INDUCED PROTECTION OF ADRENAL CORTEX AGAINST 7,12-DIMETHYLBENZ[*a*]ANTHRACENE*

INFLUENCE OF ETHIONINE. INDUCTION OF MENADIONE REDUCTASE. INCORPORATION OF THYMIDINE-H^3

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A single dose, which need not be hazardous to life, of 7,12-dimethylbenz[*a*]anthracene (7,12-DMBA)[1] invariably, selectively and totally destroys two zones of adrenal cortex of the adult rat and induces adrenal apoplexy (1); other adrenal zones survive and fatal adrenal insufficiency does not develop. The adrenal can be protected from necrosis of this sort by any of a considerable number of aromatics in small dosage provided it is given at least a few hours *before* 7,12-DMBA (2). In the work now to be described a pattern of highly efficient aromatic protectors was recognized. Evidence is presented that damage inflicted by 7,12-DMBA results from direct collision with cells highly susceptible to injury by the compound, whereas protection of the adrenal induced by aromatics is a secondary effect in which synthesis of protein has critical significance.

7,12-DMBA is unique insofar as it is the only compound known which totally destroys the susceptible zones of adrenal after a single dose; perdeuteration of 7,12-DMBA weakens the adrenocorticolytic effect of the parent compound. 2,2-*bis*(2-chlorophenyl,4-chlorophenyl)-1,1-dichloroethane (*o,p'*-DDD) also kills adrenal cells in the "susceptible" zones but, despite this common effect of the two compounds, the damage inflicted by *o,p'*-DDD differs from that induced by 7,12-DMBA both quantitatively and qualitatively (1). Far larger doses of *o,p'*-DDD than of 7,12-DMBA are required to produce adrenal necrosis which is focal and patchy when it occurs after giving the chloro compound.

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[1] The following abbreviations are used: DMBA, dimethylbenz[*a*]anthracene; 3-MC, 3-methylcholanthrene; B*a*A, benz[*a*]anthracene; BP, benzo[*a*]pyrene; *o,p'*-DDD, 2,2-*bis*(2-chlorophenyl,4-chlorophenyl)-1,1-dichloroethane; ACTH, adrenocorticotrophic hormone; tris, tris(hydroxymethyl)aminomethane; DPNH, reduced nicotinamide adenine dinucleotide; TPNH, reduced nicotinamide adenine dinucleotide phosphate; and $TdRH^3$ tritium-labeled thymidine.

7,12-DMBA induces adrenal necrosis only under appropriate conditions. The two zones of adrenal cortex which are destroyed selectively are zona fascicularis and reticularis whereas zona glomerulosa and adrenal medulla are spared from injury. The damage inflicted by 7,12-DMBA in adrenal occurs in adult but not in infant rats. Susceptibility of the adrenal cortex to injury by 7,12-DMBA is ACTH-dependent (3) and the presence of a critical level of synthesis of corticosterone is prerequisite to induction of adrenal necrosis by 7,12-DMBA.

Protection of adrenal by aromatics was discovered in work in two laboratories. Currie *et al.* (4) found that preliminary treatment of rats with 2-methyl-1,2-*bis*(3-pyridyl)-1-propanone, an inhibitor of steroid 11β-hydroxylation which interferes with synthesis of cortisol and corticosterone, prevented adrenal damage from 7,12-DMBA. Dao *et al.* (5) observed that 3-methylcholanthrene (3-MC) caused significant decrease of content of ascorbic acid and corticosterone in the adrenal and prevented adrenal injury by 7,12-DMBA (6). Moreover, protection of this sort was afforded by a single feeding of several other polycyclic aromatic hydrocarbons (7),—benzo[*a*]pyrene (BP); benz[*a*]anthracene (B*a*A); anthracene; phenanthrene,—or of aromatic amines (2),—6-aminochrysene; 2-aminofluorene; 2-naphthylamine. Significantly, a single preliminary feeding of 7,12-DMBA itself protects (7) the adrenal from damage by a large dose of 7,12-DMBA.

Enzyme Induction by Aromatics.—Substances, foreign to the organism, of widely divergent chemical structure, set in motion synthesis of enzyme systems which aid in their detoxication. Brodie and coworkers and Mitoma and coworkers (8, 9) discovered a common denominator in the metabolism of foreign compounds which was localized in microsomes of liver and required reduced nicotinamide adenine dinucleotide phosphate (TPNH) and oxygen for activity; many metabolic activities are brought about in this system including dealkylation, deamination, ether cleavage, hydroxylation, and others. A second detoxifying system is the increased synthesis of ascorbic acid (10).

Among the foreign substances inducing the synthesis of drug-metabolizing enzymes are polycyclic aromatics such as 3-MC and BP. These compounds induce the synthesis of enzymes with the following characteristics: they are localized in microsomes of the liver but not in other organs; enzyme induction is prevented by injection of actinomycin-D (11), puromycin (12), or ethionine administered *in vivo* (13); enzyme activity requires TPNH and oxygen. Enzymes, induced by 3-MC, with these properties include: (*a*) azo-dye reductase and *N*-demethylase (13); (*b*) benzpyrene hydroxylase (14); (*c*) 2-acetylaminofluorene hydroxylase (15); (*d*) zoxazolamine hydroxylase (16); (*e*) testosterone hydroxylase (17) and estradiol hydroxylase (18).

Longenecker *et al.* (10) discovered that many foreign aromatic and aliphatic compounds stimulate the excretion of ascorbic acid. 3-MC increases profoundly the excretion of ascorbic acid (19, 20) in rat and induces synthesis of enzymes in the ascorbic acid pathway (21).

Menadione Reductase.—Menadione reductase (EC 1.6.5.2) catalyzes the oxidation of reduced pyridine nucleotides by vitamin K_3 (22); it has also been designated DT diaphorase (23, 24). Williams-Ashman and Huggins (25, 26) found that the repeated feeding of 3-MC or 7,12-DMBA to rats caused an increase of menadione reductase in mammary gland and in adipose tissue, whereas the level of glutathione reductase was unaffected.

Methods

The experimental animals were normal female rats of Sprague-Dawley strain, age 46 to 49 days, weighing 140 to 160 gm. They were provided a commercial ration and water *ad libitum* and kept in an air-conditioned room at 25° ± 1°C.

The compounds to be evaluated as protectors of the adrenal cortex were recrystallized from appropriate solvents and dissolved in sesame oil, 2 ml. Solutions of hydrocarbons were administered by gastric tube, designated in this paper as *feeding*, usually 24 hours before the challenger, 7,12-DMBA. Lipide emulsions of 3-MC and B*a*A were injected intravenously. In experiments with *dl*-ethionine, 25 mg was dissolved in a minimal quantity of 0.1 N NaOH and injected into peritoneal cavity. Throughout this paper there were 5 to 8 animals tested in each group at each dose level.

With each series of compounds, 2 rats were injected with 7,12-DMBA, alone, as internal control of efficacy of the challenger itself. The challenger, a lipide emulsion[2] of 7,12-DMBA, 5 mg in 1 ml, was injected intravenously, once only; *this point of time was designated day 0.* On day 3 the rats were decapitated. The adrenals and samples of other tissues were weighed on a torsion balance. The right adrenal was prepared for histological study. The left adrenal and other samples, *ca.* 50 mg, were homogenized in 5 ml of an aqueous solution of 0.15 M NaCl and 0.003 M $NaHCO_3$ and centrifuged at 11,000 *g* for 10 minutes. The content of oxyhemoglobin in the supernatant was measured spectrophotometrically (1). An adrenal of the rat containing more than 125 μg of hemoglobin is, by our arbitrary definition, the site of hemorrhage. Adrenal apoplexy is hemorrhage visible in the gross. Complete adrenal protection signifies that no adrenal was the site of hemorrhage in any member of a group of 5 or more rats, each having been injected intravenously 72 hours previously with 7,12-DMBA, 5 mg.

Enzyme Assay and Units.—Menadione reductase was determined with a Beckman model DU spectrophotometer at 25°C using pyrex cells of 1 cm light path. The oxidation of DPNH was followed by reduction in absorbance at 340 mμ. The extinction coefficient of reduced nicotinamide adenine dinucleotide (DPNH) (liter/mole/cm) was assumed to be 6.22×10^3. Only the velocity in the first 2 minutes of the reaction was measured. The conditions yielded zero-order kinetics.

The assay was based on an earlier method (26). The reaction mixture consisted of:

	ml
0.5 M tris, pH 7.4	0.5
0.001 M DPNH	0.3
0.015 M menadione	0.02
Water	2.15
Enzyme solution (*ca.* 0.3 mg liver)	0.03

The technique of assay of lactic dehydrogenase has been described (27).

One unit of menadione reductase or lactic dehydrogenase is defined as the enzyme activity which reduced 1 μmole of DPNH/1 minute under the stated conditions. Units are expressed in terms of 1 gm of tissue, fresh weight.

Incorporation of Thymidine-H^3 into Deoxyribonucleic Acid.—The content of tritium in the washed perchloric acid–insoluble residue of adrenal was measured as in a previous study (28). In brief, $TdRH^3$, 0.75 μc/gm, was injected intravenously at 0 hours (after the animals had received hydrocarbons): the adrenals were harvested at 20 hours, pooled, weighed, and homoge-

[2] We are indebted to Paul Schurr, The Upjohn Co., Kalamazoo, Michigan, for preparing lipide emulsions. Aromatics were generously donated by M. S. Newman, J. A. Miller, N. C. Yang, and Alexander Haddow.

nized in ice-cold 0.5 N perchloric acid containing 0.05 per cent non-radioactive thymidine. Tritium was determined on the washed acid-insoluble residue by the combustion technique of Jacobson *et al.* (29).

EXPERIMENTAL

Age and pubertal status (opening of the vaginal plate; occurrence of estrus) were correlated (Table I) with susceptibility of adrenal to necrosis after intravenous 7,12-DMBA. All female rats of Sprague-Dawley strain, age 40 to 60 days, developed adrenal necrosis after intravenous injection of 7,12-DMBA, 3 mg or more. In rats younger than 40 days in which the vaginal plate had opened, the adrenal always underwent necrosis; but adrenal apoplexy also occurred in rats before the vaginal plate had become perforate. Adrenal susceptibility is not correlated closely with status of ovarian function.

TABLE I

Pubertal Status and Susceptibility of Adrenal to Damage by 7,12-DMBA

Rats were injected intravenously with 7,12-DMBA, 30 mg/kg and the adrenals were harvested 72 hours later. The occurrence of adrenal hemorrhage was correlated with opening of the vaginal plate at the time of injection and the incidence of estrus thereafter. There were 8 rats in each group.

Age	No. vaginal plates open	No. estrus	Adrenal apoplexy
days			
30	0	0	0
35	1	1	6/8
40	5	5	8/8
42	7	6	8/8
45	8	7	8/8

Whereas a single feeding of 7,12-DMBA, 30 mg, induces adrenal apoplexy in all adult rats (1), adrenal damage did not occur following a feeding, 30 mg, of 1,12-DMBA or 3,9-DMBA, or 6,8-DMBA. A feeding of the transannular peroxide of 7,12-DMBA, 50 mg, did not cause injury to the adrenals. An intravenous injection of 3-MC, 10 mg, with or without ethionine, 25 mg, immediately thereafter, did not cause adrenal necrosis. A feeding of 4-dimethylaminostilbene, 30 mg, resulted in enlarged adrenals with vascular congestion (adrenal hemoglobin 36 to 115μg) but did not induce necrosis; animals fed such a large dose of this compound have invariably succumbed within the ensuing 5 days.

Compounds Requiring Multiple Doses to Protect Adrenal.—Certain compounds which possessed hypnotic properties induced complete protection of the adrenal against 7,12-DMBA when given in repeated doses over several days while a single dose did not afford adrenal protection. A solution of each compound, 15 mg, was fed daily for 5 days. Phenobarbital (1 aromatic ring) was a protector; cyclobarbital, 5-(1-*cyclo*-hexanyl)-5-ethylbarbituric acid and barbital (no aromatic ring) did not induce protection. But chloretone (1,1,1-trichloro-2-methyl-2-propanol) induced protection of

adrenals in 7 in a group of 20 rats; this is the only aliphatic which we have found to induce protection of adrenal.

Aromatics Inducing Adrenal Protection after a Single Dose.—All compounds to be evaluated as protectors were administered once only on day −1 and 7,12-DMBA, 5 mg, was injected in a vein on day 0; the adrenals were harvested on day 3.

Eighty-seven aromatics, possessing 1 to 5 rings, were investigated by feeding at dose levels 0.5 mg to 100 mg. Forty-seven of these compounds were found to induce

TABLE II

Quantitative Evaluation of Aromatic Protectors of Adrenal

On day −1, the rats were fed a single dose of a compound dissolved in oil, 2 ml. On day 0, 7,12-DMBA, 5 mg, was injected in a vein. On day 3 the adrenals were harvested. There were 5 to 8 rats in each group. Protective dose is the smallest quantity found to prevent hemorrhage in both adrenals of all rats. Non-protective dose failed to prevent apoplexy in adrenals of all members in its group.

Aromatic	Protective dose	Non-protective dose
	mg	*mg*
3-Methylcholanthrene	0.25	0.10
3-Aminochrysene	0.5	—
Benzo[*a*]pyrene	0.5	—
3,9-Dimethylbenz[*a*]anthracene	0.5	—
2-Methyl-benz[*a*]anthracene	0.5	—
3-Methyl-benz[*a*]anthracene	0.5	—
4-Methyl-benz[*a*]anthracene	0.5	—
5-Methyl-benz[*a*]anthracene	0.5	—
6-Methyl-benz[*a*]anthracene	0.5	—
7-Methyl-benz[*a*]anthracene	0.5	—
9:Methyl-benz[*a*]anthracene	0.5	—
10-Methyl-benz[*a*]anthracene	0.5	—
12-Methyl-benz[*a*]anthracene	1.0	0.5
Benz[*a*]anthracene	1.0	0.5
7,12-Diethylbenz[*a*]anthracene	1.0	0.5
6,8-Dimethylbenz[*a*]anthracene	1.0	0.5
6-Aminochrysene	1.0	0.5
Diels hydrocarbon	1.0	0.5
Cyclopentenophenanthrene	2.0	1.0
7,12-Dimethylbenz[*a*]anthracene	2.0	1.5
7,12-Dimethylbenz[*a*]anthracene-16d	2.0	1.0

protection at some dosage[3] within the range which was investigated. A compound was regarded as a strong inducer of protection when a dose of 2 mg or smaller amount protected all adrenals of all rats in the group. Strong inducers of protection are indicated in Tables II and III; 30 compounds of this sort were identified.

[3] Dose-structure relationships are discussed in: Huggins, C., and Fukunishi, R., Molecular structure of aromatics related to their ability to induce adrenal protection, *Arzneimittel-Forsch.* 1964, **14,** in press.

A dose of 3-MC, 0.5 mg, given on day −1 in one of several ways, by feeding or injection in muscle or in vein, induced complete adrenal protection. A single feeding of 7,12-DMBA, 2 mg to 10 mg, given 1 day prior to 7,12-DMBA, 5 mg, by intravenous injection, protected adrenals completely. But when 7,12-DMBA, 0.5 to 1.5 mg, was injected intravenously as a protector on day −1 no amount was found which induced complete protection; doses larger than 1.5 mg could not be used since amounts 2 mg or more themselves cause adrenal necrosis (2). By intravenous injection, 7,12-DMBA, 1 mg, induced protection of only 5 rats in a group of 8.

Feeding the transannular peroxide of 7,12-DMBA, 10 mg, induced complete adrenal protection; a single feeding of 2 mg of this compound resulted in protection of adrenals of 3 animals in a group of 5 rats.

TABLE III

Prevention of Adrenal Apoplexy by Fluorinated 7-Methylbenz[a]anthracenes

The fluorinated compound, dissolved in sesame oil, 2 ml was fed once only on day −1; 7,12-DMBA, 5 mg, was injected intravenously on day 0; adrenals were harvested on day 3.

Fluorine substituent	Dose	Adrenals with hemorrhage
	mg	
None; 7-Methyl B*a*A	0.5	0/10
1-Fluoro-7-methyl B*a*A	1	2/6
" "	0.5	10/10
2-Fluoro-7-methyl B*a*A	0.5	0/10
3-Fluoro-7-methyl B*a*A	0.5	0/10
4-Fluoro-7-methyl B*a*A	0.5	0/10
5-Fluoro-7-methyl B*a*A	1	0/6
" "	0.5	6/10
6-Fluoro-7-methyl B*a*A	0.5	0/10
8-Fluoro-7-methyl B*a*A	0.5	0/10
9-Fluoro-7-methyl B*a*A	0.5	0/10
10-Fluoro-7-methyl B*a*A	0.5	0/10

Time-Dependence of Hydrocarbon-Induced Adrenal Protection.—This experiment was started on day 0. Hydrocarbons were given by intravenous injection. 7,12-DMBA, 5 mg, was injected at 0 hours and the biological indicator was presence or absence of adrenal apoplexy 72 hours thereafter.

Intravenous injection of the same emulsion containing 7,12-DMBA, 5 mg, *plus* 3-MC, 2, 5, or 10 mg, led to adrenal apoplexy in all of the animals. The inducers of protection had to be injected prior to the challenger in order to be effective.

The induction of the protective effect by 3-MC was determined by its dose; the smallest doses (0.5 to 2 mg) of 3-MC induced protection more rapidly than the largest doses did (Table IV). With reference to dose, the time of protection, t, was established: 3-MC, 1 mg, -2 hours $< t < -3$ hours; 3-MC, 5 mg, -4 hours $< t < -6$ hours. An intravenous injection of benz[*a*]anthracene induced complete protection in the following time: B*a*A, 10 mg, -3 hours $< t < 4$ hours.

How long did the protection last? Protection persisted longer after large doses than after small amounts. Groups of rats received intravenous injections of 3-MC, 1 mg or 5 mg; 7,12-DMBA, 5 mg, was given to one group in each set at intervals of 1 day thereafter. The adrenals of rats which received 3-MC, 1 mg, were protected for 2 days and adrenal apoplexy was observed when 7,12-DMBA was injected on day 3. The adrenals of rats receiving 3-MC, 5 mg, were protected for 6 days; adrenal apoplexy occurred in rats injected with 7,12-DMBA on day 8.

Since time was required for hydrocarbon-induced protection of adrenal against 7,12-DMBA, it seemed possible that some synthetic process was of critical importance in the induction. Did ethionine influence the protection afforded by 3-MC?

Influence of Ethionine and Methionine on Aromatic Protection of Adrenal.—This experiment started on day −1, when solutions of the amino acids were injected in

TABLE IV

Time-Dose Relationship in Aromatic Protection of Adrenal Cortex against 7,12-DMBA

On day 0, 3-MC was given prior to 7,12-DMBA, 5 mg, at 0 hours; both hydrocarbons were injected in a vein. Adrenals were harvested on day 3. +, Adrenal apoplexy; 0, no adrenal hemorrhage.

Dose of 3-MC	Time of injection of 3-MC before 7,12-DMBA, *hrs.*				
	−1	−2	−3	−4	−6
mg					
0.5	+	+	0	0	0
1	+	+	0	0	0
2	+	+	0	0	0
2.5	+	+	+	0	0
5	+	+	+	+	0
10	+	+	+	+	0

peritoneal cavity and an emulsion of the protector, 3-MC, was injected in a vein. On day 0, 7,12-DMBA, 5 mg, was injected in all rats; adrenals were harvested on day 3. Ethionine given in appropriate time relation, *t*, to the protective hydrocarbons, eliminated their protective effect; *t* was determined by the dose of 3-MC.

Groups of rats were injected with *dl*-ethionine, 25 mg, shortly before or after 3-MC, 2 mg; 7,12-DMBA, 5 mg, was injected 24 hours later. At necropsy, rats not injected with ethionine had yellow adrenals of normal appearance. Those which had been given ethionine 1 hour before, or 1 to 2 hours after 3-MC had adrenals with necrosis, scarlet with apoplexy. Those injected with ethionine 4 or 8 hours after 3-MC had yellow adrenals (Table V).

The conditions under which ethionine blocked the adrenal protective effect of 3-MC were somewhat different when big doses of the hydrocarbon were administered. The experiment was repeated except that groups of rats were injected with 3-MC, 10 mg. Single doses of ethionine, 25 mg or 50 mg, given 1 minute to 1 hour after 3-MC resulted in yellow adrenals (Table V). But ethionine, 25 mg, injected on two occasions, 1 hour

before and 1 hour after 3-MC, resulted in scarlet adrenals. Likewise, 3 doses of ethionine, 25 mg, at +1 minute, +4 hours, +8 hours after 3-MC eliminated the protective effect of 3-MC and the adrenals were scarlet.

dl-Methionine, 50 mg, given as the sole amino acid 1 hour before or 1 hour after 3-MC, 2 mg, had no influence on aromatic protection against 7,12-DMBA; the adrenals were yellow since 3-MC had induced protection. Addition of methionine, 50 mg, to the solution of ethionine, 25 mg, injected 1 hour before 3-MC, 2 mg, resulted in adrenals without apoplexy in 8 members of a group of 16 rats. Methionine had counteracted in many animals the deleterious effect of ethionine upon 3-MC protection.

Ethionine did not block the effect of 7,12-DMBA, 5 mg, in producing adrenal apoplexy. Ethionine, 25 mg or 50 mg, was injected 0.5 hours before 7,12-DMBA and adrenal apoplexy was found at necropsy in all of the rats.

No pathological findings were observed in adrenals after injection of *dl*-ethionine, 25 to 75 mg, or *dl*-methionine, 50 mg, without hydrocarbons.

TABLE V

Time Required for Ethionine to Block Induction of Adrenal Protection by 3-MC

On day −1, ethionine was injected, before or after 3-MC given at 0 hours; 7,12-DMBA, 5 mg, was injected on day 0. Adrenals were harvested on day 3 when their status was designated: +, adrenal apoplexy; 0, no adrenal hemorrhage.

3-MC dose	Ethionine dose	Time of injection of ethionine related to 3-MC, *hrs.*				
		−1	0	+1	+2	+4
mg	*mg*					
2	25	+	+	+	+	0
10	25	0	0	0	0	0

Since ethionine, in appropriate dosage at necessary time, abolished the adrenal-protective effect of 3-MC against 7,12-DMBA, it appeared possible that synthesis of protein was involved in protection. One of the proteins whose synthesis was induced by protective aromatics was menadione reductase.

Induction of Menadione Reductase by Aromatics and Influence of Ethionine Thereon.— Menadione reductase was determined on the soluble fraction of homogenized tissues. Identical values were obtained on the supernatant of homogenates centrifuged for 1 hour, respectively, at 11,000 *g* or 105,000 *g*. A single feeding of any of a number of aromatic amines or of polynuclear aromatic hydrocarbons (Table VI) resulted in an increase (as great as sevenfold) in menadione reductase of liver within 24 hours. It is noteworthy that a dose of chrysene (4 aromatic rings) induced a considerable increase whereas the same dose of pyrene (4 aromatic rings but with smaller surface area) failed to induce this enzyme; benzo[*a*]pyrene induced synthesis of the enzyme.

Following intravenous 3-MC, 2.5 mg, the level of menadione reductase was not elevated within the first 2 hours and the first rise was observed at +3 hours. There was no significant change in concentration of lactic dehydrogenase in liver of these animals (Fig. 1).

The level of menadione reductase following a single feeding was determined by the size of dose of 3-MC. A single feeding of 3-MC, 0.5 mg to 20 mg, was given; liver and lung were harvested 24 hours later and menadione reductase was determined (Fig. 2). In liver 3-MC, 0.5 mg, resulted in a twofold increase of enzyme concentration; between 0.5 mg and 10 mg the level of menadione reductase was proportional to the dose of 3-MC which had been fed; a dose of 3-MC, 20 mg, resulted in a level of menadione reductase only slightly higher than that in liver of animals which had been fed 10 mg. In lung 3-MC, 1 mg, caused an increase (150 per cent) of the enzyme level but additional doses of hydrocarbon did not result in greater increments of menadione reductase.

The kinetics of induction of menadione reductase in liver were studied following, respectively, the intravenous or oral administration of 7,12-DMBA, 5 mg. It will be

TABLE VI

Induction of Menadione Reductase in Liver by Aromatics

The aromatic was fed to female rats, age 46 to 50 days; liver was harvested 24 hours later. Controls were fed sesame oil, 2 ml, only. There were 4 rats in each group. Menadione reductase is expressed as units/gm fresh weight; mean values are given.

Aromatic	Dose of aromatic	
	1 mg units	10 mg units
3-Aminochrysene	38.8	104.0
6-Aminochrysene	22.9	100.0
3-Methylcholanthrene	51.2	83.3
Benzo[*a*]pyrene	14.3	56.0
7,12-DMBA	30.0	50.7
Chrysene	15.9	38.2
Pyrene	15.7	22.9

Values for 33 control rats: 17.5 ± 3.2 units.

recalled that 7,12-DMBA, 5 mg, given by vein invariably caused adrenal hemorrhage, whereas a feeding of the same amount of the compound not only failed to induce apoplexy, it protected the adrenal from necrosis. The intravenous injection caused a more prompt elevation of menadione reductase in liver than the oral administration of the same amount of 7,12-DMBA did, but the levels at 24 hours were similar in both cases (Fig. 3). After intravenous injection of 7,12-DMBA, 5 mg at 0 hours, the first rise in hepatic menadione reductase was detected at +3 hours; after oral administration the first elevation was noted at +8 hours (Fig. 3).

Incorporation of tritium into washed perchloric acid–insoluble residue of liver was measured after intravenous injections of 7,12-DMBA, 5 mg, and thymidine-H^3 (at 0 hours). Controls received tritium-labeled thymidine ($TdRH^3$) alone and results are expressed in percentage of incorporation in controls. 7,12-DMBA resulted in decreased incorporation. When 7,12-DMBA was given at −4 hours, incorporation was 56 per cent of control values (Fig. 3). This was the earliest lesion of 7,12-DMBA which we

detected. It was noteworthy that synthesis of menadione reductase was accelerated at a time when incorporation of thymidine into DNA was depressed.

The properties of menadione reductase induced by 3-MC were similar to those of the enzyme in normal untreated rats. For, in homogenates of liver and lung of each group (*a*) TPNH and DPNH were oxidized at precisely the same rate, and (*b*) dicumarol 10^{-9} M caused 50 per cent inhibition of the enzyme.

In addition to liver, aromatics were found to induce menadione reductase in adrenal, lung, mammary gland, and mammary cancer. Eight normal female rats, age 46 days, received intravenous injections of 3-MC, 2.5 mg, on day 0 and day 1; tissues were harvested on day 2. With respect to untreated controls the increase of menadione reductase on average was: adrenal, 159 per cent; lung, 164 per cent.

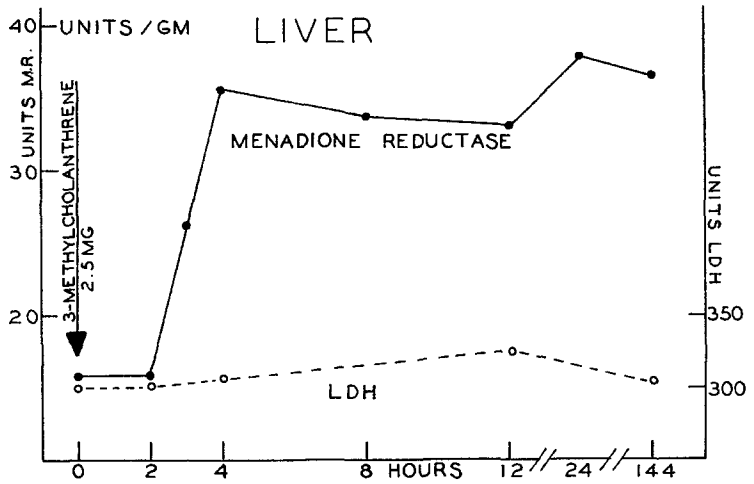

FIG. 1. Following intravenous injection of 3-MC, 2.5 mg, the first elevation in level of menadione reductase in liver was observed at 3 hours; there was no significant change in levels of lactic dehydrogenase (LDH).

Seven rats bearing mammary cancers of rather large size but without necrosis were studied; the cancers had been evoked by a single intravenous injection of 7,12-DMBA, 5 mg, 60 days earlier. On day 0, under ether anesthesia, samples of mammary gland, cancer, and liver were obtained by surgical operation and the incisions were sutured; 1 hour later, 4 of the rats were fed 3-MC, 10 mg, while 3 rats were fed sesame oil; the animals were sacrificed on day 1. Menadione reductase was determined on samples obtained at operation on day 1. In each of the three tissues of rats which had received the hydrocarbon there was an increase in the level of the enzyme. With respect to the values obtained in rats which had not received 3-MC the increases were: mammary gland, +214 per cent; mammary cancer, +275 per cent; liver, +224 per cent.

The influence of ethionine on induction of enzymes by hydrocarbons was investigated. Menadione reductase was determined in liver at serial intervals following 3-MC, 2.5 mg, in two groups of rats; one group received in addition ethionine, 25 mg,

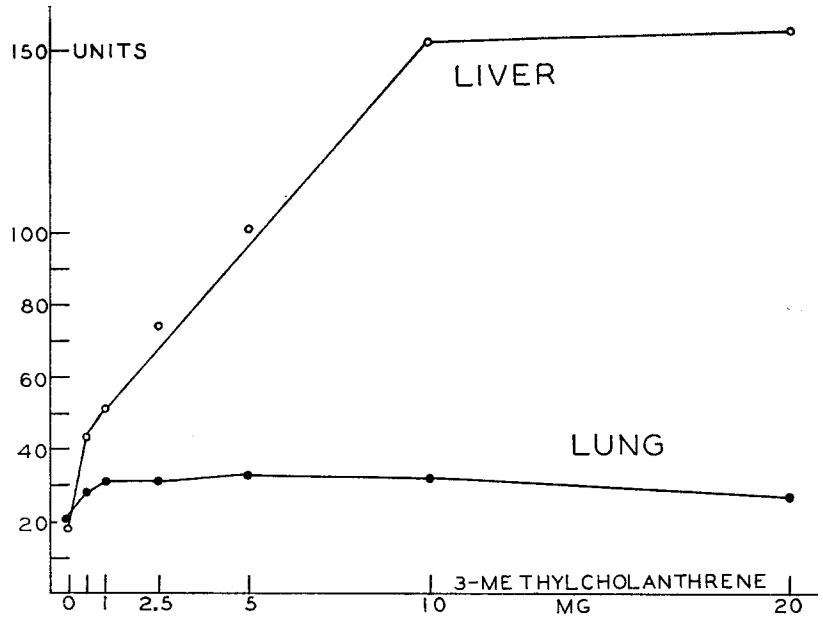

FIG. 2. Levels of menadione reductase in liver and lung 24 hours after a single feeding of various doses of 3-MC.

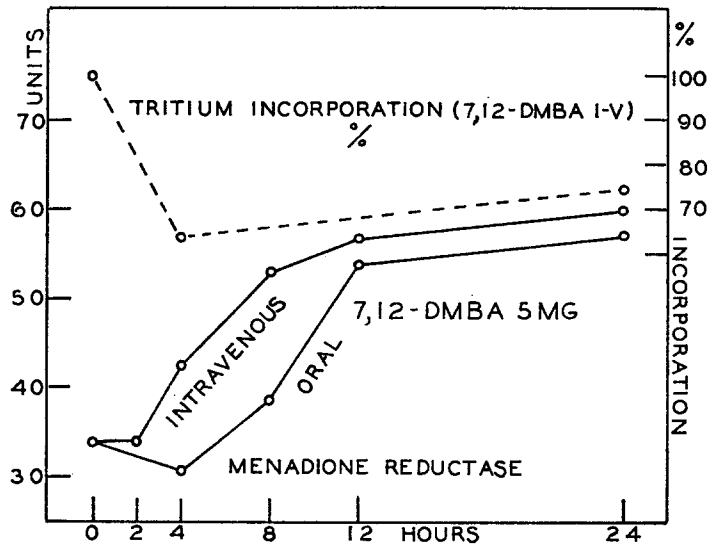

FIG. 3. Kinetics of induction in liver of menadione reductase following 7,12-DMBA, 5 mg, respectively, by feeding or intravenous injection.

Tritium concentration in washed perchloric acid-insoluble residue of liver is also indicated. 7,12-DMBA, 5 mg, was injected in a vein at −4 or −24 hours; TdRH^3, 0.75 μc/gm, was injected at 0 hours; liver was harvested at +20 hours. Incorporation is expressed in percentage of concentration in liver of controls which received TdRH^3 but not hydrocarbons.

just after 3-MC. In rats treated with 3-MC only, a significant rise in menadione reductase was found at 4 hours and its level rose progressively until +12 hours. In contrast, in rats treated with 3-MC *plus* ethionine (*a*) elevation of menadione reductase was first detected at 8 hours (Fig. 4), and (*b*) the elevation of enzyme at 12 hours was significantly lower than it was in rats which had not received ethionine.

Incorporation of Tritium in Adrenal after Administration of Hydrocarbons.—Lipide emulsions of 3-MC, 2.5 mg, and 7,12-DMBA, 5 mg, were injected prior to injection (at 0 hours) of TdRH^3; the content of tritium in washed perchloric acid–insoluble residue of adrenal (at 20 hours) was determined and compared with that of control mates receiving TdRH^3 alone (Table VII). The administration of each hydrocarbon

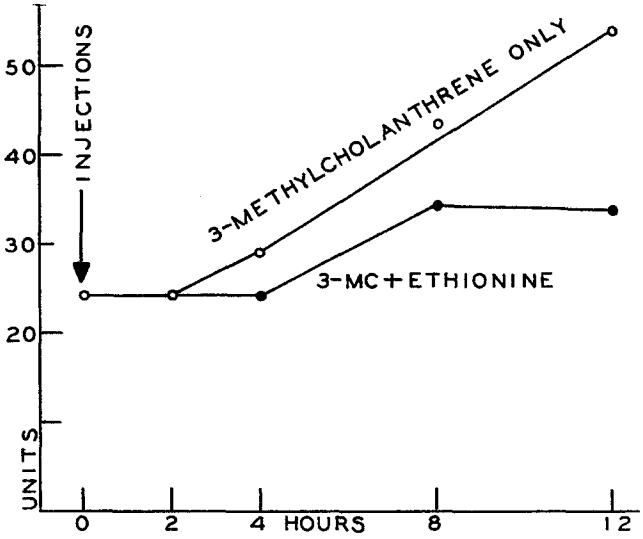

FIG. 4. At 0 hours, 2 groups of rats received intravenous injections of 3-MC, 2.5 mg; one group received in addition, ethionine, 25 mg, in peritoneal cavity. The level of menadione reductase in liver was determined at various times after the injections.

resulted in a decrease of incorporation of tritium which was more pronounced in rats receiving 7,12-DMBA (40 per cent of controls) than it was in those injected with 3-MC (72 per cent).

When 3-MC had been injected 24 hours before 7,12-DMBA, the tritium content of adrenal DNA was higher (67 per cent) than that of rats which had received 7,12-DMBA (40 per cent) as the sole hydrocarbon.

Ethionine abolished this advantageous effect upon tritium incorporation of pretreatment with 3-MC prior to 7,12-DMBA. Rats were treated sequentially with 3-MC at −24 hours; somewhat later with ethionine; 7,12-DMBA at −4 hours; TdRH^3 at 0 hours. The results of incorporation of tritium in adrenal were: ethionine 0.5 hours after 3-MC, 27 per cent; ethionine 8 hours after 3-MC, 28 per cent; no ethionine 67 per cent (Table VII).

When rats received a single feeding (10 mg) of 7,12-DMBA itself before intravenous 7,12-DMBA, 5 mg, tritium content of adrenal DNA was slightly higher than that of rats without the preliminary feeding of the hydrocarbon. The results were (Table VII); prior feeding of 7,12-DMBA, 48 per cent; no prior feeding, 40 per cent.

Effect of Sequential Administration of Hydrocarbons on Menadione Reductase.—Does an aromatic exert its beneficial protective effect by setting in operation a mechanism which rapidly destroys 7,12-DMBA?

TABLE VII

Incorporation of Tritium into Deoxyribonucleic Acid of Adrenal after Administration of Hydrocarbons

Female rats, age 46 days, were injected with $TdRH^3$, 0.75 μc per gm, at 0 hours after receiving 3-MC or 7,12-DMBA. Animals were sacrificed at 20 hours. Tritium content was determined on washed perchloric acid–insoluble residue. *dl*-Ethionine, 25 mg, dissolved in saline was injected intraperitoneally.

Treatment	Adrenal	
Controls		
	μc/gm	*per cent*
None	0.132	100
Intravenous hydrocarbons		
7,12-DMBA, 5 mg, −4 hrs.	0.053	40
3-MC, 2.5 mg, −24 hrs.	0.095	72
3-MC, 2.5 mg, −28 hrs; and 7,12-DMBA, 5 mg, −4 hrs.	0.089	67
3-MC, 2.5 mg, −24 hrs.; ethionine, −23.5 hrs.; and 7,12-DMBA, 5 mg, −4 hrs.	0.036	27
3-MC, 2.5 mg, −24 hrs.; ethionine, −16 hrs.; and 7,12-DMBA, 5 mg, −4 hrs.	0.037	28
Ethionine, −24 hrs.	0.122	92
Feeding 7,12-DMBA followed by intravenous 7,12-DMBA		
7,12-DMBA, oral, 10 mg, −48 hrs.	0.120	91
7,12-DMBA, oral, 10 mg, −48 hrs.; 7,12-DMBA, 5 mg i.v., −4 hrs.	0.064	48

The evidence was derived from a study of the effect of hydrocarbons and of the fluid in which they were emulsified (here designated lipide) given in sequence on concentration of menadione reductase in liver.

Emulsions (1 cc) were injected in a vein on two occasions, at 0 hours and again at 24 hours; menadione reductase was determined at 48 hours. The doses of hydrocarbons were: 7,12-DMBA, 5 mg; 3-MC, 2.5 mg. All of the animals received two injections of emulsions in one of the following combinations: (*a*) lipide and lipide, (*b*) hydrocarbon and lipide, (*c*) hydrocarbon and hydrocarbon.

The values (Fig. 5) for hepatic menadione reductase in rats injected with combination *a*, lipide and lipide, were similar to those of uninjected control rats. Injection with combination *b*, a single dose of either hydrocarbon at 0 hours with an injection of lipide at 24 hours, resulted in a considerable increase of menadione reductase; the values were similar with either 7,12-DMBA, 5 mg (269 per cent of controls), or 3-MC, 2.5 mg (276 per cent). Animals injected with combination *c*, hydrocarbon and hydrocarbon, had a greater increase in menadione reductase than those which had received injections in sequence of hydrocarbon and lipide. The increases resulting from two injections of hydrocarbons were not double the sum of 2 single doses. The lowest values for combinations of two hydrocarbons were found in rats injected with

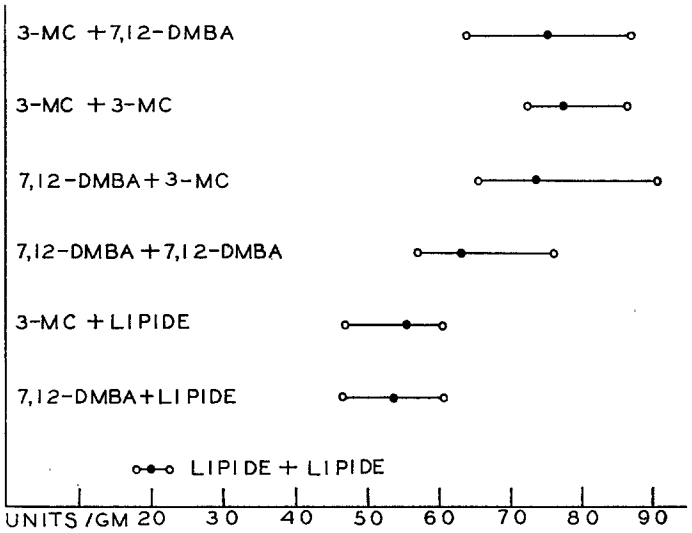

FIG. 5. Levels of menadione reductase in liver on day 2 following injections on day 0 and day 1 of sequences of 3-methylcholanthrene and 7,12-DMBA or of the fluid (lipide) in which they were emulsified.

7,12-DMBA and 7,12-DMBA (316 per cent). Excluding values found in several members of this group, each sample from every rat injected twice with the hydrocarbons in any sequence had higher values for menadione reductase in liver than that of any rat receiving hydrocarbon on only one occasion. Similar increases were found for the sequence 7,12-DMBA + 3-MC (368 per cent) and the sequence 3-MC + 3-MC (387 per cent).

Most noteworthy is the observation that rats which received 3-MC + 7,12-DMBA had elevation of hepatic menadione reductase (376 per cent) far above the value obtained in the liver of those which received 3-MC (276 per cent) as the sole hydrocarbon. This demonstrated that an earlier protective dose of 3-MC did not cause 7,12-DMBA to be inactivated insofar as its capacity to induce menadione reductase in liver was concerned.

DISCUSSION

Intravenous injection of lipide emulsions of hydrocarbons permits their rapid introduction into the blood. An advantage in feeding aromatics is a slower rate of absorption.

Whereas it emerged that there are many compounds which will induce protection of the adrenals, 7,12-DMBA is the only compound known to destroy zones of adrenal cortex completely after a single dose.

The singularity of 7,12-DMBA as an adrenocorticolytic agent in the rat is attributed to a combination of the following properties: (*a*) it is a strong electron donor; (*b*) it has close geometrical similarity to base pairs of nucleic acids and also to steroid hormones including corticosterone; (*c*) it has unusually high solubility in lipides and these are abundant in adrenal cortex. The high significance of electronic properties of aromatics in production of cancer was demonstrated by Szent-Györgyi *et al.* (30). The steric factor of hydrocarbons in biologic processes has been pointed out earlier (1, 31, 32). In contrast to the findings in many other species, corticosterone is the principal steroid (33) of rat's adrenal. The solubility w/v at room temperature of hydrocarbons in sesame oil was found to be approximately: 7,12-DMBA, 10 per cent; B*a*A, 2.5 per cent; 3-MC, 1.25 per cent. Lack of one or more of the aforementioned properties in hydrocarbons related to 7,12-DMBA would eliminate their adrenocorticolytic potency and convert them to strong adrenal protectors.

The substances which in small dosage induce protection of adrenal against 7,12-DMBA are compounds which resemble closely the challenger itself; indeed, as Dao and Tanaka (7) have shown, feeding 7,12-DMBA in small doses induces protection against itself. The highly effective inducers have common properties: (*a*) they are condensed aromatics; (*b*) they possess 4 or 5 rings; (*c*) they are flat; (*d*) their size does not exceed that of purine-pyrimidine base pairs of nucleic acids; (*e*) they are powerful electron donors. Chrysene was a rather strong inducer of menadione reductase, whereas rather large amounts of pyrene did not induce this enzyme; extension of conjugation of pyrene to form benzo[*a*]pyrene resulted in a strong inducer of enzyme and of adrenal protection. The requirements of optimal molecular size and planarity for maximal induction of soluble enzymes and for protection demonstrate the importance of a steric effect and extend the findings of Arcos *et al.* (34) with regard to induction by hydrocarbons of "drug-metabolizing" enzymes bound to microsomes of liver.

But the aforementioned molecular properties are only of significance for compounds which in small dosage induce protection. The transannular peroxide of 7,12-DMBA has very weak electron-donating properties but it induced protection. Phenobarbital possesses only 2 rings and is not flat but it induced protection when large doses were given repeatedly. Even chloretone, an aliphatic, induced protection when large doses were given repeatedly.

It has been known for some years that hydrocarbons induce synthesis in

liver of microsomal-bound enzymes. It is a novel finding that many aromatics also induce a soluble enzyme, menadione reductase, in liver and other cells, among them mammary carcinoma; to our knowledge the induction of enzymes in cancer has not been described before. Hydrocarbons induce enzymes in ascorbic acid pathway and also soluble and insoluble enzymes concerned with metabolism of compounds foreign to the organism. Since a single cause leads to production of multiple and divergent effects, it would appear that the 3 types of systems induced by aromatics are polycistronic in location.

The measurement of menadione reductase is a simple device for measuring protein synthesis induced by aromatics, with the additional advantage that the newly formed protein has enzymatic properties.

Induction of adrenal protection by aromatics against the destructive action of 7,12-DMBA was strongly influenced by the dose of the protector. Two doses of 3-MC, small and large, will be considered.

Intravenous injection of a small dose of 3-MC (2 mg) exerted these effects: (*a*) protection was induced in 2 to 3 hours; (*b*) protection lasted only 2 days; (*c*) a small dose of ethionine (25 mg) blocked protection; (*d*) this single small dose of ethionine blocked induction of protection when the amino acid was given as late as 2 hours after 3-MC; (*e*) the first elevation of menadione reductase was detected at 3 hours but only modest amounts of the enzyme were induced. Item *d* is especially noteworthy since *ethionine blocked induction at 2 hours, just before protection had been established*; administration of ethionine 4 hours after 3-MC had no effect on the process since protection had already been induced.

Intravenous injection of a large dose of 3-MC (5 mg) exerted these effects: (*a*) protection was induced in 4 to 6 hours; (*b*) protection lasted 6 days; (*c*) large doses of ethionine (50 to 75 mg) were necessary to block protection; (*d*) ethionine had to be provided repeatedly to block protection and the first dose of the amino acid needed to be given before or immediately after 3-MC; (*e*) first increase of menadione reductase was observed at 4 hours and the increase thereafter was slow in 12 hours but extremely high values were found 24 hours after injecting 3-MC.

It is well known that ethionine blocks synthesis of proteins (35, 36) and in our work it was observed that this amino acid delayed the induction and also depressed the amount of protein synthesis, specifically that of menadione reductase.

It was informative to study the influence of hydrocarbons on the incorporation of tritium into washed perchloric acid–insoluble residue (DNA) following administration of $TdRH^3$. Depressed incorporation followed protectors and challenger as well and the depression was maximal 4 hours after their administration. In the case of animals given protective doses of 3-MC (intravenous) or 7,12-DMBA (feeding) the depression was slight or moderate; in the case of

7,12-DMBA (large intravenous doses) the decrease of incorporation was severe. Administration of protective aromatics prior to the challenger was remarkably advantageous for incorporation of tritium into acid-insoluble residue; the damage to DNA inflicted by doses of 7,12-DMBA which otherwise killed adrenal cells was significantly lessened by the earlier treatment with protectors but it was not completely avoided. The design of the experiments did not permit localization of the biochemical level of the damage caused by hydrocarbons upon the thymidine incorporation apparatus.

It will be seen that there are two factors which determined, respectively, damage or protection by hydrocarbons; these are protein synthesis and extent of damage to DNA. It would appear that the protective action of a feeding of a small amount of 7,12-DMBA against the damage to the adrenal wrought by a large amount of the same compound lies in the slow absorption of the small amount permitting sufficient synthesis of protective proteins (this required some hours to be established) before great damage is caused by 7,12-DMBA. A large dose of 7,12-DMBA results in damage to DNA before proteins are induced in amounts sufficient to protect.

Can the protective effect on adrenals induced by hydrocarbons result from induction of sufficient amounts of drug metabolizing enzymes so that 7,12-DMBA is metabolized to less toxic compounds? The evidence is against such an hypothesis and it comes from study of the administration of 3-MC, 2.5 mg, followed in 24 hours by 7,12-DMBA, 5 mg. It was found that this combination of hydrocarbons induced considerably larger amounts of menadione reductase than were evoked by either hydrocarbon separately; the induction of enzyme was additive although an arithmetic sum was not attained. But it can be concluded that large amounts of 7,12-DMBA are not rapidly inactivated as a result of prior treatment with 3-MC.

SUMMARY

7,12-Dimethylbenz[*a*]anthracene (7,12-DMBA) exerts adrenocorticolytic effects which set it apart from all other polynuclear aromatic hydrocarbons and aromatic amines which have been investigated. Adrenal damage by this compound appears to be due to its steric and electronic properties together with its unusually high solubility in lipides.

Many compounds given prior to 7,12-DMBA induced protection of adrenal. The most efficient inducers of protection are flat condensed aromatics possessing 4 or 5 rings; very small doses of these compounds were required to induce protection. Other compounds devoid of these properties induced protection but large or repeated doses were necessary. All inducers of protection had to be given prior to 7,12-DMBA to prevent adrenal necrosis; when given simultaneously with, or later than, this compound adrenal apoplexy resulted.

Protective aromatics and 7,12-DMBA as well induced synthesis of menadione

reductase in liver. 3-Methylcholanthrene (3-MC) induced this enzyme in many normal organs including liver, lung, adrenal, and in mammary cancer as well. *dl*-Ethionine under appropriate conditions of time and dosage eliminated the adrenal protection induced by aromatics and also delayed the induction of menadione reductase while depressing the amount of this enzyme which was synthesized.

7,12-DMBA caused a greatly reduced incorporation of tritium from thymidine-H^3 into washed acid-insoluble residue of adrenal. 3-MC given in advance mitigated the drastic effect of 7,12-DMBA on DNA synthesis and increased considerably the amount of tritium which was incorporated.

The specific damage to adrenal by 7,12-DMBA is a direct effect on cells. Protection of adrenal is a secondary effect which requires induction of protein synthesis and it results in improvement in synthesis of DNA.

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