COO-2737-7

Temporal Aspects of Tumorigenic Response to Individual and Mixed Carcinogens

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Comprehensive Progress Report

for Period

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R. E. Albert, F. J. Burns and B. Altshuler

Institute of Environmental Medicine
New York University Medical Center
New York, N.Y. 10016

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Progress Report

Contract EY 76-S-02-2737

The U. S. Department of Energy
Division of Biology and Medicine
Washington 25, D. C.

Summary

The research proposed here is designed to obtain a better understanding of the temporal kinetics of tumor induction when one or more carcinogens are present simultaneously or sequentially for prolonged periods of time. Studies done to date under this contract have shown that carcinogenesis in mouse skin by polycyclic aromatic hydrocarbon carcinogens is consistent with the induction of dependent and autonomous cell transformations by the carcinogen followed by the conversion of autonomous tumor cells into malignancies at a rate which is determined by the level of carcinogen exposure. Dependent cell transformations remain latent in the skin unless expressed by a promoting agent. Dependent neoplasia appears to follow one-hit kinetics while malignancy is a multihit endpoint. Dose-related and time-related aspects of tumor induction are separable in the initiation-promotion system of mouse skin which

along with rat skin and hamster lung is being used as a model for testing hypotheses. Results to date provide the basis for a new interpretation of the linear non-threshold extrapolation model. The broad aim of the study is to provide a basis or rationale for estimating risks associated with prolonged exposures to carcinogens found in the environment and to predict how different tissues and species respond to the same carcinogens.

Summary of Technical Scope (50 words) - Contract EY 76-S-02-2737

The temporal kinetics of tumor induction when two or more carcinogens are present simultaneously or sequentially are studied in mouse and rat skin and hamster lung. Experimental tumor induction curves are being analyzed in terms of mathematical models that relate the probability of tumor development to carcinogen dose and time of exposure.

Introduction

This research seeks to obtain a better understanding of the dose and temporal dynamics of tumor induction by individual carcinogens, and combinations of carcinogens, through an analysis of the component processes that determine the time patterns of tumor formation as well as an intercomparison of temporal responses in different species, target organs and tissues. The broad aim of the study is to improve the basis for estimation of cancer risks in humans from exposure to environmental carcinogens.

Knowledge of dose-response relationships at very low levels of exposure is the foundation for the control of environmental carcinogens. The importance of dose-response relationships is self evident; they provide the basis for estimating the magnitude of risk at specified levels of carcinogen exposure. A number of extrapolation models have been proposed (Cornfield, 1977). Two extrapolation models are in actual use. The FDA has adopted a log-probit extrapolation model with slope = 1 for use in estimating cancer risks from carcinogenic feed additives in edible portions of food animals. The Environmental Protection Agency is using the linear non-threshold extrapolation model (Albert, et al., 1977). Other extrapolation models include notably the dt^n = constant formulation (Albert, Altshuler, 1972) and a multi-stage model with a linear dose component (Crump, et al., 1976). None of these models has an adequate basis in scientific evidence. The log-probit

model takes its justification from the fact that many biological responses including carcinogenesis conform to a log-probit pattern at relatively high response levels. There is no basis for knowing whether this holds true in the very low dose response range. The slope of one probit per log dose merely reflects an outside limit for observed animal responses. The linear non-threshold dose-reponse relationship emerged in connection with the prediction of low cancer risks from ionizing radiation. It stems from the thesis that there is a close relationship between carcinogenesis and mutagenesis and the latter has been shown to be consistent with a linear non-threshold model (McCann, et al., 1976). Furthermore, epidemiological data, particularly on the leukemogenic response to ionzing radiation is consistent with this model, although the observed responses are associated with dose levels which are far greater than those of concern with the control of environmental radiation exposures. The dt^n = constant model shows reasonable conformity with experimental and human data, however this model lacks the persuasiveness that stems from a plausible basis in biological mechanism.

The problem of characterizing low-level dose-response relationship is exceedingly difficult from the standpoint of regulatory control strategy because it is desirable to control risks down to levels where it is impossible to make direct observations in animal or human populations. It seemed to us that

one possible way out of this dilemma was to attempt to dissect the carcinogenic process into its component parts and to characterize the quantitative aspects of the dose-response relationships for each of the separate components and thus hopefully to obtain the basis for characterizing responses at levels below those which can be observed directly.

There are five components to the study: (1) mouse skin tumor experiments which are examining the interaction of various types of carcinogens as initiators and the chronic carcinogenic effects of BaP alone or in combination with the promoter, phorbol ester (TPA), (2) rat skin tumorigenesis studies with DMBA, (3) biochemical studies of BaP-DNA interactions in the mouse skin, (4) lung tumor studies with BaP in the hamster and (5) cytofluorographic analysis of epidermal cells.

Mouse Skin Tumorigenesis

New Model for Chronic BaP Tumorigenesis in the Mouse Skin

A series of experiments have been completed or are in progress involving graded single doses of BaP followed by graded durations and dose levels of TPA as well as chronic exposures to BaP alone at graded dose levels or combined with chronic exposures to TPA at different dose levels. These results tentatively provide the basis for a new conceptual framework for BaP skin carcinogenesis which clarifies the processes underlying the

actions of BaP alone or in combination with the promoting agent TPA. The mouse skin is the model which most readily lends itself to the approach of dissecting the carcinogenic process into its component parts using a polycyclic aromatic carcinogen and the promoting agent phorbol myristate acetate (PMA)[1]. We are using benzo(a)pyrene (BaP) as the primary carcinogen at least initially for this study because of its environmental importance.

The general properties of the initiation-promotion model in mouse skin have been thoroughly described (Van Duuren, et al., 1973). The characteristics of the model are the following: mouse skin can be initiated by a single carcinogen exposure at dose levels which are virtually non-tumorigenic. The presence of initiated foci can be displayed by the repeated application of a promoting agent such as phorbol myristate acetate (PMA) which in itself is virtually non-tumorigenic, but when applied after (not before) the initiator, will produce a relatively large yield of tumors in a relatively brief period. The process of initiation has been likened to the induction of somatic mutations: initiation occurs promptly and remains unchanged without further treatment for as long as a year. In a recent paper we proposed on the basis of a finding reported by Burns (Burns, et al., 1976) that most of the papillomas produced by a 60 day course of treatment with PMA after initiation with

[1]Alternative nomenclature 6-0-tetradecanoylphorbol-13-acetate (TPA)

DMBA will regress if the PMA treatment is discontinued, that the initiation and promotion process in the mouse skin can be described in the same conceptual framework and terminology as that used by Furth for endocrine tumors (Albert and Burns, 1977). Furth described tumor progression as beginning with "dependent" tumors, which are lesions that require continual growth stimulation for their maintenance (Furth, 1953). Dependent tumors progress to autonomous benign tumors which are self sustaining and no longer need the help of external growth stimuli. Autonomous tumors, in turn, progress to malignancies. There is no sharp demarcation between these stages.

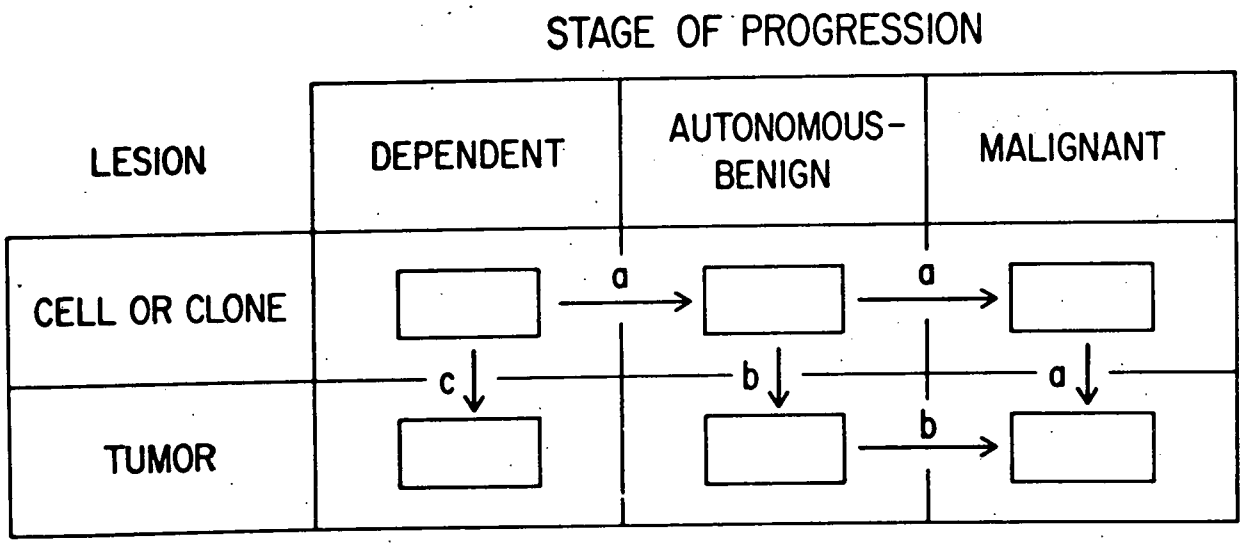


Figure 1. A conceptual framework of carcinogenesis in mouse skin. Initiators produce dependent, autonomous-benign, or malignant cell transformations and may also cause progression from lesser to more malignant stages (path a). Promotion may accelerate progression and in addition may stimulate clonal expansion of transformed cells to produce tumors. These tumors reflect the properties of their cells of origin and may progress spontaneously to carcinoma (path b) or may remain indolent or regress (path c).

The above conceptual framework involves 3 stages of cell transformation: (1) dependent, (2) autonomous-benign and (3) malignant, and 2 stages of the development of the neoplastic lesion: (1) the cell or clonal level or (2) grossly visible tumors. Within these stages there are 3 pathways for tumorigenesis: (a) progression at the cell (or clonal) level from dependent, through autonomous-benign to malignant transformation with the initial appearance of tumors as malignancies, (b) progression at the cellular level from dependent to the autonomous-benign stage of transformation with outgrowth of autonomous-benign transformations into autonomous papillomas and subsequent development of papillomas into cancers, and (c) outgrowth of dependent transformations into dependent papillomas without subsequent conversion of dependent papillomas into autonomous papillomas.

With chronic exposure to BaP alone, Pathway a is followed exclusively at low dose levels (e.g., 8 µg and 16 µg per week), i.e., carcinomas appear de novo without the precursor papilloma stage; a higher dose levels (e.g., 32 and 64 µg per week), tumorigenesis follows both Pathways a and b. Pathway a (de novo appearance of malignancies) is traversed more rapidly than at lower doses, but Pathway b (initial autonomous papilloma formation with subsequent conversion to malignancies) precedes and predominantes over Pathway a by a factor of 6-10. At very high dose rates of BaP (128 µg/wk), Pathway a (de novo cancer

formation) is speeded up more than Pathway b with the initial tumors appearing as malignancies but that eventual yield of cancers is about equal by Pathways a and b. Pathway c results from chronic high level TPA exposure with either short or prolonged BaP exposure. Here, dependent tumors (papillomas that regress with early discontinuation of TPA) are produced very rapidly and in numbers directly proportional to the dose of BaP for a given dose rate of TPA. With sustained high level TPA exposure, the dependent papillomas are converted into autonomous papillomas (non-regressible with discontinuation of TPA) and then into cancers. With combined chronic exposure to BaP and TPA at a dose level of 0.5 μg three times per week (tiw), the papilloma yield has a temporal response pattern equivalent to about 4 times the dose level of BaP alone. At minute doses of TPA (0.05 μg tiw) combined with BaP, there is marked enhancement of carcinogenesis via Pathway a alone.

The results to date indicate that the precursor lesion as a stage in the development of skin malignancy in the mouse is limited to relatively high dose levels of BaP, because at low doses the progression from the initial stages of transformation to malignancy can occur at the cellular or perhaps clonal level without the occurrence of grossly evident precursor lesions. Furthermore, the concept of a precursor lesion as an obligatory stage in carcinogenesis does not appear to be supportable in the

mouse skin, since the formation of cancers via papillomas is only one of the available pathways and is limited to the relatively high dose range. The temporal response characteristics obtained to date for skin carcinoma in the mouse with BaP are consistent with the $dt^n = c$ formulation with $n \cong 2$, where d is the dose rate and t is the time to a specific tumor yield. The dose-response relationship for tumor initiation with BaP is consistent with a linear non-threshold pattern and the process of tumorigenesis increases in efficiency with lower dose rates in the sense that a larger proportion of the initiated sites are actually expressed as tumors (Albert and Burns, 1977).

From a mechanistic standpoint, these findings are consistent with the view that dependent cell transformations are the consequence of a one-hit process with subsequent exposures converting the dependent cell transformations into autonomous cell transformations and then into malignant cell transformations. At very low dose rates the dominant action of BaP would be the one-hit process which is a non-expressed form of transformation in the absence of exposure to a promoting agent. In short, the linear non-threshold dose-response model refers only to the process of tumor initiation (dependent cell transformation) which is a static, non-progressive stage of neoplastic transformation, whereas the dose-response relationships for overt tumor induction (autonomous and malignant cell transformations) are consistent

with the occurrence of multi-hit processes.

Combined Action of Initiator and Promoter (BaP and TPA)

It seems likely that promtoers, as well as, initiators and carcinogens, have important consequences in determining the temporal pattern of cancer induction by acclerating the development of neoplasia. Promoters may act by stimulating clonal expansion of potentially neoplastic cells or they may intervene directly in the progression of normal cell to carcinoma at the cellular level. In order to determine the temporal effect of promoters, mouse skin was exposed to weekly benzo(a)pyrene either alone or in combination with various twice weekly doses of the promoter, 12-0-tetradecanoyl-phorbol-13-acetate (TPA).

The total tumor response as a function of time for BaP alone is shown in Figure 2.

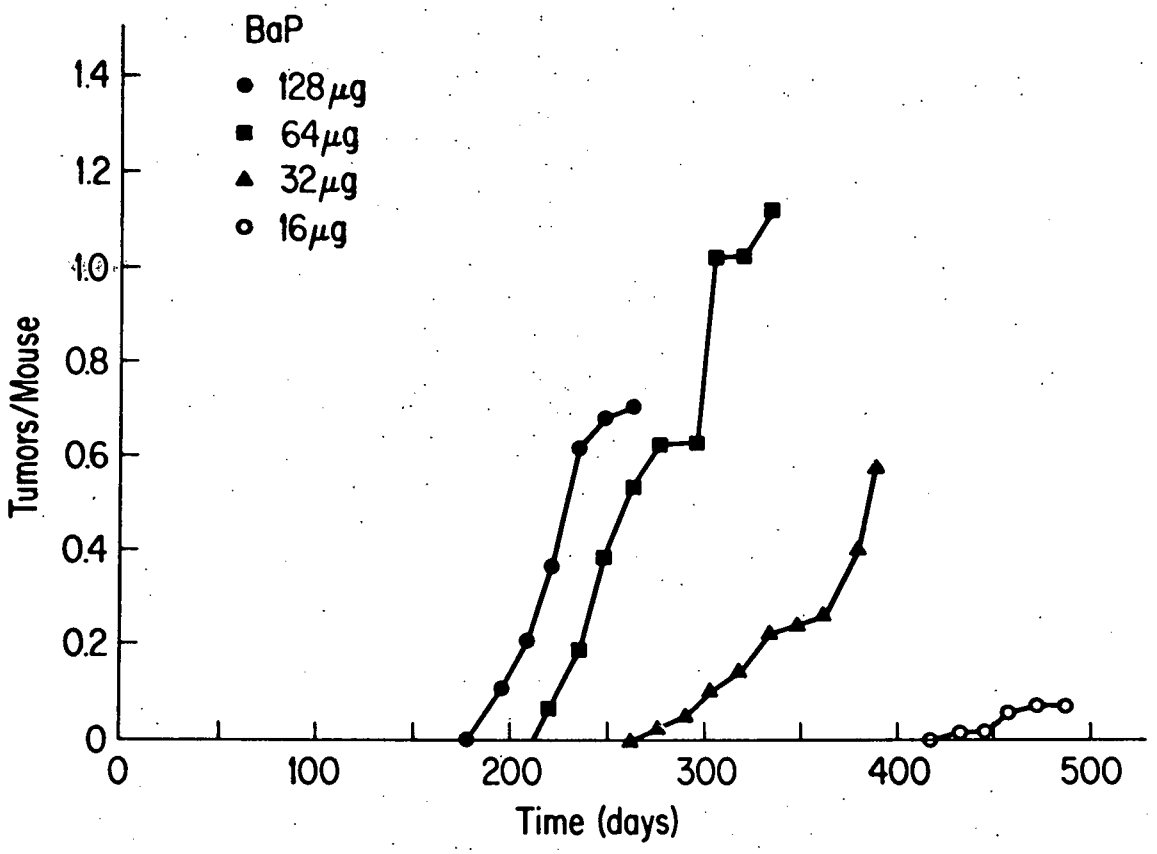


Figure 2. Cumulative incidence of skin papillomas per mouse. Numbers identifying each curve indicate the topically applied weekly dose of BaP. Time was measured from initial treatment.

As the BaP dose increased from 16 μg weekly to 128 μg weekly, the tumor curves were displaced progressively to shorter times in such a manner that n in dt^n = constant was about 2.4; where d was weekly dose and t was time to first tumor. The data in

Figure 3 were derived from the same animals as for Figure 2 and indicate that carcinomas follow a pattern quite similar to the one for total tumors.

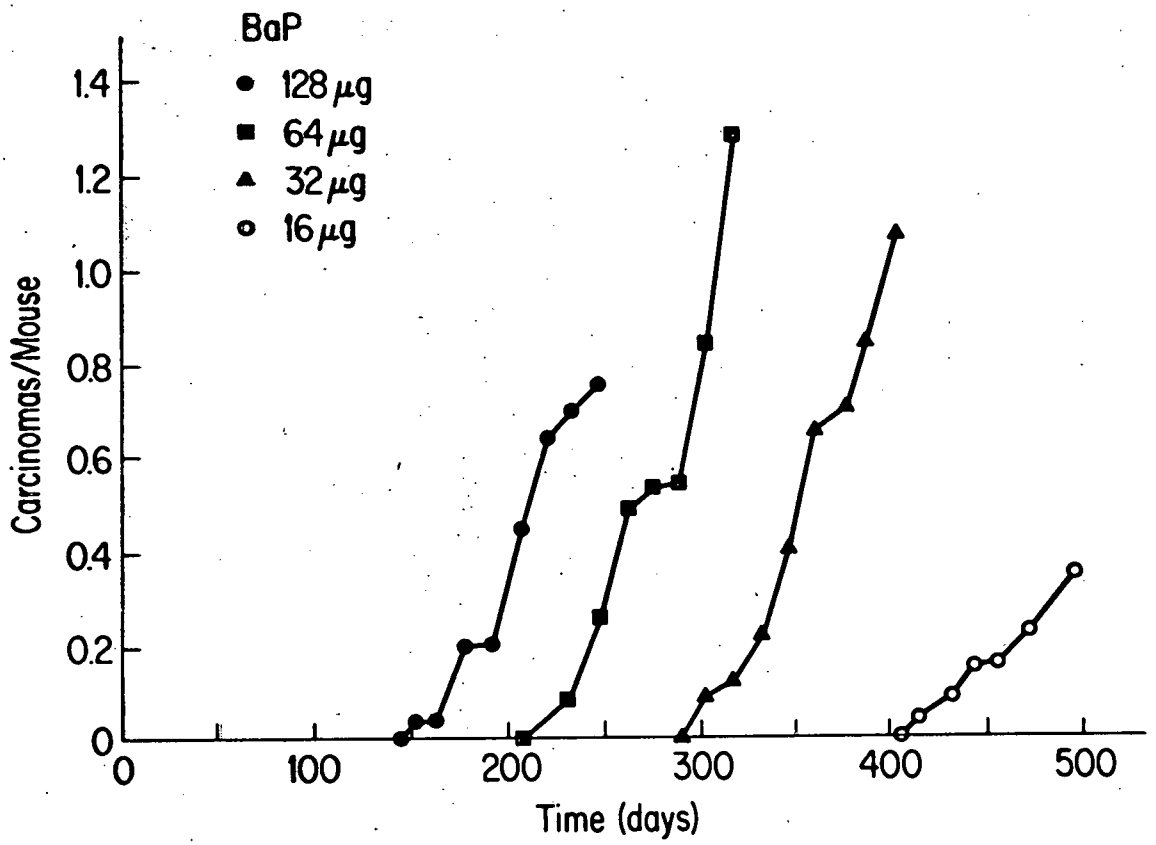


Figure 3. Cumulative incidence of skin carcinomas per mouse. Numbers identifying each curve indicate the topically applied weekly dose of BaP. Time was measured from the first BaP treatment.

The n value for carcinomas was 2.1 which is essentially the same as the n value as for total tumors (Figure 2).

In Figure 4 data on total tumors are reported for mice that received 16 µg BaP weekly (Monday) alone or in combination with various twice weekly (Wednesday and Friday) doses of TPA as indicated. These results indicate that 0.05 µg TPA only slightly accelerated the appearance of BaP-induced tumors but that 0.5 µg TPA greatly accelerated the onset of tumors.

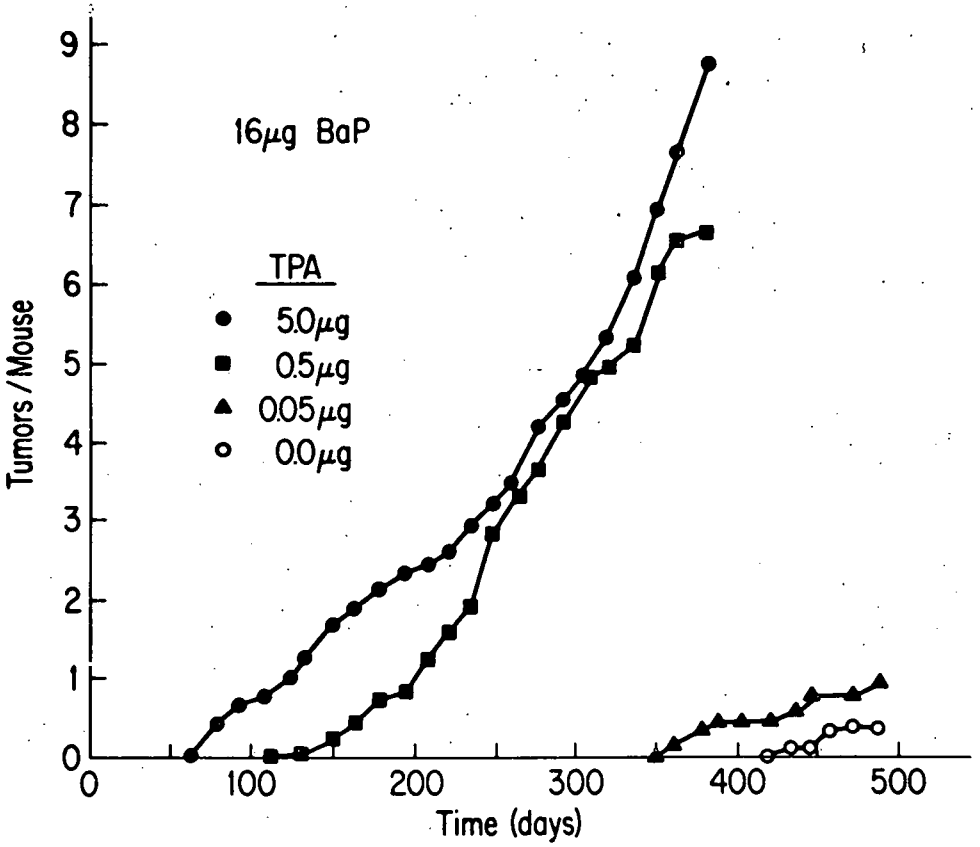


Figure 4. Cumulative incidence of skin papillomas per mouse. Each animal received weekly treatments of 16 µg BaP plus twice weekly treatments of either 0, 0.05, 0.5, or 5.0 µg TPA. Chemicals were topically applied in acetone. Time was measured from the first TPA treatment.

Similar data for the induction of carcinomas with various doses of TPA are shown in Figure 5.

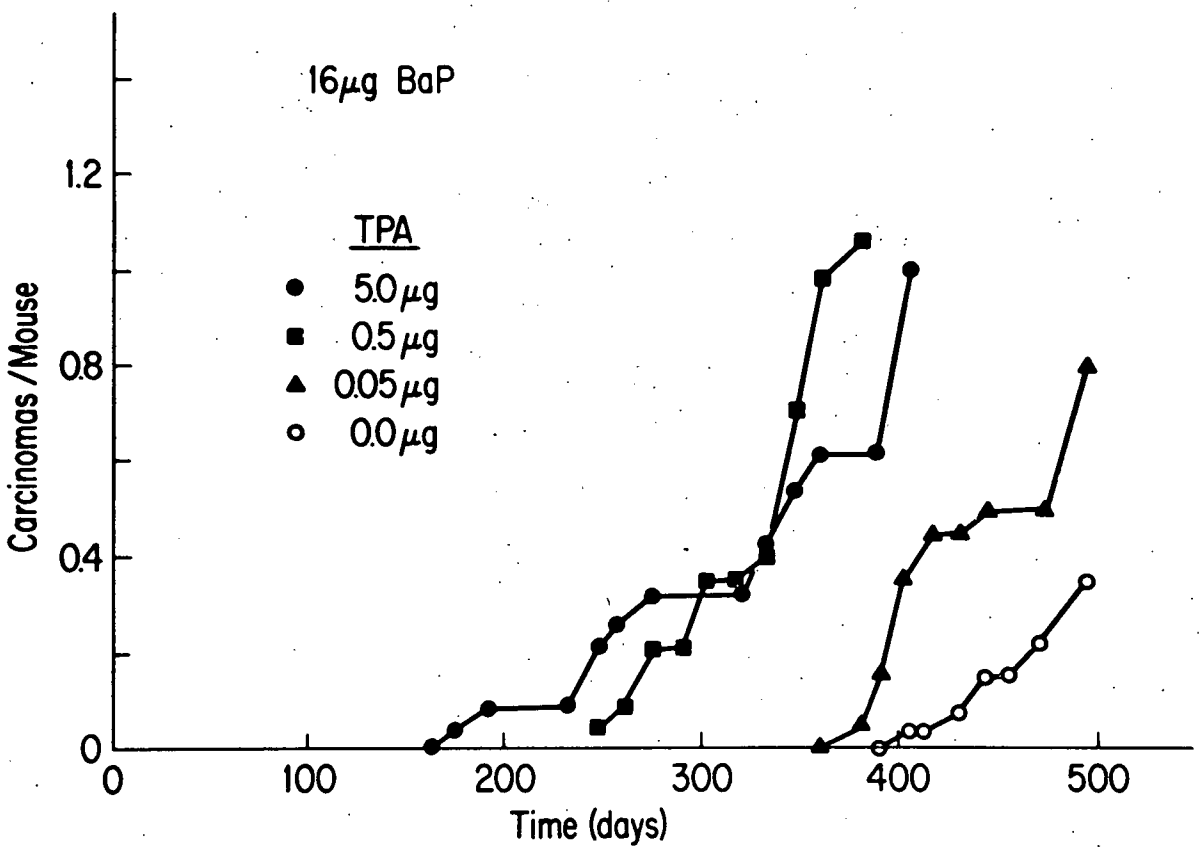


Figure 5. Cumulative incidence of skin carcinomas per mouse. Each animal received weekly treatments of 16 μg BaP plus twice weekly treatments of either 0, 0.05, 0.5, or 5.0 μg TPA. Chemicals were topically applied in acetone. Time was measured from first TPA treatment.

A comparison of total tumors in Figure 4 and carcinomas in Figure 5 indicates that the higher doses of TPA have a disproportionate effect on total tumors which are by and large papillomas. There is little doubt, however, that TPA, a promoter of papillomas, accelerated the development of carcinomas. In terms of the model in Figure 1, this could have been accomplished by a direct action at the cellular level via route a. In other words, these data indicate that TPA not only produced an expansion of cell number to produce palpable tumors but may have acted directly to accelerate progression to carcinoma. The maximum degree of temporal displacement (the higher two TPA doses) was equivalent to a 4-fold increase in BaP dose without promotion. In other words, part of the temporal displacement associated with different doses of BaP could be derived from the promoting action of BaP.

The carcinoma data has been replotted in Figure 6 on log-log coordinates in order to show the temporal onset pattern more clearly. Straight lines on such a plot represent power functions of the form ct^m were c is a dose dependent constant and m is the slope. As indicated in Figure 6, slopes range from 7.8 to 10.0 which is a measure of the steepness of these curves. Such high values of m in conjunction with n values of about 2.0 imply that the dose dependence may follow a power function form with an exponent as high as 4 or 5. These patterns are quite typical of those observed for exposure of other organs to

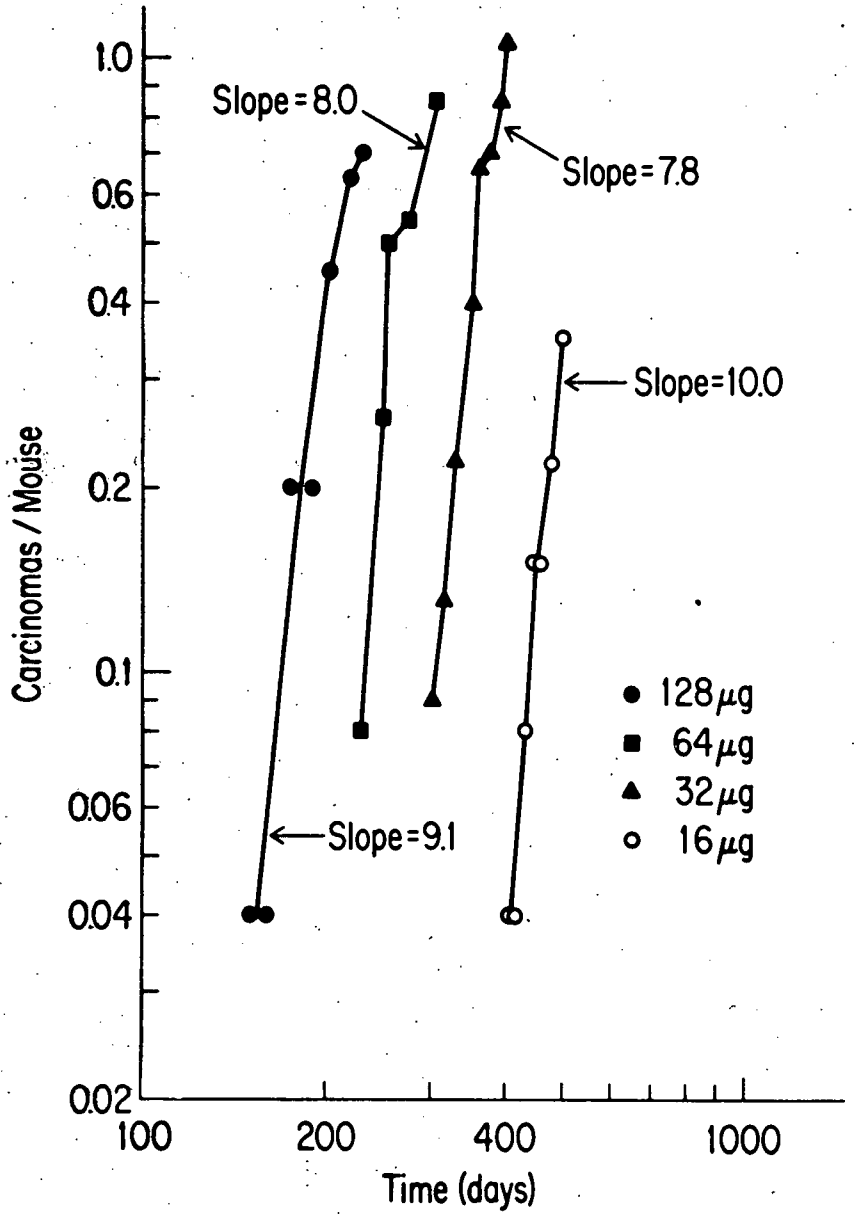


Figure 6. Log-log plot of the cumulative incidence of skin carcinomas per mouse versus time. BaP was applied topically every week at the indicated doses. Time was measured from the first BaP treatment.

carcinogens, especially, rat liver exposed to diethylnitrosamine (Druckrey, 1967) and human lung exposed to cigarette smoke (Albert and Altshuler, 1975).

Progression of Papillomas to Carcinomas

Papillomas induced by combined action of initiators and promoters differ in degree of autonomy and probability of progression to carcinoma (Burns, et al., 1976). For example, the data in Figure 7 indicate that papillomas induced by initiation with benzo(a)pyrene and promotion with TPA (tetradecanoylphorbol-acetate) regressed with a halftime of about 50 days if promotion ended at 100 days and with a halftime of about 130 days if promotion ended at 225 days. The extra persistence among papillomas after 225 days of promotion may be partly a reflection of the somewhat larger size of these lesions but may also be partly a progression to greater autonomy.

Stop experiments with BaP alone performed by Lee and O'Neil (private communication) indicate that some papillomas are truly autonomous and may grow after the BaP treatments are ended even without promotion (see Figure 8). The total dose of BaP required to produce detectible numbers of independently-growing papillomas is much greater than initiating doses of BaP given in initiation-promotion protocols. Nevertheless, our data indicate that the dose-response for induction of papillomas is essentially linear and even small doses of BaP produce various types of papillomas in proportion to dose. Promotion mainly stimulates the dependent cell transformation but may also accelerate the progression to carcinomas via route a in Figure 1.

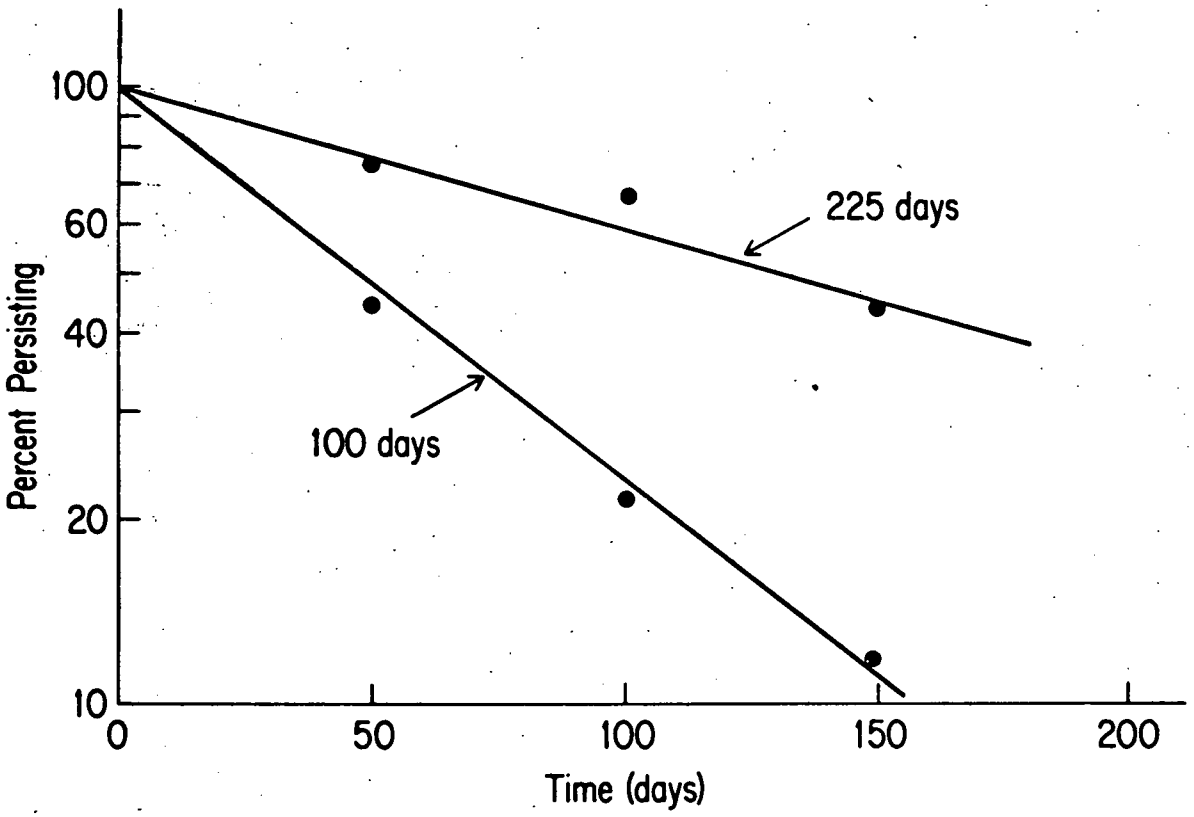


Figure 7. Percent papillomas persisting normalized to the number of tumors present at the termination of topical TPA treatments. All animals were initiated with BaP. TPA was ended after either 100 or 225 days as indicated.

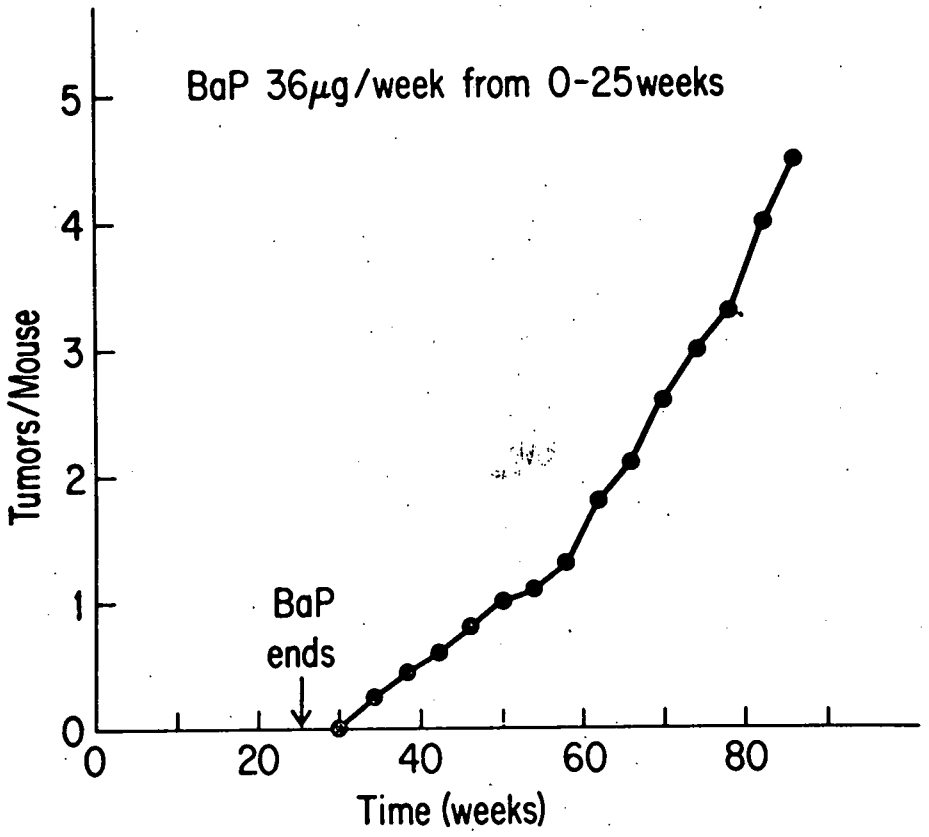


Figure 8. Cumulative incidence of skin papillomas per mouse following 26 weekly topical doses of 36 μg per week of BaP in acetone. Data from Lee and O'Neil, 1971.

Individual papillomas in mice receiving weekly BaP without promotion were identified and cancers arising from papillomas and de novo cancers were scored separately (see Figure 9).

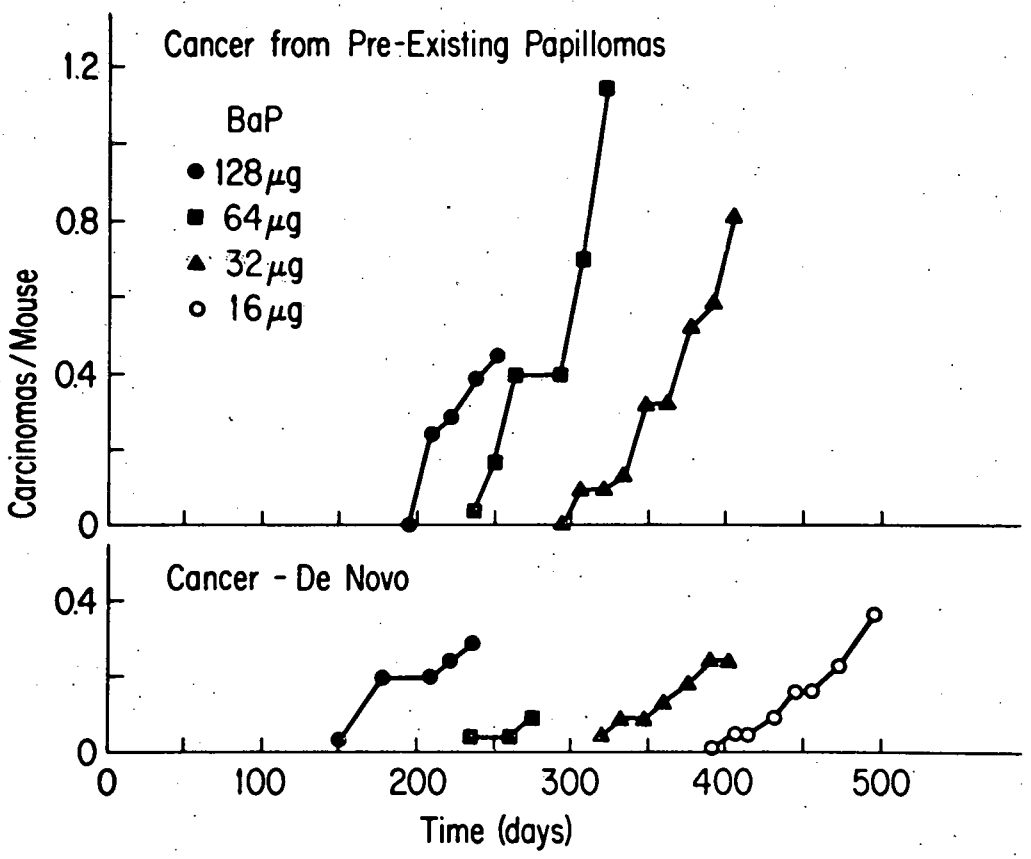


Figure 9. Cumulative incidence of skin carcinomas per mouse following topical BaP treatment. Numbers identifying each curve identify weekly dose of BaP. Upper graph shows carcinomas which arose from pre-existing papillomas. Lower graph shows carcinomas which did not have papilloma precursor lesions (de novo).

These data indicate that the majority of the cancers that arise as a result of repeated BaP treatment start as papillomas. These papillomas could be the result of weak promoting activity associated with BaP and probably represent the most autonomous end of the

papilloma spectrum. It is also clear from Figure 9 that cancers may bypass the gross papilloma state and develop apparently de novo especially at the lower doses. The greater yield of papillomas at the higher BaP doses is probably the result of weak promoting action by BaP.

The data in Figure 10 suggest that early papillomas, i.e., those occurring before 30 weeks require from 20 to 50 weeks to develop into cancer, while later papillomas, i.e., those occurring after 30 weeks require only from 1 to 10 weeks to develop into carcinomas. These results are under study but suggest that late papillomas have progressed at the cell or clone level and therefore require less time to develop into cancer. Cancer here was defined as a lesion 10 mm in diameter with raised circumferential ridges, necrotic centers and invasiveness on histology. Clearly any such lesion that developed from a presumed papilloma precursor within, say, 2 or 3 weeks could have been a de novo carcinoma. In addition, late papillomas may not have developed into carcinomas because of insufficient observation time. These studies are continuing.

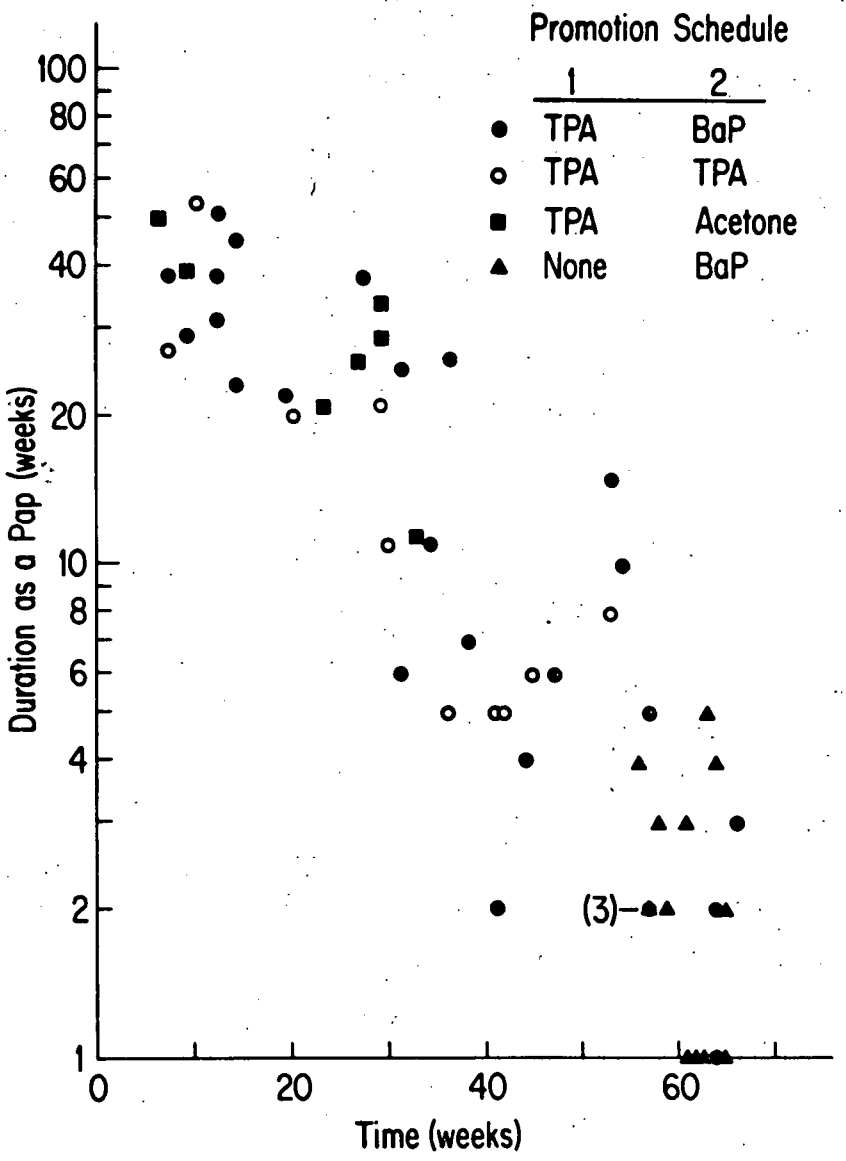


Figure 10. Time of appearance of skin papillomas (abscissa) versus duration of the lesions (ordinate) on mice initiated with 128 µg BaP and promoted with 5.0 µg TPA three times weekly for 30 weeks (promotion schedule 1). At 30 weeks treatments were changed as indicated in promotion schedule 2. BaP was applied weekly at a dose of 32 µg and TPA was applied three times weekly at 5.0 µg per application.

Dose-Response for Single and Multiple Doses of Benzo(a)pyrene on Mouse Skin

These experiments consist of applying benzo(a)pyrene (BaP) topically in acetone at several dose levels in single and multiple dose exposure patterns. One week after the final application of BaP, tetradecanoylphorbolacetate (TPA), a potent promoter, is applied three times weekly in the amount of 5.0 µg per application in acetone. In this model the BaP acts as an initiator and interacts with the epidermal cells in such a way as to cause them to grow into papillomas or carcinomas. Hence by scoring the papillomas and carcinomas one can infer the number of initiated or transformed cells that were produced as a result of interaction of the cells with the initiating chemical.

One of the first questions to be answered was whether the initiated cells were produced in proportion to the dose of benzo(a)pyrene applied to the skin. Hence groups of 25 mice 60 days of age were initiated with single doses of BaP as follows: 4 µg, 8 µg, 16 µg, 32 µg, 64 µg and 128 µg. One week later the promoter treatments (5.0 µg TPA, three times weekly) were started and were continued indefinitely until the tumor induction pattern was clearly established. Sometimes papillomas regressed so that only those that persisted for at least two weeks were scored. When lesions were suspected of being carcinomas, the tissue was prepared for microscopic examination and the diagnosis was confirmed.

on the basis of local invasiveness.

Some typical time patterns for onset of papillomas are shown in Figure 11.

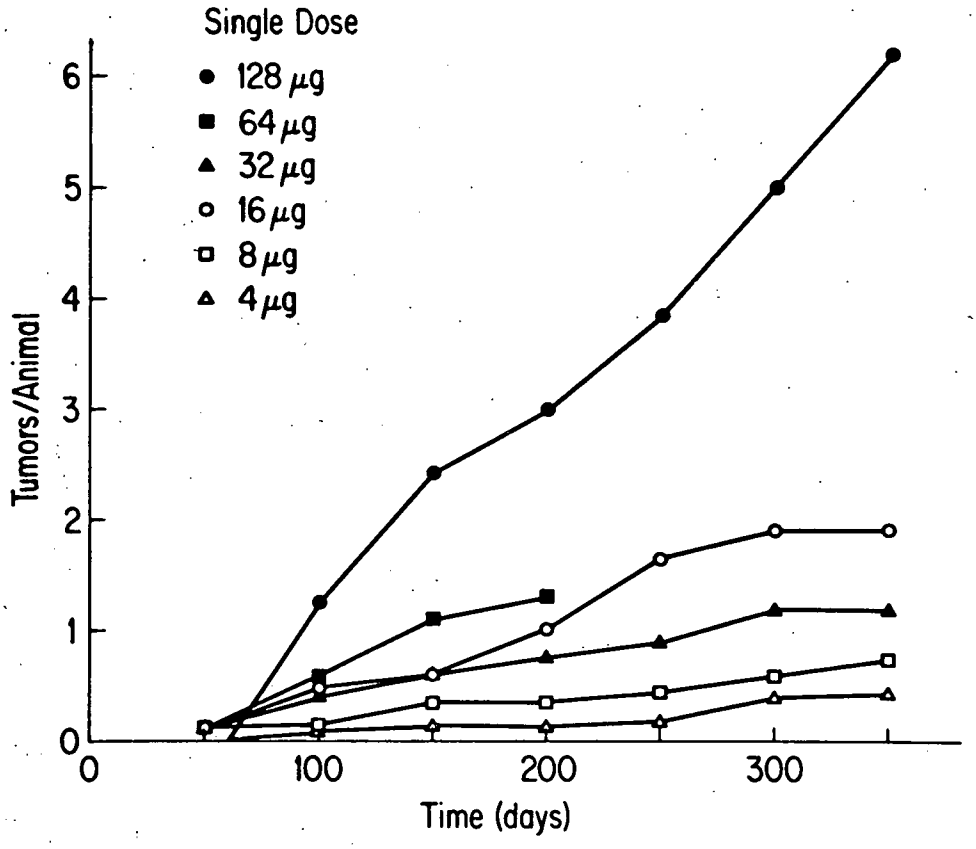


Figure 11. Incidence of skin papillomas per mouse on day 200 after treatment involving initiation with various doses of BaP, followed by promotion with 5.0 μg TPA thrice weekly.

Papillomas generally began to appear at about 50 days after the start of promotion and new papillomas continued to appear at a generally constant rate for periods up to 350d. The tumor yield at 200d was arbitrarily chosen as the point for comparison of different doses. These data are shown in Figure 12 indicating reasonably good linearity between papillomas per mouse and applied dose of initiator.

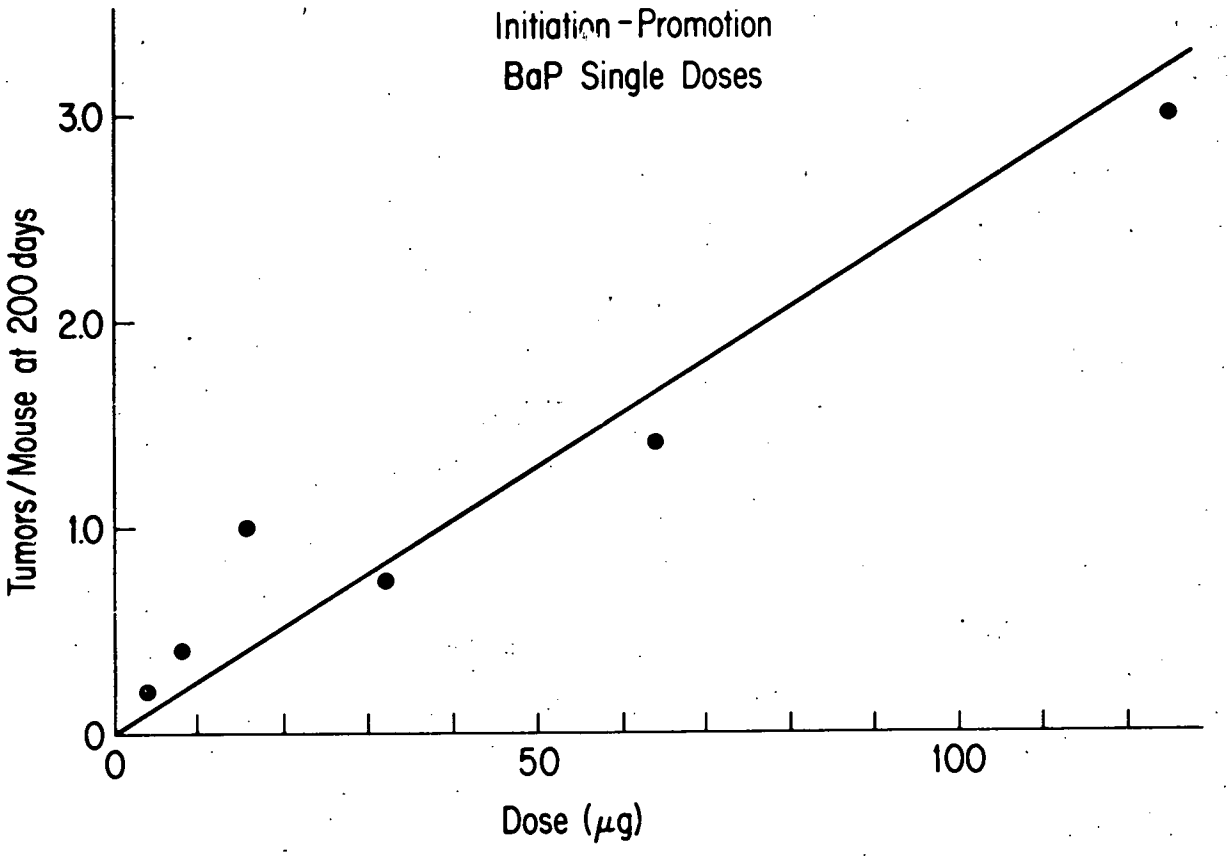


Figure 12. Cumulative incidence of skin papillomas and of skin carcinomas observed after topical carcinogen treatments. A single dose of 128 μg BaP at 56 days of age was followed by treatment three times weekly with 5 μg of TPA.

The susceptibility to initiation was somewhat dependent on the age of the animals. The initiating activity of BaP was somewhat greater in mice at 105 days of age than mice at 56 days of age or at 270 days of age. The effect of age was most apparent at the highest initiating dose of 128 µg and at present we have no explanation of the enhanced sensitivity of the 105 day old mice.

Multiple applications of BaP were applied to mouse skin in order to test whether the initiating activity was cumulative for separate doses spaced at weekly intervals. The time pattern for 32 weekly exposures (4 µg per exposure = 128 µg) is shown in Figure 13.

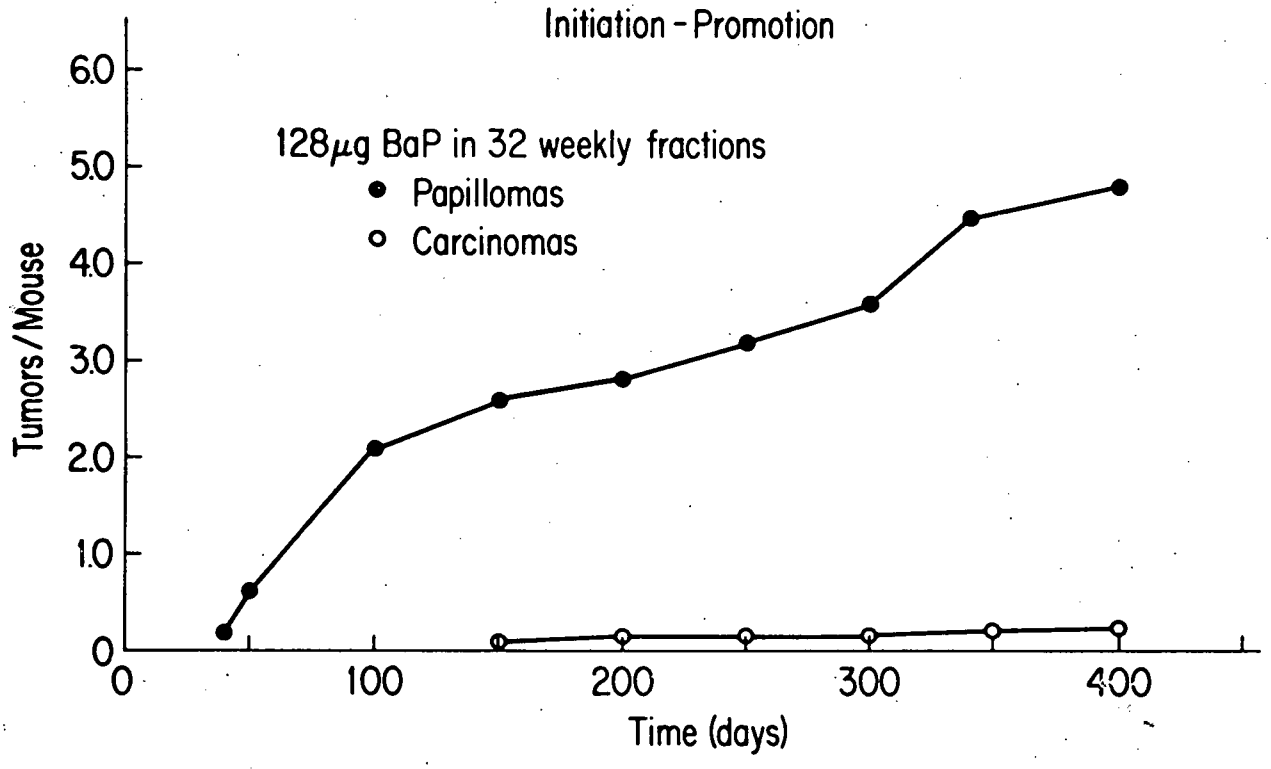


Figure 13. Cumulative incidence of skin papillomas and skin carcinomas observed after topical carcinogen treatments. A total dose of 128 µg of BaP was administered in 32 weekly fractions of 4 µg per week. This treatment was followed by three times weekly treatment with 5.0 µg TPA.

The response is essentially the same as for 128 µg in a single dose and indicates that dose fractionation has little effect on the tumor response.

A number of different fractionation patterns were performed at 64 µg and 32 µg and the temporal response is shown in Figures 14 and 15. The number of BaP applications were 1, 2, 4, 8, 16, 32, 64 and the dose per application was adjusted to give the total doses indicated. If the initiating effect of each application were in proportion to the dose applied, the papilloma yield per application could be calculated from the total papilloma yield divided by the number of applications. These data are shown in Figure 16 which is a log-log plot in order to accommodate the tremendous range of doses and responses.

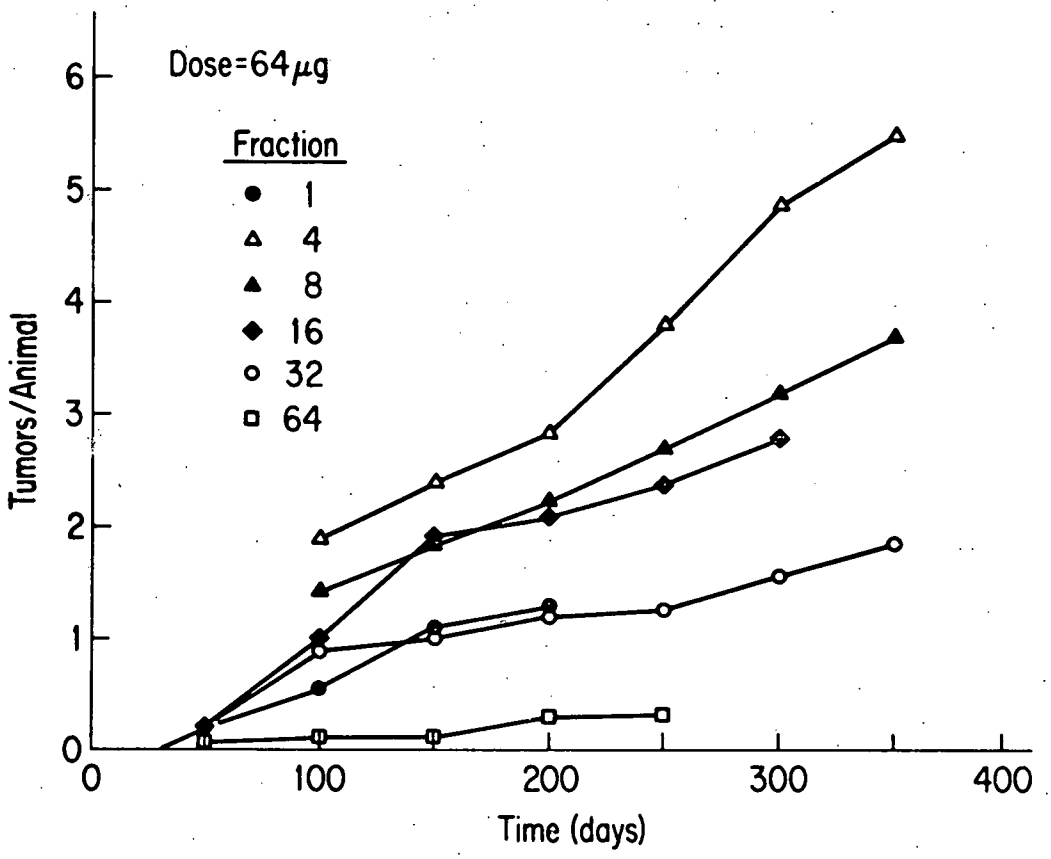


Figure 14. Cumulative incidence of skin papillomas per mouse following either single or multiple fractionated treatments with a total of 64 μg BaP. Promotion with 5 μg TPA three times weekly following initiation. Number of fractions is indicated in the legend.

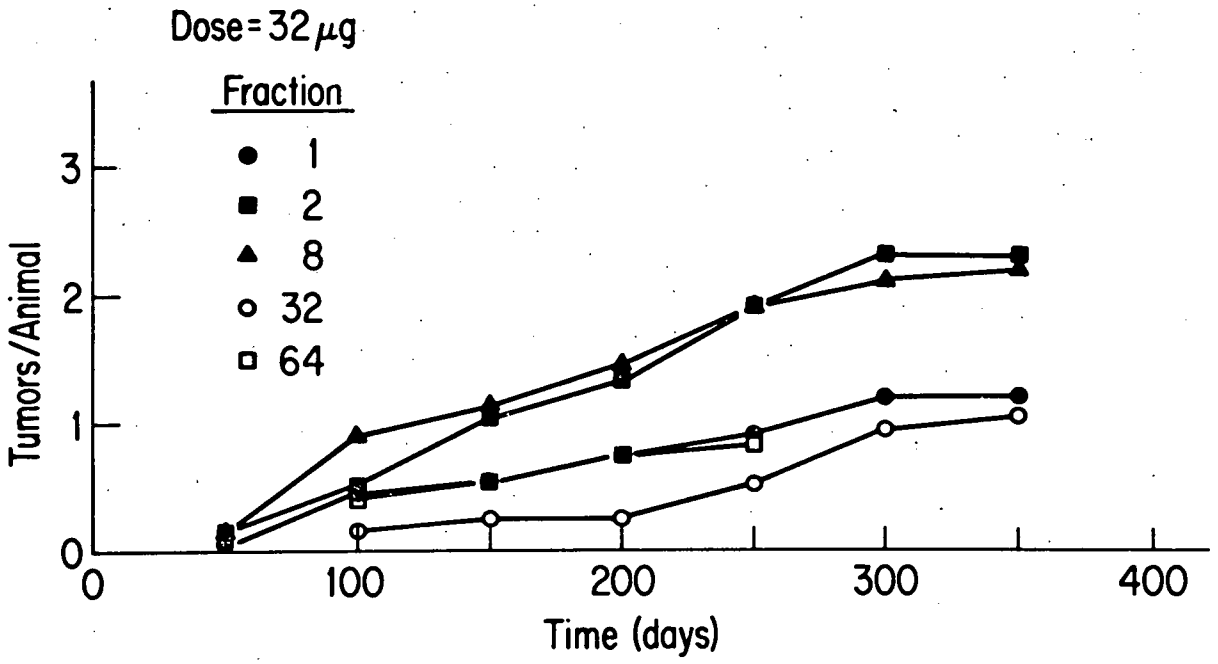


Figure 15. Cumulative incidence of skin papillomas per mouse following either single or multiple fractionated treatments with a total of 32 μg BaP. Promotion with 5 μg TPA three times weekly followed initiation. Number of fractions is indicated in the legend.

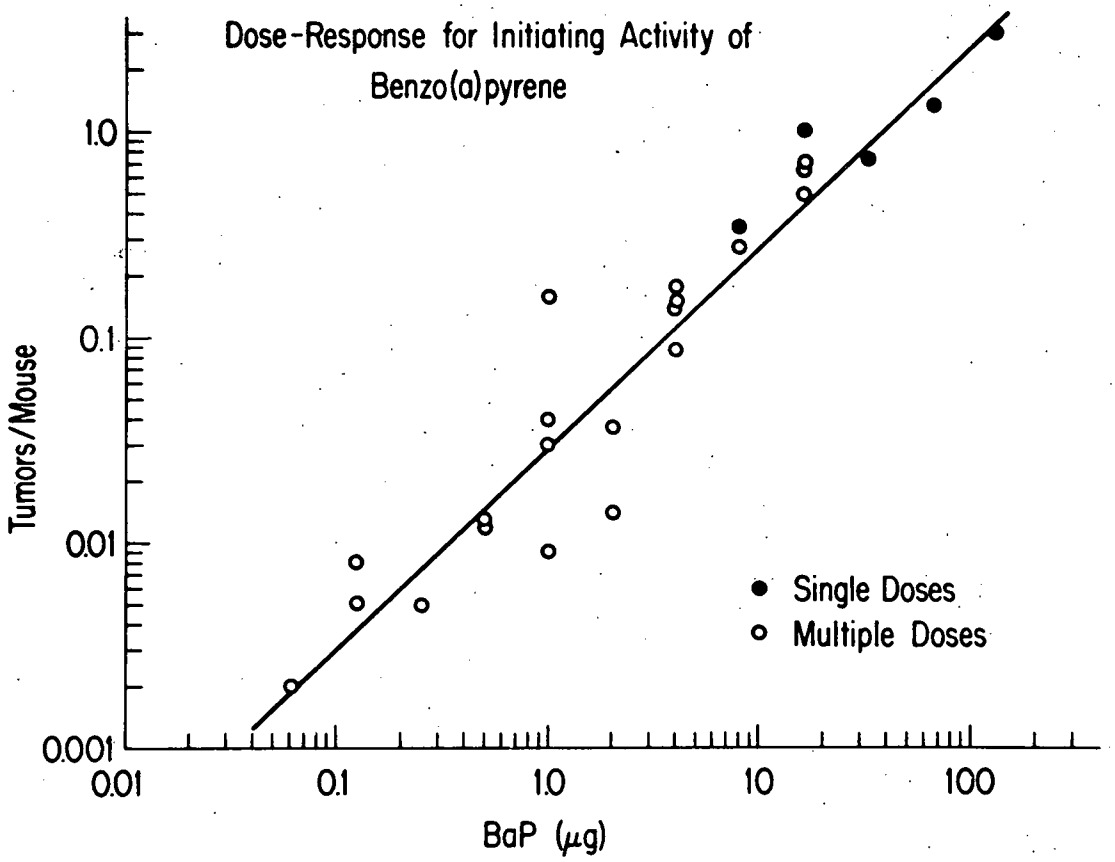


Figure 16. Incidence of skin papillomas per mouse per unit dose applied versus dose per fraction illustrating differences between single versus multiple fractionated doses of BaP. Following BaP treatment, 5 μg TPA was topically applied three times per week.

In spite of variability these data are consistent with linearity (slope 1) which means that each application of BaP contributed initiated cells in proportion to the dose applied. No systematic

departure from linearity is evident anywhere on the graph which undoubtedly represents the most comprehensive and extensive dose response curve now available in any mammalian system. Certainly no threshold is evident even for doses as low as 65 μg i.e. 65×10^{-9} g.

Cancer from Papillomas

Papillomas are the principal lesions that develop in skin treated in the conventional initiation-promotion protocol but the significance of these lesions for carcinoma induction are not clear. It is especially unclear whether the cancers that develop from papillomas are comparable in temporal onset and dose dependence to cancers that develop de novo when BaP is applied repeatedly in the absence of promotion. Information on the fate of each papilloma indicated that on the order of 350 days is necessary for their conversion to carcinomas. For example in Figure 17 the papilloma and carcinoma incidence in tumors per mouse is shown for the group subjected to 128 μg BaP followed by standard promotion of 5 μg TPA thrice weekly. These data indicate that papillomas reach 1.0 per mouse in about 90 days whereas carcinomas arising from these papillomas reach 1.0 per mouse at about 450 days. Evidence presented later indicates that later papillomas require progressively less time for progression to cancer implying the progression may proceed at the cellular or clonal level.

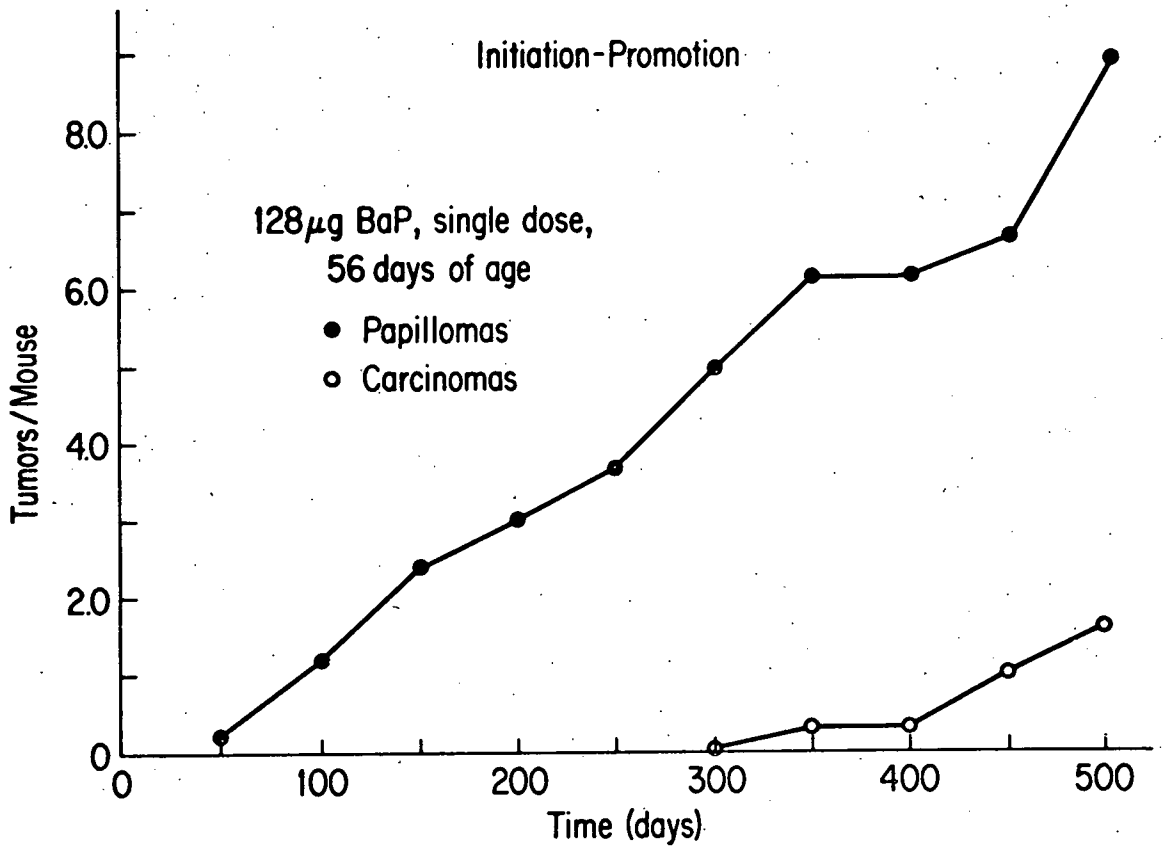


Figure 17. Cumulative incidence of skin papillomas and of skin carcinomas after topical treatments. A single dose of 128 μg BaP was administered at 105 days of age followed by treatment three times weekly with 5 μg TPA.

Similar data in Figure 18 indicate that sensitive mice, i.e., 105 day old mice, not only produce more papillomas earlier but also exhibit an earlier appearance of carcinomas as well as a lesser amount of time for progression of papillomas to carcinomas. Whatever has caused the increased sensitivity of the 105 day old mice applies comparably to carcinoma induction as to papilloma induction. The yield of papillomas was not substantially different than observed for the 60 day old mice that received the same total dose (128 μg) in a single fraction, however, the carcinoma yield may have been accelerated in the sense that carcinomas began to appear less than 200 days after the first appearance of papillomas. By 400 days, however, the yield of carcinomas was not substantially elevated above the value observed in the mice exposed to a single dose.

When the carcinoma yields at 400 days were plotted against dose of initiator (Figure 19), the resulting trend was consistent with linearity in the sense that for every 100 papillomas at 200 days there were 11.9 carcinomas at 400 days. Nearly all these carcinomas arise from papillomas so that it is possible that linearity of papilloma-induction would lead to linearity of carcinoma induction. Other evidence will indicate that cancer induction without promotion (see below) more nearly conforms to a dependence on dose squared rather than linearity.

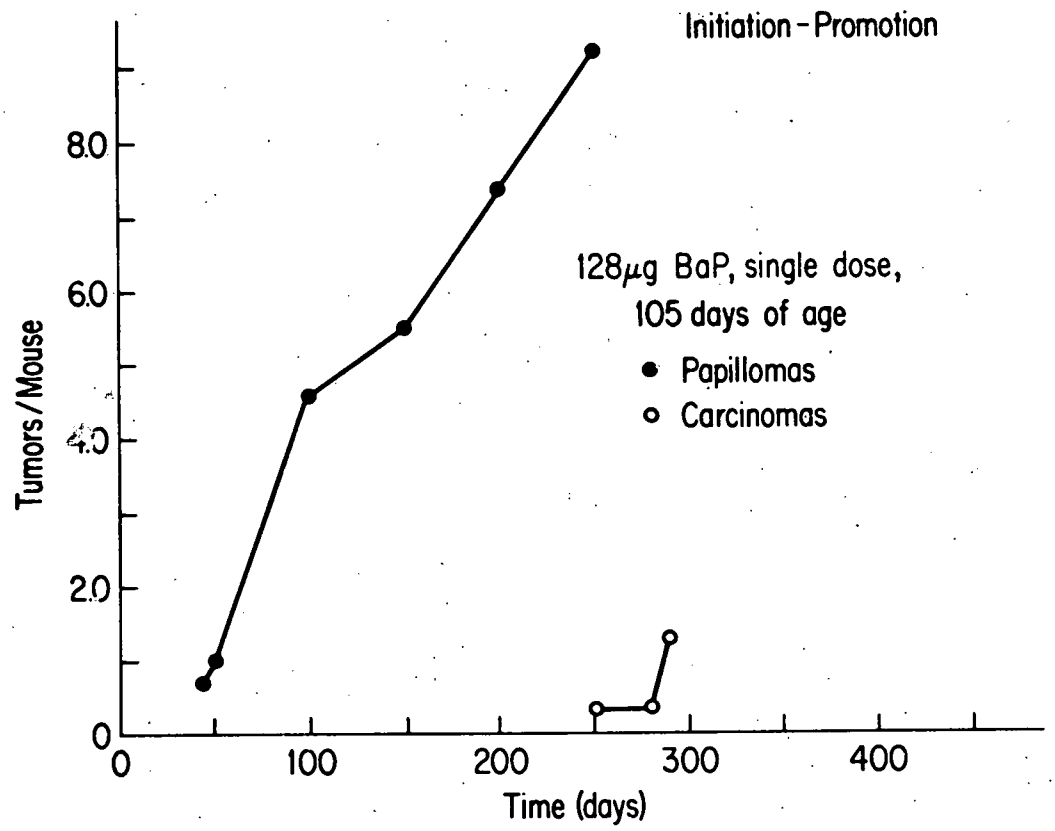


Figure 18. Cumulative incidence of skin papillomas and of skin carcinomas after topical carcinogen treatments. A single dose of 128 μg BaP was administered at 105 days of age followed by treatment three times weekly with 5 μg of TPA.

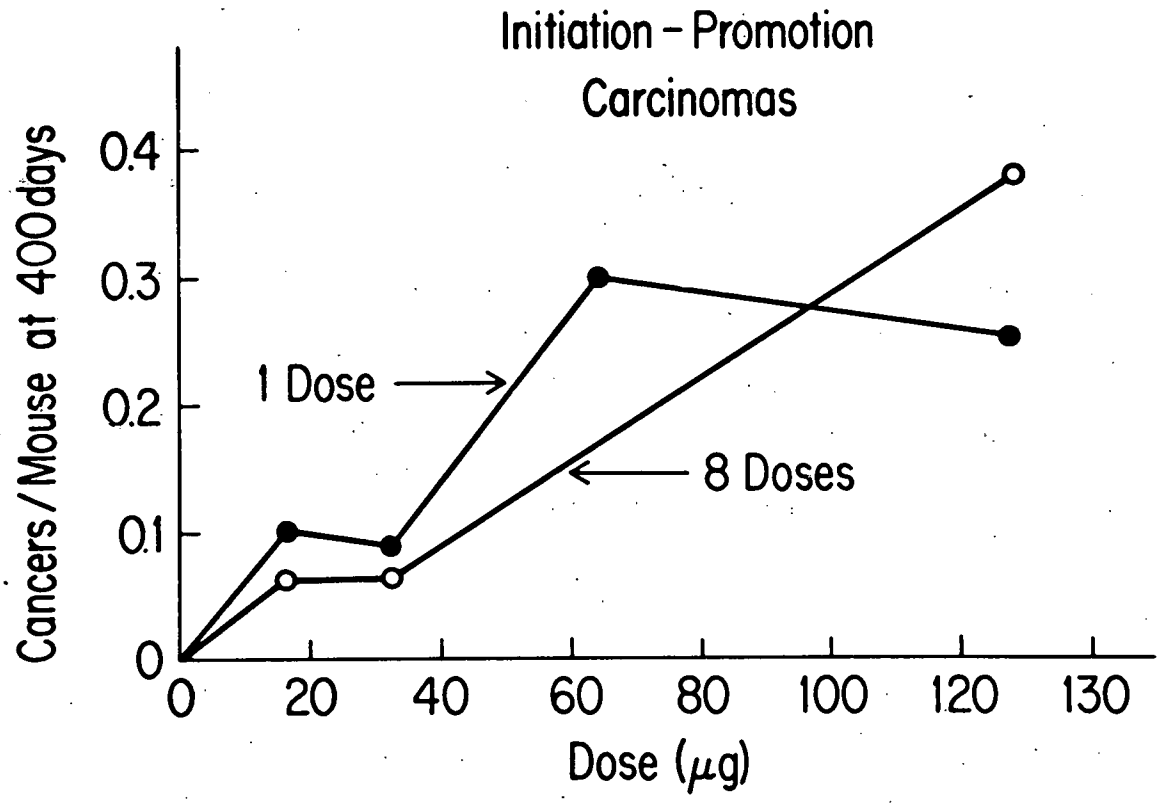


Figure 19. Carcinomas per mouse as of day 400 as a function of total doses of BaP. The BaP was topically administered as either a single dose or in 8 fractionated doses. Promotion with 5 μg TPA three times weekly began one week after the last BaP treatment.

Initiating Activity of an Alkylating Agent

The polycyclic hydrocarbons are an interesting class of carcinogens in that they are of environmental importance and they generally require metabolic conversion to an activated form. The alkylating agents, on the other hand, are believed to be direct acting and they presumably interact with relevant macromolecules directly upon entry into a cell.

Papilloma and carcinoma induction was determined in an initiation-promotion protocol for betapropriolactone, one of the more carcinogenically active alkylating agents. The results are shown in Figure 20.

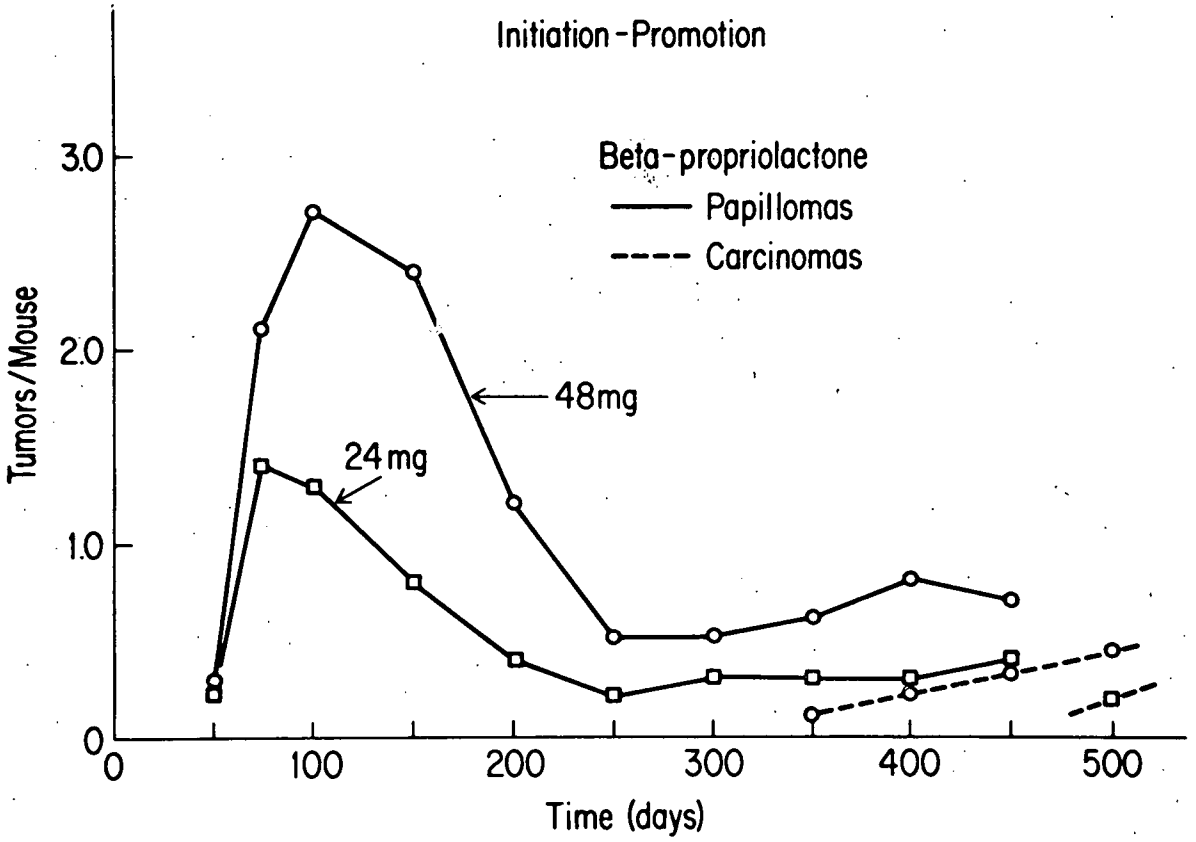


Figure 20. Incidence of skin papillomas and carcinomas per mouse appearing after a single topical application of either 24 or 48 mg of BPL. Thrice weekly promotion with 5 µg TPA began one week after initiation.

The most striking difference between these data and the data for BaP is that most of the papillomas are transient. The maximum papilloma yield occurred at about 100 days thereafter a decline with a halftime of about 40 days brought the yield by 250 days to under 20% of the peak values. In spite of the loss of many papillomas, carcinomas developed with a high probability from the survivors. The yields of unstable and stable papillomas were about proportional to the applied dose of BPL. Like the results for BaP cancers occurred about 350 days after the initial onset of papillomas at 50 days.

Ultraviolet light was tested and found to be an extremely poor initiator. The shaved dorsal skin of HA/ICR mice was exposed to 0.25, 0.5, 1.0 or 12.0 x 10^7 ergs/cm^2 in either single or splite doses followed by promotion three times weekly with 5.0 µg TPA. Only about 0.1 papilloma per mouse was observed at the highest dose and no carcinomas occurred for the duration of the experiment.

Interactions of Initiators

Although benzo(a)pyrene appears to produce linearly additive initiating effects, other carcinogens could interact differently with tissues exposed to polycyclic hydrocarbon carcinogens. A standardized protocol was developed whereby mouse skin was exposed to 16 µg of BaP and a second carcinogen 14 days prior to or 14 days subsequent to the BaP. The second carcinogens included beta-propriolactone, nitroquinolineoxide and ultraviolet light. The

sequence of administration appeared not to matter in that 12 mg BPL was more potent when given prior to BaP while 6 mg BPL was more potent when given subsequent to BaP. The data in Figure 21 include the observed results for 12 mg BPL to 16 µg BaP (average of both sequences) and the expected response based on adding the time curves for 12 mg BPl and 16 µg of BaP given as single doses.

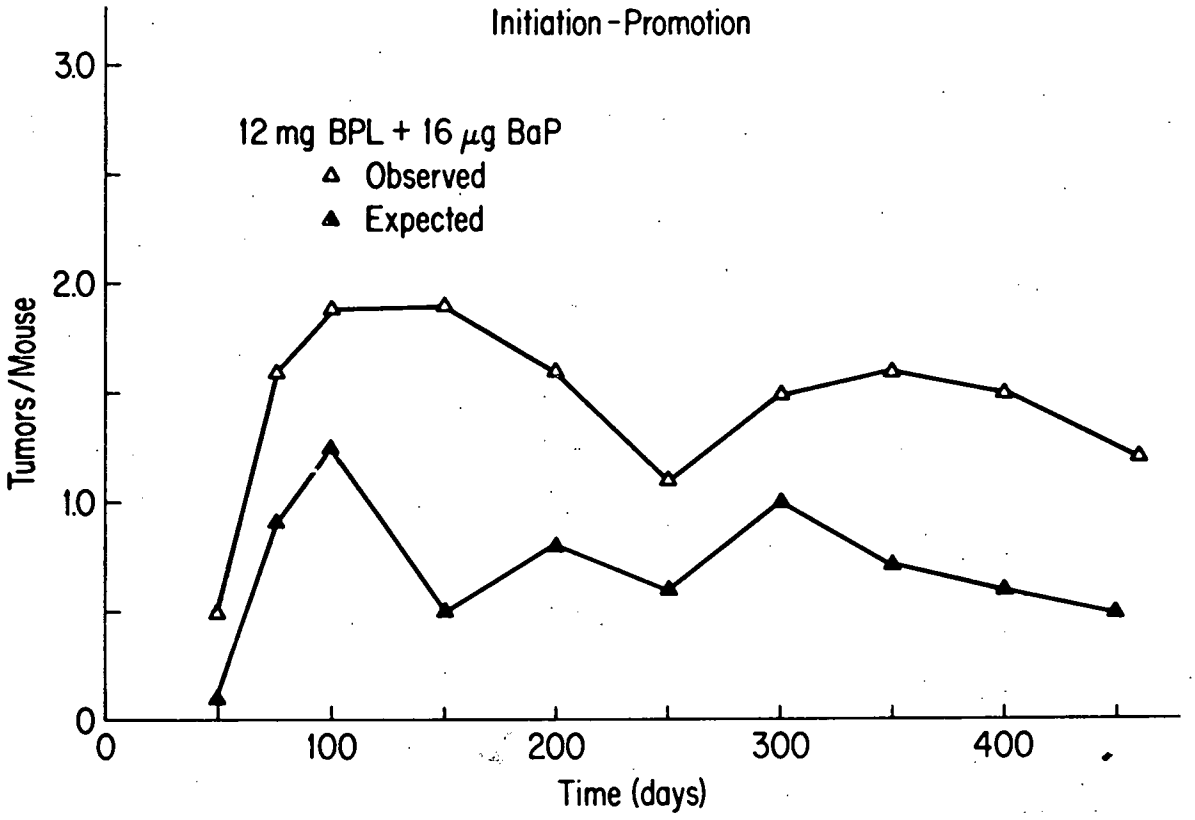


Figure 21. Curve identified as observed shows the incidence of skin papillomas per mouse following initiation with 12 mg BPL plus 16 µg BaP. Animals were promoted with 5 µg TPA three times per week after initiation. Curve identified as expected shows incidence of skin papillomas per mouse that would have been expected if the separate treatments with BPL and BaP were merely additive.

The observed curve is consistently higher than the expected curve by about a factor of two suggesting that combined exposures of these two carcinogens may not be precisely additive. A similar trend is seen in Figure 22 for 6 mg BPL.

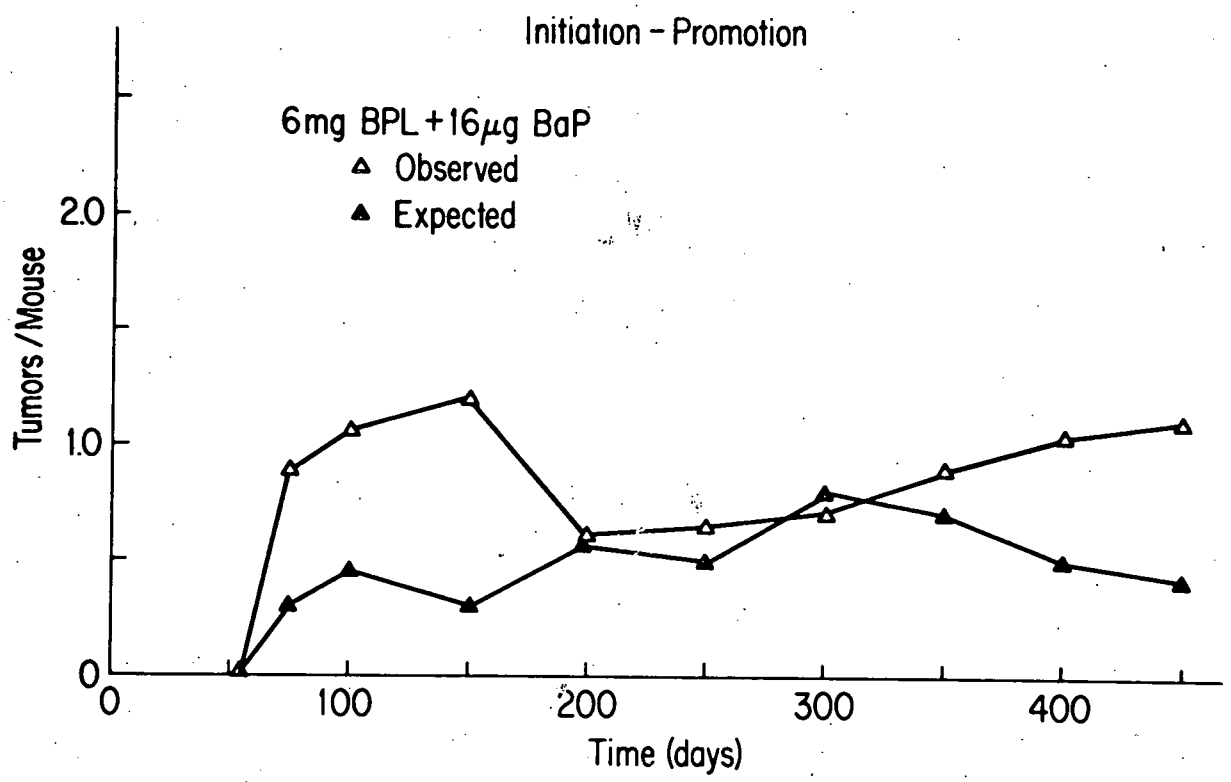


Figure 22. Curve identified as observed shows the incidence of skin papillomas per mouse following initiation with 6 mg BPL plus 16 μg BaP. Animals were promoted with 5 μg TPA three times per week after initiation. Curve identified as expected shows incidence of skin papillomas per mouse that would have been expected if the separate treatments with BPL and BaP were merely additive.

The combined action of NQO and BaP, shown in Figure 23, gave results that were surprising in two respects: First the order of administration markedly favored NQO prior to BaP for maximum initiating action and second in reverse order (BaP first) initiating activity was about equal to that for BaP alone, i.e., the NQO initiating activity was hardly detectible.

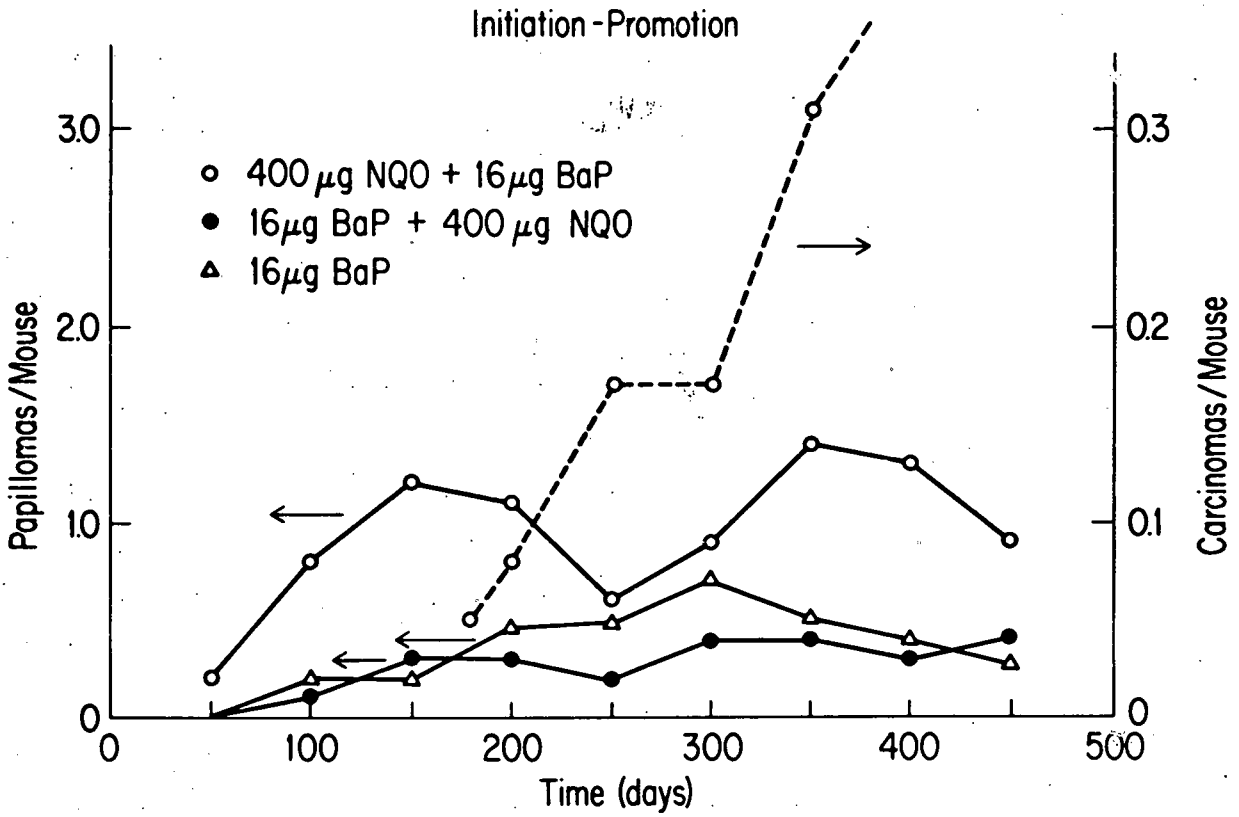


Figure 23. Incidence of skin papillomas (on left) and carcinomas (on right) per mouse observed following skin painting. Topical carcinogen treatments involved either a single dose of BaP or a single dose of either NQO or BaP followed two weeks later by another single dose of either NQO or BaP (whichever had not been applied the first time). Thrice weekly topical treatments with 5 μg TPA began one week after initiation.

A comparable dependence on sequence of administration is seen in the carcinoma data in Figure 23. Carcinomas were clearly apparent by 300 days when NQO was given first whereas in reverse sequence no carcinomas had occurred by 450 days.

Dose Fractionation

Fractionated doses of ionizing radiation generally produce less biological effect than equivalent single doses because of recovery processes occurring in the cells. Recently we have shown that such recovery processes operate to reduce the oncogenic effectiveness of radiation, and it was of interest to determine whether similar effects occur for carcinogenic chemicals. Accordingly, mouse skin was exposed to single and fractionated doses of betapropriolactone (BPL), nitroquinolineoxide (NQO) and benzo(a)pyrene (BaP). The latter experiments are reported elsewhere in this report and indicated no recovery even for multiple fractions. BPL was applied as a single dose in acetone or as two doses separated by one week. One week after the final dose, TPA promotion was begun and was continued indefinitely. The papilloma yield as a function of time is shown in Figure 20. The most striking feature of these data is the peak at 100 days. The dose-response for the cumulative number of papillomas at 200 days is shown in Figure 24 along with the carcinoma yield at 500 days. The data indicate two clear trends: First papilloma

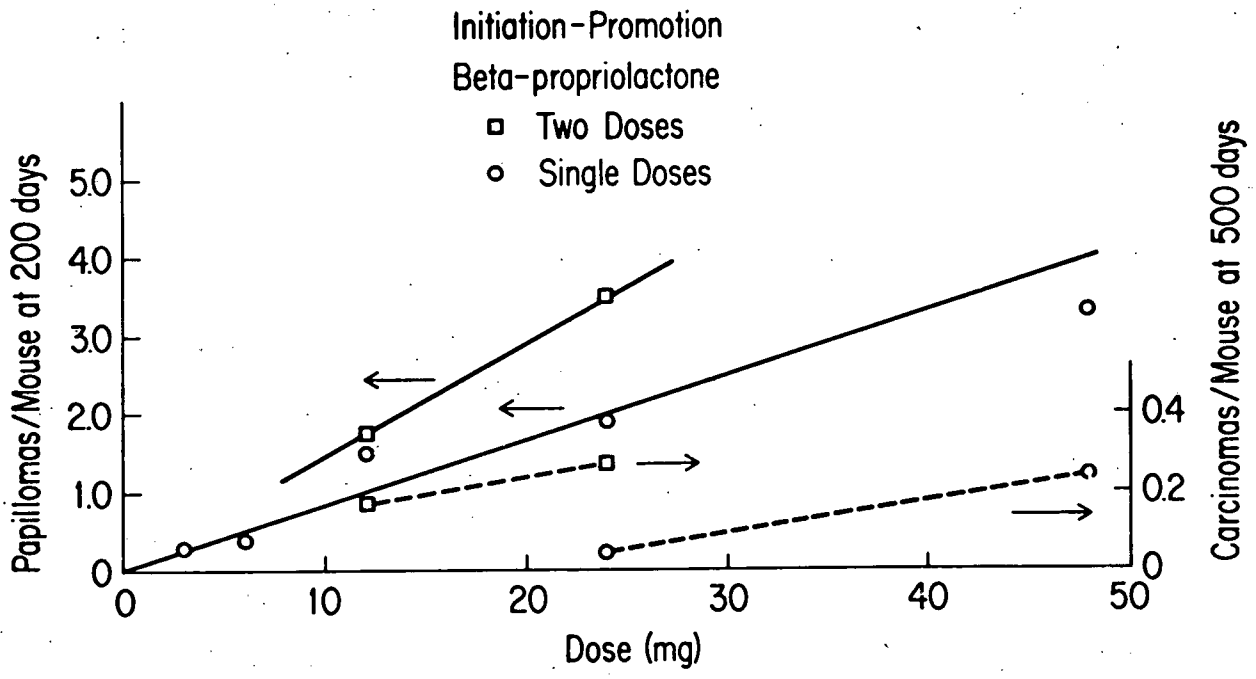


Figure 24. Incidence of skin papillomas appearing by day 200 and of skin carcinomas by day 500. BPL was topically applied as either a single dose or fractionated into two doses with a two week interval between treatments. Beginning one week after initiation, mice were promoted three times weekly with 5 µg TPA.

yield is roughly proportional to applied dose and second, fractionated exposure markedly enhanced the yield of papillomas and carcinomas.

When exposures to NQO were split into two equal doses a slight reduction in papilloma yield was noted relative to single

doses. However, the dose-response curve was not extensive for NQO and its shape was not established. The doses utilized may have been on a plateau since an increase in dose by a factor of two had relatively little effect on the yield of papillomas.

DMBA in Rat Skin

Most of the work on polycyclic hydrocarbon carcinogenesis has been done on mouse skin in the belief that other species, especially rat, were relatively refractile. Rat skin has proved to be uniquely useful for studying radiation carcinogenesis and experiments were begun to test its susceptibility to carcinogenesis by 7,12dimethylbenz(a,h)anthracene (DMBA) -- one of the more potent polycyclic hydrocarbon carcinogens in mouse skin. The DMBA was applied to rat skin in 1.0 ml acetone so that the hydrocarbon would spread over a substantial portion of the dorsal skin. Treatments were applied weekly at doses of 20 µg, 100 µg and 500 µg. The log-log time response is shown in Figure 25. These data are remarkably consistent in the sense that the steepness of the time function, which is about the 7th power of time, is maintained throughout the dose range. In addition, the time displacements associated with the change in weekly dose is consistent with the formulation:

$$dt^n = \text{constant}$$

where n was estimated experimentally to be 3.0, d is the weekly dose and $t_{1.0}$ is the time for the response to reach 1.0 tumors/rat.

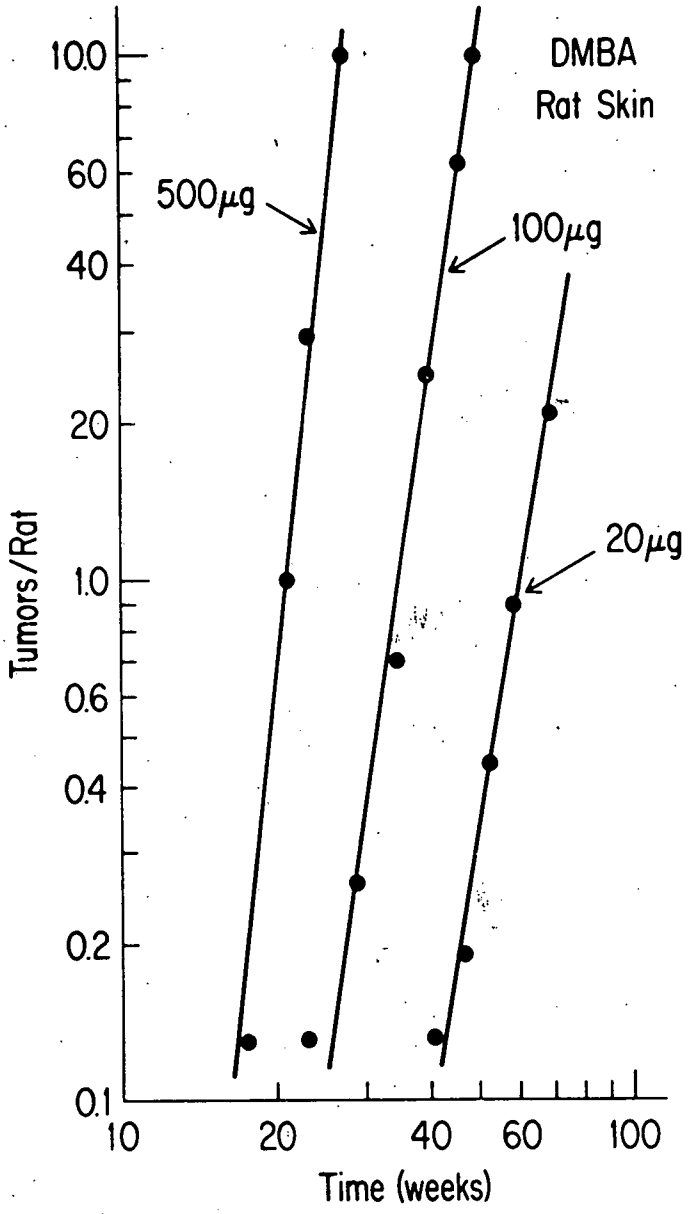


Figure 25. Incidence of cancer per rat after weekly topical application of either 20, 100 or 500 μg DMBA in 1.0 ml acetone.

The general features of this response in rat skin are remarkably similar to that observed in mouse skin treated with benzo(a)pyrene. Perhaps a similar dose-response mechanism may be operating in the

two species for polycyclic hydrocarbon carcinogenesis.

One of the important issues for carcinogenesis in rat skin was whether prior irradiation of skin would render the tissue more or less susceptible to polycyclic hydrocarbon carcinogenesis. Accordingly rats were exposed to radiation at several dose and then received weekly topical applications of DMBA. The tumors produced by the two agents were indistinguishable histologically but the radiation induced tumors were clearly additive with the DMBA tumors as indicated in Figure 26 for 1500 rads and in Figure 27 for 2250 rads.

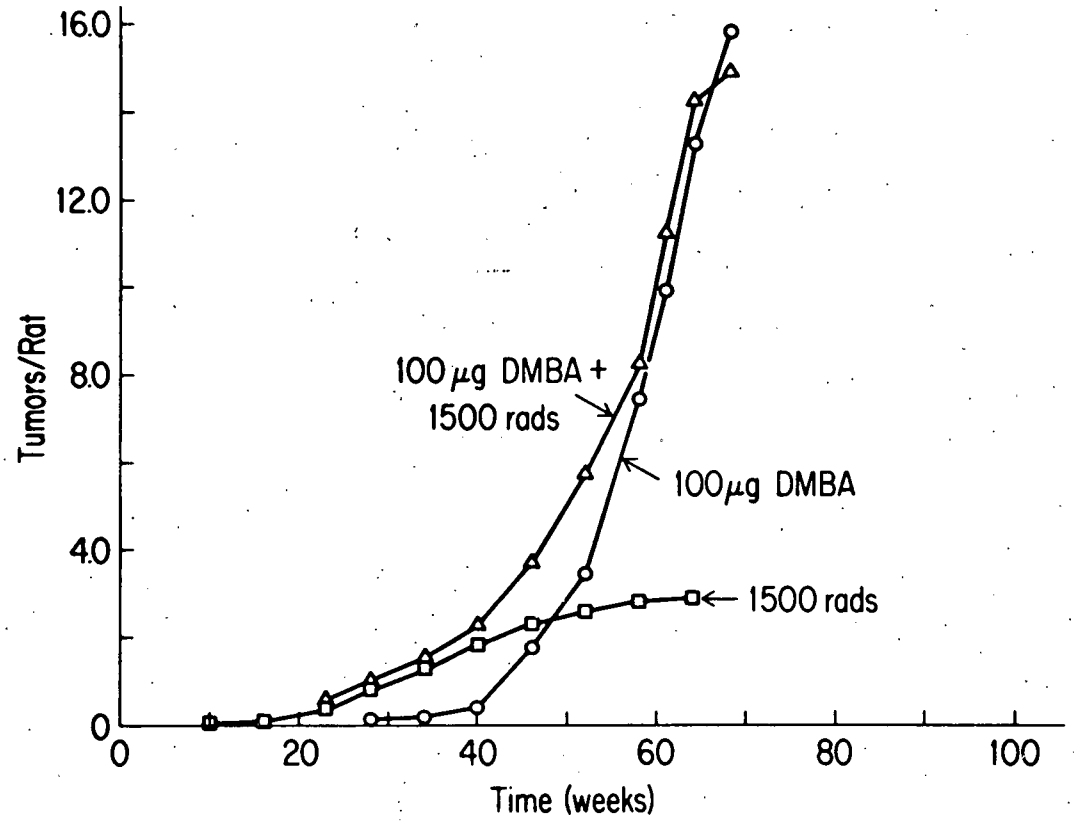


Figure 26. The 1500 rads curve represents skin tumors appearing after a single irradiation with electrons at a dose of 1500 rads. The 100 μg DMBA curve represents skin tumors appearing after weekly topical applications of 100 μg DMBA. The 100 μg DMBA and 1500 rads curve represents skin tumors appearing after a single electron irradiation at a dose of 1500 rads plus weekly topical applications of 100 μg DMBA.

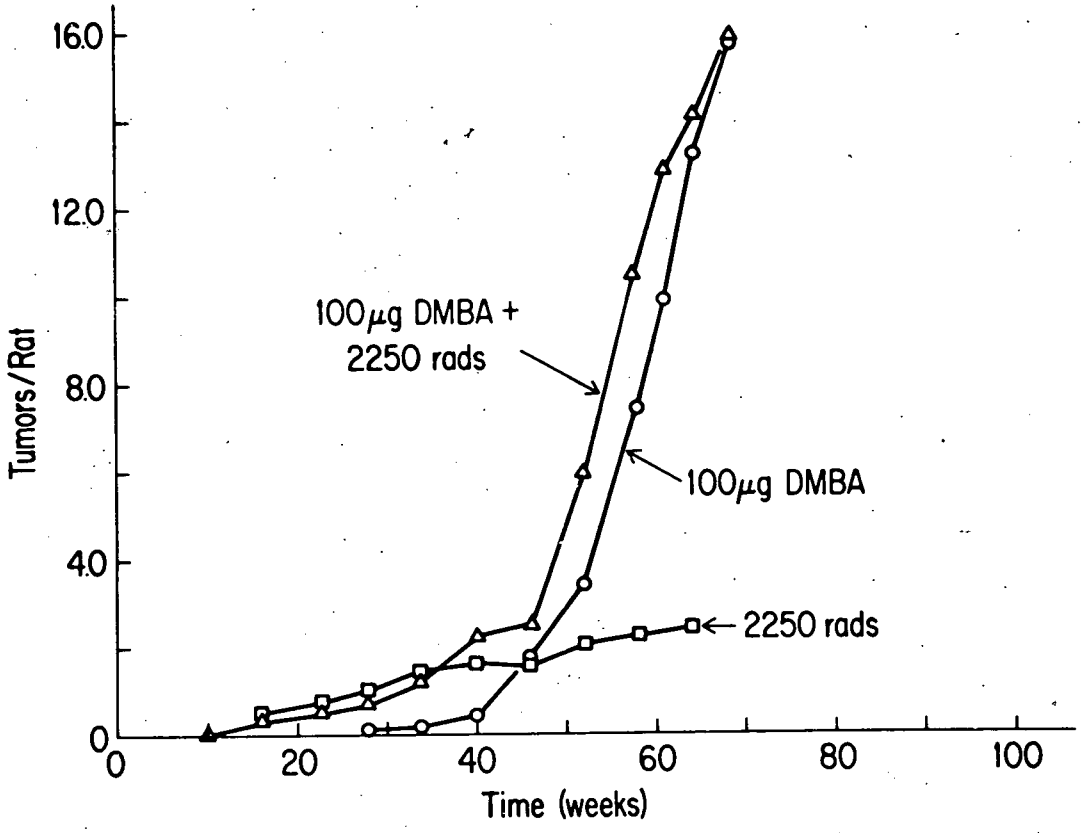


Figure 27. The 2250 rads curve represents skin tumors appearing after a single irradiation with electrons at a dose of 2250 rads. The 100 μg DMBA curve represents skin tumors appearing after weekly topical applications of 100 μg DMBA. The 100 μg DMBA and 2250 rads curve represents skin tumors appearing after a single electron irradiation at a dose of 2250 rads plus weekly topical applications of 100 μg DMBA.

The temporal pattern for radiation-induced tumors was essentially linear with elapsed time starting at about 10 weeks while the

curve for DMBA-induced tumors rose abruptly after about 40 weeks. In the combined treatment groups, the response curves for the individual treatments are discernable and indicate that under the experimental conditions employed the carcinogenic action of radiation and DMBA are independently additive.

Dose-Response for BaP Adducts in Mouse Epidermal DNA

The initiation of carcinogenesis by chemical carcinogens is believed to involve their covalent binding to cellular macromolecules. There appears to be a correlation between their carcinogenic potency and DNA binding. Therefore, the binding of chemical carcinogens to DNA might serve as a molecular marker for their biologic activity.

When BaP is applied to mouse skin (Brooks and Lawley, 1964), added to cell cultures (Duncan, et al., 1969) or incubated in the presence of microsomes and DNA (Gelboin, 1969) electrophilic metabolites are formed that covalently bind DNA. The resulting BaP-DNA adducts to deoxynucleoside bases have been isolated and characterized (King, et al., 1976). Hydrophilic BaP-DNA adducts are also present in the elution profiles obtained from the chromatography system used in the isolation of BaP-deoxynucleoside adducts that remain to be characterized (Osborne, et al., 1976). The binding of BaP radiolabeled with tritium to DNA proceeds with a significant incorporation of radioactivity that is not associated with BaP. These specific BaP-DNA adducts serve as a better molecular marker for biological potency than the total amount of radioactivity in BaP-conjugated DNA. The formation in mouse epidermis of BaP-DNA adducts was employed in the determination of the persistence and dose relationship of BaP induced alteration in the DNA.

Female HA/ICR mice were obtained from Sprague-Dawley (Madison, Wis.). Mice (7 to 8 weeks old and in the resting phase of the hair cycle) had their skin shaven 2 days prior to topical application between 7 and 9 a.m. of (^{3}H)BaP dissolved in 0.2 ml acetone. The epidermis was scraped from the frozen skin and homogenized. The DNA was extracted by the Kirby phenol procedure as described by Diamond, et al. (1967).

DNA was enzymatically degraded to deoxynucleosides with DNase I, venom phosphodiesterase and alkaline phosphatase and by mild acid treatment as described by Baird and Brookes (1973). The degradated DNA was chromatographed on a Sephadex LH-20 column (40 x 2.0 cm) equilibrated with 30% MeOH as described by Baird and Brookes with the modification that a 800 ml linear gradient was employed. The MeOH/Borate column chromatography was performed as described by King, et al. (1976) employing a 800 ml linear gradient. Radiolabeled samples were counted in a Isocap/300, Searle Corporation (Arlington Heights, Ill.) scintillation counter in 15 ml ACS (Amersham Corporation, Arlington Heights, Ill).

The DNA was enzymatically degraded to deoxynucleosides and chromatographed on a Sephadex LH-20 column. The profile of the products present in the eluate (Figure 28) contained three peaks.

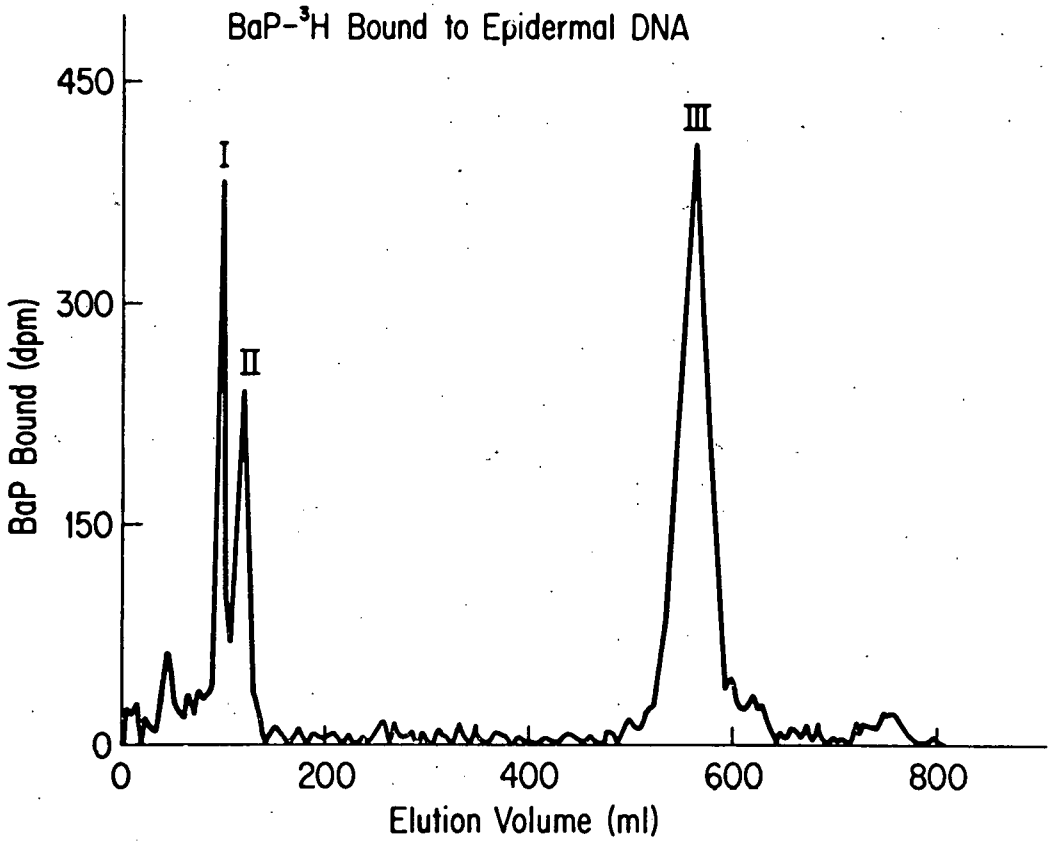


Figure 28. Sephadex LH-20 column chromatogram elution profile of BaP-^{3}H conjugated epidermal DNA after enzyme degradation to deoxynucleosides. Mice (23) were treated with 25 µg BaP-^{3}H (100 µCi) for 7 hrs. prior to sacrifice. The digest of the epidermal DNA was chromatographed on a 40 x 2.0 cm column of Sephadex LH-20 eluted with a 30% to 100% water:methanol linear gradient (total vol. 800 ml). Fractions (4.25 ml) were collected and aliquots assayed for radioactivity in 15 ml ACS by liquid scintillation.

When peak III was rechromatographed as a Sephadex LH-20 column eluted with a methanol/borate gradient as described by King, et al. for the separation of the 2 diasterioisomeric adducts, only a single peak was obtained. The single peak indicated the presence of only one of the stereoisomers.

The possibility that peak 1 is the result of incomplete hydrolysis of BaP-conjugated DNA containing N^2-(10-[7β,8α or 9β-trihydroxy-7,8,9,10tetrahydrobenzo(a)pyrene]yl)deoxyguanine was investigated. Peak 1 isolated from BaP-^{14}C conjugated calf thymus DNA was lyophilize and dissolved in water. An aliquot was subjected to the standard enzymatic hydrolysis including DNase I, venom phosphodiesterase and alkaline phosphatase treatment and used for DNA. Upon rechromatography with Sephadex LH-20 the elution profile contained only peak 1 (Figure 29) indicating the complete resistance of peak 1 to enzymatic degradation. Another aliquot of peak 1 was made 0.1 NHCl and incubated at 37^{o} for 18 hours prior to rechromatography on Sephadex LH-20. All the BaP-^{14}C was present in the hydrophilic region of the column eluate profile.

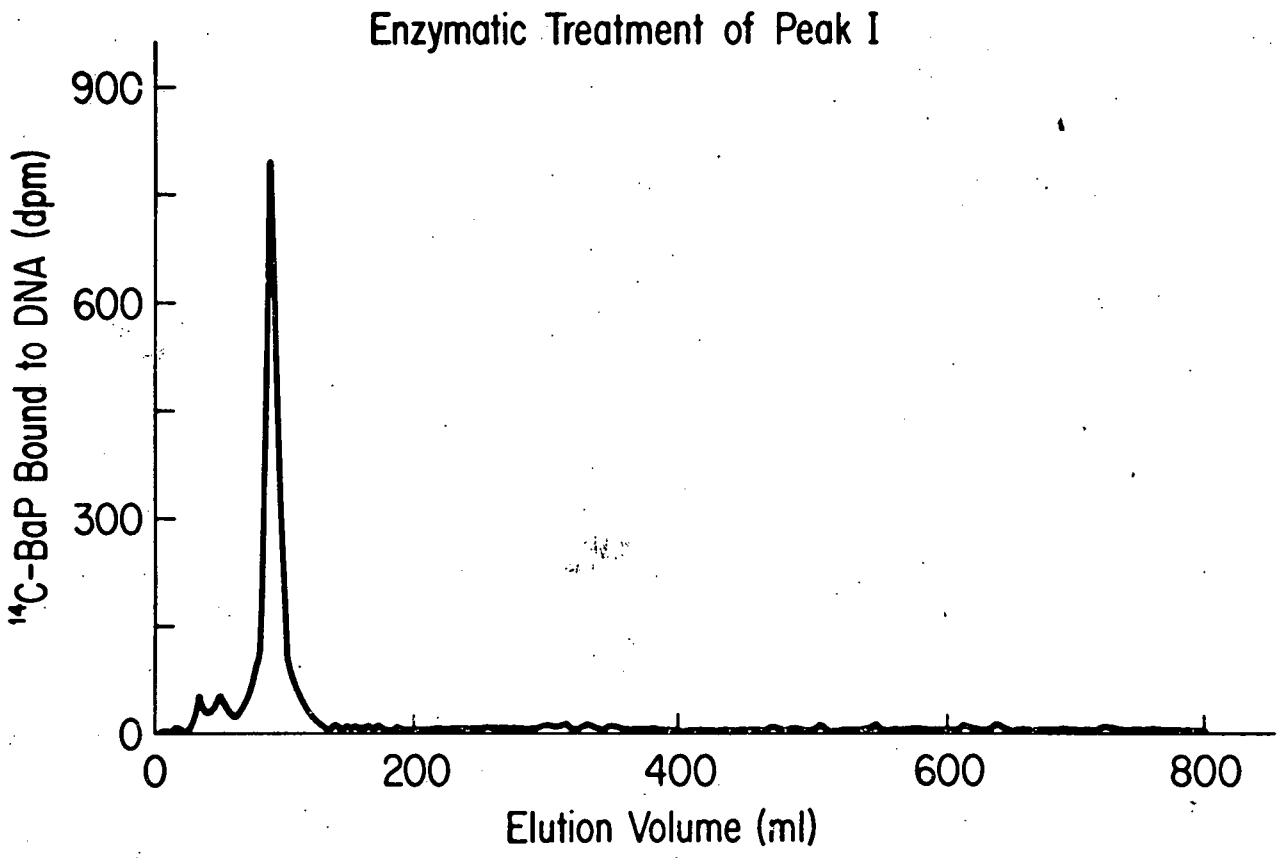


Figure 29. Sephadex LH-20 column chromatograms elution profile of peak 1 isolated by Sephadex LH-20 chromatography of enzymatically digested DNA conjugated with BaP-^{14}C by liver microsomes from 3-methylcholanthrene treated mice. The microsomal incubation contained 25 µCi BaP-^{14}C and 100 µg BaP in a total volume of 5 ml as described in "Materials and Methods". Peak 1 was isolated from an enzyme digest of the DNA as described in Chart 1. Peak 1 was lyophilized and dissolved in water. An aliquot was treated with DNase I, venom phosphodiesterase and alkaline phosphatase.

Peak 1b was eluted in the same elution volume as the original peak 1 and peak 1c, the major peak after acid treatment was also present in the very hydrophilic region. No hydrophobic peaks including peak 3 were present after either a second DNase or acid treatment of isolated peak 1 so that peak 1 is not an artifact resulting from incomplete degradation of the conjugated DNA.

Rate of Formation and Removal of BaP Adducts in Epidermal DNA

BaP conjugated epidermal DNA was isolated at various times after the application of 25 µg BaP-^{3}H (100 µCi), degraded by enzymes to deoxynucleosides, and chromatographed on Sephadex LH-20 in order to quantitate peaks 1 and 3 of the elution profile (Figure 30).

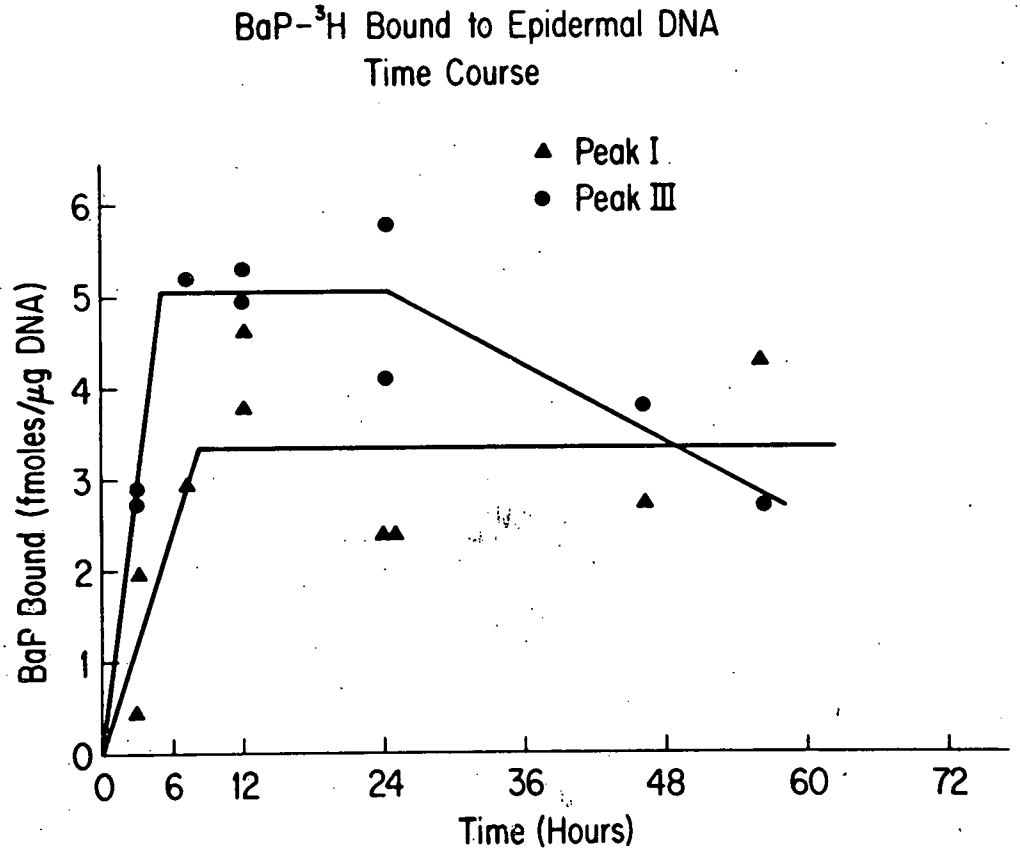


Figure 30. Time course of the formation of BaP adducts in epidermal DNA. Groups of mice, 15-23 each, were treated topically with 25 μg BaP-(^{3}H) (50-100 μCi) and sacrificed at various times later. The enzymatically digested epidermal DNA was chromatographed on a Sephadex LH-20 column as described in Chart 1a. Peak 1 (▲) and peak 3 (●) were determined from the elution profile.

The formation of peaks 1 and 3 reached a maximum in 7 hours and remained constant for 17 hours. Afterwards, the magnitude of

peak 3 decreased with a half-life of 85 hours while the amount of peak 1 remained at a constant level. The extent of peak 3 formation was greater than peak 1 for the first 24 hours after BaP application. After 48 hours post-treatment with BaP, peak 1 became the predominate BaP adduct in epidermal DNA.

Dose Dependency of the Formation of BaP Adducts in Epidermal DNA

The formation of BaP adducts identified as peaks 1 and 3 was investigated as a function of dose of BaP-^{3}H applied/mouse. Dose levels of 4-400 nmoles BaP-^{3}H/mouse was applied and the conjugated epidermal DNA isolated 24 hours after treatment, degraded enzymatically and chromatographed on a Sephadex LH-20 column. As shown in Figure 31, the degree of peak 1 and 3 formation was found to be a linear function of dose between 4-100 nmoles/mouse. The degree of both peak 1 and 3 formation at a higher dose of 400 nmoles/mouse appeared to be lower than expected by extrapolation of the linear function.

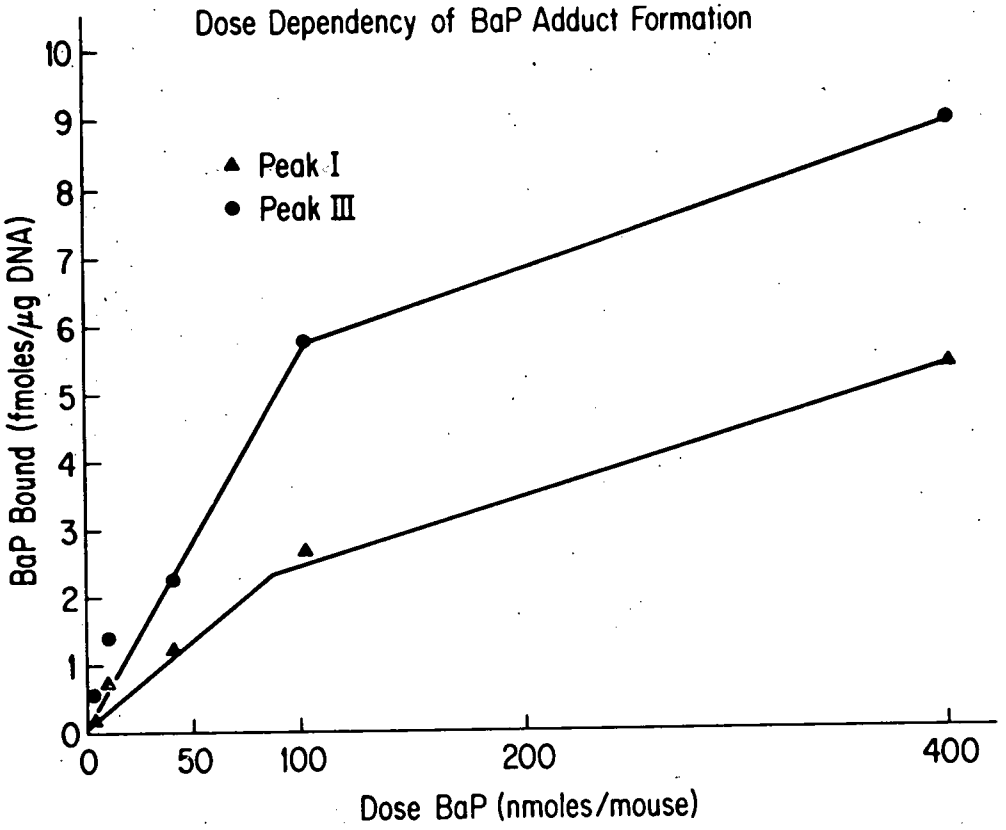


Figure 31. Dose dependency of BaP adducts formation in epidermal DNA. Groups of mice, 15-20 each, were treated topically with 1, 2.5, 10, 25 and 100 µg BaP(^{3}H) (50-100 µCi) and sacrificed 24 hours later. Enzymatically digested epidermal DNA was chromatographed on a Sephadex LH-20 column as described in Chart 1a. Peak 1 (▲) and peak 3 (●) were determined from the elution profile.

One of the products formed which BaP binds to DNA in vivo has been identified as N^2-(10-[7β,8α,9α or 9β-trihydroxy-7,8,9,10tetrohydrobenzo(a)pyrene] yl)deoxyguanosine (dGua-BaP)

(King, et al., 1976; Straab, et al., 1977). When BaP conjugated DNA is enzymatically degraded to deoxynucleosides and chromatographed on Sephadex LH-20 column, dGua-BaP is eluted in the highly hydrophobic region of the Methanol/water gradient (Baird, et al., 1975). We have confirmed that a single hydrophobic adduct of BaP corresponding to dGua-BaP is formed in vivo in epidermal DNA. DGua is present in peak 3 of the elution profile from the Sephadex LH-20 column.

However, we have also identified the formation in epidermal DNA of a very hydrophilic adduct of BaP in peak 1 of the elution profile from Sephadex LH-20. It has previously been reported that the hydrophilic region of the Sephadex LH-20 elution profile contains (1) a carbon-14 derivative of radiolabeled BaP formed by BaP-^{14}C binding to DNA in vitro (Osborne, et al., 1975) and (2) a fluorescent material formed by the binding of BaP-diol-epoxide to DNA (Osborne, et al., 1976). The carbon-14 radiolabeled adduct of BaP present in peak 1 from the Sephadex LH-20 column elution profile is resistant to further enzymatic degradation by DNase I, venom phosphodiesterase and alkaline phosphatase indicating that peak 1 is not the result of incomplete degradation of deoxynucleotides and containing dGua-BaP. Mild acid treatment of conjugated DNA results in apurination and release of dGua-BaP (Baird and Brookes, 1973). The treatment of isolated peak 1 with mild acid only slightly altered the elution of peak 1 from the

Sephadex LH-20 column so that the adduct present in peak 1 maintained its hydrophilic property. We propose that peak 1 contains a metabolite of BaP bound to phosphate of deoxynucleotides, and that the modification of peak 1 resulting from mild acid treatment produced apurinated adducts.

The alkylation of phosphate is DNA and RNA to form phosphotriestus has been demonstrated for numerous carcinogenic alkylating agents (Bannon and Verley, 1972). Shooter and Merrifield has proposed an assay for alkyl phosphotriestus in DNA that detects alkali induced chain breaks (Shooter and Merrifield, 1976). The two diastereomeric diol epoxides of BaP (± 7β,8α-dihydroxy-9α,10α or 9β,10β-epoxy-7,8,9,10tetrahydrobenzo(a)-pyrene) interact with superhelical Col E1 DNA to produce a nick in that DNA that has been proposed to result from strand scissior at an unstable phosphotriester (Gamper, et al., 1977). The two diastereomeric diol epoxides of BaP also bind polyguanylic acid at both the 2 amino group and phosphate (Koreeda, et al., 1976). We are presently investigating the possibility that the BaP adduct of DNA present in peak 1 from the Sephadex LH-20 column represents BaP bound to phosphate.

Chemical carcinogens interact with DNA producing damage and alteration in the DNA structure that might be responsible for mutagenesis and carcinogenesis. Damaged DNA can be processed by prereplication excision repair, relication or postreplication

repair. Prereplication excision repair is error free resulting in a decrease biologic potency. Persistent DNA damage has been associated with the carcinogenic potency of alkylating agents (Buecheler and Kleihues, 1977). The stability of BaP lesions in epidermal DNA might be associated with the high carcinogenic potency of BaP in mouse skin.

The BaP adducts in mouse epidermal DNA present in peaks 1 and 3 were shown to be very stable. The amount of BaP adduct present in peak 1 remained constant for 56 hours, while peak 3 (dGua-BaP) declined with a half-life of approximately 85 hours. Chemical carcinogen substitution at the 2 amino group of guanine locates the carcinogen in the small groove of the DNA helix. Having the carcinogen outside the helix produces minimum alteration in the tertiary structure so that these lesions might not be efficiently recognized by the cellular repair mechanism. The persistent of the BaP adducts in peak 1 and 3 implicates them in the inititation of carcinogenesis.

BaP is an ubiquitous environmental contaminant for which data on the potential carcinogenic risk to man due to chronic low dosing is warranted. The extrapolation of this risk from high dose mouse skin studies requires the knowledge of the dose response relationship at very low doses. The carcinogenic potency of BaP in mouse skin is a linear function between 16 and 500 nmoles. However, extrapolation of this curve to lower doses

requires the use of a molecular marker such as BaP induced DNA damage identified as BaP adducts. The amount of BaP adducts identified as peaks 1 and 3 (dGua-BaP) in mouse epidermal DNA increased as linear function of dose between and 4 and 100 nmoles/-mouse. The quantitation of polynuclear aromatic hydrocarbon adducts is epidermal DNA as molecular markers for carcinogenic potency would appear to be an useful method for extending dose response curves to doses below those suitable for carcinogenic study.

Feasibility of Isolating DNA Adducts of Carcinogens by High Pressure Liquid Chromatography

In order to determine the feasibility of separating benzo(a)pyrene adducts to DNA by high pressure liquid chromatography (HPLC), we have been permitted to run some samples on a HPLC at the New York University Medical Center in New York City, 50 miles from our laboratory in Tuxedo, New York. A Waters Associates HPLC fitted with a μ bondapak C-18 column (Waters Associates) was employed with absorbance monitored at 254 and 280 nm. Samples were eluted with the instruments concave gradient No. 7 (1% methanol in water to 100% methanol) for 30 minutes and at a flow rate of 2 ml/min.

The major benzo(a)pyrene-^{14}C adduct in epidermal DNA, a guanine adduct, was isolated by chromatography on Sephadex LH-20 as a definite peak. The collected material was lyophilized, dissolved in methanol and applied to HPLC (Figure 32). BaP-(^{3}H) conjugated epidermal DNA contained one major peak similar

to peak 3.

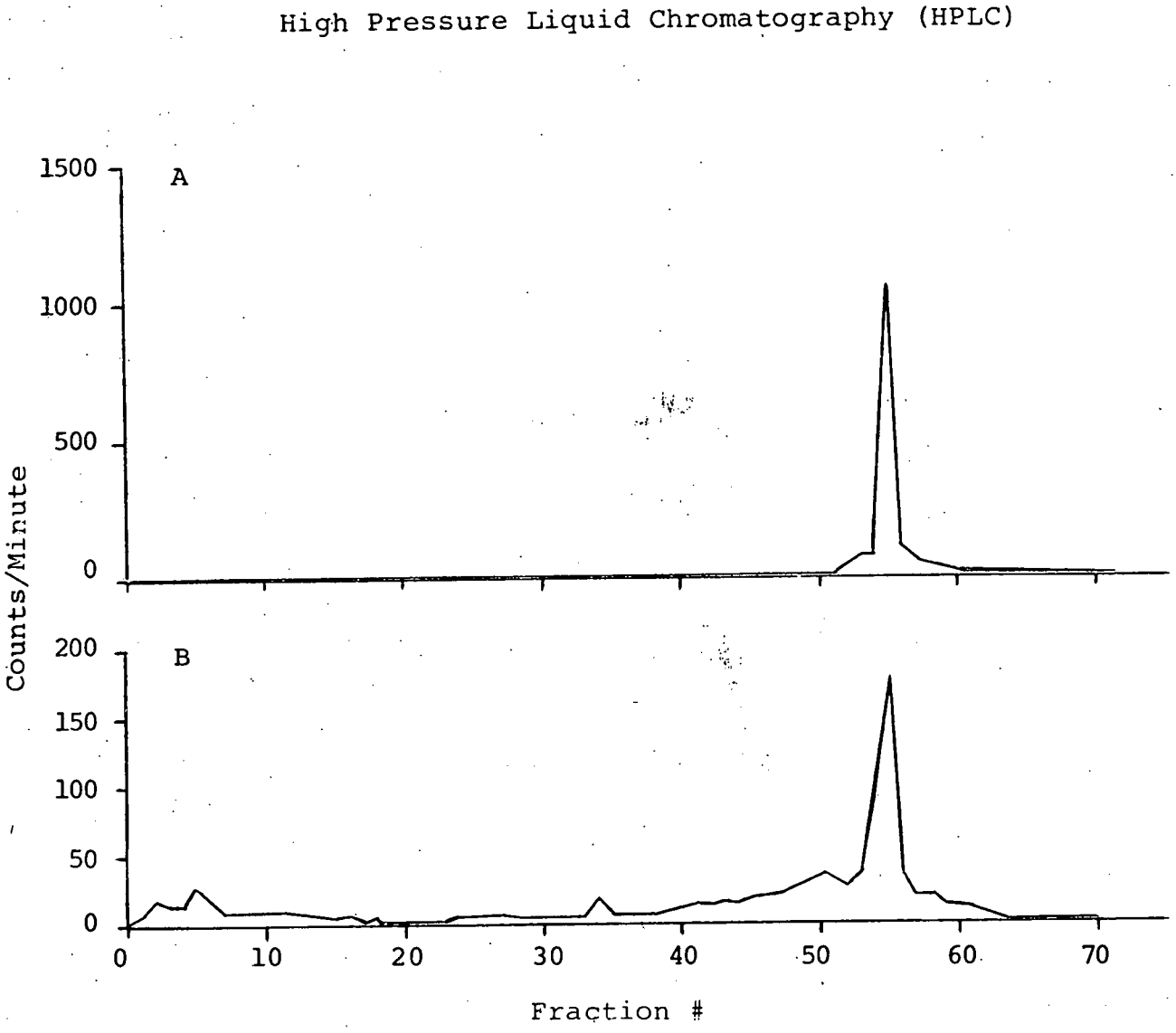


Figure 32. High pressure liquid chromatography of BaP adducts present in epidermal DNA. A. A major BaP-(^{14}C) adduct in calf thymus DNA formed by microsomal metabolism was isolated after enzymatic degradation to deoxynucleosides from a Sephadex LH-20 column in peak 3 of a 0-100% water:methanol gradient. Peak 3 formed *in vitro* corresponded to the major BaP adduct formed *in vivo* in mouse epidermal DNA. B. BaP-(^{3}H) adducts in epidermal DNA isolated from 15 mice treated topically with 2.5 µg BaP-(^{3}H) (50 µCi) for 24 hours.

Standards of deoxynucleosides and deoxynucleotides were applied to HPLC and monitored by absorbance at 254 nm. The retention times for deoxynucleosides, deoxynucleotides and the major benzo(a)pyrene adduct are presented in Table 1. On the basis of the peak shown in Figure 32, it is concluded that the procedure of HPLC can quantitatively separate the DNA adducts of benzo(a)-pyrene in a fraction of the time required for conventional liquid chromatography. The saving in time is well-worth the initial purchase cost because of greater efficiency in processing the numerous tissue samples in species and tissue comparison experiments.

Table 1. High Pressure Liquid Chromatography of Standards and Benzo(a)pyrene Adduct

Sample	Retention Time (mins.)
Deoxynucleotides	
dAMP	5.08
dCMP	2.22
dGMP	2.72
dTTP	2.68
Deoxynucleosides	
dA	12.45
dC	7.30
dG	12.8
dT	13.3
Benzo(a)pyrene Adduct	
Peak 3	26.5

Binding of Polycyclic Aromatic Hydrocarbons to Epidermal Cytoplasmic Protein

The subcellular distribution and binding of two carcinogenic polycyclic aromatic hydrocarbons, benzo(a)pyrene (BaP) and

7,12dimethylbenz(a)anthracene (DMBA), was investigated in mouse epidermis. The major site of BaP and DMBA uptake and binding was the cytoplasm where approximately 10% of the total bound carcinogen migrated as a single band on polyacrylamide gel. BaP and DMBA were bound to about the same extent to the major cytosol binding protein. Both carcinogens bound maximally at 4-6 hours after topical application. Thereafter the binding declined with a halftime of 2-3 hours. The amount of binding increased in proportion to dose up to about 200 µg for BaP and 100 µg for DMBA. Above those limits the extent of binding was independent of dose. The binding of PAH to the major cytosal binding protein might be a mechanism for transport and metabolism of PAH.

Carcinogenic polycyclic aromatic hydrocarbons (PAH) covalently bind DNA, RNA and protein in mouse skin (Brookes and Lawley, 1964; Goshman and Heidelberger, 1967; Brookes, 1975). The covalent binding to DNA has been associated with the initiation of carcinogenesis (Brookes and Lawley, 1964; Goshman and Heidelberger, 1967; Huberman and Sachs, 1977). Mouse epidermis includes a protein that binds tumor promoters, such as, TPA and dexamethasome, an inhibitor of tumor promotion (Slaga, et al., 1973; Slaga, et al., 1974). The affinity of TPA and other phorbol esters to this cytoplasmic protein correlates with their tumor promoting potency (Slaga, et al., 1973, Slaga, et al., 1974). PAH are complete carcinogens possessing tumor promoting properties

that might involve their binding to a cytoplasmic receptor.

This report describes the epidermal subcellular distribution of benzo(a)pyrene (BaP and 7,12dimethylbenz(a)anthracene (DMBA) and their binding to cytoplasmic protein. The binding of PAH to cytoplasmic proteins was demonstrated to be (1) specific for a single protein, (2) dose dependent, and (3) capable of being saturated at high doses.

Animal Treatment - HA/ICR female Swiss mice (7-8 weeks old) from Sprague-Dawley, Madison, Wis., had their dorsal backs shaven. Two weeks later mice were treated with a topical application of titriated carcinogen dissolved in 0.2 ml acetone. The epidermis was removed and homogenized. The filtered homogenate was centrifuged at 1,000g for 15 mins. to obtain a crude nuclear pellet, 10,000g for 10 mins. to obtain the mitochondrial pellet and 109,000g for 1½ hours to obtain the microsomal pellet and cytosol.

The uptake of radiolabeled carcinogen by the subcellular fractions was determined by counting 0.1 ml aliquots in a scintillation fluid. The counts were made with Nuclear Chicago liquid scintillation spectrometer (Amersham-Searle Corporation, Arlington Heights, Ill.). For the determination of the amount of carcinogen bound to the subcellular fractions, aliquots were pipetted onto Whatman filter discs and washed. The discs were air dried and counted in a scintillation vial containing 0.1 ml water.

Electrophoresis of Cytosol Protein - For electrophoresis the sample was applied to multiphasic buffer 2127 at 0°. The gels were stained with Coomaisse brilliant blue by the procedure of Fairbanks, et al. (1971) and scanned with a Gilford spectrophotometer. Unstained gels were sliced into 2 mm slices and counted by liquid scintillation.

Protein was assayed by the procedure of Lowry, et al. (1951) with bovine serum albumin as a standard.

The epidermal subcellular distribution of BaP and DMBA at 4 hours after the topical application of the carcinogen is presented in Table 2. The uptake and binding of PAH by the subcellular fractions (mitochondria, microsomes, nuclear and cytosol) reached a peak in 4-6 hours after which the subcellular content of PAH rapidly declined. The total uptake and covalent binding in the various subcellular fractions was very similar for BaP and DMBA. The cytosol contained the target amount of covalently bound PAH.

The cytosol proteins were fractionated in order to determine whether PAH was bound to a specific protein. Polyacrylamide gel electrophoresis of the cytosol proteins was performed under very mild conditions. A tracing of the absorbance of a Coomassie brilliant blue stained gel and the radioactivity present in slices of a gel are shown in Figure 33 for the cytosol from BaP-^{3}H treated mice.

Table 2. Subcellular Uptake

Carcinogen BaP µg/mouse	Mitochondrin Total	Mitochondrin Bound	Microsomes Total	Microsomes Bound	Nuclei Total	Nuclei Bound	Cytosol Total	Cytosol Bound
0.3	0.284*	0.052	0.567	0.176	0.080	0.014	0.478	0.273
1	0.628	0.123	0.943	0.208	0.284	0.057	0.932	0.518
3	2.43	0.476	2.14	0.627	1.31	0.189	3.75	2.20
10	4.22	0.819	3.56	1.11	2.08	0.426	4.91	3.03
30	36.4	4.62	19.1	4.04	17.2	2.10	27.4	11.6
100	85.1	8.79	48.1	7.41	18.0	2.66	72.8	25.0
300	373	52.1	166	14.6	15.1	1.87	127	53.3
600	599	30.7	116	12.7	46.0	1.95	201	82.2
1000	395	76.5	187	17.3	30.2	2.20	400	196
DMBA µg/mouse								
0.3	0.237	0.075	0.258	0.086	0.027	0.012	0.448	0.267
1	0.790	0.202	0.335	0.172	0.051	0.019	1.13	0.658
3	1.80	0.363	0.950	0.468	0.152	0.052	2.58	1.80
10	5.29	0.965	1.83	0.603	0.394	0.103	4.98	2.76
30	26.1	1.88	9.60	1.94	1.74	0.192	15.6	7.07
100	187	5.21	41.4	3.10	10.2	0.666	53.9	13.8
300	302	7.34	92.8	5.70	32.5	1.17	82.7	18.6
1000	999	22.8	224	11.7	44.6	0.955	337	53.0

*nanograms/mouse

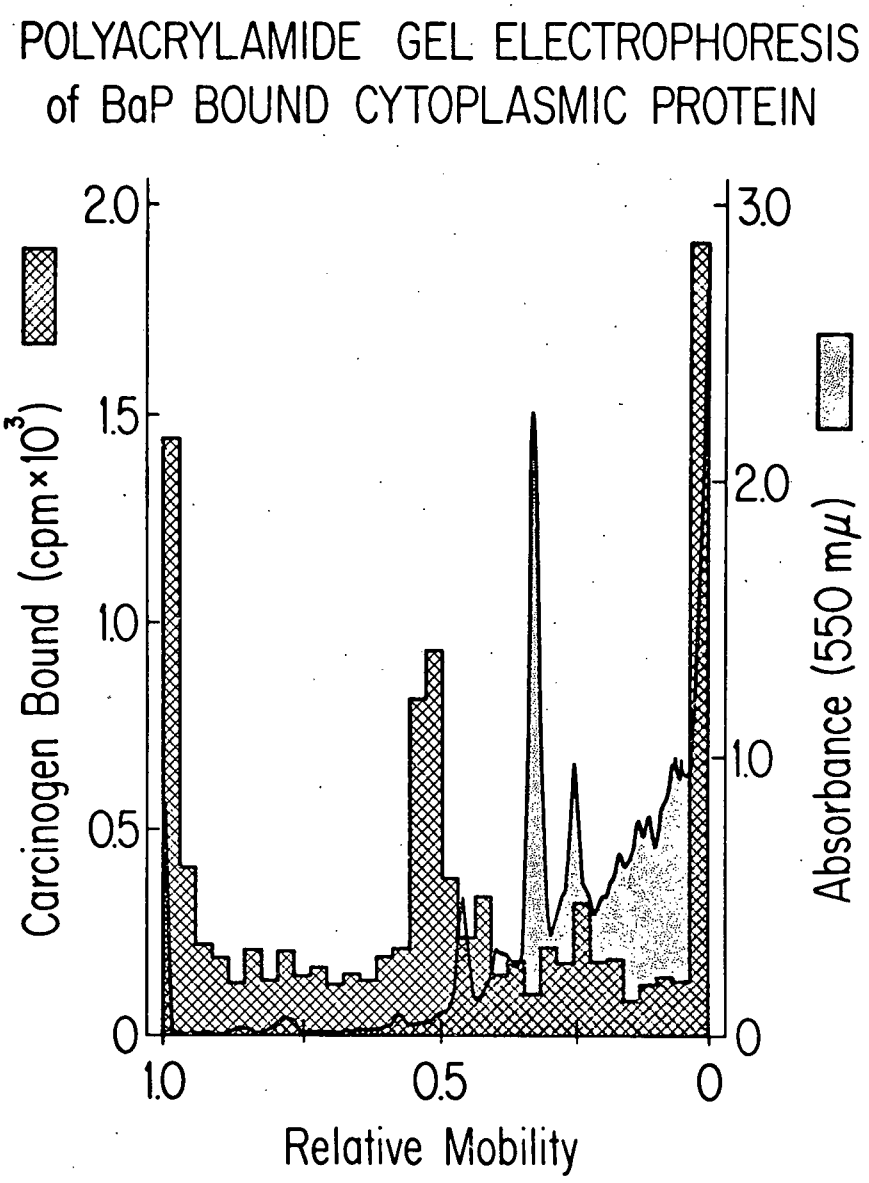


Figure 33. Polyacrylamide gel electrophoresis of epidermal cytosol from BaP-(^{3}H) treated mice. Mice (6) were treated 30 µg BaP-(^{3}H) (50 µCi) per mouse 4 hours prior to sacrifice. The epidermal cytosol was isolated and 0.2 ml applied to two gels. After electrophoresis, one gel was sliced into 1.5 mm slices and the radioactivity measured. The second gel was stained with Coomaisse brilliant blue and the absorbance at 550 nm scanned.

The majority of the bound BaP-^{3}H was present in a peak that migrated with a relative mobility of 0.53 and did not correspond to a major protein peak. The protein bound radioactivity in the cytosol from DMBA-^{3}H treated mice also migrated as a single peak with a relative mobility of 0.53.

The time course for the binding of BaP and DMBA to the major cytosol binding protein was very similar to the binding to the other subcellular fractions (Figure 34). The extent of PAH binding is expressed as pg PAH bound/µg of cytosol protein applied to the gel since the amount of absorbance under the radioactivity peak was too low to be quantified.

The dose dependence of BaP and DMBA binding to cytosol protein and the major binding protein are presented in Figure 35. PAH binding to cytosol protein was determined as ethanol and acetone insoluble radioactivity. The extent of BaP and DMBA binding was very similar until DMBA reached a plateau of maximum binding at 100 µg/mouse. Maximum binding of BaP and DMBA to the major cytosol binding protein was 33 and 14 pg/µg cytosol protein respectively. For DMBA there are 3.3×10^{10} binding sites/µg cytosol protein and 4.1×10^{12} per animal.

These results indicate the existence of an epidermal cytosol binding protein for PAH. The covalent binding of BaP and DMBA to cytosol protein accounted for half of all of the subcellular binding to macromolecules. The binding to the major binding protein

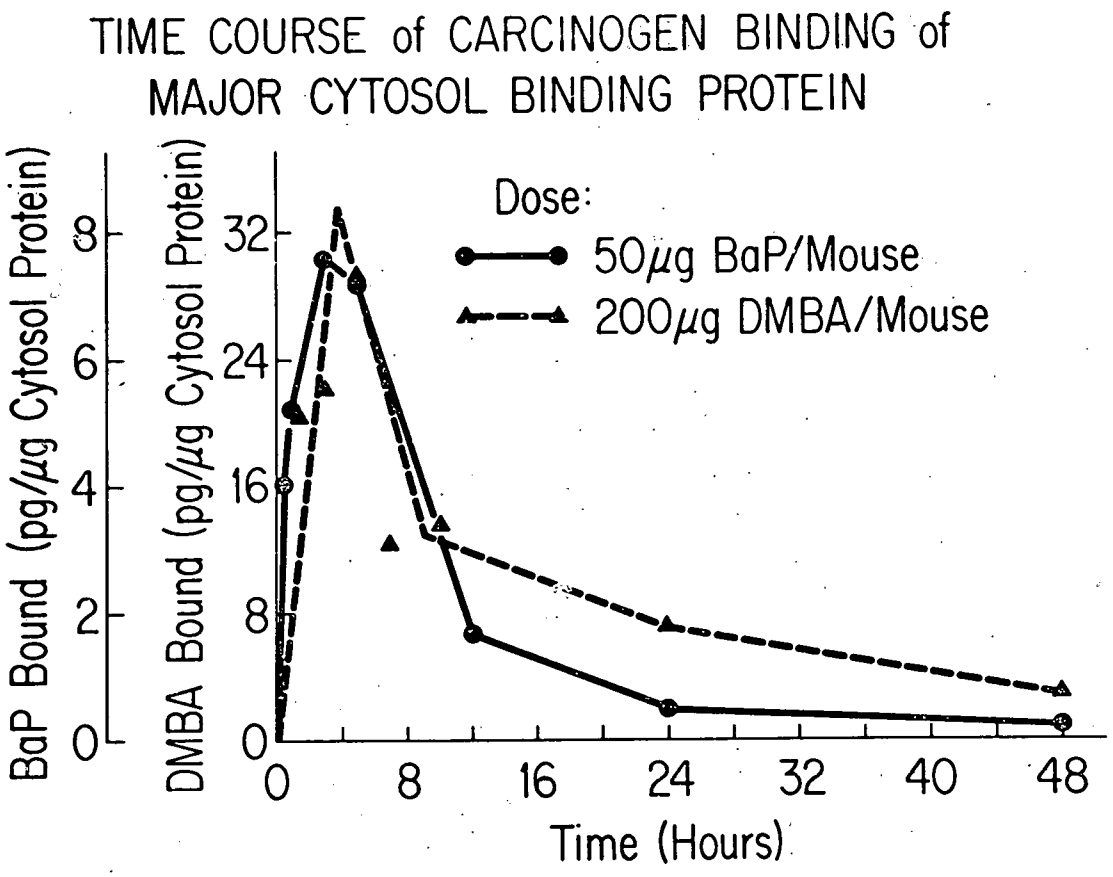


Figure 34. Time course of carcinogen binding to the major cytosol binding protein. Mice were treated with either 50 μg BaP-(^{3}H) (50 μCi) or 200 μg DMBA-(^{3}H) (100 μCi) and sacrificed in groups of 6-8 mice at various times. The epidermal cytosol was isolated and 0.1-0.3 aliquots applied to polyacrylamide electrophoresis gels. After electrophoresis, one gel was sliced and the other stained with Coomaisse brillian blue. The amount of carcinogen bound to the major cytosol binding protein was determined from the radioactivity peak (relative mobility of 0.50-0.55) in the electrophoretogram. Carcinogen bound is expressed as pg carcinogen/μg cytosol protein applied to the gel.

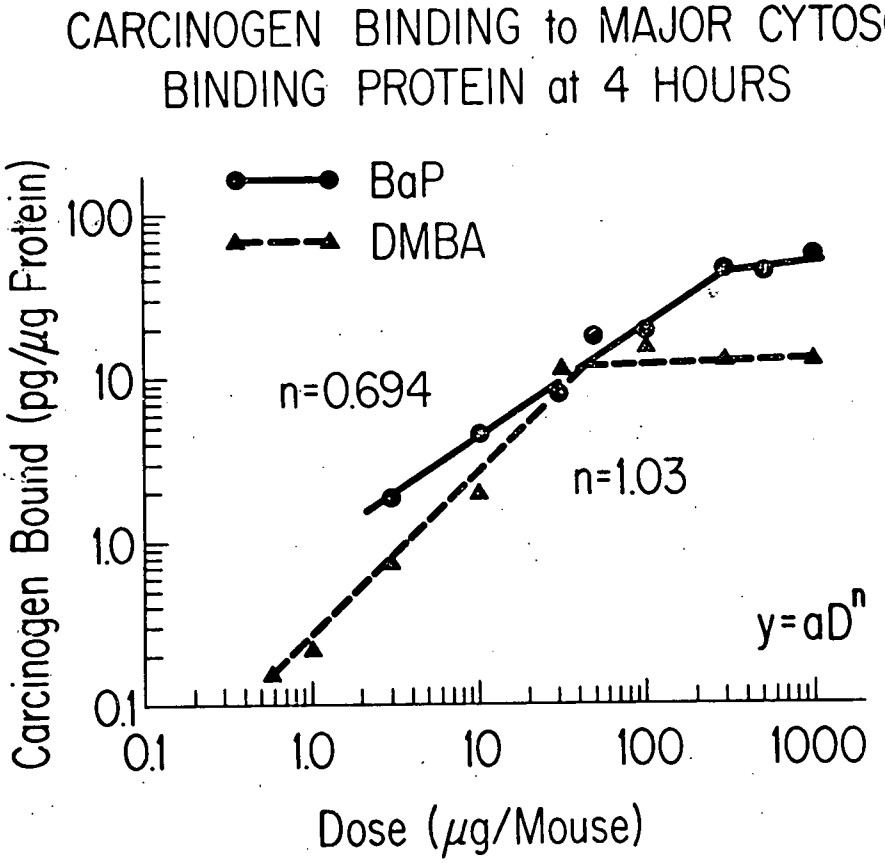


Figure 35. Dose dependency of BaP-(^{3}H) and DMBA-(^{3}H) binding to the major cytosol binding protein. Mice were treated topically with various doses of BaP-(^{3}H) (33.4-50 μCi) and DMBA-(^{3}H) (50 μCi) and sacrificed 4 hours later in groups of 6-9 mice each. BaP-(^{3}H) (●), and DMBA-(^{3}H) (▲), binding to cytosol protein was determined by polyacrylamide gel electrophoresis and expressed as pg carcinogen bound/μg cytosol protein applied to the gel.

constituted about 80% of the total cytosol binding, so that approximately 10% of all the covalently bound PAH in mouse epidermis occurred on this protein.

An epidermal cytosol binding protein for 12-0-tetradecanoylphorbol-13 acetate (TPA), dexamethasome (a potent inhibitor of tumor promotion) and 3-methylcholanthrene (MCA) has been identified and shown to also bind BaP and DMBA (Slaga, et al., 1973). The TPA binding proteins appears to be a protein distinct from ligandin and mouse skin h-protein (Slaga, et al., 1974). The degree of binding by different phorbol esters to this protein correlated with their tumor promoting potency (Slaga, et al., 1973; Slaga, et al., 1974). The major cytosol binding protein for BaP and DMBA might be the TPA receptor and be associated with their whole carcinogenic and tumor promoting property. The major cytosol binding protein for PAH might also function in the transport into the nucleus of the activated PAH metabolies produced by the mixed function oxidase of the endoplasmic reticulum.

Tracheal Intubations of BaP in Hamsters

The carcinogenic action of benzo(a)pyrene (BaP) in the hamster respiratory tract is being investigated for comparison with results obtained in mouse and rat skin. The hamster model was developed by Saffiotti, et al. A 1:1 mixture of BaP and ferric oxide in aqueous suspension was instilled weekly into the trachea by intubation. The treatment schedule included 15 or 30

weekly doses or weekly dosage for life at four weekly amounts: 2.0, 1.0, 0.5 and 0.25 mg. Appropriate BaP only, iron oxide, vehicle and colony controls were included.

In preparation for instillation, the BaP was milled and dispersed as crystalline particles, then mixed in a 1:1 ratio with Fe_2O_3 in a gelatin-saline vehicle. Increasing the Fe_2O_3 to BaP ratio above 1:1 has been shown to be effective in increasing tumor yield (Sellakumar, et al., 1973). The suspension was placed in a sonicator to assure homogeneity, and was mixed with a magnetic stirrer to maintain suspension during instillation. Doses of 0.1 ml of the BaP Fe_2O_3 suspension are given to hamsters under sodium Brevitol anesthesia.

The Fe_2O_3, an inert carrier dust of small (approximately 1 micron) particle size, provides a surface for attachment of the carcinogen particles. The carcinogen-dust aggregate penetrates the epithelium of the respiratory bronchioles and alveoli, where phagocytosis by macrophages occurs. The carcinogen is then eluted by plasma and diffuses to all lung tissues, thereby reaching cells of the bronchial mucosa (Saffiotti, 1969).

After the beginning of treatment animals were observed daily and examined at 6-week intervals by diagnostic radiology for evidence of respiratory tract nodules. At death the animals were autopsied, and organs of the respiratory system and any other organs showning gross abnormalities are examined histologically.

From these data dose-response and time-response relationships for respiratory carcinogenesis and mortality incidence were completed.

Preliminary results indicate that mortality incidence was unaffected by BaP and for Fe_2O_3 treatment as compared to vehicle and colony controls. Similarly, no difference in mortality was seen between groups with different treatment schedules, i.e., doses with BaP and Fe_2O_3 or BaP alone for 15 as compared to 30 weeks (Figure 36).

Preliminary results, based on gross observation only, indicate that respiratory nodule incidence increased in a dose-dependent manner. The lowest dose of BaP used, 0.25 mg with 0.25 mg Fe_2O_3 was ineffecitve in eliciting lung nodules when given in either 15 or 30 weekly doses. By contrast, the highest dose used, 2.0 mg BaP with 2.0 mg Fe_2O_3 induced macroscopic nodules in approximately 86% of animals treated for 30 weeks and 44% of animals treated for 15 weeks. Intermediate dose levels induced tumors at rates between the two cited (Figure 37).

An unexpected finding was that induction of lung nodules by BaP alone, without Fe_2O_3 as a carrier, was equally carcinogenic as with the Fe_2O_3 carrier. Reports in the literature (Kuschner, 1968) indicate intratracheal instillation of BaP without Fe_2O_3 produces no tumors; the only pathologic change seen was bronchio-alveolar metaplasis (Laskin, et al., 1970). However, our preliminary data again based only on gross pathology, indicates that BaP at

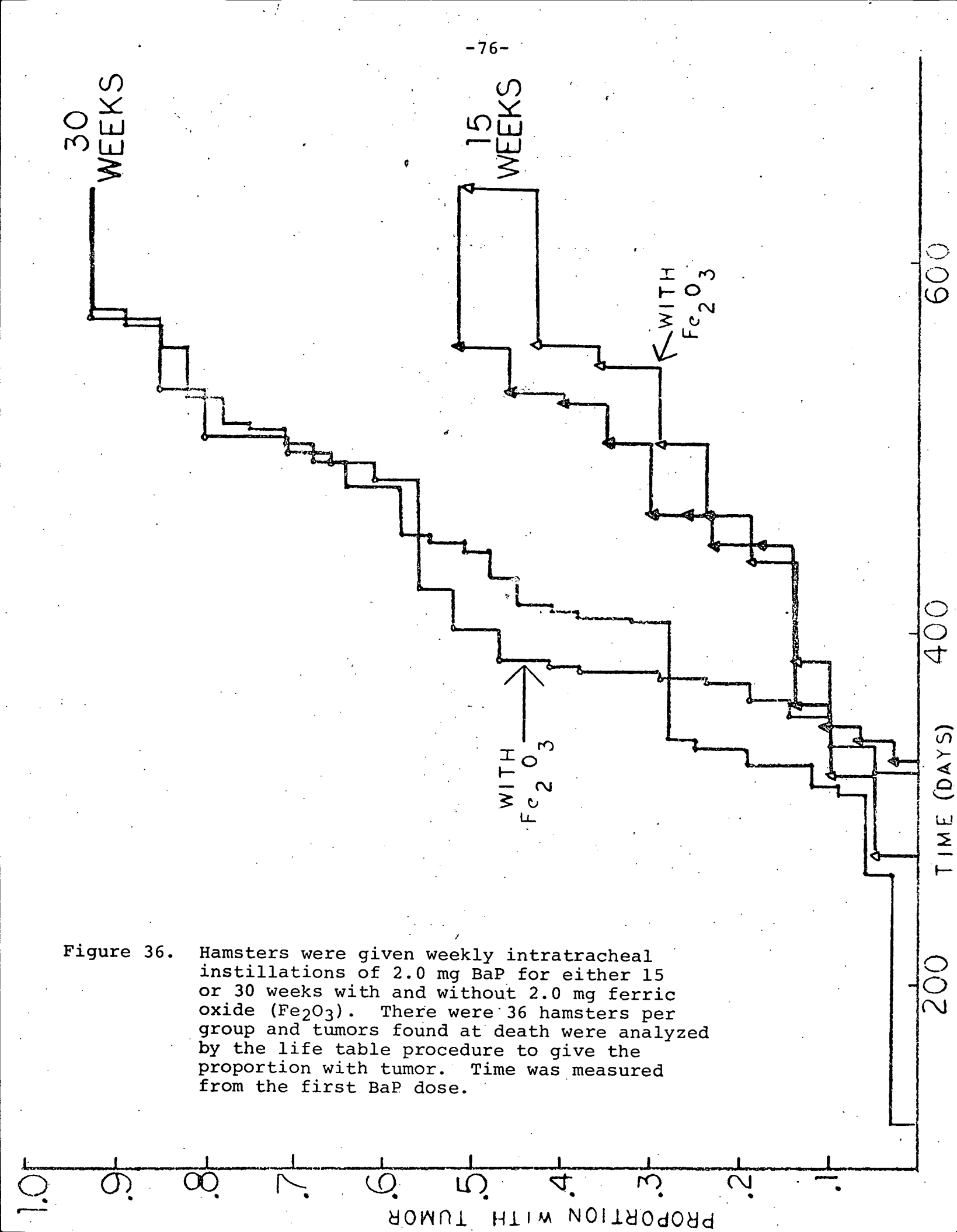


Figure 36. Hamsters were given weekly intratracheal instillations of 2.0 mg BaP for either 15 or 30 weeks with and without 2.0 mg ferric oxide (Fe_2O_3). There were 36 hamsters per group and tumors found at death were analyzed by the life table procedure to give the proportion with tumor. Time was measured from the first BaP dose.

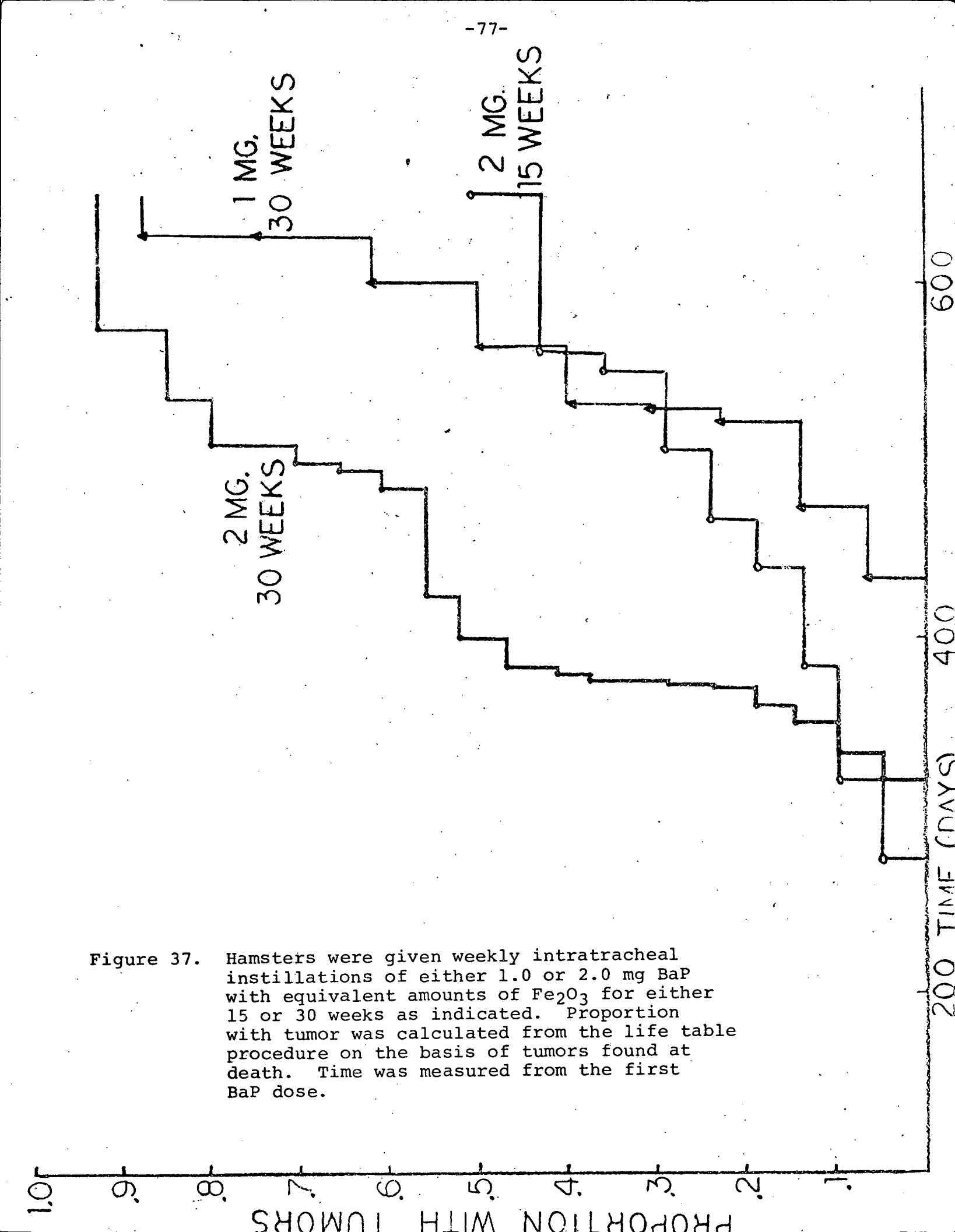


Figure 37. Hamsters were given weekly intratracheal instillations of either 1.0 or 2.0 mg BaP with equivalent amounts of Fe_2O_3 for either 15 or 30 weeks as indicated. Proportion with tumor was calculated from the life table procedure on the basis of tumors found at death. Time was measured from the first BaP dose.

weekly dose of 2 mg was equally effective as a respiratory carcinogen with or without Fe_2O_3. As stated above, 30 weekly doses of 2 mg BaP and 2 mg Fe_2O_3 produced gross lung nodules in 85% of experimentals; 30 weekly doses of 2 mg BaP alone produced nodules in 77% of treated animals. Fifteen weekly doses of 2 mg BaP and Fe_2O_3 produced nodules in 45% of the experimental population, while 15 doses of 2 mg BaP alone induced nodules in 40% of the experimental population. Data comparing the tumor induction of BaP and Fe_2O_3 and BaP alone at doses other than 2 mg per week is not available at this time.

Consistent with reports in the literature (Saffiotti, et al., 1972), our data indicates that increasing the number of carcinogen treatments increases the incidence of respiratory tract nodules. At the highest weekly dose of BaP and Fe_2O_3 (2 mg); 30 treatments induced nodules in 86% of treated animals, while 15 treatments induced nodules in only 44%. One mg doses of BaP and Fe_2O_3 were effective at nodule induction in 38% and 11% of experimentals receiving 30 and 15 doses, respectively. Doses of 0.25 mg were ineffective of both dose schedules, and weekly doses of 0.5 mg BaP and Fe_2O_3 induced nodules in only one animal per group. A thirty dose schedule induced gross nodules in 77% of treated animals while 15 doses induced nodules in 40%.

In general, tumor latency period decreased with increasing weekly dose. No clear trend is seen, however, in the comparison

of temporal response aspects of 15 and 30 week treatment schedules at the same dose. The temporal response curves for nodules induced by 2 mg BaP and Fe_2O_3, and by 2 mg BaP alone are quite similar.

The usefulness of diagnostic X-ray is indicated in Figure 38 where tumors were detected anywhere from 3 to 6 months prior to death without having to kill the animals. In future experiments diagnostic X-ray will be employed routinely to follow the development of lung tumors.

Cytofluorographic Analysis of Epidermal Cells

The use of rapid flow cell analysis systems for determining physical and biochemical properties of single cells has provided new and improved techniques for the study of cell cycle kinetics.

We have initiated a program to study the effects of chemical carcinogens and radiation on mouse and rat skin cells by means of a rapid flow microfluorometer (Cytofluorograph Model 48--A-Biophysics) coupled to a multi-channel pulse height distribution analyzer (Model 2102-Biophysics). The cytofluorograph is capable by argon laser exictation at 488 mm, of detecting cellular fluorescence in 2 spectra (green 5100 Å-5900 Å and red, 6100 Å-700 Å). By the use of fluorescent dyes one can specifically stain nuclear and/or cytoplasmic cellular constituents and rapidly show quantitatively and qualitatively relative cell size, protein and DNA content, specific antigenic properties and light scattering properties of single cells.

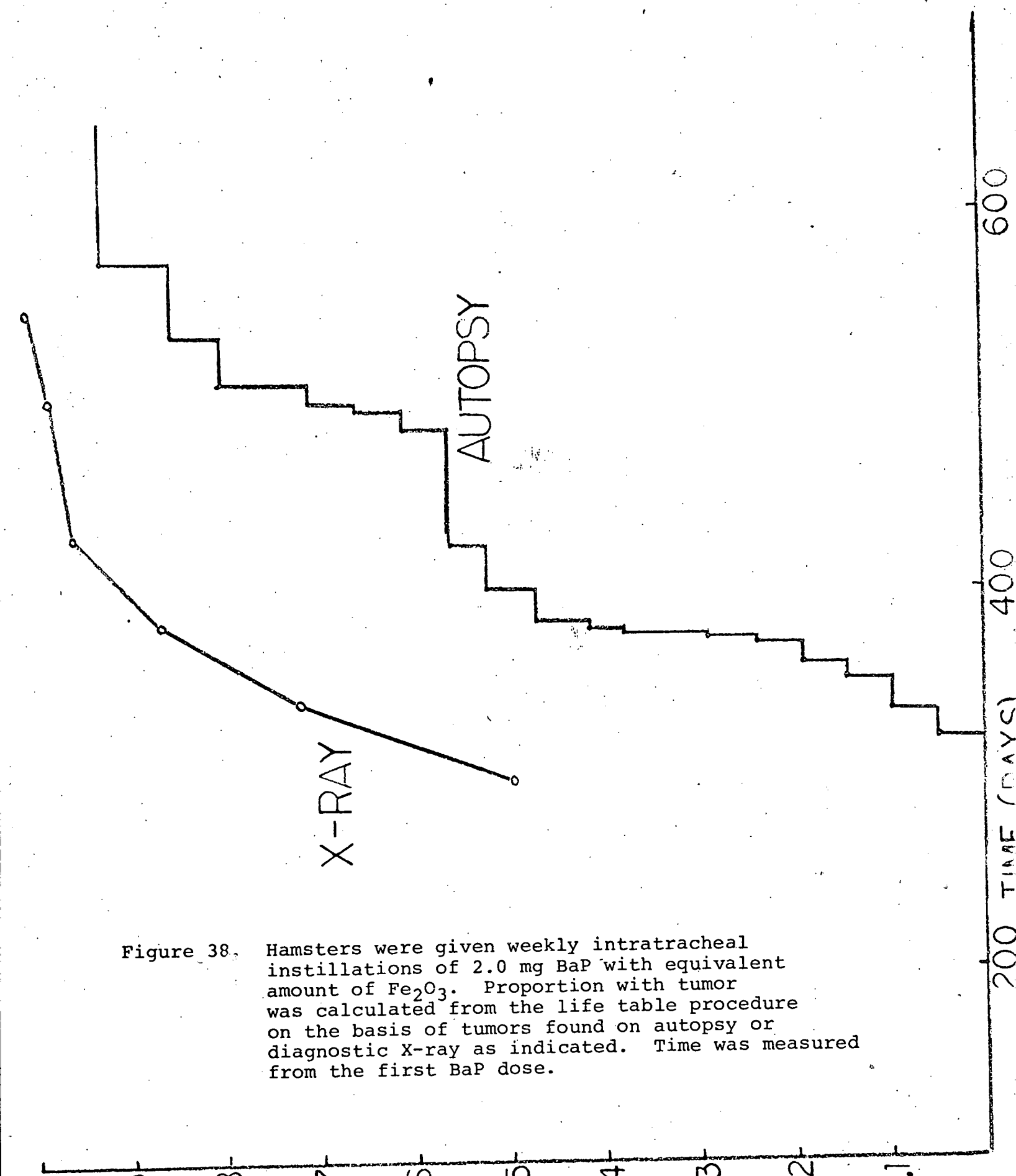


Figure 38. Hamsters were given weekly intratracheal instillations of 2.0 mg BaP with equivalent amount of Fe_2O_3. Proportion with tumor was calculated from the life table procedure on the basis of tumors found on autopsy or diagnostic X-ray as indicated. Time was measured from the first BaP dose.

Our main efforts have been focused on getting viable, single cell suspensions of primary tissue, i.e., mouse and rat epidermis to examine nuclear DNA content. Many methods now exist for getting single cell suspensions of primary liver, brain and thymus tissues, however, these techniques have proved inadequate for skin. Because of the strong intercellular desmosomes and the overlying keratin holding epidermal cells together, enzymatic digestion in addition to mechanical agitation is needed to sufficiently disperse the cells.

In attempting to increase cell yield while decreasing the CV, we experimented with varying concentration of trypsin solutions and incubation times. We maximized our cell yield (39%) and minimized our coefficient of variation (CV) (6%) by incubating mouse skin in 0.5% trypsin for 1.75 hour at 37^{o}C, scraping off epidermis, washing with PBS and vigorously (high speed) stirring magnetically (with 2 stirrers) in a small (50 mm D) glass petri dish for 45 minutes in phosphate buffered saline. The cells from this harvest were then filtered through a 63 μm metal mesh filter, pipetted 20 times, and centrifuged (163 g - 10 minutes). The resultant pellet was stained with propidium iodide.

We are continuing to use propidium iodide (P.I.) because of its rapid, intense staining - 50 ppm solution of P.I. in hypotonic 1% sodium citrate -- previously described by Freid, et al., 1976. Rapid Staining, devoid of excess washes and the

need for pre-fixing of cells permits analysis of cells about 3 hours after treatment.

In addition to experimenting with mouse and rat epidermal cells, we have prepared mouse skin carcinoma and papilloma cells and cultured mouse epidermal cells. The carcinoma cells consisted of 5 well defined populations corresponding to different DNA contents. The cultured mouse epidermal cells were prepared from confluent plates grown and prepared by Dr. Mukai's Lab. Seventy-six percent of the cells were within a DNA range expected of G_2 cells and 24% fell in a range expected of G_1 cells.

Papilloma cells were more difficult than carcinomas to disperse and as a result we had problems with interfering quantities of cellular debris and insufficient numbers of viable cells. We are currently investigating the appearance of polyploidal cells in mouse skin after ionizing radiation as a function of time and dose. Future experiments include the effects on DNA, protein and cell volume of UV and ionizing radiation and chemical carcinogens (DMBA and BaP).

Measurement of DNA Breaks as an Indicator of Dose

Experiments have been initiated to measure DNA strand breaks in rat epidermal cells after *in vivo* treatment with carcinogenic doses of ionizing radiation. The method is a modification of one developed by Rydberg (1975) and modified by Sheridan and Huang (1977) and Gutin, et al. (1977).

The procedure is based on the observation of Ahnstrom and Erixon (1973) that the rate of transformation of double-stranded DNA to single-stranded DNA can be accelerated by low doses of ionizing radiation. Ionizing radiation results in both single and double strand scissions of the DNA both of which will increase the rate of DNA unwinding in alkali. The unwinding of the DNA is due to the disruption of hydrogen bonds by high pH, and is rate limited by temperature, pH, and ionic strength of the alkali solution. The fraction of DNA remaining double stranded after various times of alkali treatment is used as a measure of the unwinding rate. In order to distinguish single strand (unwound) DNA from double stranded, the DNA is treated with single strand specific S_1 nuclease. The S_1 nuclease digested the single strand DNA leaving the double stranded DNA to be isolated and quantitated. This method is more sensitive and quantitative than previously used methods including alkaline sucrose gradient and alkaline elution analysis (Kohn and Grimek-Ewig, 1973). By varying the pH and/or the time of alkaline treatment, the alkaline unwinding method can detect DNA strand breaks at in vivo doses as low or lower than 200 rads and with a sensitivity of one break per 10^9 daltons. The development of techniques for the isolation of single cell suspensions and several adaptations of the alkaline unwinding method were made in order to measure DNA strand breaks in rat epidermal cells after in vivo treatment with ionizing radiation.

A method was developed to separate the epidermis from the dermis by stretching the whole rat skin on cardboard, dermis side down, and freezing in liquid nitrogen. Then, after removing hair by rubbing the skin with gauze, the epidermis was scraped off with a scalpel. It was noted that there was a change in the texture of the skin surface once the epidermis was removed, and it was confirmed histologically that all of the epidermis was removed at this point. This method afforded the advantage of inhibiting possible repair of the DNA breaks by freezing immediately after irradiation.

A technique was also developed to produce single cell suspensions of the epidermal cells. Conventional techniques of homogenization produced unacceptable cell suspensions by either lysing the cells or inadequately separating tissue clumps. It was found that stirring the cells in PBS at 4^{o} C at 1500 rpm on a magnetic stirrer and then filtering through 200 mesh nylon screen was adequate to isolate single cells without clumping or lysis of the cells.

Adaptations to the alkaline unwinding procedure was also made. Previously used conditions (Rydberg and Johanson, 1975) of pH 11.80 and incubation at room temperature were found to be unfavorable for the study of rat epidermis. At pH 11.80 the rate of unwinding of the DNA was too rapid to measure so it was slowed by using an alkaline solution at pH 11.35. The daily

variations in room temperature were found to be too great and therefore the incubations were run in a water bath at 23° C.

Preliminary results are shown in Figure 39. A log-linear plot of the fraction of double stranded DNA vs. time of alkali treatment is shown for four doses of electrons. The control curve is the average of each of four controls run concurrently with each dose point. A least squares line was drawn for each set of points and the slopes of these lines determined. The slopes or rate of DNA unwinding, is directly proportional to the number of DNA breaks.

Figure 40 shows that the slopes of the alkali unwinding curves are linear with electron dose between 0 and 2400 rads.

We plan to study the following using the alkaline unwinding procedure for the measurement of DNA strand breakage:

1. Dose response in rat epidermis following in vivo treatment with ionizing radiation and polycyclic aromatic hydrocarbons.
2. To study the time and dose kinetics of repair of DNA strand breaks in rat epidermis.
3. To study the sensitivity of G_1, S and mitosis phase rat epidermal cells.

Mammary Carcinogenesis with Benzo(a)pyrene

The modification of carcinogenesis by treatment with vitamin A and its natural and synthetic derivatives (retinoids)

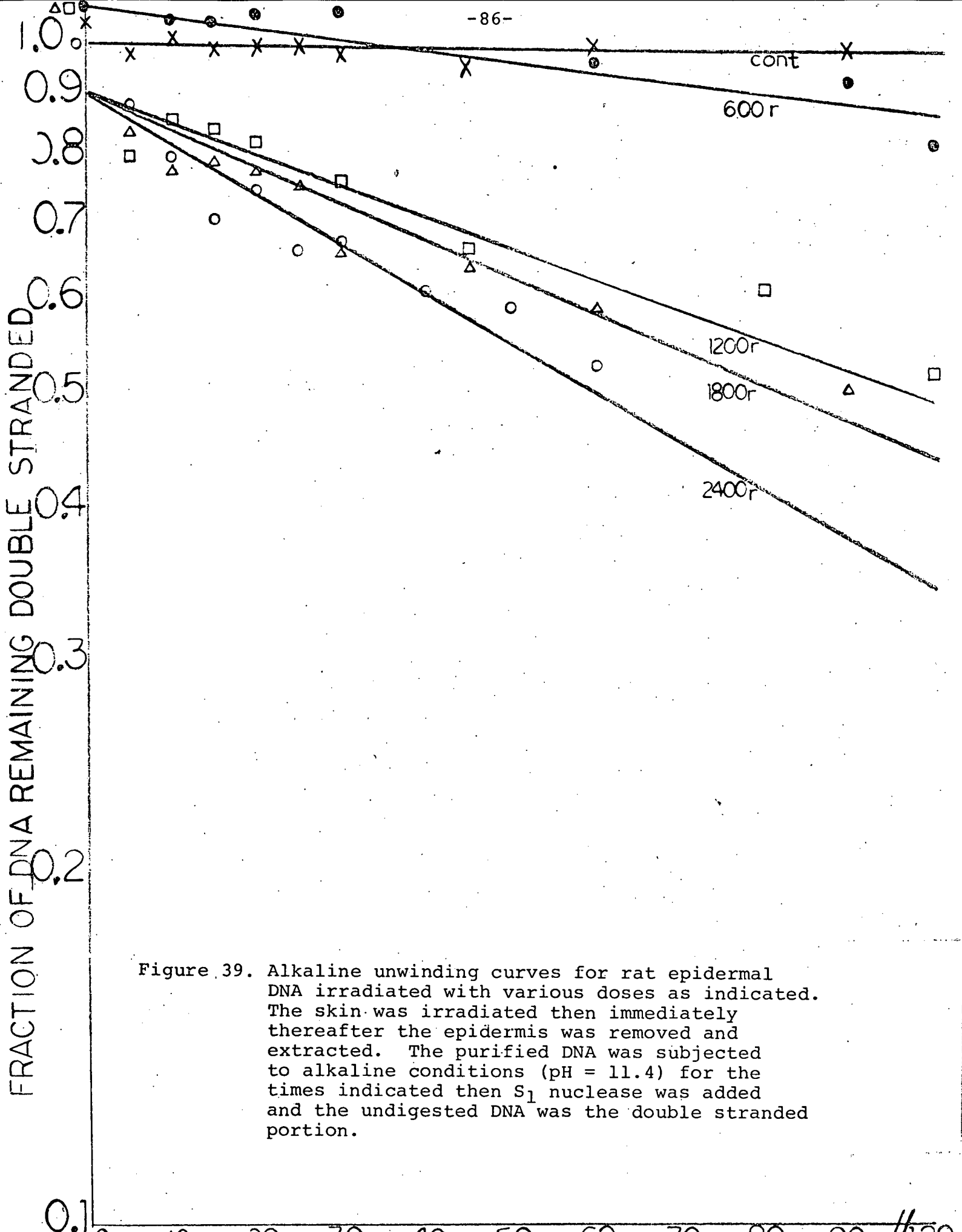
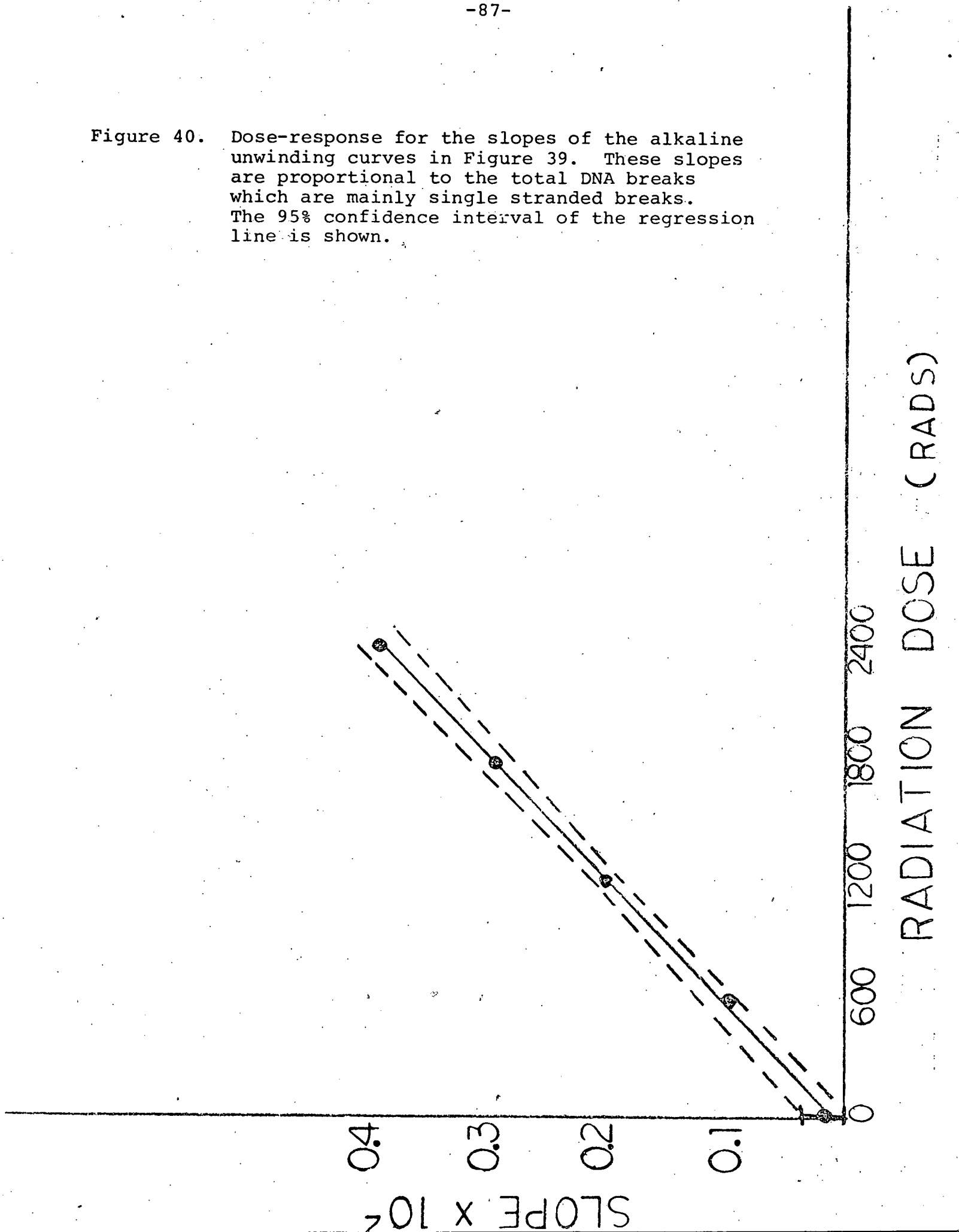


Figure 39. Alkaline unwinding curves for rat epidermal DNA irradiated with various doses as indicated. The skin was irradiated then immediately thereafter the epidermis was removed and extracted. The purified DNA was subjected to alkaline conditions (pH = 11.4) for the times indicated then S_1 nuclease was added and the undigested DNA was the double stranded portion.

Figure 40. Dose-response for the slopes of the alkaline unwinding curves in Figure 39. These slopes are proportional to the total DNA breaks which are mainly single stranded breaks. The 95% confidence interval of the regression line is shown.



is an area that has generated a large body of research over the past ten to fifteen years. Reports in the literature indicate that retinoids can have a significant antitumor effect in a large number of species and organ systems; these include mouse skin and prostate, hamster skin, cervix, forestomach and respiratory tract, rabbit skin and rat skin, bladder colon, respiratory tract, and mammary gland (for a recent review, see Sporn, et al., 1976). Clinical trials have also shown retinoids to be effective inhibitors of tumors in human skin (Bollag and Ott, 1970) and bladder (Evard and Bollag, 1972). Vitamin A treatment has been effective when used both prophylactically in the prevention of carcinogen-induced tumors, and when used to slow growth and induce regression of existing preneoplastic and neoplastic lesions. Conversely, reports indicate that vitamin A deficiency enhances susceptibility to chemical carcinogenesis.

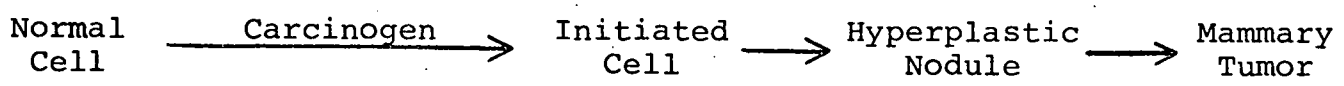
One experimental tumor system in which retinoids have only recently been shown to be effective antitumor agents is the carcinogen-induced rat mammary tumor system (Huggins, 1961). In this system, a single intragastric or intravenous dose of carcinogen is given to a female rat at approximately 50 days of age; this single treatment is followed by tumor development from one to twelve months later. Tumors induced in this manner are hormone responsive, and may be either benign fibroadenomas or malignant adenocarcinomas. They do not, however, metastasize

or invade surrounding tissues. Work by Moon, Grubbs and Sporn (1976) has shown both natural (retinyl acetate) and synthetic (retinyl methyl ether) (Grubbs, et al., 1977) retinoids to be effective in the prophylactic treatment of carcinogen-induced tumors in this experimental system. The same investigators (Moon, et al., 1977) have also shown retinyl acetate to be effective in the prevention of experimental mammary tumorigenesis in a related system, that developed by Gullino, et al. (1975). This system, using N-nitrosomethylurea as a carcinogen-induced mammary adenocarcinomas and papillary carcinomas similar to those of the Higgins system, but with the added property of metastasis and local tissue invasion.

The schedule of retinoid treatment used to achieve the tumor inhibition reported by Moon and co-workers started retinoid treatment after carcinogen exposure, and continued it for the animals' lifetimes. This schedule specifically excluded the possibility of any interaction of retinoids with the initiating event. However, it has been shown that retinoids will inhibit carcinogen metabolism by the aromatic hydrocarbon hydroxylase (AHH) system (Hill and Shin, 1974) and decrease carcinogen binding to DNA (Yuspa, et al., 1977) *in vitro*. Carcinogen metabolism to a proximal metabolite and its binding to DNA are considered control steps in the initiation of the carcinogenic process. Will these *in vitro* activities of retinoids be active *in vivo*, and thereby

influence initiation and alter tumor response? An experiment is proposed to answer this question.

Reports in the literature also indicate that mammary tumor development may proceed through a precursor lesion, the hyperplastic alveolar (Beuving, 1968) or ductal (Russo, et al., 1977) nodule. This multi-stage model can be detailed as follows:



This experimental model contains two discrete steps in the post-initiation development of the rat mammary tumor. However, the work done by Moon, Grubbs and Sporn dealt only with the post-initiation phase as a whole, and did not examine the effect of exogenous retinoid treatment on the sequential development of the mammary tumor. Will exogenous retinoids inhibit formation of hyperplastic alveolar nodules and intraductal end-bud proliferations, lesions proposed to be precursors? Will retinoid treatment of existing precursor lesions induce their regression? Will the progression from precursor lesion to tumor be slowed or stopped by retinoid treatment?

Experiments are proposed herein that seek to monitor retinoid influence on precursor lesion development and on the growth of a precursor lesion into a palpable mammary tumor. By providing the retinyl acetate dietary supplement at various stages in the development of mammary tumors, it should be possible

to determine which stage is more strongly inhibited by the retinoid.

To provide background information relevant to the questions posed above, preliminary experiments have been initiated. These experiments include dose-response studies for benzo(a)pyrene (BaP) as a mammary carcinogen in the Lewis rat using mammary tumors and mammary hyperplastic nodules as endpoints, and a repetition of the work of Moon, et al. using the Lewis rat.

As mentioned in the scientific background section, while information exists concerning retinoid influence on post-initiation rat mammary tumorigenesis, no such data is available with respect to retinoid effects on the initiation of tumors or possible effects on the multi-stage model of tumor development described above. A series of experiments is proposed to provide information on the effect of retinyl acetate on these processes.

To determine the influence of retinyl acetate on mammary tumor initiation, a retinyl acetate supplement will be provided in the experimental animals' diet for 14 days prior to and 7 days after BaP treatment. Parallel BaP and vehicle-treated controls not receiving the retinoid supplement will also be included. Animals will be palpated weekly to monitor mammary tumor development.

The influence of retinyl acetate on precursor lesion formation will be monitored by inclusion of a retinyl acetate supplement from 7 days after BaP treatment until 50 days after

BaP. By this time, numerous nodules are present in the animals' mammary tissue (see Figure 41). Groups of BaP plus retinoid-treated and BaP only animals, along with vehicle controls, will be serially sacrificed to assay hyperlastic nodule formation via microscopic observation of whole mounts. Parallel groups of animals will also be observed for their life-span to monitor the effect of this schedule of retinoid treatment in mammary tumor formation.

To monitor the influence of retinyl acetate on existing precursor lesions, the retinoid dietary supplement will be started at day 140 post BaP, and continued for the life-span of the animals. Serial sacrifice of BaP plus retinoid, BaP alone, and vehicle controls will yield information concerning possible retinoid-induced nodule regression, and concerning retinoid effect on late nodule appearance. Parallel groups of animals will again be monitored over their lifetime to assay the influence of late retinoid treatment on mammary tumor formation.

Parallel groups of retinoid-treated and untreated animls will receive multiple low doses of BaP in an attempt to more closely mimic environmental carcinogen exposure, and assay retinoid effect on this type of exposure. Animals will be observed for tumor development over their life-span.

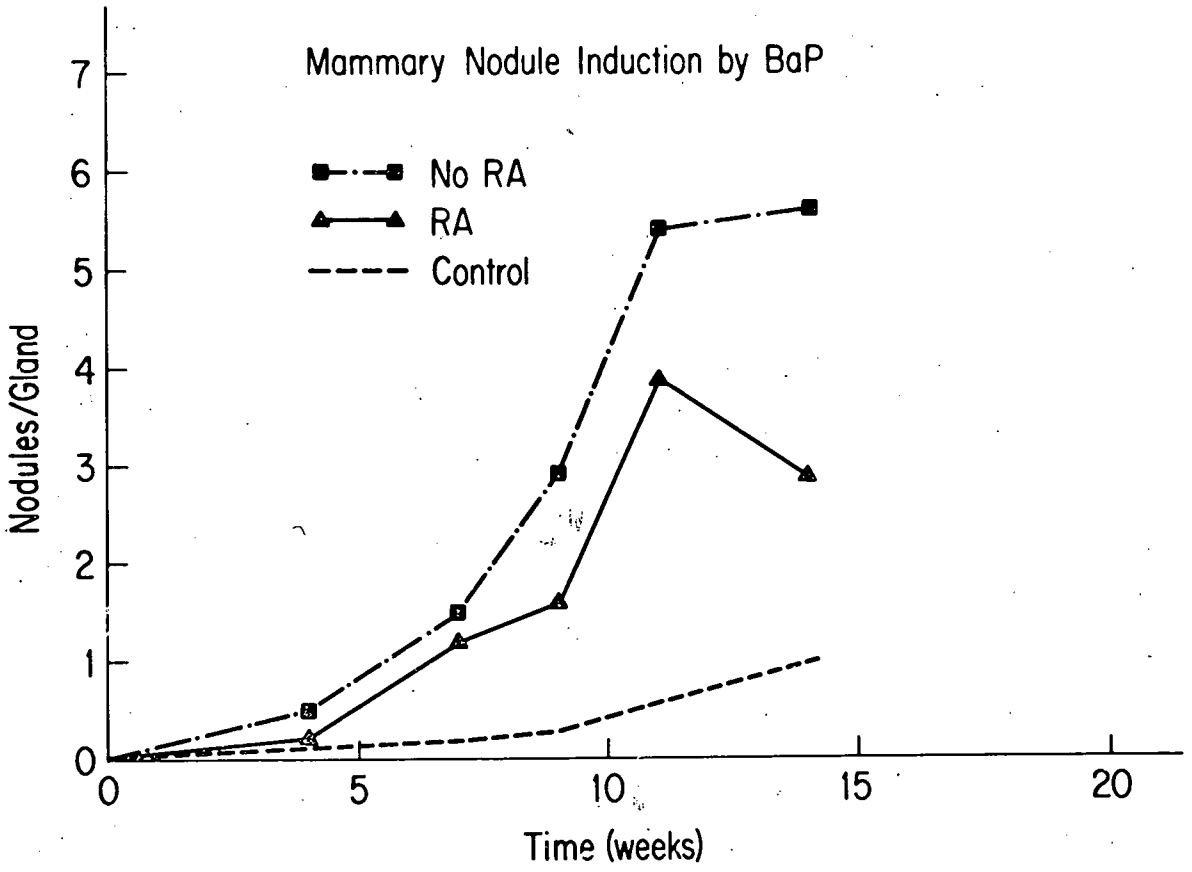


Figure 41. Female rats were given a single dose of 50 mg BaP intragastrically, and then they were given retinyl acetate (RA) in their diet or not as indicated. The mammary glands were excised, cleared, stained and nodules were counted (5 rats per point). Time was measured from when the BaP was given. Controls were given neither BaP nor RA.

Papilloma Progression to Carcinoma

The papillomas that develop in mouse skin following initiation with a polycyclic hydrocarbon, such as, 7,12dimethylbenz-(a,h)anthracene and promotion with an active promoter, such as tetradecanoylphorbol acetate have been studied extensively and yet their significance to carcinogenesis by polycyclic hydrocarbons is not precisely known (Boutwell, 1964; Berenblum, 1954; Shubik, et al., 1953; Van Duuren, 1969). Papillomas are focal, benign lesions consisting of folded layers of rapidly dividing cells that differentiate into squamous keratinizing cells almost as rapidly as they are produced (Burns, et al., 1976). Such lesions may persist for many months growing slowly; others may regress and still others may develop into invasive carcinoma (Borum, 1958). The latter papillomas are especially interesting in carcinogenesis. The present experiment was designed to determine how the various types of papillomas and their conversion to carcinoma depends on the dose of the initiating chemical and the dose and duration of application of the promoting chemical.

Chemicals were applied topically by means of pipette to the dorsal skin of female HA/ICR mice (Sprague Dawley, Madison, Wis.). The dorsal hair was clipped weekly and chemicals were applied in 0.2 ml aliquots of acetone. As tumors developed samples of individual lesions were identified by tattooed marks on the skin. The fate of identified lesions was established during a

312-day observation period. At the end of the experiment, the pulse labeling index of persisting tumors was determined by injecting i.p. 1 μc/gm (^{3}H)thymidine 30 minutes prior to death and counting labeled basal cells on autoradiographs. Groups of 20 mice were initiated with 5, 25 or 100 μg of DMBA followed by promotion with 2.5 μg TPA three-times weekly. In other groups of mice the initiator dose was held constant at 25 μg and promoter doses were varied in magnitude (2.5 μg and 10 μg) and duration (120d, 170d and indefinite).

The effect of initiator dose on papilloma yield and regression and on carcinoma incidence is shown in Figure 42. In all groups promotion was stopped at 120d. Carcinomas began to appear at about 160d, and by about 220d a sufficient percentage of animals had developed large carcinomas that tabulations of papillomas were ended because of inaccuracies associated with a dwindling population of mice. The data in Figure 42 indicate that neither the incidence of papillomas nor of carcinomas was strongly dependent on the initiator dose. About 90% of the carcinomas were found to arise in association with a preexisting papilloma.

The effect of promoter dose on the yield of papillomas and carcinomas is shown in Figure 43. Papillomas per mouse at 120 days was about the same for TPA doses of 2.5 and 10.0 μg, although the higher TPA dose produced a greater number of papillomas

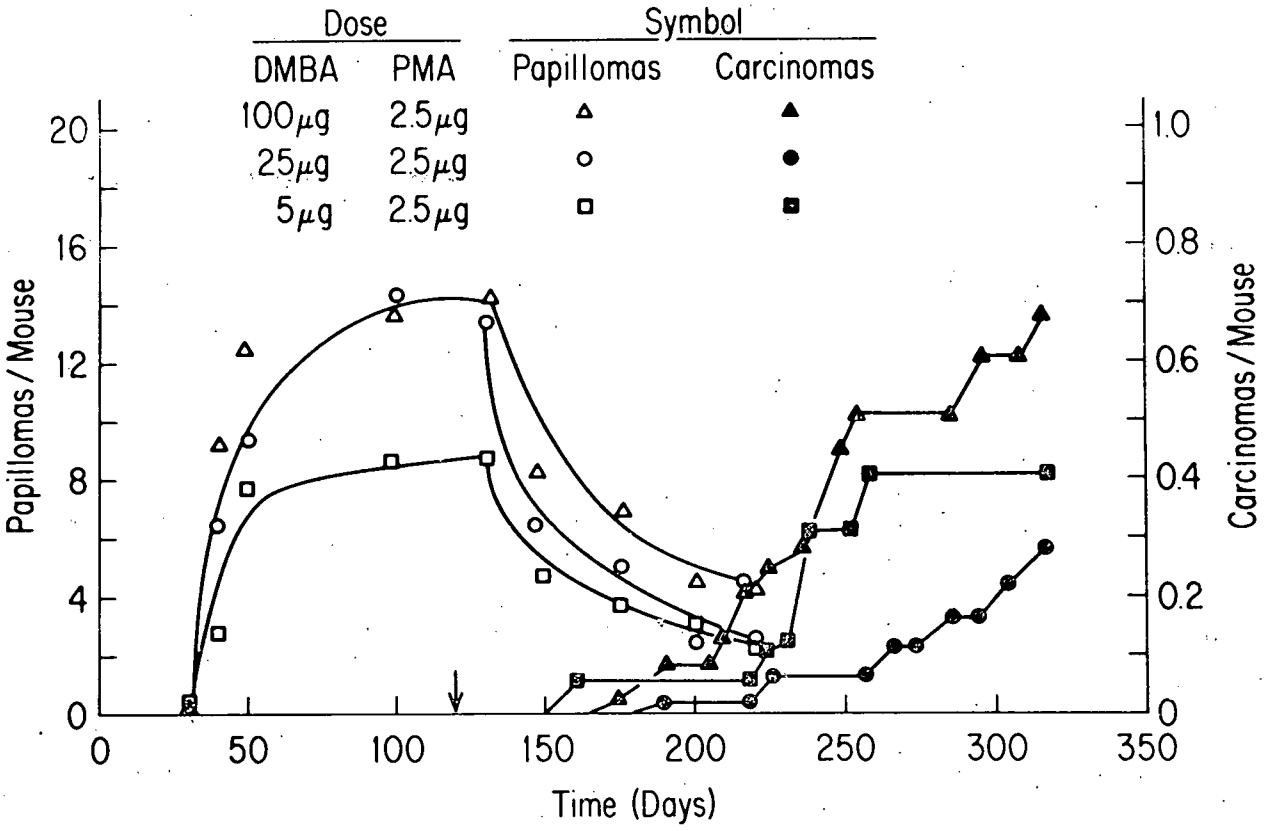


Figure 42. Mice were given single topical initiating doses of DMBA in 0.2 ml acetone as indicated. PMA treatments were begun at time zero and continued for 120 days. Carcinomas were confirmed histologically and were scored upon reaching 10.0 mm in diameter.

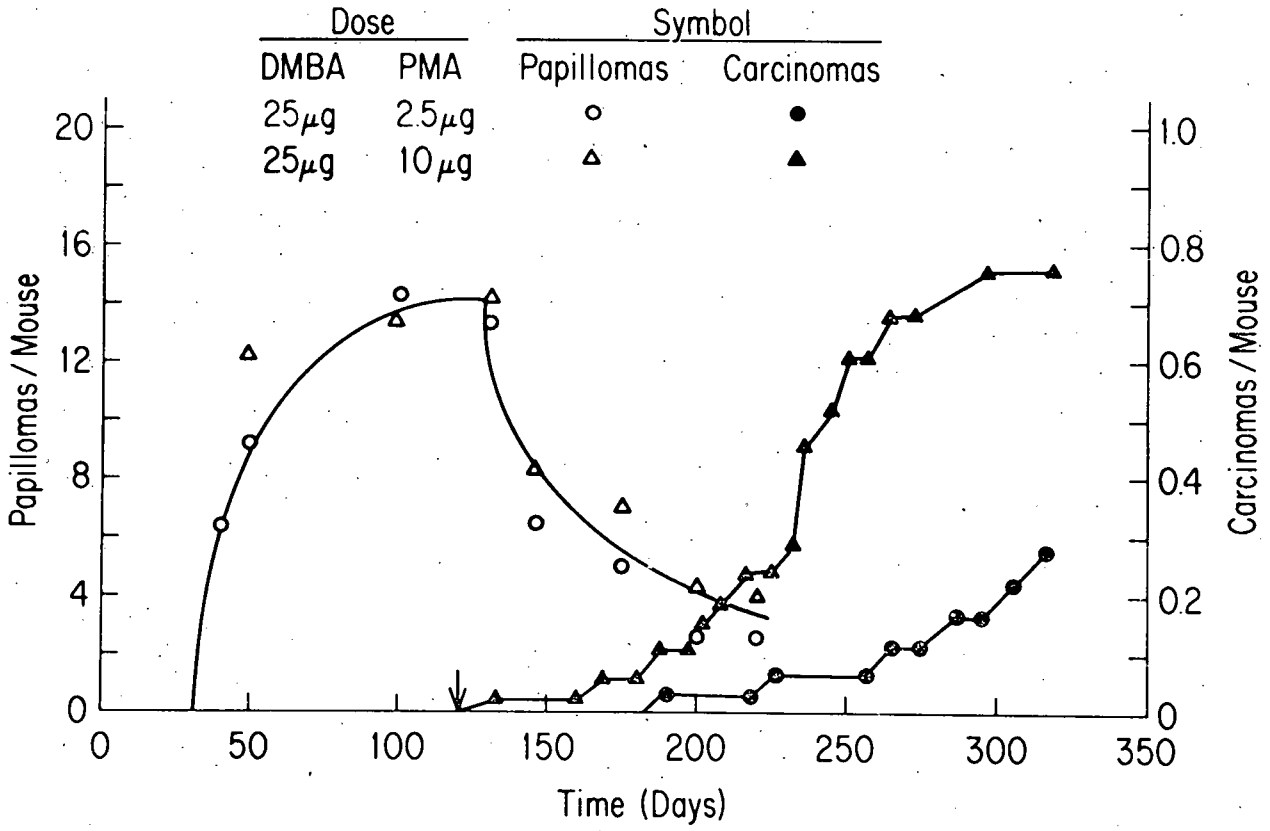


Figure 43. Mice were given single topical doses of 25 μg DMBA in 0.2 ml acetone following by thrice weekly doses of PMA as indicated. PMA treatment was stopped at 120 days and papillomas and carcinomas were scored weekly.

that had not regressed by 220d. Similarly mice receiving the higher TPA dose exhibited nearly a three-fold higher rate of carcinoma formation and about two-fold higher probability of conversion to carcinoma.

The effect of promotion duration on the yield of papillomas and carcinomas is shown in Figure 44.

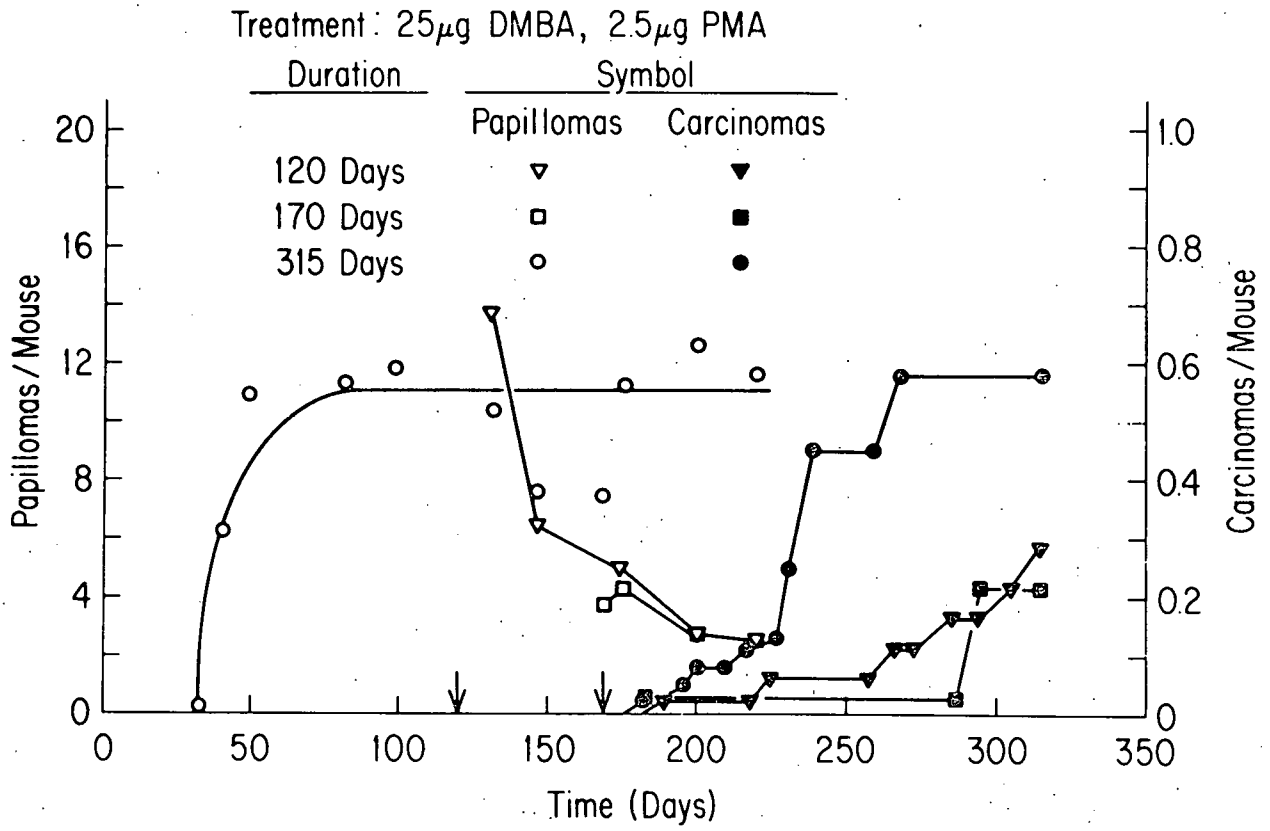


Figure 44. Mice were treated to single topical doses of 25 μg DMBA in 0.2 ml acetone on their dorsal skin. PMA treatments of 2.5 μg thrice weekly were begun at time zero (one week after DMBA) and continued for various times as indicated.

As promotion duration increased, papillomas became progressively less likely to regress and the carcinomas developed earlier. The earliest carcinomas occurred in the group that received promotion throughout the experiment. A small but definite decline in papilloma yield occurred between 130d and 170d such that in the group where promotion was stopped at 170d, not only was the papilloma yield relatively low, but there was little tendency for the surviving papillomas to regress.

Pulse labeling indices (L.I.) with (^{3}H)thymidine were determined on samples of 15 carcinomas and 15 papillomas. The average L.I. for the papillomas was 21% $\pm$ 4% ($\pm$ standard error) and the comparable value for the carcinomas was 24% $\pm$ 3%. These numbers indicate there was essentially no difference in the proportion of cells in the S-phase in the two lesions in spite of large differences in their growth rates.

The results presented here support the concept that papillomas induced by initiation-promotion of mouse skin exhibit a spectrum of neoplastic properties in terms of their ability to grow independently of the promoting chemical and their tendency to undergo conversion to carcinomas (Burns, et al., 1976). Not unexpectedly the greatest tendency for conversion to carcinomas was found among the papillomas with the greatest degree of autonomy, i.e., those having the least tendency to undergo regression when the promoting chemical was stopped.

Variability in the tendency for papillomas to grow or develop into carcinomas was observed in earlier work (Andrews, 1974). At least 80% of the papillomas induced in the present experiment were conditional and depended on the continued presence of a promoter. Conditional papillomas varied greatly in the time required to undergo regression; some regressed as early as a week after promotion ended, while others regressed months later.

Conditional papillomas probably contributed very little to the caricnoma yield, since the yield of carcinomas in the continuously-promoted animals, where the population of papillomas at 220d included autonomous and conditional types, was only slightly greater than in the groups where promotion was stopped and where only autonomous papillomas still persisted by 220d. The slight increase in carcinoma yield could mean that a few of the conditional papillomas were available for conversion to carcinomas or that new autonomous papillomas arose as a result of the continuation of the promotion.

The autonomous papillomas at 220d were converted to carcinomas with a frequency of about 6% in the subsequent 100d ending at 320d. Generally, only the first carcinoma on a given animal was observable so that the true frequency of conversion to carcinoma could have been somewhat higher than 6% since autonomous papillomas occurred with a frequency of 3 or 4 per

mouse.

The papilloma to carcinoma transition occurred without a noticeable change in the proportion of cells in DNA synthesis in the respective lesions. The substantial increase in the growth rate of the carcinomas in comparison to the papillomas could only have been achieved by a proportionate decrease in the rate of cell loss within the lesion. Of course, a carcinoma is not simply a papilloma with poor cell loss capacity. There are significant histological differences in the cells and in the way the cells are organized in these two lesions (Shubik, 1950), but it is remarkable that there is probably no difference in their capacity for producing new cells.

An initiating dose of DMBA without promotion may produce a few carciomas very late in life (Turosov, et al., 1971). With promotion the carcinomas occur much earlier and more frequently but whether these are the same carcinomas that would appear anyway if there were no promotion cannot be answered. In the absence of promotion, carcinomas may occur without passing through a noticeable papilloma stage but whether cancer cells may have existed as initiated cells not having multiplied sufficiently to become palpable is not known.

Conceptually, papillomas may be considered to be clonal expansions of initiated cells (Foulds, 1975) and the results here indicate that such cells, especially in autonomous papillomas,

have a fairly high probability to undergo malignant transition. It would not be unreasonable to postulate that the precursor cells of the papillomas, i.e., the original initiated cells, retain the same probability of malignant trnasition as the cells in the papillomas. Since papillomas contain at least 10^5 cells, their overall probability of malignant transition would be at least that much greater than that of single initiated cells and correspondingly cancers would be expected earlier and with greater frequency in the papillomatous tissue. Obviously, more work is necessary to test such ideas, but the skin papilloma clearly provides an excellent model for studying the benign to malignant transition.

Progression of Papillomas by Treatment with BaP

In this laboratory, the classic methods of initiation and promotion have been used in the past to investigate the regression of chemically induced mouse skin papillomas (Burns, et al., 1976a,b; 1978). This investigation concerns another possible fate of palpable tumors: the progression of chemically induced papillomas to the malignant state and whether this progression can be influenced by additional carcinogen or promoter treatments. In addition, evidence is presented for a stage of progressive development prior to tumor palpability which is related to Boutwell's proposed "conversion" of initiated cells (Boutwell, 1964).

Papillomas were generated by initiation with 128 µg BaP and 33 weeks of promotion with TPA, on virgin female albino mice. At the end of the 33 weeks of promotion, mice from each initiated/promoted group were assigned to either BaP, TPA or acetone treatment groups for the next phase of treatment. Also included as controls were untreated HA/ICR mice of comparable age which were now divided into BaP and continued no-treatment groups (8 and 9 mice respectively). Treatments consisted of either 32 µg of BaP applied twice weekly in 0.2 ml acetone, continued promotion with TPA, (generally this was 5 µg TPA applied thrice weekly in 0.2 ml acetone), or 0.2 ml acetone applied thrice weekly, all starting without interruption of the previous treatment schedule. The population of papillomas in existence at the time the last phase of treatment was started were referred to as "original papillomas". Any papillomas which appeared after the start of these treatments were referred to as "new papillomas".

Observations of the mice were done on a weekly basis, charting the location and history of each original and new tumor, and classifying the tumors according to the terminology developed by Shubik, Baserga and Ritchie (1953): sessile, pedunculated, conical, and carcinoma. Size of the tumors was also noted. The charted location of the tumors, especially with respect to other nearby tumors and to the mouse's tail, legs, and neck served

to confirm the identity of each individual lesion, and the appearance of new lesions. Photographs of most tumor-bearing mice were taken, starting from one-half to three months after commencement of the last phase of treatments, on approximately a monthly basis and prior to sacrifice.

Tumors were diagnosed histologically without reference to gross diagnosis or treatment group. Tumors were classified according to architecture of the tissues, cell type, cellular atypia (such as cell size and cytoplasm to nuclear ratio), keratinization and invasion of surrounding tissues. Carcinomas were judged first by the criteria of invasion of the panniculous carnosus. Additional diagnosis of carcinoma was made when cellular architecture and atypia, and appearance of a more limited invasion (breaking of the basement membrane) would not allow designation of the tumor as a papilloma.

Autoradiographic Estimation of ^{3}H-BaP in Tumors and Skin

Tumors and skin sections removed from mice which had received ^{3}H-BaP four hours prior to death were preserved in formalin, processed in the autotechnicon, and embedded in paraffin. Tumors and skins from mice which had been treated four hours prior to death with non-radioactive BaP were processed with the ^{3}H-BaP treated tissues and served as controls. Slides prepared for autoradiography were dipped in Kodak NTB-2 emulsion (diluted 1:1 with distilled water), dried in a controlled atmosphere

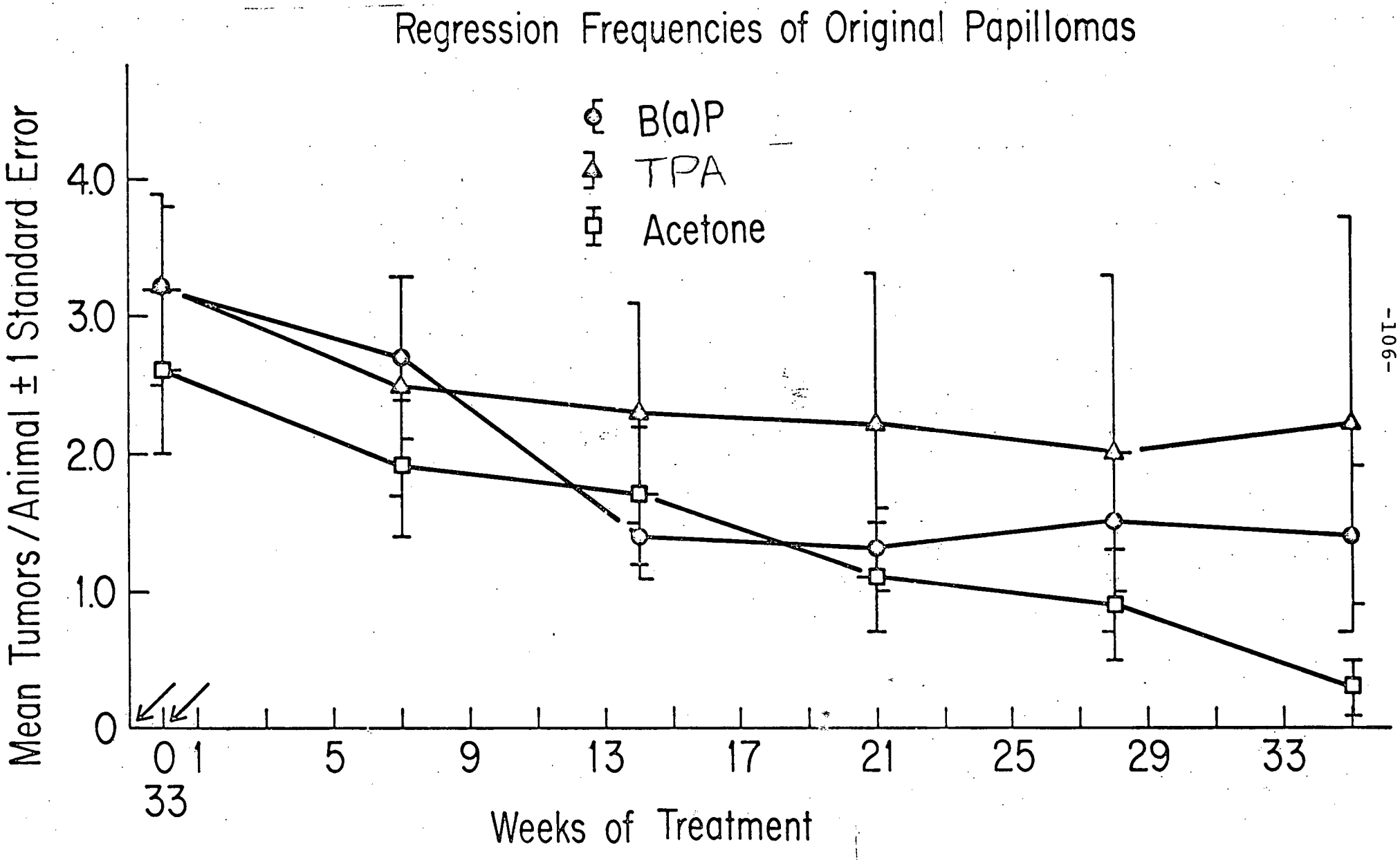
(20° C, 42% humidity), exposed in the dark for 2 weeks (at 8° C) and developed.

Carcinomas from Original and New Papillomas

A distinction was made between (1) the carcinomas which were derived from original papillomas and (2) those which were derived from new papillomas or arose "ab initio" as carcinomas. Only the first grossly diagnosed carcinoma (histologically confirmed) on each mouse was counted for the purpose of graphical description and statistical analysis. Therefore, an animal which developed a first carcinoma from an original papilloma was no longer at risk to also develop a first carcinoma from a new papilloma (and conversely, a first carcinoma from a new papilloma eliminated the animal from the "at risk" pool for original papilloma conversion).

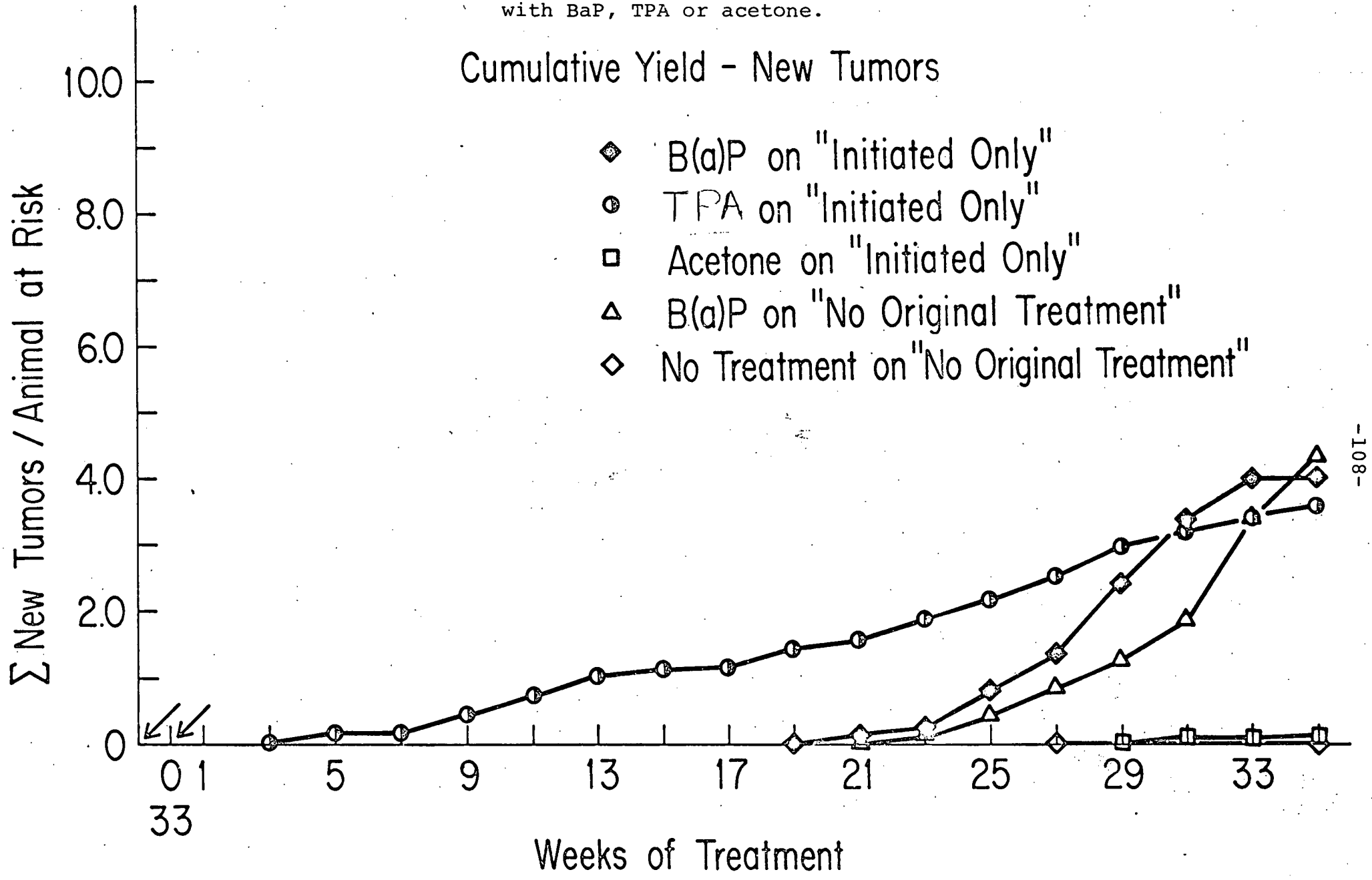
Figure 45 illustrates the cumulative yield of first carcinomas from original papillomas per animal at risk at time of death. During the 35 weeks of the last phase of treatment there was no apparent difference among the three treatment groups. Carcinoma yield was 0.66 per mouse for the BaP treatment group, 0.47 per mouse for the TPA treatment group and 0.42 per mouse for the acetone treatment group. Logrank tests which were performed on the data confirmed this finding of no significant difference among the treatment groups for conversion of original papillomas to carcinomas.

Figure 45. Arrow on left indicates the last week (32) of original treatment with TPA. Arrow on right indicates the start of treatments with BaP, TPA or acetone. Numbers in parentheses indicate the numbers of mice still at risk at the start of the last 2-week interval.



Carcinomas were suspected for the first time at 24 weeks in the BaP treated "initiated only" control group and at 30 weeks in the BaP treated "no original treatment" control group. The cumulative carcinoma yield for these groups reached 0.93 per mouse and 0.14 per mouse, respectively, by 35 weeks (see Figure 46).

Figure 46. Arrow on left indicates the last week (32) of original treatments with acetone for "initiated only" groups. Arrow on right indicates the start of tertiary treatments with BaP, TPA or acetone.



The relatively rapid rise in carcinoma yield for the BaP treated "initiated only" control group paralleled that of the main BaP treated group between 27 and 35 weeks of treatment. Prior to this rise in carcinoma yield, the difference in first carcinomas per animal between the BaP treated previously initiated and promoted group, and the BaP treated previously "initiated only" group was confired by the logrank test which gave a statistically significant value ($p < 0.05$) for the cumulative interval up to 27 weeks. The BaP treated control group which had received no original treatment was followed to week 40 instead of week 35 since the first appearance of carcinomas was delayed in this group as compared to that of the "initiated only" BaP-treated control group. The cumulative carcinoma yield was 0.14 at 35 weeks and 1.11 at 40 weeks. This relatively rapid rise in carcinoma yield is thus similar to those observed serveral weeks earlier for the BaP treated mice which comprised both the "initiated only" control group and the main BaP treatment group.

The mice which hd received no treatment at all did not develop any papillomas or carcinomas during the course of the study.

The treatment of papillomas with potent doses of the carcinogen BaP produced no greater rate of conversion to carcinoma than treatment with the promoter TPA or the solvent acetone. The progression, therefore, of these papillomas was not accelerated

by carcinogen or promoter treatments. This was demonstrated both for the original papillomas treated with either BaP, TPA or acetone and for the new papillomas that appeared after the start of these treatments.

Late papillomas converted to carcinomas more quickly than early papillomas. The mean duration as papilloma was approximately 3 times longer for the early than for the late papillomas. However, the appearance time of the carcinomas derived from both new and original papillomas was more constant when measured from the start of promotion, which implied that some events relevant to the induction of these carcinomas occurred prior to the appearance of the new papillomas. The nature of these events cannot yet be specified, although they might be similar to Boutwell's (1964) conversion step of initiated cells to dormant tumor cells. The cells which reached this advanced level of progression will be called autonomous benign. These cells required the promotion with TPA to reach this level of responsiveness to stimulation by the BaP treatments.

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