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Inhibition by Actinomycin D of DNA and RNA Synthesis and of Skin Carcinogenesis Initiated by 7,12-Dimethylbenz[a]anthracene or β-Propiolactone[1]

Henry Hennings, Harold C. Smith, Nancy H. Colburn, and R. K. Boutwell

McArdle Laboratory for Cancer Research, University of Wisconsin Medical Center, Madison, Wisconsin 53706

SUMMARY

Application of 84 μg of actinomycin D inhibits skin tumor formation initiated by either 7,12-dimethylbenz[a]anthracene (DMBA) or β-propiolactone (BPL). Treatment with this dose of actinomycin D inhibits the binding of DMBA to skin DNA by 40%, but does not affect BPL binding to DNA. Inhibition of binding of initiators to DNA cannot explain more than a small part of the inhibition of tumor formation by actinomycin D. A dose of 10 μg of actinomycin D inhibits tumor formation by 40-50% when given the same day as the initiator (DMBA or BPL) or 1 or 7 days later. Application of this dose of actinomycin D inhibited RNA synthesis only slightly for about 12 hours (6-18 hours after treatment), but inhibited DNA synthesis by 75-90% for at least 2 days (from 24-72 hours after treatment). The inhibition of tumor formation by actinomycin D may be related to its inhibition of the incorporation of thymidine-^{3}H into skin DNA. It is not known whether this block of DNA synthesis is reversible or irreversible in potential tumor cells.

INTRODUCTION

In experiments utilizing the initiation-promotion procedure (2, 5) for producing skin tumors, topical application of actinomycin D was found to inhibit the initiation of papilloma (14-16) and carcinoma (16) formation in mouse skin. Tumor formation was inhibited by actinomycin D applied more than a month after the initiator to nearly as great an extent as actinomycin D applied the same day as the initiator (16). The major metabolic effects of actinomycin D are the inhibition of the DNA-dependent syntheses of RNA and DNA (23). In addition to the expected inhibition of RNA synthesis (11, 15), treatment with high, tumor-inhibitory levels of actinomycin D caused necrosis (11, 15, 16), hyperplasia (11), an altered mitotic index (11), and an increased uptake of leucine-^{3}H (11, 15) in mouse skin. The tumor-inhibitory action of actinomycin D may be due to the destruction of initiated cells or to the inhibition of metabolic processes necessary for tumor formation in skin; the mechanism by which actinomycin D inhibits skin tumor formation is not clear.

It is the purpose of this communication to report the results of further experiments with actinomycin D. In tumor induction experiments, three parameters were varied: (*a*) the dose of actinomycin D, (*b*) the time of actinomycin application with respect to the time of initiator application and (*c*) the initiator used. In order to test some of the possible explanations for the tumor-inhibitory action of actinomycin D, the effects of actinomycin treatment on the binding of tritiated initiators to DNA and on the rates of RNA and DNA synthesis in mouse skin were investigated.

MATERIALS AND METHODS

Animals. Female STS[2] mice were obtained from the A. R. Schmidt Co., Madison, Wisconsin. In most experiments, mice 7-8 weeks old were used.

Chemicals. 7,12-Dimethylbenz[a]anthracene was obtained from Eastman Organic Chemicals. β-Propiolactone was purchased from Fellows Medical Manufacturing Co. Actinomycin D was kindly provided by Merck, Sharpe and Dohme.

Radiochemicals. Thymidine-methyl-^{3}H (3.0 c/mmole), uridine-^{3}H (8.0 c/mmole), and cytidine-^{3}H (3.0 c/mmole) were obtained from Schwartz BioResearch, Inc. Orotic acid-^{3}H (5.0 c/mmole), DMBA-^{3}H (15.1 c/mmole, greater than 97% radiochemically pure), and BPL-^{3}H (169-202 mc/mmole, greater than 95% radiochemically pure) were obtained from Nuclear-Chicago Corporation.

Radioactivity Measurements. A Packard Tri-carb liquid scintillation spectrometer (model 3375) was used for counting samples. Duplicate 0.2-ml aliquots of each sample were counted in 10 ml of ANPO (a solution containing 295.2 gm of naphthalene, 18.4 gm of 2,5-diphenyloxazole, 0.1839 gm of α-naphthylphenyloxazole, 1400 ml of xylene, 1400 ml of dioxane, and 840 ml of ethanol). Correction for quenching was made using automatic external standard ratios.

Binding Experiments. DMBA-^{3}H was diluted with nonradioactive DMBA zero- to 5-fold; the specific activity of the

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[2] Abbreviations used are: BPL, β-propiolactone; DMBA, 7,12-dimethylbenz[a]anthracene; STS, skin tumor susceptible mice, obtainable from A. R. Schmidt Co., Madison, Wisconsin; PCA, perchloric acid; mRNA, messenger RNA.

applied dose varied from 11.8 to 59 μc/μg. At zero time, 12 μg of DMBA-^{3}H per mouse were applied to the skin of the back by syringe in 0.3 ml of anhydrous acetone solution.

BPL-^{3}H was diluted with nonradioactive BPL to give a specific activity of 0.4 to 0.6 μc/μmole. At zero time, 480 μmoles of BPL-^{3}H per mouse were applied by syringe in 0.3 ml of anhydrous acetone solution.

For the experiments shown in Chart 2, the DNA was extracted by a modified Marmur procedure (18) and the specific activity determined according to methods previously described (9).

For the experiment illustrated in Table 3, the skin DNA, RNA, and protein were extracted according to a modified Kirby procedure (17) and the specific activity determined according to methods which will be described elsewhere (N. H. Colburn and R. K. Boutwell, Cancer Research, in press).

Treatment of Mice. The mice were shaved 1 or 2 days before use; only those mice in which there was no hair regrowth were used in biochemical experiments. A volume of 0.2 ml of acetone, when applied with an automatic pipetter, covered the shaved area; in most experiments solutions of initiators and of actinomycin D were prepared in acetone concentrations appropriate for the delivery of the desired amount of chemical to the mouse's back in 0.2 ml. Mice treated with 84 μg of actinomycin were given 14 μg doses at 7:00 A.M., 10:00 A.M., 1:00 P.M., 5:00 P.M., 8:00 P.M., and 11:00 P.M. Mice treated with 15 μg of actinomycin were given 5 μg doses at 11:00 A.M., 2:00 P.M., and 5:00 P.M. Zero time, the time of application of the initiator in tumor induction experiments, was 3:00 P.M. Lower doses of actinomycin (0.1, 1, 3, or 10 μg) were given as a single application; control animals were treated with 0.2 ml of acetone.

Determination of Specific Activity of DNA or RNA *in Vivo*. In experiments in which the specific activity of DNA was measured, the mice were sacrificed 20 minutes after the intraperitoneal injection of 60 μc of thymidine-^{3}H, and the DNA was extracted by the method of Orlov and Orlova (20).

In experiments in which the specific activity of RNA was determined, the mice were killed 1 hour after the intraperitoneal injection of 20 μc of cytidine-^{3}H or 60 μc of orotic acid-^{3}H. A modified Schmidt-Thannhauser procedure (24) was used for obtaining skin RNA and/or DNA hydrolysates in both *in vivo* and *in vitro* experiments. Mice were killed by cervical dislocation. The skin from each mouse's back was removed, pinned hair side down to a large cork, frozen in liquid nitrogen, and scraped with a scalpel to remove muscle and fat of the dermis (29). The scraped skin was homogenized in ice water and the nucleic acids and proteins precipitated by adding concentrated perchloric acid to a final concentration of 0.4 N. Following centrifugation, the supernatant (acid soluble fraction) was decanted. The precipitate was washed 6 times with 0.2 N PCA at 4°C, twice with ethanol, and twice with ether at room temperature; the RNA was hydrolyzed in 0.3 N KOH at 37°C for 3 hours. At 4°C, the suspension was acidified with PCA to precipitate DNA and protein; following centrifugation, the RNA hydrolysate was decanted. After 2 washes with 0.4 N PCA to remove any remaining hydrolyzed RNA, the DNA was hydrolyzed by suspending the precipitate in 0.5 N PCA and heating at 90°C for 5 minutes. After centrifugation at 4°C, the DNA hydrolysate was decanted.

A 0.2-ml aliquot of the RNA or DNA hydrolysate was counted in a scintillation counter as described above; a second aliquot was used for measuring the amount of RNA by the orcinol reaction (25) or the amount of DNA by the diphenylamine reaction (7). Amounts of RNA or DNA were determined from standard curves using yeast RNA (Nutritional Biochemicals Co.) or salmon sperm DNA (Calbiochem) as a standard. The specific activity of RNA or DNA was expressed as

$$\frac{\text{dpm}/\mu\text{g RNA or DNA}}{\mu\text{c injected/gm body weight}}.$$

Determination of Specific Activity of DNA or RNA *in Vitro*. The procedure used was based on that described by Flamm *et al.* (11, 12). After application of actinomycin D or acetone to the backs of mice *in vivo*, the mice were sacrificed, the skins removed and scraped at 4°C to remove dermal muscle and fat. The scraped skin was cut with a razor blade into strips approximately 3 mm wide. Strips from a single skin were added to a 25-ml flask containing either 2 μc of uridine-^{3}H or 5 μc of thymidine-^{3}H in 5 ml of Eagle's medium (with Earle's salts, without serum, obtained from the Grand Island Biological Co.). After 2 hours incubation at 37°C in an oscillating water bath, the flasks were placed on ice; the skin strips were removed, blotted, and frozen in liquid nitrogen until they were homogenized and processed by the modified Schmidt-Thannhauser procedure described above. The specific activity of RNA or DNA was expressed as $\frac{\text{dpm}}{\mu\text{g RNA or DNA}}$.

RESULTS

Papilloma formation was inhibited 40–45% when a single application of 10 μg of actinomycin D was given the same day as DMBA or 1 or 7 days later; carcinoma formation was inhibited at all times tested (Table 1). Doses of 1 or 0.1 μg of actinomycin D had no consistent inhibitory effect on papilloma formation; that part of the experiment was terminated before many carcinomas had developed.

Papilloma and carcinoma formation initiated by an alkylating agent, BPL, were inhibited by doses of actinomycin D of 10, 15, and 84 μg (Chart 1) to about the same extent as in experiments in which DMBA was the initiator (14–16; Table 1). There was a suggestion of inhibition of tumor formation by 1 μg of actinomycin given 1 or 7 days after BPL (Table 2); this experiment remains to be verified. No tumor-inhibitory effects were seen with 0.1 μg of actinomycin D.

The degree of binding to skin DNA of carcinogens, such as DMBA and BPL, has been reported to correlate with initiation of skin tumor formation (6, 9). In Chart 2 is shown the effect of actinomycin D, given on the same day as DMBA-^{3}H or BPL-^{3}H, on the level of tritiated initiator firmly bound to skin DNA. A dose of 15 μg of actinomycin D, which inhibits papilloma formation by 65% (15), had little effect on DMBA binding to skin DNA, but 84 μg of actinomycin D inhibited DMBA binding to DNA by about 40%. Neither high nor low tumor-inhibitory doses of actinomycin D affected the degree of binding of BPL-^{3}H to skin DNA. In an additional ex-

Table 1

Dose of actinomycin D (μg)	Time of actinomycin D	No. of mice (Week 18)	Papillomas per mouse (Week 18)	Effectual total (Week 18)	% with carcinoma (Week 34)
None		32	5.7	32	28
10	Day −2	26	6.3	26	15
10	Hour −2	28	3.3	28	18
10	Day +2	28	3.1	28	18
10	Day +7	28	3.4	28	14
1	Day −2	28	6.4		
1	Hour −2	25	5.6		
1	Day +1	29	8.1		
1	Day +7	25	5.5		
0.1	Day −2	28	6.6		
0.1	Hour −2	29	7.1		
0.1	Day +1	30	5.5		
0.1	Day +7	27	3.9		

The effect of low doses of actinomycin D on tumor formation initiated by 7,12-dimethylbenz[*a*]anthracene (DMBA). Mice were initiated by a single treatment with 15 μg of DMBA in 0.2 ml of acetone and promoted by twice-weekly treatment with 0.05 ml of 0.5% croton oil in benzene, beginning at Week 4. Single applications of 0.1, 1, or 10 μg of actinomycin D in 0.2 ml of acetone were given either 2 days or 2 hours before or 1 or 7 days after treatment with DMBA. Tumors were counted weekly in all groups. *Papillomas per mouse* is the number of papillomas divided by the number of surviving mice in each group. Effectual total is the number of mice alive in each group when the first carcinoma was seen in the experiment. *Percent with carcinoma* is the cumulative total number of carcinomas divided by the effectual total number of mice, expressed as percent.

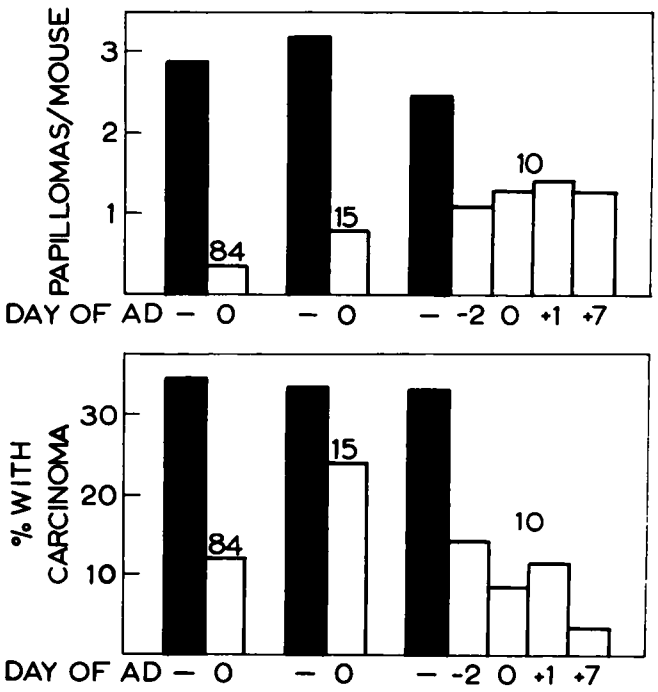

Chart 1. The effect of 10, 15, and 84 μg of actinomycin D (AD) on tumor formation initiated by β-propiolactone (BPL). Groups of 30 mice were initiated by a single treatment with 0.2 ml of 15% BPL (480 μmoles) in acetone and promoted by twice weekly treatment with 0.05 ml of 0.5% croton oil in benzene, beginning at Week 4. Doses of 84 μg (6 applications of 14 μg) and 15 μg (3 applications of 5 μg) of actinomycin D were given on the same day as the BPL; single applications of 10 μg of actinomycin D were given either 2 days or 2 hours before, or 1 or 7 days after treatment with BPL. Tumor incidence in the BPL-initiated, croton oil-promoted control groups is shown by the *solid bars*; tumor incidence in the groups which received the additional treatment with actinomycin D, at the times and doses indicated, is shown by the *open bars*.

periment, 15 μg of actinomycin D did not affect the binding of BPL-^{3}H to skin DNA, RNA, or protein (Table 3).

The degree of inhibition of tumor formation was somewhat greater when high levels of actinomycin D were given the same day as DMBA than when they were given at later times (Table 4; also see data in Reference 16). Perhaps this difference in degree of inhibition can be explained by the inhibition by actinomycin D of the binding of DMBA to skin DNA. When given 14 days or longer (16) after the initiator, actinomycin D cannot affect the initial binding of DMBA to DNA as it can when given on the day of initiation.

The effect of 84 μg of actinomycin D on the specific activity of RNA was determined after intraperitoneal injection of orotic acid-^{3}H *in vivo* or addition of uridine-^{3}H *in vitro* (Chart 3). When measured *in vivo*, the specific activity decreased about 25% at 10 hours, then increased rapidly, reached a peak 3 days after treatment, and returned to normal by 14 days (not shown). When measured *in vitro*, the specific activity was decreased by 25% at Day 1; this level of inhibition was maintained for at least 7 days.

The apparent explanation for the discrepancy between *in vivo* and *in vitro* data is shown in Chart 4, in which cytidine-^{3}H was used as precursor *in vivo*. (Results with cytidine-^{3}H duplicated those with orotic acid-^{3}H *in vivo*). Scintillation counting of acid-soluble fractions revealed that nearly twice as much tritium was taken up in the actinomycin-treated skins 3 days after treatment, paralleling the increase in specific activity of

Table 2

Dose of actinomycin D (μg)	Time of actinomycin D	No. of mice (Week 20)	Papillomas per mouse (Week 20)	Effectual total (Week 16)	% with carcinoma (Week 39)
None		27	2.4	27	31
1	Day −2	28	3.5	28	18
1	Hour −2	21	3.2	24	50
1	Day +1	28	1.5	28	11
1	Day +7	25	2.0	27	19
0.1	Day −2	29	3.1	29	31
0.1	Hour −2	20	3.2	27	44
0.1	Day +1	26	2.2	26	35
0.1	Day +7	25	3.5	26	27

The effect of 0.1 and 1 μg of actinomycin D on tumor formation initiated by β-propiolactone. Mice were initiated and promoted as described under Chart 1. Single applications of 0.1 or 1 μg of actinomycin D were given either 2 days or 2 hours before or 1 or 7 days after treatment with β-propiolactone. Column headings are defined under Table 1.

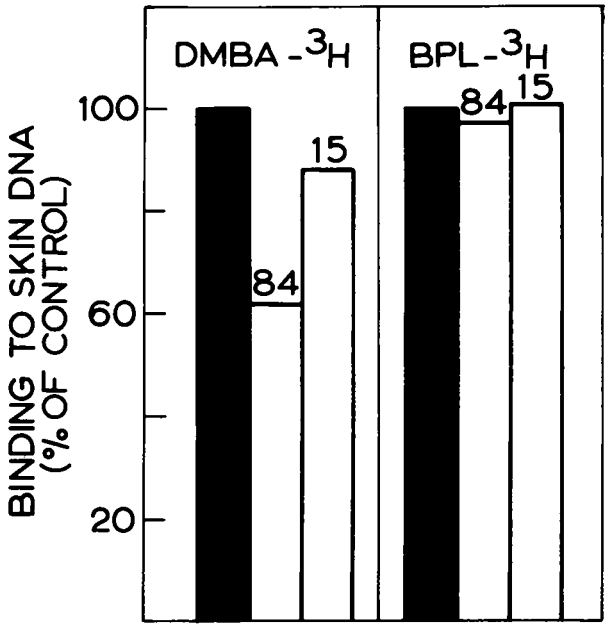

Chart 2. The effect of 84 or 15 μg of actinomycin D on the degree of binding of 7,12-dimethylbenz[*a*]anthracene-^{3}H (DMBA-^{3}H) or β-propiolactone-^{3}H (BPL-^{3}H) to skin DNA. The degree of tritiated initiator bound to DNA (calculated as μmoles of initiator per gm of DNA) is shown by the *solid bars* for the acetone-treated control groups and by the *open bars* for the actinomycin-treated groups. Mice were killed 4 hours after BPL-^{3}H treatment in the group treated with 15 μg of actinomycin D and 24 hours after tritiated initiator treatment in the other groups.

the RNA *in vivo*. This effect on the acid-soluble fraction was first seen 24 hours after actinomycin treatment. Therefore, the *in vivo* specific activity of RNA in the skins of mice treated with 84 μg of actinomycin D cannot validly be compared with the specific activity of RNA in the acetone-treated controls. In the *in vitro* experiments, there was no facilitated uptake of the tritiated precursor in the actinomycin-treated skins; the tritiated precursor available to each skin was determined by the amount added to the incubation medium. Apparently the specific activity of RNA measured *in vitro* is a valid measure of the rate of RNA synthesis.

Table 3

Macromolecule	Degree of binding: dpm/μg, Acetone	dpm/μg, 15 μg actinomycin D	% of control, 15 μg actinomycin D
DNA	5.6	6.2	111
RNA	8.3	8.9	107
Protein	12.8	14.9	116

The effect of 15 μg of actinomycin D on the binding of β-propiolactone-^{3}H to DNA, RNA, and protein in mouse skin. Mice were treated with acetone or 5 μg of actinomycin D 4 hours before, 1 hour before, and 2 hours after a single application of 480 μmoles of β-propiolactone-^{3}H. They were killed 4 hours after the β-propiolactone-^{3}H application. The degree of binding is expressed as dpm per μg of macromolecule.

The effect of 10 μg of actinomycin D on the specific activity of RNA was measured *in vivo* and *in vitro* (Chart 5). With this dose of actinomycin D, the same result was obtained regardless of the precursor or procedure used (orotic acid-^{3}H or cytidine-^{3}H *in vivo* or uridine-^{3}H *in vitro*). By 6 hours after treatment, the specific activity was reduced about 25%; this degree of inhibition was maintained for about 12 hours. Actinomycin D, at 0.1 and 1 μg, had no effect on the specific activity of RNA (Table 5).

The effect of 84 μg of actinomycin D on DNA synthesis was measured *in vivo* (Chart 6). The specific activity of DNA decreased rapidly after actinomycin treatment and remained at a low level for 5 days. By 7 days the average specific activity returned to a normal level. In *in vitro* experiments, 84 μg of actinomycin D produced a similar degree of inhibition (Table 6). Treatment with 10 μg of actinomycin D inhibited the *in vivo* incorporation of thymidine-^{3}H into skin DNA by 75–90% from 1 to 3 days after treatment (Chart 7). The specific activity of DNA was increased by 5 days and returned to normal levels by 7 days. Of lower actinomycin doses tested, only 3 μg caused an inhibition, followed by a stimulation, of DNA synthesis (Table 7); 0.1 and 1 μg did not affect DNA synthesis.

In experiments in which actinomycin D was applied *in vivo*, it was not clear whether the synthesis of RNA or DNA was inhibited first in mouse skin. In order to determine the early effects of actinomycin D on RNA and DNA synthesis in skin, strips of untreated skin were incubated in 5 ml of Eagle's

Table 4

Group	Dose of actinomycin D (μg)	Time of actinomycin D	No. of mice (Week 0)	No. of mice (Week 32)	Papillomas per mouse (Week 32)	% Inhibition
1	None		40	30	5.9	
2	84	Day 0	29	16	1.0	83
3	84	Day 14	29	16	2.3	61

The effect of 84 μg of actinomycin D, given Day 0 or Day 14, on tumor formation initiated by 7,12-dimethylbenz[*a*]anthracene (DMBA). Mice were initiated by application of 18 μg of DMBA. The mice in Group 1 were not treated with actinomycin D. The mice in Group 2 were treated with 14 μg of actinomycin D at 8, 5, and 2 hours before and 2, 5, and 8 hours after application of DMBA. An identical dose schedule of actinomycin D was used for Group 3, but the actinomycin D was applied 14 days after DMBA. All 3 groups were promoted with twice-weekly applications of 0.05 ml of 0.5% croton oil, beginning at Week 16. Column headings are explained under Table 1.

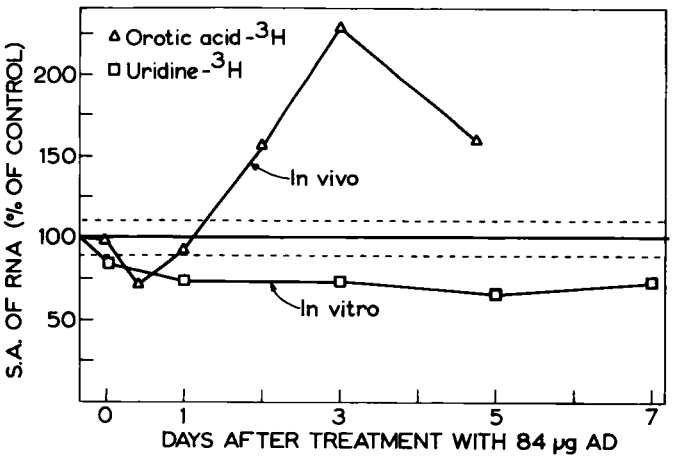

Chart 3. The effect of 84 μg of actinomycin D (AD) on the specific activity of RNA, determined *in vivo* and *in vitro*. Acetone or 14 μg of actinomycin D was applied to groups of mice 6 times in 16 hours. The mice killed at "0" time were killed 2 hours after the third application of acetone or actinomycin; the mice in all other groups were treated 6 times and killed at the indicated times after zero time. Each point on the graph represents the average specific activity (S.A.) of RNA in 4 actinomycin-treated animals, expressed as the % of the average specific activity of RNA in 4 acetone-treated control animals. The average deviation from the average for all of the control animals is indicated by the *dashed lines*. △, mice injected with orotic acid-^{3}H 1 hour before killing. □, skins incubated at 37°C for 2 hours in Eagle's medium containing 2 μc of uridine-^{3}H.

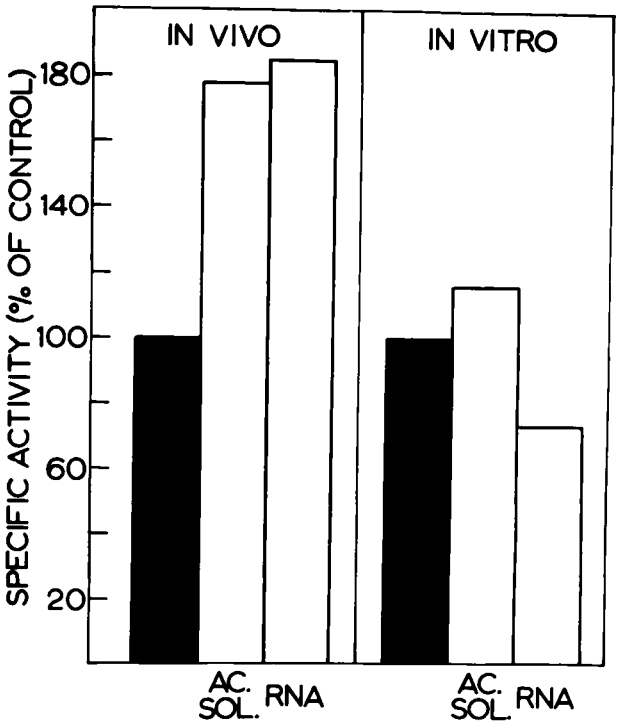

Chart 4. The effect of 84 μg of actinomycin D on the specific activity of RNA and acid soluble fractions 3 days after treatment. Mice were treated as described in Chart 3, except that cytidine-^{3}H was injected i.p. instead of orotic acid-^{3}H. The specific activity of each acid soluble (AC. SOL.) fraction was calculated as dpm/optical density at 260 mμ × 10^3. The specific activities in the skins of the acetone-treated control mice, represented by the *solid bars*, were set at 100%; the specific activities in the skins of the actinomycin-treated mice, represented by the *open bars*, were expressed as % of control.

medium containing 2.5 μg of actinomycin per ml. After 2 hours of incubation in the presence of actinomycin D, either uridine-^{3}H or thymidine-^{3}H was added and the incubation continued for an additional 2 hours. Determination of the specific activities of RNA and DNA revealed that RNA synthesis was inhibited first *in vitro*, while there was no effect on DNA synthesis (Chart 8).

As reported previously (16), 84 μg of actinomycin D given 34 days after DMBA inhibits tumor formation by about 70% only if croton oil treatments are delayed until after actinomycin treatment. If croton oil treatments are begun at Week 1, actinomycin D has little effect on tumor formation (Chart 9). To see whether there was a correlation between the degree of inhibition of tumor formation and the degree of inhibition of DNA synthesis by actinomycin D, we determined the specific activity of DNA in mice treated similarly to those in the tumor experiment shown in Chart 9. The results are shown in Chart 10. In mice treated once with DMBA, but not with croton oil, actinomycin treatment reduced the specific activity of DNA by about 95%; in mice treated once with DMBA and 8 times with croton oil prior to actinomycin treatment, the specific activity of DNA was reduced only about 15%.

DISCUSSION

In order to discuss the possible mechanisms by which actinomycin D inhibits skin tumor formation, it will be helpful to present our working hypothesis for what may be occurring at the molecular level during the initiation[3] of skin carcinogenesis. The hypothesis presented is certainly not proved, but it provides a framework within which to design and perform experiments.

A single treatment with an initiator causes a permanent alteration in mouse skin; groups of mice in which croton oil treatments were begun 10 months after application of DMBA gave a tumor incidence similar to that of groups in which croton oil treatments were begun soon after DMBA (3). If the initiator acts directly on those cells which will eventually become tumor cells, it is reasonable to assume that the initiator interacts with cellular macromolecules in such a way that the potential for development of a few cells (the initiated cells) is changed. This heritable change, caused by the initiator, can be envisioned most simply as an alteration in DNA, although

[3] The term initiation refers to the change made in a skin cell which may be revealed by tumor formation upon exposure of the skin to a promoting agent. Although initiation may be accomplished by a single exposure to an agent with an *in vivo* half-life of less than 1 hour (e.g., β-propiolactone), it is likely that a sequence of structural and metabolic events comprise initiation, and initiated cells may form over a period of a week or more after exposure to the initiating agent.

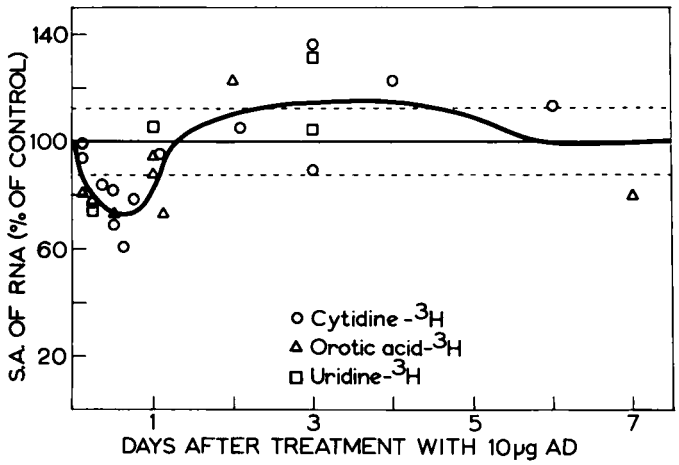

Chart 5. The effect of 10 μg of actinomycin D (AD) on the specific activity of RNA. Mice were treated with acetone or 10 μg of actinomycin D and killed at the times indicated. In the *in vivo* experiments, either cytidine-^{3}H (○) or orotic acid-^{3}H (△) was injected i.p. 1 hour before killing. In the *in vitro* experiments, skins were incubated for 2 hours in medium containing uridine-^{3}H (□). Each experimental point represents the specific activity (S.A.) of RNA in 4 actinomycin-treated animals, expressed as % of the average specific activity of RNA in 4 acetone-treated controls. The average deviation from the average, expressed as %, for all of the control animals is indicated by the *dashed lines*.

interaction of the initiator with specific proteins could produce such a change (21). Experiments on the binding of tritiated hydrocarbons (6) and of BPL-^{3}H (9) to DNA have suggested a correlation between the degree of binding of these initiators to DNA and their ability to initiate skin tumor formation. Other workers (13, 22, 26) have suggested that the rate of DNA synthesis is important in the initiation of skin tumors. Based on *in vitro* transformation experiments with hamster embryo cells, Borek and Sachs have suggested that ". . . transformation induced by irradiation, and presumably also by chemicals, requires replication of some cellular constituent that has been changed by the carcinogen, such as DNA, and that this has to occur before the change is repaired by an error-correcting mechanism of the type described for changes induced by ultraviolet light or alkylation" (4).

Based on the above considerations and others, our working hypothesis to explain the initiation of skin carcinogenesis is the following: First, the initiator interacts with DNA and alters it. The extent and type of interaction may be affected by the configuration or the rate of replication of the DNA, as well as the nature of the initiator. Second, the alteration is fixed in a form capable of being faithfully replicated. A competing process is that of repair of the alteration; fixation must occur before repair if the cells are to be initiated. In a way not yet determined, promotion accomplishes selection for the initiated cells, resulting in a visible tumor.

The data of Gelboin *et al.* (15) suggested that actinomycin inhibited the formation or establishment of initiated cells; treatment with actinomycin D was tumor-inhibitory only at times shortly after treatment with the initiator. Based on their data, one could suggest at least two possible explantations for the tumor-inhibitory action of actinomycin D:

1. Inhibition of binding of the initiator to DNA (or other target molecules).
   a. Interaction of the initiator and actinomycin D.
   b. Inhibition of the conversion of the applied initiator to its active form.
   c. Stimulation of the detoxication of the initiator.
   d. Competition for binding sites on the DNA.
   e. Alteration of the conformation of the DNA.
   f. Inhibition of macromolecular synthesis important in the interaction of the initiator and DNA.
2. Inhibition of processes, occuring while the initiator is bound to DNA, necessary for the establishment of cells in a permanently initiated state.
   a. Stimulation of a repair mechanism which would excise the altered part of the DNA molecule before the alteration was fixed in a form capable of being replicated.
   b. Inhibition of macromolecular synthesis (or cell division) necessary for fixation of an alteration in DNA (4, 28).

Killing of the potential tumor cells appeared to be unlikely as an explanation for the tumor-inhibitory action of actinomycin D since actinomycin treatment long after initiation was apparently not tumor-inhibitory (15).

It was subsequently shown (16) that actinomycin treatment inhibited tumor formation when given more than a month after initiator application, provided that croton oil treatments were not begun until after the actinomycin treatment. This result eliminated Item 1 above as an explanation for the major tumor-inhibitory action of actinomycin D; inhibition of DNA binding can only explain the slightly greater degree of inhibition found when actinomycin D was given at Day zero as opposed to actinomycin given at later times. The initiator may be bound to DNA at a low, critical level for a long period of time, similar to the binding of *N,N*-dimethyl-4-aminoazobenzene to liver DNA (28); Item 2 remains as a

Table 5

Treatment	Specific activity of RNA ± standard deviation			
	3 hours	6 hours	26 hours	50 hours
Acetone	22.8 ± 2.1	21.4 ± 5.4	28.0 ± 3.6	33.0 ± 2.9
Actinomycin D, 1 μg	22.6 ± 2.2	20.3 ± 1.8	34.6 ± 10.4	32.1 ± 10.2
Actinomycin D, 0.1 μg	30.5 ± 5.6	24.8 ± 4.9	34.1 ± 4.8	37.7 ± 9.4

The effect of 0.1 or 1 μg of actinomycin D on the rate of RNA synthesis in mouse skin. Mice were treated with acetone or 0.1 or 1 μg of actinomycin D at the times indicated. One hour before killing, mice were injected i.p. with 20 μc of cytidine-^{3}H. The specific activity of RNA was calculated for each skin; the average specific activity ± the standard deviation is indicated for each group of 4 animals.

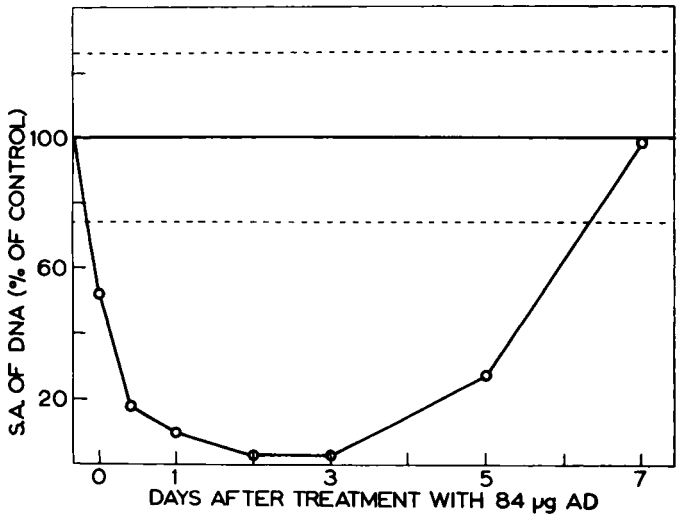

Chart 6. The effect of 84 μg of actinomycin D (AD) on the specific activity of DNA. Groups of 4 mice were treated with acetone or 84 μg of actinomycin D and killed at the times indicated. Thymidine-^{3}H was injected i.p. 20 minutes before killing. The specific activity (S.A.) of DNA in skins from actinomycin-treated mice is shown as % of the average specific activity of DNA in the skins from the 28 acetone-treated controls. (As shown in Table 7, the specific activity of DNA in skins from acetone-treated mice did not change with time after treatment.) The average deviation from the average for the control animals is shown by the *dashed lines*.

Table 6

Treatment	Specific activity of DNA ± standard deviation	
	1 day	3 days
Acetone	10.7 ± 1.0	14.2 ± 5.2
Actinomycin D, 84 μg	0.84 ± 0.7	2.13 ± 2.4

The effect of 84 μg of actinomycin D on the *in vitro* rate of DNA synthesis. Groups of 4 mice were treated with acetone or 84 μg of actinomycin D either 1 or 3 days before killing. Skins were incubated at 37°C for 2 hours in Eagle's medium containing 5 μc of thymidine-^{3}H. The average specific activity of DNA (expressed as dpm/μg of DNA) ± the standard deviation is shown for each group.

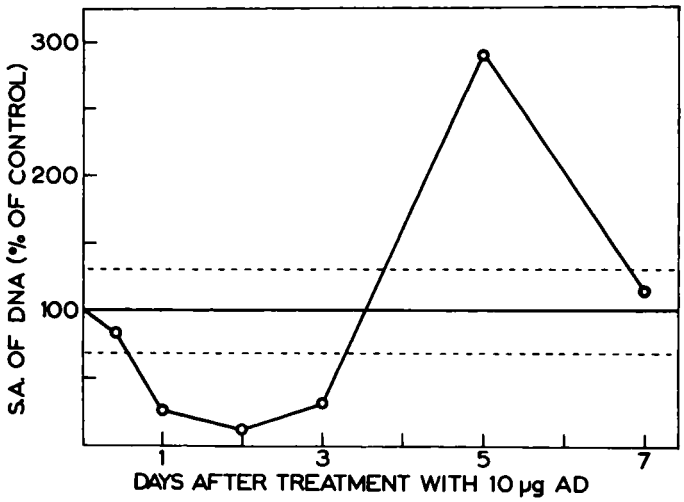

Chart 7. The effect of 10 μg of actinomycin D (AD) on the specific activity of DNA. Mice were treated with acetone or 10 μg of actinomycin D and killed at the times indicated. The specific activity (S.A.) of DNA in the skins from actinomycin-treated mice is expressed as % of the average specific activity of DNA in skins from the 24 acetone-treated mice. The *dashed lines* indicate the average deviation from the average in all control animals. The standard deviations for 5 points on the graph are shown in Table 7.

possible explanation for the action of actinomycin D. Our results (16) suggested additional possibilities for explaining the tumor-inhibitory action of actinomycin D:

3. Inhibition of macromolecular synthesis necessary for maintenance or evolution of initiated cells.
4. Prevention of division of initiated cells.
5. Destruction of initiated cells.

Some of the above possibilities have been tested experimentally; the effects of actinomycin D, at various dose levels, on RNA, DNA, and protein synthesis have been determined in mouse skin.

High doses of actinomycin D (84–90 μg) were reported to inhibit RNA synthesis by 58–90% for as long as 11 days (11, 12, 15). In STS mice, it was found that application of 84 μg of actinomycin D inhibited RNA synthesis by 25–35% for at least 7 days (Chart 3). The lowest actinomycin dose found to inhibit RNA synthesis was 10 μg; a 20–40% inhibition was maintained for about 12 hours (Chart 5). A single application of 1 μg of actinomycin D was reported to inhibit RNA synthesis by 75% for 3 days (11). In the present experiments, 1 μg of actinomycin D did not affect RNA synthesis (Table 5).

Table 7

Treatment	Specific activity of DNA ± standard deviation					
	6 hours	1 day	2 days	3 days	5 days	7 days
Acetone	15.0 ± 5.1	16.1 ± 6.4	16.3 ± 6.8	17.7 ± 0.9	13.8 ± 3.1	20.4 ± 4.2
Actinomycin D, 10 μg		4.7 ± 1.7	2.2 ± 1.0	5.7 ± 2.6	51.3 ± 6.3	23.5 ± 15.0
Actinomycin D, 3 μg	7.9 ± 3.6	12.3 ± 5.6	9.2 ± 1.2	13.7 ± 3.2	24.4 ± 3.6	11.1 ± 2.8
Actinomycin D, 1 μg		13.6 ± 4.1	15.0 ± 5.6	19.4 ± 4.5	18.4 ± 1.6	15.0 ± 5.4
Actinomycin D, 0.1 μg	18.1 ± 3.9	21.6 ± 5.8	23.5 ± 3.0	17.4 ± 5.2		

The effect of low doses of actinomycin D on the *in vivo* rate of DNA synthesis. Groups of 4 mice were treated with acetone or 0.1, 1, 3, or 10 μg of actinomycin D at the times indicated. All mice were injected i.p. with 60 μc of thymidine-^{3}H 20 minutes before killing. The average specific activity of DNA ± the standard deviation is shown for each group.

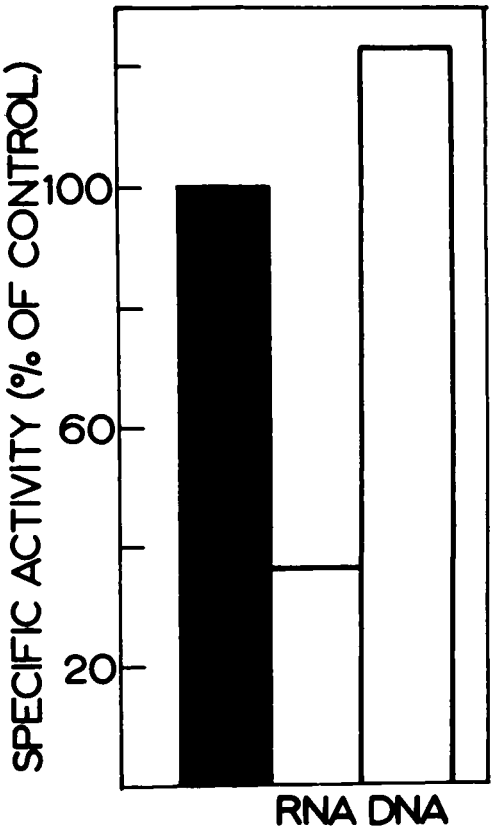

Chart 8. The effect on the specific activities of RNA and DNA of actinomycin D added to strips of mouse skin *in vitro*. The specific activities of RNA and DNA in skins incubated in medium containing no actinomycin are indicated by the *solid bar*; specific activities of RNA and DNA in skins incubated in medium containing 2.5 μg of actinomycin D/ml are indicated by the *open bars*.

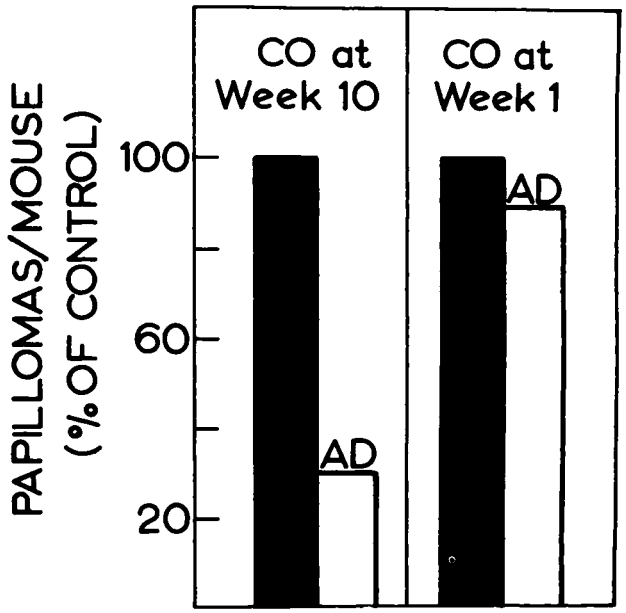

Chart 9. The effect of prior croton oil treatment on the inhibition of tumor formation by actinomycin D. Papilloma incidence in control mice initiated with 18 μg of 7,12-dimethylbenz[*a*]anthracene (DMBA) and promoted for 18 weeks with croton oil, with no actinomycin treatment, is set at 100% and is represented by the *solid bars*. Papilloma incidence, expressed as % of the control, in mice treated with 84 μg of actinomycin 34 days after DMBA treatment is shown by the *open bars*. In the part of the experiment shown in the left graph (labeled "CO at Week 10"), mice were not treated with croton oil (CO) prior to AD treatment. In the part of the experiment shown in the right graph (labeled "CO at Week 1"), mice were treated 8 times with 0.5% croton oil before treatment with actinomycin D.

This discrepancy may be due to differences in experimental procedure, the most obvious of which are the strain of mice used, the time at which skins were scraped, the amount of uridine-^{3}H added to the *in vitro* incubation medium, and the time of incubation. In the procedure of Flamm *et al.* (11), strips of full thickness skin were added to 5 ml of medium containing 50 μc of uridine-^{3}H. After incubation for 4 hours, the strips of skin were scraped prior to homogenization. In our procedure (Materials and Methods), the skins were scraped before a 2 hour incubation in 5 ml of medium containing 2 μc of uridine-^{3}H. Skins were scraped before incubation to facilitate uridine-^{3}H uptake into the skins and to minimize handling of the ^{3}H-labeled skins. A level of 2 μc of uridine-^{3}H gave sufficient counts in RNA without excessively high counts to be removed by washing repeatedly with 0.2 N PCA. The incubation time was shortened to reduce the possibility of growth of bacterial contaminants which could utilize the uridine-^{3}H.

Protein synthesis in mouse skin was stimulated by high levels of actinomycin D (12, 15) and was relatively unaffected by lower doses (12). This lack of inhibition of protein synthesis by actinomycin D, combined with the preferential inhibition of ribosomal RNA synthesis in mouse skin, led Flamm *et al.* (11) to suggest that mRNA synthesis was inhibited only slightly by actinomycin D.

In mammalian cells grown in culture (19, 27) and in tissues other than skin (1), an inhibition of RNA synthesis by actinomycin D was followed by an inhibition of DNA synthesis. These experiments have been interpreted as evidence that cells must produce certain species of mRNA in order to replicate their DNA. In mouse skin maintained for 4 hours in the presence of actinomycin D *in vitro*, RNA synthesis was inhibited while DNA synthesis was unaffected (Chart 8). Treatment with 10 μg of actinomycin D caused a slight inhibition of RNA synthesis for about 12 hours (Chart 5), followed by a 75–90% inhibition of DNA synthesis for at least 2 days (Chart 7). These results may indicate that the synthesis of a particular RNA fraction important in the replication of DNA is inhibited by actinomycin D, resulting in the inhibition of DNA synthesis. But, unless the synthesis of this RNA fraction is inhibited to a greater extent than the bulk of the pulse-labeled RNA, it seems unlikely that the slight inhibition of RNA synthesis, of short duration, can account for the more prolonged, more nearly complete inhibition of DNA synthesis.

Tumor-inhibitory doses of actinomycin D inhibit DNA synthesis by 75% or more for 2–5 days (Charts 6 and 7), while

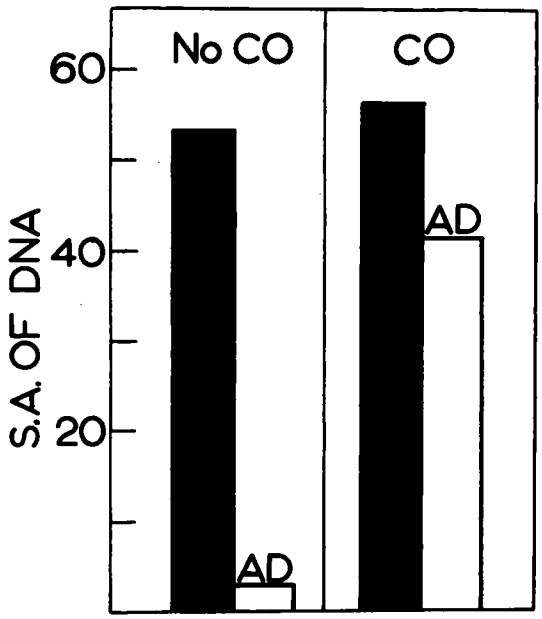

Chart 10. The effect of prior croton oil (CO) treatment on the inhibition of DNA synthesis by actinomycin D (AD). Mice were treated with croton oil and actinomycin in the manner described in Chart 9 and were killed 36 hours after actinomycin treatment. Mice were injected i.p. with thymidine-^{3}H 30 minutes before killing; the skins were processed according to the modified Schmidt-Thannhauser procedure described in Materials and Methods. The specific activity (S.A.) of DNA in control mice (± croton oil) is shown by the *solid bars*. The specific activity of DNA in actinomycin-treated mice (± croton oil) is shown by the *open bars*.

a dose which does not inhibit tumor formation (1 μg) has no effect on the rate of DNA synthesis (Table 7). While treatment with 10 μg of actinomycin D causes no visible skin damage, a stimulation of DNA synthesis (Chart 7) was seen 5 days after treatment. It is not known whether this effect is due to the recovery of the cells in which DNA synthesis was inhibited or to the hyperplasia of the remaining cells following the death of the inhibited cells.

As a result of experiments on the toxicity of alkylating agents in mammalian cells, Crathorn and Roberts concluded ". . .the lethal damage is the result of a direct effect on DNA which interferes with its replicative ability, but not with its function as a template for RNA synthesis . . ." (10). Actinomycin D, which interacts with DNA (23), is cytotoxic to HeLa cells (8) and, at high levels, causes necrosis in mouse skin (12, 16). Perhaps the skin cells in which the incorporation of thymidine-^{3}H into DNA is blocked do not survive; potential tumor cells would certainly be included in this group of skin cells capable of DNA replication and cell division.

Under environmental conditions (croton oil prior to actinomycin treatment) in which tumor formation was not inhibited by actinomycin D, the rate of DNA synthesis was inhibited only about 15%; when tumor formation was inhibited 70% (no prior croton oil treatment), the rate of DNA synthesis was inhibited 95% (Charts 9, 10). The degree of inhibition of tumor formation by actinomycin D correlates with its inhibition of thymidine-^{3}H incorporation into DNA, but since the croton oil-induced hyperplasia and hyperkeratosis (16) may merely lower the dose of actinomycin D per cell; other parameters affected by actinomycin may also correlate with the inhibition of tumor formation.

Several possibilities remain to explain the tumor-inhibitory action of actinomycin D. The possibility that actinomycin inhibits the fixation of initiation by stimulating a repair mechanism (Item 2*a*) remains untested experimentally. The most striking effect of actinomycin D on macromolecular synthesis in mouse skin is its nearly complete inhibition of DNA synthesis. This result, along with tumor induction experiments which show that actinomycin D is an effective inhibitor of tumor formation when given more than a month after the initiator (16), suggests that DNA synthesis (or cell division) may be important in the formation of initiated cells (Item 2*b*) or, more likely, in their maintenance or progression (Items 3, 4). Destruction of cells (Item 5), which may be indicated by the inhibition of thymidine-^{3}H incorporation into DNA, is evident at high tumor-inhibitory levels of actinomycin D (16) and may occur at lower tumor-inhibitory levels. Since initiated cells cannot be distinguished histologically from other epidermal cells, the viability of initiated cells in actinomycin-treated skin cannot be determined experimentally.

The cytotoxicity of actinomycin D complicates the interpretation of the results of the present experiments. Other agents which selectively affect DNA, RNA, protein synthesis, or cell division in mouse skin, without the complication of cell killing, will be more useful in elucidating the early biochemical events in chemical carcinogenesis.

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