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A Preliminary Report on the Evaluation of an In Vitro Assay for the Detection of "Dioxin-Like" Activity Using Extracts of Soot from the Binghamton State Office Building

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and

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Abstract

As a result of the involvement of an electrical transformer containing polychlorinated biphenyls (PCBs) and chlorinated benzenes in a fire in the Binghamton State Office Building (BSOB), the structure was contaminated with soot ladened with dioxin congeners and isomers, which included 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8 TCDD) and 2,3,7,8-tetrachlorodibenzofuran (2,3,7,8 TCDF). A panel of experts, which was convened to examine decontamination procedures, recommended the development of a short term assay for these compounds, based on an in vitro model of the in vivo chloracne response to dioxin exposure. This assay uses the dioxin-induced keratinization of epithelial cell cultures as an endpoint. This endpoint is evaluated by eye, using intensity of the keratin specific stain, Rhodamine B, as a marker. This report describes the evaluation of the potential utility of such an assay as a rapid, semiquantitative screen for monitoring the BSOB decontamination process.

Initial experiments using a 2,3,7,8 TCDD standard established that the keratinization model could be duplicated in this laboratory. A soot sample from the building for which levels of 2,3,7,8 TCDD and 2,3,7,8 TCDF had been determined by mass spectrometry, was tested in this in vitro system. The resulting activity was of the same order of magnitude as expected from the content of TCDD and TCDF as determined in the mass spectrometry analysis. Twelve extracts of soot from various floors of the building were tested for their activity in the assay. Activity of soot extracts from the various floors ranged over three orders of magnitude with samples from the lower floors generally having lower activity.

These studies have also lead to the observation that after exposure to 2,3,7,8 TCDD, cells in the assay undergo a change in morphology characterized by the appearance of flat, apparently nonproliferating cells. This change in morphology, when used as an endpoint, correlated well with the keratinization endpoint and thus

may form the basis of an alternative and possibly better in vitro assay for dioxin congeners and isomers.

A possible correlation between the keratinization activity and the PCB content of these soot extracts was observed. Preliminary assays on a PCB mixture (Aroclor 1254), which utilized the flat cell morphology as an endpoint, have indicated that this correlation was probably not due to a direct effect of the PCBs on this assay.

The effect of a proposed decontamination agent, Triton X-100, was also tested using this endpoint. A concentration of 0.1 µl/ml was toxic while lower levels had no activity in this assay, nor did they affect the sensitivity of the assay to 2,3,7,8 TCDD.

The keratinization assay exhibited a decrease,with time, in the magnitude of the response to the point that keratinization was characterized by the appearance of colonies of keratinizing cells on a background of nonkeratinizing cells. These cultures could be evaluated microscopically and there was no loss of sensitivity to 2,3,7,8 TCDD associated with the decline of the response magnitude. Efforts to determine the cause of the decline, and to reverse it, are discussed.

The occurrence of polychlorinated dibenzo-p-dioxins (PCDD) and dibenzofurans (PCDF) in the environment poses a serious potential threat to human health. These compounds are among the most toxic and teratogenic low molecular weight compounds known (Poland et al., 1976).

Chlorinated dibenzo-p-dioxins are formed from the condensation of two orthochlorophenates under conditions of high alkalinity, pressure and temperature. The combustion, chemical nature and combustion chamber residence time can also lead to the production of PCDDs and PCDFs. This has been verified by the analysis of flue gas emission and fly ash of some municipal incinerators in the Netherlands (Hutzinger et al., 1981). This process has also been observed in laboratory models (Langer et al., 1973). The pyrolysis of organohalogens such as chlorinated phenols and PCBs lead to the formation of PCDDs and PCDFs. This observation was evidenced under field conditions recently in New York State. On February 5, 1981, as a result of fire, the Binghamton State Office Building (BSOB) was heavily contaminated with soot; subsequent cleanup efforts were halted when chemical analysis indicated the presence of high levels of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) as well as other possibly toxic congeners (Smith et al., 1981). This contamination was traced to the involvement by fire of an electrical transformer which contained the dielectric fluid Pyranol, a mixture of PCBs composed of Aroclor 1254 and chlorinated benzenes.

A number of human exposures to dioxin congeners and isomers have been reported. An acne outbreak in Germany in 1954 was traced to trichlorophenol contaminated with TCDD and TCDF (Poland et al., 1976). Similar outbreaks of chloracne, the most common manifestation of dioxin exposure in humans, were noted in 2,4,5-T production plants in France, Germany and the United States in the 1950's and 1960's. In 1971 the use of waste oil contaminated with TCDD for dust control in a riding arena lead to the death of a large number of horses and other small animals, as well as some cases of chloracne in children who played in the area (Kimbrough et al., 1977). The contamination of rice oil with dibenzofuran analogs lead to many cases of chloracne in Japan (Bradlaw et al., 1979). The explosion of a chemical plant in Seveso, Italy

in 1976 lead to exposure of the population to TCDD which was thought to be responsible for the occurrence of chloracne in humans and a number of livestock losses (Hay, 1980; Pocchiari et al., 1979; Reggiani, 1978). The use of Agent Orange (a mixture of n-butyl esters of 2,4-D and 2,4,5-T), a defolient, in Vietnam between 1962 and 1969 lead to the contamination of large areas by TCDD (Poland et al., 1976).

Recent work by Knutson and Poland has demonstrated that the hyperkeratinization response to TCDD exposure which occurs upon exposure to TCDD in vivo could be reproduced in an in vitro model system (Knutson et al., 1980). The induction of keratinization is thought to be responsible for the in vivo induction of chloracne. They found that although TCDD did not produce an acute toxic response in various cell types in culture (Knutson et al., 1980a), XB cells, a cloned epithelial line of mouse teratoma cells (Rheinwald et al., 1975) when co-cultured at high density with irradiated 3T3 mouse fibrablast cells exhibited a keratinization response when exposed to TCDD. Spontaneous keratinization occurs in these cells when they are seeded at low density (Rheinwald et al., 1975). High density XB/3T3 cultures not treated with TCDD did not show this response.

Other halogenated aromatic hydrocarbon congeners including dibenzo-p-dioxins, dibenzofurans, biphenyls and azo(xy)benzenes also induced keratinization in the XB/3T3 system although to a lesser extent than 2,3,7,8 TCDD. Keratinization could also be induced by some non halogenated aromatic hydrocarbons such as benz(a) anthracene and 5,6-benzoflavone (Knutson et al., 1980). Since 2,3,7,8 TCDD is the most potent, but not the only inducer of this hyperkeratinization response, this effect will be referred to here as "dioxin-like" activity. Unrelated toxins were studied to determine if the keratinization response was due to nonspecific cell damage. The direct acting alkylating agents N-acetoxy-2-acetylaminofluorene and N-methyl-N'-nitrosoguanidine; inhibitors of nucleic acid synthesis, actinomycin D, bleomycin and cordycepin; the spindle microtubule inhibitor, colchicine; kepone, a chlorinated hydrocarbon producing a toxic syndrome distinct from that of TCDD; and 2,6-dichlorobenzo-nitrile, and acnegen considered to have a different mechanism

than TCDD all failed to produce keratinization in the XB/3T3 culture system (Knutson et al., 1980). Furthermore, a correlation has been demonstrated between the degree of toxicity of various TCDD isomers and congeners and the extent of enzyme induction, receptor binding and hyperkeratinization response (Knutson et al., 1980; Poland et al., 1977; Poland et al., 1976a; Kende et al., 1974).

The XB/3T3 system is extremely sensitive, giving an induction of keratinization with as little as 3.2 pg of TCDD (Knutson et al., 1980). In addition, this system is particularly relevant as a model since it deals with an induced differentiation of the intact cell using an endpoint (hyperkeratinization) known to be relevant to human exposure (i.e., chloracne).

This report describes studies which have been carried out to determine the feasibility of using the in vitro XB/3T3 keratinization model as a mass screen of environmental samples for "dioxin-like" activity, i.e., the ability to induce a hyperkeratinization response. Such an assay could be applied to the detection of "dioxin-like" activity in extracts of samples collected at the Binghamton State Office Building. The evaluation and verification of this assay system is being done by the testing of 12 specific soot samples from various floors of the BSOB building and comparing the results with those obtained from high resolution mass spectroscopy of the same samples when they become available.

METHODS

Sample Preparation

A soxhlet extract of the soot sample in benzene (Smith et al., 1981) at a volume of 0.5 - 2.0 ml was mixed with 100 µl of dimethylsulfoxide (DMSO) and evaporated at room temperature for 24 hours in darkness to allow for solvent exchange from benzene to DMSO with a final volume of 100 µl. A solvent exchange was also done with the positive controls (2,3,7,8 TCDD in DMSO) and solvent controls (DMSO). The extract, now in DMSO, was diluted 1:1000 in tissue culture medium (Dulbecco's modified Eagles medium supplemented with 20% fetal calf serum) and four more 10-fold dilutions were made in this medium for application to the cell cultures. This dilution series was freshly made for each of the twice weekly refeedings.

Cells

The XB-2 cell line and the 3T3 feeder cells were obtained from H. Green (M.I.T.)(Rheinwald et al., 1975). The cultures were grown in Dulbecco's modified Eagles medium with 100 U/ml penicillin and 100 µg/ml streptomycin. The XB line was cultured in medium conditioned by a 24 hour exposure to confluent 3T3 cells (25 ml/ 75 cm^2). The medium was supplemented with 20% fetal calf serum for the XB cells and 10% for the 3T3 cells. No contamination with mycoplasma was detected by the Hoechst fluorescence staining method (Chen, 1974).

Keratinization screen assay for "dioxin-like" activity

Initial screening was done essentially as described by Knutson and Poland (Knutson et al., 1980) for the in vitro keratinization model. Target cells were the XB epithelial clone derived from mouse teratoma cells by Rheinwald and Green (Rheinwald et al., 1975). When these cells are plated at low cell density (250 cells per 60 mm dish) along with lethally irradiated 3T3 cells (3 X 10^5 cells per 60 mm dish), the XB cells form epithelial colonies which stain red with Rhodanile blue. Rhodanile blue stains most mammalian tissue blue but keratinized tissues are stained red (MacConaill et al., 1964). When XB cells are plated at high density (10^5 cells/dish) in the pre-

sence of irradiated 3T3 cells (3×10^5 cells per dish), the XB cells replicate but keratinization is not seen and therefore the red staining component of Rhodanile blue (Rhodamine B) is absent. Addition of TCDD to cultures of XB cells at high cell density which are refactory to spontaneous keratinization, produces a dose-related keratinization with the associated red staining with Rhodanile blue. TCDD was shown to produce maximal red staining at concentrations of 5×10^{-11} to 10^{-7}M; 1×10^{-11}M TCDD produces an intermediate response while cultures treated with 5×10^{-12}M TCDD and untreated control cultures do not keratinize. This response reaches a maximum by day 10-13. When XB/3T3 cells were plated in 16 mm wells containing 1 ml of medium, as little as 3.2 pg of TCDD produced observable keratinization (Knutson et al., 1980).

Confluent cultures of 3T3 cells were lethally irradiated with 4000 rads by a cesium source and both the irradiated 3T3 and XB cells were seeded together in Costar Multiwell dishes (15 mm) at 5×10^4 XB cells and 5×10^4 irradiated 3T3 cells per ml per well. These dishes were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was replaced twice weekly.

The dilution series of the sample was added to the assay cultures 24 hr post seeding and with each medium change. Appropriate solvent controls were run as well as 2,3,7,8 TCDD reference samples.

After 12 days, the cultures were rinsed with phosplate buffered saline (PBS) fixed for 30 min. in 10% formalin-PBS and stained with 1% Rhodanile blue or 1% Rhodamine B (Knutson et al., 1980). The cultures were then observed for the occurrence of red staining material indicative of the keratinization response. The highest dilution of sample capable of inducing keratinization greater than background levels was taken as the endpoint. The endpoint of the 2,3,7,8 TCDD standard was compared to the endpoints derived from the extracts in order to determine the TCDD equivalency of the sample.

The range of activity of a sample was calculated from the endpoint as follows: It has been shown (Knutson et al., 1980) that 10^{-11}M TCDD produces only a moderate keratinization response, (compared to that produced by 10^{-10}M and higher).

Furthermore, one half this amount, i.e. 5×10^{-12}M TCDD produces no response at all. Therefore, in all cases production of such a moderate endpoint was taken to indicate the presence of between 5×10^{-12} and 1×10^{-11}M TCDD equivalent. The assumption was made that one fifth the amount of sample which gave the moderate response would give a negative response. Thus the amount of sample which contains between 5×10^{-12} and 1×10^{-11}M dioxin-like activity was taken to be between 20% and 100% of the actual amount of sample which gave the moderate response.

Safety Considerations

It is recognized that the safe handling, containment, and disposal of the substances to be used in this study are essential. Some of the precautions include prohibition of mouth pipetting, subtraction weighting, use of glove box storage cabinets vented with HEPA and charcoal filters, restriction of traffic in hazard area, no food, smoking or drinking in hazard area, accurate record keeping of hazardous materials from receipt to disposal, use of filter vented biohazard hoods (BioGard Type II B) for medium changes and use of commercial hazardous waste disposal facilities.

RESULTS

Assay of BSOB Extracts

Preliminary studies were performed to attempt to duplicate the in vitro keratinization model in this laboratory. XB/3T3 cultures were exposed to 10^{-9}M TCDD in DMSO or to DMSO alone at 0.01% final concentration to correspond to the level in the TCDD solution. After 12 days of incubation, with refeeding every 3 to 4 days, these cultures were fixed and stained. Cultures exposed to 10^{-9}M TCDD showed a marked increase in keratin deposition, as shown by intense Rhodamine B staining, as compared to the DMSO negative control (Fig. 1).

A 10-fold dilution series of authentic 2,3,7,8 TCDD from 10^{-8} to 10^{-12}M was tested. The minimal concentration capable of inducing a keratinization response greater than background levels was found to be 10^{-11}M (Fig. 2b). This is equivalent to 3.2pg/ml and is in general agreement with published results for this compound (Knutson et al., 1980). A BSOB soot extract which had been analyzed by mass spectroscopy for TCDD and TCDF (Smith et al., 1981a) was also tested in this experiment for its ability to induce a hyperkeratinization response. A dilution series of this extract was applied to the XB/3T3 cell cultures as described in methods. Figure 2a shows that the DMSO solvent control dilution series induced no keratinization. The highest concentration of the DMSO soot extract (1 µl/ml, equivalent to 820 µg soot/ml) caused cytotoxicity. The lowest concentration of extract which induced keratinization was 10^{-3} µl/ml, which is equivalent to 0.82 µg of soot (Fig. 2c). From this result the concentration of dioxin-like activity in the extract was calculated (as described in Methods) to be in the range of 2-20 ppm. Analysis of this sample by mass spectrometry (Smith et al., 1981b) indicated the presence of 1.2 ppm 2,3,7,8 TCDD and 48 ppm 2,3,7,8 TCDF. The latter is 20-fold less potent then 2,3,7,8 TCDD (Knutson et al., 1980); therefore, 48 ppm of this compound is equivalent in keratinization activity to 2.4 ppm of 2,3,7,8 TCDD. Thus, the total keratinization activity, based on the mass spectrometry data for these two compounds, should be that of 3.6 ppm 2,3,7,8 TCDD. This is within the concentration limits actually obtained in the keratinization assay.

In order to further test the ability of the assay to predict the results of mass spectrometric analysis, benzene extracts of 12 soot samples from different floors of the BSOB were tested for their ability to induce keratinization. Cells were seeded and exposed to dilutions of the extracts in culture medium. The cultures were refed every 3-4 days with fresh dilutions of the extract. After 21 days, the cultures were fixed and stained with Rhodamine B. It became obvious, however, that a decrease in the magnitude of the hyperkeratinization effect in response to TCDD exposure had occurred, apparently with time, such that induced keratinization could be seen clearly only under microscopic observation. Colonies of keratinizing cells, similar to those described by Rheinwald and Green (1975) could be seen on a background of non-keratinizing cells. (Attempts to determine the reason for this decrease and to regain the earlier level of responsiveness seen in Figure 1 will be described later.) This decrease in magnitude of the keratinization response did not however, decrease the sensitivity of the assay as seen in studies with authentic TCDD. Accordingly, blind microscopic evaluation was carried out on each of three replicates for each sample by two observers for the occurrence of keratinizing colonies. The results, shown in Table 1, are an average of 6 values per sample and showed good reproducibility with variation rarely being greater than one dilution for any of the replicates of any single sample. The solvent controls and blanks showed no false positive response. Calculations for dioxin-like activity were made as described in Methods. The results show a variation of about 3 orders of magnitude in the "dioxin-like" activity of the various samples. There appears to be something of a trend in that the samples from the lower floors seem in general to have less activity than those from the higher floors. The results of this study will be compared to the mass spectrometric analysis of these samples when this becomes available.

The average keratinization results in terms of dioxin-like activity in ppm were plotted against the available PCB analysis data for these samples generated by gas chromatography (Pause, 1981). Figure 3 shows a double log plot of these two sets of data. There appears to be a possible correlation between the PCB content

and the keratinization inducing activity of these soot extracts. Possible reasons for this correlation are examined in the discussion section.

Attempts Made to Reverse the Decrease in the Magnitude of the Hyperkeratinization Effect

A personal communication from Dr. A. Poland confirmed that a decline in magnitude was also experienced in his laboratory and was alleviated by the use of reconstituted frozen stocks of early passage XB cells. However, such a strategy in this laboratory failed to increase the magnitude of the keratinization endpoint. It has also been shown that the ratio of XB cells to the irradiated 3T3 feeder cells can affect the magnitude of the response (Rheinwald et al., 1975). Experiments done to optimize this ratio also failed to restore the response to the early levels, as did changing the irradiation time of the feeder cells, changing the refeeding schedule, using culture vessels of different manufacture and lot numbers, changing incubators, altering the pH of the cultures, using stain from various sources or using medium conditioned by unirradiated 3T3 cells rather than co-cultivation of XB cells with irradiated 3T3 cells. Current attempts to increase the magnitude of the keratinization response will be addressed in the discussion section.

Morphological Alteration - A Possible Alternative Endpoint

The appearance of an altered cell morphology was observed with those cells which had been exposed to TCDD as well as the various soot extracts in the experiment described in table 1. This change was first seen after 7 days of exposure to the samples and was characterized by a flat cell morphology as compared to the more fibroblastoid cells in the unexposed cultures or those exposed to less than 10^{-11}M TCDD (Figure 4). An apparent cessation in cell growth was also seen in these flat cells, while the unexposed cells continued to proliferate, resulting in more dense cultures. The minimal concentration of each sample capable of inducing the flat cell response was recorded on the 13th day of exposure of the cultures to the extracts. Replicate dilution series were evaluated by phase microscopy of the living cells by two observers generating 10 values for each sample. The agreement between replicates was excellent with variation rarely being greater than one dilution

for any one sample. One half of the replicates were fixed and stained for a record, while the remaining cultures were later evaluated for the previously described keratinization (table 1).

The high correlation between the induction of the keratinization response and that of the flat cell morphology (Figure 5) indicates that it may by possible to use this flat cell morphological change as an alternative and perhaps improved endpoint in the assay for dioxin-like activity. Preliminary experiments suggest that the induction of the flat cell appearence by TCDD is not dependent upon the irradiated 3T3 feeder layer, thus simplifying any proposed assay procedure using this endpoint. The induced flat cell morphology has been found to be apparent after only 6-7 days of exposure to authentic 2,3,7,8 TCDD, thereby allowing the assay to be run without the necessary refeeding needed in the keratinization assay.

Effects of Triton X 100 on the Assay

Triton X100 (TX) has been proposed for use in the decontamination of the BSOB. Since TX is soluble in benzene which is currently used in the soxhlet extraction of the samples and could therefore be encountered in samples to be assayed, a preliminary experiment was carried out to deterimine whether TX has any effect on the assay. A concentration range of TX from 10^{-1} - 10^{-3}µl/ml was tested with the standard concentration series of the TCDD standard (10^{-8} - 10^{-12}M). It was found that TX at 10^{-1} µl/ml is cytotoxic while levels of 10^{-2} and 10^{-3} µl/ml are not. No synergistic or antagonistic effect of TX in regard to the TCDD induced flat cell endpoint was observed. There was also no induction of a false positive result by TX alone at any concentration tested. Experiments to determine the effect of TX on the keratinization endpoint are in progress.

DISCUSSION:

The fire at the Binghamton State Office Building and involvement of an electrical transformer containing a mixture of PCBs (Araclor 1254) and chlorinated benzenes lead to subsequent contamination of the structure with dioxin and dibenzofuran laden soot. A need was established for a rapid semiquantitative assay for "dioxin-like" activity. This would allow prioritization of samples for high resolution quantitative and qualitative analysis in order to monitor the progress of decontamination. This preliminary report has dealt with the evaluation and verification of such an assay which is based on an in vitro model of the in vivo keratinization response to dioxin exposure.

Preliminary studies demonstrated that the published in vitro model was reproducible in this laboratory and that this model was sensitive to levels of dioxin (2,3,7,8-TCDD) which corresponds to that in the literature (Knutson et al, 1980) (approximately 10^{-11}M). Assay results of a single soot extract for which mass spectrometry data were available indicated a good correlation between these data and the data resulting from the keratinization model. This initial verification lead to the application of this assay to 12 soot extracts for comparison with results of mass spectrometry of the same samples as they become available.

However, the decrease in the magnitude of the TCDD induced keratinization effect may reduce the usefulness of this particular endpoint in spite of the readily apparent, microscopically observable, induction of keratinizing colonies. Attempts are currently being made to reclone the XB cell line for the desired characteristics of hyperkeratinization upon TCDD exposure. Successful monitoring of the cell line, cell storage and recloning would insure a stable cell population upon which the keratinization assay could be based. Cloning is being done by identification and isolation of spontaneously keratinizing colonies of XB cells in sparse culture, as well as keratinizing colonies of XB cells induced by TCDD

exposure in dense cultures. New XB and 3T3 feeder cell starter cultures will be obtained, since the reconstitution and use of our early passage frozen stocks has failed to increase the magnitude of the keratinization response to that seen in the initial assays.

The subjective reading of the endpoint by visual estimation of the minimal concentration of extract capable of keratin induction based on the intensity of Rhodamine B staining or identification of keratinizing cell colonies limits the degree of quantitation possible. Therefore, several other methods of achieving better quantitation of the induced keratin production are being considered and evaluated. One involves extraction of the Rhodamine B stain from the cells and quantitation by fluorometric measurement. Another involves a quantitative radio-immunoassay method for mammalian epidermal keratin protein which has recently been published (Yuspa et al, 1980). Another possible method for detection of increased keratin production to be explored is based upon the observation (Steinert et al, 1978) that in keratinizing cells, keratin represents 20% of the total urea extractable cellular protein. Thus, keratin may represent the predominent protein being synthesized. Furthermore, keratin has been shown to be relatively rich in histidine, methionine and cysteine (Belanger, 1956; Clark, 1968; Hambrick, 1966). Thus, it may be possible to quantitate keratin production by measurement of incorporation of these (radiolabeled) amino acids into cellular protein.

The verification of the flat cell morphological change as an indicator of "dioxin-like" activity would allow a second and perhaps improved biological endpoint to be used in a routine screen assay in addition to or in place of the keratinization assay. The endpoint is apparent after only 6-7 days and can be readily assessed by microscopic observation of living cultures. Another advantage of the short development time of this endpoint is the lack of the necessity to refeed the cultures, thus reducing both the sample requirement and handling of the cultures. The homogeneity of these cells may allow use of smaller culture vessels, reducing even further

the amount of sample needed. In addition recent experiments have suggested that the irradiated 3T3 cell feeder layer may not be needed for the development of this endpoint.

Further studies will be carried out to establish whether the induction of the flat cell morphology is a valid endpoint for the detection of dioxin-like activity. These will include a double blind study to test the ability of the endpoint to quantitate various concentrations of authentic 2,3,7,8-TCDD and a test of the relative activities of other halogenated polycyclic compounds, e.g. TCDF, biphenylene, PCB, azo(xy)benzenes, as well as nonhalogenated aromatic hydrocarbons. Compounds that inhibit cell proliferation by acting on macromolecular synthesis will also be tested to determine if the flat cell response in these cells is simply due to the coincidental inhibition of cell growth. Attempts to establish cloned cell lines exhibiting the flat cell characteristic to insure homogeneity are currently underway.

The possible correlation which we have observed between the in vitro assay results and the PCB content of the soot samples (Figure 3) lead to concern that the PCBs were responsible for the induction of keratinization. In fact, it has been reported that 2,3,4,2',3',4' polybrominated biphenyl can induce keratinization in the XB/3T3 system at a concentration 2,000 fold higher than 2,3,7,8-TCDD (Knutson et al, 1980). To examine this possibility, Araclor 1254, the PCB mixture present in the transformer coolant and thus the presumed source of PCBs in the soot samples, was tested for the induction of the flat cell morphology. The lowest concentration of Araclor 1254 for which the flat cell induction was observed was 10 µg/ml. The highest concentration of PCBs found in any of the soot samples tested was 23,000 µg/gm (Pause, 1981). The lowest amount of this sample which gave an endpoint was 1.5 µg of soot per ml which contains 0.035 µg of PCBs per ml. Based upon the results with Araclor 1254, this is insufficient to induce the flat cell response. Thus, the observed induction of the flat cell morphology by the soot extracts is unlikely to have been caused by the PCBs contained in the

samples. Experiments are in progress to determine if similar results are obtained for PCB induced keratinization.

Another explanation for the correspondence between the PCB data and the in vitro assay results may be a synergistic or additive interaction between PCBs and low levels of TCDD or some other substance in the soot samples. Studies designed to detect such interaction between Araclor 1254 and 2,3,7,8-TCDD are being carried out and studies using PCBs to spike actual soot extracts are planned. The correspondence could also be the result of a similar deposition pattern of the PCBs and the dioxin congeners and isomers in the building.

Triton X-100, the proposed decontamination agent for the BSOB, was found to be toxic to the in vitro system at 0.10 µl/ml but not at 0.01 µl/ml. TX is soluble in benzene, and a relatively low, non toxic, concentration in a sample could be concentrated by a benzene extraction procedure to toxic levels and preclude use of the in vitro assay. This possibility should be considered in the development of decontamination and sample collection procedures. The lack of effect of TX on the induction of the flat cell response does however, decrease the concern of a false positive result from low levels of this compound.

In summary, the in vitro keratinization model developed by Knutson and Poland (Knutson et al, 1980) has been examined for use as a screen assay for the detection of "dioxin-like" activity in extracts of BSOB soot samples. After initial verification with authentic TCDD, a preliminary assay was done on the one sample for which TCDD and TCDF data from mass spectrometry were available. The results of this in vitro assay compared well with the quantitative analysis data. Based on this result, a screen for keratinization induction was carried out on 12 extracts of soot from various floors in the BSOB. These results will be compared to the mass spectrometry data on the same samples, thus allowing a more rigorous validation. A high correlation would indicate that this model may be

used as a semiquantitative mass screen assay for "dioxin-like" activity.

The decrease in the magnitude of the keratinization response which we have observed with time gives reason for concern, as the cause for this decline has not been determined. The problem may be solved by the acquisition of new starter cultures, or stabilized for a time by recloning the line for the desired characteristics and/or using reconstituted frozen stocks of those cultures which currently exhibit TCDD induced keratinizing colonies. Alternatively, the TCDD-induced change in morphology (which is characterized by the appearance of flat, non proliferating cells) which seems to correlate very well with the keratinization endpoint may have the potential for use as an additional or alternative (and possibly better) endpoint for use in mass screening for "dioxin-like" activity.

REFERENCES

Belanger, L.F. (1956) Autoradiographic Visualization of the Entry and Transit of S^{35} Methionine and Cystine in the Soft and Hard Tissues of the Growing Rat. Anat. Rec., 124:555-563

Bradlaw, J.A., and Casterline, J.L. (1979) Induction of Enzyme Activity in Cell Culture: A Rapid Screen for Detection of Planar Polychlorinated Organic Compounds. J. Assoc. Off. Anal. Chem., 62:904-915

Chen, T.R. (1974) Utilization of Fluorescent Hoechst Stain to Effectively Detect Mycoplasma Contamination. In Vitro 10:390

Clark, S.L. (1968) Incorporation of Sulfate by the Mouse Thymus: Its Relation to Secretion by Medullary Epithelial Cells and to Thymic Lymphopoiesis. J. Exp. Med., 128, 927-957.

Fukuyama, K. and Epstein, W.L. (1966) Epidermal Keratinization: Localization of Isotopically Labeled Amino Acids. J. Inves. Derm., 47, 551-559.

Hambrick, G.W., Lamberg, S.I. and Bloomberg, R. (1966) Observations on Keratinization of Human Skin In Vitro. J. Inves.Derm., 47, 541-550.

Hay, A. (1980) Health monitoring endangered at Sevèso. Nature, 283, 237.

Hutzinger, O., Olie, K., Lustenhouwer, J.W.A., Okey, A.B., Bandiera, S., and Safe, S. (1981) Polychlorinated Dibenzo-P-Diozins and Dibenzofurans: A Bioanalytical Approach. Chemosphere 10, 19-25.

Kende, A.S., Wade, J.J., Ridge, D. and Poland, A. (1974) Synthesis and Fourier Transform Carbon-13 Nuclear Magnetic Resonance Spectroscopy of New Polyhalodibenzo-p-dioxins. J. Org. Chem., 39, 931-937.

Kimbrough, R.D., Coleman, D.C., Liddle, J.A., Cline, R.E. (1977) Epidemiology and Pathology of a Tetrachlorodibenzodioxin Poisoning Episode. Arch. Environ. Health, March-April, 77-85.

Knutson, J.C., and Poland, A. (1980) Keratinization of Mouse Teratoma Cell Line XB Produced by 2,3,7,8-Tetrachlorodibenzo-p-dioxin: an in vitro model of toxicity. Cell, 22, 27-36.

Knutson, J.C. and Poland, A. (1980) 2,3,7,8-Tetrachlorodibenzo-p-dioxin: Failure to Demonstrate Toxicity in Twenty-three Cultured Cell Types. Toxicol. and Applied Pharmacol., 54, 377-383.

Langer, H.G., Brady, T.P. and Briggs, P.R. (1973) Formation of Dibenzodioxins and other Condensation Products from Chlorinated Phenols and Derivatives. Environ. Health Perspect., 5, 3-7.

Lowery, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem., 193, 265-275.

MacConaill, M.A., and Gurr, E. (1964) The Histological Properties of Rhodanile Blue. Ir. J. Med. Sci., June, 243-250.

Pause, R. (1981) Personal Communication, N.Y. State Department of Health.

Pocchiari, F., Silano, V. and Zampieri, A. (1979) Human Health Effects from Accidental Release of TCDD at Seveso, Italy. Ann. N.Y. Acad. Sci. 320, 311-320.

Poland, A. and Kende, A. (1976) 2,3,7,8-Tetrachlorodibenzo-p-dioxin: environmental contaminant and molecular probe. Fed. Proc., 35, 2404-2411.

Poland, A., Glover, E., Kende, A.S., DeCamp, M., and Giandomenico, C.M. (1976a) 3,4,3',4'-tetrachloroazoxybenzene and azobenzene: Potent Inducers of Aryl Hydrocarbon Hydroxylase. Science, 194, 627-630.

Poland, A. and Glover, E. (1977) Chlorinated Biphenyl Induction of Aryl Hydrocarbon Hydroxylase Activity: A Study of the Structure-Activity Relationship. (1977) Mol. Pharmacol., 13, 924-938.

Reggiani, G. (1978) Medical Problems Raised by the TCDD Contamination in Seveso, Italy. Arch. Toxicol., 40, 161-188.

Rheinwald, J.G. and Green, H. (1975) Formation of a Keratinizing Epithelium in Culture by a Cloned Cell Line Derived from a Teratoma. Cell, 6, 317-330.

Smith, R.M., O'Keefe, P.W., Hilker, D.L., Jelus-Tyror, B.L., and Aldous, K. (1981) Chemical analysis of 2,3,7,8-tetrachlorodibenzo-p-dioxin and 2,3,7,8-tetrachlorodibenzofuran in a soot sample from the transformer explosion in Binghamton, New York. N.Y. State Department of Health Report. February 20, 1981.

Smith, R.M., Hilker, D.L., O'Keefe, P.W., Kumar, S., O'Brien, J., Jelus-Tyror, B.L., and Aldous, K. (1981a) Analysis of a Binghamton soot sample for tetrachlorodibenzofurans and tetrachlorodibenzo-p-dioxins. N.Y. State Department of Health Report, October 1, 1981.

Steinert, P. and Yuspa, S.H. (1978) Biochemical Evidence for Keratinization by Mouse Epidermal Cells in Culture. Science, 200, 1491-1493.

Yuspa, S.H., Poirier, M.C., Harness, J.R., Olsom, D.R. and Steinert, P.M. (1980) Specific Quantification of Mouse and Human Keratin Proteins by Radioimmunoassay. Biochem. J., 187, 281-284.

Table I

Keratinization Assay of BSOB Soot Samples

Floor number	Lowest active concentration of extract (µg soot equivalent/mℓ)[a]	TCDD equivalent (ppm)[b]
1	114.2	0.01 - 0.10
3	1.3	1.2 - 12
4	14.0	0.11 - 1.1
6	15.9	0.10 - 1.0
7	0.3	5.3 - 53
8	1.0	1.6 - 16
9	1.5	1.1 - 11
10	0.5	3.2 - 32
12	0.7	2.3 - 23
14	12.9	0.12 - 1.2
15	0.4	4.0 - 40
17	0.4	4.0 - 40

a) Activity was determined by the appearance of keratinizing colonies. This result is the average of values from 3 replicates evaluated by two observers.

b) Calculated as described in Methods.

Figure Legends

Figure 1 Effect of 2,3,7,8 TCDD on XB/3T3 Cell Cultures

Cultures were seeded and cultivated as described in methods. Figure 1a is representative of an XB/3T3 culture exposed to 10^{-9} M 2,3,7,8 TCDD in the presence of 0.01% DMSO. Figure 1b is representative of a replicate culture exposed to 0.01% DMSO alone. The bright red rhodamine B stain, indicative of keratin deposition, shows as dark grey in these photos.

Figure 2 Keratinization assay of a BSOB soot extract

Figure 2a shows the negative response of the DMSO control. Figure 2b shows the effect of 2,3,7,8 TCDD on this system with keratinization first evident with 10^{-11}M. Figure 2c shows the toxic effect of a BSOB soot extract at the highest concentration (1 µl/ml) and the induction of keratinization at lower concentrations, with the effect first detected at the fourth highest dilution (10^{-3} µl/ml), equivalent to 0.82 µg soot/ml.

Figure 3

Log-log plot of the amount of PCBs detected in BSOB soot extracts from various floors by gas chromatography (abscissa) and the calculated values of "dioxin-like" activity generated by the keratinization assay of the same samples (ordinate). The values for "dioxin-like" activity were derived by dividing the amount of soot per ml in the lowest soot extract concentration capable of inducing keratinization into 3.2 pg/ml which is the lowest concentration of 2,3,7,8 TCDD found to induce keratinization.

Figure 4 TCDD induced flat cell morphology

Figure 4a: XB/3T3 culture after 12 days of incubation with 0.1% DMSO, showing high density and fibroblast-like organization. Figure 4b: a similar culture exposed to 10^{-8}M TCDD in 0.1% DMSO, showing flat morphology and relative low density. Phase, 400X magnifiifcation.

Figure 5

Log-log plot of calculated values of "dioxin-like" activity of BSOB soot extracts from various floors generated by the keratinization assay (ordinate) and by the induction of the flat cell response (abscissa) calculated as in Figure 3.

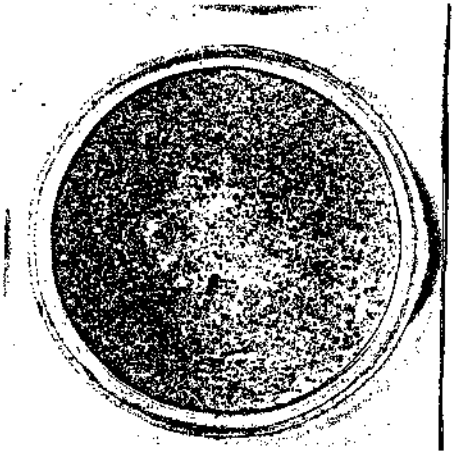

Figure 1a

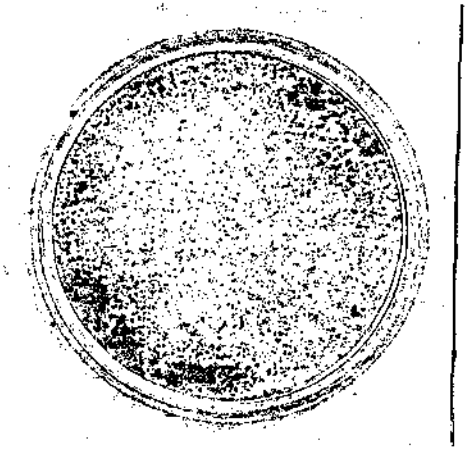

Figure 1b

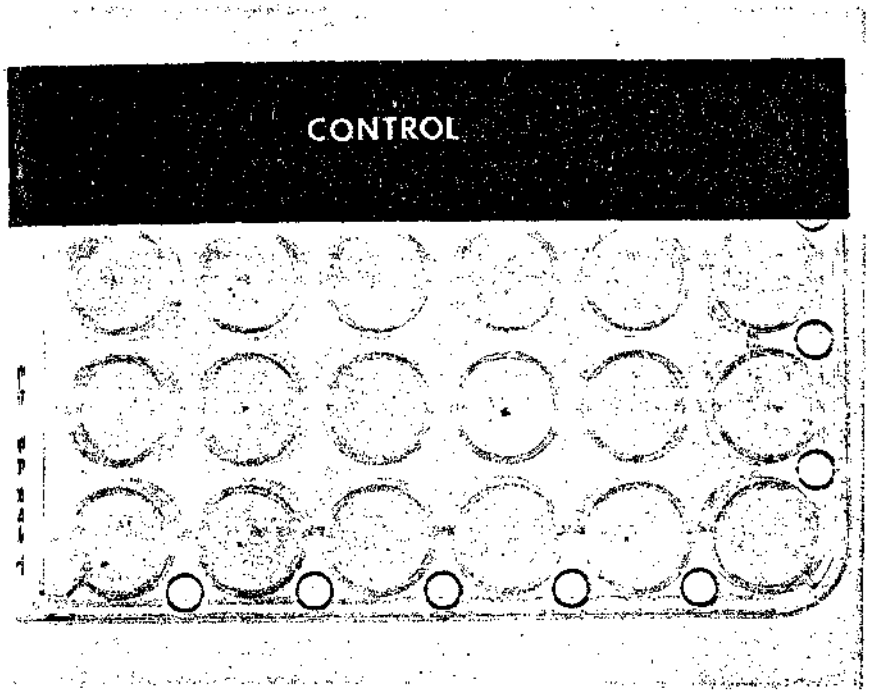

Figure 2a

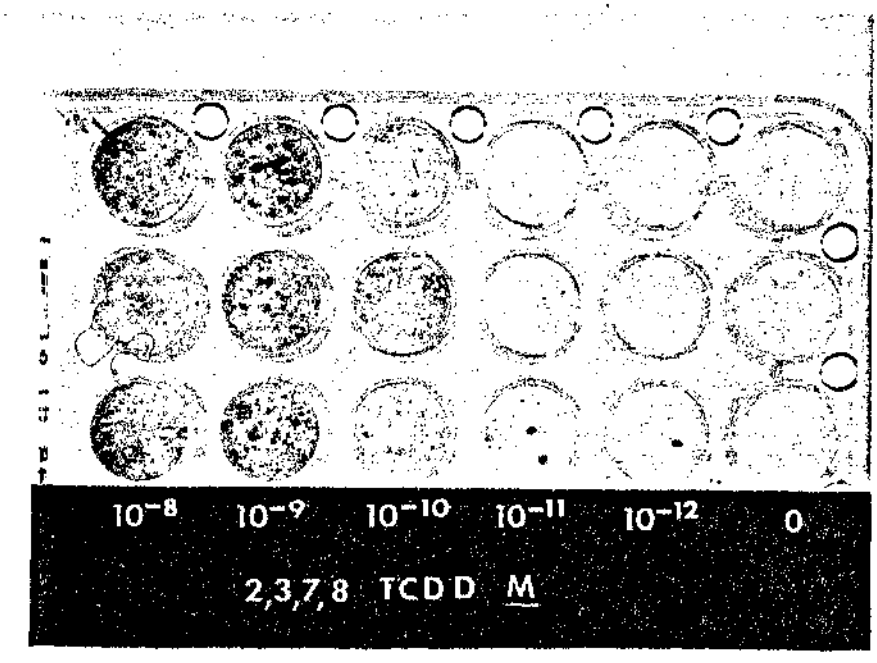

Figure 2b

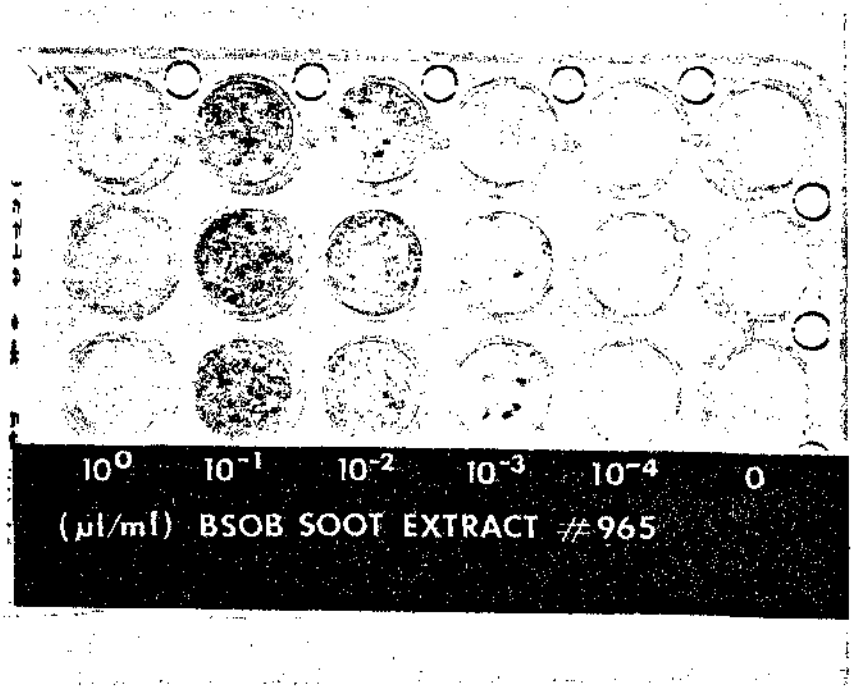

Figure 2c

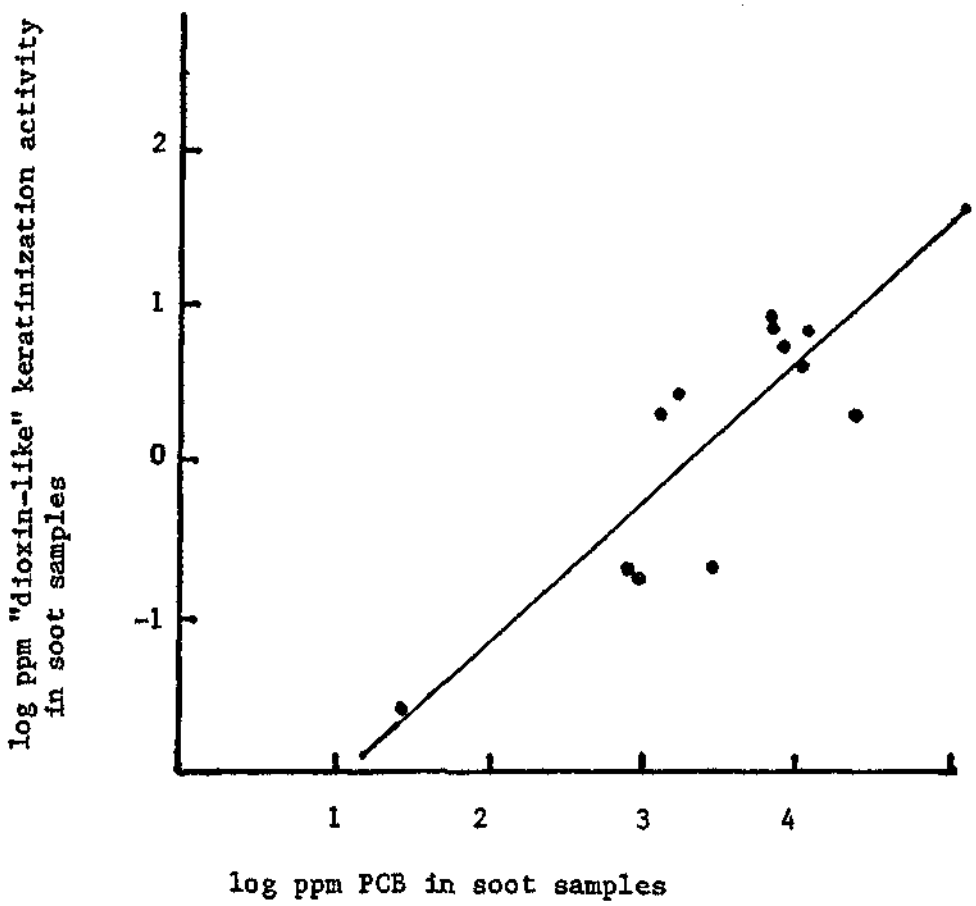
log ppm "dioxin-like" keratinization activity in soot samples
2
1
0
-1
1
2
3
4
log ppm PCB in soot samples

Figure 3

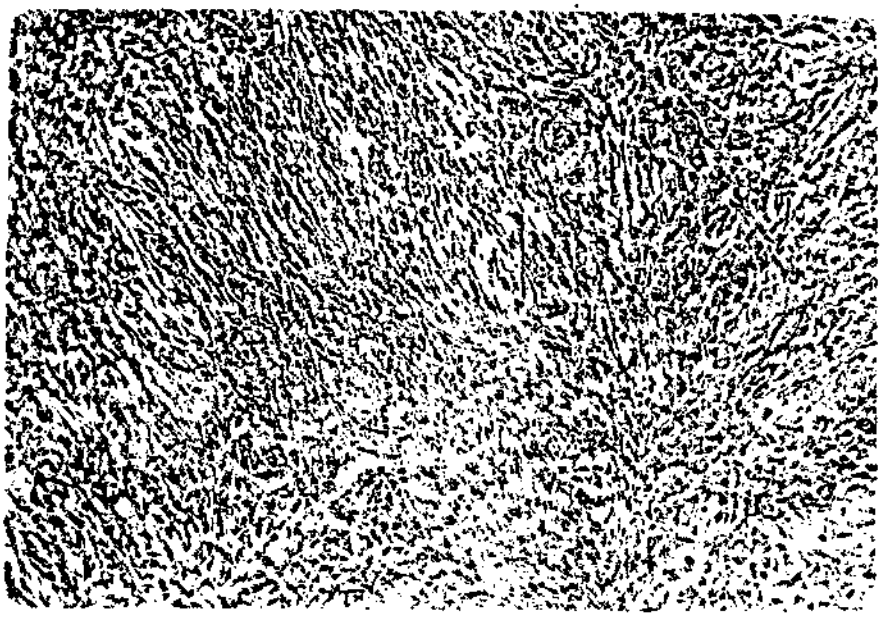

Figure 4a

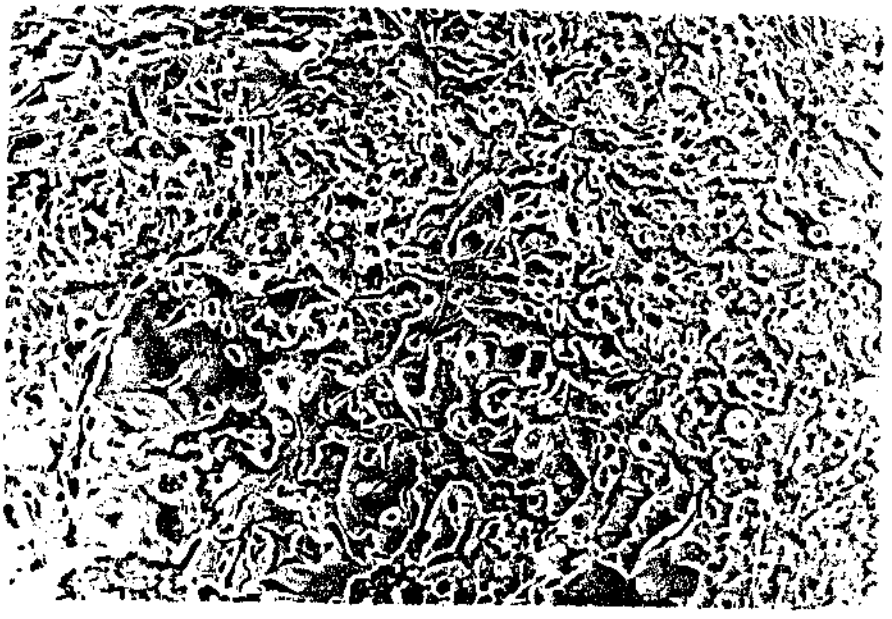

Figure 4b

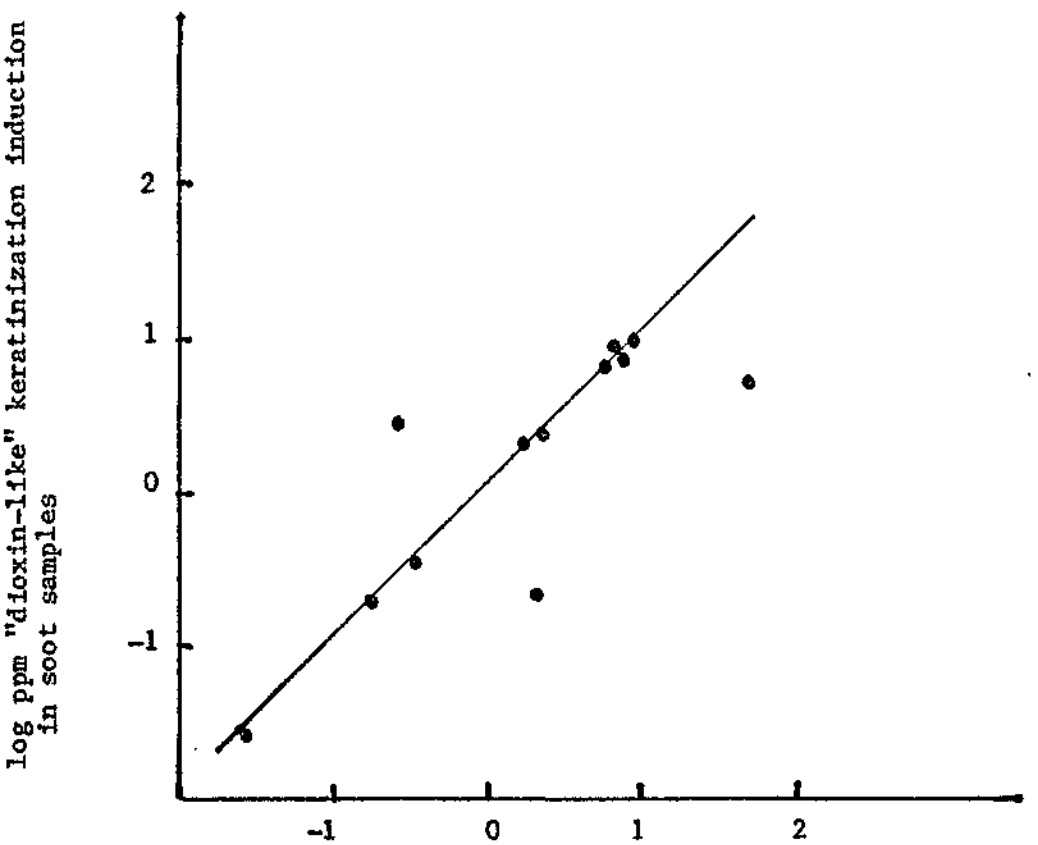
log ppm "dioxin-like" keratinization induction in soot samples
2
1
0
-1
-1
0
1
2
log ppm "dioxin-like" flat cell induction in soot samples

Figure 5