The Effect of Croton Oil Pretreatment on Skin Tumor Initiation in Mice

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SUMMARY

A single application of 0.5% croton oil to mouse skin stimulates nucleic acid and protein synthesis in the treated area. The maximum rates of synthesis were attained at 6 hours for RNA, 12 hours for protein, and 18 hours for DNA. By 48-72 hours, the rate of synthesis of RNA and protein was returning to normal, but the rate of DNA synthesis was still 2-3 times the control value. In order to test the hypothesis that cells synthesizing DNA are more susceptible to initiation of skin tumor formation, mice were given a single, preliminary application of croton oil either 18 or 48 hours before initiation with 7,12-dimethylbenz[a]anthracene (DMBA), β-propiolactone (BPL), or urethan. Tumors were subsequently elicited by multiple applications of croton oil. Croton oil pretreatment did not affect initiation by BPL, caused a slight increase in tumor yield after initiation by DMBA, but clearly increased tumor incidence in mice initiated with urethan. The overall binding of DMBA-3H to mouse skin DNA, RNA, and protein, or to RNA fractionated on a methylated albumin kieselguhr column, was not greatly affected by the croton oil pretreatment.

INTRODUCTION

Chemical carcinogenesis in mouse skin can be divided into two stages, initiation and promotion (4, 6). A single application of an agent such as DMBA, BPL, or urethan (the 3 initiators chosen for the present study), initiates the process of tumor formation. Subsequent repeated applications of croton oil promote the formation of papillomas and carcinomas. The number of tumors formed presumably indicates the number of epidermal cells which were initiated by the single treatment with the carcinogen. By altering the metabolism of skin macromolecules in a specific way at the time of initiator application and, after an appropriate interval, applying the promoter to reveal the number of cells initiated, one can determine the effect of the metabolic alteration on the process of initiation. This experimental approach to the study of the mechanism of initiation was first used by Mottram (21).

Comparisons of the binding to mouse skin DNA, RNA, and protein of DMBA and related hydrocarbons (7), and BPL and related alkylating agents (10), have suggested the importance to carcinogenesis of the interaction of these initiators with DNA. Studies on the diurnal variation of susceptibility to initiation (12, 23), the enhancement of tumor formation by croton oil treatment prior to initiation (26, 31), the inhibition of tumor formation by actinomycin D (1, 15), and the inhibition of binding of carcinogenic hydrocarbons to skin DNA by hydroxyurea (32) have suggested the possibility that DNA synthesis is important in the formation of initiated cells.

In order to test the hypothesis that cells synthesizing DNA are more susceptible to initiation, mice were initiated either 18 hours or 2 days after a single application of croton oil, which stimulates DNA synthesis (16, 31). The pretreatment time of 2 days was chosen based on the effects of 0.5% croton oil on the synthesis of DNA, RNA, and protein in mouse skin. At 2 days, the time of initiator application in 4 of the experiments, the synthesis of DNA was stimulated to a much greater extent than the synthesis of RNA or protein. The effect of croton oil pretreatment on tumor incidence and on the binding of DMBA-3H to mouse skin DNA, RNA, and protein was determined.

MATERIALS AND METHODS

Animals. Female skin tumor susceptible (STS) mice, purchased from the A. R. Schmidt Co., Madison, Wisconsin, were used in all experiments. Mice 6-8 weeks old were shaved with surgical clippers 1-2 days before use; those mice in the resting phase of the hair cycle were selected for biochemical experiments. Mice 7-12 weeks of age were used in the tumor induction experiments.

Chemicals. 7,12-Dimethylbenz[a]anthracene was purchased from Eastman Organic Chemicals; β-propiolactone from Fellows Medical Manufacturing Co., Detroit, Michigan; croton oil from S. B. Penick and Co., New York, New York; and urethan from Merck and Co., Rahway, New Jersey.

Radiochemicals. Thymidine-methyl-3H (3.0 c/mmole), cytidine-5-3H (6.0 c/mmole), and L-leucine-4,5-3H (6.0 c/mmole) were obtained from Schwarz BioResearch, Inc. DMBA-3H (8.3 c/mmole) was obtained from Nuclear-Chicago Corporation.

1This work was supported in part by grants from the American Cancer Society (E-6) and the USPHS (T01-CA-5002 and CA-07175).

2Abbreviations used are: BP, benzo[a]pyrene; BPL, β-propiolactone; DMBA, 7,12-dimethylbenz[a]anthracene; MAK, methylated albumin kieselguhr; PCA, perchloric acid; STS, skin tumor susceptible; TCA, trichloroacetic acid.

Received August 29, 1968; accepted June 6, 1969.
Extraction of DNA, RNA, and Protein Hydrolysates from Mouse Skin. Precursors used were thymidine-\(^3\)H for DNA, cytidine-\(^3\)H for RNA, and leucine-\(^3\)H for protein. Mice were killed by neck fracture 30 minutes after the intraperitoneal injection of 60 \(\mu\)c of thymidine-\(^3\)H, or 1 hour after injection of 20 \(\mu\)c of cytidine-\(^3\)H or 100 \(\mu\)c of leucine-\(^3\)H. As described previously (15), DNA and RNA hydrolysates were prepared by a modified Schmidt-Thannhauser procedure (30) and their specific activities determined. RNA was hydrolyzed from a PCA-insoluble precipitate in 0.3 N KOH at 37°C for 3 hours. DNA was then hydrolyzed in 0.5N PCA at 90°C for 5 minutes.

A modification of this procedure has been used to obtain protein hydrolysates. After the skin was removed from a mouse’s back, pinned to a large cork, frozen in liquid nitrogen, and scraped with a scalpel, the skin was homogenized in ice water in a glass homogenizer with a loose-fitting glass pestle. Skin macromolecules were precipitated by the addition of concentrated PCA to a final concentration of 0.4 N. The precipitate was washed 6 times with 0.2 N PCA at 4°C and twice with ethanol at room temperature. Nucleic acids were hydrolyzed in 0.5 N PCA for 5 minutes at 90°C and discarded as the supernatant after centrifugation at 4°C. The precipitate, containing denatured protein, was washed twice with 0.5 N PCA at 4°C and once with ethanol at room temperature. The protein was hydrolyzed in 0.5 N NaOH at 80°C for 30 minutes. After centrifugation the protein hydrolysate was decanted. Aliquots (0.2 ml) of the hydrolysate were counted in a liquid scintillation counter. An estimation of the amount of protein was made by determining the optical density of the hydrolysate at 280 \(\mu\)m in a Beckman DB spectrophotometer. The specific activity of protein was expressed as dpm/optical density at 280 \(\mu\)m.

Phenol Extraction of DNA, RNA, and Protein from Mouse Skin. For experiments in which the binding of tritium from \(0.5\%\) croton oil in acetone on the rates of synthesis of skin DNA, RNA, and protein were increased at the earliest times tested, 2 hours.

Fractionation of RNA on MAK Columns. The MAK column was prepared as described by Monier et al. (20). Bovine albumin was methylated according to Mandell and Hershey (19). The column dimensions were 1.2 x 30 cm; from 250 \(\mu\)g to 1 mg of RNA could be fractionated conveniently on the column. Elution with a 200-ml linear gradient of NaCl between 0.3 M and 1.2 M (buffered with 0.05 M phosphate, pH 6.7) was carried out at room temperature at a flow rate of approximately 0.7 ml per minute. Fractions of 3 ml were collected and the optical density of each fraction read at 260 \(\mu\)m on a Beckman DB spectrophotometer. The concentration of NaCl in the eluate was estimated by refractometry. For counting, the RNA was precipitated by adding 75 \(\mu\)g of albumin and 25% TCA to a final concentration of 5%. Each sample was filtered on a Millipore filter (pore size, 0.65 \(\mu\)). The filter was washed with 2 ml of cold 5% TCA, allowed to dry, and counted in a scintillation counter. Essentially all of the RNA and more than 95% of the radioactivity were eluted from the column by the NaCl gradient.

Assay of Radioactivity. A Packard Tri-carb liquid scintillation spectrometer was used for counting samples. Duplicate 0.1 or 0.2 ml aliquots of each sample were counted in 10 ml of ANPO (15). Correction for quenching was made using automatic external standard ratios. Samples precipitated on Millipore filters were counted using 10 ml of toluene-2,5-diphenyloxazole as the scintillation fluid. Disintegrations per minute were not calculated for the samples on filters.

RESULTS

The effects of a single application of 0.2 ml of 0.5% croton oil in acetone on the rates of synthesis of skin DNA, RNA, and protein are shown in Chart 1. The specific activities of RNA and protein were increased at the earliest times tested, 2 hours.

\(^{3}\) ANPO: a medium containing 295.2 gm of napthalene, 18.4 gm of 2,5-diphenyloxazole, 0.1839 gm of \(\alpha\)-napthylphenyloxazole, 1400 ml of xylene, 1400 ml of dioxane, and 840 ml of ethanol.
Croton Oil Pretreatment and Skin Tumors

Chart 1. The effect of a single application of 0.5% croton oil on the specific activity of mouse skin DNA, RNA, and protein. Groups of 4 mice were treated with 0.2 ml of acetone or 0.2 ml of 0.5% (v/v) croton oil in acetone and killed at the times indicated. Incorporation times for the tritiated precursors were 30 minutes for thymidine-3H (○) and 1 hour for cytidine-3H (△) and leucine-3H (■). Each point represents the average specific activity of the macromolecule in the croton oil-treated mice, expressed as the percent of the average specific activity in the acetone-treated mice. Acetone treatment did not affect the specific activity of skin macromolecules. Calculated as described in Reference 17 and in Materials and Methods, the average specific activity ± the standard deviation in the acetone-treated groups was 49.4 ± 11.7 for DNA, 37.6 ± 8.4 for RNA, and 23.3 ± 7.1 for protein.

Chart 2. The effect of an application of 0.5% croton oil on the amount of DNA or RNA extracted per mouse skin. Groups of 4 mice were treated with 0.2 ml of acetone or 0.2 ml of 0.5% croton oil in acetone and were killed at the times indicated. Each point represents the average yield of DNA (○) or RNA (△) in the croton oil-treated mice, expressed as the percent of the yield in the acetone-treated mice. Although a constant area of skin was not taken from each mouse, the variation in amount of skin used was not great. In the acetone-treated groups, the standard deviations from the average amounts of DNA and RNA were 14.5% and 11.5% respectively.

for RNA and 3 hours for protein. The peaks of specific activity were reached at 6 hours for RNA and 12 hours for protein. The specific activity of DNA was decreased 65% at 2 hours, had begun to increase at 12 hours, and reached a peak 18 hours after croton oil application.

As shown in Chart 2, the amounts as well as the specific activities of DNA and RNA isolated from mouse skin increased after croton oil treatment. The increase in yield of RNA parallels the degree of epidermal hyperplasia (16) and may be accounted for primarily by an increased number of skin cells. The yield of DNA increased sharply beginning at 6 hours, which was about 7 hours before the specific activity of DNA increased to a level greater than the control. This early increase in the amount of DNA, which cannot be due to DNA replication in skin, is probably the result of the migration of leukocytes into the dermis, which has been reported to occur as early as 4-6 hours after croton oil application (16). At later times, when epidermal hyperplasia as well as inflammation is evident, the increase in amount of DNA is due to an increase in the number of epidermal cells and of inflammatory cells. Autoradiographs have shown that very few of the leukocytes are labeled in a 30-minute incorporation time of thymidine-3H; these cells are contributing primarily DNA, not radioactivity, to the skin samples. Since the specific activity of DNA is expressed in terms of dpm per microgram DNA, an increase in the amount of nonepidermal DNA lowers the specific activity of DNA and results in a low estimate of the rate of DNA synthesis after croton oil treatment. The extent of stimulation of DNA synthesis shown in Chart 1 should be regarded as a minimal value.

The data shown in Chart 1 were used to determine a suitable pretreatment time of croton oil in tumor induction experiments. The rates of synthesis of RNA and protein were returning to normal by 2 days, but the increased rate of DNA synthesis remained at a level more than twice that of the control for at least 3 days after croton oil treatment. In order to determine the effect on tumorigenesis of an increased rate of DNA synthesis at and soon after the time of initiator application, a croton oil pretreatment time of 2 days was selected in 4 of the 7 tumor induction experiments.

The results of 3 experiments with DMBA as initiator, 2 experiments with BPL as initiator, and 2 experiments with urethan as initiator are shown in Table 1. A single application of croton oil 2 days before DMBA treatment of the skin (Experiments I and II) clearly caused an early increase in the number of papillomas formed. Considering the maximum number of papillomas per mouse that developed late in the experiment, the croton oil-induced increase in tumor incidence was only about 25%, an increase which is repeatable although of borderline statistical significance. In the experiment in which DMBA was injected i.p. 18 hr after croton oil pretreatment (Experiment III), the increase in the number of papillomas per mouse was nearly 3-fold at early stages of the

The effect on skin tumor incidence of croton oil pretreatment either 18 hr or 2 days prior to initiation with 7,12-dimethylbenz[a]anthracene (DMBA), urethan, or β-propiolactone (BPL). The 0.5% croton oil for pretreatment and solutions of the initiators for skin application were prepared in acetone; 0.2 ml was applied to each mouse with an automatic pipet. The initiators were dissolved in acetone for skin application; 0.2 ml was applied to each mouse once. DMBA for i.p. injection was dissolved in a 1:1 solution of dimethyl sulfoxide:propylene glycol, 300 μg/0.1 ml/mouse, once. Urethan for i.p. injection was dissolved in water, 1 mg/0.01 ml. Solutions of 0.5% croton oil for promotion were prepared in either benzene or acetone: 0.5 ml of croton oil in benzene was given twice per week in Experiments I and IV; 0.1 ml of croton oil in benzene was given once per week in Experiments II and V; and 0.2 ml of croton oil in acetone was given once per week in Experiments III, VI, and VII.

The number of mice is the number surviving at the time the early papilloma yield was recorded. The early papilloma incidence, expressed as papillomas per surviving mouse, is recorded at 16 weeks for Experiments I, II, and VII, 14 weeks for Experiment III, 12 weeks for Experiments IV and VI, and 18 weeks for Experiment V. The late papilloma incidence is recorded at 26 weeks for Experiment I, 22 weeks for Experiment II, 24 weeks for Experiment III and VII, 20 weeks for Experiment VI, 28 weeks for Experiment V, and 18 weeks for Experiment VI. The percent with carcinomas is the cumulative total number of carcinomas divided by the number of mice alive when the first carcinomas were seen. The carcinoma incidence is given for the final week of the experiment, the 40th week after initiation in Experiment I, and the 32nd week in Experiment VI. The tumor induction time, or latent period, is defined as the number of weeks until 50% of the mice developed papillomas or until 20% of the mice developed carcinomas. The tumor induction time of 18 weeks in this control group was unusually long. In 5 other experiments initiated and promoted identically to Experiment IV, the tumor induction times varied from 11 to 16 weeks, with an average of 13 weeks.

experiment. However, as was the case in Experiments I and II, after the number of tumors had plateaued in the pretreated group, the tumor incidence continued to increase in the control group. Furthermore, the incidence of carcinomas was nearly

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<th>Initiator (route of administration)</th>
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<th>Pretreatment and (time)</th>
<th>Number of mice</th>
<th>Papillomas per mouse (early)</th>
<th>Papillomas per mouse (maximum)</th>
<th>Percent with carcinoma</th>
<th>Tumor induction time (weeks)</th>
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doubled when croton oil pretreatment preceded initiation by DMBA, and the tumor induction time for both papillomas and carcinomas initiated by DMBA was shortened in all cases by the croton oil pretreatment.

The number of skin tumors elicited in mice that were treated once with urethan and followed by croton oil applications was increased 3- to 12-fold by a single preliminary application of croton oil 18 hours before urethan treatment (Experiments VI and VII, Table 1). The induction time for both papillomas and carcinomas was shortened several weeks in the croton oil pretreated groups.

In contrast, neither the papilloma incidence nor the tumor induction time was affected by croton oil treatment prior to initiation by BPL (Experiments IV and V). This result suggests that there are differences in the requirements for initiation by a carcinogenic hydrocarbon, urethan, and a carcinogenic alkylating agent.

Since a croton oil application prior to DMBA initiation resulted in an increased papilloma and carcinoma incidence (Table 1, Experiments I and II), the effect of the croton oil pretreatment on the binding of DMBA-3H to skin macromolecules was determined. As shown in Chart 3, the overall binding to skin macromolecules 24 hours after the tritiated DMBA application was not greatly affected by croton oil pretreatment. The level of binding to DNA and RNA was slightly higher, while binding to protein was slightly lower in croton oil-pretreated mice. These data do not exclude the possibility that croton oil pretreatment may increase the level of carcinogen bound in basal cells or to particular regions of DNA, specific RNA molecules, or specific proteins. After fractionation of the RNA from croton oil-pretreated mouse skin on MAK columns, no major differences in the binding to different RNA fractions were apparent (Charts 4, 5). Transfer RNA was eluted from the column first, followed by DNA and ribosomal RNA. Fractionation on MAK columns confirmed the firm binding of tritium from DMBA-3H to skin RNA (7) and demonstrated that the binding to ribosomal RNA was greater than the binding to transfer RNA.

Mottram (21, 22) utilized the initiation-promotion regimen in experiments designed to test the effect on tumor yield of an alteration of the state of the skin at the time of initiator application. Five applications of croton oil, given at 2, 4, 6, 8, and 10 days before a single application of benzo[a]pyrene, greatly increased the yield of tumors. At the time of BP application, the mitotic count was increased about 7.5-fold, which led Mottram to conclude that BP was acting on dividing cells. Since his experiments were performed on small numbers of animals, confirmation of the results was necessary. Berenblum and Shubik (3) found no effect of 2 weeks of croton oil treatment prior to initiation by BP. By regulated starvation, Bielschowsky and Bullough (5) varied the number of epidermal mitoses present at the time of initiation by BP and found that the number of papillomas formed did not correlate with the number of mitoses. Later work showed that multiple croton oil treatments prior to hydrocarbon initiation can result in an increased tumor yield (33). Mottram (23) also found a greater tumor yield when mice
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Chart 5. Methylated albumin kieselguhr column fractionation of mouse skin RNA prepared 24 hours after application of 15 μg (486 μc) of DMBA-3H. The mice were treated with 0.2 ml of 0.5% croton oil in acetone 2 days before application of the initiator. The amount of RNA fractionated was 558 μg. The optical density at 260μm, shown by the solid circles, and the counts per minute, shown by the open circles, are plotted vs the fraction number. Note that the scales in Charts 4 and 5 are not the same; about twice as much RNA was extracted and fractionated from the croton oil-pretreated skins.

were initiated with BP at midnight than when initiated at noon. Ritchie et al. (28), and Frei and Ritchie (12) confirmed this diurnal variation in susceptibility to initiation, showing that more papillomas developed in mice initiated with DMBA at 10:00 P.M. than in those initiated at 10:00 A.M. Since the mitotic frequency was lower and the frequency of epidermal cells synthesizing DNA higher in the hours following 10:00 P.M. than in the hours following 10:00 A.M., these authors suggested that DMBA may act on cells synthesizing DNA.

Shinozuka and Ritchie (31) subsequently found that pretreatment with a single application of croton oil 23 hours before DMBA application resulted in a small increase in the number of papillomas per mouse (4.9 to 6.6) with no apparent effect on the tumor induction time. Since the frequency of epidermal cells synthesizing DNA was increased 3-4 times at the time of initiator application, their findings are compatible with the hypothesis that DMBA acts on epidermal cells which are synthesizing DNA. Frei and Harsono (11) verified the effect of croton oil on DNA synthesis, but their observed effect of croton oil on initiation by DMBA was very slight.

In the experiments reported here, with DMBA as initiator (Table 1), croton oil pretreatment increased the rate of tumor formation, leading to a substantial early stimulation of the number of papillomas per mouse. However, the maximum yield of papillomas per mouse recorded late in the experiment was only increased from 8.3 to 10.1 and from 6.1 to 7.7 in two separate experiments in which the hydrocarbon was applied locally. This increase was comparable to that found by Shinozuka and Ritchie (31). The rate of carcinoma formation and the carcinoma incidence at 40 weeks were both substantially increased by croton oil pretreatment (Experiment I). Unfortunately, the effect on the final carcinoma incidence was not determined.

Although data are lacking, it is likely that systematically administered DMBA is more pulse-like in nature than locally applied DMBA, which is known to remain in the basal cells for at least 3 days and in the skin appendages and surface keratin for as long as 10 days (2, 24). While the inhibiting effects on macromolecular metabolism of locally administered DMBA at the 10- to 16-μ level are not great (1, 14), it is possible that fewer effects irrelevant to initiation might result from systemically administered DMBA, and, therefore, antagonism to the stimulating effect of croton oil pretreatment might be less. The stimulus to early tumor formation by croton oil pretreatment was greater in the experiment in which DMBA was administered i.p. (Experiments III and II) than in those in which DMBA was applied locally (Experiments I and II). This difference may be due to the mode of administration of DMBA but could also be due to the different croton oil pretreatment time (18 hours in Experiment III and 48 hours in Experiments I and II). However, it is clear that differences in the tissue half-life of the free form of the initiator did not correlate with the observed effects; both BPL and urethan are rapidly cleared from tissues, while DMBA is more slowly removed.

After croton oil pretreatment, the degree of binding of tritium from locally applied DMBA-3H to skin DNA, RNA, and protein, and to RNA fractionated on a MAK column was not much different from normal. This result might have been anticipated since croton oil pretreatment had a rather small effect on the maximum papilloma yield, with the greatest effect being the acceleration of tumor formation (Table 1).

One possible explanation for this acceleration is that the mitotic stimulus from croton oil persisted until after the initial interaction of the initiators with DNA or other critical macromolecules. The increased rate of multiplication of the potential tumor cells could result in the earlier formation of a tumor cell clone of sufficient size to develop into a visible tumor. Laws (17) has found that partial hepatectomy of rats at the start of the feeding period of the liver carcinogen, 2-acetylaminofluorene, caused a reduction of the latent period for liver carcinogenesis.

Croton oil pretreatment 2 days before initiation by BPL did not affect tumor formation (Table 1). In contrast to this lack of effect on BPL initiation and the slight stimulation of papilloma formation initiated by DMBA, croton oil pretreatment caused a marked stimulation of tumorigenesis initiated by urethan (Experiments VI and VII). In a series of papers by Pound and coworkers (25-27), it was demonstrated that applications of 0.5% croton oil from 12 hours to 3 days before an initiating injection or topical application of urethan results in a greatly enhanced tumor yield. Application at other times (from 3 to 9 hours and from 4 to 14 days) did not affect the papilloma yield. It is of interest that the time period after croton oil treatment at which urethan initiation was stimulated corresponded closely to the time period during which DNA synthesis was stimulated by 0.5% croton oil in our mice,
and it bears no apparent relationship to the rate of RNA or protein synthesis.

The differences in the effect of croton oil pretreatment on initiation by DMBA, BPL, and urethan suggest that the detailed mechanism by which these initiators act may differ. DNA has been considered by several workers to be the molecular target of each of these initiators (7, 8, 10, 29). Assuming that an alteration in DNA is necessary for initiation, there are at least 3 mechanisms by which an increased rate of DNA synthesis could result in an increased extent of initiation: (a) The initiator may interact with a DNA precursor; the altered precursor is dependent on DNA synthesis for incorporation into DNA. (b) Replicating DNA may be more accessible than nonreplicating DNA to the initiator. (c) After the initial interaction of the initiator with DNA, the alteration must be replicated and thus fixed in a stable, replicable form before repair occurs.

One or more of these mechanisms may be involved in the enhancement by croton oil pretreatment of tumor initiation by DMBA and urethan but appear not to be involved in initiation by BPL.

The enhancement of DMBA or urethan initiation by croton oil pretreatment is consistent with the hypothesis that the rate of DNA synthesis is important for initiation by these compounds. The major effect of croton oil which has been discussed is the stimulation of DNA synthesis. However, many other effects of croton oil, such as stimulations of RNA and protein synthesis (Chart 1), increases in the amount of nucleic acid (Chart 2), induction of inflammation and influx of leukocytes (13, 16), and stimulation of cell division (16) more or less parallel the effects on DNA synthesis. In order to determine whether DNA synthesis is in fact important for initiation, specific inhibitors of DNA synthesis such as hydroxyurea should be tested alone and in conjunction with croton oil for their effect on initiation by DMBA, BPL, urethan, and other initiators. In addition, other ways of stimulating DNA synthesis at the time of initiator application should be tested. The correct interpretation of the croton oil pretreatment experiments depends on the results of the additional experiments.

ACKNOWLEDGMENTS

The authors acknowledge the valuable technical assistance of Miss Jackie Negri, Mrs. Kari Haugli, and Mrs. Julie Corbett.

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