*AROMATIC AZO DERIVATIVES PREVENTING MAMMARY CANCER AND ADRENAL INJURY FROM 7,12-DIMETHYLBENZ(a)ANTHRACENE**

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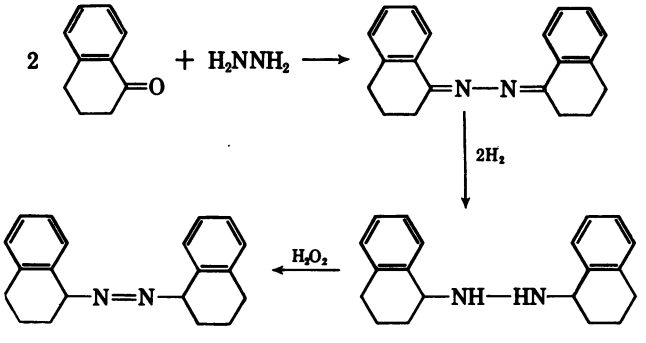
Many polycyclic aromatic hydrocarbons and aromatic amines share in common the ability to evoke cancer in certain animal species; the most powerful of these carcinogens is 7,12-dimethylbenz(a)anthracene.[1] A single pulse dose of 7,12-DMBA evokes mammary cancer invariably and rapidly in Sprague-Dawley rats; the yield of cancers per rat is greatly enhanced when several pulse-doses are given at intervals of a few days.[2]

In addition to evoking cancer in a dramatic way, 7,12-DMBA causes massive hormone-dependent necrosis of two zones of the rat's adrenal;[3] this effect is only exerted by congeners whose structure is very closely related to 7,12-DMBA. It has been found that adrenocorticolysis of this special sort can be prevented by any of a large number of cyclic compounds,[4–6] provided that it is given prior to 7,12-DMBA. Prevention of hydrocarbon-induced adrenal apoplexy is useful as a rapid screen to identify compounds which also will suppress aromatic-induced cancer.[2]

In the present work, series of aromatic azo and ethylene derivatives were investigated for their ability to induce protection against adrenal injury and cancer induced by 7,12-DMBA. The most effective inducers in this regard were found to be 1-(*p*-phenylazo-phenylazo)-2-naphthol (sudan III) and compounds closely related to it; these exceed by an order of magnitude all protectors that have been described earlier. Moreover, dose-response in the induction of an enzyme, menadione reductase, runs in parallel with efficacy of azo dyes in the induction of adrenal protection.

Experimental.—Chemical: Some of the compounds were purchased and these were recrystallized from appropriate solvents. Compounds IV, VI, VII, X–XII, XIV–XIX, XXI, XXIV–XXXV, XXXVII–XLII, were synthesized by known procedures and purified by chromatography.

Compounds VIII and IX, the *d-*, *l-*, and *meso-* forms of 1,1′-azotetralin were prepared by the ollowing reaction scheme:



All compounds were dissolved in sesame oil by heating gently.

Biological: In studying adrenal protection, there were in each group 5 normal female Sprague-

Dawley rats, age 46–49 days, weighing 140–160 gm. On day − 1, the animals were fed by gastric intubation a single dose of compound to be evaluated as a protector dissolved in sesame oil, 2 ml. On day 0, 1 ml of a lipide emulsion[7] containing 5 mg of 7,12-DMBA was injected in a caudal vein. On day 3 adrenals were harvested, weighed, and the content of hemoglobin in right adrenal was measured. The adrenal was homogenized for 1 min in 3 ml of 0.15 *N* NaCl and the homogenate was centrifuged for 10 min at 11,000 *g* at 4°C. One ml of the supernatant was diluted with 2 ml of water; absorbance at 416 mμ was determined in a spectrophotometer and from this density the absorbance at 600 mμ was deducted to correct for nonspecific turbidity. The pigment expressed as hemoglobin was calculated from measurements made in parallel to a reference standard of hemoglobin diluted with water. Under conditions of the experiment, an adrenal was considered to be protected from damage by 7,12-DMBA when it contained less than 125 μg of hemoglobin.

In investigating the possible protective value of a compound, a preliminary series of dose levels (e.g., 100 mg, 10 mg, 1 mg) was given. Subsequently, the protective dose was determined within ever narrower limits of dosage. Minimum protective dose is the smallest quantity of a compound which prevents apoplexy in all adrenals in a group; adrenal protective unit is the reciprocal of minimum protective dose.

With each experimental series harvested on a given day, 2 additional rats were injected on day 0 with 7,12-DMBA alone as an internal control of efficiency of the challenging effect of the lipide emulsion as adrenocorticolytic agent.

Sudan III (XXII) was investigated as a protector of rats against 7,12-DMBA-induced mammary cancer by a method[2] described earlier.

Menadione reductase (EC 1.6.5.2) was determined on the supernatant of homogenized liver after centrifugation. One unit of menadione reductase is defined as the enzyme activity which oxidized 1 μmole of reduced nicotinamide adenine dinucleotide/1 min under stated conditions.[8]

Results.—(1) *Azo dyes:* All control rats injected with 7,12-DMBA had massive adrenal necrosis with hemorrhage on day 3.

Many azo dyes induced adrenal protection (Table 1). The least effective compounds (I–IV) caused methemoglobin formation evident in their black spleens.

TABLE 1

AROMATIC AZO DERIVATIVES INDUCING ADRENAL PROTECTION

No.	Compound	Minimum Protective Dose (mg)	(units/mg)
I	Azobenzene	20*	0.05
II	*p*-Phenylazoaniline	10*	0.1
III	N,N-dimethyl-*p*-phenylazoaniline	10*	0.1
IV	1-N-(4-phenylazo)-phenylformimidoyl-2-naphthol	10*	0.1
V	4-Amino-3-methylazobenzene	5	0.2
VI	2-Phenylazonaphthalene	2	0.5
VII	4-Phenylazo-1-naphthylamine	2	0.5
VIII	1,1′-Azotetralin, mp 95–97	2	0.5
IX	1,1′-Azotetralin, mp 118–120	2	0.5
X	4-(1-Naphthylazo)-1-naphthol	2	0.5
XI	2,2′-Azonaphthalene	1.5	0.7
XII	1-Phenylazonaphthalene	0.5	2
XIII	1-Phenylazo-2-naphthol	0.5	2
XIV	1-(*p*-Aminophenylazo)-2-naphthol	0.5	2
XV	N,N′-di-(1-naphthyl)-hydrazine	0.25	4
XVI	1-(2-Naphthylazo)-2-naphthol	0.25	4
XVII	1,1′-Azonaphthalene	0.1	10
XVIII	1-(1-Naphthylazo)-2-naphthol	0.1	10
XIX	1,2′-Azonaphthalene	0.1	10
XX	1-(*o*-Tolylazo-*o*-tolylazo)-2-naphthol	0.05	20
XXI	1-(*p*-Phenylazo-phenylazo)-1-naphthalene	0.05	20
XXII	1-(*p*-Phenylazo-phenylazo)-2-naphthol	0.01	100

* Black spleens resulted.

With increase in number of rings from 2 to 4 there was a progressive increase in protective potency: I < XII < XVII < XXI. But IV was anomalous insofar

as it possesses 4 rings, yet it was a weak protector. Since methemoglobinemia was extensive in rats treated with compound IV, it is reasonable to assume that its azine linkage was cleaved *in vivo* to yield II.

Azo derivatives of 1-naphthalene were more potent protectors than those derived from 2-naphthalene: XII > VI; XVII > XI. It is of interest that there was equivalence in potency in 1,2′-azonaphthalene (XIX) and 1,1′-azonaphthalene (XVII).

Two isomers of 1,1′-azotetralin (VIII; IX) were less potent than 1,1′-azonaphthalene (XVII). The reduction of the azo bridge of 1,1′-azonaphthalene to form the hydrazine results in decrease of efficacy: XV < XVII.

Introduction of a hydroxyl group in naphthalene *ortho* to the diazo linkage either had no effect on potency of the parent compound (XIII = XII; XVIII = XVII) or its potency was increased (XXII > XXI). A substituent, $-NH_2$ or $-OH$, in naphthalene *para* to the azo bridge resulted in decrease of potency: VII < XII; X < XVII. But equivalent in potency were 1-(*p*-aminophenylazo)-2-naphthol (XIV) and 1-phenylazo-2-naphthol (XIII). Sudan III (XXII) was more potent than sudan IV (XX). Adrenal protection against 7,12-DMBA was induced by intravenous injection of sudan III, 10 μg, dissolved in ethanol and diluted with water to make the final concentration of alcohol 10 per cent. Intravenous injection and feeding of the dye were quantitatively equivalent in the induction of protection.

Sudan III-induced adrenal protection was abolished by ethionine. On day -1, *dl*-ethionine, 25 mg, was injected in peritoneal cavity 1 hr prior to intravenous injection of an alcoholic solution of sudan III, 20 μg; on day 0, 7,12-DMBA was injected intravenously; at harvest on day 3 adrenals were scarlet with hemorrhage. In animals treated similarly, except that ethionine was omitted, normal yellow adrenals were found.

(2) *Ethylenes:* All of the active aromatic derivatives of ethylene with *trans* configuration around the bridge were more potent than their *cis* isomers (Table 2).

TABLE 2

EFFECT OF AROMATIC ETHYLENE DERIVATIVES ON INDUCTION OF ADRENAL PROTECTION

No.	Compound	Adrenal protection	Minimum Protective Dose (mg)	Minimum Protective Dose (units/mg)
XXIII	Stilbene (100 mg)	No	—	—
XXIV	*cis*-Phenyl-2-naphthylethylene (25 mg)	No	—	—
XXV	*trans*-Phenyl-2-naphthylethylene	Yes	2.5	0.4
XXVI	*cis*-Phenyl-1-naphthylethylene	Yes	15.0	0.07
XXVII	*trans*-Phenyl-1-naphthylethylene	Yes	1.0	1.0
XXVIII	*cis*-1-(1-Naphthyl)-2-(2-naphthyl)-ethylene	Yes	15.0	0.07
XXIX	*trans*-1-(1-Naphthyl)-2-(2-naphthyl)-ethylene	Yes	1.0	1.0
XXX	*cis*-1,2-Di-(1-naphthyl)-ethylene	Yes	2.0	0.5
XXXI	*trans*-1,2-Di-(1-naphthyl)-ethylene	Yes	0.25	4.0
XXXII	*cis*-1,2-Di-(2-naphthyl)-ethylene (25 mg)	No	—	—
XXXIII	*trans*-1,2-Di-(2-naphthyl)-ethylene (10 mg)	No	—	—

trans-Stilbene (XXIII) did not protect but *trans*-phenyl-1-naphthalene (XXVII) induced protection, and *trans*-1,2-di-(1-naphthyl)-ethylene (XXXI) was a strong protector. Derivatives of 1-naphthalene were more potent than their isomers

derived from 2-naphthalene: XXVII > XXV: XXXI > XXXIII. Compound XXXIII was only slightly soluble in vegetable oil.

(3) *Pyridine compounds:* Stilbazoles were able to induce adrenal protection (Table 3); of these compounds 3-stilbazole (XXXV) was more potent than 2-stilbazole (XXXIV) or 4-stilbazole (XXXVI).

In a series of azopyridine compounds (Table 3) only 3-phenylazopyridine (XXXIX) and 3,3′-azopyridine (XL) were active as protectors. Both of these compounds, in contrast to 2- and 4-phenylazopyridine, caused extensive formation of methemoglobin (Table 3).

TABLE 3

EFFECT OF PYRIDINE COMPOUNDS ON INDUCTION OF ADRENAL PROTECTION

No.	Compound	Adrenal protection	Minimum Protective Dose (mg)	(units/mg)
XXXIV	2-Stilbazole	Yes	15	0.07
XXXV	3-Stilbazole	Yes	5	0.2
XXXVI	4-Stilbazole	Yes	15	0.07
XXXVII	2-Phenylazopyridine (25 mg)	No	—	—
XXXVIII	2,2′-Azopyridine (25 mg)	No	—	—
XXXIX	3-Phenylazopyridine*	Yes	15	0.07
XL	3,3′-Azopyridine*	Yes	15	0.07
XLI	4-Phenylazopyridine (25 mg)	No	—	—
XLII	4,4′-Azopyridine (25 mg)	No	—	—
XLIII	1-(2-Pyridylazo)-2-naphthol	Yes	10	0.1

* Black spleens resulted.

Adrenal protection was induced by 1-(2-pyridylazo)-2-naphthol (XLIII), whereas 2-phenylazopyridine (XXXVII) was inactive in this regard.

Induction of menadione reductase in liver: Groups of rats were fed azo dyes dissolved in sesame oil, and liver was harvested in 24 hr; the activity of menadione reductase was measured in the soluble fraction of liver homogenate. With increase of molecular size there was a progressive increase in enzyme induction (Fig. 1) by these compounds: azobenzene < 1-phenylazonaphthalene < 1,1′-azonaphthalene < 1-(*p*-phenylazo-phenylazo)-2-naphthol.

Protection by sudan III against cancer: Three groups, of 10 rats each, were fed a diet *ad libitum*, injected with 7,12-DMBA, and given sesame oil, 2 ml, by gastric tube daily for 26 days; 2 of the groups received, in addition, sudan III dissolved in the oil.

The control rats did not receive sudan III: each rat developed mammary cancers, and 108 carcinomas (Table 4) were found in the group of 10 rats at necropsy performed in 70 days after the first injection of 7,12-DMBA.

TABLE 4

SUDAN III-INDUCED PROTECTION AGAINST MAMMARY CANCER IN RATS INJECTED WITH 7,12-DMBA

Protective hydrocarbon	Dose (mg × days)	Rats with cancer	Appearance of cancer (days)	Active Centers Total	Mean*
None: controls	—	10/10	28–36	108	10.8 ± 4
Sudan III	1 × 26	5/10	43–83	9	1.8
Sudan III	5 × 26	4/10	45–63	4	1

* Total number cancers per group/number of rats with cancer; ±, standard deviation. Beginning at age 47 days, sudan III dissolved in sesame oil, 2 cc, was fed by gastric tube daily for 26 days; controls were fed sesame oil, 2 cc. All animals received intravenous injections of lipide emulsion of 7,12-DMBA 2 mg, at age 50, 53, and 56 days. Necropsy was performed on controls at age 120 days and on rats fed protective hydrocarbon at age 150 days.

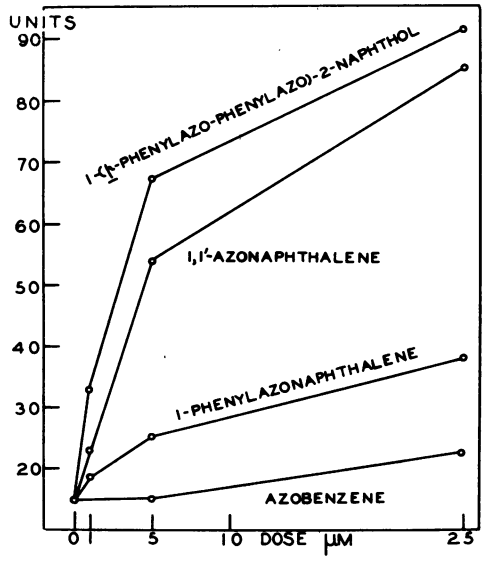


FIG. 1.—Induction of menadione reductase in liver 24 hr after a single feeding by gastric instillation of azo dyes dissolved in sesame oil. Each point represents mean value, in units/gm fresh weight, obtained from 3 rats.

The results were far different in the two groups which were given sudan III. One group received sudan III, 1 mg, daily; 5 rats developed cancer, and a total number of 9 cancers were found in the group. Another group received sudan III, 5 mg, daily; 4 of the rats developed mammary carcinoma, and the total number in the group was 4 cancers in 10 rats in 100 days (Table 4).

Discussion.—Quantitatively, the amounts of a given azo dye required to induce adrenal protection ran in parallel to its efficacy in the induction of menadione reductase in liver. Induction of each sort was prevented by treatment of the rat with ethionine prior to administration of the azo dye. Protein synthesis[8] is crucial in significance in protection against the highly damaging aromatics.

From the present work it appears that significant attributes of the inducer molecule are its size, geometry, charge distribution, and solubility in lipides.

A simple expression of molecular size[9, 10] is the surface area of the minimum rectangular envelope enclosing the molecule (incumbrance area). As inducers of adrenal protection, in ascending order of potency, are the following compounds with their incumbrance area in $Å^2$: azobenzene, 92.1; 1-phenylazonaphthalene, 114.4; 1,1'-azonaphthalene, 124.7; 1-(*p*-phenylazo-phenylazo)-1-naphthalene, 182.7. But in the series of aromatic azo dyes, it is merely a first approximation that the largest molecules exceed smaller ones in efficiency in exerting biological effects.

As an example of the influence of molecular geometry, it is noteworthy that 1,1'-azonaphthalene, 124.7$Å^2$ is 15-fold more active in inducing adrenal protection than is 2,2'-azonaphthalene, 124.1$Å^2$.

As an example of the influence of charge distribution upon protective capacity, consider stilbene which did not protect, whereas 3 isomers of stilbazole were inducers of adrenal protection. It is of interest that 3-stilbazole and 3-phenylazo-

pyridine are considerably more active in inducing adrenal protection than their isomers with N-atom at either positions 2 or 4.

Methemoglobinemia developed in rats fed pyridine derivatives whose nitrogen was in *meta* position and did not develop when N-atom was in positions 2 or 4. Aside from the 3-azopyridines, as inducers of adrenal protection, aromatic azo derivatives were somewhat more effective than their analogues possessing an ethylene bridge.

In the present series, sudan III was the most potent protector against injury from 7,12-DMBA—against adrenal apoplexy and against induction of cancer. Adrenal protection resulted when a single dose of sudan III, 10 μg or a larger amount, was fed or given by vein. Supplementing the diet with small daily amounts of sudan III resulted in reduction of the number of cancers which were evoked to 4 per cent of the controls, and many rats which received the azo dye were completely protected.

Richardson *et al.*[11] found that the administration of 3-methylcholanthrene to rats inhibited the development of hepatic tumors in rats fed an azo dye, 3′-methyl-4-dimethylaminoazobenzene. The present work is the counterpart of the Richardson effect insofar as azo dyes prevented biologic damage by an exceedingly powerful polynuclear aromatic hydrocarbon.

Summary.—In the induction of protection by aromatic azo dyes and ethylene derivatives against adrenal apoplexy and mammary cancer in rat injected with 7,12-dimethylbenz(a)anthracene, size, geometry, charge distribution, and solubility of inducer molecule are of high importance.

Of compounds which have been investigated, 1-(*p*-phenylazo-phenylazo)-2-naphthol (sudan III) is the most efficient in (*a*) induction of menadione reductase in liver, (*b*) protection of adrenal against 7,12-DMBA, and (*c*) prevention of hydrocarbon-induced mammary cancer.

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[1] The following abbreviation is used: 7,12-DMBA, 7,12-dimethylbenz(a)anthracene. Roman numerals refer to compounds in Tables 1–3.

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