OLEIC ACIDS AND THEIR CORRESPONDING GLYCEROL ESTERS

Oleic Acid			Glycerol Ester		
Sample No. (from Table I)	PE activity ratio	PE activity score	Sample No. (from Table II)	PE activity ratio	PE activity score
	pos./total	*percent*		*pos./total*	*percent*
5 (75%)	3/10	133}			
11 (25%)	0/10	0}	2	6/10	13[illegible]
5	3/10	133	3	25/31	23[illegible]
1	0/21	0	4	0/11	[illegible]
3	1/10	33	5	1/14	1[illegible]

SUMMARY

A number of samples of commercially-prepared (U.S.P.) oleic acids and glycerol esters of oleic acids were shown to contain a material which produces pericardial edema in chicks. The active material was concentrated from either the oleic acid or the glycerol mono-ester by molecular distillation.

Four of the 11 samples of oleic acid were inactive; this indicates that the pericardial edema is not caused by the fatty acid itself but by an incidental material in the fatty acid.

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Appreciation is expressed to Louis J. Lee and Richard E. Ardell for conducting the molecular distillations, to William C. Lyman and Gary Brooks for correlation of samples, to Herbert W. Rawlings of the Products Control Laboratory for chemical analyses and to David C. Herting, Philip L. Harris, and Norris D. Embree for advice.

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STUDIES OF THE CHICK EDEMA FACTOR. II. ISOLATION OF A TOXIC SUBSTANCE

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A crystalline halogen containing material producing chick edema symptoms at 0.1 part per million in the diet has been isolated from a sample of triolein which was toxic to monkeys. This material is similar to that reported by Harman *et al.* (4) but differs somewhat in ultraviolet spectral properties.

In a symposium on the chick edema disease in October, 1958, several laboratories (1,2,3) presented reports on their progress toward the isolation and elucidation of the toxic factor responsible for the occurrence of this unusual syndrome. It was established that the disease is caused by a toxic factor in the unsaponifiable fraction of a fatty by-product of industrial stearic and oleic acid manufacturing operations, and it was further suggested that the factor might posses a polynuclear or steroidal structure. A note, later published as an addendum to the contribution from this laboratory (2), reported that the toxic factor was associated with eluates from alumina and "silane-treated Celite" chromatographic columns which exhibited the ultraviolet absorption spectra of polysubstituted naphthalenes (λ_{Max} at 236 mμ, secondary λ_{Max} at 286 and 296 mμ). Neighboring cuts from these chromatograms showed the characteristic spectra of phenanthrene derivatives (λ_{Max} at 259, 282, 292, 300 mμ) and of simpler naphthalene derivatives (λ_{Max} 228–233 mμ, secondary λ_{Max} 270–280 mμ).

Subsequent purification of substances that had an absorption peak at 236 mμ demonstrated that they were not the most toxic fractions in our materials. Furthermore our computations showed that the toxic factor must be potent when present in the diet at levels of a fraction of one part per million. Recently Harman *et al.* (4) have reported the isolation of the chick edema factor in crystalline form from a feed-grade tallow. Their substance was toxic to chickens at 0.1 p.p.m. in the diet and had an ultraviolet absorption spectrum with a major peak at 244 mμ, a lesser peak at 312 mμ, and a shoulder at 238 mμ. A private communication from Tishler of the same laboratory (5) disclosed that the crystalline substance contains chlorine to the extent of about 47%.

Ames *et al.* (6) have observed the presence of the

ingredient in a series of dietary treatments involving changes in the level and types of fats to which a group of Cebus monkeys had been subjected.

The following summary of experimental results relative to these monkeys was received from O. W. Portman and S. B. Andrus of the Department of Nutrition, Harvard School of Public Health.

Of a group of nine monkeys that received this triolein in their diets at a level of 25% by weight, one died at one month and four at three months. After three months on the triolein diet corn oil was substituted for the triolein. The other four monkeys died from three weeks to five months later even though triolein had been discontinued and replaced by corn oil. Of 14 monkeys in the colony that did not receive troilein but were supplied other fats and oils at 25% of the diet by weight, there was only one spontaneous death. Eight of the nine monkeys fed triolein were autopsied and showed the following findings: jaundice (4.8?); pancreatic atrophy and fibrosis (6); hemosiderosis (6); fatty liver (5); bile duct proliferation (3); extramedullary erythropoiesis (3); necrosis of liver (2); gross hemorrhage in gastrointestinal tract (2); and erythrocytophagocytosis (1). Several features including marked anemia in several instances suggested the possibility of a hemolytic process. Pancreatic changes were most pronounced in the two monkeys that survived longest (seven to nine months from the beginning of triolein feeding). The severity of the lesions in the pancreas was unrelated to that of the hepatic changes. With the exception of fatty changes in the liver, the above findings have not been reproduced in rats. These observations are from an experiment not designed to study a toxic principle, and it would be unwise to draw firm conclusions with respect to the toxicity of the triolein from these limited data.

The fact that, in our laboratory, marked symptoms of chick edema disease were produced by this sample of triolein suggests the possibility that the chick edema factor may have been responsible for the toxic effects noted in the triolein-fed monkeys.

We now wish to report the ioslation of a highly toxic crystalline substance from this triolein and to describe its properties.

EXPERIMENTAL

The triolein sample was of excellent quality with an unsaponifiable content of 0.87% and a steroidal hydrocarbon (1) absorbance of 0.07. Only 0.01% of oxirane oxygen (epoxide) was detected. Examination of the fatty acids as the ethyl esters by gas chromatography showed that oleic acid constituted 70.9% of the total acids with 3.6% linoleic, 0.3% linolenic, 13% palmitoleic, 9.4% palmitic and shorter-chain fatty acids, and 1.8% of a C_{17} fatty acid containing one double bond (by inference from relative retention time). Urea filtrate fatty acids were present to the extent of 4.73% (6a).

When fed to chicks at a level of 15% in the diet, the triolein produced the symptoms of chick edema disease with a severity approximately equivalent to that observed with a diet containing the toxic fatty product used in our original studies (2) at the 5% level. The unsaponifiable fraction of the triolein was proportionately richer in the toxic factor than any other materials available to us.

The toxic factor in 17.6 kg. of triolein was concentrated by molecular distillation under pressures of 10–20 microns at temperatures up to 200°C. The distillate (289 g.), which contained all of the toxic factor, was saponified, and 166 g. of unsaponifiable material were recovered. The portion soluble in petroleum ether (155 g.), was chromatographed on 3 kg. of Fisher Alumina A 540 in a 4-ft. x 3½-in. column, using petroleum ether as eluent. Two-liter fractions were collected, and substances with the absorption spectra of naphthalene and phenanthrene derivatives were eluted in fractions 6–15. The bulk of the material containing cholestadiene and related hydrocarbons (129 g.) was eluted in the first three days, and the remainder was recovered by the use of more polar solvents. These foreruns and tailings were devoid of potency in the chick edema test.

Fractions 6 to 15 were combined (668 mg.) and chromatographed on 2,500 g. of the more retentive Merck Alumina No. 71707. No material was eluted with petroleum ether. Foreruns were eluted with 1–2% ethyl ether in petroleum ether, and fractions exhibiting the spectra of naphthalene and phenanthrene derivatives (239 mg.) were obtained when 5% ethyl ether in petroleum ether was employed as eluent. This material was again chromatographed on Merck

alumina, using 250 g. in a 42 x 4.5-cm. tube. Several fractions which eluted with 5% ethyl ether in petroleum ether exhibited an absorption maximum near 245 mμ as well as the peaks previously observed at 234–236 mμ and 255–260 mμ. There fractions were combined, and the eluted material was partitioned on 5 g. of silane-treated Celite, by reverse-phase chromatography, using 4 ml. of iso-octane in the immobile phase, and 80% alcohol saturated with iso-octane as the mobile solvent.

Fractions exhibiting maximum absorption at 245 mμ, but not at 235 mμ or 255μ, were combined for two further chromatographic purifications on alumina. Merck alumnia was deactivated with 3% its weight of water, and the ratios of alumina to sample and column size 2,000:1 in a 25 x 1-cm tube and 25,000:1 in a 25 x 2-cm. tube, respecively. Iso-octane, redistilled and chromatographed over silica gel, was employed as eluent, and fractions were again monitored by ultraviolet spectral absorbance fractions devoid of inflection and 255 mμ were combined and evaporated to dryness. The solid residue was dissolved in a small volume of boiling iso-octane and stored over-night in the refrigerator. White crystals weighing 2.64 mg. were obtained.

The ultraviolet absorption spectrum of the crystals dissolved in iso-octane was characterized by maxima at 245 mμ ($E^{1\%}_{1 cm.} = 718$) and 300 mμ ($E^{1\%}_{1 cm.} = 80$) and inflections at 240 mμ ($E^{1\%}_{1 cm.} \simeq 655$) and 310 mμ ($E^{1\%}_{1 cm.} \simeq 76$). The substance sublimed on the hot stage about 239°C. The infrared absorption spectrum of the substance, incorporated into a potassium bromide disc, showed evidence of aliphatic and aromatic linkages but no bands characteristic of oxygen or nitrogen functions.

The reported presence of chlorine in the toxic substance isolated by the Merck group (5) prompted a Beilstein test, which showed the presence of halogen. The presence of halogen in the crystalline material being reported here was confirmed by examination in a microcoulometric gas chromatograph.[1] (In this instrument the sample is fractionated by gas-liquid chromatography, and, as they are eluted from the column, the compounds are pyrolyzed at 800°C. under oxidizing conditions. The halogen acid formed from halogenated materials is titrated in a microcoulometer.)

In the chick biossay the substance, when fed at *ca.* 1 p.p.m. in the diet, produced severe hydropericardium, hydroperitoneum, and liver damage, with death occurring within 12 days. At 0.1 p.p.m. in the diet marked hydropericardium was evident at autopsy after a three-week feeding period.

The isolation of the toxic substance had been complicated by the presence of another material with an absorption maximum at 248 mμ. This second substance was isolated by the same techniques of chromatography, monitored by ultraviolet spectrophotometry, and a yield of white crystals was obtained. The ultraviolet spectrum of this material was identical with that of the toxic substance except that it was shifted 3 mμ so that the major absorption peak was at 218 mμ. It behaved similarly to the toxic substance in the microcoulometric gas chromatograph, showing a similar retention time and a similar halogen content. However, it was completely inactive in the chick edema test when fed at 1 p.p.m. in the diet.

In order to obtain additional crystalline material the portion remaining from nondestructive testing, fortified with the adjacent fractions from the final chromatography (of high potency, judging from the ultraviolet spectrum), was further chromatographed according to the previous isolation scheme. Small quantities of phenanthrene derivatives were separated with a consequent diminution in the ultraviolet absorption in the 250–300 mμ region. Furthermore, although there was no indication of nonhomogeneity by paper chromatography, the ratio of absorbances of the shoulder at 240 mμ to the peak at 245 varied from fraction to fraction, suggestion the presence of an additional component.

The extinction values reported above may be in error because of inaccuracies in weighing on account of the difficulties in handling the small amount of material involved. There is no doubt however of the validity of the relative absorbances at various wave-lengths. The spectrum of the substance reported here differs somewhat from that reported by Harman *et al.* (4), particularly in the ratio of the absorbance at the maximum (244 or 245 mμ) to the absorbance at the inflection at 238 or 240 mμ.

[1] Dohrmann Manufacturing Company, Palo Alto, Calif.

DISCUSSION

The toxic substance which we have isolated from triolein resembles that recovered by Harman *et al.* (4) from animal feed tallows. However the divergences in their properties suggest either that we are dealing with two different but closely related compounds, or that one or both of the preparations is still a mixture of related compounds despite the fact that only a single spot could be obtained on paper chromatography in a number of solvent systems. In view of the manifest difficulties involved in isolating a pure compound in minute quantities from a myriad of substances with similar properties it would be hazardous, as Harman warns, to infer chemical structures from spectral data. Nevertheless it should be pointed out that the spectra obtained by us and by Harman *et al.* are strongly reminiscent of those exhibited by highly substituted naphthalenes (7). Furthermore the toxic factor occurs in association with a bewildering array of aromatic naphthalene and phenanthrene derivatives, as we have previously noted (2). The detection of chlorine in large proportions in a toxic preparation, and the ultraviolet spectrum observed, suggest a possible relationship with chlorinated naphthalenes. Pentachloronaphthalene possesses an an absorption maximum at 243 mμ and a secondary maximum at 312 mμ (8), and it has been shown to cause hyperkeratosis in cattle (9) and several other species of animals including chickens (10). Other chlorinated naphthalenes also are toxic (11).

The possibility that the chick edema factor is a chlorinated napthalene derivative cannot be ignored. Samples of tetrachloronaphthalene and hexachloronaphthalene, kindly provided by Engel and Bell of the Virginia Polytechnic Institute, who had demonstrated that these compounds could produce hyperkeratosis in cattle, were without effect in the chick edema test. Furthermore these compounds, despite the similarity of their ultraviolet spectra and their chromatographic behavior to the toxic substance, showed considerable difference in the microcoulometric gas chromatograph. For instance, chlorinated pesticides, aldrin and heptachlor, showed retention times of 10 min., tetrachloronaphthalene 9 min., and hexachloronaphthalene 14 min., whereas the toxic substance, as well as its inactive analogue with the absorption maximum at 248 mμ, had retention times of 37–38 min. It is tempting to speculate that the greater retention-time of the toxic material is related to a greater molecular weight or to a substituent conferring different solubility and polarity properties.

We are continuing our studies toward the isolation of the toxic factor. It is necessary that the chemical nature of this substance be elucidated to make possible a rapid chemical test for its detection, to clarify its origin, to verify the suggestion of its severe toxicity to primates, and to study its action in other species.

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Collaborative Bioassay for Chick Edema Factor*

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A substance contained in certain processed fats and fatty products used in poultry feeds has been implicated in the outbreak of the condition called "chick edema disease" which occurred in 1957 (1). A number of laboratories are actively working to isolate and identify the agent responsible for the disease (2–5) and each has developed its own bioassay, which in each case has admirably served its specific purpose.

Ames and co-workers (6), early in 1960, reported this factor in oleic acid samples destined for human consumption. The pres-

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ence of this highly poisonous substance in human food products immediately resulted in a regulation from the Food and Drug Administration specifying that all such products must be free of "chick edema factor" in order to be incorporated in food. From the point of view of the regulated industry and the regulatory agency, the need to develop an assay method of adequate specificity and sensitivity, the results of which are comparable from laboratory to laboratory, can hardly be overemphasized. Since the chemical characterization of the substance has not proceeded far enough to provide the basis for a chemical or physical assay, it is necessary to use the bioassay. This method has successfully proved itself capable of providing for the detection and semiquantitative estimation of small amounts of this factor.

When the need for the standardization of a method became acute, investigators in eight laboratories that are currently engaged in some phase of work on the chick edema factor were invited to participate with us in a collaborative study and to submit suggested procedures. All graciously responded, and from the procedures which they forwarded, the authors selected what they considered the most desirable features of each and combined them into the procedure presented here.

Three collaborative test samples were prepared by thoroughly mixing a fat, known to be toxic, with cottonseed oil in such proportions that the amount of toxic fat in the samples was in the ratio of 1:2:4.

Each of the collaborators was sent the procedure given below as well as four collaborative test samples identified by number. Sample 1 was USP cottonseed oil; Samples 2, 3, and 4 contained, respectively, 1, 2, and 4 grams of toxic fat in 16 grams of sample. Report sheets suitable for recording the following specific information were also sent: chick number, initial body weight, final body weight, weight gain, heart fluid volume, presence or absence of peritoneal and subcutaneous fluid, and dates of early deaths.

Noteworthy features of the assay procedure are the use of white leghorn cockerels as the assay animal; a semi-synthetic ration in which the test samples are contained at 16%; and a 21 day feeding period. We included a request that groups be assigned to provide for observations to be made at the 14th day as well. The collaborators were asked to score the results visually, by use of a suggested scale of values, as well as by measurement of the pericardial fluid volume.

METHOD

Reagents

(a) *Salt mixture.*—

	G/60 G MIXT.
$CaCO_3$	15
$Ca_3(PO_4)_2$	14
K_2HPO_4	9
NaCl	8.5*
Na_2HPO_4	7.3
$MgSO_4.7H_2O$	4.86
$MnSO_4.4H_2O$	0.42
Fe citrate	0.4
$ZnCO_3$	0.2
$CuSO_4.5H_2O$	0.02
$Na_2SeO_3.5H_2O$	1.2 mg

(b) *Vitamin mixture.*—

	AMOUNT, G
Folic acid (1.0% triturated in powd. glucose)	10
Biotin (0.1% triturated in powd. glucose)	7.5
Vitamin B_{12} (0.1% triturated in powd. glucose)	2.5
Niacin	2.0
Ca pantothenate	0.50
Thiamine	0.50
Riboflavin	0.375
Pyridoxine·HCl	0.200
Menadione	0.025
Celluflour (Alphacel), to make	500

(c) *Fat-soluble vitamin mixture.*—

	AMOUNT/KG MIXT.	AMOUNT/KG DIET
Vitamin A acetate, cryst.	900,000 USP Units	9,000 USP Units
Vitamin D_3, cryst.	100,000 IC Units	1,000 IC Units
D-α-Tocopheryl acetate	2.00 g	20 mg
Corn oil, to make	1.00 kg	10 g

Table 1. Summary data of the collaborative assay for chick edema factor

Coll.	Diet	Assay Period (Days)	No. of Chicks	No. of Early Deaths	Average Weight Gain	Average Hydropericardium			Edema Incidence		
					Total (g)	Visual Score	Fluid Volume (ml)	Score From Volume	Ascites	Subcutaneous	Hydropericardium*
1	1	14	12	0	39.5 ± 3.2	0	0.054 ± .004	0	0/12	0/12	0/12
	1	21	12	2	73.0 ± 4.3	1+	0.12 ± .025	1+	1/12	0/12	1/12
	2	14	12	1	52.8 ± 4.3	0	0.11 ± .009	0	0/12	0/12	0/12
	2	21	12	1	78.2 ± 4.9	12+	0.26 ± .039	9+	0/12	0/12	7/12
	3	14	12	0	47.0 ± 3.2	7+	0.23 ± .09	3+	1/12	1/12	1/12
	3	21	12	2	69.4 ± 4.6	20+	0.92 ± .29	21+	3/12	2/12	10/12
	4	14	12	3	20.2 ± 3.3	20+	0.71 ± .25	15+	1/12	2/12	6/12
	4	21	12	5	39.4 ± 5.1	27+	1.58 ± .72	22+	6/12	3/12	10/12
2	1	14	6	1	26.2 ± 6.7	1+	0.028 ± .013	0	0/5	0/5	0/5
	1	21	5	0	59.6 ± 12.6	0	0.072 ± .013	0	0/5	0/5	0/5
	2	14	7	1	32.7 ± 9.2	9+	0.095 ± .021	0	0/6	0/6	0/6
	2	21	5	0	70.0 ± 4.3	10+	0.59 ± .24	7+	0/5	0/5	4/5
	3	14	6	0	39.8 ± 2.9	7+	0.155 ± .079	2+	1/6	1/6	1/6
	3	21	5	1	63.2 ± 4.7	15+	1.43 ± .49	14+	2/5	1/5	5/5
	4	14	7	3	39.3 ± 7.6	20+	2.05 ± .78	16+	5/7	6/7	5/7
	4	21	5	4	57.0 ± 9.6	15+	1.80 ± .66	15+	4/5	3/5	5/5
3	1	14	12	0	46.1 ± 2.0	0	0.023 ± .001	0	0/12	0/12	0/12
	1	21	12	0	104.1 ± 3.3	0	0.085 ± .016	0	0/12	0/12	0/12
	2	14	12	0	51.4 ± 2.5	4+	0.101 ± .018	1+	0/12	0/12	1/12
	2	21	12	0	118.2 ± 6.3	3+	0.145 ± .036	4+	0/12	0/12	3/12
	3	14	12	0	48.7 ± 2.6	12+	0.39 ± .13	9+	2/12	6/12	4/12
	3	21	12	1	89.5 ± 4.9	7+	0.28 ± .12	7+	1/12	8/12	5/12
	4	14	12	0	45.4 ± 3.3	14+	0.54 ± .21	14+	3/12	4/12	6/12
	4	21	12	1	86.1 ± 4.4	23+	1.46 ± .45	25+	8/12	9/12	9/12
5	1	14	12	0	42.3 ± 1.6	0	0.059 ± .008	0	0/12	0/12	0/12
	1	21	12	0	91.3 ± 8.9	0	0.081 ± .034	0	0/12	0/12	0/12
	2	14	12	0	43.5 ± 2.9	0	0.127 ± .006	3+	0/12	0/12	3/12
	2	21	12	2	41.8 ± 5.1	10+	0.306 ± .052	10+	1/12	1/12	8/12
	3	14	12	0	28.1 ± 2.1	1+	0.102 ± .021	2+	0/12	2/12	2/12
	3	21	12	4	35.0 ± 1.4	16+	0.66 ± .29	17+	10/12	8/12	11/12
	4	14	12	0	22.3 ± 1.8	11+	0.37 ± .17	9+	1/12	5/12	6/12
	4	21	12	8	32.3 ± 5.1	19+	0.80 ± .25	20+	8/12	9/12	9/12
7	1	14	12	0	22.6 ± 5.9	0	0.05 ± 0	0	0/12	0/12	0/12
	1	21	12	0	33.4 ± 10.0	0	0.054 ± .004	0	0/12	0/12	0/12
	2	14	12	0	11.2 ± 6.3	0	0.058 ± .005	0	0/12	0/12	0/12
	2	21	12	1	16.2 ± 7.2	8+	0.196 ± .093	3+	1/12	4/12	1/12
	3	14	12	2	25.4 ± 5.2	4+	0.166 ± .078	2+	1/12	2/12	1/12
	3	21	12	2	35.9 ± 10.3	10+	0.427 ± .203	9+	1/12	2/12	4/12
	4	14	12	1	12.4 ± 7.2	10+	0.391 ± .14	9+	3/12	5/12	5/12
	4	21	12	9	22.0 ± 15.6	6+	0.204 ± .078	6+	3/12	6/12	5/12
8	1	14	12	0	42.9 ± 3.8	0	0.061 ± .015	0	0/12	0/12	0/12
	1	21	12	0	100.8 ± 7.3	3+	0.093 ± .013	0	0/12	0/12	0/12
	2	14	12	0	54.5 ± 3.2	9+	0.125 ± .019	2+	0/12	0/12	2/12
	2	21	12	1	78.1 ± 4.7	15+	0.183 ± .023	4+	0/12	2/12	4/12

(*Continued*)

(d) *Assay Ration.*—

	%(w/w)
Sucrose, commercial	20.3
Corn starch, commercial	20.3
Casein (vitamin-free)	20
Fat (USP cottonseed oil or assay fat)	16
Gelatin	13
Salt mixt., (a)	6
Vitamin mixt., (b)	2
Salt, iodized	1.2
Fat-sol. vitamin mixt., (c)	1
Choline chloride, 25% aq. soln	0.2

Treatment of Experimental Animals

Use day-old white leghorn, single comb cockerels. On day of receipt of chicks, tag individually, record body weights, and place in brooder cages equipped with heater. Use room with controlled temp. and humidity.

Offer control ration contg 16% cottonseed oil as fat and H_2O *ad libitum*. After 48 hr, weigh chicks and do not use any chick which is outside limit of mean body wt by ±5 g. Place 12 chicks in each brooder cage and record body wt and date of beginning feeding regimen.

Assay Period

Continue 1 group on control ration contg 16% cottonseed oil and substitute test fats for all or part of the 16% cottonseed oil in assay rations. Check chicks daily for fatalities and for presence of adequate food and H_2O. Record all deaths and cause of deaths. For each death due to other than accidental cause, autopsy and record presence of hydropericardium, hydroperitoneum, subcutaneous edema, and amount of heart fluid to 0.01 ml. Autopsy remaining chicks on 21st day and record findings as above.

Postmortem Examination for Quantity of Heart Fluid

(a) *Sacrifice of chicks.*—Sacrifice by means of cervical dislocation and proceed as follows:

(b) *Exposure of heart.*—With dissecting scissors make small transverse cut in skin over full diam. of abdomen. Peel skin toward head and lay skin fold over head. This cutaneous incision permits wide field exposure of subcutaneous area over thoracic and abdominal cavities for examination for subcutaneous edema. Skin may be reflected caudally to afford wider field of vision. Record (+ or −) evidence of subcutaneous edema.

Insert blunt tip of scissors thru body wall and make transverse incision of musculature to lower rim of rib cage, avoiding cutting into organs of peritoneal cavity.

Lift breastbone with fingers, insert blunt tip of scissors, and carefully enlarge incision by cutting on each side of chest cavity thru rib joints up to clavicles. (Do not cut into subclavian vessels.) With sufficiently wide cut, fingers may be used to protract incised chest cavity and thus permit clear observation of substernal attachment of pericardium. Firmly clasp pericardial attachment with fingers and reflect flap of sternum so as to expose heart with pericardium intact.

Estimate visually and record severity of pericardial edema according to following table:

Pericardial Edema	*Score*
Absent	0
Slight	+
Moderate	++
Severe	+++
Very severe	++++

(c) *Withdrawal of heart fluid.*—*(1) For vols estimated as <1.0 ml.*—Firmly clasp apex of pericardium with small forceps and make small incision in heart sac. Insert small spatula into incision of pericardium and push heart to one side. Carefully aspirate fluid into 1.0 ml tuberculin syringe. Blunt-end 18 gauge needle permits more complete aspiration of small vols. To reduce formation of air bubbles during aspiration, prerinse needle and syringe with *n*-butyl alcohol. Do not include vol. of *n*-butyl alcohol in measurement of fluid.

(2) For vols estimated as >1.0 ml.—Insert sharp hypodermic needle on 10 ml syringe into intact pericardium. Aspirate as much as possible of the heart fluid (*n*-butyl alcohol rinse *is unnecessary*). Collect and measure remainder with blunt-end tuberculin syringe. Record total vol. heart fluid.

(d) *Other observations.*—Observe and record other obvious changes such as peritoneal edema (ascites), liver changes, kidney changes, etc.

Results

Seven of the nine cooperating laboratories submitted results in time for inclusion in this report. Their observations are compiled in Table 1.

It is seen that mortality due to the toxic factor does not become appreciable until the third week on the diet containing the highest level of the toxic fat. Note that three of the

this value falls at the high end of the normal range of the volumes of pericardial fluid for chicks of the age and weight used in the bioassay.

From the observations reported by the collaborators it may be generalized that of the three anatomical sites in which fluid may accumulate, hydropericardium occurs with the greatest frequency, followed in order by peritoneal and subcutaneous accumulation. This is in agreement with earlier observations.

The appended charts (Fig. 1) show graphically the response of the volume of peri-

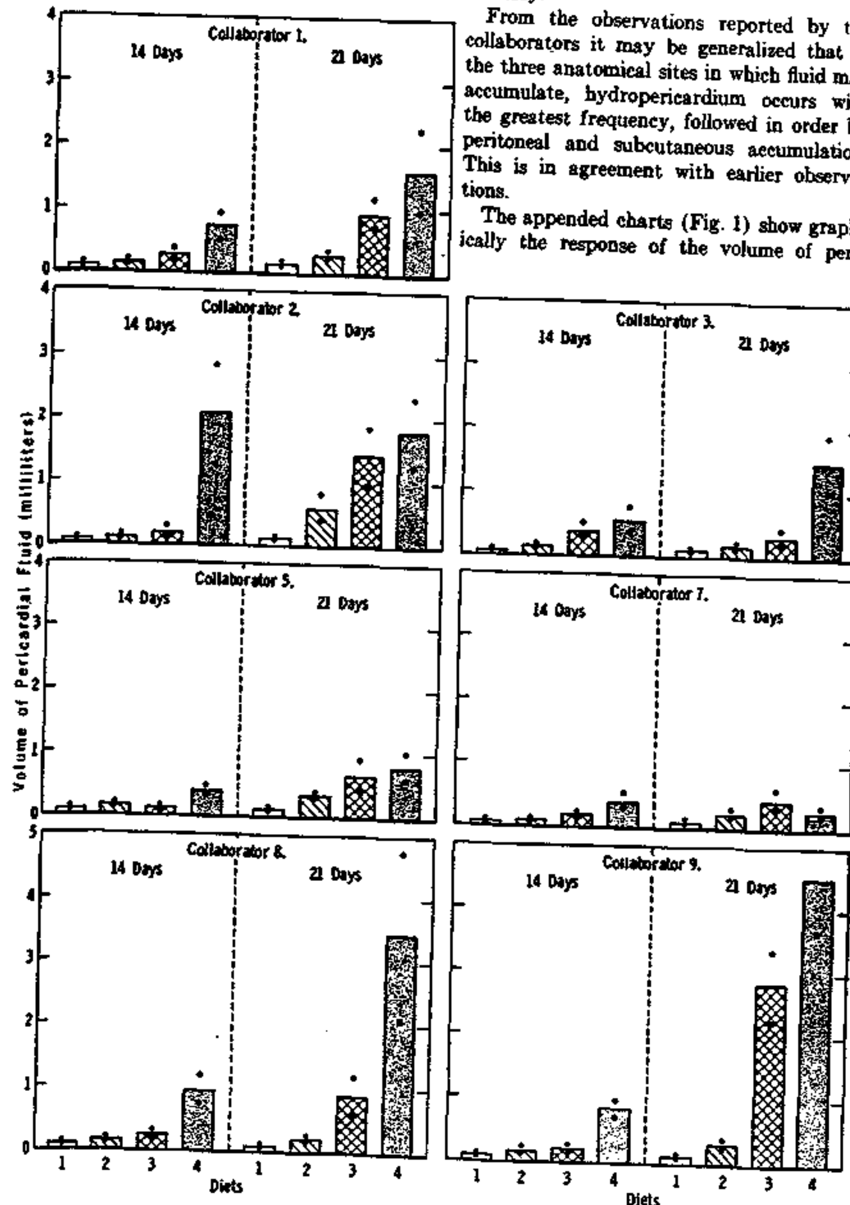

Fig. 1—Volume of pericardial fluid (± S.E. of the mean) versus level of toxic fat for 14 and 21 day feeding periods.

Table 1. *(Continued)*

Coll.	Diet	Assay Period (Days)	No. of Chicks	No. of Early Deaths	Average Weight Gain	Average Hydropericardium			Edema Incidence		
					Total (g)	Visual Score	Fluid Volume (ml)	Score From Volume	Ascites	Sub-cutaneous	Hydro-pericardium*
	3	14	12	0	45.1 ± 2.8	13+	0.203 ± .066	5+	1/12	1/12	4/12
	3	21	12	2	72.5 ± 8.3	28+	0.878 ± .339	17+	4/12	7/12	8/12
	4	14	12	1	48.3 ± 5.4	28+	0.903 ± .303	20+	5/12	8/12	8/12
	4	21	12	5	75.1 ± 6.4	26+	3.44 ± 1.40	19+	6/7	6/7	7/7
9	1	14	12	0	54.5 ± 3.3	0	0.09 ± .01	0	0/12	0/12	0/12
	1	21	12	1	104.4 ± 9.6	0	0.10 ± .015	0	0/12	0/12	0/12
	2	14	12	0	57.3 ± 5.6	1+	0.15 ± .07	2+	0/12	0/12	1/12
	2	21	12	0	108.0 + 7.1	10+	0.29 + .05	10+	0/12	0/12	0/12
	3	14	12	0	63.3 ± 3.0	4+	0.10 ± .045	5+	0/12	0/12	4/12
	3	21	12	1	104.0 ± 11.8	36+	2.86 ± .55	42+	9/12	5/12	12/12
	4	14	12	0	60.4 ± 4.8	21+	0.84 ± .24	22+	6/12	2/12	11/12
	4	21	12	5	89.6 ± 6.7	35+	4.52 ± .86	34+	11/11	9/11	11/11

* Hydropericardium incidence: based on measurement of pericardial fluid.

collaborators reported deaths of four birds on the control diet. Since these deaths could not have been due to the factor, this and the foregoing observation indicate that mortality measurement alone is not a reliable index of the presence of the chick edema factor. It has been found that signs of chick edema disease are the development of hydropericardium, hydroperitoneum, and subcutaneous edema, in that order, as the dose of toxic material is increased. At higher dosage levels, death will occur from the 10th day. Birds dying of chick edema disease invariably exhibit these signs. Deaths unaccompanied by any of these signs cannot be attributed to the toxic agent.

Weight gains of the chicks were somewhat depressed at the higher levels of toxic fat. In the control groups, the average weight gains at the end of the assay periods were quite variable among the laboratories. This variability may be due to borderline nutritional inadequacies of the basal diet that possibly were aggravated by hereditary or environmental factors or a combination of both, as may have happened in the case of Collaborator 7's group. Perhaps it would have been wise to follow the suggestion of certain of the collaborators and to have an antibiotic incorporated in the test ration.

Visual subjective scoring of the degree of hydropericardium according to the instructions given in the collaborative procedure is seen to agree fairly well with a score calculated from the actual measurement of pericardial fluid as shown in Table 2. The figures given in Table 1 represent the sum of the plus scores for individual chicks within the group. It appears that there was greater accuracy in visual scoring of large toxicity responses than in the smaller responses. While visual scoring may be adequate in judging the presence of a large amount of toxic material, it is inadequate in those borderline cases where controversy is most likely to develop.

We have arbitrarily adopted 0.2 ml as the upper limit of normal heart-sac fluid volume. This choice is justified on the basis of the work of Shue and Gallo (7), who report that

Table 2. Scoring by Referee of pericardial edema

Volume of Pericardial Fluid	Toxicity Score
<0.20	0
0.21–0.40	+
0.41–1.00	++
1.01–2.00	+++
>2.01	++++

cardial fluid to the level of toxic fat in the diet. It is seen to vary directly with this level. It is likewise seen that an appreciably greater response to the dose is obtained after a three-week feeding period than after a two-week period. Although there is a high incidence of negative responses in a group of chicks on a low level of toxic fat, the average response of the group as measured by the volume of pericardial fluid is proportional to the dose level within the dose limits of this experiment.

Comments of Collaborators

Most of the comments were directed at the nutritional adequacy of the basal ration. One investigator has suggested that dry, stabilized vitamins A, D, and E be used rather than the crystalline products used in the procedure. This suggestion appears to be meritorious and has been incorporated in the recommended procedure as optional. Another objected to the mode of addition of choline to the diet and suggested a 25% dry, free-flowing preparation which is commercially available. For the sake of convenience, this has likewise been incorporated as optional. Objection was made by another collaborator to adding the water-soluble vitamins on Celluflour as the carrier. Since many laboratories routinely use other of the dietary ingredients as carriers for the vitamin mixture, we see no reason why any of the major components cannot be used for this purpose.

Weight gains obtained on the diet are not optimal. This, however, is not critical from the standpoint of the assay except, as one collaborator points out, that it is easier to carry out the procedure of withdrawing the heart-sac fluid from a larger bird than from a smaller one. Since the diet has given excellent pericardial fluid responses, major changes cannot be justified at this time.

Other collaborators have suggested that an antibiotic be incorporated in the ration, that crude casein be substituted for the vitamin-free casein, and that commercially available salt and vitamin mixtures be used. These appear to be desirable from the standpoints of economy and convenience and will form the basis of a modification of the diet to be used in further collaborative work. The level of sodium chloride was likewise the subject of question on the basis that 2% of salt is too high and subjects the chick to extra "stress." It has been found in our laboratory that this level favors the development of the edema. Selye and Stone (8) have found that extra sodium chloride in the diet of chicks accentuates the development of edema produced by certain steroids. Biester and Schwartz (9) state that 6–8 grams of sodium chloride per day is not harmful to 9 week-old chicks. We cannot see that a change here is justified.

One collaborator reported that the ration was unpalatable to his chicks. The weight gains reported by him were much lower than in any other laboratory. We have no explanation for this effect. It is suggested that this laboratory may have some peculiar problem and that the performance of these chicks is not typical.

Several of the collaborators preferred to use heavy breeds of chicks rather than leghorns. We have retained leghorns for the reasons of uniformity of response, widespread availability, convenience, and the demonstrated sensitivity of this breed.

Summary and Recommendations

A successful collaborative study of a procedure for the detection and assay of the chick edema factor in fats and fatty materials has been carried out. The results indicate that the method as studied is satisfactory for the intended purpose. Although it is demonstrated here that the method studied is capable of differentiating contaminated from uncontaminated fats, it would be desirable to carry out additional collaborative studies on samples of lower potencies than those used here.

It is recommended[1]—

(1) That the method for bioassay of chick edema factor, presented in this report, be adopted as first action.

(2) That collaborative studies be continued.

References

(1) Editor's Note, *This Journal*, 42, 120 (1959)

[1] These recommendations were approved by the General Referee and by Subcommittee C, and were adopted by the Association. See *This Journal*, 44, 70 (1961).

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(8) Selye, H., and Stone, H., *Proc. Soc. Exptl. Biol. and Med.*, 52, 190 (1943).

(9) Biester, H. E., and Schwartz, L. H., *Diseases of Poultry*, 4th Ed., The Iowa State University Press, Ames. Iowa, p. 112.

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Progress in the Chick Edema Problem

By Dr. Leo Friedman, Food and Drug Administration

It has been almost four years since I first became aware of the problem that we know today as "chick edema disease." Because of our activity on this problem, my colleagues and I have had the opportunity to become acquainted with many scientific groups and individuals working in the same area with whom we have enjoyed a fruitful cooperation and pleasurable association. We hope that they feel as kindly toward us as we do to them, but sometimes, I known, they wish as we do that they had never heard of chick edema disease.

This problem has been most difficult and progress frustratingly slow. Despite the meager amount of new information that can be added at this time, especially since the most recent advances were reported by Dr. Artman recently, it is nevertheless worthwhile to review the several aspects of this problem and see its present status in full perspective.

As you recall, during 1957 an epidemic disease caused millions of dollars in losses among broiler flocks throughout a large part of the U.S. After elimination in succession of all other possibilities, attention was focused on the fat ingredient of the feed as the etiologic agent. A series of reports in 1958 from several laboratories described the manifestations of the disease and definitely implicated a toxic fat or a toxic substance in fat as the cause. The characteristic symptoms were droopiness, ruffled feathers, labored breathing and high morbidity and mortality. Autopsy findings revealed hydropericardium, abdominal ascites (water belly), subcutaneous edema, swollen liver, swollen and pale kidneys, etc. In laying hens the toxic fat caused a rapid drop in egg production. Pullets receiving toxic fat during the full growing period did not come into production, and mortality was very high. Hydropericardium, the most common lesion found in young birds, was not found in birds of laying age.

Symptoms Different

These differences in susceptibility and symptoms in different age groups of the same species should be noted. The feeding of toxic fat to other species has not produced such striking results as with young chicks, with the exception possibly of monkeys. However, every species that has been tested has shown evidence of deleterious effects. Very little work has been done with rats. Our very limited experience indicates that they are much more resistant than chicks in short-term feedings, but that when fed in sufficient dosage, extracts of the toxic fat produce definite deleterious effects as shown by growth depression, enlarged and fatty livers, and marked involution of the thymus.

I recall few reports of the effects of toxic fat on swine, but again, in our own limited experience we have seen depressed growth . . . and have demonstrated the presence of toxic factors in the meat of hogs that had been fed toxic fat.

I am indebted to Dr. Wilcke of Ralston Purina for reports of studies on guinea pigs and dogs. Guinea pigs fed 2½% toxic fat stopped growing after six weeks, and death losses occurred after eight weeks. At a level of 4½% toxic fat weight losses occurred after three weeks and deaths after four weeks. Control groups receiving non-toxic fats did not show weight loss or deaths. The only observed pathology at the conclusion of the experiment was congestion of the lungs and mottled livers.

In experiments with three different breeds of dogs, using Purina Dog Chow in which 10% of toxic fat was substituted for the usual normal fat, there was poor reproduction and lactation performance. The females on the toxic fat ration whelped pups that were dead or weak, and, furthermore, the mothers seemed to have an insufficient milk supply. When the pups were removed before weaning and fed a normal ration the increase in growth was immediate and dramatic. Also, the females on the toxic fat ration tended to lose hair on their backs and shoulders. With the ration containing toxic fat, post-weaning growth tests (6–18 weeks) with five litters of pups demonstrated inferior growth performance using either weight gain or increases in body length as the criterion.

Work with Other Species

In these experiments with other species, fat that had first been proved to be toxic to chicks had been used. In the case of monkeys, a sample of triolein

that had produced irreversible toxic symptoms for no apparent reason later was proved toxic to chicks and was the source from which we isolated a highly purified crystalline "chick edema" factor. At the present time, then, "toxic fat" that produces "chick edema disease" has been demonstrated also to produce deleterious effects in rats, guinea pigs, swine, dogs and monkeys. It should be emphasized that the toxic fat undoubtedly contains many other substances that may have effects in these other species. Definitive information as to the effect of "chick edema factor" (CEF) in other species must await tests with purified CEF. Triolein-fed monkeys probably received the "purest" source of CEF. However, purified CEF should be administered to monkeys to verify the implication that the severe toxic symptoms observed with triolein were due to CEF.

Relatively little has been done to throw light on the mechanism of the toxic effect. Flick and Gallo in our laboratories have reported that in young chicks showing symptoms of the disease, the intra-cellular water was not changed. Neither were the total blood and plasma volumes altered so that the observed edema was primarily interstitial. Hemoglobin and hematocrit levels were low and blood glucose levels were decreased in advanced stages of the disease. Plasma sodium, potassium and chloride were not affected. In the liver, neutral fat was decreased and phospholipid increased. Preliminary experiments by Flick give some indication of increased membrane permeability, but these must be repeated under much more rigorous conditions.

DISSERTATION ABSTRACT

A most interesting study in chicks has been reported by J. R. Allen, Jr. Part of the results were presented at the federation meetings in March, 1961, and the complete study is available as the dissertation of J. R. Allen, Jr., University of Wisconsin, 1961. I will quote portions from the dissertation abstract [Dissertation Abstracts 22, (2), 545 (Aug., 1961)]:

"Experiments conducted to determine the effects of 'toxic fat' on mice, pigeons and turkeys demonstrated a reduction in growth without hydropericardium or ascites."

In chicks, "Microscopic examination of the tissues of the test animals revealed lymphocytic foci in the epicardium and myocardium. Edematous fluid separated the myocardial fibers. Edema of the lungs was a frequent observation in the experimental birds.

". . . Blood pressure indicated the test birds had an elevated average mean pressure in the right ventricle of 6 cm. water and 2 cm. water in the vena cava. Electron micrographs of the myocardium revealed shrunken, vacuolated mitochondria in the test animals.

"'Toxic fat' produces a reduction in growth rate of experimental animals. This reduction depends on the age of the animal and the level of 'toxic fat' added to the diet. Hydropericardium and ascites are a frequent lesion in the animals receiving from 1.0 to 5.0% 'toxic fat.' When this level was reduced to 0.25% in the diet, reduced testicular development was a more sensitive criterion than hydropericardium, ascites or weight gain for evaluating chronic toxicity.

"The mechanism by which 'toxic fat' induces hydropericardium and ascites appears to be associated with degeneration and edema of the myocardial fibers. These data would tend to eliminate the kidneys, liver and endocrines as the primary cause of edema. The early development of hydropericardium, increased venous pressure, enlarged hearts, mitochondrial changes in the myocardium and generalized edema suggest that the myocardium may be directly inhibited; however, altered capillary permeability has not been excluded. It is believed that cardiac decompensation and increased capillary permeability act together in producing the excessive extravascular fluid collection and the demise of the animal."

The wide range of susceptibility within and among species and the variety of toxic effects that have already been noted would make it appear logical that some primary unit of structure and function such as the mitochondrion, may be the target of the toxic factor and that the observed differences may be explained by factors such as absorption, specific binding, transport, detoxication, etc., that determine the local concentration of any substance in a specific site.

Another possible mechanism that had suggested itself quite early is that the toxic factor interferes with the normal regulation of electrolyte and water balance. Selye and Stone, back in 1943, described the production of edema symptoms in chicks by certain steroids and the accentuation of these symptoms by increasing the salt intake of the chicks. Alexander has shown that it is possible to produce hydropericardium in chicks by increasing the NaCl in the ration and to prevent its occurrence even with CEF by eliminating NaCl from the diet. Work in our own and other laboratories has illustrated beautifully the interaction of nutritional factors on the susceptibility of the chicks to a toxic agent. For example, the present AOAC bioassay diet for "chick edema factor" is probably four times more sensitive than the assay diet we used originally, although both have approximately the same NaCl content.

A point to remember is that hydropericardium as a symptom of toxicity in chicks is not new. In addition to NaCl and certain steroid hormones, chapter 40 in Biester and Schwarte on *Poisons and Toxins* indicates that:

(1) Zinc phosphide, used as a rodenticide produces "various degrees of congestion with the accumulation of some serous fluid in the pericardial sac as well as in the abdominal cavity in some cases."

(2) Alpha naphthyl thiourea, the rodenticide ANTU, shows in poisoned chicks evidence of lung edema and excessive quantity of fluid in the pericardial sac.

(3) Sodium monofluoroacetate, compound 1080, another very effective rodenticide, produces in chicks distention of the pericardial sac with clear straw-colored fluid, in addition to other marked pathological changes on the heart and lungs.

(4) Chlordane. "The primary lesions found in all fatal cases were in the heart. Excessive quantities of fluid were found in the pericardial sacs. . . ."

The weed "corn cockle" and several species of Crotalaria produce seeds which are toxic, and in chicks the toxic symptoms include hydropericardium.

However, in each case other characteristic pathology is usually present, and in no case is the purified toxic principle of the same high order of activity as the toxic substances that have been isolated from toxic fats.

It is well known now that chicks can efficiently utilize large amounts of fat in properly balanced rations. The use of fat as a standard ingredient of poultry feeds grew as the price of fat calories dropped and became competitive with calories derived from corn. The chick edema disease epidemic of 1957 was a totally unexpected consequence of this growing practice. Many of you are familiar with the story of how the toxicity was associated with a fatty by-product of stearic and oleic acid manufacture that had been blended with feed grade fats.

Very large quantities of fatty acids are used industrially in the manufacture of lubricants, rubber, paints, asphalts, roofing, chemicals, and to a much smaller extent in foods. Relatively low grades of fat are split into fatty acids and glycerol at high temperatures and pressures, sometimes with the aid of catalysts. The glycerol is recovered and the fatty acids are distilled under vacuum. The first distillate may be used directly as the highest grade of mixed fatty acids or it may be separated by a low temperature crystallization process into stearic (saturated) and oleic (unsaturated) fractions. The residue from this distillation is resplit and redistilled. The second distillate yields a lower grade of fatty acids. The residue from the second distillation is usually suitable for use on highways or in rubber manufacturing, but occasionally it is again recycled to obtain a third distillate and another residue. It was this third residue that had been blended with feed grade fat for use in feeds.

Every sample of residue of this type from several manufacturers of fatty acids proved to be rich in chick edema toxicity. Our first impression, therefore, was that the toxic factor was produced during the splitting and distillation steps and that it was concentrated in the residue along with other non-volatile unsaponifiable substances. Closer study of the various stages of fatty acid production soon revealed that the toxic factor was distillable and was present to some extent in the first distillates which were used for the production of the best grades of fatty acids, and those intended for food purposes. This discovery was made independently and reported by Ames, et al., who had found several samples of oleic acid and a monoglyceride made from such an oleic acid to be contaminated.

All this happened just after the passage of the Food Additives Amendment. The fatty acid manufacturing firms were very cooperative in providing

with information and samples. However, they sincerely believed that they were not part of the food business, that the bulk of their production went for non-food industrial purposes. In our visits and talks with their technical people we advised them to study the application of the new legislation to their industry. They realized that a substantial proportion of their highest grade of materials did enter food channels when their customers started asking for guarantees that the fatty acids met the requirements of the Federal Food and Drug Law. At that point the technical committee of the Fatty Acid Producers Council became actively engaged in a study of this problem, and we have enjoyed the whole-hearted cooperation of the fatty acid industry. While they made studies to determine what part of their processes were responsible for the production of the toxic material, studies were continuing on the isolation and chemical characterization of the active substances. Three years ago at the AOAC meeting a symposium on chick edema was held at which reports from the Purina laboratories, the Quaker Oats laboratory, the Procter & Gamble research laboratories and the Food and Drug laboratories described the progress made up to that time. The Merck group had recently entered the problem, and, although they did not report, they also had traveled a similar road.

Every step and experiment had to be followed by the chick bioassay. However, during the first year all the groups had made steady progress at about the same rate. First it was demonstrated that the "chick edema" toxicity was entirely in the unsaponifiable portion of the fat. Then, in our work, the unsaponifiable was separated by chromatography on alumina into three fractions of increasing polarity by elution with petroleum ether alone, then with a mixture of ethyl and petroleum ether and finally with 100% ethyl ether. The first, or hydrocarbon, fraction contained 83% of the unsaponifiable, and its ultra-violet absorption spectrum indicated the presence of cholestadiene. The second fraction was characterized as ketonic and infrared absorption spectra suggested the presence of dipalmitone. The third fraction consisted of sterols and oxidized materials. Simultaneous bioassay of these three fractions and synthetically prepared samples of cholestadiene and dipalmitone showed that only fraction 2, but not the dipalmitone, was toxic.

Several approaches were tried simultaneously to purify fraction 2. I will not take the time to describe our experiments with a 500 tube counter-current distribution between iso-octane and methanol, or the separation of carbonyls with Girard-T reagent or the consecutive rechromatography on alumina and silane treated Celite. Suffice it to say that as our fractions became more potent we relied more and more on ultra-violet spectrophotometry as an indication of concentration and purity. Two distinct types of fractions were obtained, one with the ultra-violet spectra characteristic of naphthalene compounds and the other of phenanthrene compounds. Both fractions contained the toxic factor. However, the sum of their toxicities was not comparable to that of the starting material in the final purification steps despite the fact that practically all of it was accounted for by weight in the eluates. Since only the fractions that had a significant weight had been tested biologically, the insignificant, hardly visible residue of less than 0.5 mg. in the practically "empty" beaker that represented the cut between the two major fractions was rinsed into a chick diet and to our surprise was very active at approximately 0.1 ppm., representing the most potent material we had obtained. At this stage we had practically exhausted our raw material, and we had to start from the beginning once again.

During the course of these studies we had learned that Drs. Portman and Andrus in the department of nutrition at the Harvard School of Public Health had lost a number of monkeys in a nutritional study. They had used a synthetic triolein as the source of fat and had to abandon the experiment. At a Gordon Research Conference I had the opportunity to discuss this experience with Dr. Portman. Fortunately about 40 lb. of the triolein was still available. It proved to be toxic in the chick edema assay. The triolein was of excellent quality, containing only 0.9% of unsaponifiable material. This unsaponifiable proved to be the richest source of the toxic material we had ever examined.

The toxic fraction was separated from the triglyceride by molecular distillation. The distillate from 17.6 Kg. of triolein was saponified, and the unsaponifiable was chromatographed on alumina to remove the cholestadiene fraction. The naphthalene and phenanthrene-containing cuts were collected, yielding 670 mg. of material. This fraction was rechromatographed on more retentive alumina with 5% ether in petroleum ether, and 240 mg. of naphthalene-phenanthrene material was separated. This was rechromatographed in the same system and three distinct types of U.V. spectra began to emerge in the fractions: Naphthalene (235 mμ), phenanthrene (260 mμ) and a new peak at 245 mμ. These materials were combined and chromatographed on a silane treated Celite-iso-octane column, with 80% alcohol saturated with iso-octane as the mobile phase. The cuts with maximum absorbence at 245 mμ were further purified by two more chromatographic treatments on alumina at a very high sample: adsorbent ratio (1:2,000 and 1:25,000). The final fraction was completely free of absorption peaks near 235 and 260 mμ. This fraction was evaporated to dryness, dissolved in a small volume of boiling iso-octane and stored overnight in the refrigerator. White crystals were obtained, weighing 2.6 mg. and representing a concentration over the original triolein of three million-fold. This material at 1 ppm. in the diet killed the chicks in 12 days and produced typical hydropericardium when fed at .05 ppm. in a 21-day test.

During the last stages of this work the Merck group [Harmon, et al., JACS 82, 2078 (1960)], announced the isolation of a crystalline chick edema factor from a toxic fat. On July 19, 1960, the Merck group informed us that they had found 47% chlorine in their crystalline material. The presence of chlorine in our material was quickly confirmed by the use of the Dohrmann microcoulometric gas chromatograph.

In this test, a few micrograms of sample is injected into a gas chromatograph and the components as they emerge are pyrolyzed at 800° under oxidizing conditions. The halogen acid formed from halogenated materials is titrated automatically in a microcoulometer.

Of interest also is the isolation of a crystalline material that had an ultra-violet absorption spectrum identical with that of the toxic material but shifted 3 mμ so that the major peak was at 248 mμ instead of 245 mμ. It behaved like the toxic substance in the microcoulometric gas chromatograph, showing a similar retention time and halogen content. However, it was completely inactive in the chick edema test when fed at 1 ppm. in the diet.

The finding of chlorine in the toxic substance was a major breakthrough. It was no longer necessary to think in terms of a naturally occurring material that had changed chemically under the conditions of industrial fatty acid production. The possibility of contamination with one of many familiar chlorinated hydrocarbons was obvious. Samples of tetrachloronaphthalene and hexachloronaphthalene, kindly provided by Engel and Bell of Virginia Polytechnic Institute, who had demonstrated that these compounds produced hyperkeratosis in cattle, were without effect in the chick edema test. We have tested a long list of chlorinated compounds including aldrin, dieldrin, lindane, DDT, DDE, BHC, chlordane, toxaphene, methoxychlor, and a series of Halowaxes, without any definitely positive indication. Furthermore these have all been heated with oleic acid at 250° C for long periods and then fed to chicks, with negative results. Also, on the theory that tallow may sometimes be bleached with active chlorine materials which may chlorinate a sterol nucleus, we have chlorinated cholesterol, squalene, estone and equilenin with negative results. To test the theory that the toxic substance is a metabolite of a chlorinated insecticide we have fed large doses of chlordane, methoxychlor, heptachlor, aldrin and dieldrin to rats for a month and are feeding the unsaponifiable extract of the rat carcass in the chick test. The results to date show that chlordane and methoxychlor produce no response.

INDUSTRIAL EXPERIMENTS

Similar experiments have been carried out and are still in progress by at least two industrial laboratories under conditions of fatty acid production. The results so far have been largely negative or at best only suggestive but not clear cut. Dr. Artman has reported that chlorination of naphthalene and phenanthrene by substitution reactions has produced CEF active products. The chlorinated phenanthrene is particularly promising, since preliminary fractionation experiments indicated the possibility that a highly toxic compound was produced.

Dr. Boyd O'Dell and colleagues at the University of Missouri observed hydropericardium and other symptoms of chick edema disease in chicks housed in freshly painted cages. They traced the responsible agent down to one of the paint ingredients, a chlorinated biphenyl sold under the name

Arochlors of different chlorine content. Some were not toxic; others produce the disease but only at relatively high feeding levels, e.g., 200 ppm. It may be that an impurity in these compounds is the toxic agent, or that they are innately toxic at the high levels fed. Of interest is the use of one of these Arochlors in some insecticide formulations. At the present time there has been no real evidence developed to implicate any product or compound. However, the circumstantial evidence is stimulating considerable speculation and activity.

The feed industry has managed by careful control of ingredients to avoid a recurrence of the 1957 epidemic. The color test developed by Brew, et al., at Purina has been very useful in screening out toxic fats. This test is not specific but is useful since the presence of large amount of steroidal compounds that give this test usually indicates still residue that may be toxic. It is useless, however, for fatty acids and other products such as triolein, monogylcerides, etc., that give no response in this test, but which sometimes are quite toxic.

GEORGIA OUTBREAK

Last year, at this time, an outbreak of the chick edema disease occurred in Georgia. Although considerable chlordane residues were found in the feed, we do not believe they were responsible for the symptoms observed. Furthermore, the best information we have indicates that only rendered fat was used in this feed and no product of the fatty acid industry was involved. With each new development the scope of the problem increases. First we were concerned only with still residues, then also with fatty acid distillates and their derivatives. For a long time it was felt that only the fat derived from animals was involved. Recent evidence from sources in the fatty acid industry and our own studies indicates that some vegetable fat sources may yield fatty acids contaminated with CEF.

Still another development has occurred to further complicate the picture. Early this year in the course of our regulatory activities, we examined a sample of oleic acid. All the test chicks died by the end of the second week, with symptoms of severely stunted growth, ascites, jaundice and pathology of the liver and other organs, but with no hydropericardium. This sample had been tested by the manufacturer last year before the adoption of the present AOAC test procedure. The testing laboratory had used the procedure we ourselves had used in our earlier work, and had found the sample negative for chick edema. Repeat of the test in both laboratories by both procedures confirmed both findings. The sample is free of chick edema disease factor when tested on a diet of natural ingredients, and the chicks survive in apparent good health. On the casein-sucrose diet of the AOAC chick edema test, the chicks fail to grow and die early with the described symptoms but no hydropericardium. Furthermore, the dose response curve for this effect is very steep, since the ratio of the dose that gives a maximum effect to the dose that produces a minimum effect is less than two, as compared to a ratio of four to five for chick edema factor. Evidence from preliminary fractionation studies also indicates that this is an entirely different substance. There is no other information as to its characteristics at this time. There is evidence that this toxic contamination has occurred in different places from time to time and in a variety of fatty acid samples and may occasionally occur together with CEF. Here again we must anticipate that this material may occur elsewhere independently of the fatty acid industry.

For whatever comfort we may derive, it should be noted that chick edema disease has been observed in England. In a letter to the editor of the Veterinary Record of June 10, 1961, C. C. Wannop of the Houghton Poultry Research Station draws attention to a condition apparently identical with that reported by Sanger, et al., and Schmittle, et al., in 1958, that has appeared in several broiler flocks. In a personal note dated Sept. 29, he says that the condition has disappeared for the time in his country.

At the present time fatty acids can be used in the manufacture of foods or food ingredients only if they are free from CEF. This requirement made necessary the development of a bioassay which has been accomplished by collaborative work and is now being adopted as official by the AOAC.

I have tried to review the history of this troublesome problem, what little is known of its toxicology and its physiological aspects, the speculations as to the origin of the contamination, and attempts to track down its sources. I have sketched quickly our own attempts at isolation and identification of the toxic factor and have alluded to the most recent developments along this line that were reported at the AOAC Section of Fats and Oils by Dr. Artman.

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Occupational Intoxication Occurring in the Production of Chlorophenol Compounds by H. Bauer, K. H. Shulz and U. Spiegelberg, Hamburg

May 20, 1961

INTRODUCTION, GENERAL

In the last few years, professional work in the production of chlorinated phenols has been the cause of group outbreaks ...

German chemical works, the acne persisting over a long period and being associated with other effects on the health.

Diseases in a group of 17 workers from a company in North Rhine/Westphalia were reported by BAADER and BAUER, as well as by BRINKMANN in 1950/51. The workers in that company were engaged in the production of pentachlorophenol. Apart from comedone acne with various degrees of secondary pustular infection and boils, most of the workers, whilst still in the first stages of the skin diseases, also experienced pain and weakness in the lower limbs, mild paraesthesia, heart complaints and indeterminate psycho-vegetative disturbances. Subsequent examination of the records of 17 cases[1] revealed the following findings:

All 17 were suffering from an acne, 4 of these being very severe, 8 fairly severe, and 5 moderately severe to mild. In almost all cases there were extensive pustular infections and boils, 4 with bursitis on the elbow.

Other disturbances amongst the workers included 11 cases of bronchitis, 5 of myocardiac damage, 2 of cirrhosis of the liver (one of which proved fatal), 9 of neuritis symptoms (severe pains in the lower extremities in 7 patients, sensibility disturbances in 4 cases, mild paresis without atrophy in 2 patients, and 2 cases of weakening of the Achilles' reflex). Seven workers complained of physical conditions such as continuous fatigue, depression, lack of vitality, nervousness, slight headaches, disturbed sleep, and decrease in libido and potency.

A larger number (about 60 cases, Prof. Hergt) of similar conditions occurred in two Mid-Rhenish companies amongst workers who had been engaged for long periods, generally several years, in the production of trichlorophenol (saponification of 1,2,4,5,-tetrachlorobenzene to 2,4,5-trichlorophenol by treatment with methanolic caustic soda solution). These trichlorophenol workers, like those in a third group of affected persons from the Hamburg region who will subsequently be dealt with in more detail, suffered from further disturbances to health, these often not occurring until a fairly long time after occupational exposure had ceased. In the course of a discussion on a paper by SPIEGELBERG, who referred briefly to our Hamburg cases in a lecture at the 1960 North-West German Neurologists' and Psychiatrists' Congress in Lüneberg on psychopathological delayed and chronic damage following occupational intoxication. Janzarik described largely identical disturbances amongst workers from the Mid-Rhenish companies.

The third group comprised 31 workers in a Hamburg company. Those of this group who were affected were engaged in the trichlorophenol department of the company, in which the herbicide 2,4,5,-trichlorophenoxyacetic acid was manufactured from technical 2,4,5-trichlorophenol by heating trichlorophenol together with caustic soda solution and monochloroacetic acid in autoclaves. After completion of this esterification process, the end product was purified by double recrystallization. The task of the workers consisted first of all in charging the autoclaves, for which purpose the trichlorophenol in flake form had to be removed by shovel from open barrels. In this operation, a fine dust formed and dispersed throughout the room.

Other operations were concerned with filling and controlling centrifuges and regulating feed and outlet pipes. Since it was the workers most exposed to contact with trichlorophenol who suffered from the severest skin conditions, it was logical from the outset to suspect the causal noxa to be present in the trichlorophenol. The extent to which this assumption was valid is discussed later in this paper in connexion with etilogy.

SOME CLINICAL OBSERVATIONS

Of the 31 workers of this Hamburg company, 9 are still receiving medical attention 5 years after the termination of occupational exposure, this being due to residues of their acne, chronic neuromuscular weakness of the leg musculation, vaso-vegetative lability and, most especially, marked psychopathological disturbances. Details of the established complaints and damage to health are given in the table. The development of the skin conditions in the patients followed, by and large, a uniform pattern. Numerous comedones formed, first on the face, especially on the cheeks above the malar bones, forehead, temples, chin and ears, after which folliculitis, pustules, boils and retention cysts occurred as a result of secondary infections. As the disease progressed, these symptoms spread in the majority of patients, especially to the sides of the neck, back of the neck, upper half of the back, chest, forearms, genitals and thighs. Numerous boils formed, particularly on the back of the neck and on the back. The efflorescences were generally located so closely together that scarcely any follicles remained unchanged.

[1] Our thanks are due to the Berufsgenossenschaft der Chemischen Industrie for placing their records and other documents at our disposal, and also for their understanding in the sometimes lengthy clinical examinations.

In certain workers who had apparently been more strongly exposed, the development of these acne-like symptoms preceded a dermatitis associated with erythema and swelling, this extending to the region of the eyes, the cheeks and the forehead. At about the same time, blepharoconjunctivitis occurred in several patients, this, like the skin symptoms, becoming chronic in some cases.

As the table of findings shows, spots or, in certain cases, patches of pigmentation occurred in the faces of some patients, these giving the skin a dirty, greyish-brown appearance.

The overall clinical picture was identical to the symptoms occurring after working with chlorinated naphthalenes, diphenylenes and other aromatics as first described by HERXHEIMER (1899) and subsequently by several other authors (BETTMANN, HOLTZMANN, TELEKY, HERZBERG, BRAUN, GRIMMER, etc.). (For further details, see W. BRAUN and A. RISSE-SUNDERMANN (1959)). Although not a completely exact description, the designations "chloracne" and "perna disease" have become the most popular for these forms of occupational intoxication.

TABLE OF FINDINGS

Skin and mucous membranes: Dermatitis of the face in initial stage; comedones, retention cysts, nodules, pustules, boils; patches of pigmentation; blepharoconjunctivitis.

Internal organs: Loss of appetite; abdominal complaints; loss of weight; reduction in general condition; altered acidity of the gastric juice; gastritis; damage to liver; pulmonary emphysema, dyspnea; myocardiac damage; blood pressure; edema; pathological urine finding (renal damage).

Nervous system.—Neurological: Muscular pains; weakness in legs; (general) fatigue; increased sleep requirements; paresthesia; headaches; attacks of giddiness; orthostatic collapse tendency; paresis (implicit); coordination disturbances; hypaesthesia; reflex irregularities; vegetative hyperexcitability; EEG finding[1]; EMG finding.[2]

Nervous system.—Psychopathological: Decrease in initiative and interests; hyperaesthetic-emotional traits; pronounced fluctuations in intensity; disturbances in memory and concentration; disturbances in libido and potency; alcohol intolerance; depressions; decrease in impulsion; affective disturbances in the restricted sense of the term; experimental weakness in mental capacity; organic Rohrschach psychogram; individual neurotic traits.

n. = normal, abn. = abnormal, p. = pathological, n.s.p. = not definitely pathological, v.mU. = premature fatigue in the electromyographic series stimulus test.

The course of the dermatological manifestations proved to be extremely obstinate in our cases. The therapeutic measures employed (drainage of the comedones, external keratolytic and antibacterial measures, as well as the internal administration of antibiotics in severe cases) could not prevent the reformation of comedones, retention cysts and boils in the first year or two, although there was no further contact with the causal noxae. Only after a long time did the tendency to relapses cease. A residual condition now to be found, particularly amongst the serious cases, is closely arranged pitted scars which have a disfiguring effect, especially where localization occurs in the face (pseudo-atrophodermia vermiculata).

All affected workers reported pronounced fatigue and weakness in the legs, often with pain, especially in the region of the proximal leg musculation. These conditions were marked, even in the early stage of the disease, and in some cases even before the development of skin changes. Paraesthesia was reported in the records or in spontaneous information in only 2 of 9 cases.

[1] Our thanks are due to Doz. Dr. BOCHNIK and Dr. BUSCHART for conducting the electro-encephalographic investigations, and to Dr. PUFF and Dr. RUEDAS for the electro-myographic investigations.

Implicit paresis or atrophy, weakening of the reflexes, or absence of muscle expansion reflexes as a sign of toxic polyneuropathy was not established in any of the cases. Two of the patients examined indicated a decrease in sensibility with isolated epicritical disturbances in the lower limbs. No definite signs of neurogenic damage that could have been expected with peripheral nerve lesions were established electro-myographically; premature fatigue, which was recorded in the series stimulus test, requires further confirmation regarding both the method and the raised findings. As these findings show, the neuromuscular disturbances do not fit in with the typical picture of toxic polyneuritis or polyneuropathy.

The electroencephalographic examination produced an abnormal electroencephalogram in 6 cases, with frequency lability and dysrhythmic groups of a partly asymmetrical character. In one of the patients examined, accentuated dysrhythmia and raised cerebral excitability were revealed after photostimulation. The electroencephalographic changes found were uncharacteristic and afforded no diagnostic viewpoints of any real consequence.

Some of the workers examined complained of headaches, attacks of giddiness and orthostatic collapse tendency. In 5 of the 9 patients examined, there were distinct signs of vegetative hyperexcitability, fine tremor of the hands, increased perspiration on the hands and legs, axillary perspiration, raised dermagraphism and suggestions of Chvostek's sign. The blood pressure value measured during out-patient check-ups were all in the normal region, though at the lower limit of the norm in 5 of the 9 patients examined. Orthostatic collapse tendency was not established either during out-patient visits or during in-patient observation by an internist. In 2 cases, myocardiac damage was suspected.

Abdominal complaints such as a feeling of fullness, pressure in the stomach and liver region, and slight pain, were reported by 5 of the 9 patients. There were 4 reports of disturbances in the gastric secretions, 3 of subacidity, one of hyperexcitability and one radiographic finding of gastritis.

Very thorough investigations were conducted as part of repeated out-patient examinations and in-patient observation to ascertain any liver damage.[3] Whilst the liability reactions were uncharacteristic in all cases, the bromphthalein test indicated slight delay in the dyestuff excretion in 2 instances. In 3 cases, the liver biopsy produced pathological findings, these comprising 2 cases of slight perihepatitic changes and in one case a fatty liver with inflammatory symptoms and slight fibrosis of the liver. Owing to the clinical and histological findings, it was suspected that a condition following virus hepatitis existed in this instance. Deposits of ferrous and non-ferrous yellow-brown pigment were established in this case, although these did not correspond to the grey, non-ferrous pigment discovered by KALK and WILDHIRT in chlorophenol intoxication. The excretion of erythrocytes in the urine of one worker whose renal findings were otherwise normal remained unaccounted for.

The psychopathological changes in the chlorophenol workers who were all psychiatrically and psychopathologically examined were especially remarkable. In 6 cases the course could be observed over a 2-year period and there was an opportunity for objective anamnesis investigation and experimental psychological examinations.[4] With a very large degree of agreement, a subjective syndrome of complaints was reported by the patients under investigation, this syndrome extending from the psychoneuropathic complaints in the region of the extremities, cardiovascular and abdominal symptoms to the mental/spiritual sphere, especially in the modes of behaviour associated with the vital forces (BÜRGER-PRINZ). Considered in detail, there were reports of disturbances in the vital senses such as general sense of weakness, feeling of fatigue, indisposition, sense of insecurity, inner restlessness and a feeling of illness. The basic mental mood was reported to be deteriorated and lowered towards behaviour characterized by dissatisfaction or sullenness and irritation. Not infrequently, a mood component of fear and unease was present. Changes in affectivity in the restricted sense of the term were reported by the patients in the form of increased emotional reactions, irritability, tendency to fits of temper and also a certain hebetude.

[3] Prof. Dr. HORNBOSTEL and Dr. SCHONFELDER, I. Med. Univ.-Klinik, Hamburg-Eppendorf.

[4] Our thanks are due at this point to Dipl. Psychologist W. von SCHUBERT, for conducting the tests (Rorschach psychogram and Hamburg-Wechsler intelligence test for adults).

General loss of strength and reduced inner vitality and impulsion were symptoms noted in each of the cases observed. The probands described reduction in initiative and interests, weak willpower, reduced efficiency, and more rapid exhaustion in physical and mental/spiritual matters.

The subjective and objective anamnestic psychopathological picture is further complicated by a number of additional symptoms that are present with a greater or lesser degree of regularity. Disturbances of the instincts occurred in practically all cases. Thus, probands suffered from a sharp reduction in potency, and most also from decreased libido. The appetite had deteriorated and occasionally there were substantial fluctuations in weight. The sleep and sleep requirements of most probands were distributed in that there was an increase in disturbances to sleep itself and concurrently, but less often, to the process of falling asleep.

In certain cases, a decrease in mental capacity, especially disturbances of the memory and perception, were mentioned. In the majority of instances, alcohol intolerance was noted. Finally, mention should be made of hyperaesthetic traits with hypersensitivity to light and noise. Marked fluctuations of the psychopathological syndromes described were reported in nearly all cases.

Changes were described in the intensity of the symptoms, daily patterns of fluctuation occurring in favour of early morning, late morning or evening hours. In many probands, there were intervals of some days or weeks in which they were practically free from complaints.

For completeness' sake, a further two less common phenomena are described. Two probands stated that the general symptoms and the polyneuropathic symptoms were relieved for a varying length of time by the use of cold media (cold showers and washing with cold water). One proband reported abnormal, constantly changing eating habits, such as a wish for nothing but black bread, milk soup, or three litres of milk daily, there being no desire for food of other types at these times.

Compared with the multifarious polysymptomatic subjective pictures, the objective psychopathological signs can be recorded at less length. In exploratory conversations, the majority of the probands displayed a distinct, slightly depressed and subdued mood, which could be brightened only slightly or not at all. So far as impulsion was concerned, the patients examined gave an impression of lifelessness; their psychomotivity was feeble and fatigued. The impression was rather one of slight cerebral organic impulsion reduction than of inhibition. The affective modes of behaviour were occasionally notable for their reduced reactivity and oscillation capacity, though also because of lability and decompensability. In 2 cases, pronounced hypochondria and, in one case, slight but distinct alienation of the total personality were recorded.

The psychological tests are significant for the discovery of finer intellectual performance shortcomings and psycho-organic disturbances in affectivity. In the majority of the probands tested by the Hamburg-Wechsler intelligence test for adults (HAWIE), there was a significantly raised percentage of degeneration, this providing a certain indication of an acquired decrease in mental capacity. In the Rorschach psychogram, coartation of the experiential type, signs of weakened emotional reactivity, poor concentration, reduction in tempo, sluggishness of the mental processes and a tendency to perservation point to cerebro-organically governed changes.

DISCUSSION

On the basis of the findings in three independent groups of chlorophenol workers, which together included more than 100 affected by diseases, a characteristic clinical picture is provided, the most important features of this being the following disturbances:

1. Following initial dermatitis of the face and symptoms of irritability on the part of the conjunctiva: often together with gradually developing acne primarily in the region of the face, then the back of the neck, shoulders and upper trunk, and in severe cases on the entire body, with comedones, pustules, boils and patches of pigmentation. In several cases with severe irritation of the mucous membranes of the face and the upper respiratory tract: sometimes with continuing blepharoconjunctivitis.

2. In several cases, disturbances connected with the internal organs, especially damage to the liver, with deposits of a nonferrous pigment as a

characteristic biopsy finding. In some cases, chronic bronchitis and isolated instances of myocardiac damage.

3. In all cases, general fatigue and weakness principally affecting the proximal muscles of the lower limbs, often with pain in the musculation and in some cases paraesthesia and slight hypaesthesia. In isolated cases only, more pronounced disturbances of sensitivity, slight paresis (implicit) and weakening of reflexes.

4. A psychovegetative syndrome with the following disturbances: Subjective: Disturbances of the vital senses, disturbances in the basic mental mood and affectivity, disturbance in impulsion, weakness of memory and concentration, hyperaesthetic traits, vegetative dysregulation, tendency to orthostasis, sleep disturbances, much increased sleep requirements, disturbances of the instinct sphere, reduction in libido and potency, and alcohol intolerance.

Objective.—psychopathological: Reduction in impulsion, subdepressive traits of a type characterized by genuine vital moments of depression, disturbances in affectivity in the sense of a certain levelling-out, increased excitability; occasionally hebetude, hypochondria and personality alienation.

Experimental psychological. HAWIE: Increased degeneration percentage; Rorschach psychogram: Coartation of the experiential type, signs of weakened emotional reactivity, weakness of concentration, reduction in tempo, sluggishness of mental processes, tendency to perseveration.

The dermatological picture of the chlorophenol intoxication described shows extensive agreement with the disease caused by chlorinated aromatic hydrocarbons as first described by HERXHEIMER and later by several authors (see BRAUN, RISSE-SUNDERMANN). On the basis of the observations that chlorinated naphthalenes were principally responsible, WAUER, and later TELEKY, suggested the designation "perna disease" (*PER*chlorinated *NA*phthalene). TELEKY pointed out that the chloracne already described by HERXHEIMER in 1899 was produced not by pure chlorine but by chlorinated hydrocarbons or the simultaneous action of chlorine and tar. Further observations on perna disease made by MITTELSTADT, FLINN and JARWIK, DRINKER and collaborators, and GREENBURG and collaborators, indicated that not only the skin symptoms but also fatigue, loss of appetite, giddiness, and severe liver damage with acute yellow atrophy of the liver leading to death can result from work with chlorinated naphthalenes. BAADER mentions epidemics at American shipyards during the Second World War. In his description of the cases occurring in America and Great Britain, sometimes with a fatal outcome, TELEKY refers to the report of BROWN, President of the Halowax Co., New York (1937), that only the manufacture of the higher stages of chlorination and the combination with chlorinated diphenyls and other substances led to severe damage to the health and in some cases to fatal acute yellow atrophy of the liver. TELEKY also refers to the animal experiments by C. K. DRINKER and collaborators to support the view that only the higher chlorinated diphenylamines produce serious damage.

The general symptoms in occupational chlorophenol intoxications are apparently more pronounced than those occurring with the lower chlorinated naphthalenes employed earlier. This fact was also observed by TRUHAUT and collaborators amongst workers who had been using pentachlorophenol for wood preservation, as well as KUBOTA in Japan, who mentions multifarious disturbances of the autonomous nervous system and who observed several fatal cases. In all three German groups of chlorophenol intoxication, liver damage was established, the damage that was most pronounced and studied most intensely being that found amongst the cases of disease occurring in two Mid-Rhenish companies (HERGT, KALK and WILDHIRT). In all the groups, several cases of chronic emphysema bronchitis and myocardiac damage were found, although these disturbances did not occur nearly so regularly as the pronounced fatigue and neuromuscular weakness, which we observed in all our patients. The psychosyndrome described was equally regular, this being found not only by us but also by JANZARIK and RICHERT to a completely identical degree amongst the Mid-Rhenish workers.

The psychopathological syndrome could be distinguished with a sufficient degree of certainty by differential diagnostics from endogenic psychosis, especially mild cyclothymic diseases, neurotic personality developments and organic psychosyndromes of different etiology, and somewhat presenile or

cerebrosclerotic processes of degeneration. Phenomonologically, the relationships to the pseudoneurasthenic syndrome, which is described in connexion with a large number of occupational intoxications such as those caused by lead, carbon monoxide, manganese, thallium, arsenic, carbon disulphide, trichloroethylene, etc. (for relevant articles, see BORBELY, von HATTINGSBERG MEGGENDORFER, MOESCHLIN, PENTSCHEW, TELEKY)—and especially the relationships to particular endogenic mood conditions—are obvious. Lowering of the vital level, moments of depression, vegetative symptoms, and, not least, fluctuations in intensity can be observed *predominantly* in endogenic-depressive conditions. On the other hand, alcohol intolerance, hyperaesthetically excitable and polyneuropathic traits influence the differential diagnostic aspect more in the direction of an apparently exogenic condition. The somewhat older psychiatric literature should be borne in mind in this connexion (MEGGENDORFER, STERTZ), this placing the neurasthenic syndrome quite definitely in the pattern of exogenic symptom complexes. Not least, reference should be made to the phenomenological relationship of our observations to the (exogenic) hyperaesthetic-emotional conditions of weakness of BONHOFFER, which, from the psychopathological aspect, have significantly been designated by EWALD as no longer heteronomous but homonomous in the sense propounded by KLEIST.

Despite the phenomenological relationships discussed, the psychopathological delayed syndrome of the chlorophenol workers scarcely corresponds completely with any of the known clinical pictures. In any case, the question of a special psychic-vegetative delayed intoxication syndrome, which was discussed by SPIEGELBERG in connexion with observations on persons suffering chronic occupational damage from military poison gas, also demands consideration in view of the observations mentioned in this paper.

It has been possible to rule out psychogenic-neurotic moments so far as our subjects are concerned, provided that individual neurotic conditions, i.e. characterogenic and experiential situative data are involved. Two of the nine probands exhibited considerable psychopathic or neurotic structural elements. However, it was easily possible to separate these two probands from the other completely or largely *non-neurotic* cases. Certain "collective-neurotic" factors have, in our opinion, to be taken into account as an unfortunate but practically unavoidable fact in all group investigations but especially those involving etiological evidence (SPIEGELBERG). Reactions of this type have also been observed in our cases in the sense of a superimposed psychogenic accessory with, as it were, "physiological" but not inadequate, individual-neurotic (complex-determined) idemnification wishes. The psychopathological analysis of the individual case and the comparison of the findings in each instance with such independent collectives of the same etiology afford sufficient protection from authoritative and scientific false assessments. Despite the long course, the prognosis of the psychopathological *intoxication* results appears favourable. Although technical aspects of the pension situation have not yet been finally clarified, there was, on the whole, a certain subjective improvement in the symptoms, or else they remained static. We have not observed any objective deteriorations, except for the momentary intensity fluctuations. The experience of the Mainz Nerve Clinic (RICHER) and the impressions of works medical staff (KNECHT) suggest a benign course of acute and chronic intoxications, provided no toxic parenchyma damage, as such, influences the prognosis unfavourably.

It seemed appropriate to attribute the toxic action to the high-chlorinated chlorophenols, this view being supported by animal experiments conducted by MACHLE and THOMAS, H. KITZMILLER, a series of other investigators (KEHOE, DEICHMANN, GRUEBLER, BOYD, McGAVACK, TERRANOVA, PICCIONE cited acc. to von OETTINGEN) and also our own animal experiments. KIMMIG and SCHULZ were, however, able to show that the use of non-industrial, analytically pure, high-chlorinated chlorophenols (trichlorophenols, pentachlorophenols) does not lead to the characteristic symptoms of chlorophenol intoxication.

Animal experiments were carried out with a view to discovering the noxae causing the symptoms. The rabbit's ear proved to be a suitable test object since it is possible to produce the changes on this with the substances causing chloracne, these changes closely resembling those of human chloracne (HOFMANN and NEUMANN, BRAUN, LANDES, etc.). Brushing with a

substance which is active in this respect leads at first to patches of dermatitis in conjunction with reddening, swelling and flaking: then, some days later, hyperkeratosis linked with the follicles and also small cysts occur, these being easy to record histologically, as well. In addition to the brushing experiments, tests were carried out on rabbits to determine the general toxicity, whilst cats, too, were used for testing a number of substances. In these tests, it was found, in corroboration of findings derived by OETTEL and also HOFMANN from similar cases of intoxication in a large chemical works in southern Germany, that the substances producing chloracne possess marked liver toxicity in rabbits. It was possible to trace effectively the damage to the parenchyma of the liver intra vitam with the micro-modification of the bromsulphthalein test given by HOFMANN and OETTEL. In autopsies, diffuse steatoses and extensive necrosis of the parenchyma of the liver were found. The investigations, which have already been reported (SCHULZ 1956; KIMMIG and SCHULZ 1957) led to the following results:

The effective substances must have occurred in the alkaline hydrolysis of 1,2,4,5-tetrachlorobenzene to 2,4,5-trichlorophenol, this having been carried out technically under pressure at about 180°C in the presence of methanol and caustic soda solution. However, it was not the trichlorophenol itself but the by-products that formed in small quantities in the course of the pressurized phenol process that were regarded as the causal noxae; for it was not possible to produce any of the above-named changes on the rabbit's ear with pure, repeatedly distilled 2,4,5-trichlorophenol or with 1,2,4,5-tetrachlorobenzene, although they did occur with the trichlorophenol used technically.

Since the isolation of defined compounds from the residue occurring in the distillation of technical trichlorophenol was not possible at first, compounds were synthesized by chemical means and given to us for testing on animals where there was a certain likelihood that these substances may occur as by-products in the saponification of tetrachlorobenzene to trichlorophenol. The substances initially available were various chlorination products of the diphenyl ether and the dibenzofuran (diphenylene oxide).

Although the diphenyl ether and its 1× to 4× chlorinated derivatives, and also dibenzofuran and monochlorodibenzofuran were ineffective in experiments on animals, 3× and 4× chlorinated dibenzofurans, even in concentrations as low as 0.05%, produced the symptoms mentioned on the rabbit's ear. Single doses of 0.5 to 1 mg/kg administered orally produced severe liver damage in rabbits, this leading to the death of the animals in most instances.

The clinical observation of a laboratory assistant engaged elsewhere, who fell ill with severe chloracne after exposure to tetrachlorodibenzodioxine, indicated the chlorine derivatives of the dibenzodioxine.

Tetrachlorinated dibenzodioxines, especially 2,3,6,7-tetrachlorodibenzodioxine, were highly effective on the rabbit's ear, even in low concentrations. Three brushed applications with 0.01–0.005% solutions (in polyglycol) were sufficient to cause severe areas of inflammation and follicularly arranged hyperkeratosis. When administered orally, single doses of 0.05–0.1 mg/kg body weight led to severe liver damage and generally the death of the animals.

The assumption that 2,3,6,7-tetrachlorodibenzodioxine is actually of considerable importance in causing the chloracne diseases occurring in the chemical works received further substantial support from the chemical angle. It was possible to prove that this compound is formed from two molecules of sodium trichlorophenolate in association with the cleavage of NaCl under the pressure and temperature conditions prevailing in the autoclave.

It was, moreover, possible to isolate the named tetrachlorodibenzodioxine from the by-product occurring in the technical pressurized phenol process (alkaline saponification from tetrachlorobenzene to trichlorophenol). To prove that 2,3,6,7-tetrachlorodibenzodioxine is capable of producing alterations in the form of chloracne not only on the rabbit's ear but also on human skin, one of us (SCHULZ) carried out a test on his own body. Two brushed applications of a 0.01% solution on a circumscribed skin area of the forearm led within two days to a mild dermatitis, then some days later to a follicular hyperkeratosis and comedones, these also being easy to record histologically. The etiological significance of this substance for the diseases described here seems to us to be sufficiently evidenced by this experiment. However, it is not impossible that other chlorinated aromatic compounds with highly toxic characteristics may occur in this technical process, these possibly not having been so far identified or tested in experiments on animals.

The experimental and clinical findings are an impressive example of the fact that, in works pathology, substances occurring in small quantities as by-products in chemical processes can be of importance. In experiments to discover the origin of frequency occurring occupational intoxications, this viewpoint should not be ignored. If it is possible to prove that impurities attached to the main product should be regarded as the causal noxae of an occupational disease, this constitutes an important prerequisite for successful prophylaxy.

In our own special case, it was possible, by changing the plant chemistry aspect of the manufacturing process, to prevent the formation of the highly toxic, multi-chlorinated dibenzodioxines and dibenzofurans. Since than, 2,4,5-trichlorophenol is again being manufactured in this works and processed into the herbicide 2,4,5-trichlorophenoxyacetic acid, without symptoms of intoxication of any kind occurring amongst the workers.

A Technic for Testing Acnegenic Potency in Rabbits, Applied to the Potent Acnegen, 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin*

E. Linn Jones, M.D. and Helen Krizek, Ph.D.

Follicular hyperkeratosis is an important feature of the occupational disease known as chloracne, which is characterized by the appearance of papules, comedones and cysts after exposure to industrial materials containing highly chlorinated diphenyls, highly chlorinated naphthalenes, and other chlorinated aromatic compounds. A characteristic epithelial hyperplasia and hyperkeratosis can be produced on the inner surface of the rabbit ear by such compounds (1, 2), and a difference in intensity of response has been noted and suggested as a basis for comparative tests (2). Experimental studies heretofore (1–7) have been, however, directed chiefly to the ability or the failure of various materials to produce this effect in experimental animals and in man, and in delineating its gross and its histological features; these studies have used either material of unstated origin, or else mixtures (*e.g.* Halowax, 1014). We have attempted to study the phenomenon of acnegen-induced hyperkeratinization on the rabbit ear in a quantitative fashion by recovering and weighing the keratin formed after applying known amounts of a single, well-characterized chemical compound under controlled conditions. To recover keratin a new technic was developed based on the resistance of this material to digestion by pepsin. As test compound we have chosen 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, which has been reported to be so potent that painting the rabbit ear three times with a 0.05%-0.001% solution was sufficient to produce the acneform response (7). With a compound of such potency, the expected effects could be produced without the necessity of applying the material in an ointment or as a crust, circumstances which would have made very uncertain the quanity actually in contact with the skin.

EXPERIMENTAL

Preliminary gross and histologic observation indicated that 0.3 micrograms applied to the rabbit's ear gave, by gross observation, a minimal follicular plugging whereas 0.02 micrograms caused no observable effect. Accordingly, 0.3 micrograms was chosen as the lowest dose; in addition dose levels of 1.0, 3.0 and 10.0 micrograms were studied.

Seven mature, white, male rabbits were used for each dose level, except in the first studied (0.3 micrograms) for which only six were used. Seven days after wax epilation †, of the inner surface of the ears, 1 ml. acetone solution of the compound was applied to one ear of each of the rabbits, and 1 ml. acetone to the other. Special effort was made to distribute the liquid uniformly over

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† With "Improved Zip."

the entire inner surface, and to aid in securing uniformity the dose was divided into three applications, made on successive days. The right ear was used as control for some rabbits in each group, and the left for others. Fourteen days after the first application, three biopsy samples, extending through the cartilage, were taken under procaine anesthesia, with a 9 mm. punch. One sample was taken from the middle, one from the posterior and one from the anterior area of the ear, at a level about 15 mm. distal to the notch of the ear. (Fig. 1) The discs were washed free of blood without delay. Under a dissecting microscope at 9× magnification, the moist samples were plucked free of any adhering hairs and clots; and the cartilage was removed with sharp #10 Bard-Parker scalpel. A thin coating of white petrolatum was applied to the epidermis. Each disc was floated in a 50-mm. petri dish containing 10 ml. 0.1% pepsin (Worthington Biochemicals "2× Crystallized" product) in 0.24 N HCl and incubated 4 hours at 37°C., at the end of which period the keratin disc was gently lifted out, free of any undigested dermis by inverting and gently irrigating it, and resuspended in 10 ml. 1:1 V/V ethanol: diethyl ether mixture. A light aluminum-foil cup 9 mm. in diameter, with perforated bottom, previously washed with ether and weighed to 0.02 mg., was brought close to the disc, which was then gently transferred, with follicular projections up, with the aid of a scalpel handle, into the cup. After four hours at room temperature in the covered petri dish, the solvent was aspirated. A similar leaching with 10 ml. ether was made, after which the cup in the covered petri dish was dried in vacuo overnight. The cup and sample were weighed to the nearest 0.02 mg.

Additional biopsy samples were taken on about half the animals and examined histologically after routine hematoxylin and eosin staining.

The following method was found convenient for preparing a small quantity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.[1] The sodium salt of 2,4,5-trichlorophenol was prepared by dissolving 1.6 g. metallic sodium in 25 ml. absolute ethanol in a 100-ml. round-bottomed flask, adding 20 g. of 2,4,5-trichlorophenol (Eastman "Practical Grade," recrystallized from petroleum ether) and distilling off the ethanol. The salt was cautiouly heated until the copious evolution of acid fumes which took place at 200–250° C. subsided, after which the flask was kept at 350–400° C. for 30 hours. Two zones were found in the distilling head. The lower of these, a dense, compact mass, had a melting point of 230–300° C. and was only slightly soluble in chloroform. This was the crude acnegen. The upper zone of coarse crystals soluble in chloroform was probably a tetrachlorobenzene formed in a competing reaction. Two batches of the crude acnegen were combined and recrystallized twice from anisole to yield 0.25 g. product. Its synthesis is represented in Fig. 2.

Analysis. Calculated for $C_{12}H_4O_2Cl_4$: C, 44.76%; H, 1.25%; Cl, 44.05%. Found: C, 44.31%; H, 1.40%; Cl, 44.18%.[2] The melting point was 295–300° C.; literature values for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin prepared by chlorination of dibenzo-*p*-dioxin: 295° C. (8), 320–325° C. (9). The infrared spectrum showed a doublet at 1310–1322 $cm.^{-1}$ in the range reported for a series of dibenzo-*p*-dioxin derivatives (10). The ultraviolet absorption spectrum of the compound in absolute ethanol had a maximum at 233 mμ and one at 307 mμ; the respective molecular extinction coefficients were 46,500 and 4,250.

RESULTS

Histological sections showed a characteristic hyperkeratosis of the follicular epithelium and a marked hyperplasia of the surface epidermis. At low dose level some sebaceous cells were present: at high dose level, only a few were seen, and the follicle was filled with a keratinous mass. (Figs. 3 and 4). There was marked thickening of the epidermal keratin, although this was only rarely observed on the slides, presumably because it was lost in the cutting and processing.

[1] Following the practice of *Chemical Abstracts* we describe our command as 2,3,7,8-tetrachlorodibeno-*p*-dioxin. From a comparison of its preparation with the mode of origin in the Industrial process discussed by Kimmig and Schulz (7) we conclude that it is the same as their 2,3,6,7-tetra-chlorodibenzo-*p*-dioxin.

[2] Analyses by Micro-Tech Laboratories, Skokie, Ill.

The complete removal of all non-keratinized tissue from the biopsy by pepsin digestion was confirmed by histological section of a keratin disc. (Fig. 10).

In general, the keratin discs recovered from the treated animals were less fragile and thicker than the controls. At lower dosages the follicular keratin was usually observed as tree-like forms representing casts of the multiolobated sebaceous glands. (Figs. 5A, 6, and 7). These follicular projections were not present in the controls. (Fig. 5B). At higher doses the keratin in the follicles appeared as a larger structure of smooth, oval shape typical of comedones. (Fig. 8).

Weights of keratin recovered from the biopsy samples are given in Tables 1 and 2. In Table 1 are listed average weights for the three biopsy specimens taken from each ear. In subsequent experiments biopsy specimens taken from the anterior, the middle and the posterior portion were distinguished, in an effort to assess the importance of the site of biopsy removal with respect to the weight of keratin recovered.

DISCUSSION

For each rabbit there was calculated a value for the relative increase in weight, $(T - C)/C$, where T = average weight for the three biopsy samples from the treated ear and C = the corresponding average for the control. These values and their average for each dose level are plotted (Fig. 9) against the dose. The averages vary approximately linearly with the logarithm of the dose, but individual $(T - C)/C$ values at each dose deviate widely. The deviation may be due, in part, at least, to failure to secure uniform spreading of the aenegen, in spite of the precautions taken. Loss of keratin during manipulation of the samples may be another source of deviation; however, such an error would be expected to be most important for smallest amounts of keratin handled (*i.e.* the controls). An inspection of the control weights does not reveal a corresponding spread of values. Further, the probability of losing keratin was greater for the rabbits showing greatest response, because these tended to have some loosely adhering scales and large comedones which might be expressed and lost in the course of tissue removal and subsequent processing; actually, therefore, the spread of response may be larger than our results indicate. In addition, gross observation indicated that some rabbits gave a weaker response than others at the same dose level. We feel, therefore, that the values reflect a real individual variation. Inspection of Tables 1 and 2

TABLE I.—RECOVERY OF KERATIN FROM EARS OF RABBITS AFTER APPLICATION OF 0.3 MICROGRAMS 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN

Rabbit	Milligrams of keratin per 9-mm biopsy sample [1]	
	Treated ear	Control
31	0.98	0.97
49	1.07	0.70
47	1.91	1.31
66	1.88	1.18
84	1.58	1.20
57	1.13	1.31

[1] Average of three samples from, respectively, anterior, middle, posterior.

reveals some tendency for greater response to occur in animals for which control values were high, but this tendency is by no means clear-cut. We found no correlation between intensity of response and weight of the animal.

There appears to be no consistent difference in the three positions, anterior, middle and posterior, along the line of section, either for the control or for the treated ear, with respect to weight of keratin recovered. Average value for keratin recovered from the controls was remarkably constant: 1.11 ± 0.19, 0.85 ± 0.18, 1.02 ± 0.26 and 1.02 ± 0.16 mg. for the rabbits treated with, respectively, 0.3, 1.0, 3.0 and 10.0 micrograms.

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The technic developed in the present study might be used for a comparison of acnegenicity of various materials if the test substance is applied to one ear, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to the other. Such a procedure might avoid the complications introduced by individual differences, and reduce the number of animals necessary. Control values could be secured independently from untreated animals. Three biopsy samples may not be necessary; an analysis of the data showed that values calculated from only one (the middle) biopsy sample were not significantly different from those based on the average for three.

A fairly good correlation was found between gross observations and intensity of response as assessed by weight of keratin, but there were some deviations. Thus all the animals at 0.3 micrograms dose level were assessed grossly as showing follicular dilatations. However only four showed increase in weight of keratin. At 3.0 micrograms large comedones were observed on four rabbits, and these showed the greatest relative increase in weight of keratin; on one

TABLE 2.—RECOVERY OF KERATIN FROM EARS OF RABBITS AFTER APPLICATION OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN

Rabbit	Milligrams of keratin per 9-mm. biopsy sample							
	Treated ear				Control			
	Anterior	Middle	Posterior	Average	Anterior	Middle	Posterior	Average
Dose: 1 microgram								
16	1.24	0.54	0.90	0.89	0.65	0.54	0.46	0.55
15	2.14	1.82	1.70	1.89	0.94	0.70	0.64	0.76
30	1.60	1.14	1.26	1.33	0.88	0.76	0.72	0.79
17	2.16	1.52	2.52	2.07	0.80	0.82	0.58	0.73
11	2.04	1.36	1.26	1.55	1.14	1.08	1.14	1.12
7	3.82	2.08	2.52	2.81	1.08	1.08	1.04	1.07
27	3.44	2.86	2.06	2.79	0.88	1.16	0.86	0.97
Dose: 3.0 micrograms								
18	0.86	1.22	0.96	1.01	0.90	0.64	0.92	0.82
19	1.76	0.94	0.84	1.18	0.78	0.90	1.32	1.00
20	1.80	1.08	1.58	1.49	1.08	0.54	0.92	0.85
21	7.00	8.54	5.64	7.06	1.80	0.88	1.26	1.31
22	2.82	2.04	1.38	2.08	0.88	0.64	0.74	0.75
23	3.00	2.88	5.86	3.91	1.24	0.98	0.78	1.00
24	5.86	6.14	5.56	5.85	1.94	1.30	0.90	1.38
Dose: 10.0 micrograms								
32	3.28	3.30	2.02	2.87	0.88	0.96	0.80	0.88
33	2.68	2.48	2.60	2.59	1.20	1.08	1.20	1.16
34	1.26	1.62	1.18	1.35	0.78	1.16	0.78	0.91
35	6.56	7.12	5.84	6.51	1.32	0.80	1.08	1.07
36	5.14	2.90	3.84	3.96	0.98	1.08	1.16	1.07
37	1.64	1.34	2.18	1.72	1.18	1.28	0.52	0.99
38	6.28	4.02	4.90	5.07	1.08	1.06	1.08	1.07

other, comedones were observed; but the response in terms of weight recovered was less than for two which gave the gross impression of follicular papules.

Attention should be called to the great toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Kimmig and Schulz (7) reported that 0.5–1.0 mg. per kg. orally was lethal to most of their rabbits. In our preliminary experiments a rabbit receiving topically 0.3 micrograms in acetone, died at the end of 24 days, and two others receiving, respectively, 30 and 2 micrograms died within a week. However, there were undoubtedly other contributing factors, because in all animals treated subsequently no toxic symptoms were observed.

SUMMARY

1. Hyperkeratinazation induced on the rabbit ear by the aenegen 2,3,7,8-tetrachlorodibenzo-*p*-dioxin is studied by a new technic based on weighing keratin recovered after careful pepsin digestion.

2. When applied in acetone solution to the rabbit ear, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin is effective at microgram levels. Effect of dose and individual differences in response are discussed.

3. The new technic, using 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, is suggested for comparing acnegenicity of various substances.

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DISCUSSION

DR. PETER FLESCH, Philadelphia, Pa.: Since the criterion of acnegenic activity appears to be the conversion of the sebaceous cells into keratin-forming cells, I would like to ask, what did you see in the histologic sections?

DR. E. LINN JONES, (in closing): In the paper we will have histologic sections of treated glands and the digested keratin disc.

In regard to histology there is varying response, depending on the amount applied. With lower doses there is conversion of the cells in the follicle to keratinizing squamous cells, with occasional remnants of selbaceous cells in pockets here and there. With larger doses no sebaceous cells can be found. The entire follicle is converted into a large keratin-filled papule.

STUDIES OF THE CHICK EDEMA DISEASE

2. PREPARATION AND BIOLOGICAL EFFECTS OF A CRYSTALLINE CHICK EDEMA FACTOR CONCENTRATE

BY

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2. PREPARATION AND BIOLOGICAL EFFECTS OF A CRYSTALLINE CHICK EDEMA FACTOR CONCENTRATE

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INTRODUCTION

FOLLOWING the early reports of the chick edema disease (CED), Sanger *et al.* (1958), Potter *et al.* (1959), Allen (1961) and Allen and Lalich (1962) reported toxicological studies which established that the disease differed from known poultry diseases. A number of investigators reported on the purification, isolation and partial chemical characterization of toxic factor (Brew *et al.*, 1959; Friedman *et al.*, 1959; Wootton and Alexander, 1959; Ames *et al.*, 1960; Harman *et al.*, 1960; Yartzoff *et al.*, 1961; Wootton *et al.*, 1962; and Wootton and Courchene, 1964).

Brew *et al.* (1959) reported that broiler chicks developed the disease when fed a fraction purified 3200-fold from the original starting material. A fraction purified 10,000-fold which elicited CED was reported by Friedman *et al.* (1959). Yartzoff *et al.* (1961) reported that a fraction purified

3,000,000-fold from a low potency commercial triolein produced hydropericardium (HP) when fed to day-old chicks at 50 p.p.b. in the diet, and resulted in death at 1 p.p.m. Wootton and Courchene (1964) found one fraction (designated α 3.02) which was highly toxic for the chick. These workers estimated that ingestion of 5 µg. of the α 3.02 fraction was enough to kill one chick. Firestone *et al.* (1963) reported that signs of the disease were elicited by one fraction fed at 0.1 p.p.m., which substantiated the report by Yartzoff *et al.* (1961).

The purpose of this paper is to report our recent studies on the purification and biological effects of a concentrate of the chick edema factor (CEF) isolated from a crude toxic fatty material (TFM) known to produce the chick edema disease (Flick *et al.*, 1962, 1963). Preliminary data are included on the effects of CEF-containing material on egg hatchability and development of the chick embryo.

MATERIALS AND METHODS

Preparation of CEF Concentrate. Eighteen pounds of unsaponifiable material was isolated from 180 pounds of toxic fatty material (TFM) by saponification and extraction of the unsaponifiable fraction with petroleum ether:diethyl ether (1:1, v./v.). The petroleum ether was redistilled and the solvent boiling between 40-60°C. was collected and used. The unsaponifiable fraction was chromatographed on Fisher Alumina (Cat. No. A 540) essentially as described by Yartzoff *et al.* (1961). Fractions with ultraviolet absorption spectra that matched those of naphthalene and penanthrene derivatives were combined and chromatographed on Merck Alumina (Cat. No. 71707). Foreruns were eluted with petroleum ether. Additional fractions that eluted with 5% diethyl ether and that exhibited the spectra of naphthalene and phenanthrene derivatives were collected, combined and concentrated. The concentrate was chromatographed on columns of Celite:H_2SO_4:fuming H_2SO_4 (1:1:1) and eluted with CCl_4. The foregoing procedure was a modification of AOAC method 24.111 (a) (Horwitz, 1960). The CCl_4 eluates were re-chromatographed on Merck Alumina, and individual fractions were collected and checked by ultraviolet spectrophotometry. Fractions eluting with 10% diethyl ether with absorption spectra of naphthalenes (absorption maxima in the range of 240-250 mµ.) were combined and re-chromatographed on Merck Alumina with isooctane as the eluant. Individual fractions were collected and bioassayed, and a highly toxic crystalline fraction (CEF concentrate) weighing 79 mg. was obtained.

The CEF concentrate was examined by microcoulometric and electron capture gas chromatography. A microcoulometric gas chromatograph (Dohrmann Manufacturing Company, Palo Alto, California) was used at a column temperature of 250°C. with a 6-foot × ¼ inch (i.d.) aluminum column packed with 20% Dow-Corning High Vacuum Grease on acid-washed Chromosorb W. Details of this technique were described previously by Firestone *et al.* (1963). For electron capture detection, an Aerograph Hy Fi (Model 600B) gas chromatograph (Wilkins Instrument and Research Inc., Walnut Creek, California) was used. This instrument was equipped with a tritium source electron capture detector. A stainless steel column, 5 feet × ⅛ inch, packed with 5% SE-52 silicone gum rubber on 60-80 mesh Chromosorb W, was used at a column temperature of 202°C. and a nitrogen (carrier gas) flow rate of 50 ml./minute.

Feeding Procedure and Biochemical Methods. Day-old Single Comb White Leghorn

TABLE 1.—*Composition of basal ration*

Component	Level
	% (W/W)
Corn meal, yellow	36
Wheat flour middlings	15
Alfalfa meal	1
Linseed meal	8
Protein, soya (Drackett)	16
Salts, A.O.A.C.[1]	3
Yeast	3
NaCl, iodized[2]	1
Cod liver oil	1
Cottonseed oil	16
	100

[1] For composition of A.O.A.C salt mixture, see report by Flick *et al.* (1963).
[2] This addition gave a total of 1.99% NaCl in the diet.

cockerels were fed *ad libitum* and provided with fresh water at all times. They were handled and maintained as reported previously (Flick *et al.*, 1963).

The composition of the basal diet used is shown in Table 1. The crystalline CEF concentrate, dissolved in chloroform:ether, was added to the cottonseed oil, and the oil was warmed with stirring to remove solvent. The CEF concentrate was fed at levels of 50 and 200 p.p.b. for three weeks.

Body weights and feed intake were determined and at weekly intervals chicks were anesthetized with diethyl ether, volume of hydropericardium (HP) was measured according to the procedure outlined by Douglass and Flick (1961), and postmortem observations were recorded. Blood samples were withdrawn from the right ventricle with needles and syringes moistened with heparin, and the following tests were performed: microhematocrit (Natelson, 1961), whole blood glucose (Somogyi, 1952) and total plasma proteins and plasma protein fractions (Gornall *et al.*, 1949). Other blood samples were allowed to clot, and copper determinations[1] were made on the sera.

Hatchability Study. For this preliminary study, commercially available White Leghorn fertile eggs were injected with the test materials into the yolk sac prior to incubation according to the technique described by McLaughlin *et al.* (1963).[2] Control eggs were injected with 0.1 ml. corn oil. Experimental eggs were injected with 10, 20 or 50 µl. of the unsaponifiable fraction of crude TFM from which the crystalline CEF concentrate was prepared. Eggs were candled each day after the 5th day of incubation. Dead embryos were removed and examined for gross malformations. After incubation, unhatched eggs were opened for gross observation. Chicks which hatched were observed for 3 days.

RESULTS AND DISCUSSION

GLC Analysis of CEF Concentrate. Gas chromatographic separations of crystalline CEF concentrate were obtained by using both the microcoulometric and electron capture techniques. A typical electron capture chromatogram of our purified prepara-

[1] Samples of serum were collected in acid-rinsed tubes, frozen under carbon dioxide and sent to Dr. E. W. Rice, Presbyterian Hospital, Pittsburgh, Pennsylvania for determination of copper

[2] Injection directly into the yolk has been studied in over 70,000 eggs (personal communication from Dr. McLaughlin) with approximately 300 chemicals. India ink and various dyes were injected in conjunction with several different studies Injected materials were found to consistently remain in the yolk and no great difficulties were encountered which were not amenable to correction.

The unsaponifiable fraction is a liquid residue of the toxic fatty material which is a still bottom residue resulting in the commercial production of fats The unsaponifiable fraction comprised 10.1% of the crude starting material and contained hydrocarbon residues and fatty acid degradation products in addition to the chick edema factor. When centrifuged 1000 × g for 15 minutes at 24°C, the unsaponifiable material contained <0.5% (v./v) of precipitable material See Brew *et al.* (1959), Friedman *et al.* (1959 and Wootton and Alexander (1959).

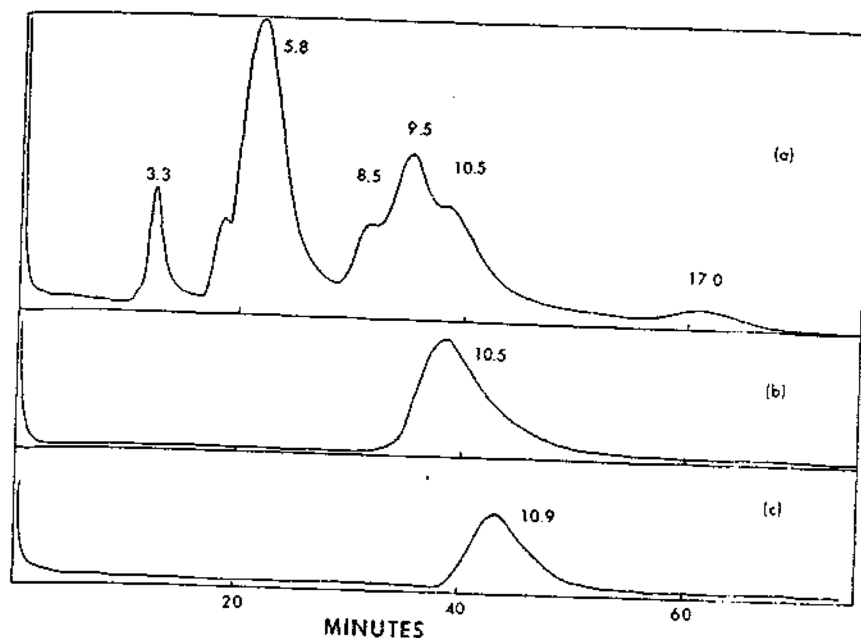

FIG. 1. Gas chromatograms of (a) toxic concentrate, (b) Procter & Gamble α 3.02, and (c) Procter & Gamble α 3.17. See text for details on instrumentation. The numbers adjacent to chromatographic peaks are retention times relative to aldrin.

tion is shown in Figure 1(a). The chromatogram revealed that within a 70 minute run at least eight components were present in the preparation. Wootton *et al.* (1962) isolated two components, which were designated *α 3.02* and *α 3.17* (gas chromatographic retention time relative to methyl arachidate). Each of these two components *contained a high-melting fraction* that produced edema and a low melting fraction that did not produce edema. Our toxic concentrate was chromatographically compared to the low melting fractions of Wootton *et al.*[3] The peaks of the latter two compounds, obtained with the electron capture technique, are shown in Figure 1(b) and 1(c). The chromatogram of our CEF concentrate shows a peak (retention time of 10.5 relative to aldrin) having the same retention time as the α3.02 inactive isomer.

The relative peak area (% of total area of the chromatographic peaks) of the 10.5 component was estimated by using the retention × peak height method of Carroll (1961). By this method the 10.5 peak obtained in the microcoulometric chromatogram represented about 20% of total components.

Chick Response. Results obtained on weekly weight gain, food consumption, feed/gain ratio and calculated consumption of the CEF are tabulated in Table 2. The weight gains among control birds increased at a fairly constant rate each week. Weight

[3] Kindly supplied by Dr. N. R. Artman, Procter & Gamble Company, Cincinnati, Ohio.

TABLE 2.—*Weekly weight gain, feed consumption, feed/gain ratio and ingestion of crystalline CEF concentrate*

CEF level	No. of chicks	Mean weight gain[1]	Diet consumed[1]	No. of deaths	Feed/gain ratio	CEF concentrate consumed	
p.p.b.		g.	g.			Total μg.	μg./100 g. B.W.
			Week 1				
0	52	27.4±1.1[2]	38.5	0	1.41	0	0
50	47	29.8±1.4	42.0	0	1.41	2.1	3.2
200	47	28.0±1.4	42.7	0	1.53	8.5	13.1
			Week 2				
0	39	36.7±1.6	82.6	0	2.25	0	0
50	36	29.3±2.0	80.5	0	2.75	4.0	4.1
200	38	39.0±2.2	72.1	1[3]	1.85	14.4	14.4
			Week 3				
0	23	58.4±2.1	139.3	0	2.39	0	0
50	20	27.5±3.0	112.0	0	4.07	5.6	4.4
200	22	40.6±2.7	133.7	3[4]	3.29	26.7	18.3

[1] Mean value/cage (8-10 chicks/cage in 5-6 cages/treatment).
[2] S.E. of the mean.
[3] This chick died on 11th day with edema.
[4] These chicks were severely edematous.

gains among birds fed 200 p.p.b. CEF were similar to those of controls for the first two weeks but were less than those of controls for the third week. The birds fed 50 p.p.b. CEF grew less during the second and third weeks than the controls or the birds fed 200 p.p.b. Weight gains were equivalent among all groups during the first week.

There were a few deaths, all of which were associated with feeding CEF concentrate (Table 2). The chicks that died had edema. Birds with severe edema always had gross organ changes. These pathological changes were not so severe as those observed among chicks fed the crude TFM (unpublished observations). This observation may be an indication that the purified CEF preparation has been at least partially separated from substances in the crude fat that not only enhance edema formation but also may lead to extensive and more severe pathological changes.

From the data in Table 2 on diet consumption, it is apparent that the CEF concentrate did not alter the amount of diet ingested during the first week. CEF concentrate at 200 p.p.b. caused a moderate decrease in diet consumption during the second week, and at 50 p.p.b. led to a decrease in diet consumption during the third week. The feed/gain ratio was elevated during the second week among birds fed 50 p.p.b. CEF and was further increased during the third week. The feed/gain ratio for the birds fed 200 p.p.b. CEF concentrate was elevated during the third week but it was lower than the ratio for the birds fed 50 p.p.b. From the data, however, it is apparent that feeding of the concentrate was accompanied by a moderate decrease in feed utilization.

From determination of feed intake and knowledge of the amount of crystalline CEF concentrate incorporated into each diet, the amount of purified material ingested was calculated. These calculations are given in Table 2. The total μg. CEF concentrate consumed was 11.7 μg./chick for those fed p.p.b. and 49.6 μg./chick for those fed 200 p.p.b. The amount of CEF concentrate ingested in μg./100 g. of body weight/chick is shown also in Table 2. The total amount of purified concentrate ingested (49.6 μg.) was about ninefold greater than the amount (5 μg.) that Wootton *et al.* (1962) calculated could kill one

TABLE 3.—*Weekly levels of plasma proteins of S. C. White Leghorn cockerels fed CEF concentrate*

CEF level	Albumin	Globulin	A/G ratio	Total protein
p.p.b.	g. %	g. %		g. %
		Day-old		
—	2.19±.05[1]	1.76±.13	1.27±.12	3.95±.10
		Week 1		
0	2.03±.07	1.28±.09	1.66±.19	3.31±.09
50	1.98±.09	1.39±.12	1.42±.10	3.37±.07
200	1.96±.13	1.28±.09	1.59±.19	3.23±.08
		Week 2		
0	2.21±.11	1.07±.04	2.08±.11	3.31±.11
50	2.51±.05	0.98±.06	2.63±.16	3.50±.10
200	2.12±.17	0.95±.08	2.29±.21	3.08±.22
		Week 3		
0	2.29±.11	1.39±.11	1.76±.20	3.68±.11
50	1.99±.20	1.87±.31	1.17±.18	3.88±.37
200	1.96±.16	1.19±.12	1.77±.26	3.15±.20

[1] S.E. of the mean.

chick. The difference between these values may be due to a number of reasons. First, Wootton *et al.* (1962) did not report the amount of food ingested by their birds fed the α 3.02 fraction. Second, they used a heavy breed of chick, while we have used a light breed. Third, though both diets were synthetic, they were not identical. Fourth, their α 3.02 fraction was essentially homogeneous, while our preparation contained perhaps as many as eight components (compare a, b and c, Figure 1). Nevertheless, the prediction of Brew *et al.* (1959) that the chick edema factor may be detectable when fed to chicks at levels of 2.0 p.p.m. or less has been confirmed by our studies and by those of Wootton *et al.* (1962).

The results obtained for plasma albumin, globulins, A/G ratio and total proteins are presented in Table 3. In general, there were no appreciable differences in either the plasma protein fractions or the total plasma proteins when considered on the group basis. Some chicks with severe edema had marked hypoalbuminemia and hypoproteinemia, in confirmation of the observations of Alexander *et al.* (1962). From the data it seems unlikely that the massive edema formed was the result of such modest alterations in circulating proteins. These data support the hypothesis of Flick *et al.* (1963) that the mechanism of CEF toxicity resulting in edema formation may be the effect of CEF on capillary permeability, and perhaps more specifically, on causing chemical alterations in endothelial intercellular cement substance, as postulated by Allen (1961). Even though there was no appreciable depression of circulating albumin, at least sufficient to result in hypoalbuminemic edema (Best and Taylor, 1955; and Smith and Jones, 1961), it may be that even slight decreases in albumin could enhance fluid transfer across endothelial membranes damaged by the chick edema factor.

The degree of development of HP in response to the ingestion of the CEF concentrate is shown in Table 4. The concentrate fed at the 200 p.p.b. level produced a marked increase in fluid which accumulated within the pericardial sac. The magnitude of this response is of the same order as reported previously by Flick *et al.* (1963), using 4% crude TFM in a similar diet. The HP group scores show that the 50 p.p.b. level of CEF did not result in abnormal volumes of pericardial fluid according to the ranges of volumes reported by Shue and Gallo (1961). The HP incidence shows that all the birds fed 200 p.p.b. CEF had abnormally high levels of fluid in the pericardial sac. Also shown in Table 4 are values for hematocrit, serum copper and whole blood glucose. The packed cell volumes were decreased by the two levels of CEF concentrate fed. These decreased hematocrits were not so low as those reported previously by Flick *et al.* (1963) from feeding 5% crude TFM with a practical type of ration.

Serum copper levels (Table 4) were elevated at the 50 p.p.b. level but no statistically significant change from normal occurred at the 200 p.p.b. level of CEF concentrate. Since it had been observed that the liver, heart and kidney were adversely in-

TABLE 4.—*Hydropericardium (HP), hematocrit, serum copper and whole blood glucose values of S. C. White Leghorn cockerels fed CEF concentrate for 3 weeks*

CEF level	Hydropericardium			Hematocrit	Serum Copper	Glucose[2]
	Mean volume	Score[1]	Incidence			
p.p.b.	ml.			% cells	µg. %	mg. %
0	0.060±0.012 (8)[3]	0	0/8	34.2±1.5 (5)	51± 6 (7)	226±11 (8)
50	0.138±0.018 (4)[4]	0	0/5	28.0±0.8 (8)	95±11 (6)	240± 7 (8)
200	4.68 ±1.64 (6)	30+	6/6	27.1±1.4 (8)	41± 8 (5)	217±10 (8)

[1] HP score determined from HP volumes according to the following scale: <0.20, 0; 0.21–0.40 1+; 0.41–1.00, 2+; 1.01–2.00, 3+; 2.01–3.00, 4+; 3.01–4.00, 5+; 4.01–5.00, 6+; 5.01–6.00, 7+; and >6.01, 8+.

[2] These levels are considerably higher than glucose levels among birds fed crude TFM in a purified ration (Flick *et al.*, 1963) in another experiment which resulted in rather marked hypoglycemia (controls: 146±5 mg. % *vs.* severe disease: 110±8 mg. %).

[3] S.E. of the mean (number of observations).

[4] One sample of HP fluid lost from this group.

volved in the chick edema disease (Allen, 1961; Alexander *et al.*, 1962; and Flick *et al.*, 1963), and that changes in these organs had been involved in the mechanism of edema formation (Smith and Jones, 1961), it was thought that determination of the levels of serum copper in the birds with the disease might be of some diagnostic value. Scheinberg and Sternlieb (1963) reported that human patients with Wilson's disease had severe liver disease, and many patients had serum copper dyscrasias caused by excessive urinary excretion, malabsorption from the intestine, decreased protein synthesis (particularly in severe malnutrition) and severe hepatic dysfunction. It may be that the elevated serum copper among the chicks fed 50 p.p.b. CEF concentrate was associated with either decreased liver utilization of copper or decreased renal excretion. The adverse effect on serum copper of CEF concentrate fed at 50 p.p.b. is not clear, but may be indicative that CEF toxic activity is oligodynamic and more specifically oligotoxic (more toxic at low levels than at higher levels).

Whole blood glucose levels were not appreciably altered by feeding the concentrate (Table 4). Crude TFM, however, fed in a purified ration, frequently elicited a marked hypoglycemia among chicks in advanced stages of the disease (see footnote 2, Table 4).

From what is known to date, the chick edema factor elicits a number of signs of intoxication which not only accompany the feeding of crude TFM but are more severe when the crude material is fed. The finding that our CEF concentrate contained 8 peaks by electron capture gas chromatography (Figure 1) indicates that at least 8 compounds were present in the purified preparation fed in these studies. It may be that only one, or a few, of these compounds possess the necessary molecular configuration to produce signs of the disease equivalent to the estimated potency of the α 3.02 fraction.

Hatchability Study. The preliminary results obtained from injection of White Leghorn eggs with the unsaponifiable fraction of TFM, which contained CEF, are shown in Table 5. The percent hatch of control eggs was within the expected range reported by McLaughlin *et al.* (1963). Chicks which hatched appeared to be normal in size and development by gross observation. When fertile eggs were yolk-injected with 10 µl. of the undiluted unsaponifiable fraction containing CEF, the hatch was 40%; injection of 20 µl. resulted in

TABLE 5.—*Effect of CEF-containing unsaponifiable fraction of TFM on embryonic development and egg hatchability*

Unsap. injected	No. eggs injected	Hatch	Observations	
			Embryo	Chick
µl.		%		
0[1]	20	93[2]	—	Normal
10	20	40	Malformations of right cerebral hemispheres, legs and beaks; small embryos	Weight retardation, sparse and deformed feathers.
20	20	20		
50	5	0		—

[1] Controls were injected with 100 µl. of corn oil.

[2] *Normal expected % hatch (McLaughlin et al., 1963).*

20% hatch and 50 µl. completely inhibited hatching. Embryos which failed to hatch exhibited one or more of the following developmental anomalies: malformed beak, lack of development of the right mesencephalon, eye defects, growth retardation or leg deformities. The deformities observed were not studied further. The deformities found were common to the embryopathies which occurred and were not related to level of CEF-containing fraction injected. Embryos which hatched after injection with 10 or 20 µl. of unsaponifiables exhibited sparse and defective feathering (down) and were small compared to the controls. This study revealed that CEF-containing unsaponifiables are capable of interfering with normal embryonic development and hatchability.

SUMMARY

The following studies were performed: gas-liquid chromatographic (GLC) separation of a purified crystalline concentrate containing the chick edema factor (CEF); feeding of the concentrate at 50 or 200 parts per billion (p.p.b.) in a semi-synthetic diet to day-old S. C. White Leghorn cockerels for three weeks and determination of growth, feed intake, feed/gain ratio, mortality, total intake of CEF concentrate, plasma proteins, hydropericardium (HP), hematocrit, serum copper, whole blood glucose; injection of CEF-containing unsaponifiable fraction and determination of its effects on embryonic development and on egg hatchability.

The following observations were made: 1) presence of approximately eight components (GLC separation) in crystalline material, 2) moderate growth depression at 50 p.p.b. level of CEF concentrate, 3) essentially normal feed consumption, 4) moderately increased feed/gain ratio, 5) increased HP at 50 p.p.b. level of CEF concentrate, 6) severe HP at 200 p.p.b. level of CEF concentrate, 7) essentially no group changes in plasma proteins, 8) moderate decrease in hematocrit, 9) no change or moderate elevation of serum copper (50 p.p.b. CEF concentrate), and 10) normal blood glucose levels.

A preliminary hatchability study revealed that a CEF-containing material led to a decreased hatch of injected eggs and to development of embryonic deformities.

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NUTRITIONAL ADJUNCTS

Chick Edema Factor. III. Application of Microcoulometric Gas Chromatography to Detection of Chick Edema Factor in Fats or Fatty Acids

By DAVID FIRESTONE, WALID IBRAHIM, and WILLIAM HORWITZ (Division of Food, Food and Drug Administration, Washington 25, D.C.)

A rapid screening test for detecting chick edema factor in fats consists of adsorption chromatography of extracted unsaponifiables on alumina, followed by analysis of specific fractions by a microcoulometric gas chromatograph which is sensitive only to halogens. This chromatographic method appears to be more sensitive than the chick bioassay.

Toxic fat yielded gas chromatographic peaks with retention times relative to aldrin of 5 or more. All samples which failed to reveal those chromatographic peaks have been shown to be nontoxic in the chick bioassay.

The widespread occurrence of chick edema disease in 1957 resulted in the deaths of millions of young chickens. The toxic materials causing this disease have been found to be chlorinated aromatic hydrocarbons, occurring in toxic fats in association with a large number of relatively nontoxic aromatic materials with similar chemical and physical properties. This paper describes a screening procedure for detection of such toxic fats. Specific fractions of unsaponifiable matter isolated from the fats are examined by using a microcoulometric gas chromatograph, an instrument which can detect submicrogram amounts of halogen. The presence of slow-eluting substances is an indication of the chick edema factor in the fat.

The precise structure of the substances causing chick edema disease have yet to be determined. Preliminary work on the detection, isolation, and characterization of the toxic agents was reported in 1959 by several laboratories (1-3). Subsequently,

Harmon and co-workers (4) isolated a toxic substance in crystalline form from a feed grade tallow. A private communication from Tishler of the same laboratory (5) disclosed that the crystalline substance contained about 47% chlorine.

Yartzoff and co-workers (6) isolated a crystalline halogen containing material that produced chick edema symptoms at 0.1 ppm in the diet from a sample of triolein. This triolein was toxic to monkeys, producing changes in the liver, kidney, pancreas, and other organs. More recently, Wootton and co-workers (7) isolated three compounds from a toxic fat which produced chick edema disease. Mass spectra of two of the compounds indicated a molecule which has a molecular weight of 391 and contains six chlorine atoms. Ultraviolet spectra were consistent with the concept that these materials are highly chlorinated aromatic compounds.

Ames and co-workers (8) and Firestone and co-workers (9) reported the occurrence of chick edema disease factor in oleic acid samples destined for human consumption. A food additive regulation[1] of the Food and Drug Administration now requires that food grade fatty acids be "free of chick edema or other toxic factor." At present, the detection and assay of chick edema factor in fats is carried out by a bioassay procedure (10–12) that requires 21 days to complete.

We observed that unsaponifiable matter from toxic fats contained a number of chlorinated components which had greater retention times than chlorinated pesticides when examined in a microcoulometric gas chromatograph;[2] our observation prompted *this investigation of the use of microcoulometric gas chromatography for detecting the* presence of chick edema factor in fats. Chick edema factor is presumed to be present if one or more gas chromatographic peaks with retention times relative to aldrin of 5 to 20 are found; its absence is presumed if analysis of the equivalent of 100 g of a fat or fatty acid fails to reveal the presence of these gas chromatographic peaks.

METHOD

Extraction of unsaponifiable matter (modification of AOAC method 26.064(5).—Reflux 111 g sample with 270 ml alcohol and 55 ml 50% (w/w) KOH for 1 hour. Transfer mixture to 2 L separator, rinsing flask with 325 ml H_2O, and add rinsings to separator. Add 300 ml petroleum ether, A.C.S. (redistilled, retaining cut with b.p. 40–60°C), and shake vigorously. Let layers separate, breaking emulsions that may have formed by adding 10 ml alcohol and swirling gently. Draw off lower layer, and transfer upper layer to another separator. Repeat extraction 3 times with 300 ml portions of petroleum ether and combine extracts. Wash extracts twice with 60 ml portions of H_2O by swirling gently. Wash petroleum ether extracts first with 60 ml H_2O and then with 60 ml of an alkaline dilute alcohol soln (dissolve 28 g anhydrous K_2CO_3 in 600 ml H_2O and then add 400 ml alcohol), and repeat washings in same order. Wash extracts with 60 ml portions of H_2O until neutral to phenolphthalein. Transfer extract to a 2 L erlenmeyer and dry by adding 20 g anhydrous Na_2SO_4, swirling vigorously, and letting the solution stand a half hour. Decant solution through a glass funnel containing a pledget of cotton in the neck and holding 20 g anhydrous Na_2SO_4 into another 2 L erlenmeyer. Wash first erlenmeyer and funnel with three 10 ml portions of petroleum ether, transferring washings from the erlenmeyer through the funnel and into the filtered solution. Evaporate most of solvent on steam bath, and transfer extract to 100 ml tared fat flask containing several boiling chips. Evaporate solvent on steam bath and complete drying under a gentle current of air, or by evacuating flask to 0.5 cm of mercury while swirling on steam bath. Determine weight of unsaponifiable matter.

Fractionation of unsaponifiable matter by alumina chromatography.—To a chromatographic column, 25 mm o.d. × 300 mm long, fitted at the bottom with a coarse porosity fritted glass disk and Teflon stopcock, add redistilled petroleum ether, dried prior to use with anhydrous Na_2SO_4, until column is ⅔ full. Weigh 50 g aluminum oxide (Merck reagent, No. 71707), and transfer to column. Store the alumina in tightly closed bottle, and close bottle as soon as possible after weighing out portions for chromatography. Let alumina settle, and when air bubbles stop rising to the

[1] Code of Federal Regulations, Title 21, Section 121.1070.

[2] Dohrmann Manufacturing Company, Palo Alto, Calif.

surface of the solvent, place a disk of coarse filter paper on top of the alumina. Cover the disk with 20 g anhydrous Na_2SO_4. Drain the excess petroleum ether so that it is level with the upper surface of the Na_2SO_4.

Transfer unsaponifiable matter to the chromatographic column, using a total of 20 ml petroleum ether. Allow liquid level to fall *so that it is just below the top of the* Na_2SO_4. Elute sample with 400 ml portions of each of the following solvents (dried prior to use by shaking with anhydrous Na_2SO_4): Petroleum ether (fraction 1), 5% ethyl ether in petroleum ether (fraction 2), and 25% ethyl ether in petroleum ether (fraction 3). Collect eluates in 500 ml erlenmeyer flasks, add several boiling chips, and evaporate to small volume on steam bath. Transfer residues to tared fat extraction flasks, evaporate solvent, and weigh. Transfer to 2 g short style vials having screw cap with tin liner, and evaporate solvent.

Microcoulometric gas chromatography.—Dissolve 4 g silicone grease (Dow Corning High Vacuum Grease) or Dow Corning DC 200 silicone fluid (12,500 centistokes) in 200 ml chloroform on steam bath. Add 16 g acid-washed Chromosorb W (Johns-Manville Co.), and stir continuously until most of solvent evaporates (about half an hour). Let stand on steam bath 1 hour, and place in vacuum oven at 50°C overnight to remove residual solvent.

Pack the coated Chromosorb W into a 3′ length of 0.25″ o.d. aluminum tubing plugged at one end with glass wool, using a Burgess Vibratool. (Two 3′ columns may be prepared from 20 g coated Chromosorb W.) Add a plug of glass wool to the open end of the column and bend it into a tight spiral, using a 3″ diameter mandril. Condition the column at 275°C for 48–72 hours, passing nitrogen through at 20 ml per minute.

Prepare a 1.00×10^{-3}% solution (10 mg/L) of aldrin in hexane or benzene and chromatograph 100 µl portions in a Dohrmann microcoulometric gas chromatograph at 216–218°C, using a nitrogen flow rate of 50–100 ml per minute so that aldrin elutes in 2.3–3 minutes. Use the 128 ohm range setting.[3] Determine area of aldrin peak by triangulation, or with a disc chart or electronic integrator installed on the strip chart recorder, and calculate recovery of aldrin using the following equation (applicable to chlorinated compounds):

µg Aldrin = (peak area, in.^{2}) × [recorder sensitivity (min./in.)(mv/in.)] × (35.5 g/eq.) × (60 sec./min.) × (10^6 µg/g)(10^{-3}v/mv) (10)/(sensitivity range, ohms) × (% chlorine in compound) × (96,500 coulombs/eq.).

For a 0.1 mv/in. recorder sensitivity, 2 min./in. chart speed, and 128 ohms sensitivity range resistance, the equation above reduces to:

µg Aldrin = (area × 34.5)/% chlorine.

The number of strokes of a disc chart integrator coupled to a chromatography recorder[4] equivalent to each square inch of area can be determined as follows: (*a*) Remove the fuse from the strip chart amplifier; (*b*) move the pen upscale on the strip chart a known distance from the baseline; (*c*) run the chart a known distance; and (*d*) divide the calculated area (height × distance traversed by the pen) by the number of strokes obtained.

By using the formula calculated as described above, a recovery of at least 70% of the aldrin injected should be obtained.

Dissolve fractions 2 and 3 from alumina chromatography separately in benzene to give 100 µl solution, and chromatograph each solution in the Dohrmann instrument. (For analysis of more than about 60 mg of each fraction, approximately 50% benzene solutions of up to 250 µl volume should be prepared and injected. Do not inject more than 125 mg material into column). First chromatograph ⅒ of the fraction, and if no chromatographic peaks with R_A = 5 or greater are observed, chromatograph the remaining 9/10 of the fraction (equivalent to 100 g starting sample). Chromatograph a portion of aldrin before each sample, and calculate R_A value (retention time relative to aldrin) of each peak in the sample chromatogram, using a millimeter rule to measure retention times. Record R_A of gas chromatographic peaks in the range R_A = 5–20. Peaks in this range are indicative of the presence of chick edema factor. The presence of broad bands with no definite peaks is not indicative of the presence of chick edema factor.

(*Note:* Types of samples which are found from experience to be generally free of components characteristic of toxic fats may be examined as described above in 100 g portions, the sample saponified by refluxing with 210 ml alcohol and 50 ml 50% (w/w) KOH, and all of each of the polar alumina fractions gas chromatographed.)

[3] This setting will have a resistance of 12.8 ohms when the chromatograph is used with a 1 mv strip chart recorder.

[4] Minneapolis-Honeywell Model Y 153X (Minneapolis-Honeywell Regulator Co., Philadelphia, Pa.), or equivalent.

Results and Discussions

Relative Retention Times of Chlorinated Pesticides and Chlorinated Materials from Toxic Fats.—A number of chlorinated pesticides and several chlorinated materials isolated from toxic fats were chromatographed in the Dohrmann instrument at 248°C with the 3′ column. Retention times relative to aldrin (R_A) are shown in Table 1. The pesticides are representative of the whole range of retention times displayed by chlorinated pesticides. A toxic factor isolated from triolein (6), an inactive analogue, and a concentrate prepared from a toxic fat, all yielded chromatograms with peaks of R_A = 5 or greater whereas the pesticide peaks were all less than R_A = 4. The toxic factor from triolein as well as the toxic fat concentrate produced chick edema when fed to young chicks at a level of 0.1 ppm in the diet.

Table 1. Relative retention times of chlorinated pesticides and materials isolated from toxic fats

(3 foot, ⅛ in. diameter column, 20% silicone grease, 80% Chromosorb W; carrier gas flow rate, about 60 ml/min.; column temperature, 248°C; injection block temperature, 270°C)

Sample	Retention Time vs. Aldrin (R_A)
Chlordane	1.0
Heptachlor	0.9
Kepone	2.2
Mirex	3.6
Strobane	0.5–3.5
Tedion	3.4
Toxaphene	0.6–3.8
Toxic factor from triolein	5.0
Inactive analogue from triolein	9.0
Concentrate from a toxic fat	2.3, 3.6, 5.4

The chick edema-producing factors isolated by Wootton and co-workers (7) had retention times relative to methyl arachidate of 1.17, 3.02, and 3.17 when chromatographed at 250°C on a 20% silicone column. Since aldrin elutes twice as fast as methyl arachidate under these conditions, it would be expected that these toxic factors would have R_A values of about 2.4, 6.0, and 6.3 at 250°C on silicone columns. When a low-melting inactive isomer[5] having the same retention time (R_A = 6.0) as one of the toxic factors was chromatographed in the Dohrmann instrument, the following R_A values were obtained at 246°, 248°, and 250° respectively: 6.0, 6.4, and 6.2.

Preliminary Analysis of a Group of Toxic and Nontoxic Fats. Microcoulometric Analysis of Unsaponifiable Matter Without Prior Fractionation on Alumina.—A group of 7 toxic and 7 nontoxic fats were examined initially. The fats are described in Tables 2 and 3, respectively. Presence of chick edema disease was determined by bioassay (2) using a special basal ration. Organic halogen in the unsaponifiable matter (assumed to be chlorine) was determined by microcoulometric gas chromatographic analysis of 50 mg portions of unsaponifiable matter without prior fractionation by alumina chromatography. Both toxic and nontoxic fats contained widely variable amounts of unsaponifiable matter and organic chlorine. Sources refer to individual manufacturers. Gas chromatograms of unsaponifiable matter from two of the toxic fats (Nos. 1 and 2) show peaks with R_A values of 14. Gas chromatograms of unsaponifiable matter from the other toxic fats and from nontoxic fats obtained without prior fractionation by alumina chromatography were similar; most of the organic halogen eluted in 2–4 minutes, and there were no peaks with R_A values greater than 3. Since components with R_A values of 5 or more are usually present in the unsaponifiable matter of toxic fats at very low levels, a concentration step by alumina chromatography is necessary prior to microcoulometric analysis.

Table 2. Data on toxic fats

Component	Manufacturer	% Unsaponifiable Matter	Organic Cl in Unsaponifiable Matter, ppm
1. Tallow acids, still distillate	1	18.3	10
2. Tallow acids, still distillate	1	14.8	47
3. Tallow acids, still distillate	2	10.1	14
4. Tallow acids, still distillate	3	2.9	25
5. Tallow acids, still residue	1	4.5	2000
6. Tallow	2	5.1	39
7. Fat from broiler feed	4	2.5	8000

[5] Supplied by Dr. N. R. Artman, Procter and Gamble Co., Cincinnati, Ohio.

Table 3. Data on nontoxic fats

Component	Manufacturer	% Unsaponifiable Matter	Organic Cl in Unsaponifiable Matter, ppm
1. Cottonseed oil (CSO)	8	0.6	80
2. Cottonseed oil	8	0.5	13
3. CSO foots, still residue	6	12.1	5
4. CSO fatty acids	2	0.4	31
5. Vegetable oil foots	5	2.2	28
6. Tallow fatty acids, still residue	6	2.2	7
7. Corn oil	7	0.6	150

Fractionation of Unsaponifiable Matter on Alumina Prior to Microcoulometric Analysis.—The 7 toxic and 7 nontoxic fats were then analyzed by procedures essentially as described above. At first, 6-foot, and later, 3-foot chromatographic columns were used in the microcoulometric gas chromatograph. With shorter columns, faster elution permitted analysis of 12 samples each working day, and results were comparable to those obtained with the conventional 6-foot columns. Because 10–100 mg portions of sample were repeatedly injected, all components of the microcoulometric gas chromatograph were cleaned every 2–4 weeks as required. Gas chromatographic columns were replaced each 1–2 months. In cases where samples contained large amounts of unsaponifiable matter, larger alumina columns were used for the column chromatography so that the ratio of alumina to unsaponifiable matter was at least 20 to 1. In these cases appropriately larger amounts of eluting solvents were also used.

Fractions obtained by adsorption chromatography on alumina of unsaponifiable matter were analyzed by microcoulometric gas chromatography. Gas chromatograms of polar fractions from toxic fats (eluted with 5% and 25% ethyl ether in petroleum ether) all showed peaks with R_A values greater than 5. No peaks with R_A values greater than 5 were found in gas chromatograms of these fractions from the nontoxic fats.

Portions of alumina fractions 2 and 3 equivalent to only about 50 g of the nontoxic still residues (Table 3, samples 3 and 6) were chromatographed in the Dohrmann instrument because of the presence of a large amount of crystalline material in these fractions. The infrared spectrum of this material (isolated by recrystallizing from petroleum ether) resembled that of dipalmitone. An additional cleanup procedure must be developed for routine analyses of 100 g samples of such still residues. Additional examination of unsaponifiables from 2000 g portions of three nontoxic vegetable oils (Table 3, samples 1, 2, and 7) failed to reveal chromatographic peaks with R_A values greater than 3.

Table 4. Slow-eluting peaks in microcoulometric gas chromatograms from analysis of toxic fats

No.	Toxic Fat	Manufacturer	$R_A \geq 5$
1	Tallow acids, still distillate	1	6, 9, 12, 21
2	Tallow acids, still residue	1	6, 9, 10, 14
3	Tallow acids	1	6, 9, 10, 18
4	Tallow	2	6, 9, 12
5	Tallow acids, still distillate	2	6, 9, 10, 18
6	Tallow acids	3	6, 10

Table 4 lists the slow-eluting peaks found in gas chromatograms from 6 of the 7 toxic fats. Figure 1 shows chromatograms of alumina fraction 3 from three still distillates, each of which was obtained from a different manufacturer of commercial fatty acids. A similar pattern of slow-eluting peaks sug-

gests that a common complex contaminant may be responsible for the presence of chick edema factor in fats. Each peak probably represents a complex mixture of closely related compounds. In fact, when polar alumina fractions from several toxic fats were further fractionated by additional column chromatography on alumina, such purification often resulted in partial resolution of the corresponding peaks into at least 2 components.

The fat from a chick edema-producing sample of broiler feed (toxic sample 7) contained over 400 ppm chlordane, and this sample required special treatment because large quantities of chlordane eluted in alumina fractions 2 and 3. Although the R_A of chlordane = 1, the large amounts present produced overloaded chromatograms which interfered with gas chromatographic detection of other components. A portion of combined fractions 2 and 3 was molecularly distilled in a "cold finger" pot still for 2 hours at 55°C and 50 μ pressure. The chlordane was volatile under these conditions and was eliminated from the residue which was analyzed in the Dohrmann instrument. Peaks with R_A values greater than 5 were then found in the chromatograms.

These results indicated that alumina chromatography of unsaponifiable matter followed by microcoulometric gas chromatography of appropriate fractions might be used as a screening procedure to detect chick edema factor in fats and fatty acids. Additional work on cleanup procedures is required before this technique can be applied routinely to examination of 100 g samples of low-grade fats, such as still residues containing large amounts of material that elute in alumina fractions 2 and 3.

Effect of Alumina Activity and Column Dimensions on Adsorption Chromatography.—Alumina activity was found to affect the rate of elution of substances from toxic fats which are responsible for the gas chromatographic peaks of $R_A = 5$ and greater which are characteristics of toxic samples. Various batches of Merck alumina used for this work were found to vary in activity from

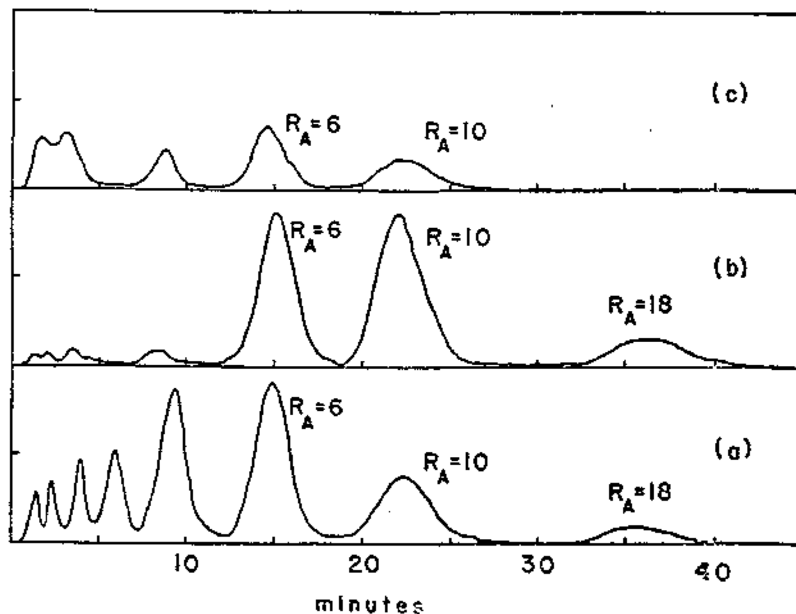

Fig. 1— Microcoulometric gas chromatograms of alumina fraction 3 isolated from toxic batch still distillates obtained from 3 manufacturers of commercial fatty acids. The fractions were isolated from (a) 5 g, (b) 5 g, and (c) 20 g of fat.

Brockmann activity I to activity II. Activities were determined by observing the rate of travel of solutions of specific pairs of azo dyes (13, 14). Using activity I alumina, unsaponifiable matter from toxic fat or from toxic fat added to USP cottonseed oil was eluted from the columns so that the characteristic slow-eluting peaks were found in gas chromatograms of alumina fraction 3. With activity II alumina, these peaks were found in chromatograms of alumina fraction 2.

Generally, the alumina used in this work was not standardized; it required gas chromatographic analysis of alumina fractions 2 and 3. Standardization of the alumina should permit elution of the slow-eluting compounds in one fraction, reducing the number of samples required for gas chromatography. For example, Merck alumina heated 48 hours at 200°C had a Brockmann activity I, and all the slow-eluting peaks from several toxic fats examined were found in chromatograms of fraction 3. Work is continuing on a procedure for standardizing alumina in a simple and reproducible manner.

Column dimensions also were found to affect the elution of characteristic substances from toxic fats. When activity I alumina was used, these substances were eluted in fraction 3 from a 25 × 300 mm column, whereas they eluted in fraction 2 from a 30 × 300 mm column.

Effect of Column Temperature and Flow Rate on Gas Chromatography of Slow-eluting Components of Toxic Fats.—Studying the gas chromatographic behavior of chlorinated pesticides, Burke and Johnson (15) found that varying the column temperature and/or the carrier gas flow rate resulted in variations of relative retention times of the pesticides. Similar variations in relative retention times were observed with the slow-eluting components of toxic fats. R_A values, however, were affected more by variations in temperature than in flow rate. A toxic substance isolated from triolein (6) had the following R_A values at 246°, 250°, and 252°C: 5.6, 4.9, and 4.7. An inactive analogue isolated from the sample had the following R_A values at these temperatures: 9.7, 8.0, and 7.8.

Because of the design of the microcoulometric gas chromatograph used for this work, whereby oven temperatures are controlled only by a variable transformer, line voltage fluctuations result in continuous variation of column temperature. A variation of ± 1°C within a 1-2 hour period is the best stability to be expected. Even with these variations, however, the instrument is suitable for detection of slow-eluting materials in toxic fats because of the large difference in retention times between these slow-eluting materials and the chlorinated pesticides and other fast-eluting chlorinated materials found in all fats examined.

Analysis of Bioassay Collaborative Samples.—A recent collaborative study of the AOAC bioassay method for detection of chick edema disease (12) indicated that the lower limit of sensitivity was obtained with a test sample containing 1.56% of a toxic fat in USP cottonseed oil. One hundred g portions of this sample, the toxic fat, and the original cottonseed oil were analyzed. Extracted unsaponifiables were chromatographed on 50 g alumina as described above, and the 5% and 25% ethyl ether eluates (fractions 2 and 3) were gas chromatographed. Chromatograms of fraction 2 from the cottonseed oil without and with added toxic fat are shown in Figs. 2 and 3, respectively. Peaks with R_A values greater than 1.5, including the $R_A = 6$ and $R_A = 10$ peaks, are due to the toxic fat. A chromatogram of alumina fraction 2 from 100 g of test sample containing 0.78% toxic fat in

Table 5. Analysis of USP cottonseed oil containing various levels of added toxic fat; microcoulometric gas chromatography of alumina fraction 2

Toxic Fat Added, %	Disc Integrator Response (No. of Pen Strokes)	
	$R_A = 6$	$R_A = 10$
0.00	0	0
0.78	6	2
1.56	14	6
3.12	32	13
4.68	32	12
6.24	47	28

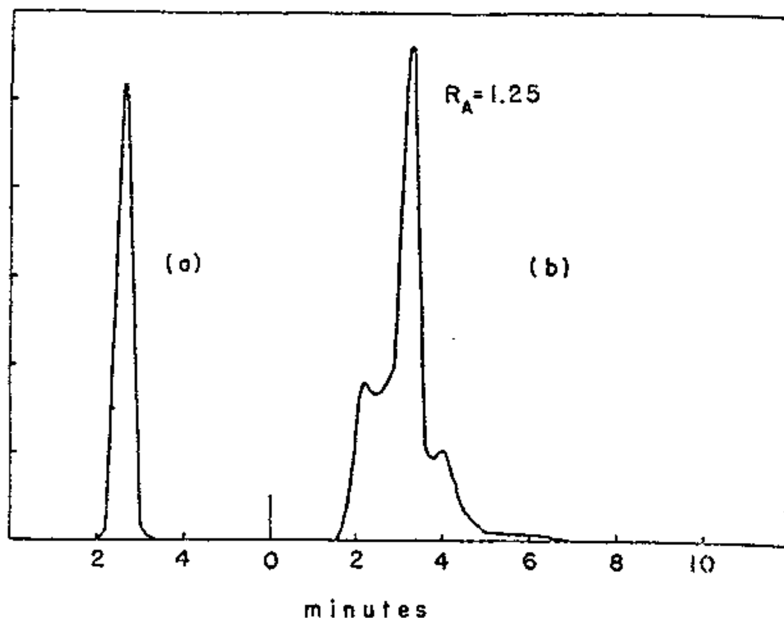

Fig. 2—Microcoulometric gas chromatograms of (a) aldrin standard (1 μg), and (b) alumina fraction 2 from USP cottonseed oil without added toxic fat.

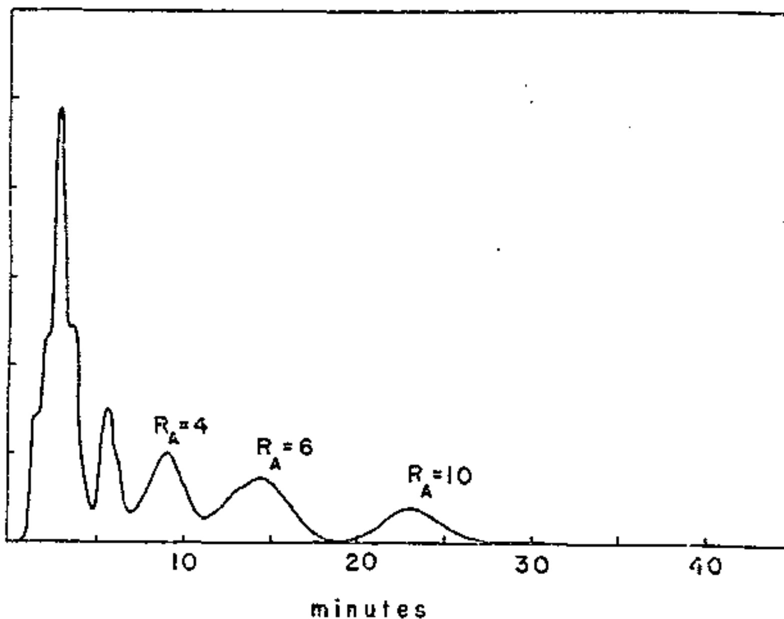

Fig. 3—Microcoulometric gas chromatogram of alumina fraction 2 from USP cottonseed oil containing 1.56% toxic fat.

the USP cottonseed oil is shown in Fig. 4. The $R_X = 6$ and $R_X = 10$ peaks can still be definitely detected at this level of toxic fat, which was half of that found to be at the lower limit of detection of the AOAC bioassay.

Samples of USP cottonseed oil containing various levels of added toxic fat up to 6.24% were analyzed. The disc integrator response of the $R_X = 6$ and $R_X = 10$ peaks are shown in Table 5. The integrator response (number of pen strokes) is approximately proportional to the level of added toxic fat in the cottonseed oil. Each stroke is equivalent to about 0.05 μg of organic halogen.

Analysis of Commercial Oleic and Stearic Acids and Derivatives.—Twelve food grade oleic acids were examined by the chromatographic method. The examination of 9 of these samples for chick edema toxicity was reported earlier (9). Gas chromatograms of alumina fractions from a nontoxic and a toxic acid are shown in Figs. 5 and 6, respectively. Chromatographic peaks of $R_X = 6$ and greater from the toxic sample are shown in Fig. 6. These peaks were present in alumina fractions 2 and 3. The "peak" with $R_X = 6$ in the chromatogram from alumina fraction 1 is believed to be an artifact due to overloading of the coulometer.

Results of analysis of the 12 oleic acids, compared with the chick bioassay using a special basal ration (2), are shown in Table 6. Hydropericardium activity, the primary index of the presence of chick edema factor, was estimated as described by Firestone and co-workers (9). Six samples were positive by both the bioassay and chromatographic methods. One sample (No. 9) was negative by the regular bioassay when fed at the usual level of 16% in the diet. However, a positive response was obtained when extracted unsaponifiable matter was fed at a level equivalent to 6 times that present in the normal test diet.

Sample 10 was negative by the chick bioassay, but gave a weak positive response by the chromatographic method. A small chromatographic peak with $R_X = 10$ was obtained from alumina fraction 2. In comparing bioassay activity and peak areas of slow-eluting components of the other toxic oleic acids, it would be expected that the level of chick edema factor in sample 10

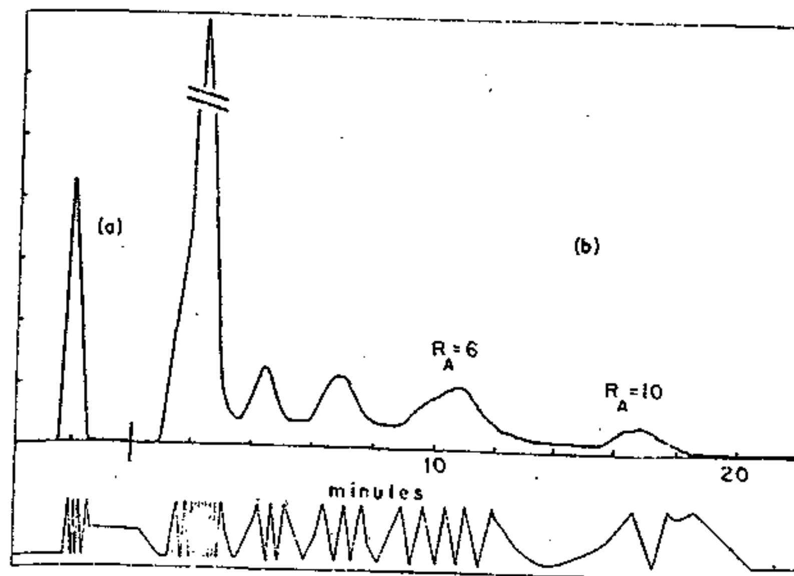

Fig. 4—Microcoulometric gas chromatograms of (a) aldrin standard (1 μg), and (b) USP cottonseed oil containing 0.78% toxic fat. Also shown are disc integrator pen strokes.

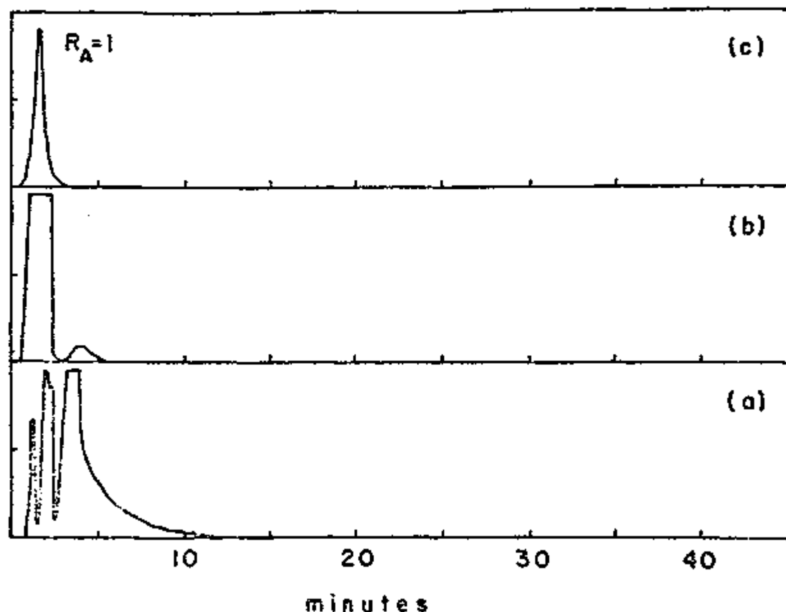

Fig. 5—Microcoulometric gas chromatograms of (a) alumina fraction 1, (b) alumina fraction 2, and (c) alumina fraction 3 from a non-toxic commercial oleic acid.

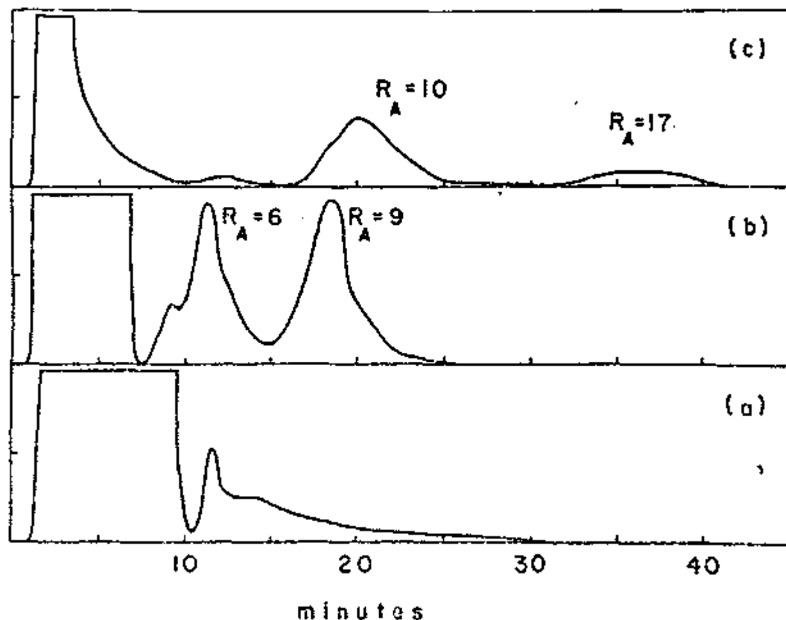

Fig. 6—Microcoulometric gas chromatograms of (a) alumina fraction 1, (b) alumina fraction 2, and (c) alumina fraction 3 from a toxic commercial oleic acid.

Table 6. Analysis of commercial oleic acids

Sample No.	Bioassay: Hydropericardium Activity (θ)	Chromatographic Analysis, Relative Peak Area (Integrator Strokes[a])		
		R_A = 5-6	R_A = 9-10	R_A = 15-18
1	+0.2	74	150	9
2	+3	340	90	8
3	+0.3	98	196	104
4	+0.1	118	156	9
5	+0.2	46	88	14
6	—			
7	—			
8	—			
9	+0.1[b]	106	105	25
10	—		0.6	
11	—			
12	—			

[a] Each stroke is equivalent to about 0.05 μg organic halogen.
[b] Unsaponifiable matter fed at a level equivalent to 6 times that present in the normal test diet.

might be below that *detectable* by the bioassay.

In comparing hydropericardium activity and gas chromatographic response (relative peak area), no parallel relationship was found between the bioassay response of the toxic oleic acids and the chromatographic response. It should be emphasized, however, that the slow-eluting compounds in toxic samples represent both toxic and relatively nontoxic materials, and most of the gas chromatographic response is probably due to relatively nontoxic substances. Nevertheless, microcoulometric gas chromatographic detection of slow-eluting compounds appears to be an effective screening tool for segregating questionable samples and diverting them to nonedible uses or for further testing by bioassay.

The gas chromatographic response of the R_A = 9 peak present at widely different levels in 2 oleic acids was checked by analysis of individual samples on different days, using different gas chromatographic columns for each run. Results are shown in Table 7. Sample 1 contained a low level of material responsible for the R_A = 9 peak. The differences in integrator response are due both to variation in coulometer response and to inaccuracies of disc integrator response at low halogen levels. The larger amounts of material in sample 2 responsible for the R_A = 9 peak were distributed in alumina fractions 2 and 3 to varying extents depending upon the alumina activity. The total integrator response from both alumina fractions was fairly constant, however, varying from 136 to 169 integrator pen strokes.

In addition to the oleic acids, a number of derivatives of oleic acid (triolein, glycerol monooleate, etc.) were analyzed. Nine of ten samples were negative by both the bioassay and chromatographic analysis. One toxic sample, a triolein, gave gas chromatograms with peaks of R_A = 6, 10, and 19.

Ten stearic acids and derivatives examined were negative by the bioassay and chromatographic method.

Analyses of Animal and Vegetable Fats and Commercial Vegetable Oil Fatty Acids.—Fifteen animal and vegetable fats were examined. Three toxic animal tallows were positive by the chromatographic method.

Table 7. Response[a] of R_A = 9 peak from 2 oleic acids

Run No.	Sample 1		Sample 2		
	Fraction 2	Fraction 3	Fraction 2	Fraction 3	Total
1	0.2	0	20	116	136
2	0.8	0	96	48	144
3	0.6	0	168	0.5	169
4	5.0	0	100	50	150

[a] Disc integrator response in number of disc integrator pen strokes.

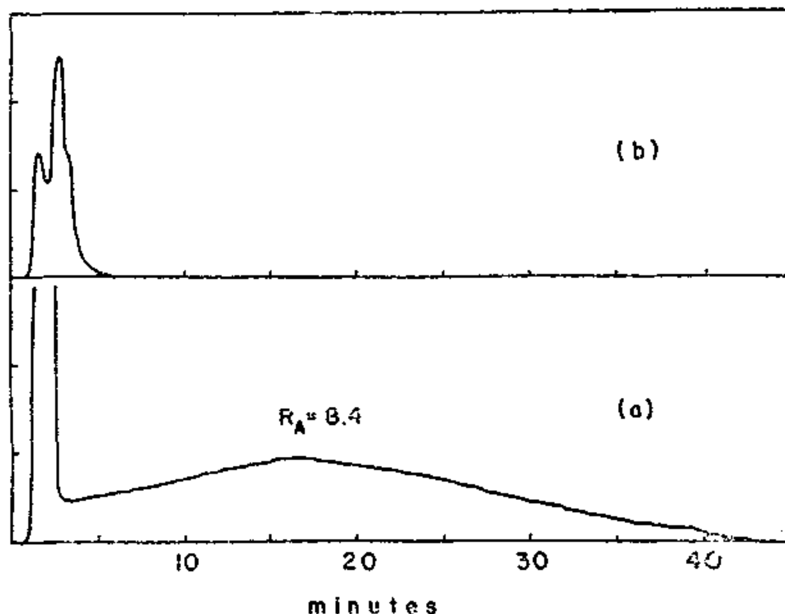

Fig. 7—Microcoulometric gas chromatograms of (a) alumina fraction 2, and (b) alumina fraction 3 from a growth-depressing cottonseed oil which did not produce chick edema disease.

Ten vegetable fats (including cottonseed oil, corn oil, peanut oil, safflower oil, and soybean oil) were examined, and were negative by the bioassay and chromatographic method. Chromatograms from several of the vegetable oils showed broad bands with no definite peaks. Chromatograms of alumina fractions 2 and 3 from one of these samples, a USP cottonseed oil, are shown in Fig. 7. The broad band in chromatogram (a) is typical of that found in the other oils. This sample depressed the growth of young chickens, but did not produce symptoms of chick edema disease. The broad band in chromatogram (a) with a maximum at 8.4 is not characteristic of a toxic fat. No additional work has been done to identify the substances causing these broad bands.

Twelve commercial vegetable oil fatty acids, nontoxic by the chick edema bioassay, were examined. These samples included fatty acids from coconut, cottonseed, corn, palm, soybean, and tall oils. Eleven of the samples were negative by the chromatographic method, but one of the samples (a tall oil fatty acid) gave a small chromatographic peak with $R_A = 5$, indicative of a toxic sample.

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The Injection of Chemicals into the Yolk Sac of Fertile Eggs prior to Incubation as a Toxicity Test

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The increasingly large number of food additive chemicals introduced into the market each year has necessitated the development of rapid and reliable methods for the evaluation of their toxicity. Toxicologic studies of all these chemicals by the usual methods using animals are very difficult, and such studies sometimes give inconclusive results.

The toxicity of some chemicals, and especially of food additives, may be determined by injection of the chemical into the yolk sac of fertile eggs prior to incubation and subsequent observation of the effects of the chemical on the embryonic development of the chick. This appears to be a promising method in that it may be carried out much more economically in terms of money and space than would be possible with larger animals. Hundreds of chicken embryos may be observed in a minimum of space, and over a comparatively short period of time. The feasibility of using such large numbers is valuable also in the statistical evaluation of toxicity data.

A review of the literature shows how little work has been done in this field except in a fragmentary way on isolated cases. Most of the reports refer to injections of chemicals made after the fourth or eighth day of incubation and examination of the embryos killed before they hatch.

The earliest work that we have found in the literature was by Féré (1893). During ten years after this date he published about sixty-seven papers; a review article (Féré, 1899) contains a summary of many of his studies. His work consisted mainly of injection before incubation. but the eggs were usually opened on the third day of incubation. His interest was mainly in the teratogenic effect of chemicals.

Since 1900 other articles have appeared in the literature, but most of them concern the effects of one or two chemicals on a small number of embryos. One of the most informative papers is that of Ridgway and Karnofsky (1952) in which they report experiments on compounds containing fifty-five of the elements, mainly the metallic ones, generally injected at either the fourth or eighth day of embryonic development to study toxicity and teratogenic effects. Hamburger and Hamilton (1951) have described an elegant method for determining the stages of development of the chick embryo. More recent investigations employing the chick embryo to study the toxicity of various chemicals have been reported by Kemper (1962), Platt *et al.* (1962), and Goerttler (1962). Finally, the classic books of Romanoff and Romanoff (1949) and Romanoff (1960) contain a wealth of information on the avian embryo.

Preliminary reports of this investigation have been presented by Marliac (1962) and McLaughlin and Mutchler (1962).

EXPERIMENTAL

The fertility and hatchability of eggs and the livability of chicks are dependent on a complex interrelationship of ecological factors, among which are the genetic background and the age of the mated birds, the nutritional status and general management of the flock, and seasonal variations. In view of this the initial phase of our work, which started in 1959, was devoted to a study of the hatchability of our supply of White Leghorn eggs[1] under conditions existing in our laboratories. The data accumulated during two years for control eggs showed that the hatchability of these eggs was, in fact, consistent, reproducible, and very high. The possibility of a seasonal variation occurring in responses to compounds introduced into the eggs was also examined by repeated testing of several chemicals at all seasons of the year. No important variation was detected.

Selection of eggs. Eggs to be injected are first candled in order to discard those that are defective and to outline with a pencil the exact location of the air cell. In our laboratory 9% of 5000 eggs had to be discarded: 2% cracked, 4% with improperly calcified shells, 1.5% with a tremulous air cell, 1% with the air cell in the wrong place, and 0.5% with blood clots. After the elimination of such defective eggs, the batch of control eggs averages 95%. A further restriction is based on the weight of the eggs: all those weighing less than 52 g or more than 63 g are rejected. After

[1] Truslow Eggs, Chestertown, Maryland.

candling, the eggs are randomized in order to avoid series of infertile eggs in any one experiment.

The initial experiment with a given chemical is for range-finding and is performed at two or more concentrations of the chemical with 10 eggs per level. On the basis of this information, 20 or more eggs are injected with the appropriate amount of the chemical.

If the chemical proves to be nontoxic, the experiment is repeated with the minimum number of eggs that will give a reliable and reproducible value for the hatchability. In the case of a toxic chemical, additional eggs are injected to determine the specific effects of the chemical. The total number of eggs used for a chemical depends upon the data obtained initially and upon the kind of information desired. Hence, data for some chemicals are based on less than one hundred eggs, whereas data for others are based on several hundred eggs.

Technique of injection. The injections of pure chemicals, chemical solutions, or suspensions are made at volumes up to 0.10 ml. When necessary, dilutions are made with solvents such as water, propylene glycol, corn oil, peanut oil, or other nontoxic solvents.

In order to avoid contamination, the injections are carried out in an Isolator Box[2] with a sterile atmosphere created by using formaldehyde vapors (produced by mixing 2 g of potassium permanganate and 50 ml of 37% formalin). During the period of a year, more than fifteen hundred noninjected eggs were exposed to formaldehyde vapors; no toxic effect was noticed. After exposure to these vapors for 30 minutes, the eggs are ready for injection.

The large end of the egg is wiped with a sterile gauze pad moistened with a 70% alcohol solution, and a hole is drilled in the shell in the center of the surface over the air cell (Fig. 1). Care must be taken not to damage the shell membrane with the point of the drill[3]; this is to avoid, if possible, contact of the air with the egg membrane. Fine particles of shell are removed with an aspirator to prevent the needle from carrying them into the yolk.

Immediately before the injection, each egg is shaken with a quick twist of the wrist. Since the germinal disc occasionally sticks to the air cell and it is possible to damage it with the needle, this movement will allow the disc to float free in the egg.

[2] Kewaunee Scientific Company, Adrian, Michigan.

[3] Burgess Vibrocrafters Inc., Grayslake, Illinois.

The needle (hypodermic, 1 inch long, either no. 22 or no. 27, depending on the viscosity of the liquid to be injected) is inserted horizontally through the air cell into the yolk (Fig. 1). Care must be taken in withdrawing the needle to avoid damaging the vitelline membrane since such damage could cause the yolk to spread out in the albumen. If the end of the needle has some yolk on it, the injection is not satisfactory. The needle should be wiped with a sterile gauze pad between each injection. As soon as the egg has been injected, the hole in the shell is covered with a small piece of Scotch tape, care being taken not to cover the entire air cell.

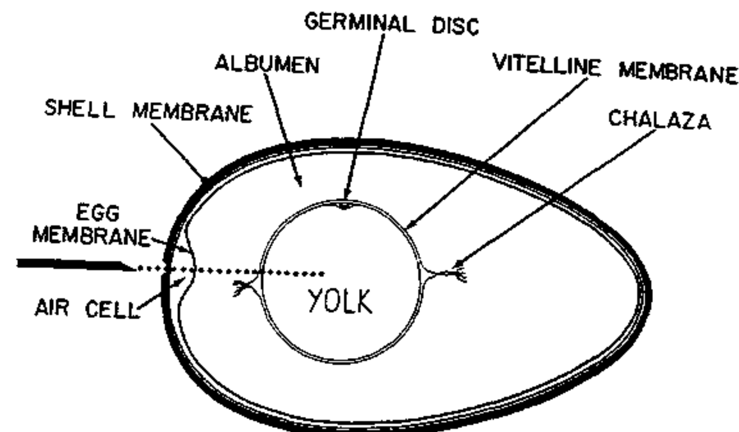

Fig. 1. Diagram of egg and position for injection.

Incubation and hatching. The injected eggs are put into the incubator trays with the large end up; the trays are placed in the incubator,[4] which automatically rotates hourly and is maintained at an optimum temperature of 38°C and a relative humidity of 60%. The eggs are candled on the fifth day of incubation and every day thereafter. Clear eggs and dead embryos are removed for examination. On the seventeenth day of incubation the fertile eggs are transferred to the hatcher[5] and kept at a temperature of 37°C until they hatch.

EVALUATION OF DATA

The injection of the chemical into the egg may produce one of four possible results: (1) the chemical is highly toxic at the level injected, and

[4] Humidaire Incubator Co., New Madison, Ohio; model no. 50, capacity 450 eggs.

[5] Brower Manufacturing Co., Quincy, Illinois.

all the embryos are killed during the first 20 hours of incubation (before the two-somite stage); (2) the chemical is toxic but allows a number of embryos to develop only up to a certain point, and some possibly even to hatch; (3) the chemical has little effect on the hatch; and (4) the chemical has no effect on the hatch or on the posthatch development of the chick ("no effect" level).

If the chemical appears to be highly toxic and all the embryos are killed, the experiment is repeated with smaller doses of the chemical until some hatch is obtained. If the chemical is toxic, but allows the embryos to develop for a longer period of time, dead embryos are examined pathologically and the chicks that do hatch are examined for eye damage, color of the feathers, weight, length of the legs, form of the beak and of the rump, hematologic changes, and condition of the internal organs (liver, kidneys, heart, gall bladder, and spleen).

It is advisable in all cases to observe the chicks for a period of a few weeks in order to detect any delayed effects. Since as much as 30% of the yolk remains at the time of hatching and is absorbed during the first 7 days thereafter, effects of a toxic chemical may first be observed at this time. There may be weight retardation, death during the first week, or the appearance of nerve damage occurring as late as 2–6 weeks after hatching.

The toxicity of a chemical is evaluated mainly from the percentage of hatch at varying dosages of the chemical as compared to noninjected (control) eggs, from a study of the embryonic development of the eggs that fail to hatch, and from a study of the appearance and development of the chicks that do hatch. However, several other factors must also be considered in this evaluation: these are specific gravity, solubility, coagulating effect, pH, and the ionic concentration of the chemical tested.

If the chemical has a high specific gravity, there is the possibility of its settling out in the bottom of the egg and thereby giving a value of apparently low toxicity.

The solubility is quite important since the availability of the chemical for utilization in the chick embryo is partially dependent on its solubility in the egg. However, since egg yolk is an emulsion, solubility problems are somewhat minimized. In the case of insoluble chemicals that are injected as suspensions, it is also necessary to consider particle size in the evaluation of toxicity data.

In order to have a toxic effect, the chemical must come in contact with the embryo either directly or indirectly through the bloodstream. Chemicals such as the lower aliphatic alcohols have a coagulating effect on the protein, and this coagulation may decrease the availability of the chemical as well as some yolk nutrients, and thereby alter the response of the embryo.

If the pH is highly acidic or basic, a pH effect differing from the true toxic effect of the chemical may be obtained due to interference with the normal acid-base equilibrium in the egg.

The ionic concentration is also important for a similar reason. The observation of increasing toxicity with increasing concentration of a chemical should be interpreted cautiously, since highly concentrated solutions may upset the physical equilibrium of the yolk by causing osmotic effects.

Finally, the introduction of a chemical into the yolk may cause a special type of toxicity because it destroys, alters, or combines with essential nutrients such as vitamins and minerals.

EXPERIMENTAL DATA

Twenty-five thousand eggs have been used in our laboratory during the past three years to test more than 100 chemicals with the following results: (1) nontoxic chemicals injected at an appropriate level allowed the embryo to develop and to hatch as did the controls; (2) toxic chemicals produced effects at dose levels which may be compared to those producing effects in feeding experiments using animals; and (3) this technique often provided toxicologic information which had not been shown by conventional methods.

TABLE 1
NONTOXIC CHEMICALS

Chemical	Solution injected		Per cent hatch
	Concentration	Quantity (ml)	
Water (boiled)	—	0.05	95
Propylene glycol	Undiluted	0.05	95
Corn oil	Undiluted	0.05	90
Peanut oil	Undiluted	0.05	90
Sodium chloride	0.9% in water	0.05	90
	5.0% in water	0.05	70
	5.0% in water	0.10	30
	10.0% in water	0.05	20
Dextrose	5.0% in water	0.05	90

Table 1 lists the results obtained with some chemicals in common use in food and shows the dosages used and the percentages of hatched chicks.

TABLE 2

Chemicals with a High Order of Toxicity

Chemical	Solution injected: Concentration	Solution injected: Quantity (ml)	Per cent hatch	Remarks
Lead acetate	10% in water	0.05	0	—
	2% in water	0.05	0	Hydrocephalus in embryos that failed to hatch
Mercuric chloride	0.5% in water	0.05	20	—
	1% in water	0.05	0	All embryos died at the beginning of incubation
Thiourea	10% in water	0.05	0	Some embryos lived up to 34 days
	5% in water	0.05	20	Hatched on the 28th day of incubation; growth retardation
Triorthocresyl phosphate	Undiluted	0.02	0	Most of the developing embryos died on the 15th day of incubation
	Undiluted	0.01	40	Hatched chicks showed growth retardation and developed paralysis 2–6 weeks after hatching
p,p'-Diaminodiphenylmethane	10% in ethanol	0.05	30	Leg damage; beak deformity (short mandible)
Sodium selenite	0.2% in water	0.05	0	—
	0.1% in water	0.05	20	—
	0.1% in water	0.02	80	—
Dibutyl-tin-dilaurate	Undiluted	0.01	0	Beak deformity
Aroclor 1242 (chlorinated polyphenyls)	Undiluted	0.025	0	—
	Undiluted	0.01	5	Beak deformity (short upper beak); edema; growth retardation

These data, supplemented by an examination of the nonviable eggs and an autopsy of the chicks that hatched, have not indicated any hazard from their use in food. However, it must be pointed out that any chemical, added at a sufficiently high concentration, may have some toxic effect. Since our data and those reported in the literature indicated safety, this phase of the study was not carried beyond a preliminary examination to ascertain that nontoxicity also was shown for these chemicals by the chick embryo technique. This is of theoretical as well as practical importance for the evaluation of any new technique to be used in toxicologic studies.

Table 2 lists our results with several chemicals which have been shown to be highly toxic to animals. In each case there is not only the low percentage of hatch at a low level of the chemical tested, but there are also congenital abnormalities and other responses that raised extremely serious questions as to the safety to the consumers of any food contaminated with these chemicals.

Lead acetate resulted in no hatch at a level of 1 mg per egg. Autopsy of the dead embryos showed extensive brain damage, as has been reported by de Franciscis and Bocalatte (1962) and by Karnofsky and Ridgway (1952).

Mercuric chloride showed no hatch even at a level of 0.5 mg per egg.

Thiourea is a known carcinogen with a basic effect on the thyroid gland. The hatch time was delayed with increasing amounts of this chemical. At a level of 5 mg per egg no chicks hatched and the embryo required 35 days to develop to the stage normally attained at 20 days. At 2.5 mg per egg some chicks hatched, but most of them had to be helped out of the shell. This effect has been reported also by Yushok (1950).

Cavanagh (1954) reported that triorthocresyl phosphate (TOCP), a well-known plasticizer for nonfood use, causes paralysis when fed to adult chickens. We observed this paralysis in some chicks which hatched from eggs injected with 10 mg of the undiluted chemical.

The compound *p,p'*-diaminodiphenylmethane has been reported by Zylberszac (1951) to cause cirrhosis of the liver in rats. We found this chemical to be extremely teratogenic at a level of 5 mg per egg; more than 90% of the chicks had a short mandible and leg damage consisting of a severe bending of the tibia and a general shortening of the bones of the leg.

Sodium selenite proved to be highly toxic. At a level of 0.1 mg per egg, no embryos developed to more than the 5-day stage.

Dibutyl-tin-dilaurate showed no hatch at a level of 10 mg per egg. The majority of the embryos, which did not live more than 15 days at this

TABLE 3

Chemicals with an Intermediate Order of Toxicity

Chemical	Solution injected		Per cent hatch	Remarks
	Concentration	Quantity (ml)		
Acetone	Undiluted	0.05	70	* At these levels: corneal opacity, cataract, cleft palate, nerve and kidney damage
n-Butanol	Undiluted	0.01	95	
		0.02*	70	
		0.03*	20	
		0.04	0	
Carbon tetrachloride	Undiluted	0.05	40	
		0.10	0	
Diethylene glycol	Undiluted	0.05	80	
		0.10	55	
Ethyl acetate	Undiluted	0.05	35	
		0.10	15	
Ethyl alcohol	Undiluted	0.05	95	
		0.10	60	
		0.30	0	
Ethylene glycol	Undiluted	0.05	80	
		0.10	65	
Di-2-ethylhexyl phthalate	Undiluted	0.05	95	
Heptachlor	1% in propylene glycol	0.05	75	
		0.10	65	
Hydrochloric acid	4% in water	0.05	80	
Isopropanol	Undiluted	0.05	35	
		0.10	15	
Malathion	Undiluted	0.05	60	Short legs; bleaching effect on chick down
		0.10	0	
Methanol	Undiluted	0.05	90	
		0.10	65	
Styrene	5% in 80% ethanol	0.05	95	

level, had a short and/or flexed mandible; in addition, some embryos showed subcutaneous edema.

Aroclor 1242 gave no hatch at a level of 25 mg per egg. At a level of 10 mg per egg, one chick hatched out of 20 injected eggs, but died 2 days later. Some embryos, which were examined after they died, showed beak deformities (often a short upper beak), edema, and growth retardation.

Table 3 lists preliminary data on the toxicity of some chemicals which have been shown to be toxic in some degree to animals, and which may be found in some processed foods. Included in this group are various solvents, plasticizers, and insecticides. Some of the chemicals on this list require further study, including observation of the chicks until they reach maturity, before they may be classified as to low or high toxicity.

DISCUSSION

The injection of chemicals into the yolk of fertile eggs prior to incubation is a method which can be advantageously used as an element in the evaluation of the safety of food additive chemicals and drugs, and which could be used to screen new products and eventually to correlate their toxicity with that of similar products already tested.

If one considers that a chemical injected into the yolk may be compared to a substance which has the power to cross the placental barrier, this technique, in addition to being an embryonic feeding study, assumes further importance in that it is also a reproduction study. The unfortunate experiences recently suffered with chemicals that have teratogenic effects in humans, and the failure of conventional testing methods to produce this effect in animals, emphasize the urgent necessity for new methods of analysis. Preliminary work that we have done in this area has given satisfactory results (Verrett and McLaughlin, 1963).

Since this represents a system in which the chemical is in direct contact with the embryo throughout development, it is more than likely that any toxic or teratogenic effects would be readily observed. However, there is always the possibility that the chicken will not be a species susceptible to a particular compound, just as it has been shown that the other commonly used species of animals do not respond to all chemicals in a similar manner. It is also possible for the chicken to be more sensitive to a chemical than other species.

Finally, this technique may be applied also to the study of the synergistic effects of chemicals. Results of experiments in our laboratory on

the potentiation of a few pesticides have been very encouraging (Marliac and Mutchler, 1963).

SUMMARY

An evaluation of toxicity by injection of the chemical into the yolk sac of fertile eggs prior to incubation gave the following results:

Water, propylene glycol, corn oil, peanut oil, isotonic saline solution, and isotonic glucose solution showed no toxicity or a very low order of toxicity.

Mercuric chloride, lead acetate, selenium, triorthocresyl phosphate, *p,p'*-diamino-diphenylmethane, thiourea, Aroclor 1242, and dibutyl-tin-dilaurate showed a high order of toxicity and/or teratogenic effects at certain levels.

Acetone, methanol, ethanol, *n*-butanol, diethylene glycol, ethylene glycol, iso-propanol, di-2-ethylhexyl phthalate, hydrochloric acid, carbon tetrachloride, ethyl acetate, malathion, heptachlor, and styrene showed an intermediate order of toxicity.

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Use of the Chicken Embryo in the Assay of Aflatoxin Toxicity

By M. JACQUELINE VERRETT, JEAN-PIERRE MARLIAC, and JOSEPH McLAUGHLIN, JR. (Division of Toxicological Evaluation, Food and Drug Administration, Washington, D.C. 20204)

The possibility of using the chicken embryo as a test organism for the assay of aflatoxin toxicity has been investigated and found to be feasible. The injections of test solutions were made before incubation, in fertile White Leghorn eggs, by either of two routes: yolk or air cell. The development of the embryos was observed for the full 21 day incubation period. The vehicle for all injections was propylene glycol.

The injection of solutions of pure aflatoxins B_1 and G_1, and of extracts of aflatoxin-producing mold cultures indicated that the chicken embryo was sensitive to these compounds. A dose-response was exhibited in that the toxicity of the samples was related to the mortality at the time of hatching.

Extracts of aflatoxin-free peanut products were found to be nontoxic to the chicken embryo. The addition of aflatoxin B_1 to such uncontaminated extracts produced the expected toxicity in the embryos. The injection of extracts from contaminated peanut products resulted in a toxic response that correlated well with that obtained by injection of pure aflatoxin B_1 solutions at the same dose levels, and in most instances the chemical analysis was confirmed. The presence of aflatoxins G_1, B_2, and G_2 had no apparent effect on the toxicity due to aflatoxin B_1, at the levels at which they occurred in the particular samples tested.

The separation of aflatoxin B_1 from contaminated extracts by thin-layer chromatography, and its subsequent elution from the plates and injection into the eggs, confirmed that the toxicity of these extracts was due primarily to their aflatoxin B_1 content.

The sensitivity of the chicken embryo to aflatoxins was reported in 1962 by Platt, *et al.* (1) who observed that preparations of "groundnut toxin" injected into the yolk of 5-day old chicken embryos caused deaths, and that the quantities required were about 1/200th of those required for a positive result in the day-old duckling.

An investigation of the feasibility of using the chicken embryo for a bioassay of aflatoxin toxicity is currently underway in our laboratories. The preliminary results reported here consider only the general systemic toxicity of the aflatoxins to the chicken embryo. At the present time, the studies are not sufficiently complete to verify whether the aflatoxins produce any specific pathological lesion in the embryos.

Experimental

The sensitivity of the chicken embryo to aflatoxins was studied by injecting the following: (*1*) solutions of pure aflatoxin B_1 and pure aflatoxin G_1; (*2*) extracts of aflatoxin-producing mold cultures; (*3*) extracts of raw peanuts, roasted peanuts, peanut meal, and peanut butter; and finally (*4*) aflatoxin B_1 obtained from crude extracts by elution from thin-layer chromatographic plates.

The solutions were injected into fertile White Leghorn eggs before incubation. Groups of at least 20 eggs were used at each dose level of a sample, and each sample was injected at two or more levels when there was sufficient material available. More than 8,000 eggs have been used in these studies to date.

The injections into the eggs were made by one of two routes: into the yolk, or into the air cell. The technique for injection into the yolk has been described previously (*2*). The volume injected into the yolk was 0.05 ml or less in all cases. For injections into the air cell, a hole of about 5 mm diameter was drilled in the shell over the air cell. The solution was then deposited on the egg membrane by a syringe, and the hole was sealed with adhesive cellophane tape. The eggs were allowed to remain undisturbed in a vertical position (air cell up) for about an hour to let the material disperse. The volume injected into the air cell was restricted to 0.04 ml or less.

The solvent used for all injections was propylene glycol, which was known, from previous investigations (*3*), to be nontoxic in the eggs at the levels used. However, eggs were periodically injected with this solvent in appropriate amounts and incubated with the aflatoxin-injected eggs. Noninjected controls and drilled-only controls were also included in the experiments to provide the necessary data on the background mortality.

The eggs were incubated (2) and candled daily from the fourth incubation day on, at which times all nonviable embryos were removed and examined grossly.

Results and Discussion

Purified Aflatoxin Solutions and Mold Culture Extracts.—The toxicity of solutions of crystalline aflatoxin B_1 and aflatoxin G_1 to the chicken embryo was first determined.

The toxicity of aflatoxin B_1 to the chicken embryo was greater when injected via the air cell route than when injected into the yolk. Figure 1 contains plots of the mortality at 21 days due to the injection of several dose levels of aflatoxin B_1 by both the yolk and the air cell routes. The LD_{50}s obtained were 0.048 and 0.025 μg for the yolk and air cell routes, respectively.

Aflatoxin G_1, which was injected into the yolk only, showed a lower toxicity to the chicken embryo than that obtained with

aflatoxin B_1. The injection of 1.0 μg of aflatoxin G_1 produced a mortality of 60% (at 21 days), while 2.0 μg was required to produce a mortality of 90%.

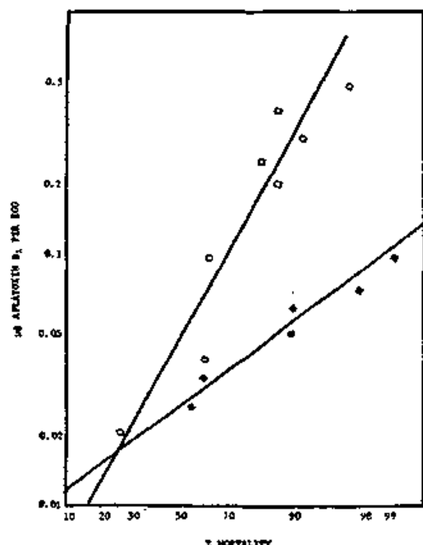

Fig. 1—Toxicity of aflatoxin B_1 in the chicken embryo: mortality at 21 days. LD_{50}: yolk, 0.048 μg; air cell, 0.025 μg. Open squares: yolk injection. Closed squares: air cell injection.

Examination of nonsurviving embryos from eggs injected with aflatoxin B_1 by either route revealed a severe growth retardation in most cases. In addition, edema, hemorrhage, underdevelopment of the mesencephalon (in embryos that died before the seventh day), mottled and granular liver surface, short legs, and slight clubbing of the down were also observed in many of these embryos.

Extracts of several cultures of aflatoxin-producing molds were used to determine the sensitivity of the chicken embryo to combinations of aflatoxins B_1, G_1, B_2, and G_2. The concentrations of these four constituents in the extracts were known from prior chemical analysis. The extracts were injected into eggs by both routes, in amounts designed to contain specific levels of aflatoxin B_1, to compare their toxicities to those of solutions of the pure aflatoxin B_1 at the same dose levels.

The toxicities of these extracts to the embryos showed a good correlation with the standard solution of aflatoxin B_1. There was no apparent alteration in the toxicity of aflatoxin B_1 due to the presence of aflatoxins B_2, G_1, and G_2 at the levels at which they occurred in these extracts.

Aflatoxin-free Extracts of Peanut Products.—The usefulness of the chicken embryo in a bioassay of aflatoxins in peanut products depends on whether the constituents of aflatoxin-free peanut product extracts are inherently toxic to the embryos.

To determine the toxicity of these materials, extracts of raw peanuts, roasted peanuts, peanut meal, and peanut butter, which were found to be free of aflatoxins by chemical analysis, were injected into eggs by both the yolk and air cell routes. In most of these experiments the equivalent of original peanut product injected ranged from 1 to 3 g per egg. The toxicity was low for all of these extracts; in general, it was equal to or only slightly higher than that of the background, which ranged from 0 to 20% mortality.

One experiment was carried out with an extract of aflatoxin-free raw peanuts, injected by both the yolk and air cell routes, in quantities corresponding to a raw peanut equivalent of 1, 2, 4, and 8 g per egg. The toxicity observed for the 8 g level was not significantly higher than that observed for the 1 g level or that of the background.

In the same experiment, known quantities of the pure aflatoxin B_1 were added to this raw peanut extract, and injected at the same levels and by both routes. This was done to determine whether the aflatoxin B_1 toxicity could be masked or enhanced by the presence of increasing amounts of peanut material. Separate groups of eggs were injected with corresponding amounts of the standard aflatoxin B_1 solution for comparison. The results of this experiment indicated that as little as 25 ppb of aflatoxin B_1 in the original raw peanuts could be easily detected by administration of the extract by either injection route, and that the toxicity was not significantly different from that obtained with the solution of pure aflatoxin B_1 of the same concentration.

Aflatoxin-contaminated Peanut Products.—Extracts from raw or roasted peanuts, peanut meal, and peanut butter, which were shown to contain aflatoxins by chemical analysis, were injected into eggs by both routes. The amount of extract to be injected in the egg was calculated on the basis of the aflatoxin B_1 content determined chemically, irrespective of the amounts of aflatoxins B_2, G_1, and G_2 present. In most instances the results corroborated the chemical analysis, since the toxicity was comparable to that obtained with the injection of equivalent amounts of the pure aflatoxin B_1 solution.

Aflatoxin B_1 Obtained by Thin-Layer Chromatography (TLC).—In order to confirm that the toxicity of the contaminated extracts was primarily due to their aflatoxin B_1 content, separate portions of some extracts were subjected to TLC and the resulting aflatoxin B_1 spots were removed from the plates, eluted, dissolved in propylene glycol, and injected into eggs.

Separate experiments were performed to confirm that no toxic materials were derived from the TLC process itself. TLC "blanks" injected into eggs in a similar manner had a very low toxicity and were comparable to background.

The toxicities of these eluted aflatoxin B_1 spots from more than 20 extracts of a variety of peanut products correlated very well with that of standard aflatoxin B_1 solution injected at the same dose level, and verified that the toxicity of these extracts was, in fact, primarily due to aflatoxin B_1.

Embryo Age and Aflatoxin Toxicity.—The toxicity of aflatoxin B_1 to the chicken embryo at various stages of incubation was also investigated. Single injections into the yolk were made up to the fourth incubation day, and air cell injections were made in embryos from 1 to 18 days old.

These experiments revealed that, with both injection routes, the embryonic sensitivity to the aflatoxin decreased rapidly as the embryo age increased, and the maximum toxic effect was obtained with pre-incubation injections.

Evaluation of Sample Toxicity.—In the course of these studies it was also observed that the toxicity of aflatoxin B_1 injected at the higher dose levels was apparent early in the incubation period, since most of the embryos did not survive beyond the eighth to tenth day. With lower dose levels, it is necessary to continue observations for the duration of the 21 day period, since many embryos survive longer than the tenth day but fail to hatch. In these instances an evaluation of the toxicity of a sample on the basis of survivors at 8 or 10 days would be premature, and the true toxicity of a sample might be underestimated.

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The Role of "Toxic Fat" in the Production of Hydropericardium and Ascites in Chickens

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Summary

When "toxic fat" was added to the diet of experimental birds at concentrations from 0.25 to 5.0% for 35 to 150 days, edema of the myocardium, skeletal musculature, and lungs; hydropericardium; ascites; and foci of lymphoytes in the myocardium and epicardium were observed. Appreciable changes were not observed in the total serum protein levels, albumin: globulin ratio, electrolyte balance, or in the nonprotein nitrogen levels of the blood. There was dilation, edema, and lymphocytic infiltration of the heart. The myocardial mitochondria were vacuolated and shrunken. An increase in venous pressure was also noticed. The fluid imbalance observed in birds that consumed toxic fat did not result from a decrease in total blood proteins or from an alteration in the albumin: globulin ratio, but was associated with cardiac decompensation and increased capillary permeability.

Schmittle *et al.*[19] were the first to incriminate some fats as responsible for the production of hydropericardium and ascites in young chickens. Subsequent studies[3,12] have demonstrated a reduction in growth rate, retarded sexual development, and increased mortality in pullets that have consumed toxic fat. A marked reduction was observed in the hatchability of eggs from hens fed toxic fat.[14] Turkeys and ducks appeared to be less susceptible than chickens to the detrimental effects of this fat in the diet.[4] Fat-soluble tissue extracts from chickens fed toxic fat were capable of producing hydropericardium and ascites when added to the diet of other birds.[6] Associated with the transudate, Sanger *et al.*[18] observed necrosis of the liver, subepicardial hemorrhage, and lymphocytic infiltration of muscle fibers in chickens consuming toxic fat. In addition to these changes, Simpson *et al.*[20] also noticed bile duct hyperplasia and proliferation of the endothelium in the parenchymal tissues. The unsaponifiable portion of some batches of fat was found responsible for the toxicity.[2,16,17,22] Repeated passages of this fraction through alumina and silica gel columns led to the isolation of a crystalline factor of unknown structure which was able to produce anasarca.[8]

The primary aim of this study was to investigate the mechanism by which toxic fat produces anasarca. Preliminary studies suggested that a vascular or cardiac injury may be responsible for the transudation of fluid into tissues. In the hope that some clarification of the mechanism might be obtained, arterial perfusions, recordings of hemodynamic changes, and ultrastructural myocardial studies were undertaken.

Materials and Methods

In experiment 1 (acute), 100 White Leghorn cockerels, 1 day old, were separated into 5 groups of 20, placed in heated batteries, and fed rations containing 0, 0.5, 1.0, 3.0, and 5.0% toxic fat* for 35 days. Because the exact chemical nature of toxic fat is not known, the amount of the toxic material undoubtedly varies in fats from different sources. The fat used in these experiments was from the same source, identical shipments, and had the samd LD_{50} when fed to day-old chickens.

In experiment 2 (chronic), forty-eight 4-week-old cockerels of comparable weight were given, for 150 days, a ration** containing 0, 0.25, and 1.0% toxic fat. The birds were fed and watered daily. Throughout the trial period the general appearance, food consumptions, and mortality were spleen, pancreas, kidneys, adrenal glands, skeletal muscle, brain, and bone marrow were fixed in 10.0% neutral formalin, embedded in paraffin, sectioned at 7 μ, and stained with hematoxylin and eosin. Frozen sections were prepared from the livers and kidneys and stained with Sudan IV to demonstrate neutral fats. At the termination of the trials, blood was obtained from the cephalic vein, complete

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* Emery Industries, Cincinnati, Ohio.
** McMillen Feed Mills, Fort Wayne, Ind.

blood counts were conducted on a portion of the sample, and the remainder was allowed to clot. After centrifugation the serum was saved for total protein,[9] electrolyte concentration,[7] and nonprotein nitrogen determinations[6] and electrophoretic studies.[21]

In experiment 1, surviving birds were used for vascular perfusion studies. Immediately after they were killed, the thoracic aorta was perfused with 5.0% dextrose to remove blood, and 1.0% silver nitrate was injected to outline the "cement substance" between the endothelial cells of the mesenteric capillaries. Slides were prepared of the mesenteric vessels and examined for alteration of interendothelial silver precipitate in birds consuming toxic fat.

Five control and 5 test birds given the ration containing 1.0% toxic fat were selected for hemodynamic studies after 150 days on trial. Venous pressures were determined by making an incision through the skin over the jugular vein. The vein was dissected away from the surrounding tissue, and 2 ligatures were placed around the vessel. The superior legature was made secure, and a small incision was made into the vessel. A No. 5 cardiac catheter was placed in the vein and made secure by the inferior ligature. The birds were placed under a fluoroscope and the exact position of the catheter determined. The catheter was attached to a 3-mm. water manometer filled with physiologic saline solution, thus enabling pressures to be determined in the venae cavae and right ventricles.

At the termination of experiment 2, tissues were obtained for electron microscopy by severing the cervical spinal cord and exposing the heart by removal of the sternum. Sections were taken from the left ventricle and cut into blocks of approximately 1 mm. with a razor blade. The tissues were immediately fixed in 1.0% osmium tetroxide, dehydrated, and embedded as outlined by Palade.[15] Thin sections were cut on a cicrotome.[1] Tissues were examined with an electron microscope[2] operating at 50 kv.

Results

In experiment 1, birds given rations containing 0 to 1.0% toxic fat survived, whereas those consuming rations with 3.0 and 5.0% toxic fat had a 25.0 to 55.0% mortality, respectively (Table 1). As the level of toxic fat in the diet

TABLE 1.—EFFECTS OF TOXIC FAT CONSUMPTION ON CHICKENS

No. birds	Toxic fat in diet (percent)	Days on trial	No. died	Ascites	Hydropericardium
20	0	35	0	----	----
20	0.5	35	0	----	----
20	1.0	35	0	----	----
20	3.0	35	2	20	20
20	5.0	35	11	20	20
12	0	150	0	0	0
12	0.25	150	1	1	4
12	0.5	150	4	0	8
12	1.0	150	6	2	11

was increased, there was a corresponding decrease in growth rate. The control birds averaged 348 Gm., whereas those given rations with toxic fat averaged 176 Gm., at the end of 35 days. Hydropericardium and ascites were not observed in the chickens fed rations containing 0 and 0.5% toxic fat. Pericardial fluid volumes ranged from 0.5 to 5.0 ml. in the birds given rations with 1.0 to 0.5% fat. Ascites was also observed in these groups, but the volume was not determined due to the partial coagulation of the transudate.

In experiment 2, 6 birds fed 1.0%, 4 fed 0.5%, and 1 fed 0.25% toxic fat in the ration died. The mean survival time for the groups was 80 ± 20, 100 ± 14, and 135 days, respectively. The control group gained an average of 16.5 Gm., whereas the survivors of the group fed the ration containing 1.0% toxic fat gained 14.9 Gm. per day. The weight gain of the surviving birds of the

[1] Porter-Blum microtome, Ivan Sorvall, Inc., Norwalk, Conn.
[2] RCA, EMU-3G, Radio Corporation of America, Camden, N.J.

groups fed rations containing 0.5 and 0.25% toxic fat was almost comparable to that of the controls. Hydropericardium was observed in 11 and ascites in 2 birds fed rations containing 1.0% toxic fat. Eight of 12 birds on the 0.5% toxic fat ration had hydropericardium but were free of ascites. The average volume of pericardial transudate in the test birds was 13.5 ml. No appreciable difference was noticed in the organ weights of the test and control birds, with the exception of the testes and hearts of the birds in experiment 2. The average weight per testis of birds in the control group was 14.9 Gm., whereas that of the 0.25% group was 6.6 Gm., the 0.5% group was 4.1 Gm., and the 1.0% group was 3.9 Gm. The hearts of birds of the control group averaged 15.0 Gm., and those of birds in the test groups averaged 23.0 Gm. and were markedly dilated in most cases (Fig. 8).

Tissue sections of the various organs were examined microscopically. The birds with hydropericardium had fibrinous deposits and foci of lymphocytes on the visseral pericardium. The muscle fibers were separated by edematous fluid. A number of the small myocardial artieries were surrounded by lymphocytes. There were also foci of lymphocytes between the myocardial fibers (Fig. 12). The lungs were congested and had a moderate amount of peribronchial lymphoid hyperplasia. Pulmonary edema was found in those birds which died during the experiments. Livers from birds with marked ascites frequently had thickened capsules. In many cases there was coagulation of the transudate on the convex surface of the liver which formed a false capsule (Fig. 3). There was moderate fatty infiltration of the hepatic cells in birds given toxic fat in experiments 1 and 2 (Fig. 4). Extensive lymphoid hyperplasia around the periportal areas was found consistently (Fig. 5). Five of the test birds in experiment 2 also had myeloid hyperplasia scattered throughout the parenchymal tissue of the liver.

Microscopic examination of the testes of the birds given toxic fat in experiment 2 revealed a reduction in size of the seminiferous tubules. The Sertoli cells and spermatogonia appeared normal, but there was a reduction of the primary and secondary spermatocytes. These maturing cells were reduced to the point where no spermatids and spermatozoa were observed. Cells in the testes of the test birds appeared normal. The major difference between testes

TABLE 2.—SERUM PROTEIN STUDIES ON CHICKENS FED TOXIC FAT

Toxic fat in diet (percent)	Serum protein (Gm./100 cc.)	Albumin (Gm./100 cc.)	Globulin A/G (Gm./100 cc.)		Nonprotein nitrogen (mg./100 cc.)	Sodium (mEq./liter)	Potassium (mEq./liter)
0	3.36	1.06	2.30	46	21.7	143	5.5
0.5	3.24						
1.0	3.14						
3.0	3.08						
5.0	3.06	0.94	2.12	44	20.7	146	5.3

of birds in test and control groups was a lack of spermatogenesis and a marked reduction in tubular size (Fig. 6,7) in testes of test birds.

Total blood proteins were determined by the micro-Kjeldahl method. The control group of experiment 1 had an average value of 3.36 Gm./100 cc. The 0.5, 1.0, 3.0 and 5.0% groups had values of 3.24, 3.14, 3.08, and 3.06 Gm./100 cc. of protein, respectively (Table 2). In experiment 2, it was found that the groups given rations containing 1.0% toxic fat had an average of 4.1 Gm./100 cc., the 0.25% group averaged 4.5 Gm./100 cc., and the controls had an average value of 4.8 Gm./100 cc. of serum protein. The protein level of the ascitic fluid of the experimental birds was 1.7 Gm./100 cc., with no appreciable difference in the levels of the various groups. The albumin: globulin ratio was determined by paper electrophoresis, and little difference was found in the protein ratio of the various groups.

Sodium, potassium, and nonprotein nitrogen levels were determined on serum samples from 6 birds given rations containing 5.0% toxic fat and 6 control birds. No appreciable difference was found in the nonprotein nitrogen levels of the 2 groups, with the controls averaging 21.5 mg./100 cc. and the test birds 20.7 mg./100 cc. The sodium values were 146 mEq./liter in the experimental

and 143 mEq./liter in the control birds. The potassium levels were 5.3 mEq./liter in the test birds and 5.5 mEq./liter in the control birds.

When 1.0% silver nitrate solution was perfused in the thoracic aorta and the mesenteric capillaries examined microscopically, a difference was found in the cement substance located between the endothelial cells of the test and control birds in experiment 1. The control group had uniform diamond-shaped bands of silver precipitate between the capillary endothelial cells, whereas the bands in the test birds were irregular and indistinct (Fig. 9,10).

Catheterization of the heart revealed the mean right ventricular pressure of the control birds to have an average value of 15.3 Cm. of water, whereas the test birds had an average of 21.3 Cm. of water. The pressures in the inferior vena cava averaged 5.5 cm. of water in the control birds and 7.1 cm. of water in the test birds (Table 3).

TABLE 3.—HEMODYNAMIC ALTERATIONS DUE TO TOXIC FAT CONSUMPTION IN CHICKENS

Toxic fat in diet (percent)	Mean right ventricular pressure (cm. H_2O)	Mean superior vena cava pressure (cm. H_2O)	Mean inferior vena cava pressure (cm. H_2O)
0	15.4	5.6	5.2
0	15.8	5.4	5.3
0	15.2	5.2	5.6
0	15.0	5.8	5.8
0	15.0	5.4	5.7
5.0	22.2	7.4	7.0
5.0	20.0	7.0	7.1
5.0	21.0	7.2	6.9
5.0	25.5	6.5	7.2
5.0	20.4	6.8	7.4

Preliminary data obtained from electron micographs of heart muscle from 10 test and 10 control birds of experiment 2 indicted that the major changes occurred in the mitochondria of the cardiac muscle cells. In some cases the mitochondria were markedly shrunken and vacuolated with indistinct cristae. In the birds with more advanced cases, many of the mitochondria had disappeared, leaving large areas devoid of any organelle.

Discussion

There was normal development of all organs with the exception of the testes of birds in experiment 2, which were markedly reduced in size. Kumaran and Turner[10] outlined the tubular and spermatogenic development in chickens at various states of maturity. It would appear that the consumption of toxic fat in these experiments retarded testicular development by approximately 2 months in the 6-month-old roosters. Despite the reduction in testicular size, secondary sex characteristics such as comb size and body conformation were unaffected.

In earlier studies,[1] young birds were killed every other day for 3 weeks to determine when hydropericardium or ascites developed. Hydropericardium was usually noticed about the 16th day after feeding toxic fat in the ration was initiated, without ascites or pulmonary edema. Fluid accumulation in the lungs and peritoneal cavity invariably developed later. When the concentration of toxic fat in experiment 2 was reduced to a low level, the birds failed to develop ascites, but hydropericardium was a consistent observation.

The most prominent lesions at necropsy were hydropericardium, ascites, and pulmonary edema. A number of procedures were conducted to resolve what caused the anasarca. Birds with ascites and hydropericardium in many cases had higher serum protein levels than those in the control group. The albumin: globulin ratios of experimental and control birds were approximately comparable. The nonprotein nitrogen and electrolyte levels of the blood were within normal range in the test groups. There was a reduction in the cement substance in the experimental birds with hydropericardium and ascites. Since a large quantity of fluid escapes between the endothelial cells rather than through them[13] any alteration in the cementum would affect the permeability of the vessels.[14] Whether this alteration in the cement substance of the capil-

laries was associated with hypoxia of the endothelial cells resulting from cardiac decompensation or from a direct effect of toxic fat on the capillary membrane remains to be clarified.

Hemodynamic studies revealed an increase in right ventricular and vena cava pressures in the experimental birds. Associated with these pressure changes were dilatation and hypertrophy of the right side of the heart of birds given toxic fat. Pulmonary edema, cardiac dilatation, and increased venous pressure are indicative of cardiac decompensation.

What do these data reveal in regard *to* the causes for the excessive extravascular fluids? The lack of any appreciable change in the total blood protein and albumin: globulin ratio would indicate that the liver was producing adequate amounts of albumin. There were no marked alterations in kidney function, because the serum albumin and nonprotein nitrogen levels were comparable in the 2 groups, which suggests that the kidney tubules and adrenal cortex were not affected by toxic fat ingestion. The microscopic observations fortify the biochemical data, because the alterations in the liver, kidneys, and adrenal glands of test birds did not appear of sufficient magnitude to account for the anasarca.

Since myocardial fibers are very active, there is a constant demand on the respiratory enzymes located in the numerous mitochondria between the myofibrils. A rather high metabolic demand is implied, because the ratio of mitochondria in cardiac muscle to skeletal muscle is approximately 500:1.[illegible] Any significant alteration in the mitochondria would affect the respiratory enzymes and sooner or later lead to cardiac failure. Certainly this would appear to be a logical explanation for what occurs in birds consuming toxic fat. As the heart becomes less efficient, there will be an increase in hydrostatic pressure in the veins and capillaries, with a predisposition for the extravasation of fluid into the tissues and body cavities.

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Summario in Interlingua

Le Rolo de "Grassia Toxic" in le Production de Hydropericardio e Ascites in Gallinas

Quandro "grassia *toxic*" esseva addite al dieta de aves experimental a concentrationes de 0,25 a 6,0 pro cento durante 35 a 150 dies, le sequente alterationes esseva observate: (1) Edema del myocardio, del musculatura skeletic, e del pulmones; (2) hydropericardio; (3) ascites; e (4) focos de lymphocytos in le myocardio e le epicardio. Appreciabile alterationes non esseva observate in le nivellos de proteina total in le sero, in le proportion albumina a globulina, in le balancia electrolytic, e in le nivellos de nitrogeno non ligate a proteina in le sanguine. Esseva notate dilatation, edema, e infiltration lymphocytic del corde. Le mitochondrios myocardial esseva vacuolate e contrahite. Un augmento del tension venose esseva etiam notate. Le imbalancia de liquido observate in aves que consumeva grassia toxic non resultava ab un declino in total proteinas de sanguine o ab un alteration in le proportion albumina a globuina sed esseva associate con discompensation cardiac e un augmento del permeabilitate capillar.

Industrially Acquired Porphyria

Twenty-nine patients working in a chemical factory engaged in the manufacture of 2,4-dichlorophenol (2,4-D) and 2,4,5-trichlorophenol (2,4,5-T) exhibiting features of chloracne were studied for the presence of porphyria cutanea tarda. In 11 cases urinary uroporphyrins were elevated.

Two of these patients who showed evidence of acquired porphyria with chloracne were hospitalized. The features of chloracne as well as the clinical and laboratory features of acquired porphyria have been discussed. There appeared to be an etiologic but not quantitative relationship between the chloracne in workers engaged in the manufacture of 2,4-D and 2,4,5-T and porphyria cutanea tarda of the acquired type. It is our feeling that either the finished chemicals or some intermediate are responsible for both diseases.

Since Waldenstrom first implied that porphyria cutanea tarda might be acquired, a growing number of chemials have been implicated in the pathogenesis of this disease. These chemicals have included alcohol, sedatives, fungicides, etc.[1-13] While treating a severe outbreak of chloracne in a factory which manufactures 2,4-D and 2,4,5-T, a number of workers were noted to have hyperpigmentation, hirsutism, fragility of the skin and vesiculobullous eruptions on exposed areas of skin, together with cutaneous findings of chloracne. Investigation revealed evidence of porphyria cutanea tarda of varying degrees of severity in 11 out of 29 workers investigated. Porphyria cutanea tarda has never before been described as related to chloracne, nor has it been ascribed to

industrial exposure in the United States. This outbreak is therefore of interest in adding more evidence to the growing concept that porphyria cutanea tarda may be an acquired disease occurring after various insults to the liver. Three cases were studied in detail.

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REPORT OF CASES

CASE 1.—A 48-year-old white male who was employed at the factory for three years as a chemical operator. His work brought him into intimate contact with the suspected chemicals. His past history included two attacks of biliary colic prior to 1953. He was never a heavy user of alcohol. A diagnosis of cholecystitis had been made and a cholecystectomy was performed early in 1953. After this he came to work at the factory in question. In 1956 he began to notice some darkening of his skin and suffered right upper quadrant pain. A diagnosis of common duct obstruction was made, and this patient was operated on again in January of 1956. An unsuccessful attempt was made to probe the common duct, and no further operative procedure was done. During the postoperative course, this man received 2 gm of barbiturates. The patient stated that his urine had turned "the color of Coca-Cola" at least one year prior to the second operation. That spring, an eruption of bullae appeared on the face, ears, and hands. These lesions could be produced either by exposure to the sun or by pressure. In addition to the vesicular eruption, the patient noted progressive darkening of his skin and marked hirsutism, especially over the temples. Inspection of the urine revealed a Coca-Cola coloration and, under the Wood's Light, a brilliant red fluorescence. The exact laboratory data on this patient are no longer available except for the presence of quantitatively markedly increased excretion of urinary porphyrins including uroporphyrins, coproporphyrins, and porphobilinogen.

This man is now alive and well and apparently is suffering minimal if any symptoms of porphyrim cutanea tarda. His present job does not entail the use of any chemicals. He has failed to present himself for further testing.

CASE 2.—This is a 60-year-old white male who has been employed in the factory for seven years as a welder. In the course of his work, which consisted of welding tanks and pipes, he was brought into frequent and prolonged contact with chemicals. He was admitted to the Newark Beth Israel Hospital for investigation. He stated that three months prior to admission, he had noted an increased darkening of the skin, thickening of the eyebrows, and a darkening and reddening of his urine. His family history and his past medical history were unrevealing, except for moderately heavy alcohol intake for some years.

Physical examination revealed numerous comedones and small epidermoid cysts and furuncles the face, chest, and shoulders. There was intense grayish-brown hyperpigmentation with a purplish tint on the exposed surfaces of the face, neck, chest, and hands and moderate hypertrichosis of the temples. The scalp hair showed a lusterless, dull silver color change. The liver edge was palpable about 3 cm below the right costal margin and was smooth and nontender. The remainder of the physical examination was within normal limits. A casual urine specimen revealed a strong tea color with a deep fluorescence, reddish, under the Wood's light.

Laboratory studies revealed increased urinary uroporphyin, coproporphyrin, and urobilinogen excretion. There was no demonstrable porphobilinogen. The feces showed increased uroporphyrins and coproporphyrins. All porphyrin determinations were qualitative and done by the Watson-Schwartz method. Other significant findings included an elevated serum glutamic oxaloacetic transaminase ranging between 41 and 51 units on five different days. Serum glutamic pyruvic transaminase on corresponding days ranged between 53 and 64 units. The sulfobromophthalein retention was 6% in 30 minutes. The erythrocyte sedimentation rate (Westergren) was 94 mm in the first hour. All other studies, which included complete blood count, bleeding and clotting time, urinalysis, glucose tolerance test, serum bilirubin, blood urea nitrogen, total

proteins, albumin-globulin ratio, serum cholestrol, alkaline phosphatase, cephalin flocculation, and thymol turbidity, serum electrolytes, and CO_2 combining power, as well as serological tests for syphilis were all within normal limits. Electrocardiograms and chest x-rays were normal. A liver biopsy was performed and the specimen immersed in isotonic saline. It fluoresced intensely under the Wood's light. The red pigment diffused out into the saline, so that the entire tube fluoresced. The microscopic examination of the liver specimen revealed parenchymal cell regeneration and hemofuscin deposition. A skin biopsy from clinically hyperpigmented postauricular skin showed a normal epidermis except for the dermoepidermal border, where there was a striking deposition of brown granular pigment. In addition, there was mild infiltrate of small round cells in the dermis. No sebaceous glands were visible in the sections. Shortly after discharge from the hospital in June, 1963, the patient was treated for a chromic trichophytosis of the feet with griseofulvin 0.5 gm twice daily. About four days after the onset of this treatment, a severe vesiculobullous eruption on the dorsal surface of the hands appeared. The griseofulvin was stopped, but the eruption progressed for another two weeks. Healing time was very prolonged, and at present, residual atrophic scarring is visible on both hands (Fig 1). In the scars occasional milia are seen.

SUMMARY OF DATA FOR 26 WORKERS WHOSE URINE WAS TESTED FOR PORPHYRINS

Patient	Chloracne [1]	Hyperpigmentation	Hirsutism	Urine Uroporphyrins	Chemical [2] Contact	Skin Fragility
1	Severe	Mild	Moderate	Pos.	Moderate	Pos.
2	Mild	None	None	None	Severe	Neg.
3	do	Mild	do	do	do	Do.
4	Severe	do	Mild	do	do	Pos.
5	Mild	None	do	Pos.	do	Do.
6	do	do	None	None	Moderate	Neg.
7	do	Mild	do	Pos.	Moderate	Do.
8	Severe	Moderate	Severe	None	do	Do.
9	Mild	Mild	Mild	do	do	Do.
10	Moderate	Moderate	Moderate	do	Severe	Do.
11	Severe	do	do	do	Moderate	Do.
12	None	None	None	do	Severe	Do.
13	do	do	do	do	do	Do.
14	do	do	do	Pos.	Moderate	Do.
15	Moderate	Mild	Moderate	None	do	Pos.
16	do	Moderate	Marked	do	Severe	Do.
17	Mild	Mild	None	do	Mild	Neg.
18	Moderate	Moderate	Marked	do	do	Do.
19	Severe	Marked	None	Pos.	do	Do.
20	Mild	Mild	Moderate	Pos.	do	Do.
21	None	None	None	None	do	Do.
22	do	do	do	do	do	Do.
23	Mild	do	do	do	do	Do.
24	None	do	do	do	do	Do.
25	do	do	do	Pos.	do	Do.
26	do	do	do	Pos.	do	Do.

[1] Severity of chloracne is judged on the presence of comedones, epidermoid cysts, and furuncles and pustules.
[2] The extent of exposure is difficult to truly judge because of such variables as personal hygiene and work habits.
[3] Brief period of employment.

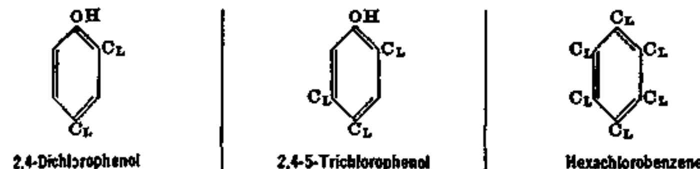

Figure 3.—Comparison of structural formulae of weed killer manufactured and fungicide responsible for acquired porphyria cutanea tarda. Note the similarities

CASE 3.—This is a 48-year-old white male employed at the factory for eight years mixing batches of chemicals. During the past two years, he had developed hyperpigmentation of the exposed skin of the face and hands. There was

marked hirsutism which involved the temples. The dull silvery tint of the hair was visible. He stated that in the past he had had episodes of blistering of the exposed skin. He also had noticed that his urine was dark on voiding. His family history was noncontributory. The physical examination revealed an intense hyperpigmentation of the face, neck, and hands. There was severe hirsutism involving the eyelids, eyebrows, and lateral aspects of the forehead (Fig 2). Comdones and small epidermoid cysts were very prominent, and there were numerous furuncles scattered over the entire body. The remainder of the physical examination was within normal limits except for prolapsed hemorrhoids. The following laboratory studies were within normal limits: complete blood cell count, urinalysis, bleeding and clotting time, prothrombin time, blood glucose tolerance test, urea nitrogen, cholestrol, bilirubin, alkaline phosphatase, total protein and albumin-globulin ratio, cephalin flocculation, thymol turbidity, serum electrolytes including sodium potassium, chlorides, CO_2 combining power, calcium, and phosphorus. The serum glutamic oxaloacetic transaminase on five successive days ranged between 39 and 56 units while the serum glutamic pyruvic transaminase on corresponding days ranged between 47 and 72 units. The sulfobromophthalein retention was 8% in 30 minutes. The electrocardiogram was normal. The chest x-ray revealed a diffuse nodular infiltration of both lungs due to pneumoconiosis. This was consistent with the patient's history of having worked a number of years as a coal miner. The plain film of the abdomen was negative. The urine revealed a negative Watson-Schwartz test. The urine failed to fluoresce under the Wood's light. The erythrocyte sedimentation rate (Westergren) was 24 mm in the first hour.

A liver biopsy was performed and the specimen immersed in saline. Under the Wood's light the specimen and saline in which it was immersed fluoresced faintly. On microscopic examination, the liver biopsy showed evidence of liver cell regeneration and hemofuscin deposition. A skin biopsy showed brown granular pigmentation as the basal margin of the epidermis. There was a mild chronic inflammatory infiltrate scattered through the dermis. No sebaceous glands were visible in the sections.

Since the man's chloracne has been so severe, he had been removed from contact with chemicals two years prior to his admission to the hospital. This probably was responsible for the failure to prove qualitative chemical evidence of porphyrins in the urine. It also may indicate that acquired porphyria cutanea tarda is reversible.

SCREENING TESTS

Twenty-six additional men working at this chemical factory were studied on an ambulatory basis. In addition to routine urinalysis, each urine specimen was tested for uroporphyrin by the Watson-Schwartz method. Eight out of the 26 manifested significantly increased excretion of urinary uroporphyrins by the Watson-Schwartz method. If the three cases described in the case reports above are added, this is a total of 11 cases of porphyria cutanea tarda of varying degrees of severity out of 29 patients tested, or 37+% (Table).

COMMENT

Hyperpigmentation in these workers was limited to the sun-exposed areas of the head, neck, and hands. It was more frequently observed in the Negro patients involved. The degree of hyperpigmentation was roughly proportional to the severity of the chloracne. The hyperpigmentation varies from mild redness in extremely fair individuals to dark gray intense dusky bronzing of the skin. The degree of hirsutism was also proportional to the severity of the chloracne. This too was quite variable in degree but always involved the temples between the lateral half of the eyebrow and the temporal hair of the scalp. The hirsutism in a few cases, notably case 3, extended beyond this and involved both the upper and lower eyelids. The hair was of approximately the same texture and density as that of the eyebrows.

The occupational environment of these men consists of a group of basic chemicals including acetic acid, phenol, monochloracetic acid and sodium hydroxide, plus the finished products 2,4-D and 2,4,5-T as well as many unknown intermediary products. It is known that one of the intermediaries is a highly volatile chlorinated phenolic ether which contains six chlorine atoms. This particular compound, because of its volatility, is strongly suspected of being a possible causal agent. Porphyria has been described in many cases as a

result of ingestion of hexachlorbenzene,[2,3] chemically a closely related compound (Fig 3). This would lend support to the concept that porphyria cutanea tarda is not necessarily genetically produced, unless the genetic defect is an extremely common one.

An analysis of the table of the 26 surveyed workers (Table 4) reveals: the severity of chloracne does not usually correspond to the degree of exposure to chemicals (patients 1, 8, 11, 19 or patients 2, 3, 5, 10, 12, 13, 16). The severity of prophyria does not usually correspond to the degree of chemical exposure (patients 7, 20, 25, 26 or patients 2, 3, 4, 10, 12, 13, 16). The severity of chloracne does not usually correspond to the presence of porphyria (patients 1, 4, 8, 11, or patients 5, 20, 25, 26). Therefore it would appear that there is some individual susceptibility to these disease. It has been observed in general that: (1) Patients with adolescent acne tend to get worse chloracne: (2) Possibly previous liver damage (alcoholism, etc) predisposes to porphyria. Also there must be in these cases some etiologic relationship between chloracne and porphyria since a relatively large number of both diseases began to appear and have persisted at the same time.

On the basis of the elevated transaminase levels and the histological signs of liver cell regeneration in the liver biopsies, it may be assumed that the basis of the distrubed porphyrin metabolism is a hepatotoxic effect of one or more of the chemicals in this factory environment. The synergistic roles of other known liver toxins such as alcohol and barbiturates, or griseofulvin (1 case), cannot be overlooked.

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Electron Microscopic Alterations in the Liver of Chickens Fed Toxic Fat*

Toxic fat is the name applied to certain fats that produce hydropericardium, hydrothorax, and ascites when added to the diet of chickens.[9,18,20] It has been demonstrated that the toxic fraction is associated with the unsaponifiable portion of the fat.[10,13] The toxicity can be increased by repeated passages through silica and alumina gel columns.[6,21] Crystalline preparations of the toxic fraction have been prepared, but the chemical nature of the compound remains to be identified.[12]

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The following alterations that resulted from the consumption of toxic fat have been reported. Sanger, Scott, Hamdy, Gale, and Pounden[19] observed focal necrosis of the liver, epicardial hemorrhage, and lymphocytic infiltration of the cardiac muscle. Simpson, Pritchard, and Harms[21] reported bile duct hyperplasia and proliferation of the endothelial lining of the smaller blood vessels. Allen and Lalich[4] observed testicular hypoplasia. Marked dilation and edema of the myocardium and altered right ventricular and vena cava pressures have been demonstrated.[3]

The present experiment was initiated to determine the morphologic effect of toxic fat upon the liver of chickens and to correlate these findings with the development of anasarca.

EXPERIMENTAL PROCEDURE

One hundred sixty-eight 1-day-old White Leghorn-New Hampshire chickens were divided into groups of 48 and 120 chickens. One-half of the chickens from each group were given a commercial diet containing 3.0 per cent toxic fat (Emery Industries, Inc., Cincinnati, Ohio), while the remaining chickens received a comparable diet which contained 3.0 per cent corn oil. In the group of 48 chickens, three control and three experimental chickens were killed every other day for 16 days and sections of the liver were obtained for electron microscopic evaluation.

The group of 120 chickens remained on the toxic fat diet until the LD_{50} was established. From the survivors, blood was obtained for hematocrit,[15] hemoglobin,[8] total serum protein,[12] serum electrophoretic pattern,[22] and electrolyte studies.[2] The ascitic fluid was also collected for total protein and electrophoretic pattern determinations. All chickens that died during the course of the experiment as well as those sacrificed at its completion were necropsied. Portions of the liver were fixed in 10 per cent buffered formalin for 24 hours, dehydrated, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Other portions of the liver were sectioned on a cryostat and stained with Sudan IV for neutral fats. Additional paraffin-embedded tissues were stained with aniline blue and phosphotungstic acid-hematoxylin.[5]

Small sections of liver from the 48 chickens of the first group plus 20 control and 20 experimental chickens of the second group were obtained at the time of death, cut in small cubes, and fixed in Caulfield's[7] and Millonig's fixatives.[16] These tissues were dehydrated through a graded series of ethanol and embedded in an Araldite-Epon mixture.[17] Thin sections of approximately 0.5 μ were cut on an ultramicrotome for light microscopy and stained by the toluidine blue method.[23] Ultrathin sections were placed directly on 400-mesh, uncoated copper grids, stained with uranyl acetate, and examined with an RCA EMU-3G electron microscope.

TABLE 1.—ALTERATIONS IN THE PERIPHERAL BLOOD OF CHICKENS CONSUMING TOXIC FAT

No. of chicks	Toxic fat in diet	Hematocrit	Hemoglobin	Na	K	Serum protein	Serum [illegible]
	Percent	*Percent*	*gm. per 100 ml.*	*mEq./liter*	*mEq./liter*	*gm. per 100 ml.*	*Percent*
30	0.0	31.0	10.0	160	5.4	3.4	[illegible]
30	3.0	18.0	6.0	162	5.9	2.3	[illegible]

RESULTS

When 3.0 per cent toxic fat was added to the diet of young chickens 50 per cent died within 15 days. Approximately 24 hours prior to death, the chickens became listless and moved only when agitated. Because of the marked abdominal distention the chickens assumed a ducklike gait when forced to walk. Moist rales were present in the lungs and considerable amounts of clear to blood-tinged fluid were observed in the oral cavity of the experimental chickens at the time of death.

On the 15th day, blood studies were made on the surviving chickens. Those fed toxic fat showed a reduction in several of the blood components (Table 1). Hemoglobin levels decreased from 10.0 gm. per 100 ml. to 6.0 gm. per 100 ml. and hematocrits were reduced from 31.0 per cent to 18.0 per cent. There was a decrease in total serum protein from 3.4 gm. per 100 ml. to 2.3 gm. per 100 ml. and a shift in the albumin-to-globulin ratio of the treated chickens. Similar electrophoretic patterns were obtained on the ascitic and pericardial fluids, although the total protein of these fluids was only 1.3 gm. per 100 ml. Blood electrolytes were not appreciably altered.

GROSS AND MICROSCOPIC OBSERVATIONS

A large quantity of colorless, semiclotted fluid accumulated in the subcutaneous tissue and the pectoral, thigh, and lower leg muscles were pale and edematous. The abdominal cavity of each experimental chicken contained approximately 40 ml. of ascitic fluid. The liver was somewhat mottled with rounded margins and a thick gelatinous material resembling coagulated plasma was firmly attached to the capsule in 25 per cent of the chickens that had received toxic fat. The kidneys were pale and swollen. All of the organs within the abdominal cavity appeared edematous. There was marked distention of the pericardial sac with a clear, slightly yellow fluid. Five to 10 ml. of pericardial fluid were obtained from each chicken. There was a noticeable dilation and hypertrophy of the right side of the heart and the myocardium was pale, with no gross lesions evident. The lungs were extremely edematous and the tracheobronchial tree was filled with blood-tinged, foamy fluid. The brain was edematous but free of other gross lesions.

The major microscopic alterations were observed in the liver. Its capsular surface was covered by a thick layer of homogeneous, eosinophilic material containing numerous fibrin strands. Although the general architecture of the liver parenchyma was maintained, numerous foci of necrosis were observed (Fig. 1). These lesions varied in size, from only two or three cells to a major portion of the lobule. There was a fairly sharp line of demarcation between the viable and dead cells. Immediately adjacent to these necrotic foci, the cytoplasm of the parenchymal cells was quite vacuolated, but otherwise not remarkable.

THIN SECTIONS FOR LIGHT MICROSCOPY

Better visualization of the histologic alterations was obtained from tissues embedded in the Araldite-Epon mixture than was possible using the conventional paraffin embedded preparations. The parenchymal liver cells of the control chickens stained uniformly with toluidine blue. Their nuclei were dark blue and the cytoplasm was much lighter and granular. The degenerating parenchymal liver cells of the toxic fat chickens were of two types on the basis of their affinity for toluidine blue. One cell type had a decided affinity for the dye so that both the cytoplasm and nucleus were very dark (Fig. 2). Many of these cells were shrunken, distorted and contained large morphologic features of the organelles were fairly well maintained in the cells obtained during the first 5 days of the experiment. However, the organelles were in very close apposition as a result of what appeared to be shrinkage of the cells and loss of the cytoplasmic matrix. Although the external mitochondrial membranes were quite irregular, the shape and distribution of the cristae remained unaltered. The membranes of the endoplasmic reticulum were in close apposition and had abundant ribosomes along the outer surface. Free ribosomes were dispersed throughout the cytoplasm. In the dark cells, the Golgi complex, lysosomes, and microbodies were usually sparse and only vaguely discernible. Only isolated areas within the cytoplasm contained glycogen granules. Numerous myelin-like structures appeared to arise from the cytoplasmic membranous systems of these cells. The nuclei of these dark cells had approximately the same electron density as the cytoplasm. The paired nuclear membranes appeared as one wide, irregular dense band containing few discernible pores. Except for the extreme density of the nucleoplasm, the chromatin material and nucleoli resembled those in the control cells.

From the 7th through the 15th day of the experiment, the dark parenchymal liver cells became increasingly electron-dense, the intercellular spaces wider and myelin-like bodies more abundant along the plasmalemmae. The cytoplasmic organelles became more difficult to visualize. Numerous oval to oblong clear vacuoles of variable size developed in the cytoplasm. Myelin-like bodies, identical to those along the plasmalemma, were present throughout the cytoplasm. At this time it was difficult to visualize the existence of a distinct nuclear and cytoplasmic separation because of the electron density and the

absence of any distinct nuclear membrane. Eventually these cells developed into dark, shrunken, fairly homogeneous masses which exhibited little morphologic resemblance to liver parenchymal cells (Fig. 6).

The second type of parenchymal cell in the degenerative areas was large and very electron-lucent. An isolated cell of this variety was seen most frequently within a group of the previously described dark cells. The plasmalemmae of these cells were in close apposition to the adjacent dark cells. The microvilli projecting into the space of Disse were shorter and less abundant than those of the dark cells (Fig. 7). Many of the organelles were markedly distorted. Small segments of the endoplasmic reticulum were scattered throughout the cytoplasm. The majority of these membranes contained ribosomes along their outer borders. Numerous free ribosomes were also scattered throughout the cytoplasm. The mitochondria were large and disorted. Their external membranes were quite irregular and formed many bulblike projections from the surface of the mitochondria. Small vesicles were apparent between the external and internal mitochondrial membranes. The enlarged cristae practically filled the interior of the mitochondria (Fig. 6). Large, clear vacuoles and cytoplasmic degenerative bodies were abundant throughout the cytoplasm while glycogen granules were very sparse in the light cells. The Golgi complex consisted of collapsed vesicular sacs dispersed in small groups throughout the cytoplasm. The mitochondria were large and distorted. Their external chromatin material quite evenly dispersed throughout the nucleoplasm.

During the terminal portion of the experiment, the large electron-lucent cells developed openings in their plasmalemmae. Their cytoplasmic organelles were observed in the sinusoids, space of Disse, and in the intercellular spaces.

The endothelial cells which line the sinusoids showed similar changes to those previously described in the dark parenchymal cells. The endothelial changes were most frequently observed adjacent to a large focus of dark cells. The long, slender cytoplasmic processes of the endothelial cells became extremely dark with the cytoplasmic and nuclear structures becoming indistinguishable. Large fragments of these endothelial cells were frequently seen in the sinusoidal spaces. Similar changes were observed in the bile duct epithelium. It was not uncommon to see two or three extremely electron-dense cells which had lost all their internal structure adjacent to a number of seemingly normal epithelial cells (Fig. 8).

DISCUSSION

The gross lesions produced in chickens fed toxic fat have been well documented, yet the mechanism by which this fat produces ascites, hydropericardium, hydrothorax, and edema has not been clarified. Vascular lesions and cardiac decompensation have been mentioned as possible causes of the massive accumulation of extravascular fluid in chickens that have eaten toxic fat.[1] In this experiment the liver was investigated to determine its association with the accumulation of extravascular fluid in chickens whose diets contained toxic fat.

There was a decided reduction in the total blood protein of the toxic fat chickens. The electrophoretic patterns of their sera showed the drop in protein resulted from a decrease in the serum albumin. When the liver tissues were examined microscopically the explanation for the decrease in serum albumin became apparent. There were numerous areas of focal necrosis throughout the liver of the experimental chickens.

There is no clear explanation of why some parenchymal liver cells shrink and become extremely electron-dense, while others appear to swell and become very electron-lucent. Undoubtedly each of these changes is a degenerative process which eventually terminates in the death of the cell. One could postulate that these two cell types perform different functions and because of this functional difference, the reactions of these cells to this toxic material were manifested differently. Such changes in size and lucency, however, are not unique for toxic fat but resemble those observed in hepatic lesions produced by monocrotaline and x-irradiation.[4] Further studies will be necessary to clarify the differing responses of various parenchymal cells to this toxic substance.

The accumulation of large quantities of extravascular fluid in the tissues and body cavities of chickens that have consumed toxic fat can be attributed at least in part to an alteration in the permeability of the vascular bed. The high level of protein in the ascitic and pericardial fluid and the electrophoretic

pattern which resembled that of blood serum would be substantiating data for the above statement. Extensive necrosis of the endothelial cells of the liver would also be related to altered capillary permeability. These observations would confirm a previous report[3] regarding the toxic effect of this fat upon the vasculature of chickens.

SUMMARY

Three per cent toxic fat was added to the diet of 1-day-old chickens. Fifty per cent of the chickens died within 15 days. There was a reduction in the hemoglobin, hematocrit, serum protein, and a shift in the albumin-to-globulin ratio of these chickens. The gross lesions in these chickens included hydropericardium, dilation of the heart, pulmonary edema, ascites, and subcutaneous edema. When the tissues were examined microscopically the most distinct lesions were necrosis of the parenchymal cells, bile duct epithelium and endothelial cells of the liver. The liver lesions were evaluated electron microscopically and the morphologic changes reported.

The reduction in total serum protein and shift in the albumin-to-globulin ratio of the chickens that consumed toxic fat were attributed to liver necrosis. The decrease in serum albumin may have predisposed to a portion of the extravasated fluid. However, altered permeability of the vascular bed following toxic fat consumption appeared to be the major predisposing factor for the accumulation of large quantities of extravascular fluid.

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Chick Edema Factor: Some Tissue Distribution Data and Toxicologic Effects in the Rat and Chick.* (31029)

The chick edema factor (CEF), responsible for a large number of deaths in the broiler industry in the fall of 1957, was traced to the unsaponifiable matter (unsap) of the fat used in the broiler rations. It since has been crystallized (1,2,3) and its structure proposed as that of a hexachlorohexahydrophenathrene (2). It is known that in the toxic fat, a mixture of related compounds can be found, some toxic and some relatively nontoxic.

To lay the groundwork for a study of the specific physiological effects of pure CEF compounds, a few short studies have been completed to learn the distribution of the toxic material in the body and which organs were primarily affected.

Experimental. Adult rats and day-old White-Rock chicks were used. Because the pure material was not available in sufficient quantity, we used, from the toxic fat, the unsap which represented 38% of the original toxic fat and was estimated to contain at least 10 ppm CEF. The unsap was force fed because the animals' food intake was drastically curtailed when it was mixed in the diet. All animals were offered water and commercial feed *ad libitum*.

Table I shows the experimental plans and dosage levels employed for all 3 Trials. Feed consumption and fecal and urinary excretion were measured for the rats. Body weights were recorded in all experiments. All animals, upon sacrifice, were examined grossly for pathology and selected organs were weighed and frozen. Hydropericardial fluid (HPF) volume was measured in the chicks.

In addition to the examination for gross effects, the presence of CEF material in various tissue was determined. Adrenals, kidneys, and livers were assayed in the rats; livers only have been analyzed in the chicks. To obtain a picture of the amount of material being absorbed and perhaps excreted, analyses of the feces and urine of rats and of the combined chick excreta were made.

TABLE I.—EXPERIMENTAL PLAN OF TRIALS

Trial	Species	Number of animals	Group	Dosage (per kg) Unsap (ml/day)	CEF (estimated (µg/day)
I. 14 days	Rat	2	High	2.0	18
		2	Low	1.0	9
II. 6 days	do	4	High	2.0	18
		4	Low	1.0	9
		4	Control [1]	0	0
III. 6 days	Chick	6	High	5.4	48
		6	Low	1.1	10
		6	Control [1]	0	0

[1] Control animals were not intubated.

The assay method for CEF is being reported in detail elsewhere.‡ In short, the sample is homogenized with water and saponified with alcoholic KOH. The unsaponifiable portion is extracted with petroleum ether and chromatographed first on an alumina column. The eluate obtained with 25% ethyl ether in petroleum ether, following prior elution with petroleum ether and 5% ethyl ether in petroleum ether, is concentrated and chromatographed on 500 µ thin layer silica gel plates with 3% ethyl ether in petroleum ether. The silica gel in the area of R_f 0.80–1.00 is removed and eluted with ethyl ether, the solvent removed, and the residue is redissolved in isooctane for gas chromatography. We used an F & M Model 400 gas chromatograph with an electron capture detector and a U-tube column, 3 ft × 6 mm (o.d.) × 4 mm (i.d.), packed with

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‡ T. C. Campbell and L. Friedman in press, J. AOAC, "Chemical Assay and Isolation of Chick Edema Factor."

1% SE-30 on ANAKROM ABS (Analabs). The operating conditions were: temperatures, column oven 180°C, EC detector 200°C, flash heater 240°C; gas flows, helium carrier gas, 60 cc/min; argon, 10% methane purge gas 180 cc/min. The limit of detection by this method is approximately 0.2 ppb, assuming a sample size of 5 g and a sensitivity of the EC detector of 5×10^{-11} g.

The so-called CEF components can be seen as a gas chromatographic pattern of peaks shown in Fig. 1. This pattern is that of a highly concentrated material isolated from the toxic unsap. It is similar to a material isolated by Yartzoff *et al* and kindly supplied to us by Firestone (3). We have numbered the peaks 1 through 8 as shown. Peak 4a was not seen in the Firestone preparation.§

Results and discussion. Table II shows the effects upon body weights, feed conversion, and feed consumption for the rats. The results for the vital organ weights and HPF volume are shown in Table III. There was no apparent gross pathology in either species with the exception of HPF and some ascites and subcutaneous edema in the chicks.

While only the chicks develop hydropericardium, apparently both are equally sensitive to liver weight increase, as shown in Table III. In the case where each species was maintained on CEF for 6 days, (Trials II and III), the rats

TABLE II.—GROSS EFFECTS IN RATS

Trial	Group	Percent body wt. change	Feed intake depression [1] (percent)	Dry matter dig. coeff. (percent)
I. 14 days	High	−15	—	76
	Low	−8.5	—	78
II. 6 days	High	−6.6	−38	72
	Low	−3.7	−29	75
	Control	−1.0	—	77

[1] Depression below control animals.

TABLE III.—ORGAN WEIGHTS EXPRESSED AS PERCENT OF BODY WEIGHTS

Trial	Group	Heart	Liver	Spleen	Kidney	Adrenals
II. Rats	High	.34	[2]4.08	.20	.90	[3].21
	Low	.32	[2]4.08	.20	.82	.18
	Control	.31	3.36	.19	.76	.14
					HPF (ml)[1]	
III. Chicks	High	.86	[2]3.85	.61	[3].65	
	Low	.70	3.15	.54	[3].12	
	Control	.78	2.73	.65	.04	

[1] HPF—hydropericardial fluid (ml).
[2] Significant statistically at 1 percent probability level.
[3] At 5 percent probability level (Hogben L test)(5).

(9 µg/kg/day) showed an increase over controls of 21% while the chicks (10 µg/kg/day) showed an increase of 15%. (The increase in liver weight in the chicks was not due to moisture or fat.)

Also, in Table III, neither heart nor spleen weights in either species are significantly affected. In rats, there appears to be a slight increase in kidney weights, though not statistically significant, and a highly significant increase of 50% in adrenal weights for animals on the high level. Whether this adrenal weight increase is simply a non-specific stress effect from intubation is not known, although it would seem that this cannot be entirely responsible, since the high level group showed an increase of nearly twice that of the low level group.

Some other observations which have been made on previously studied birds in this laboratory are of interest. Hematocrit values are depressed. Of a total of 43 birds, we have observed that, with an average of 0.08 ml HPF in control

§ The chromatogram for the Firestone preparation is presented elsewhere.

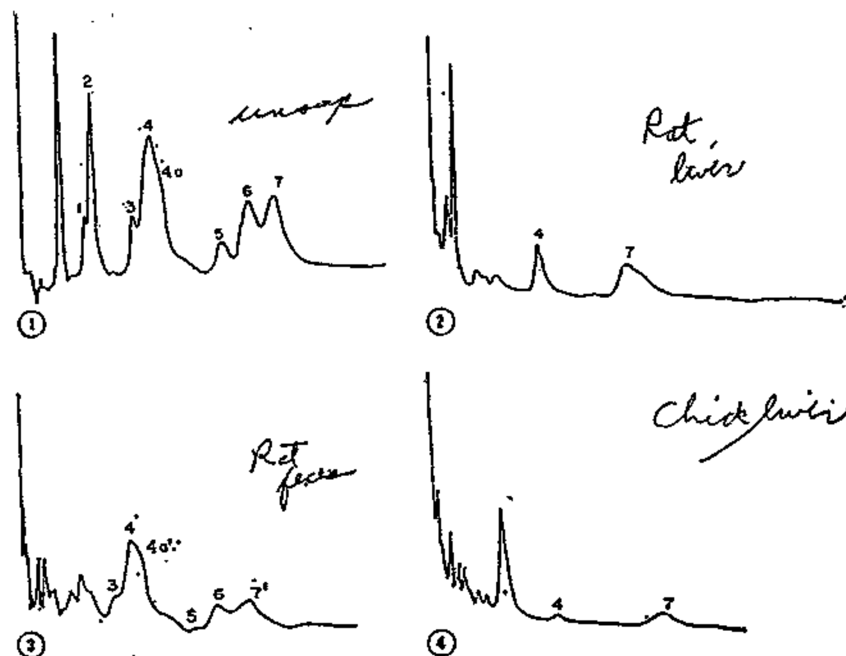

FIGURE 1.—Toxic CEF components found in unsap used in this study.
FIGURE 2.—Rat liver extract showing 2 CEF peaks.
FIGURE 3.—Rat feces extract showing CEF peaks, with altered Nos. 4a and 7.
FIGURE 4.—Chick liver extract showing 2 CEF peaks. (Large peak just before No. 4 found to be contaminant leached from liner of sample vial.)

birds (considered normal), there was a packed cell volume of 33.0%, while for diseased birds having 0.73 ml HPF, the packed cells volume was 27.9%.

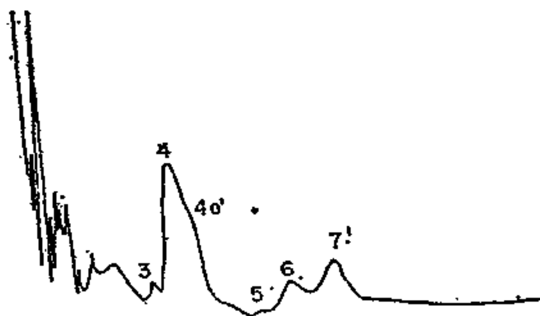

FIGURE 5—Chick feces extract showing CEF peaks, with altered Nos. 4a and 7.

Also, we observed that whereas control birds will show disappearance of a given amount of an I.V. injected dose of T-1824 dye (Evans Blue) from their vascular system of approximately 1%/min, poisoned birds will show a disappearance of approximately 2%/min, supporting the observation of Allen (4), that the permeability of the vascular wall appears to be increased.

Distribution of the CEF in animal body.—Of the rat tissues and samples examined, CEF was detected only in the liver and feces. Fig. 2 through 5 show typical chromatograms of purified extracts of feces and liver of both species.

Control animals not receiving CEF did not show these peaks. These chromatograms show that only peaks 4 (or 4a?) and 7 were present in the liver. In the fecal extracts all peaks were found, with the exception that instead of peaks 4a and 7 showing their original retention times, these were slightly increased in each case and have been designated 4a′ and 7′.

These retention times increases in the fecal components were measured by noting their retention times in relation to their neighbors, as shown in Table IV. Whether the 2 components of the liver are the products of components 4 and 7 or the original unaltered substances cannot be accurately determined from these chromatograms, since the rest of the CEF chromatographic pattern is missing. It may be concluded, however, that there is a selective absorption of peaks 4 (or 4a) and 7, with metabolism by the liver and excretion into the intestine.

It appears, on the basis of the tissues analyzed, that the liver is the target organ. Furthermore, the pattern of CEF chromatographic peaks presented here, Nos. 4 and 7 are the key components. We have succeeded in partially separating the CEF components such that 4a and 7 crystallized together, indicating a certain chemical as well as physiological similarity.‡ There is no way of differentiating 4 from 4a with regard to its absorption and metabolism on the basis of the available evidence.

Summary. Unsaponifiable matter isolated from a toxic fat and containing an estimated 10 ppm chick edema factor (CEF) was force-fed daily to adult rats at levels of 2.0 cc and 1.0 cc/kg body weight/day in 2 studies of 14 and 6 days, respectively. Feed consumption, body weight, and digestibility were depressed. Heart and spleen weights were unaffected, kidney weights seemed to be slightly increased, and adrenal and liver weights were significantly increased. In the chick, typical hydropericardium, ascites, and subcutaneous edema were observed. There were no significant changes in heart or spleen weights. Liver weights were significantly increased. The rat was as sensitive as the chick to CEF according to increase in liver weight. Of the 8 or 9 CEF components shown to be in this unsap, 2 (Nos. 4 and 7) were found to be absorbed and located in the liver, while the other components were not detected. In place of Nos. 4 and 7, there were 2 new peaks in the feces with slightly increased retention times. This suggests that the 2 active CEF components are *metabolized in the liver and excreted into the intestine via* the bile, both in the chick and the rat. No CEF-like material was found in kidneys, adrenals, or urine.

TABLE IV.—CHANGES IN CHROMATOGRAM RETENTION TIMES OF CEF PEAK NOS. 4a AND 7

Material analyzed	No. of runs	Relative retention times	
		$R_{7:5}$	$R_{4a:3}$
Firestone Standard	31	[3] 1.10±.01	4a not found
Unsap [1]	2	1.11±.01	1.22±.01
Feces [2]	13	1.14±.03	1.27±.04

[1] Unsaponifiables administered to animals in this study.
[2] Includes feces of 11 individual rats and the composite feces of 2 groups of 6 chicks each.
[3] The deviation includes the total range of values.

Studies on the Metabolism of Chick Edema Factor: Distribution in Chick Tissues

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The distribution of chick edema factor in chick tissues following consumption of rations containing toxic fat has been of considerable interest. Chick

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edema factor was detected in chicken tissues by electron capture gas chromatography, and an estimation of the concentration in various tissues was made by evaluation of gas chromatographic response.

Thirty samples consisting of homogenized tissues and parts from three groups of chickens, submitted by the Division of Nutrition, were examined for chick edema factor by a recently developed method.[1] One group had been fed a ration containing 3% of a reference toxic fat. Another group had been fed a ration containing unsaponifiables equivalent to 3% of the toxic reference fat. The last group received a ration free of toxic fat. The weights of tissue samples ranged from 0.4 to 93.4 grams. The samples were received in glass stoppered Erlenmeyer flasks, in ethanol.

DETERMINATION

(1) Extraction of unsaponifiable matter from animal tissue (Modification of AOAC Official Method 26.071).

Quantitatively transfer an alcoholic solution of the homogenized tissues to a 24/40 round bottom flask, add ethyl alcohol to give a final volume of 4 ml/gram tissue, but not less than 50 ml. Add 2 ml KOH solution (3 + 2) per gram of tissue.

Saponify by boiling with occasional swirling on a steam bath for one hour under reflux air condenser. Transfer alcohol soap solution while still warm to separator using water (equivalent to twice the volume of ethyl alcohol). Rinse saponification flask with the same volume of ethyl ether and transfer to separator. Shake vigorously, let layers separate and clarify, breaking any emulsion by adding up to 1/20 volumes of alcohol and swirling gently. Drain lower layer and pour ether layer through top into a second separator containing water (2 ml/g tissue), but not less than 20 ml. Make two more extractions of soap solution with ethyl ether (8 ml/g tissue). Rinse pouring edge with ethyl ether and add rinsings to second separator.

Rotate combined ether extracts gently with the H_2O (violent shaking at this stage may cause troublesome emulsions). Let layers separate and drain aqueous layer. Wash with two additional portions of H_2O (2 ml/g tissue), shaking vigorously. Then wash ether solution two times with alternate portions (2 ml/g tissue) of K_2CO_3 and H_2O. If emulsion forms during washing, drain as much of aqueous layer as possible, leaving emulsion in separator with ether layer and proceed with next washing. Wash final solution with H_2O until washings are neutral to phenothalein.

Transfer ether solution to erlenmeyer, rinsing separator and its pouring edge with ether, adding rinsings to main solution. Dry ether solution by adding anhydrous Na_2SO_4 (1 g/g tissue) and swirling vigorously ca 1 min. Let solution stand 10 min. Decant ether solution through glass funnel containing pledget of pre-rinsed cotton in neck and holding 25 gr anhydrous Na_2SO_4 into another erlenmeyer containing boiling chips. Wash first erlenmeyer with ether and transfer to second erlenmeyer.

Evaporate most of solvent on steam bath and transfer to 100 ml (preweighed) extraction flask. Evaporate residual solvent on steam bath under N_2. Dry flask to constant weight and obtain weight of unsaponifiable matter.

Proceed with fractionation of unsaponifiable matter on alumina and cleanup of alumina fraction 3 as directed in method (1), analyzing the residue by electron capture gas chromatography as directed.

RESULTS AND DISCUSSION

All residues except for liver samples were initially taken up in 100 ul of iso-octane and 5 ul injected. The liver samples were taken up in 250 ul of iso-octane and 1 ul injected. Examination of the chromatograms indicated the presence of a small contaminant (Ra 11.1) in the reagents used in the cleanup of the negative control samples.

Six out of 10 samples were "toxic" from each group of chicks fed toxic fatty material. The liver sample in each group was the most "toxic", containing roughly 83% of the total "toxicity" as indicated in Table I. The terms "toxic" or "toxicity" as used here to describe results of gas chromatographic analyses refers to the amount of material in an individual tissue which produces characteristic peaks at Ra 12 and 22 resulting from feeding the toxic fat. Of the pooled adrenals, bone, brain, heart, intestine, kidney, liver, skeletal muscle, skin, and testes that were examined, only the bone, heart, intestine, kidney,

liver, and skin showed peaks characteristic of the presence of chick edema factor. No characteristic peaks were found in the adrenals, brain, skeletal muscle, and testes; perhaps the concentration was too low to be detected in these tissues.

The toxic fat as well as the toxic unsaponifiables used for this study exhibited 4 characteristic GLC peaks of Ra = 10.6, 12.4, 18.6, and 21.6 (See Table 2). The intestine chromatograms exhibited greatly diminished 10.6, 18.6 and 21.6 peaks, and a peak appeared at 12.0 in place of the 12.4 peak of the toxic fat and toxic unsaponifiables. The 10.6 and 18.6 peaks were not evident in chromatograms from the other tissues exhibiting characteristic peaks, and the 12.0 peak was the major characteristic peak. In addition, the bone, heart, kidney and skin extracts from the chicks fed toxic fat (Group A) exhibited a 12.4 peak which occurred as a shoulder on the major 12.0 peak.

These results suggest a selective absorption of the chlorinated components of the toxic fat; this is most clearly indicated by the diminished 10.6 peak in the intestine extract which is completely absent in the other tissue extracts. The appearance of peaks at 12.0 or 12.0 and 12.4 in place of the 12.4 peaks of the toxic fat can also be explained by selective absorption and deposition of individual components if we recognize that the GLC peaks observed each represent more than a single component. Campbell and Friedman (2) have also observed selective absorption of chick edema factor components in rats as well as in chicks. However, these authors only detected chick edema factor in the liver and feces.

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TABLE I.—RELATIVE % OF CHICK EDEMA FACTOR IN POSITIVE TISSUES[1]

Tissue	Group A (3 percent toxic fat)	Group B (unsap.～3 percent toxic fat)
Bone	7.1	4.8
Heart	0.6	1.2
Intestine	2.4	2.4
Kidney	2.4	1.8
Liver	82.8	83.6
Skin	4.7	7.2

[1] The relative percent of CEF in each positive tissue was estimated by adjusting the volume of each sample so that a 5 ul injection of each would yield a chromatogram exhibiting the major peak (Ra 12) with a height of ca 6 cm. The following formula was used to calculate percentages:

$$\% \text{ CEF} = \left(\frac{\text{volume of sample}}{\text{Total volume of all samples in group}}\right) \times 100$$

TABLE II.—RETENTION TIMES (RA)[1] OF CHARACTERISTIC OF CHICK EDEMA FACTOR COMPONENTS IN TOXIC FAT ADDED TO DIET AND ISOLATED TISSUES

[Barber Colman Model 5360 gas chromatography; 7 foot 1/4 id glass column packed with 2½% SE52 silicone gum rubber on 60–80 mesh Gas Chrom Q; 3 x 10-8 amperes full scale; detector voltage, 30; injector temp., 240° C; column temp. 200° C; detector temp., 210° C]

Sample	Group A (Fed 3% toxic fat)	Group B (fed unsap. ≅ (3% toxic fat)
Toxic fat	10.6, 12.4, 18.6, 21.6	
Unsaponifiables (from toxic fat)		10.6, 12.4, 18.6, 21.6
Adrenals	None	None.
Bone	12.0, 12.4, 21.6	12.0, 21.6
Brain	None	None
Heart	12.0, 12.4, 21.6	12.0
Intestine	10.6, 12.0, 18.6, 21.6	10.6, 12.0, 21.6
Kidney	12.0, 12.4, 21.6	12.0, 21.6
Liver	12.0, 21.6	12.0, 21.6
Skeletal Muscle	None	None
Skin	12.0, 12.4, 21.6	12.0, 21.6
Testes	None	None.

Ra=
[1] Retention time relative to aldrin.

LIGHT AND ELECTRON MICROSCOPIC OBSERVATIONS IN *Macaca mulatta* MONKEYS FED TOXIC FAT

J. R. Allen, D.V.M., Ph.D., and L. A. Carstens, B.S.

SUMMARY

Thirty-six *Macaca mulatta* monkeys were given a diet that contained 0.125 to 10.0% of a fat capable of producing hydropericardium, ascites, and death in chickens. There was an inverse relationship between the concentration of toxic fat in the diet and the survival time of the monkeys. The monkeys given the greatest level of toxic fat had the mean survival time of 91 days, and the monkeys given the lowest level and the mean survival time of 445 days. During the last 30 days of life, the monkeys developed generalized subcutaneous edema, ascites, hydrothorax, and hydropericardium. There were decreases in erythrocytes, leukocytes, total serum protein values, and altered albumin:globulin ratios. There was also cardiac dilatation and myocardial hypertrophy and edema. Experimental monkeys had reduced hematopoiesis and spermatogenesis, degeneration of the blood vessels, focal necrosis of the liver, and gastric ulcers. It was proposed that toxic fat exerted its injurious effects upon the parenchymal cells of the liver, endothelium, and myocardium with subsequent development of generalized anasarca.

Fats from plant and animal sources have been used to increase the caloric level of diets for animals. As a result of increased demands by feed manufacturers for low-cost fats, almost every available source of these products has been utilized. Certain fats were found to be extremely toxic to poultry, and hundreds of thousands of chickens died or were killed after they were fed diets containing these fats. Results of experiments indicated that young chickens developed hydrothorax, pulmonary edema, ascites, and subcutaneous edema in 1 or 2 weeks when there was toxic fat in their diet.[1,3,15-17] The accumulation of large quantities of extravascular fluid in chickens seemed to result from altered permeability of the vascular bed, cardiac decompensation, and liver necrosis.[1,3]

Chemical studies on toxic fat indicated that the toxic fraction was located in the unsaponifiable portion.[6,20] By repeated passages through alumina and silica gel columns, crystalline preparations of the toxic fraction were prepared[6]; however, the chemical composition of the compound was not determined.

Before the cause of this intoxication of poultry was established, many chickens that had been fed toxic fat were processed for human consumption. Since that time, the clinical, histologic, and electron microscopic changes that occurred in the intoxicated chickens have been enumerated.[1,3,15,17] Data are not available, however, concerning the effects of toxic fat on primates. Since the chemical composition of this fat is unknown, the possibility exists that it may once again adulterate various edible fats. This is a report on experiments undertaken to determine the effect of toxic fat on lower primates. The results of the experiments may be helpful in postulating the effects that toxic fat might have in man.

MATERIALS AND METHODS

Since the chemical nature of toxic fat was unknown and chemical procedures were not available to determine the toxicity, a biological assay was performed on the fat used in the experiments. When the diet of 1-day-old chickens contained 3.0% toxic fat, 50.0% died within 15 days.

In the initial experiment, 16 *Macaca mulatta* monkeys (av. weight, 4.2 kg.) were allotted to 4 groups and fed diets containing 0 (control), 1.0, 5.0, and 10.0%, respectively, of toxic fat. In the 2nd experiment, 20 *M. mulatta* monkeys (av. weight, 6.0 kg.) were allotted to 4 groups and fed diets containing 0 (control), 0.125, 0.25, and 0.5%, respectively, of toxic fat. The toxic fat was combined with corn oil to obtain similar fat intake levels in all groups of monkeys. The following data were obtained at 1-month intervals: total serum protein value,[7] serum electrophoretic pattern,[18] complete blood count,[19] prothrombin time,[13] serum bilirubin value,[10] cholesterol level,[4] serum electrolytes,[9] blood urea nitrogen value,[14] and body weight. Observations were made each day on the general appearance and amount eaten. Needle biopsies of the liver were performed each month throughout the experiment to study sequential changes that occurred in hepatic tissues.

All monkeys were necropsied immediately after death. When possible, the monkeys were killed immediately prior to their anticipated death to ensure procurement of fresh tissue for light and electron microscopy. Tissue sections from heart, lung, liver, spleen, mesenteric lymph node, sternal bone marrow, skeletal muscle, testis, gastrointestinal tract (3 zones of stomach and every 10 cm. of intestinal tract), skin, adrenal gland, pancreas, kidney, cerebrum, cerebellum, pituitary gland, thyroid gland, parathyroid gland, and urinary bladder were fixed in 10.0% neutral formalin, dehydrated, embedded in paraffin, sectioned at 6 μ, and stained with hematoxylin and eosin stain. Frozen sections from liver and heart were stained with Sudan IV for neutral fats.

Sections of liver and heart were taken from each monkey, cut into small cubes, and fixed in Millonig's[11] and Caulfield's[5] fixatives. These tissues were subsequently dehydrated through a graded series of ethanol and embedded in a plastic resin mixture.[12] Sections of the tissues were cut on an ultramicrotome, placed on 400-mesh uncoated copper grids stained with uranyl acetate, and examined with an electron microscope.*

TABLE 1.—TERMINAL HEMATOLOGIC CHANGES IN MONKEYS FED DIETS CONTAINING TOXIC FAT

Group	No. of monkeys	Total serum protein (Gm./100 ml.)	Serum albumin (%)	Packed cell volume (%)	White blood count $\times 10^3$/cmm.	Red blood count $\times 10^6$/cmm.
Controls	9	7.5	61	41	6.8	6.5
Fed toxic fat	27	5.4	35	16	3.0	2.5

RESULTS

In the initial experiment, monkeys in the groups fed diets containing 5.0 and 10.0% of toxic fat had the mean survival time of 91 days. The monkeys in the group fed the diet with 1.0% toxic fat had the mean survival time of 169 days.

In the 2nd experiment, the monkeys survived for a much longer period. The monkeys in the groups fed 0.5, 0.25, and 0.125% toxic fat had mean survival times of 202, 274, and 445 days, respectively.

There were considerable differences in survival times of monkeys in the various groups; however, the major clinical and pathologic changes were similar and occurred during the terminal 30 days regardless of whether the monkey survived for less than 4 months or longer than 1 year. Therefore, the data from the experimental monkeys will be presented collectively.

HEMATOLOGIC EVALUATIONS

Total serum protein values of the monkeys were reduced approximately 2.0 Gm./100 ml. during the experiment (Table 1). The mean percentage of serum albumin in the monkeys fed toxic fat on the last determination prior to death was 35%, whereas that of the control monkeys averaged 61%. There was a gradual decrease in the cellular elements of the blood during the experiments. Packed cell volumes were reduced from 32% to 16% during the last 30 days of life. These findings were substantiated by the total red blood cell counts which averaged 2.5 million/cmm. of blood immediately before the monkey died. The hemoglobin values followed a similar course, with the mean value of 6.0 Gm./100 ml. of blood being obtained in the last 30 days of life. Comparable observations were recorded for white blood cell counts; these averaged 3,000 white cells/cmm. of blood on the last hematologic evaluation of the experimen-

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* RCA EMU-3G Electron Microscope, Radio Corporation of America, Camden, N.J.

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tal monkeys. Prothrombin times, serum bilirubin values, serum electrolytes, blood urea nitrogen values, and cholesterol levels of serum were not changed appreciably during the experiment.

CLINICAL OBSERVATIONS

The major changes in the monkeys were the development of generalized alopecia and subcutaneous edema 1 to 2 months before death. Edema, first noticed around the lips and eyelids, progressed to the remainder of the face and eventually involved the subcutaneous tissue of the trunk and extremities. Especially obvious was the marked edema of the scrotum and sheath which developed during the last few weeks of life and, in some monkeys, partially obstructed the flow of urine. During the last month of life there was decreased food consumption and the subsequent loss of body weight was frequently as much as 1 kg. Diarrhea developed in 75% of the experimental monkeys during the last few days of life. Results of bacteriologic cultural examinations of feces were negative for pathogenic enteric organisms.

GROSS AND MICROSCOPIC FINDINGS

The findings at necropsy substantiated the presence of extensive subcutaneous edema in over 75% of the monkeys fed toxic fat.

Heart.—Dilatation of the heart was especially obvious on the right side. This was further clarified when the circumference of the valves was determined. The mean tricuspid and mitral valve circumference of the experimental monkey hearts was 55 and 45 mm., respectively; in contrast, the tricuspid and mitral valves of the control monkey hearts averaged 40 to 36 mm., respectively. Hypertrophy of the cardiac muscle was also apparent in the experimental monkeys. The hearts of the experimental monkeys were 0.55% of the body weight, whereas hearts of the control monkeys were 0.30%. Microscopically, the muscle fibers were distinctly separated by fluid. Individual muscle cells were hypertrophic, and their nuclei were enlarged, distorted, and hyperchromic (Fig. 1). There were no distinct valvular lesions in hearts of the experimental monkeys.

Lungs.—Lungs of experimental monkeys were not altered appreciably. Isolated areas of atelectasis, congestion, edema, and fibrosis were observed. The proliferation of fibrous connective tissue was associated with the presence of lung mites (*Pneumonyssis simicoli*).

Liver.—Livers of experimental monkeys were small, firm, and moderately yellow. On microscopic examination, moderate distortion of the architecture was found. Many parenchymal cells were enlarged, multinucleated, and had only moderate affinity for stain, whereas other cells were small and markedly hyperchromic. There was also focal necrosis of the parenchymal cells in the centrilobular zone (Fig. 2). Many parenchymal cells contained vacuoles in their cytoplasm which stained positively for neutral fat when frozen sections were prepared. Small areas of fibrous connective tissue occurred in the periportal area; however, they did not alter the architecture appreciably.

Spleen.—Spleens of experimental monkeys averaged only 0.074% of the body weight, and those of the control monkeys were 0.13%. Microscopically, the germinal centers were surrounded by only a narrow zone of lymphocytes, the blood sinuses were practically devoid of cells, and the trabeculae were especially prominent.

Mesenteric Lymph Nodes.—Lymph nodes were light tan and edematous. Microscopically, the germinal centers were surrounded by a narrow band of lymphocytes. The medullary cords were indistinct, and the sinuses were filled with proteinaceous fluid.

Sternal Bone Marrow.—Grossly, the bone marrow resembled coagulated plasma. Microscopically, only a small number of hematopoietic cells were seen in the marrow, and those were approximately equally divided between the

myeloid and erythroid series (Fig. 3). Most of the bone marrow was composed of fatty tissue and proteinaceous fluid. The blood vessels and sinuses contained only a limited number of cells.

Skeletal Musculature.—The skeletal muscle was pale and edematous. Microscopically, the muscle bundles and fibers were widely separated by fluid, but otherwise seemed normal.

Testes.—Grossly, the testis seemed normal; however, when examined microscopically, active spermatogenesis was not found. The seminiferous tubules had abundant spermatogonia and Sertoli cells, but only a limited number of primary spermatocytes. There were no spermatids or mature spermatozoa. Interstitial tissue was moderately edematous, but the Leydig cells did not seem affected.

Gastrointestinal Tract.—In 18 of the 27 experimental monkeys, marked hypertrophy of the gastric mucosa occurred in the fundic and pyloric regions. In the same areas, small gastric ulcers penetrated the mucosal layer. (Fig. 4). In 6 monkeys, inflammatory changes in the intestinal tract were seen. Results of bacteriologic evaluations of these lesions indicated no pathogenic organism that could have been associated with the gastrointestinal disturbance. Microscopically, the hyperplastic gastric mucosa was seen to form many large, interdigitating folds. Adjacent to these proliferative areas, the mucosal lining was eroded, and the underlying tissue was necrotic. Large numbers of polymorphonuclear leukocytes were in the necrotic tissue and underlying musculature. Blood vessels of the edematous submucosal and muscular layers of the stomach adjacent to the ulcerated areas were free of obstruction.

Enteritis in 6 monkeys had caused moderate denudation of the intestinal mucosa and considerable hemorrhage into the lumen. The mucosal lining near the base of the crypts was intact, and the underlying musculature was normal.

Skin.—There was marked edema of the dermal layer of the skin, causing disarray of the collagen fibers. The epidermal layer was comparable in the control and experimental monkeys. There was an absence of any detectable change in the hair follicles of the monkeys given toxic fat.

The adrenal gland, pancreas, kidneys, cerebrum, cerebellum, pituitary gland, thyroid gland, and urinary bladder of the experimental and control monkeys were comparable grossly and microscopically.

ELECTRON MICROSCOPIC CHANGES

The extent of electron microscopic change in the liver correlated well with the level of toxic fat in the diet and the duration of toxic fat consumption. An early change in the parenchymal cells was the disruption of the orderly arrangement of the granular endoplasmic reticulum. The cisternal spaces were dilated, and the loss of ribosomes from the outer surfaces of the cisternae resulted in an apparent increase in the smooth endoplasmic reticulum. As a result of mitochondrial swelling, the cristae seemed shorter and less abundant than those of the control monkey hepatic cells (Fig. 5 and 6). Cytosomes of variable content and size were moderately prevalent in the cytoplasm. Small vesicles and flattened lamellae comprised the relatively small Golgi complex. Fat vacuoles were abundant throughout the cytoplasm (Fig. 6). The nuclei contained distinct nucleoli and abundant chromatin dispersed throughout the nucleoplasm.

Results of hepatic biopsies made a few weeks before death, and experimental tissues obtained at the time of death, indicated distinct alterations. Many parenchymal cells were shrunken and electron dense (Fig. 7). These dark cells were seen in various stages of degeneration. In some cells, the cytoplasmic organelles were still visible despite the extremely dense matrix. Vacuoles were dispersed between the cellular organelles. Myelin bodies were abundant in the cytoplasm, along the plasmalemma, and in the intercellular spaces. The nuclei were also electron dense, and the nuclear envelope was only vaguely discernible. Other dark cells had lost all resemblance to normal parenchymal cells,

being devoid of discernible cytoplasmic organelles and nuclei. Microvilli were extremely prominent and abundant along the plasmalemma adjacent to Disse's space.

The lighter cells (*Fig. 8*) seemed to follow an entirely different course in their degenerative process. The cytoplasmic organelles were quite distinct in the abundant matrix. There was marked disruption of the endoplasmic reticulum, with only short fragments being scattered throughout the cytoplasm. Abundant free ribosomes were quite evenly dispersed between the organelles. The external contours of the mitochondria were frequently irregular, and the matrix was moderately electron dense. Occasionally, bulblike projections were formed by the external mitochondrial membrane. The plasmalemmae were irregular, and the microvilli were short and sparse. Occasionally, there were myelin bodies along the plasmalemmal surface. In some instances, the light cell plasmalemmae had ruptured, and organelles were dispersed throughout the extracellular space.

Bile duct epithelium was affected markedly in monkeys fed diets containing toxic fat. Many of the epithelial cells were so electron dense that the cytoplasmic organelles were difficult to visualize, and their nuclear membranes were irregular and extremely dense. The interlocking plicae of adjacent cells were widely separated. As a result of the shrunken condition of the epithelial cells, microvilli on the luminal surface of the plasmalemmae seemed thin and elongated.

Endothelial cells in some areas of the liver had a distinct resemblance to the dark parenchymal and bile duct epithelial cells. The dark endothelial cells were shrunken, and their internal structures were distorted. There were widened fenestrations between the endothelial cells. Changes in the cytoplasmic organelles were comparable with those observed in the parenchymal cells. Occasionally, large cytoplasmic sequestra from the endothelial cells were observed in the lumen of the vessels.

Heart.—The main differences between hearts of control and experimental monkeys were the dilatation of the intercellular spaces and the wide dispersal of the myofibrils in the latter. Between the widely separated groups of myofibrils (Fig. 9), there were mitochondria, occasionally a segment of sarcoplasmic reticulum, and abundant matrix. Usually, only the Z lines could be readily visualized. Cytosomes were much more abundant in the muscle cells of experimental monkeys and were usually found near the nucleus (Fig. 10). In more than 75% of the hearts of the experimental monkeys there was a distinct swelling of the mitochondria. The cristae were widely separated, and the mitochondrial matrix was abundant (Fig. 11). Large myelin figures were observed within and surrounding many mitochondria. Myocardial nuclei, components of the transverse tubular system, as well as elements of the sarcoplasmic reticulum, seemed unaffected. There was a noticeable separation of intercalated disks in many of the experimental monkeys (Fig. 12). The various bands formed by the myofibrils were often masked due to the abundance of edematous fluid in the tissue.

Many of the endothelial cells appeared electron dense and shrunken, with distortion of the nuclei and cytoplasmic organelles (Fig. 13). In most instances, the organelles were in close apposition as the result of the cell shrinkage. There were cytoplasmic myelin bodies in many of the cells. Occasionally, intercellular stromal cells had changes similar to those in the endothelial cells.

DISCUSSION

Toxic fat consumption had a decided effect upon hematopoiesis and spermatogenesis. Myeloid, erythroid, and lymphoid elements of the peripheral blood were markedly reduced. Germinal centers in the lymph nodes and spleen and the islands of hematopoietic cells in the marrow were extremely sparse. Inhibited spermatogenesis was also observed in the monkeys fed toxic fat. Similar observations have been reported in chickens fed toxic fat.[2] The exact mechanism by which toxic fat exerts its inhibitory effects upon hematopoiesis and spermatogenesis has not been determined, and additional research will be required to elucidate these points.

The relationship of toxic fat consumption by monkeys and the development of moderate to extensive alopecia has not been established. Periodically, under normal conditions, there is a loss of hair by most monkeys. However, only in isolated instances has alopecia become as extensive as that of the monkeys given toxic fat.

An interesting and not well-understood lesion was the development of gastric ulcers in more than 66% of the monkeys fed toxic fat. The microscopic appearance of the ulcers was similar to that observed in man and lower animals, and determination of the etiologic factors was equally evasive. The direct effect of toxic fat upon the gastric mucosa and underlying vasculature must be considered; however, vascular lesions associated with the ulcers were not observed in the monkeys examined.

The most noticeable lesion produced by toxic fat consumption was the accumulation of large quantities of fluid in the tissue spaces and body cavities. When the blood protein values were tested, a decrease in total serum protein and a reversal in the albumin:globulin ratio were found (Table 1). The decrease in serum protein values, particularly albumin, could not be attributed to altered renal function because there was no increase in blood urea nitrogen or albumin in the urine. The most logical explanation for the decrease in serum protein would be related to the changes observed in the liver. Focal areas of necrosis and degeneration were observed in the livers of all experimental monkeys. Examination of electron micrographs of these degenerative areas substantiated the light microscopic observations. As a result of the altered functional status of the hepatic parenchymal cells, there was a decrease in albumin production. This would, in turn, alter the osmotic pressure of the blood sufficiently to produce stasis of fluid in the extravascular spaces.

Another important aspect of the problem was the effect of toxic fat upon the vasculature. Many of the hepatic and myocardial vessels were dark, shrunken, and seemed to have undergone degenerative changes. Since these vascular changes were not detected when the tissues were examined with the light microscope, it is likely that other vessels throughout the body had undergone similar changes. These observations would correlate well with the vascular changes recorded in chickens that have consumed toxic fat.[1] As a result of cellular shrinkage, the intercellular spaces were widened and there was a subsequent increase in the porosity of the vessel. This porosity would eventually lead to an increase in vessel permeability and predispose to the accumulation of extravascular fluid. There was cardiac dilatation and hypertrophy in all of the monkeys that consumed toxic fat. When the myocardium was examined microscopically, there was hypertrophy of the individual muscle cells and marked interstitial edema. Both of the latter have been commonly reported in patients with cardiac insufficiency. As the heart became less efficient, there was likely a gradual increase in hydrostatic pressure on the venous side which eventually produced stasis of fluid in the tissue spaces. An increase in right side of the heart and vena cava pressure similar to that postulated for the monkeys has been reported in chickens fed toxic fat.[1]

Reduced osmotic pressure of the blood, increased capillary permeability, and cardiac insufficiency have been incriminated as the causes for development of anasarca in monkeys fed toxic fat. It is difficult to determine at this time which of these entities was responsible. The most likely explanation is that all 3 play a major role, with each becoming the paramount entity at a particular stage of the disease.

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CALCULATED DIETARY INTAKES OF CHICK EDEMA FACTOR (CEF) FROM DATA PUBLISHED BY ALLEN AND CARATONS (AM. J. VET. RES, 28, 1513-26 (1967))

Dietary level of toxic fat (Percent)	Mean survival time (days)	CEF intake per animal per day (ug.)	Total CEF intake (ug.)
MONKEYS (MACACA MULATTA)[1]			
0.125	445	0.225	[illegible]
0.25	274	0.45	[illegible]
0.50	202	0.90	[illegible]
1.0	169	1.80	[illegible]
5.0	90	8.0	[illegible]
10.0			
CHICKS (DAY-OLD)[2]			
3.0	15	0.36	[illegible]

[1] Calculations based on average daily food consumption of 225 gm. for a 5.0-kg. monkey (F. Sperling, personal, communication, June 12, 1969).
[2] Calculations based on average daily food consumption of day-old chicks (personal studies, see Flick, et al., Poultry Science, 45, 630-36 (1966)).

Note on an Improved Cleanup Method for the Detection of Chick Edema Factor in Fats and Fatty Acids by Electron Capture Gas Chromatography

By PAUL NEAL (Division of Food Chemistry, Food and Drug Administration, Washington, D.C. 20204)

The electron capture GLC screening test for chick edema factor (1, 2) has been modified by replacing the saponification step with a sulfuric acid cleanup (3, 4) which permits a 50% reduction in sample cleanup time. The modified procedure involves treatment of 25 g sample with sulfuric acid, fractionation of the petroleum ether extract from the sulfuric acid treatment on an alumina column, and sulfuric acid cleanup of the third alumina fraction, followed by electron capture GLC. Gas chromatographic peaks with retention time versus aldrin of 10-25 are indicative of the presence of chick edema factor.

Table 1. Comparison of sulfuric acid cleanup with saponification for detection of chick edema factor by electron capture gas chromatography (ECGLC)

Sample[a]	ECGLC Analysis[b]	
	Saponification	H_2SO_4 Cleanup
Low positive reference fat (1.5% toxic fat in USP Cottonseed Oil)	10.1 (45), 11.8 (48), 17.6 (65), 20.4 (60)	10.1 (18), 11.8 (14), 17.6 (40), 20.4 (34)
Toxic animal tallow	10.2 (74), 11.9 (82), 18.8 (94), 20.6 (98)	10.2 (56), 11.9 (42), 18.8 (85), 20.6 (90)
Toxic oleic acid	10.1 (147), 11.8 (23), 17.8 (>500), 20.4 (200)	10.1 (98), 11.8 (16), 17.8 (>500), 20.4 (154)
Toxic glyceryl monooleate	10.1 (50), 11.8 (23), 17.8 (200), 20.4 (82)	10.1 (48), 11.8 (14), 17.8 (258), 20.4 (98)
Vegetable oil soapstock (nontoxic)	10.4 (trace), 13.5 (trace)	10.4 (trace), 13.5 (trace)
Oleic acid (nontoxic)	10.4 (trace), 13.5 (trace)	10.4 (trace), 13.5 (trace)
Cottonseed oil (nontoxic)	10.4 (23), 13.5 (22)	10.4 (trace)
Blank	10.4 (trace), 13.5 (trace)	10.4 (trace), 13.5 (trace)

[a] Toxic and nontoxic refer to results of AOAC chick bioassay (AOAC *Official Methods of Analysis*, 10th Ed., 1965, 26.087–26.091).
[b] The first values (without parentheses) refer to retention time of peaks at 200°C vs. aldrin; the values in parentheses refer to peak area which is equal to retention time (cm) × peak height (cm).

Method

Reagents and Apparatus

Rinse all glassware with appropriate solvent before use. Do *not* use polyethylene containers to store solvents (5).

(a) *Petroleum ether*.—Reagent grade; redistill in glass between 30° and 60°C (available from Burdick and Jackson Laboratories, Muskegon, Mich.).

(b) *Carbon tetrachloride*.—Distilled-in-glass.

(c) *Celite*.—Johns-Manville #545, acid-washed. Wash well with petroleum ether and dry.

(d) *Filter paper*.—#549 S&S Blue Ribbon, or equivalent.

Determination

Sulfuric acid cleanup.—Dissolve 2.5 g fat in 10 ml CCl_4 in 400 ml beaker (heat, if necessary). Add 10 ml concentrated H_2SO_4 and then 20 g Celite; mix with heavy glass stirring rod during additions and stir until homogeneous mixture is obtained. Add 125 ml petroleum ether, mix well, let solids settle, and filter the supernatant liquid through filter paper in 90 mm conical funnel. Repeat with additional 125 ml portion of petroleum ether. Evaporate combined petroleum ether filtrate to 5 ml for alumina column fractionation.

Complete determination as outlined in the method of Higginbotham *et al.* (1).

Results and Discussion

GLC retention times and peak areas for negative, positive, and blank samples were compared for both the sulfuric acid and the saponification methods; see Table 1. Results are comparable as indicators of toxic material. Gas chromatographic peak heights were lower in some cases with the sulfuric acid cleanup; however, the presence of toxic factor was clearly indicated in the low positive reference material. The nontoxic cottonseed oil sample would have been judged toxic by the saponification method because of the relatively large GLC peak of R_a 13.5, a peak detected at low levels in gas chromatograms from blanks and other nontoxic samples; see Table 1. Small peaks of R_a 10–25 were observed in both procedures in the blank and nontoxic samples. However, they did not interfere with identification of the toxic fats and differentiation of toxic from nontoxic samples. Gas chromatograms of blank and low positive reference samples after saponification and preliminary sulfuric acid treatment are shown in Figs. 1 and 2.

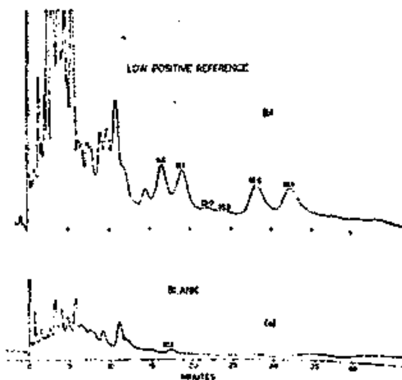

Fig. 1—Gas chromatograms of (a) blank and (b) low positive reference fat after cleanup with saponification.

GLC conditions: 7′ × 4 mm i.d. glass column packed with 2.5% SE-52 on 60–80 mesh Gas Chrom Q at 205°C. Amount injected: 1/50th of alumina column fraction 3.

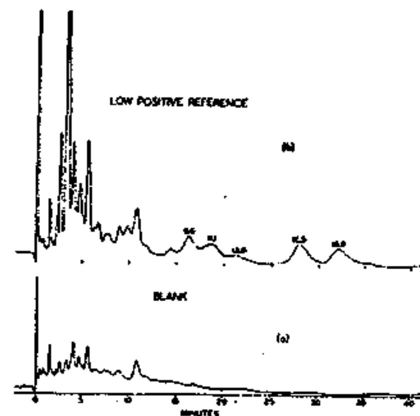

Fig. 2—Gas chromatograms of (a) blank and (b) low positive reference fat after cleanup with sulfuric acid treatment. See Fig. 1 for GLC conditions.

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Reprinted from the *Journal of the Association of Official Analytical Chemists*, Vol. 50, December 1967.

(1) The official, first action GLC-microcoulometric method for chick edema factor, 26.092–26.096, was changed by adding the following to 26.092:

(g) *Ethyl ether for alumina chromatography.*—Ether (not >2% alcohol) or absolute ether (not >0.01% alcohol) (available from Burdick and Jackson Laboratories).

(2) The official, first action electron capture method for detection of chick edema factor, *This Journal* 50, 216–218(1967) was changed by addition of the following to (b) in the *Determination* section:

After ". . . 26.094" in line 6 add "(using ether specified in 26.092(g)" (item (1) above).

(3) The following rapid screening method for detection of chick edema factor was adopted as official, first action:

PRINCIPLE

Samples are subjected to preliminary H_2SO_4 cleanup and extd with petr. ether. Ext. is purified on Al_2O_3 column and examined by electron capture GLC, after addnl H_2SO_4 cleanup. Gas chromatographic peaks with retention time relative to aldrin of 10–25 are indicative of chick edema factor.

REAGENTS AND APPARATUS

(a) *Petroleum ether.*—Redistd in glass, b.p. 30–60° (available from Burdick and Jackson Laboratories, 1953 S. Harvey St., Muskegon, Mich. 49442).

(b) *Ethyl ether for alumina chromatography.*—Ether (not >2% alcohol) or absolute ether (not >0.01% alcohol) (available from Burdick and Jackson Laboratories).

(c) *Carbon tetrachloride.*—Redistd in glass (available from Burdick and Jackson Laboratories).

(d) *Celite.*—No. 545, acid-washed. Wash well with petr. ether and dry at room temp.

(e) *Aldrin standard soln.*—0.1 µg/ml. See *Reagents and Apparatus,* section (a), JAOAC 50, 216(1967).

(f) *Chick edema factor low positive reference sample.*—1.5% reference toxic fat in USP cottonseed oil. (Available from Division of Food Standards and Additives, Food and Drug Administration, Washington, D.C. 20204).

(g) *Activated alumina.*—See *Reagents and Apparatus,* revised 26.092(b), JAOAC 50, 216(1967).

(h) *Alumina chromatographic column.*—To *dry* chromatographic tube, 17 mm o.d. (14.5 mm i.d.) × 250 mm long, fitted at bottom with coarse porosity fritted glass disk and Teflon stopcock (tube without fritted disk but holding glass wool plug in bottom may be used), add redisd petr. ether, *dried* before use with anhyd. Na_2SO_4, until column is ⅔ full. Weigh 15 g Al_2O_3 and transfer to column in small portions, tapping tube as Al_2O_3 settles. When last portion of Al_2O_3 settles and air bubbles stop rising to surface, add 5 g anhyd. Na_2SO_4. Drain excess petr. ether so that it is just above upper surface of Na_2SO_4.

(i) *Gas chromatographic column.*—Glass, 5–7′ × ¼″ i.d., packed with 2.5% SE-52 silicone gum rubber on 60–80 mesh Gas Chrom Q (Applied Science Laboratories, State College, Pa. 16801). Coat support with substrate as follows: Weigh 2.5 g silicone gum rubber stationary phase and dissolve in 300 ml CH_2Cl_2-toluene (1 + 1), heating to dissolve. Add 97.5 g Gas Chrom Q and let stand 10 mm with occasional gentle stirring. Dry in rotary evaporator held in 50° bath. Pack coated material into chromatographic column by adding small amts while vibrating column at packing level with Vibro-graver tool (Fisher Scientific Co., Pittsburgh, Pa. 15219). Fill to within 1″ on exit side and 3″ on entrance side, and fill remaining space with silanized glass wool. Condition column at operating pressure 2–5 days at 250°.

(j) *Gas chromatograph with electron capture detector.*—See *Reagents and Apparatus,* (e), JAOAC 50, 216(1967).

DETERMINATION

(a) *Preliminary sufuric acid cleanup.*—Dissolve 2.5 g fat in 10 ml CCl_4 in [illegible] ml beaker; mix with heavy glass stirring rod while adding 10 ml H_2SO_4. Add 5 g anhyd. Na_2SO_4 and stir well while adding 20 g Celite, until homogeneous mixt. is obtained. Add 125 ml petr. ether, mix well, let solids settle, and filter supernatant thru paper in 90 mm conical funnel. Repeat with addnl 125 ml petr. ether. Evap. combined petr. ether filtrate to 5 ml for Al_2O_3 column fractionation.

(b) *Fractionation of petroleum ether filtrate by alumina chromatography.*—Dry solvents prior to use by shaking with anhyd. Na_2SO_4. Transfer petr. ether filtrate from (a) to Al_2O_3 chromatographic column, using total of 15 ml petr. ether. Let liquid level fall to just above top of Na_2SO_4. Elute sample with 100 ml petr. ether (fraction 1), 50 ml 5% ether in petr. ether (fraction 2), and 100 ml 25% ether in petr. ether (fraction 3). Relatively fast flow rates of ca 8–9 ml/min give satisfactory results. Keep liquid level above top of Na_2SO_4 at all times. Discard fractions 1 and 2, and collect fraction 3 in 125 ml erlenmeyer. Add several boiling chips and evap. to dryness on steam bath. Transfer residue with petr. ether to 10 ml g-s. graduate and evap. petr. ether soln to 3 ml.

(c) *Sulfuric acid cleanup of alumina fraction 3.*—See *Determination,* section (c), JAOAC 50, 217(1967).

(d) *Electron capture gas chromatography of petroleum ether extract.*—See *Determination,* section (d), JAOAC 50, 217(1967).

(4) The official, first action method for methyl esters of fatty acids, 26.055–26.059, with changes in *This Journal* 49, 231–232 (1966), was revised as follows:

(A) 26.058(c), revised third paragraph, *This Journal* 49, 232(1966), after ". . . to obtain calibration factor." insert "Reference mixts simulating most fats and oils may be obtained from Applied Science Laboratories, Box 440, State College, Pa. 16801; Supelco, Box 581, Bellefonte, Pa. 16823; and Lipids Preparation Laboratory, Hormel Institute, Austin, Minn. 55912)."

(B) 26.059, change to read as follows: "Two single detns of major components (>5%) performed in 1 laboratory shall not differ by >1.0 percentage unit. Two single detns performed in different laboratories shall not differ by >3.0 percentage units."

(5) The official, final action lead-salt ether method for determination of saturated and unsaturated fatty acids, 26.040, was changed as follows:

(A) Change first paragraph, first sentence to read: "Accurately weigh 10 (for plant fats used in common household cooking oils) or 20 g sample into 200. . . ."

(B) Add the sentence "Reserve ether filtrate (contains ether-sol. Pb soaps)." to the end of the second paragraph.

(C) Fifth paragraph, line 8, after ". . . HCl-free", insert "(no ppt with $AgNO_3$)."

(D) Revise the sixth sentence of the fifth paragraph to read: "Distill ether, avoiding any loss of fatty acids, and heat over steam bath to constant wt under controlled flow of N to prevent oxidation of fatty acids. Cover steam bath with towel to prevent splashing H_2O into erlenmeyer."

(E) Paragraph 6, line 1, insert "reserved" after "Transfer". Line 6, add "Repeat HCl hydrolysis until no more $PbCl_2$ is pptd." after ". . . into beaker."

(F) Change paragraph 7, lines 2 and 3 to read, ". . . until HCl is removed (no ppt in wash H_2O with $AgNO_3$). Dehydrate ether with ca 2 g anhyd. Na_2SO_4 and transfer ether soln . . . ."

(G) Change paragraph 8 to read: "Det. in duplicate I numbers of 0.2–0.3 g all from unsatd fatty acid fraction, and from entire satd fatty acid fraction. (I number of satd acid fraction is due to presence of some unsatd acid.)"

(6) The following gas-liquid chromatographic method for butylated hydroxyanisole (BHA) (121006) and butylated hydroxytoluene (BHT) (128370) in corn and rice breakfast cereals was adopted as official, first action.

APPARATUS

(a) *Gas chromatograph.*—Barber-Colman Model 5000, or equiv., with H flame ionization detector and strip chart recorder. Establish following operating conditions: temps—column 160°, detector 210°, flash heater 200°; N flow rate, sufficient to elute BHT in 3–4 min from QF-1 column and elute BHA in 3–4 min from Apiezon column; H flow rate, ca 40 ml/min for Apiezon L and ca 25 ml/min for QF-1; air flow rate, ca 340 ml/min; electrometer sensitivity; 300 × (5×10^{19}) amp full scale deflection) with 5 mv recorder. Adjust H and air flow rates, if necessary.

Adjust electrometer sensitivity so 0.1 µg BHA gives ca 50% deflection. Repeat injections until constant peak heights are obtained on successive injections *of identical* vol. of std mixt.

Order of appearance on Apiezon column (4′): BHA, BHT, di-BHA. Order of appearance on QF-1 column (6′): BHT, BHA, di-BHA.

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Chemical and Toxicological Evaluations of Isolated and Synthetic Chloro Derivatives of Dibenzo-*p*-dioxin

ONE of the toxic compounds known as hydropericardium factor (chick oedema factor) is 1,2,3,7,8,9-hexachlorodibenzo-*p*-dioxin[1]. Because it is highly improbable that these very toxic compounds are naturally occurring components of the fats from which they have been isolated, the question of their origin has aroused speculation.

A clue to the possible origin of hydropericardium factor is found in a report by Tomita *et al.*[2] of the synthesis of polyhalodibenzo-*p*-dioxins: chlorophenols and their salts, when heated, undergo condensation reactions and form chlorinated derivatives of dibenzo-*p*-dioxin. Because of this observation, we studied the pyrolysis of a number of commercially available chlorophenols which are widely used in agriculture and industry, and present results of some preliminary experiments which suggest that hydropericardium factor could arise from certain chlorophenols.

The commercial chlorophenols used in this work were pyrolysed in accordance with the general procedures described[2]. Benzene extracts of the reaction mixtures were fractionated by passing them through an alumina column. Residues of the benzene effluents were extracted with petroleum ether at room temperature, and the petroleum ether was then removed. The resulting products were sufficiently pure in most cases for examination by electron capture gas–liquid chromatography (GLC) and biological testing by the chicken embryo assay[3]. Table 1 shows retention times of the GLC peaks on a 7 foot, 2·5 per cent '*SE*-52' column at 200° C and the results of the chicken embryo assay. Individually isolated hydropericardium factors of known chemical structures were not available for use as standards; consequently, *a concentrate from the unsaponifiable fraction of a certain* commercial toxic fatty acid material was used as a GLC reference. The unsaponifiable fraction of the fatty acid material was known to contain trace amounts of the hydropericardium factor. The toxic fatty acid material, a by-product obtained from the manufacture of oleic and stearic acids, has been used as a reference in all our previous chemical and biological work[4].

Table 1. EXAMINATION OF CHLOROPHENOL PYROLYSATES

Sample pyrolysed*	R_x at 200° C of components† in pyrolysate	Chicken embryo bioassay μg/egg‡	Per cent mortality§
2,4-Dichlorophenol (R)	1·0	600·0	[illegible]
2,4,5-Trichlorophenol (T)	8·5, 6·5	0·35	100
2,4,6-Trichlorophenol (R)	2·4	6·0	[illegible]
2,3,4,6-Tetrachlorophenol (T)	8·9, 10·0, 11·2, 17·4, 20·0, 35·0	1·0	100
Pentachlorophenol (R)	35·0	5·0	[illegible]
Chlorinated dibenzo-*p*-dioxin	1·0, 1·8, 3·5, 6·5	0·06	100
Reference toxic fat components	1·0, 1·8, 3·5, 6·5, 8·9, 10·0, 11·2, 17·4, 20·0, 35·0	2·0	100

* (R) = reagent grade; (T) = technical grade.
† R_x = retention times of peaks relative to the retention time of aldrin.
‡ 1 μg ≅ 20 p.p.b. in the egg. Sample injected into the air cell of fertile eggs before incubation. Solvents used: ethanol, acetone and chloroform.
§ Embryonic mortality at 21 days. The mortality of non-injected and solvent-injected controls was 10–15 per cent.

The chromatogram of components from the toxic fatty acid material contained a number of peaks of widely varying retention times; four of these peaks had R_x values greater than 10. According to a current electron capture GLC test[4], the presence of one or more GLC peaks with R_x values of 10 or more indicates that hydropericardium factor is present in a fat. The pyrolysate from technical grade 2,3,4,6-tetrachlorophenol was the only product that showed a peak pattern indicating the presence of hydropericardium factor; the peak pattern resembled that displayed by the long retention time components of the reference toxic fat. When tested by the official chick bioassay[5] for hydropericardium factor, the mixture produced the disease at a dietary level of 0·1 p.p.m.

In addition, dibenzo-*p*-dioxin was chlorinated directly in order to prepare 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. This material proved to be extremely toxic in the chicken embryo assay (Table 1), and provided a source for isolation of a trichloro as well as the tetrachlorodibenzo-*p*-dioxin.

The technical grade 2,3,4,6-tetrachlorophenol pyrolysate, chlorinated dibenzo-*p*-dioxin, and components from the reference toxic fat were each fractionated by preparative GLC (thermal conductivity detection), using a 12 foot × 4 mm column at 250° C packed with 10 per cent 'DC-200' on 60/80 mesh 'Gas Chrom Q'. Isolated components from the 2,3,4,6-tetrachlorophenol pyrolysate and from the chlorinated dibenzo-*p*-dioxin were examined by electron capture GLC and infrared spectroscopy, and were also analysed by mass spectrometry to determine molecular weight and number of chlorine atoms per molecule. Results are recorded in Table 2. The components isolated from the reference toxic fat have not yet been examined spectroscopically.

Table 2. COMPONENTS ISOLATED FROM CHLORINATED DIBENZO-*p*-DIOXIN AND 2,3,4,6-TETRACHLOROPHENOL PYROLYSATE

Mixture	GLC peak No.	R_x at 200° C	Molecular weight	No. of Cl atoms
Chlorinated dibenzo-*p*-dioxin	1	1·8	286	3
	2	3·5	320	4
2,3,4,6-Tetrachlorophenol pyrolysate	1	8·9	388	6
	2	10·0	388	6
	3	11·2	388	6
	4	11·6	388	6
	5	17·4	422	7
	6	20·0	422	7

Infrared spectra of individual components exhibited characteristic absorption bands in the region of 1,330–1,280 cm^{-1} attributed to asymmetric stretching vibration of C—O—C in the dibenzo-*p*-dioxin ring. The two principal compounds isolated from the chlorinated dibenzo-*p*-dioxin were 2,3,7-trichlorodibenzo-*p*-dioxin and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. The infrared spectrum of the trichloro compound has bands at 870 cm^{-1} and 805 cm^{-1} (1,2,4-substituted benzene), and 850 cm^{-1} (1,2,4,5-substituted benzene). The infrared spectrum of the tetrachloro compound has a strong band at 808 cm^{-1} (1,2,4,5-substituted benzene). The infrared spectrum of the tetrachloro compound matched the spectrum of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin published by Tomita *et al.*[2]. Chicken embryo assay showed that the tetrachloro compound was more toxic than the trichloro compound.

The first four peaks in the chromatogram from the tetrachlorophenol pyrolysate were caused by positional isomers, each containing six chlorine atoms; the fifth and sixth peaks were caused by positional isomers, each containing seven chlorine atoms. A seventh peak in the chromatogram, observed when the sample injection was overloaded, had a *retention time identical* to that of a sample of octachlorodibenzo-*p*-dioxin prepared by pyrolysis of pentachlorophenol. The presence of pentachlorophenol in technical grade 2,3,4,6-tetrachlorophenol accounts for the formation of the two heptachloro isomers and the octachlorodibenzo-*p*-dioxin.

A fourth hexachloro isomer, in addition to the three expected from pure 2,3,4,6-tetrachlorophenol, is probably caused by the presence of a tetrachlorophenol other than the 2,3,4,6-isomer in the starting material. Infrared spectra of peaks 3 and 4 from the 2,3,4,6-tetrachlorophenol pyrolysate were very similar to published spectra of two hydropericardium factors isolated from a contaminated fat[6].

The components isolated by preparative GLC from the reference toxic fat, the chlorinated dibenzo-*p*-dioxin, and the 2,3,4,6-tetrachlorophenol pyrolysate were analysed by electron capture GLC at 200° C and by the chicken embryo assay. These results are shown in Table 3. The quantity of material in each fraction from the reference toxic fat was estimated on the basis of the weight of sample injected into the preparative gas chromatograph and the number of fractions collected. The amounts of material isolated from the chlorinated dibenzo-*p*-dioxin and 2,3,4,6-tetrachlorophenol pyrolysate were rough estimates based on comparison of GLC peak areas of isolated components and the starting mixtures. Amounts of individual components in the mixtures were estimated from normalized peak area calculations. The data suggest that one hexa and one hepta-isomer are less toxic than the other isomers. Work is now under way to prepare individual compounds of high purity in sufficient quantities for additional chemical and biological testing.

Table 3. GLC ANALYSIS AND EGG EMBRYO ASSAY OF ISOLATED COMPONENTS

Reference toxic fat components: R_x at 200° C	Chicken embryo assay* per cent mortality	Components from chlorinated dibenzo-*p*-dioxin and 2,3,4,6-tetrachlorophenol pyrolysate: R_x at 200° C	Chicken embryo assay† μg/egg	Per cent mortality
1·0	20			
1·8	20	1·8‡	0·5	75
3·3	100	3·5‡	0·2	100
5·1	40			
6·5	100			
8·9	60	8·9	2·0	30
10·0	40	10·0	1·0	100
		11·2	1·0	100
11·6	100	11·6	0·5	100
17·4	40	17·4	2·5	16
20·0	100	20·0	1·25	100
35·0	60			

* It was estimated that about 20 μg of material was injected into each egg.
† See footnotes ‡ and §, Table 1.
‡ Components from chlorinated dibenzo-*p*-dioxin.

Reference toxic fat components with low R_x values (less than R_x 8·9) could be due to chloro-organic pesticides and/or lower chlorodibenzo-*p*-dioxins. Additional work is required to identify the compounds responsible for the peaks with low R_x values. The peaks with high R_x values (those equal to or greater than 8·9) correspond to those of the hexachloro and heptachloro positional isomers isolated from the pyrolysate of 2,3,4,6-tetrachlorophenol. The overall results of this work suggest that chlorophenols could be precursors of hydropericardium factor. Commercial chlorophenols are widely used for such diverse applications as contact herbicides, defoliants and termite control agents, as well as for control of microbial attack in the manufacture of a number of products. There are many opportunities for fats and fatty acids, the only products in which hydropericardium factor has been found, to become contaminated. When crude fats and tallows are subjected to heating operations (hydrolysis, distillation) in the production of commercial fatty acids, residues of commercial chlorophenols present in the fat might be converted in part to chick oedema factors. Additional work is required to determine whether hydropericardium factors are formed from residual chlorinated phenols during production of commercial fatty acids. This laboratory is developing methods for detecting chlorophenols in fats and fatty acids.

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Internal Preliminary Report Analysis of Commercial Chlorophenols for Trace Amounts of Their Condensation and Plymerization Products

By G. R. Higginbotham and John Ress

Laclair (1) has reported an ultraviolet absorption study of the components of technical pentachlorophenol, separated by vacuum sublimation, and disclosed only pentachlorophenol (PCP), 2.3.4.6-tetrachlorophenol, and an unidentified, dark brown, high melting, "chlorophenol" containing 58.3% chlorine. The unidentified material was presumed to be a polymerization product produced during process of manufacture. Tomita *et al.* (2) have demonstrated that chlorphenols and their salts, when heated, undergo condensation reactions and form chlorinated derivatives of dibenzo-p-dioxin (chick edema factors, CEF).

Recently, we examined the unsaponifiable fraction of a number of commercial chlorophenols and obtained results which suggest that the impurities of chlorophenols consists of chloro derivatives of dibenzo-p-dioxin in addition to other components which have not been identified.

Thirteen chlorophenol samples were analyzed for chick edema factor. The samples were carried thru the 2.5 gram saponification procedure and examined by electron capture GLC after Al_2O_3 column chromatography and H_2SO_4 extraction [JAOAC, *50*, 884 (1967)]. GLC peaks indicative of chick edema factor were observed in most of the chromatograms from the thirteen samples.

Six of the thirteen samples were selected for further study. The unsaponifiables from each sample were extracted and chromatographed on an Al_2O_3 column, according to A.O.A.C. Method 26.092–26.094. The unsaponifiable fractions were submitted to the egg embryo biassay. Two components (Ra 6.3 and Ra 11.6) were isolated by preparative GLC from the unsaponifiable fraction of Eastman's technical grade 2,3,4,6-tetrachlorophenol. The components were examined by mass spectrometry and submitted to the egg embryo bioassay. Results are tabulated below.

Table I shows the amount of unsaponifiables isolated from 100 g samples of six representative commercial chlorophenols. In every case the amount of CEF in the total sample is estimated to be less than 0.3%. If a lipid sample is contaminated with pre-formed chick edema factor,* high levels of poly chlorophenols would also be expected to be present in the sample.

Results from the chick embryo assay are recorded in Table II. Table III summarizes the results obtained on two components isolated from 2,3,4,6-tetrachlorophenol. The molecular weight and the number of chlorines for each component are not consistent with a chloro derivative of dibenzo-p-dioxin.

The Ra values of these components are not consistent with known chick edema factors. The compounds may be photodecomposition products of technical grade 2,3,4,6-tetrachlorophenol.

Further work will be required to characterize the unsaponifiables from commercial chlorophenols. The overall results of this preliminary study emphasize the need for a rapid method for polychlorophenols in fats and fatty acids. The unsaponifiable and CEF (hexa-, hepta- and octachlorodibenzo-p-dioxin) content of a number of reagent and technical grade commercial chlorophenols are shown in Table 4. CEF content was determined by GLC using a synthetic CEF mixture as a reference material. CEF was found in all the chlorophenols examined, varying from a trace (ca 0.001 $\mu g/g$) to 205 $\mu g/g$.

* Pre-formed chick edema factor is defined here as a group of chloro derivatives of dibenzo-p-dioxin initially present in a sample before it is subject to any type of heat treatment.

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TABLE 1.—EXAMINATION OF CHLOROPHENOLS FOR PRE-FORMED CHICK EDEMA FACTORS

Sample (100 g.)	Wt. Unsap., mg.	Wt. Al_2O_3 Fr. 3	Percent CEF[1]
2,3,4,6-Tetrachlorophenol (Eastman)	[2] 126.5	109.2	0.11
2,4,5-Trichlorophenol (Pract.)	223.7	11.9	0.012
Pentachlorophenol (tech.)	323.8	228.5	0.23
2,4,6-Trichlorophenol (Reagent)	2.0	0.9	0.001
2,3,4,6-Tetrachlorophenol (Baker)	[3] 135.8	120.2	0.12
2,4-Dichlorophenol (Baker)	12.2	0.3	----------

[1] Estimate based on weight of Al_2O_3 Fr. 3 and assuming that the fraction consists only of CEF.
[2] A second extraction of the unsaponifiables (after acidification) of the soaps afforded 5.0 mg of material.
[3] A second extraction of unsaponifiables gave 9.8 mg of material.

TABLE 2.—CHICK EMBRYO ASSAY

Sample (unsaponifiables)	μg/egg	Percent mortality
2,4,5-Trichlorophenol (T)	10.0	100
	0.55	30
2,3,4,6-Tetrachlorophenol (Baker)	10.0	100
	0.60	15
Pentachlorophenol (T)	10.0	100
	0.6	55
2,3,4,6-Tetrachlorophenol (Eastman)	4.8	70
2,4,6-Trichlorophenol (R)	50.0	40
2,4-Dichlorophenol (T)	15.0	13

TABLE 3.—EXAMINATION OF TWO COMPONENTS FROM THE UNSAPONIFIABLE FRACTION OF TECHNICAL 2,3,4,6-TETRACHLOROPHENOL

R_a of GLC component	Mol. wt.	No. of Cl atoms	Chicken embryo assay	
			μg/egg	Percent mortality
6.23	408	7	2.0	0
11.6	442	8	1.0	0

TABLE 4.—EXAMINATION OF COMMERCIAL CHLOROPHENOLS FOR CHICK EDEMA FACTORS (HEXA-, HEPTA- AND OCTACHLORODIBENZO-P-DIOXINS)

No.	Compound[a]	mg. Unsap.	μg/g. of CEF[b]
1	2,4-Dichlorophenol (R)	2.0	0.018
2	2,4-Dichlorophenol (T)	2.4	0.219
3	2,5-Dichlorophenol (R)	0.8	0.008
4	2,4,5-Trichlorophenol (T)	32.5	trace
5	2,4,6-Trichlorophenol (T)	12.3	trace
6	2,4,6-Trichorophenol (R)	1.9	0.013
7	2,3,4,6-Tetrachlorophenol (T)	22.5	205
8	2,4,5-Trichlorophenol (R)	3.0	0.021
9	2,3,4,6-Tetrachlorophenol (T)	35.0	96.5
10	Pentachlorophenol (T)	67.1	121
11	Pentachlorophenol (R)	1.4	0.167
12	Dowcide B (sodium 2,4,5-trichlorophenate (T)	1.8	trace
13	Dowcide G (sodium pentachlorophenate (T)	25.8	47.0
14	Reference toxic fat	223	3.0

[a] R=reagent grade. T=technical grade.
[b] Hexa-, hepta- and octachlorodibenzo-p-dioxins.

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The Identification and Crystal Structure of a Hydropericardium-Producing Factor: 1,2,3,7,8,9-Hexachlorodibenzo-*p*-dioxin

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A crystalline material, isolated from a contaminated animal feed fat, and capable of producing hydropericardium in chicks, was shown by solution of its crystal structure to be 1,2,3,7,8,9-hexachlorodibenzo-*p*-dioxin ($C_{12}O_2H_2Cl_6$). The triclinic unit cell has the dimensions $a=7.952\pm0.005$, $b=9.379\pm0.01$, $c=9.433\pm0.01$ Å, $\alpha=92.35°\pm0.20°$, $\beta=92.39°\pm0.20°$, $\gamma=109.92°\pm0.30°$. The calculated density is 1·958 g.cm^{-3} for $Z=2$, compared with 2·01 g.cm^{-3} measured for the bulk material. A statistical treatment of the 1158 measured reflections indicated a center of symmetry; the space group was therefore assumed to be $P\bar{1}$. The structure was solved by the symbolic addition method of Karle & Karle. The nearly planar molecules are almost parallel to the (0$\bar{1}$1) crystallographic planes. No unusual bond lengths or angles were found. The structure was refined to $R=10.5\%$.

Introduction

The isolation, chemical analyses, and spectroscopic data on the hydropericardium toxic factor (HPTF) material have been described by Wootton, Artman & Alexander (1962), and by Wootton & Courchene (1964). One of the active fractions of material isolated was that called α-3·17, where this nomenclature refers to the vapor phase chromatographic behavior as described by Wootton *et al.* (1962). Wootton and his colleagues proposed that HPTF was a chlorinated hexahydrophenanthrene with the empirical formula $C_{14}H_{10}Cl_6$. Following the molecular identification herein reported, Wootton (1966) showed that a synthetic hexachlorinated dibenzo-*p*-dioxin, whose physical properties are remarkably similar to the isolated α-3·17 material, does indeed produce the hydropericardium condition in chickens.*

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Experimental

Two types of crystals were isolated from a warm benzene–hexane solution of the α-3·17 material. The bulk of the crystalline material appeared to differ in phase from the material used for this study. No crystals of the bulk phase were found to be satisfactory for single-crystal studies, and only two crystals of the studied phase were isolated. Measured *d*-spacings of X-ray powder patterns taken of the bulk phase material did not match *d*-spacings calculated from the unit cell of 1,2,3,7,8,9-hexachlorodibenzo-*p*-dioxin. However, when the bulk phase was heated to just below the melting point (230°C) a phase change occurred. Measured *d*-spacings from X-ray powder patterns of the transformed bulk phase match the calculated *d*-spacings of 1,2,3,7,8,9-hexachlorodibenzo-*p*-dioxin reasonably well. Therefore, it was assumed that the material used for this crystal structure determination was a high temperature phase of the bulk crystalline material known as α-3·17 HPTF.

The single crystals used were diamond shaped and had the approximate dimensions 0·18 × 0·10 × 0·08 mm ($a\times b\times c$).

The unit-cell parameters were determined from single-crystal data using a General Electric single-crystal orienter and Ni-filtered Cu $K\alpha$ radiation ($\lambda=1.5418$ Å). The parameters of the triclinic cell chosen according to Dirichlet (Balashov & Ursell, 1957) are as follows:

$a=7.952\pm0.005$ Å	$\alpha=92.35\pm0.20°$	$\varrho_c=1.958$ g.cm^{-3}
$b=9.379\pm0.01$	$\beta=92.39\pm0.20$	$\varrho_o=2.01$ g.cm^{-3}
$c=9.433\pm0.01$	$\gamma=109.92\pm0.30$	$Z=2$
		$V=662.8$ Å^3

where ϱ_o was measured for the bulk phase by flotation.

Two-dimensionally integrated equi-inclination Weissenberg data were collected for the *a*-axis zones, $0kl$–$4kl$, and for the *b*-axis zones $h0l$–$h5l$ using the multiple-film technique (one pack each of four films, Eastman Kodak No-Screen). Intensity data were recorded for both crystals, reduced separately, then compared, edited, and averaged. Absorption corrections were made separately for each crystal using Busing & Levy's general absorption correction program as modified by Jeffrey (1964).

Owing to the very tiny crystals, and in part to the integration, very long exposures of approximately 150 hours were required to obtain satisfactory multiple-film data. The entire Weissenberg camera was placed inside a plastic bag and a helium atmosphere was provided to reduce background due to air scattering. Of the 3030 possible reflections, 1158 (38%) were recorded; 397 of these reflections had intensities less than a minimum threshold value and were classified as 'less-thans'. The intensities of most of the reflections were measured by a Joyce Loebl microdensitometer scanning at right angles to the longer integration direction. The weakest reflections were estimated visually. A standard intensity strip was prepared and used for the visually estimated intensities. To ensure that both types of intensity data were on the same scale, a sufficient number of medium intensities were measured both visually and by the densitometer. Radiation damage effects were found to be negligible by retaking data for earlier crystal settings.

Statistical treatment of the intensity data by Ramachandran & Srinivasan's (1959) modification of the method of Howells, Phillip & Rogers (1950) indicated a center of symmetry. The space group was assumed, therefore, to be $P\bar{1}$ (C_i^1) and this assumption was confirmed during the direct method calculations.

* The composition for the structure reported here, namely $C_{12}O_2H_2Cl_6$, agrees well with unpublished microchemical analyses performed by Professor Wolfgang J. Kirsten, University of Uppsala, Uppsala, Sweden, at a very early stage of this structure work.

Solution and refinement of the structure

Initially we knew the weight of the molecule and the number of chlorine atoms per molecule, and we knew that the molecule possessed some aromatic character. Attempts to solve the structure from the three-dimensional Patterson map were not successful. The symbolic addition method of Karle & Karle (1963, 1966) was then employed.

The phases were determined for the 251 most intense reflections in terms of four algebraic quantities, a,b,c,g. A summary of the calculation of the unitary structure factors or *E*-values used for this determination is compared with theoretical values and is as follows:

Quantity	Non-centrosymmetric	Centrosymmetric	$C_{12}H_2O_2Cl_6$	Karle *et al.* (1964) 3-Indolyl-acetic acid
$\langle\lvert E\rvert\rangle$	0·886	0·798	0·772	0·769
$\langle\lvert E^2-1\rvert\rangle$	0·736	0·968	0·970	0·934
$\langle\lvert E\rvert^2\rangle$	1·000	1·000	1·000	1·031
$\lvert E\rvert>3.0$		0·3%	0·4%	0·2%
$\lvert E\rvert>2.0$		5·0%	4·5%	3·3%
$\lvert E\rvert>1.0$		32·0%	30·8%	36·1%
			1·158 reflections 761 non-zero 397 unobserved ('less-than')	1·289 reflections 865 non-zero 424 zero

The overall temperature factor was 2·24.

A summary of the sixteen cases that resulted from the sign permutation of the four algebraic assignments is as follows:

		Case 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	a	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
	b	+	+	+	−	−	−	−	+	+	+	+	−	−	−	−	+
	c	−	+	−	+	−	−	+	+	−	+	−	+	−	−	+	+
	g	−	−	+	+	+	−	−	+	−	−	+	+	+	−	−	+
No. refl. generated	+	119	183	114	153	142	116	118	251	121	121	115	125	121	122	128	122
	−	132	68	137	98	109	135	133	0	130	130	136	126	130	129	123	129
No. incorrect signs		117	132	125	132	133	129	130	129	121	128	104	130	35	61	120	117
% wrong sign		46·6	52·6	49·8	52·6	53·0	51·4	51·7	51·4	48·3	51·0	41·5	51·7	13·9	24·3	47·8	46·6
Objections to choice		✓	✓	✓	✓	✓	✓	✓	✓†					*			

✓ Electron density peaks were found at centers of symmetry when the special sections $x=0$ and $x=\frac{1}{2}$ were computed.
† It is very unlikely that all 251 most intense reflections would be positive.
* This correct case was selected after examining three-dimensional electron density maps for cases 9 to 16.

Case 13 was chosen over the other seven acceptable choices because of the appearance of a chemically reasonable structure in the electron density plot in an $(0\bar{7}4)$ plane. It had already been determined that the molecule had to be oriented approximately in alternate $(0\bar{7}4)$ planes, based on (*a*) the early analysis of the Patterson map, (*b*) the very high intensity of $0\bar{2}2$, (*c*) electron densities calculated from models based on the Patterson vectors, and (*d*) packing considerations. A nearly planar hexachlorinated phenanthrene structure was initially fitted to the *E*-map peaks located near an $(0\bar{7}4)$ plane. An *R* value of 50% was obtained for the initial trial coordinates and a three-dimensional electron density map suggested that the phenanthrene skeleton should be changed to an anthracene one with some changes made in carbon and chlorine assignments resulting in, ideally, a planar 1,2,3,7,8,9-hexachloroanthracene with *mm* symmetry. This change in the structure dropped the *R* value to 35%. The plane of the molecule was originally in alternate $(0\bar{7}4)$ planes but required some tilting and when these changes were made the *R* value dropped to 24%. One cycle of least squares reduced the residual to 19%. At this point the two bridging atoms were recognized as oxygens, since their relative electron densities were 30% higher than those for the carbon atoms and the individual temperature factors for these atoms had gone negative. Substituting oxygen atoms for these carbon atoms in the proposed model resulted in an *R* value of 16%. There was a careful editing of the data, especially of those reflections classified as 'less-thans', at the *R* = 16% stage of refinement. This editing consisted of deleting a few doubtful reflections recorded near the edge of a film, correcting transcription errors, and reclassifying some of the 'less-thans' to observed reflections on the basis of visual estimations of the weakest reflections.

Table 1. *Final parameters and their standard deviations*

The fractional coordinates have been multiplied by 10^4 and the temperature factors by 10^2; the standard deviations are in parentheses. The anisotropic temperature factors of the chlorine atoms are in the form

$$\exp[-\tfrac{1}{4}(B_{11}h^2a^{*2}+2B_{12}ha^*kb^*+B_{22}k^2b^{*2}\ldots)]$$

	X	Y	Z	B or B_{11}	B_{22}	B_{33}	B_{12}	B_{13}	B_{23}
Cl(1)	−0099 (6)	3158 (6)	5185 (6)	287 (20)	376 (29)	487 (26)	149 (21)	−035 (17)	−088 [illegible]
Cl(2)	−0145 (6)	0579 (6)	2931 (5)	362 (23)	378 (30)	417 (27)	025 (22)	−162 (19)	−043 [illegible]
Cl(3)	3314 (7)	−0257 (6)	2621 (5)	487 (26)	411 (30)	376 (26)	163 (24)	−056 (21)	−064 [illegible]
Cl(4)	9493 (6)	7375 (6)	10547 (5)	372 (23)	457 (30)	347 (24)	174 (23)	−018 (18)	−105 [illegible]
Cl(5)	6188 (6)	8434 (6)	10752 (5)	395 (24)	375 (29)	309 (23)	076 (22)	034 (18)	−024 [illegible]
Cl(6)	2919 (6)	6936 (6)	8629 (5)	282 (21)	421 (29)	473 (27)	148 (21)	028 (18)	−102 [illegible]
C(1)	1762 (24)	2601 (22)	4879 (20)	354 (41)					
C(2)	1718 (21)	1502 (20)	3979 (18)	262 (35)					
C(3)	3286 (25)	1118 (22)	3853 (20)	386 (43)					
C(4)	4753 (24)	1920 (23)	4673 (21)	370 (42)					
C(6)	7712 (23)	5516 (21)	8394 (19)	321 (39)					
C(7)	7706 (20)	6572 (19)	9368 (16)	219 (34)					
C(8)	6219 (27)	7051 (24)	9503 (22)	427 (45)					
C(9)	4754 (21)	6238 (19)	8539 (17)	249 (35)					
C(11)	3292 (23)	3471 (21)	5778 (19)	325 (39)					
C(12)	4776 (24)	2934 (22)	5629 (19)	360 (42)					
C(13)	6226 (21)	4811 (19)	7461 (18)	240 (34)					
C(14)	4752 (20)	5271 (19)	7591 (17)	213 (32)					
O(5)	6312 (15)	3742 (14)	6496 (13)	355 (27)					
O(10)	3266 (15)	4591 (14)	6713 (13)	334 (27)					

The *R* value dropped to 13% during the next least-squares refinement. Fixing the chlorine atoms and refining only the carbon skeleton resulted in an *R* value of 11·8% at which time the anisotropic temperature refinement on the chlorine atoms was undertaken and gave the final *R* of 10·5%. Of the 397 'less-than' reflections only 42 calculated were larger than their threshold values and none by more than 36%. A weighting function similar to that given by Hughes (1941) was chosen so as to have little dependence on F_o; it was taken as $\sqrt{w}=K/(F_o+53{\cdot}4)$ for $F_o>F_m$ and $\sqrt{w}=K/(F_m+53{\cdot}4)$ for $F_o\le F_m$, where $F_m=21{\cdot}4$, and the quantity minimized was $\Sigma w(F_o-F_c)^2$. 'Less-than' reflections having $F_c>F_o$ (threshold) contributed like an ordinary reflection, but those having $F_c<F_o$ did not influence the refinement at all. A full-matrix least-squares procedure was used throughout the refinement, and in the last cycle all parameter shifts were less than $\frac{1}{3}\sigma$. In a difference map calculated at the $R=11{\cdot}8\%$ stage, the residual electron density ranged from −0·8 to +0·6 e.Å^{-3}, and the maxima and minima did not show any chemically or structurally significant features.

The final position and thermal parameters are given in Table 1. The observed and calculated structure factors are listed in Table 2. In this work, the scattering factors are taken from *International Tables for X-ray Crystallography* (1962); those for carbon are by Freeman (1959); those for oxygen by Berghuis, Haanappel, Potters, Loopstra, MacGillavry & Veenendaal (1955); and those for chlorine by Dawson (1960).

Table 3. *Distances of atoms from least-squares planes*

Distances for atoms not defining the plane are marked with an asterisk; s.d. is the standard deviation of the atoms defining the plane. Under the $(0\bar{7}4)$ heading are listed the deviations from the $(0\bar{7}4)$ plane containing the molecule. The planes are defined in direct space by equations $Px+Qy+Rz=S$.

	All	C+O	$(0\bar{7}4)$
Cl(1)	−0·10	−0·10*	0·33*
Cl(2)	−0·11	−0·12*	0·10*
Cl(3)	0·00	−0·03*	−0·25*
Cl(4)	−0·16	−0·18*	−0·45*
Cl(5)	−0·05	−0·06*	0·12*
Cl(6)	0·13	0·13*	0·54*
C(1)	−0·02	−0·03	0·15*
C(2)	−0·06	−0·08	0·02*
C(3)	−0·02	−0·05	−0·16*
C(4)	0·06	0·03	−0·17*
C(6)	0·00	−0·03	−0·25*
C(7)	−0·05	−0·07	−0·21*
C(8)	−0·01	−0·03	0·03*
C(9)	−0·01	−0·02	0·13*
C(11)	0·06	0·05	0·13*
C(12)	0·05	0·03	−0·09*
C(13)	0·05	0·02	−0·10*
C(14)	0·06	0·05	0·12*
O(5)	0·08	0·05	−0·17*
O(10)	0·09	0·08	0·25*
Origin	1·57*	1·55*	1·67*
s.d.	0·07	0·05	0·00
P	−1·1589	−1·1086	0·0000
Q	−5·9655	−6·6031	−6·6618
R	6·8500	6·8404	6·6618
S	1·5675	1·5497	1·6655

Discussion

The molecule is nearly planar and the molecular plane is tilted 8° to the $(0\bar{1}1)$ plane. Deviations of atoms from the $(0\bar{7}4)$ plane essentially containing the molecule and

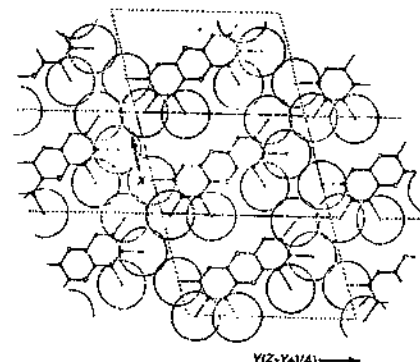

Fig. 1. Molecular packing in the $(0\bar{7}4)$ plane containing the molecule. Large shaded circles are Cl, solid circles are C, and open circles are O.

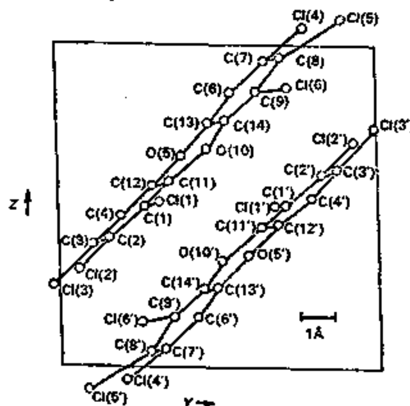

Fig. 2. Projection onto YZ plane.

from the least-squares planes of the entire molecule and of the carbon–oxygen skeleton are given in Table 3. The molecule appears to be *slightly bowed* in the middle and slightly twisted about a line from Cl(3) to Cl(5). The packing arrangement of chlorines 4, 5, and 6 appears to be more crowded than that for chlorines 1, 2, and 3. This packing difference could account for the slight twist of the molecule.

Fig. 1 pictures the molecular packing in the $(0\bar{4}4)$ plane containing the molecule, and Fig. 2 gives a projected view of two adjacent molecules related by the center at $(\frac{1}{2},\frac{1}{2},\frac{1}{2})$. Intermolecular distances in this $(0\bar{4}4)$ plane of less than 4·0 Å are shown in Fig. 3. Between centrosymmetrically related molecules there are a number of Cl(n)–Cl(m') and equivalent Cl(m)–Cl(n') distances of 4·0 Å or less. From the parent molecule to the one related by the center at $(\frac{1}{2},\frac{1}{2},\frac{1}{2})$ the distances are Cl(2)–Cl(4′) = 3·85 Å, Cl(3)–Cl(5′) = 3·66 Å, and Cl(3)–Cl(6′) = 3·83 Å; by the center at $(0,0,\frac{1}{2})$–Cl(1)–Cl(3′) = 3·84 Å; by the center at $(0,\frac{1}{2},\frac{1}{2})$–Cl(1)–Cl(1′) = 3·39 Å and Cl(2)–Cl(6′) = 3·98 Å; and by the center at $(\frac{1}{2},\frac{1}{2},1)$–Cl(4)–Cl(6′) = 4·00 Å. The least-squares planes of the two adjacent molecules related by the center at $(\frac{1}{2},\frac{1}{2},\frac{1}{2})$ are 3·13 Å apart; between these two molecules the shortest interatomic distance is 3·30 Å from a C(12) to an O(10).

Fig. 3 indicates the bond distances and angles. The mean standard deviations are as follows: σ_{C-C} = 0·025 Å, σ_{C-Cl} = 0·019 Å, σ_{C-O} = 0·022 Å; for angles σ = 2·0°. The bond distances are not significantly different from those found by Davydova & Struchkov (1962) and Gafner & Herbstein (1962) for 1,4,5,8-tetrachloronaphthalene where molecular over-crowding results from the presence of many chlorine atoms substituted on adjacent aromatic positions. This compound belongs in group (I) according to the classification due to Harnik, Herbstein, Schmidt & Hirshfeld (1954) for compounds that are affected by molecular over-crowding.

An electron density map plotted in the $(0\bar{4}4)$ plane containing the molecule is shown in Fig. 4.

Fig. 3. (*a*) Interatomic distances. Primed atoms are on neighboring molecules in the same plane. σ_{C-C} = 0·025 Å; σ_{C-Cl} = 0·019 Å; σ_{C-O} = 0·022 Å. View corresponds to Fig. 1. (*b*) Bond angles. σ = 2·0°.

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We wish to express our appreciation to Drs Jerome and Isabella Karle who provided assistance in applying the direct method for determining the phases of a number of the most intense reflections.

Thanks are due to Mr Robert Gloss who obtained part of the data and provided the computer programs used in generating the relations between reflections necessary for applying the direct method.

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Fig. 4. Electron density in the $(0\bar{4}4)$ plane containing the molecule. Contours are at 1 e.Å^{-3} starting at 2 e.Å^{-3}. The × marks are projections onto $(0\bar{4}4)$ from the electron density maxima, which in most cases are a short distance from $(0\bar{4}4)$.

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Clinical Picture and Etiology of Chloracne

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Chloracne is the name for forms of occupational acne which develop as a result of intoxication with certain chlorinated aromatic compounds. The name dates back to Herxheimer who described the first case in 1899 and still assumed that in analogy to bromine and iodine, the acneiform eruptions are the result of free chlorine as the etiological toxin. This view proved to be incorrect. The proposed designation "perna disease" of Wauer, Teleky and others based on the finding that this clinical picture occurred more frequently under the influence of *per*chlorinated naphthalenes also does not go to the heart of the matter, since other chlorinated aromatics in addition to chlorinated naphthalenes are also etiologically important.

The *clinical picture of symptoms* primarily affects the skin. Beyond this, internal organs may be affected and nervous system and emotional disorders may appear.

The skin symptoms are in the regions of the follicles. Comedones, resulting from a follicular hyperkeratosis, predominate and frequently are so numerous that hardly a single follicle remains untouched and the affected region of the skin obtains a dirty-gray appearance. In addition, at the peak of the disease, fairly large sebaceous cysts, inflammatory nodules, pustules and furuncles appear and in some of the patients, large spots or patches of pigmentation appear in regions exposed to light. Preferential sites are the face as well as the exposed areas of the throat and neck. Frequently, the external ear, especially, the ear lobes, are involved where small cysts can be easily palpated. In more pronounced forms, changes can also be found on the back, chest and extremities and in males, on the genitalia. Hands and feet usually are not involved. It is not rare that the symptoms of acne are preceded by a dermatitis with erythema and edema. In this phase of the condition, photosensitivity frequently exists which evidently contributes to the development of dermatitis and the mentioned pigmentations (S. Braun, Grimmer).

Generally, a *differential diagnosis* is not particularly difficult. The primary problem is to define the condition compared to other forms of occupational acne and acne vulgaris, which is generally possible with consideration of the clinical aspect, localization and especially the patient's history. Acneiform dermatoses caused by tars, pitch and mineral oils are found primarily on the extremities and trunk, while the face is more rarely involved. The predominance of inflammatory changes, such as folliculitis and furuncles in oil and tar acne and of comedones in chloracne are other characteristic features. Drug-caused acneiform exanthemas due to iodine, bromine or cortisone also have a picture differing from chloracne.

The *course* is eminently chronic. In spite of intensive local and general therapy, recidivism may occur even years after the elimination of the causal toxins. Healing frequently takes place with extensive pitted, permanently disfiguring cicatrization (Schmidt and Boslet).

The skin is not the only indicative region of intoxication with chloracne-causing substances. Damage of internal organs is not rare, with the *liver* being in the foreground. Several authors have reported on grave damage of hepatic parenchyma accompanied by icterus and functional disorders, including a number of fatal cases of acute atrophy of the liver (see reviews of W. Braun and A. Risse-Sundermann). The pronounced liver-toxocity of chloracne-causing substances was also confirmed in animal experiments (Bennett, Drinker and Warren; Hofmann, Oettel; Schulz).

In addition to liver damage, changes in the kidneys, pancreas, gastrointestinal tract and myocardium can also be observed, although much more rarely.

Nervous system and psychological disorders were found primarily among workers occupied in the production and processing of chlorinated phenols (Trubant et al.). General fatigue, weakness of the legs, headache, attacks of vertigo, paresthesias, muscle pain, tendency to orthostatic collapse, local paresis and disturbed sensibility, anomalies in reflexes as well as an autonomic syndrome with lowered drive, depression, reduced power of recall and concen-

tration, disturbed sleep, irritability, loss of appetite, reduced libido and impotence have been reported as the most frequent neurological and psychopathological symptoms of intoxication which become manifest often only several months after it occurs (Bauer, Schulz and Spiegelberg). With regard to the question of distinguishing the latter from psychoneurotic obsessions (wish for compensation), reference is made to the discussions of Spiegelberg.

Etiology.—When we review the literature, we find that periods of greater incidence of chloracne have existed in the last 60 years, which can be correlated with industrial development. W. Braun has described these relationships in his monograph.

The first cases were observed near the turn of the century when chlorine and hydrochloric acid began to be produced by the electrolytic route. At that time, the condition occurs primarily among workers having the assignment to clean the so-called hydrochloric acid towers, but only when tar was used as the protective coating of the walls. Although the causal toxin could not be determined at the time, it can be assumed on the basis of our present knowledge that the reaction products of chlorine and aromatic components of the tar must be considered as etiological factors of this condition.

The next period of increased chloracne frqucy coincides with the introduction of so-called halogenated waxes. These are mixtures of highly chlorinated naphthalenes and diphenyls with a waxy consistency and a number of valuable properties. They are water-repellent, nonflammable, resistant to acids, are a good dielectric and are not *pest-promoting*. The halogenated waxes developed during the first world war at that time were used primarily for the manufacture of gas masks. Numerous cases of chloracne occurred in the manufacturing plants.

In the middle twenties, these halogenated waxes were used in the mining industry as a water-repellent and nonflammable insulation for detonators. The high incidence of diseases observed in detonator manufacturing plants has been described by Teleky.

The next massive occurrence is related with the rise of the electrical and radio industry. Chlorinated naphthalenes and diphenyls were in more widespread use for the insulation of wires and condensors at the start of the thirties. Several hundred cases including one fatality with liver atrophy became known especially in the United States.

With the entry of the United States in the second world war, the field of application of these materials expanded also into ship building; this had the following reason: It was found that the halogenated waxes were in the position to insulate ships from the dangerous weapon of German magnetic mines. Consequently, large quantities of these materials were used in American shipyards for the impregnation of ship hulls. Mass incidences of chloracne with several fatalities were the results.

In spite of all negative experiences, the use of chlorinated naphthalene waxes did not stop after the last war. Chloracne cases of greater or lesser frequency were repeatedly observed in the electrotechnical and cable industry (Braun, Grimmer, Risse-Sundermann). From the pathogenic aspect, it is of interest to note an observation of Herzberg of 7 patients who developed intestinal symptoms and acneiform dermatoses following the use of industrial chlorinated greases for frying.

The question of the relationships between chemical structure and acne-producing effects of chlorinated naphthalenes is the subject of several experimental studies. Teleky as well as Drinker and Warren arrived at the conclusion about thirty years ago that the toxicity of the molecules increases with an increasing number of chlorine atoms on the ring. Later experiments conducted by Sehley and Kligman with human subjects and with the use of several chlorinated naphthalenes showed that penta- and hexachloronaphthalenes produced the strongest effects; compounds with 1 to 3 as well as 7 and 8 chlorine atoms were far less toxic of inactive. We confirmed this finding in animal experiments using rabbit ears (Schulz 1965).

In the last 10–15 years, halogenated waxes have become less important as etiological factors of chloracne. Evidently, this is related with the fact that they are no longer as important industrially and have been replaced by synthetics of the most diverse nature in most fields of application.

In the fifties, the incidence of chloracne was observed in entirely different sectors of industry i.e. in the *production and processing of chlorinated phenols.* Reports of group involvements have been published from at least three

industrial plants in Western Germany. Baader and Bauer as well as Brinkman described 17 workers of a plant in Nordrhein-Westfalen who developed the typical skin symptoms as well as damage of the internal organs and central nervous system disorders in the production of pentachlorophenol.

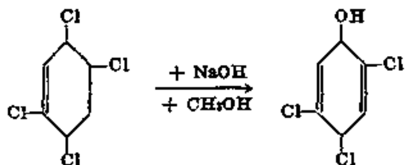
A larger number (about 60 cases) of similar disorders were recorded several years later in the region of southwest Germany among workers occupied in the production of of trichlorophenol (Hergt, Oettel, Hofmann). Approximately at the same time, 31 workers in a Hamburg plant became ill after working with industrial 2,4,5-trichlorophenol, an intermediate of the synthensis of trichlorophenoxyacetic acid, a weed killer.

Siliar high incidences of the disease occurred a few years ago in chemical plants of the Netherlands and the U.S. during analogous production processes.

The high frequency of cases in Hamburg led to studies of the etiology. They were conducted by us together with Dr. Sorge, the former manager of the chlorophenol plant.

Rabbit ears were used as the biological substrate on which symptoms corresponding to human chloracne can be produced by local painting as demonstrated by Hofmann and Neumann with chloronaphthalenes. The results, which have been reported earlier (Schulz 1957; Kimmig and Schulz 1957), can be briefly summarized as follows:

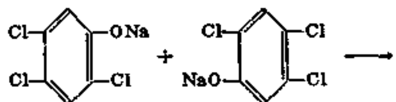
First, it was found that it was not possible to produce changes in the rabbit ear in the form of chloracne with the use of the chemically pure compound in contrast to the technical grade of 2,4,5-trichlorophenol used in the plant. Pure 1,2,4,5-tetrachlorobenzene also was inactive. The toxic factor therefore must have formed as a byproduct during the alkaline hydrolysis of tetrachlorobenzene into trichlorophenol.



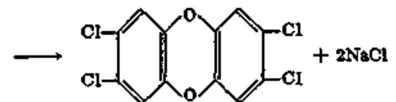
1,2,4-tetrachlorobenzene 2,4.5-trichlorophenol

Alkaline hydrolysis of 1,2,4,5-tetrachlorobenzene into 2,4,5-trichlorophenol.

Since the isolation of well-defined compounds from the distillation residue of trichlorophenol was unsuccessful, a number of especially synthesized substances were investigated which might have formed as a byproduct of the cited saponification process on the basis of theoretical considerations. The majority of investigated compounds proved to be inactive. Only dibenzofurans with 3 and 4 chlorine atoms (diphenylene oxides) and 2,3,6,7-tetrachlorodibenzodioxine (tetrachlorodiphenylene dioxide) led to the characteristic changes on the rabbit ear already in low concentrations. Moreover, it was demonstrated that 2,3,6,7-tetrachlorodibenzodioxine had formed by the following reaction route in the industrial process of alkaline hydrolysis of 1,2,4,5-tetrachlorodibenzene.



Sodium salt of 2,4,5-trichlorophenol



2,3,6,7-tetrachlorodibenzodioxine
(2,3,6,7-tetrachlorodiphenylene dioxide)

Under the conditions of a salt fusion in a solvent-free state, 2 molecules of sodium trichlorophenolate form 1 molecule 2,3,6,7-tetrachlorodibenzodioxine with the elimination of 2 molecules of NaCl. Dr. Sorge synthesized the compound and in addition, isolated it from the distillation residue of industrial trichlorophenol.

Animal experiments conducted with tetrachlorodibenzofuran and 2,3,6,7-tetrachlorodibenzodioxine showed an extremely high toxicity of these compounds. Even concentrations of 0.001–0.005% of tetrachlorodibenzodioxine led to severe reactions on the rabbit ear after local application. On human skin in a self-experiment, two applications of 10 γ of the substance produced the symptoms characteristic of chloracne. On the rabbit ear, tetrachlorodibenzofuran showed an activity which was about 10 to 20 times less pronounced. Moreover, the unexpectedly high hepatotoxic action is worthy of note, particularly after tetrachlorodibenzodioxine. Single oral doses of 20–50 γ/kg body weight regularly produced lethal liver necrosis, while doses of 10 γ/kg were lethal for about 50% of the rabbits.

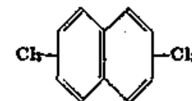
On the basis of these chemical and toxicological findings, it is justified to conclude that 2,3,6,7-tetrachlorodibenzodioxine played an important role in the etiology of the cases of chloracne which occurred during the industrial production of trichlorophenol. It cannot be ruled out, however, that other, as yet unknown chlorinated aromatics of highly toxic properties may form during the industrial process under certain conditions. The results of the study are an example that materials which form only in small amounts as byproducts of large-scale syntheses can be of importance in occupational medicine. If such toxic byproducts can be uncovered and their mechanism of formation can be elucidated, this will create an important prerequisite for successful prophylaxis. In our special case, the plant succeeded in avoiding the formation of highly toxic byproducts by modifying the production process.

Our animal experiments were extended to other chlorinated aromatics to which other authors ascribed a chloracne-causing action on the basis of clinical observations. (Reviews of these compounds in the monograph of W. Braun.) Neither benzenes and phenols with 1 to 6 chlorine atoms nor chlorinated diphenylethers produced an effect in animal experiments. It seems indicated to assume. therefore, that neglected toxic byproducts were of decisive etiological importance in these cases rather than the main products.

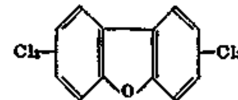
In connection with the acne produced by chloronaphthalenes, the question arose whether toxic byproducts rather than the chloronaphthalenes themselves might not be considered as the true toxins (Oettel). In the production of industrial naphthalene by fractional distillation of tar, the presence of other aromatic compounds deriving from the tar apparently cannot be ruled out. In the following chlorination process, such substances might then also undergo CL-substitution. These question prompted us to carry out animal experiments on rabbit ears using chemically pure chloronaphthalenes of different degrees of chlorination specially synthesized for this purpose.*

In agreement with the findings of Shelley and Kligman, we found that naphthalenes containing 5-6 chlorine atoms have a chloracne-producing effect. The necessary concentrations, however, were about 100 times higher than those of tetrachlorodibenzofuran (diphenylene oxide) about about 1000 times higher than for 2,3,6,7-tetrachlorodibenzodioxine (tetrachlorodiphenylene dioxide).

On the basis of the present state of the art, therefore, the chloracne-producing activity of the following compounds appears to be sufficiently demonstrated or at least highly probable:



Naphthalenes containing 5–6 chlorine atoms:



Dibenzofurans (diphenylene oxides) with higher degrees of chlorination:

2,3,6,7,-tetrachlorodibenzodioxine:

In conclusion, it should be noted that the toxicodermatosis represented by chloracne results from intoxication with certain chlorinated aromatics. The causally responsible compounds partly involve highly toxic substances which can cause damage in various internal organs, especially the liver and nervous system, in addition to the skin. Since the skin does not always represent the only manifestation site, it is recommended that thorough internal and neurological as well as psychiatric examinations be made in cases of suspected chloracne.

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Report on Methodology for Chlorinated Aromatics in Fats, Oils, and Fatty Acids

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ABSTRACT

The official, first action electron capture GLC (EC-GLC) method for chick edema factor (polychlorodibenzo-p-dioxins) have been reviewed. This general procedure, which underwent collaborative study in 1967, has undergone several minor modifications which result in better recoveries of polychlorodibenzo-p-dioxins and increased specificity in interpretation of the gas chromatographic results. The EC-GLC method can be used as a screening test, or where a typical pattern of GLC peaks is obtained as a preliminary test, but confirmatory tests are needed to demonstrate structure and toxicity of polychlorodibenzo-p-dioxins. Preliminary work with combined GLC-Mass Spectrometry indicated that this technique might provide a suitable test, if adequate sample cleanup can be accomplished. A chicken embryo assay has been developed to the point where toxicity can be observed in three to five days after injection of eggs.

A preliminary procedure has been developed for isolation and gas chromatography of chlorophenols in fats and fatty acids. Polychlorophenols have been found to be precursors of chlorodibenzo-p-dioxins. The use of a non-specific microbiological test for chlorophenols employing the *Bacillus megaterium* was evaluated. Chlorophenols were found to produce uniformly graded growth inhibition of the test organism in the range 1–100 μg.

The widespread use of toxic organochlorine compounds in agriculture and industry requires development of sensitive methods for their detection in a wide variety of commodities. In addition, it is equally important that methods be developed to detect toxic breakdown or conversion products of organochlorine compounds. One of the most urgent needs in the fat and oil industry is for a rapid and specific method for polychlorodibenzo-p-dioxins (chick edema factors) in fats, oils, and fatty acids. The official, first action, microcoulometric and electron capture methods for chick edema factors (CEF) are essentially screening procedures (1,2,3). Both methods, at present, require a rather time consuming three-week chick bioassay (4) for confirmation.

The purpose of this report is to review the current status of chemical and biological methods for chlorophenols and chlorinated dibenzo-p-dioxins in fats, oils, and fatty acids.

CEF consists of a mixture of chlorinated dibenzo-p-dioxins which occur occasionally as a trace contaminant in fats. Recently, a communication (5) from this laboratory reported the results of a preliminary study which demonstrated the possibility that CEF could arise from residues of pentachlorophenol and 2,3,4,6-tetrachlorophenol in fats and fatty acids. Chlorophenols and their salts, when heated, undergo condensation reactions and form chlorinated derivatives of dibenzo-p-dioxin. The following equation illustrates this condensation reaction.

Technical grades of pentachlorophenol contain ca 10% of 2,3,4,6-tetrachlorophenol which also undergoes thermal condensation reactions and forms hexachloro derivatives of dibenzo-p-dioxins. The condensation of 2,3,4,6-tetrachlorophenol with pentachlorophenol forms two heptachloro derivatives of dibenzo-p-dioxin.

An electron capture GLC method has been developed for pentachlorophenol and 2,3,4,6-tetrachlorophenol in fats, oils, and fatty acids (6). However, recov-

eries of the two volatile polychlorophenols were low and varied over a wide range; nevertheless, the method appears to be satisfactory for qualitative measurements at the 0.5 ppm level. Several samples of oleic acid known to contain CEF were analyzed and were found to be contaminated with residues of pentachlorophenol. The method requires further study.

The official electron capture and microcoulometric methods for CEF were developed before their chemical structures were known. The methods are screening procedures and are based on the observation that toxic fats contain a number of chlorinated components (now known to be polychlorodibenzo-p-dioxins) which have greater retention times than chlorinated pesticides. The electron capture method has received wide acceptance. It is approximately 2000 times as sensitive and requires less sample than the microcoulometric method. In addition, electron capture gas chromatographic equipment is simpler and is in general use in many laboratories.

Recently, it has come to our attention that a number of laboratories that routinely use the electron capture method for control work are not aware of improvements (3) that have been made in the original procedure (2). Recent changes which have not been published include a minor modification of the H_2SO_4 cleanup step and a slight modification of the procedure for packing the GLC column. The modified method, which includes these changes, replaces all existing GLC methods for the chemical assay of CEF.

METHOD

Reagents and Apparatus

Rinse all glassware with appropriate solvents before use. Do not use polyethylene containers to store solvents.

(1) Concentrated H_2SO_4.—Reagent grade.

(2) Petroleum Ether.—Reagent grade, redistilled in glass between 30 and 60°C (Available from Burdick and Jackson Laboratories, 1953 S. Harvey St., Muskegon, Mich. 49442).

(3) CCl_4.—Distilled in glass. (Available from Burdick and Jackson Laboratories, Muskegon, Mich.)

(4) Anhydrous Na_2SO_4.—Analytical reagent grade.

(5) Ethyl ether.—Analytical reagent grade (not >2% alcohol) or absolute ether (not >0.01% alcohol).

(6) Iso-octane.—Distilled in glass (Available from Burdick and Jackson Laboratories, Muskegon, Mich.)

(7) Standard aldrin solution.—Dissolve aldrin in iso-octane to make 0.05 µg/ml solution.

(8) Chick edema factor low positive reference sample.—1.5% reference toxic fat in USP cottonseen oil or other suitable vegetable oil. (Prepare from reference toxic fat available from the Division of Pesticides, Bureau of Science, Food and Drug Administration, Washington, D.C. 20204).

(9) Activated Al_2O_3 (Fisher No. A-540, do not substitute).—Activate 100 g portions by heating 4 hours at 260°C. Transfer without cooling to dry container and close tightly. Check activity of Al_2O_3 by analysis of the low positive reference sample, examining Al_2O_3 fractions 2 and 3. With sufficiently activated Al_2O_3, chick edema factor elutes predominantly or entirely in Al_2O_3 fraction 3 as indicated by the gas chromatograms. (Chromatogram should show a series of GLC peaks with Ra values between ca 8 and 45).

(10) Al_2O_3 chromatographic column.—To a *dry* chromatographic column, 22 mm o.d. x 250 mm long, fitted at bottom with coarse porosity fritted glass disk and Teflon stopcock (a column without the fritted disk but holding a glass wool plug in the bottom may be used), add redistilled petroleum ether, dried prior to use with anhydrous Na_2SO_4, until column is ⅔ full. Weigh 15 g Al_2O_3 and transfer to column in small portions, tapping the column as Al_2O_3 settles. When last portion of Al_2O_3 settles and air bubbles stop rising to surface of solvent, add 5 g anhydrous Na_2SO_4. Drain excess petroleum ether so that it is just above upper surface of Na_2SO_4.

(11) Gas chromatographic column.—Glass, 6-7′ long x ¼″ i.d., packed with 2½% SE 52 silicone gum rubber on 60/80 mesh Gas Chrom Q (Applied Science Laboratories, State College, Pa. 16801). Coat the support with substrate as follows: Weigh 2.5 g of the silicone gum rubber stationary phase and dissolve in 200 ml of 1:1 methylene chloride-toluene, heating to dissolve. Add 97.5 g of support material to liquid mixture and let stand 10 minutes with occasional gentle stirring. Dry in rotary evaporator. Apply vacuum to the chromatography column, and pack the coated material into the column by adding small amounts while tapping the column at the packing level after each addition. Fill to within 1″ on the exit side and 3″ on the entrance side, and fill the remaining space with silanized glass wool. Condition the column at operating pressure at 250°C for 2-5 days.

(12) Gas chromatograph with electron capture detector.—A tritium source concentric type detector is recommended. Operate instrument in accordance with instructions of manufacturer, and obtain a stable baseline before carrying out analyses. Choose an operating voltage (ca 50-80 volts) that will cause between 0.6 and full scale deflection for 0.1 mg of aldrin (2 µl of standard aldrin solution) at a sensitivity setting of 1 x 10^{-9} AFS. Keep the column temperature at 200° ±1°C, and adjust nitrogen flow rate so that aldrin elutes in 1-1.5 min. (3-4 min. per in. chart speed). Inject 2 µl of the standard aldrin solution before injection of each reference or test sample.

DETERMINATION

(a) Analysis of 1.5% reference toxic fat in USP cottonseed oil.—Dissolve 2.5 g of the 1.5% reference toxic fat in 10 ml of CCl_4 in a 500 ml glass stoppered Erlenmeyer flask. Proceed with determination as described below in sections (b), (c) and (d). Take up residue from (d) in 250 µl iso-octane and inject 5 microliters of reference solution (equivalent to 50 mg of the original sample) into calibrated gas chromatograph. The resulting gas chromatogram should exhibit a series of GLC peaks with Ra ca 8-45, depending on operating conditions. Peaks at Ra 8-13 are due to hexachlorodibenzo-p-dioxin isomers, 2 peaks at Ra 17-22 are due to the 2 heptachlorodibenzo-p-dioxin isomers, and a peak at Ra 35-45 is due to octachlorodibenzo-p-dioxin.

(b) Preliminary sulfuric acid cleanup.—Dissolve 2.5 gm of fat in 10 ml CCl_4 in 500 ml glass stoppered Erlenmeyer. Add 10 ml conc. H_2SO_4, stopper, and shake for 30 sec. Add 125 ml of petroleum ether, stopper and shake vigorously for ca one minute. Allow layers to separate and decant supernatant liquid into a 500 ml erlenmeyer, avoiding transfer of lower layer. Repeat extraction with additional 125 ml portion of petroleum ether. Evaporate the combined petroleum ether extracts to 5 ml for Al_2O_3 column fractionation.

(c) *Fractionation of Petroleum ether filtrate by alumina chromatography.*—Solvents must be dried prior to use by shaking with anhydrous Na_2SO_4. Transfer petroleum ether filtrate from (b) to Al_2O_3 chromatographic column using total of 15 ml petroleum ether. Let liquid level fall to just above top of Na_2SO_4. Keeping liquid level above top of Na_2SO_4 at all times, elute sample with 100 ml of petroleum ether (fraction 1), 50 ml of 5% ethyl ether in petroleum ether (fraction 2), and 100 ml of 25% ethyl ether in petroleum ether (fraction 3). Relatively fast flow rates of ca 8-9 ml/min give satisfactory results. Ground glass stoppered separatory funnels (250 ml) are satisfactory reserves for eluting solvents. Discard fractions 1 and 2, and collect fraction 3 eluate in 125 ml Erlenmeyer. Add several boiling chips and evaporate solvent to dryness on steam bath. Transfer residue with petroleum ether to 10 ml graduate cylinder equipped with a ground glass stopper, and evaporate petroleum ether solution to 3 ml.

(*d*) *Sulfuric acid cleanup of alumina fraction 3.*—Add 2 ml of concentrated H_2SO_4 to graduated cylinder containing 3 ml petroleum ether solution from (c), stopper and shake vigorously for 30 sec. Allow layers to separate and decant petroleum ether layer into 10 ml beaker avoiding transfer of H_2SO_4 layer. Add 2 ml petroleum ether to cylinder, swirl vigorously, allow layers to separate, and decant petroleum ether layer into beaker. Add ca 0.5 g of solid naHCO₃ to beaker and stir ca ½ min. Let stand five minutes and decant petroleum ether layer into clean 2 or 4 dram vial. Wash $NaHCO_3$ with 2 ml petroleum ether and decant washing into vial. Evaporate solvent under N.

(*e*) *Electron capture gas chromatography of petroleum ether extract.*—Take up residue in 250 µl iso-octane (redistilled in glass), stopper vial tightly and rotate so that solvent wets sides of vial. Inject 1 microliter of sample solution (equivalent to 10 mg fat) into calibrated gas chromatograph. Gas chromatographic peaks with Ra 8–45 are indicative of the presence of chick edema factor. Compare Ra values of sample peaks with Ra values of peaks from reference toxic fat. See (a) for identification of peaks. If peaks indicative of chick edema factor are not observed, inject 5 µl of sample solution (equivalent to 50 mg fat) into gas chromatograph. Check reagents for possible interferences by running a blank with each set of samples. The chromatogram from the blank should show a smooth low baseline from Ra 8—Ra 45. (Types of samples found by experience to be generally free of components characteristic of toxic fats may be examined by initial injection of 5 µl of solution.)

DISCUSSION

Analysis of the low positive reference sample serves as an overall check on instrument performance and sample cleanup. The chromatogram from the low positive reference fat should show a distinct peak pattern as illustrated in Figure 1. The lower chromatogram (B) represents an injection equivalent to 50 mg of the original low positive reference fat. Chromatogram (A) represents a mixture of synthetic polychlorodibenzo-p-dioxins prepared by pyrolysis of 2,3,4,6-tetrachlorophenol and pentachlorophenol. As stated previously, peaks 1 through 4 are due to four positional isomers of hexachlorodibenzo-p-dioxins. The isomer associated with the small shoulder (peak 3) is probably caused by the presence of a tetrachlorophenol other than the 2,3,4,6-isomer in the starting material. Peaks 5 and 6 are due to two positional isomers of heptachlorodibenzo-p-dioxin. Peak 7 is due to octachlorodibenzo-p-dioxin.

Aldrin is used to calibrate the instrument sensitivity for chick edema factor analyses. The similarity of detector response vs. applied voltage for aldrin and an extract from the 1.5% reference toxic fat (low positive reference sample) is illustrated in Figure 2.

CHEMICAL AND BIOLOGICAL CONFIRMATORY METHODS

The need for rapid chemical and biological confirmatory tests has led to investigation of mass spectrometry as well as two biological toxicity assays. Preliminary work has suggested that rapid EC-GLC screening for chick edema factors can be carried out initially; if the presence of chick edema factors is indicated, then larger portions of sample would be fractionated and cleaned up for chemical and biological confirmation.

MASS SPECTROMETRY

Results of a preliminary investigation of combined GLC-mass spectrometry (GLC-MS) indicated that the use of this technique might be suitable if adequate sample cleanup can be accomplished. A 7-foot coiled glass column packed with 2.5% SE-52 on 60–80 mesh Gas Chrom Q was used with an Atlas CH4 mass spectrometer and single-stage Llewellyn (silicone membrane) separator to examine standards and an extract from a 2.5 g sample (positive for chick edema factor by EC-GLC). The GLC oven temperature was 220°C with a helium flow at 75 ml/minute. Injection temperature was 235°C and silicone membrane temperature was about 150°C. MS sensitivity setting was 32 x at 40 PA.

GLC retention times as well as molecular weight and number of chlorine atoms in the molecule were determined for a standard mixture and an extract from the test sample. Two µl of 10µl solution from the test sample was injected; it was estimated that 2 µl test sample contained 0.4 µg of hexa-, hepta- and octachlorodibenzo-p-dioxins in addition to other unidentified constituents. A summary of results are in Table 1. Comparison of fragmentation pattern and relative abundance of the ions from standards and sample might afford additional specificity; impurities in the test sample prevented such evaluation at this time.

CHICKEN EMBRYO ASSAY

Extracts from a reference toxic fat and several test samples were subjected to the chicken embryo assay (7); 111 g samples of fat were fractionated according to AOAC (1965) 26.093–26.094, and alumina fraction 3, and cleaned up with sulfuric acid (JAOAC (Changes in Methods) *50*, page 217, section (c) (1967)). Small portions of each extract was retained for EC-GLC analysis and the remainder, in chloroform solution, was subjected to the chicken embryo assay (10–15 eggs per sample were tested by injection of portions of the sample extract in the air cell) at three levels equivalent to ca 40, 30 and 10 g starting sample. Assay results are shown in Table 2. These results indicate that the chicken embryo test can provide a sensitive indication of toxicity as well as a measure of specificity due to observations of localized and genralized edema. In many instances evidence of toxicity can be observed (by periodic candling) in 3–5 days.

B. megaterium TOXICITY TEST

The use of a non-specific biological test employing the *Bacillus megaterium* was evaluated (8). This test involves observation of inhibition of a seeded petri dish holding a *B. megaterium* spore suspension in agar medium. Filter paper discs of sample extracts are placed on the surface of the agar plates and inhibition zones are observed after 18 hour incubation at 37°C.

Five samples (111 g each) were fractionated according to the general procedure of AOAC (1965) 26.093–26.094. These test samples consisted of two highly toxic fats, one low toxic reference material (1.5% TEF in USP cottonseed oil), one nontoxic oil, and a reagent blank. In addition to the three alumina fractions obtained with petroleum ether, 5% ethyl ether in petroleum ether and 25% ethyl ether in petroleum ether, a fourth fraction eluted with 400 ml of 100% ethyl ether was obtained. The four alumina fractions were cleaned up twice with sulfuric acid according to JAOAC (Changes in Methods) *50*, 0.217 Sect. C (1967). The residues of fractions 2,3, and 4 were spotted on filter discs (7.5 mm diameter) at two levels equivalent to 2.5 and 54 g starting sample. EC-GLC analysis of alumina extracts indicated that chick edema factors (polychlorodibenzo-p-dioxins are predominantly concentrated in alumina fraction 3. In addition, solvent blanks, a synthetic reference standard consisting of hexa-, hepta- and octachlorodibenzo-p-dioxin (CDPD), and a sample of technical grade 2,3,4,6-tetrachlorophenol (2,3,4,6-TCP) were spotted at the following concentrations: 0.01 µg, 0.1 µg, 0.5 µg, 1.0 µg, 10.0 µg, and 100 µg.

After incubation, inhibition was observed in six cases as shown in Table 3. The sensitivity of the *B. megaterium* test for CEF appears to be limited to 100 ppm and even then only a very small zone of inhibition was noticed. It appears, however, that a rapid confirmation test for chlorophenols can be developed at levels of ca 2–3 ppm or less. These are the low levels at which aflatoxin B_1 shows inhibition of *B. megaterium*.

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TABLE 1.—MASS SPECTROMETRIC ANALYSIS OF ISOLATED COMPONENTS

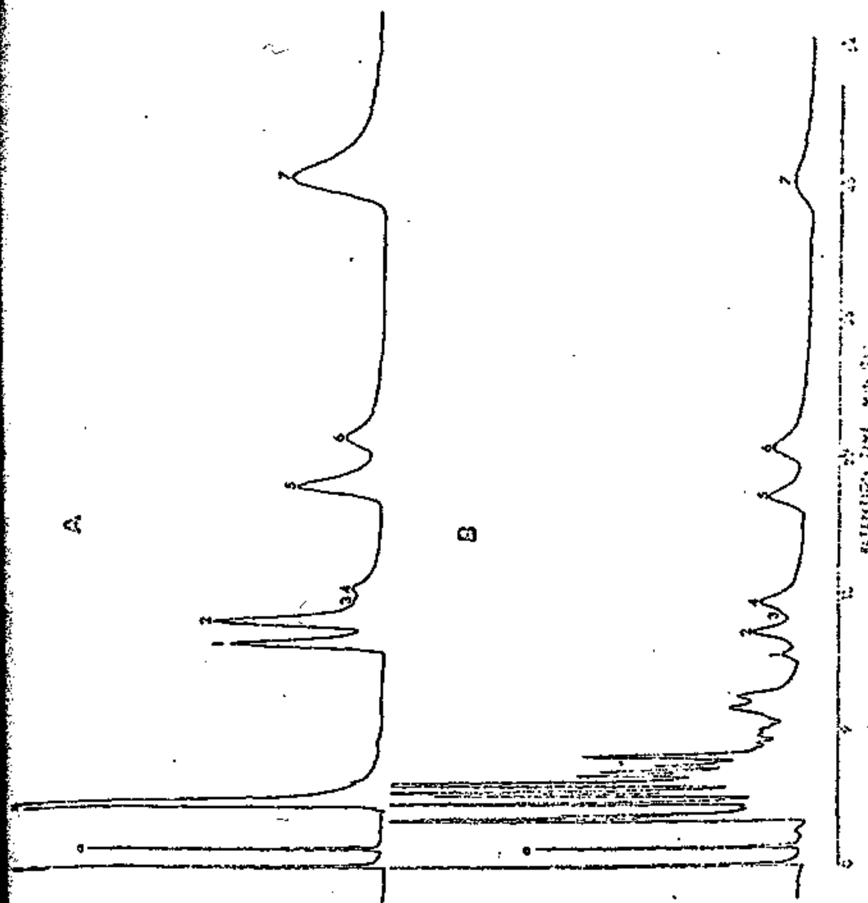
	GLC peak no.	Identity	Molecular weight found	No. of Chlorine atoms indicated
Standard mixture	1	Hexachlorodibenzo-p-dioxin	388	6
	2	do.	388	6
	3	Heptachlorodibenzo-p-dioxin	422	7
Test sample	1	Hexachlorodibenzo-p-dioxin	388	6
	3	Heptachlorodibenzo-p-dioxin	422	[illegible]

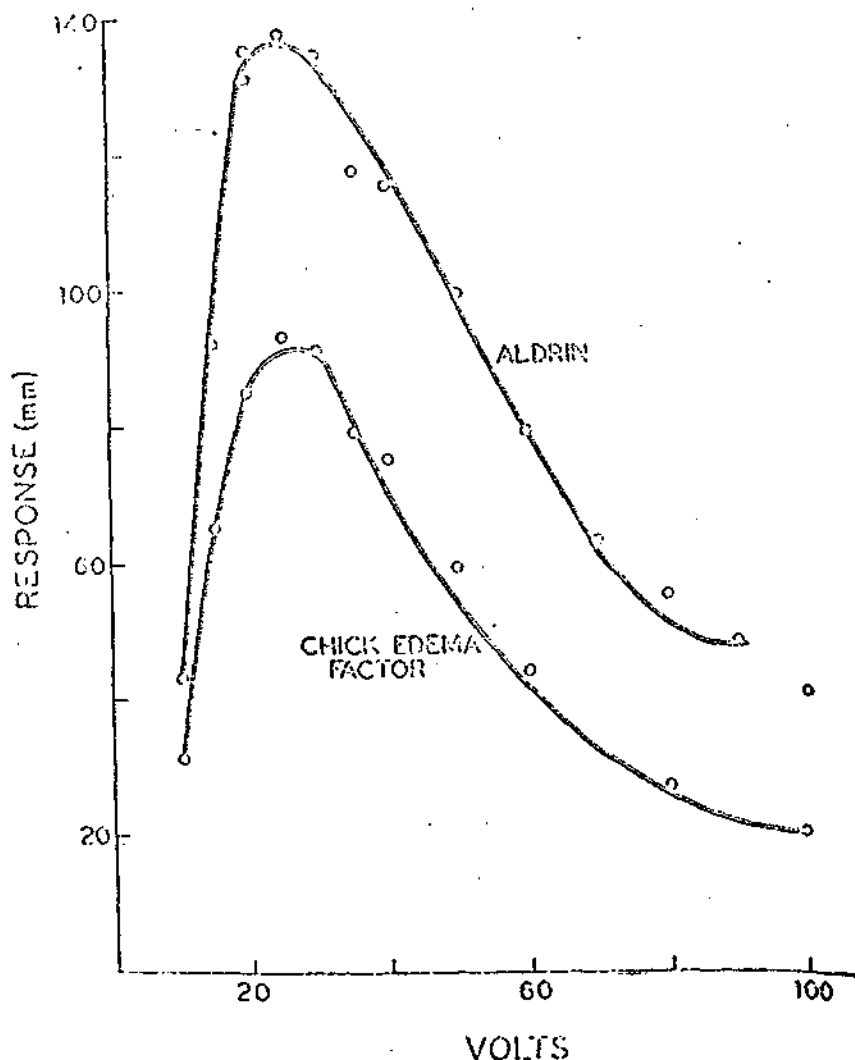
TABLE 2.—CHICKEN EMBRYO ASSAY OF EXTRACTS

Sample	EC-GLC analysis for chick edema factor	Estimated level of hexa-, hepta- and octachlorodibenzo-p-dioxins in fat, in ppm	Percent mortality chicken embryo assay	Assay observations
Reference toxic fat	positive	2.4	100	edema observed.
Test fat No. 1	do.	0.1	73	Do.
Test fat No. 2	do.	0.7	100	Do.
Test fat No. 3	do.	0.6	93	Do.
Reagent blank	negative		30	
Chloroform solvent	do.		20	
Control eggs	do.		7	

TABLE 3.—RESULTS FROM B. MEGATERIUM TEST

Sample	Observation
Nontoxic USP cottonseed oil Fr. 4 (2.5 g extract)	Barley visible around disc.
TEF-F797,- Fr. 2, (2.5 g extract)	Do.
100 μg synthetic (CDPD) reference standard	Do.
1 μg 2,3,4,6–TCP	Do.
10 μg 2,3,4,6–TCP	16 mm inhibition zone.
100 μg 2,3,4,6–TCP	36 mm inhibition zone.





SECTION 121.1070 FATTY ACIDS

The food additive fatty acids may be safely used in food and in the manufacture of food components in accordance with the following prescribed conditions:

(*a*) The food additive consists of one or any mixture of the following straight-chain monobasic carboxylic acids and their associated fatty acids manufactured from fats and oils derived from edible sources: Capric acid, caprylic acid, lauric acid, myristic acid, oleic acid, palmitic acid, and stearic acid.

(*b*) The food additive meets the following specifications:

(1) Unsaponifiable matter does not exceed 2 percent.

(2) It is free of chick-edema factor or other factors toxic to chicks, as evidenced during the bioassay method for determining the chick-edema factor as prescribed in paragraph (c) (2) of this section.

(*c*) For the purposes of this section:

(1) Unsaponifiable matter shall be determined by the method described in section 26.049 of the Official Methods of Analysis of the Association of Official Agricultural Chemists, Ninth Edition (1960).

(2) Chick-edema factor shall be determined by the bioassay method described in the Journal of the Association of Official Agricultural Chemists, Volume 44, page 146 (1961). The presence of chick-edema factor shall be determined by a comparison between the mean log of the pericardial fluid volumes of a test group and of a concurrent negative control group. The significance of the difference in pericardial fluid volumes between the test group and the negative control group is determined by calculating a t value according to the formula: The test sample is judged to contain chick-edema factor if the calculated t exceeds 1.8 and the mean log of the pericardial fluid volume obtained from the negative control group multiplied by 100 is less than 1.1461.

$$t=\frac{\bar{x}_t-\bar{x}_c}{\sqrt{\frac{s_t^2}{n_t}+\frac{s_c^2}{n_c}}}$$

where:

$\bar{x}_t$ and $\bar{x}_c$ are the means of the log of the pericardial fluid volumes of the test and control groups, respectively;

n_t and n_c are the number of chicks in the respective groups;

s_t^2 and s_c^2 are the variances of the test and control groups, respectively.

The variances are calculated as follows:

$$s^2=\frac{n\sum x^2-(\sum x)^2}{n(n-1)}$$

where:

$\sum x$ is the sum of the logs of the pericardial fluid volumes;

$\sum x^2$ is the sum of the squares of the log of the pericardial fluid volumes for either the test t or control c group date.

(3) "Other factors toxic to chicks" referred to in paragraph (b) (2) of this section shall be determined during the course of the bioassay test described in subparagraph (2) of this paragraph on the basis of chick deaths or other abnormalities not attributable to chick-edema factor or to the experimental conditions of the test.

(*d*) It is used or intended for use as follows:

(1) In foods as a lubricant binder and as a defoaming agent in accordance with good manufacturing practice.

(2) As a component in the manufacture of other food-grade additives.

(*e*) To assure safe use of the additive the label and labeling of the additive and any premix thereof shall bear in addition to the other information required by the act the following:

(1) The common or usual name of the acid or acids contained therein.

(2) The words "food grade," in juxtaposition with and equally as prominent as the name of the acid.

SECTION 121.1071 SALTS OF FATTY ACIDS

The *food additive salts of fatty acids* may be safely used in food and in the manufacture of food components in accordance with the following prescribed conditions:

(*a*) The additive consists of one or any mixture of two or more of the aluminum, calcium, magnesium, potassium, and sodium salts of the fatty acids conforming to § 121.1070.

(*b*) The food additive is used or intended for use as a binder, emulsifier, and anticaking agent in food in accordance with good manufacturing practice.

(*c*) To assure safe use of the additive, the label and labeling of the additive and any premix thereof shall bear in addition to the other information required by the act the following:

(1) The common or usual name of the fatty acid salt or salts contained therein.

(2) The words "food grade," in juxtaposition with and equally as prominent as the name of the salt.

Title 21—FOOD AND DRUGS

Chapter 1—Food and Drug Administration, Department of Health, Education, and Welfare

SUBCHAPTER B—FOOD AND FOOD PRODUCTS

PART 121—FOOD ADDITIVES

Subpart D—Food Additives Permitted in Food for Human Consumption

FATTY ACIDS

The Commissioner of Food and Drugs has received a petition (FAP 6A2003) from Fatty Acid Producers' Council. Division of the Soap and Detergent Association, 295 Madison Avenue, New York, N.Y. 10017, proposing that § 121.1070, the food additive regulation providing for safe use of fatty acids in food and in the manufacture of food components, be amended:

A. To provide for the use of a screening method for determining the presence of chick-edema factor in the fatty acids that, within certain conditions, may be used in lieu of the bioassay method prescribed by paragraph (c) (2), and

B. To delete references to "other factors toxic to chicks" from the section.

From the available information it can be concluded that the anomalies presently identified as due to other toxic factors, which may be evidenced during the bioassay method for determining chick-edema factor, are directly associated with the same conditions or substances producing chick-edema factor, and the proposed physicochemical method is adequate as a screening test for detecting the chick-edema factor complex of toxicants.

Based on the information sbumitted in the petition, and other relevant material, the Commissioner has concluded that the regulation should be amended as petitioned. In addition, the references identifying the chick-edema bioassay procedure was updated to refer to the Official Methods of Analysis of the Association of Official Agricultural Chemists.

Therefore, pursuant to the provisions of the Federal Food, Drug, and Cosmetic Act (sec. 409(c) (1), 72 Stat. 1786; 21 U.S.C. 348(c) (1)), and under the authority delegated to the Commissioner by the Secretary of Health, Education, and Welfare (21 CFR 2.120; 31 F.R. 3008), § 121.1070 (b) (2) and (c) (2) and (3) are amended to read as follows:

SECTION 121.1070 FATTY ACIDS

* * * * *

(b) * * *

(2) It is free of chick-edema factor:

(i) As evidenced during the bioassay method for determining the chick-edema factor as prescribed in paragraph (c) (2) of this section; or

(ii) As evidenced by the absence of chromatographic peaks with a retention time relative to aldrin (R_A) of five or more using the gas chromatographic-microcoulometric method prescribed in paragraph (c) (3) of this section. If chromatographic peaks are found with R_A values of five or more, it shall meet the requirements of the bioassay method prescribed in paragraph (c) (2) of this section for determining chick-edema factor.

(c) * * *

(2) Chick-edema factor shall be determined by the bioassay method described in Official Methods of Analysis of the Association of Official Agricultural Chemists, 10th Edition (1965), sections 26.087 through 26.091.

(3) The gas chromatographic-microcoulometric method for testing fatty acids for chick-edema shall be the method described in Official Methods of Analysis of the Association of Official Agricultural Chemists, 10th Edition (1965), sections 26.092 through 26.096, except that the following procedure is substituted for that described in section 26.092(b):

Activated alumina.—(Fisher No. A540 or equivalent.) Activate 250-gram portions by heating 4 hours at 260°C. Transfer without cooling to dry container and close tightly. Use within 1 week after preparation. Check activated Al_2O_3 by analysis of a reference standard by examining fractions 2 and 3. Chick-edema factor should elute in Al_2O_3 fraction 3 as indicated by the gas chromatogram. (A sample of the reference standard may be obtained on request from the Bureau of Science, Food and Drug Administration, Washington, D.C. 20204.)

* * * * *

Any person who will be adversely affected by the foregoing order may at any time within 30 days from the date of its publication in the FEDERAL REGISTER file with the Hearing Clerk, Department of Health, Education, and Welfare, Room 5440, 330 Independence Avenue SW., Washington, D.C. 20201, written objections thereto, preferably in quintuplicate. Objections shall show wherein the person filing will be adversely affected by the order and specify with particularity the provisions of the order deemed objectionable and the grounds for the objections. If a hearing is requested, the objections must state the issues for the hearing. A hearing will be granted if the objections are supported by grounds legally sufficient to justify the relief sought. Objections may be accompanied by a memorandum or brief in support thereof.

Effective date. This order shall become effective on the date of its publication in the FEDERAL REGISTER.

(Sec. 409(c) (1), 72 Stat. 1786; 21 U.S.C. 348(c) (1))

Dated: August 18, 1966.

J. K. KIRK,
Acting Commissioner of Food and Drugs.

[F.R. Doc. 66-9263; Filed, Aug. 24, 1966; 8:47 a.m.]

Table 12 — Embryotoxicity of Chlorophenols, Dibenzo-p-dioxins (Chick edema)

Estimated Dose Levels to Produce Indicated Mortality (Percent)

↓ Compound / Mortality →	Phenols (Dose in mg)			Pyrolysis* Products (Dose in mg)			Unsaponifiable* Fraction (Dose in mg)		
	100	50	0	100	50	0	100	50	0
2,4-Dichlorophenol	>2	1.5	0.4	>0.5	0.25	0.01	-	-	$1.5x10^{-2}$
2,4,5-Trichlorophenol	-	0.05	0.01	$2.5x10^{-5}$	$5x10^{-6}$	$2.5x10^{-6}$	~ $5x10^{-3}$	$2x10^{-3}$	$5x10^{-4}$
2,4,6-Trichlorophenol	-	1.0	0.2	0.05	0.005	$2.5x10^{-3}$	-	.05	-
2,3,4,6-Tetrachlorophenol	2.5	1.0	0.2	$1x10^{-3}$	$2x10^{-4}$	$1.0x10^{-4}$	$6x10^{-3}$	$1.2x10^{-3}$	$6x10^{-4}$
Pentachlorophenol	1.5	0.7	0.4	-	$2.5x10^{-2}$	-	>$1x10^{-2}$	$6x10^{-3}$	$1x10^{-3}$
2,4-Dichlorophenoxyacetic acid		1.0	<0.1						
2,4,5-Trichlorophenoxy-acetic acid									
Bionetics	1.0	0.25	< .05						
Dow #120449		1.0	~0.5						
Polychlorodibenzo-p-Dioxins *									
Dibenzo-p-dioxin					0.5				
Chlorinated dibenzo-p-dioxin				$2.5x10^{-5}$	$1x10^{-5}$	$2.5x10^{-6}$			
Trichloro dibenzo-p-dioxin				$2x10^{-3}$	$2x10^{-4}$	$5x10^{-5}$			
Tetrachloro dibenzo-p-dioxin				$2x10^{-4}$	$5x10^{-5}$	-			
Hexachloro isomers (4)									
a. Most toxic				$5x10^{-4}$	$1.25x10^{-4}$	-			
b. Least toxic				-	$2x10^{-3}$	$1x10^{-3}$			
Heptachloro isomers (2)									
a. Most toxic				$1.25x10^{-3}$	$2.5x10^{-4}$	-			
b. Least toxic				-	-	$1.25x10^{-3}$			

Unpublished data of J. Verrett
* Many of these are mixtures
Code: 1 mg = 20 ppm
1 μg = 20 ppb

Air Cell Injections
Pre-incubation

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PRELIMINARY REPORT ON TERATOLOGY STUDIES WITH DIOXIN USING GOLDEN HAMSTERS

Compound	Amount Intubated Days 6 - 10	Feti Litter	Total # of Feti	# Born Alive	Terata	# Dead	Avg. Wt. of Fetus
Control	0	8.6	43	41	None	2 - 1 ED, 1 LD	1.35
Chlorodibenzo-p-dioxin (21% trichloro, 53% tetrachloro, synthesized in FDA)	9.1 μg/kg/day	10.3	62	11	eye anomalies	51 - 4 ED, 47 LD	1.38
	2.0 μg/kg/day	10.8	43	41	gastro-intestinal hemorrhage	2 - 2 ED	1.47
	0.5 μg/kg/day	10.5	63	62	gastro-intestinal hemorrhage	1 - 1 ED	1.66
	0.13 μg/kg/day	11.6	58	56	None	2 - 2 ED	1.53

ED - Early Dead, LD - Late Dead

FROM: Dr. T.F.X. Collins, Mr. W.H. Hansen, Dr. C.H. Williams
Residue Toxicology Branch
Division of Pesticide Chemistry & Toxicology
Office of Pesticides & Product Safety

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PRELIMINARY REPORT ON TERATOLOGY STUDIES WITH 2,4,5-T SAMPLES USING GOLDEN HAMSTERS

Compound	Amount Intubated Days 6 - 10	Feti Litter	Total # of Feti	# Born Alive	Terata	# Dead	Avg. Wt. of Fetus
Control	(16)	12.3	211	198	None	13 - 12 ED, 1 LD	1.70
2,4,5-T (Pure) (Recrystallized)	100 mg/kg/day (9)	12.4	87	38	3a/	49 - 49 ED	1.46
	80 mg/kg/day (5)	13.2	66	50	gastro-intestinal hemorrhage	16 - 6 LD, 10 ED	1.52
	40 mg/kg/day (6)	14.0	84	79	gastro-intestinal hemorrhage	5 - 1 LD, 4 ED	1.51
2,4,5-T (Pure) (Recrystallized and Extracted)	100 mg/kg/day (4)	13.3	53	16	None	37 - 36 ED, 1 LD	1.66
	150 mg/kg/day (4)	10.3	41	10	None	31 - 29 ED, 2 LD	1.66
2,4,5-T Dow) (0.5 ppm dioxin)	100 mg/kg/day (5)	10.8	54	11b/	gastro-intestinal hemorrhage	43 - 43 ED	1.64

a/ One had right hind limb deformity, in 2 the fusion in the skull was not complete.
ED - Early Dead, LD - Late Dead.
b/ All alive were from one litter.

FROM: Dr. T.F.X. Collins, Mr. W.H. Hansen, Dr. C.H.Will
Residue Toxicology Branch
Division of Pesticide Chemistry & Toxicology
Office of Pesticides & Product Safety

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE,
PUBLIC HEALTH SERVICE,
Research Triangle Park, N.C., December 4, 1969.

DR. J. MCLAUGHLIN,
Food and Drug Administration,
Washington, D.C.

DEAR DR. MCLAUGHLIN: I am sending you under separate cover a sample of 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) from the supply that was used at Bionetics Research Labs, Inc. for the Teratogen Screening Study for NCI.

Sincerely,

K. DIANE COURTNEY, Ph.D,
Pharmacology and Toxicology Branch,

THE DOW CHEMICAL COMPANY,
Midland, Mich., February 9, 1970.

M. JACQUELINE VERRETT, *Bureau of Science, Division of Toxicology, Department of Health, Education, and Welfare, Food and Drug Administration, Washington, D.C.*

DEAR DR. VERRETT: The complete assay on the sample of 2,4,5-trichlorophenoxyacetic acid (Dow production batch 120449) which we recently sent to you is as follows:

PERCENT
2,3,7,8-tetrachlorodibenzo- p-dioxin = (0.5 ppm)
2,6-dichlorophenoxyacetic acid = <0.02
2,5-dichlorophenoxyacetic acid = 0.42
2,4-dichlorophenoxyacetic acid = 0.05
2,3,6-trichlorophenoxyacetic acid = 0.55
2,4,6-tichlorophenoxyacetic acid = <0.1
Bis-(2,4,5-trichlorophenoxy) acetic acid = 0.4
3 isomers of dichloromethoxy phenoxyacetic acid = 2.9
2,4,5-trichlorophenol = 0.23 (Max.)
sodium chloride = 0.035
2,4,5-trichlorophenoxyacetic acid = Balance
Freezing Point = 152.9°C
Total assay by acid titration = 100%

Sincerely,

GEORGE E. LYNN
Government Regulatory Relations,
Dow Life Sciences.

MARCH 2, 1970.

CHLORINATED DIBENZO-P-DIOXIN STANDARD (F768)

1. We have supplied Dr. C. Rilliams with a mixture of tri- and titrachlorodibenzo-p-dioxins (F768) prepared by direct chlorination of dibenzo-p-dioxin at room temperature for two and one-half hours (see memo of Ress to Campbell 1/30/70 and memo of Firestone to Campbell 2/5/70).

2. This mixture, previously analyzed by EC-GLC and found to contain about 50% tetrachlorodibenzo-p-dioxin (GLC peak area), was reexamined by GLC with flame ionization detection (6 foot glass column; 200°C; 3% OV-101; Packard GC model 871), which gives a more accurate determination of composition. By this latter analysis, it was determined that the chlorination mixture F768 consists of two components as follows:

(a) 38% 2,3,7-trichlorodibenzo-p-dioxin
(b) 62% 2,3,7,8-tetrachlorodibenzo-p-dioxin

MARCH 26, 1970.

SAMPLES FOR CHICKEN EMBRYO TESTING

1. The following samples were delivered to Dr. Verrett on 3/17/70 for chicken embryo testing:

Our. No.	Identification	Remarks
F871	2,3,7,8-tetrachlorodibenzo-p-dioxin, Dow, pure (ca. 95% by GLC)	0.0878 mg in 10 ml of acetone.
F877	2,7-dichlorodibenzo-p-dioxin, Dow, pure (ca. 99% by GLC)	0.3806 mg in 10 ml of acetone.
F883	2,3,7,8-tetrachlorodibenzo-p-dioxin, FDA, prepared by Dr. Pohland (99.5% pure by GLC).	0.1735 mg in 10 ml of acetone.
F881-A-1	2,4,5-trichlorophenoxyacetic acid; Dr. Williams' purified sample; further extracted 3 x with petr. ether and 4 x with 1+1 petr. ether—diethyl ether.	1.712 grams.
F881-B	Sylvex; Dr. Williams' purified sample; further extracted 3x with petr. ether and 4x with 1+1 petr. ether—diethyl ether.	2.519 grams.

D. FIRESTONE, *Head,*
Fats and Oils Section.

MARCH 26, 1970.

CHLOROPHENOL SAMPLES

1. About 3 grams each of the following chlorophenols were delivered on 3/23/70 to Dr. Verrett (at her request) for chicken embryo testing.

Our No.	Identification	EC-GLC anal PPB *CEF
F888-sub A	Pentachlorophenol (purified) Aldrich Chemical Co.	167.
F888-sub B	2,3,4,6-Tetrachlorophenol (technical) Eastman Chemicals	96,500.
F888-sub C	2,4-Dichlorochlorophenol (purified) Eastman Chemicals	18.
F888-sub D	Ortho-chlorophenol (purified) Fisher Scientific	No analysis.
F888-sub E	2,4,5-Trichlorophenol (technical) Eastman Chemicals	Trace.
F888-sub F	2,4,5-Trichlorophenol (reagent) Dow Chemical Co.	Trace.

*Hexa-, hepta-, and octachlorodibenzo-p-dioxine.

J. RESS,
Fats and Oils Section.

I. Cleft Palate. 21-day Embryo. 0.02 micrograms 2,3,6,7-Tetrachloro-dibenzo-p-dioxin.

II. Abnormal (incomplete) Development of Eyelid. 21-day Embryo. 0.0025 micrograms 2,3,6,7-Tetrachloro-dibenzo-p-dioxin.

III. Edematous Cyst Covering Rump. 21-day Embryo. 0.02 μg (micrograms), 2,3,6,7-Tetrachloro-dibenzo-p-dioxin.