SELECTIVE ADRENAL NECROSIS AND APOPLEXY INDUCED BY 7,12-DIMETHYLBENZ(a)ANTHRACENE*

By CHARLES HUGGINS, M.D., and SOTOKICHI MORII, M.D.

(From the Ben May Laboratory for Cancer Research, the University of Chicago, Chicago)

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In the work now to be described, it was observed that a single dose of 7,12-dimethylbenz(a)anthracene[1] caused extraordinary changes in the rat consisting of adrenal apoplexy and massive necrosis in the two inner zones of the cortex while other regions of the adrenal glands were uninjured. In addition to the selectivity of the anatomic site of damage, there is high specificity of the molecular structure of the polynuclear aromatic hydrocarbon exerting this adrenocorticolytic effect.

Earlier it was found (1, 2) that a single dose of any of a number of polynuclear aromatic hydrocarbons, under special circumstances, selectively induced mammary cancer in every rat with very great rapidity. DMBA was the most effective of these compounds and tumors arose following a solitary feeding or an intravenous injection. At necropsy of rats with cancer of the breast induced by a solitary dose of DMBA which had been administered a few weeks previously, it was observed that the adrenal glands of most of the animals were calcified. The adrenals of rats bearing mammary cancer induced by hydrocarbons other than DMBA were not calcified. It was soon found that adrenal apoplexy and necrosis were produced invariably with DMBA soon after giving the hydrocarbon. This is a newly recognized property of DMBA.

Several methods were employed in these experiments for detection and measurement of damage to the adrenal glands. These included: (*a*) direct inspection of the gland; (*b*) histological observation; (*c*) estimation of the amount of blood pigments; (*d*) determination of enzyme content of the adrenals; (*e*) roentgenologic detection of adrenal calcification.

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[1] The following abbreviations are used: DMBA, 7,12-dimethylbenz(a)anthracene; DDD, 2,2-*bis*(2-chlorophenyl,4-chlorophenyl)-1,1-dichloroethane; G-6-PD, glucose-6-phosphate dehydrogenase; 6-PGD, 6-phosphogluconic dehydrogenase; ICD, isocitric dehydrogenase; LDH, lactic dehydrogenase; MDH, malic dehydrogenase; DPNH, dihydrodiphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; *tris*, tris (hydroxymethyl)-aminomethane.

The enzyme studies demonstrated that estradiol-17β has a particular role in modifying the concentration of isocitric dehydrogenase in the adrenal cortex.

Adrenal Necrosis.—Several experimental methods have been found for damaging the adrenals. Roux and Yersin (3) in their classic paper on diphtheria toxin, described redness in the adrenal glands of guinea pigs whose mucous membranes had been excoriated and inoculated with diphtheria bacilli. Tonutti (4) found that lethal doses of diphtheria toxin caused edema, hemorrhage, and necrosis of the adrenal cortex of normal guinea pigs; but if hypophysectomy were performed either just before, or up to 11 hours after the administration of the toxin, the adrenals were spared from toxic damage (5).

The continuous intravenous injection of large doses of ACTH for prolonged periods caused necrosis of the adrenal cortex and medulla with bleeding (6).

György *et al.* (7) found that a nutritional deficiency leading to panmyelophthisis caused adrenal hemorrhage in rats. The dietary deficiency causing adrenal damage has been related to deficiency in some fraction of the vitamin B complex (8, 9).

Nelson and Woodard (10) found that the repeated feeding of the commercial insecticide DDD to dogs for 1 to 33 months caused atrophy of the adrenal cortex. The fraction of crude DDD which is active in damaging the adrenal glands is *o,p'*-DDD (11); the daily feeding of *o,p'*-DDD, 4 mg/kg, for 4 days caused massive necrosis of the adrenal glands of dogs. Vilar and Tullner (12) found that repeated large doses of *o,p'*-DDD caused *focal* degeneration in the inner zones of the adrenal cortex of dogs in 2 to 6 days.

Enzymes.—Glock and McLean (13) found high levels of glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase in the adrenal glands of the rat. G-6-PD, 6-PGD, isocitric dehydrogenase, and the malic enzyme occur almost exclusively in the supernatant fraction of centrifuged homogenates of adrenals (14). It has been found that ACTH caused an increase (almost twofold) in G-6-PD and a less marked increase in 6-PGD with no increase in concentration of ICD of human adrenals (14).

Methods

The experimental animals consisted of albino rats of Sprague-Dawley strain. Most of them were fed a commercial ration; hypophysectomized rats were kept on a synthetic high (18 per cent) protein diet. Experiments on rats from which endocrine glands had been removed were not begun until 3 weeks after the operations which had always been performed under ether anesthesia.

The polynuclear aromatic hydrocarbons were recrystallized from appropriate solvents, usually acetone–ethyl alcohol and dissolved in sesame oil. The solutions were protected from light. Most of the hydrocarbons were administered by stomach tube which was a small soft rubber catheter. In other experiments DMBA was administered as a fine emulsion[2] *via* caudal

[2] We are indebted to Paul Schurr, The Upjohn Company, Kalamazoo, Michigan, for preparing an emulsion of DMBA. Professor A. Haddow kindly provided 4-dimethylaminostilbene. Dr. Waro Nakahara donated 4-nitroquinoline-*N*-oxide, and Dr. Roy Hertz furnished *o,p'*-DDD.

vein. The hydrocarbons were administered once only to rats, age 50 to 60 days, and the day of administration was designated day 0.

Preparation of Enzymes.—The rats were killed by decapitation. The tissues to be analyzed were excised rapidly, weighed, and homogenized for 3 minutes in an ice-cold solution (3 ml) of 0.15 M NaCl containing 0.003 M $NaHCO_3$; the enzymes were kept in an ice bath thereafter until the assays were made. The homogenates were centrifuged at 11,000 *g* for 15 minutes in a refrigerated centrifuge. Usually the right adrenal was homogenized for measuring enzyme activity and the content of pigments; the left adrenal was preserved for histological study.

Enzyme Assays and Units.—Spectrophotometric determinations were made with a Beckman model DU spectrophotometer at 25°C in an air-conditioned room using pyrex cells of 1 cm light path. The reduction (or oxidation) of pyridine nucleotides was followed by measurement of change in absorbence at 340 mμ. The molar extinction coefficient of reduced pyridine nucleotides was assumed to be 6,220. Only the initial velocity of the reaction was measured. The conditions yielded zero order kinetics for all of the dehydrogenases.

The assay methods for 6-PGD, G-6-PD, and LDH have been described earlier (15).

The assay of ICD was based on the method of Ochoa (16). The reaction mixture consisted of:—

0.5 M *tris*, pH 7.4	0.5 ml
0.01 M $MnCl_2$	0.1 "
0.005 M TPN	0.1 "
0.1 M sodium isocitrate	0.1 "
Distilled water	2.1 "
Enzyme solution (*ca.* 1 mg adrenal)	0.1 "

The assay of MDH was adapted from Ochoa *et al.* (17). The reaction mixture consisted of:—

0.05 M *tris*, pH 7.4	0.50 ml
0.001 M DPNH	0.30 "
0.03 M sodium oxalacetate	0.10 "
Distilled water	2.05 "
Enzyme solution (*ca.* 0.05 mg adrenal)	0.05 "

One unit of 6-PGD, or G-6-PD, or ICD is defined as the enzyme activity which reduced 1 μmole of TPN/1 minute under the stated condition. One unit of LDH or MDH is defined as the enzyme activity which oxidized 1 μmole of DPNH/1 minute. The units are expressed in terms of 1 gm of fresh tissue (wet weight).

Blood Pigments in Adrenal.—The blood pigments in the saline homogenate (1 adrenal in 3 ml) were converted to cyanmethemoglobin with ferricyanide-cyanide (18). The absorbence at 540 mμ was determined and the results expressed in terms of hemoglobin. A disadvantage of this method is the development of an interfering turbidity due to precipitation of proteins in some of the samples.

A more serviceable method was the dilution of 1 ml of the saline homogenate of the adrenal with 2 ml of water. Absorbence at 416 mμ was determined and from this the absorbence at 600 mμ was deducted to correct for non-specific turbidity. The pigment expressed as hemoglobin was calculated from measurements made in parallel of a reference standard of hemoglobin diluted with water.

Alkaline Phosphatase in Plasma.—Blood, 0.2 ml, was obtained by cardiac puncture without anesthesia using a 26 gauge needle moistened with a minute drop of heparin. For the determination of alkaline phosphatase 0.1 ml of blood was diluted with 0.9 ml of saline, centrifuged, and the supernatant removed for analysis. The reaction mixture consisted of:—

0.1 M sodium barbital, pH 9.3	1.4 ml
0.02 M *p*-nitrophenylphosphate	0.5 "
Plasma supernatant	0.1 "
Incubate at 38° for 1 hour. Add: 0.1 M sodium hydroxide	2.0 "

The amount of *p*-nitrophenol liberated is measured by absorbence at 400 mμ. One unit is defined as the enzyme activity which liberated 1 μmole of *p*-nitrophenol/1 hour under the stated conditions. The units are expressed in terms of 1 ml of plasma.

RESULTS

Following a single feeding of DMBA, 20 mg, to rats there was a decline in body weight, evident on day 1 and maximal on day 3; on average the total loss was 10 gm. Many rats had soft feces. Growth recommenced on day 4.

There was a decrease in the number of leukocytes in the blood which reached its low point on day 7. Moreover, there was a decline in the level of alkaline phosphatase in plasma (Text-fig. 1), slight on day 1 and profound on day 3; thereafter, there was a gradual return of alkaline phosphatase to the pretreatment level.

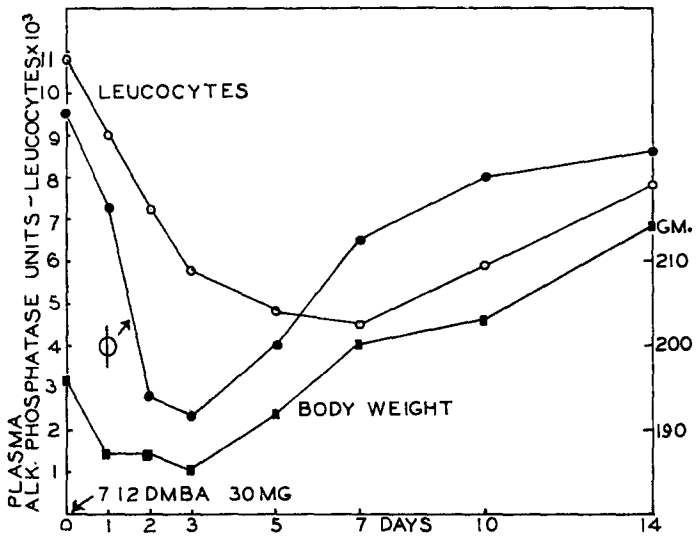
It is known (19) that there is a remarkable decrease of alkaline phosphatase in the plasma of rats during fasting which reaches its low point within 24 hours after food is withheld. The low plasma alkaline phosphatase of fasting rises abruptly within 24 hours after a complete diet is fed. The plasma alkaline phosphatase after feeding DMBA differs in its dynamic aspects from that of fasting. The levels both decline and subsequently recover more slowly after feeding DMBA than in fasting but equally low levels are reached in both states. It would appear that in addition to diminished utilization of food (evident from presence of mild diarrhea and loss of weight) DMBA exerts additional effects of a toxic nature which contribute to the decline in alkaline phosphatase.

The estrus cycle, determined by the cytology of the vagina, was scarcely modified by a single feeding of DMBA, 20 to 30 mg. 10 rats were fed DMBA, 20 mg, and all maintained a normal 4 day estrus cycle thereafter. 10 rats were fed DMBA, 30 mg; 6 of these had normal 4 day cycles, 3 had slight irregularity of estrus, and 1 rat developed anestrus which persisted for 10 days.

Pyridine Nucleotide-Linked Dehydrogenases of Adrenal and Liver.—The levels of dehydrogenases in the adrenals of female rats were compared with those in their own livers (Table I). In both organs, MDH and LDH levels outranked those of the other dehydrogenases which were studied but the concentrations of these enzymes were about twofold higher in liver than in adrenal. The high concentration of G-6-PD observed earlier (13, 20) in adrenal was confirmed. The concentration of ICD was higher in liver than in adrenal.

Steroid Influences on ICD of Adrenal.—Steroid levels of the body were found to exert interesting effects on ICD levels of the adrenal. Estradiol-17β was

found to lower the adrenal concentration of ICD and physiologic states compatible with low levels of estradiol-17β caused an elevation of ICD in the adrenal glands.



TEXT-FIG. 1. Following a single feeding of DMBA, 30 mg, at day 0, there was a decline in plasma alkaline phosphatase, circulating leukocytes (per 1 mm³), and body weight. The subsequent recovery is also shown. ϕ, Alkaline phosphatase.

TABLE I

Dehydrogenases in Liver and Adrenal Glands

The results are expressed in units per gram, wet weight. There were 15 normal female rats, ages 50 to 60 days.

Dehydrogenases				
6-PGD	G-6-PD	ICD	LDH	MDH
		Liver		
3.4 ± 0.5	2.4 ± 1.0	25.1 ± 3.5	173.3 ± 15.2	208.2 ± 37.5
		Adrenal Glands		
6.4 ± 1.5	11.2 ± 1.3	4.5 ± 0.6	59.8 ± 3.0	92.5 ± 4.1

±, Standard deviation.

The level of ICD in the adrenals of adult females (Table II) was lower than that of adult males. In the female, ovariectomy or the administration of dihydrotestosterone, resulted in a twofold increase in concentration of ICD; the level of no other dehydrogenase in the adrenal was modified by these

procedures. Orchiectomy had no effect on ICD levels of adrenal of male rats. The administration of estradiol-17β to ovariectomized female rats reduced the level of ICD to that of normal females (Table II).

Hypophysectomy resulted in an increase in concentration of ICD and a decline in the level of 6-PGD in the adrenal; other dehydrogenases were little affected (Table II) by removal of the pituitary.

TABLE II

Dehydrogenases in Adrenal Glands

The results are expressed in units per gram of adrenal, wet weight. Surgical operations were performed 2 to 3 weeks before enzymes were studied.

No. of rats	Dehydrogenases				
	6-PGD	G-6-PD	ICD	LDH	MDH
	Females: normal age 50 to 65 days				
36	6.1 ± 0.9	10.1 ± 1.3	4.4 ± 0.8	50.9 ± 6.5	95.0 ± 10.3
	Females: ovariectomy				
14	5.7 ± 0.8	10.9 ± 1.1	9.2 ± 1.8	49.2 ± 3.3	100.2 ± 9.8
	Females: ovariectomized; estradiol-17β*				
5	7.7 ± 1.0	10.8 ± 0.4	4.9 ± 1.0	67.7 ± 0.5	100.2 ± 6.0
	Females: hypophysectomy				
15	3.4 ± 0.4	12.5 ± 0.9	9.7 ± 1.3	53.4 ± 5.3	103.0 ± 16.2
	Females: intact, dihydrotestosterone‡				
6	7.8 ± 1.6	10.8 ± 2.4	7.1 ± 0.9	57.0 ± 3.7	123.3 ± 6.4
	Males: intact				
13	6.1 ± 1.0	10.0 ± 1.0	10.0 ± 1.2	47.6 ± 4.4	76.2 ± 9.0
	Males: orchiectomy				
5	7.0 ± 1.2	13.4 ± 1.2	10.1 ± 0.9	51.7 ± 6.0	80.0 ± 5.4

±, Standard deviation

* Estradiol-17β, 2μg, was injected for 14 days.

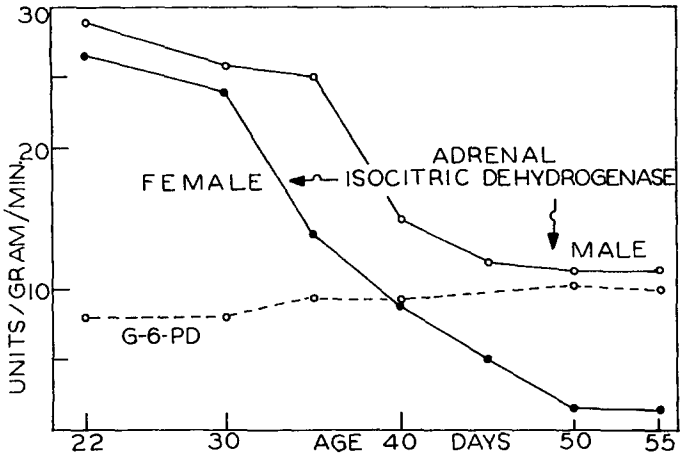
‡ Dihydrotestosterone, 3 mg, was injected for 14 days.

Sexual development of immature rats was found to influence the level of ICD in adrenals. Between ages 22 to 30 days, high levels of ICD were found in the adrenals of male and female rats (Text-fig. 2). After age 30 days, there was a decline in the concentration of ICD, pronounced in females and much less in males. Not all dehydrogenases of the adrenals are influenced in this way by the onset of puberty; G-6-PD did not decline with onset of sexual maturity (Text-fig. 2).

Effect of DMBA on Adrenal Dehydrogenases.—A single feeding of DMBA,

20 to 30 mg, or an intravenous injection of 1.5 to 10 mg, caused a decline of the levels of all of the adrenal dehydrogenases which were studied. These dosages were provided to rats, 10 animals at each level, and they were sacrificed at various intervals thereafter when the concentration of dehydrogenases was studied.

There were no changes in levels of enzymes in the adrenal on day 1. A decline in the level of all of the dehydrogenases was evident on day 2, and it was most pronounced on day 3 (Text-fig. 3). The dehydrogenases, while reaching very low levels in the adrenals, were not completely eliminated by DMBA. An increase in the levels of G-6-PD, 6-PGD, and ICD was evident on day 6 and



TEXT-FIG. 2. During puberty the concentration of ICD in adrenals declines considerably in females and to a lesser extent in male rats, whereas the concentration of G-6-PD remains unchanged.

recovery nearly, but not quite, to the levels of the enzymes in untreated mates was evident on day 14.

The decline in the levels of pyridine nucleotide–linked dehydrogenases of the adrenal and the subsequent increase ran in parallel with histologic evidence of damage and repair, respectively to be described below.

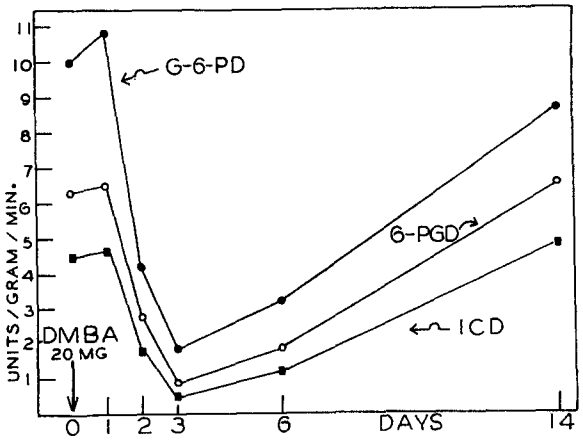
Dosage of DMBA and Adrenal Damage.—

A single feeding of DMBA dissolved in sesame oil was provided to groups of rats, age 50 days, in doses graduated from 10 to 100 mg by gastric tube; there were 10 rats or more at each dose level. All of the rats receiving a feeding of DMBA, 60 mg or greater, succumbed in 7 days or less. The dosage causing death of one-half the rats was calculated by the probit method of Gaddum (21). The LD_{50} for a single feeding of DMBA to Sprague-Dawley rats was 27 mg/100 gm.

A single feeding of DMBA, 20 mg (12 mg/100 gm), to female Sprague-Dawley

rats, age 50 days, produced adrenal necrosis in *ca.* 79 per cent of the rats (Table III). The optimal dose for inducing adrenal necrosis by a single feeding was 30 mg (18 mg/100 gm). For, no rat succumbed following this dosage and every animal developed adrenal hemorrhage and necrosis. All rats fed the optimal dose developed diarrhea which was mild. On average the rats lost 13 gm of body weight. The mean leukocyte count of the blood was 4,800/mm^3, *ca.* 60 per cent of values of untreated mates.

Graduated dose levels of DMBA, 1 to 20 mg, in finely emulsified form[2] were injected intravenously only once into female rats, age 50 days; there were 10 rats at each dose level. The LD_{50} of DMBA for a single intravenous injection in Sprague-



TEXT-FIG. 3. Following a single feeding of DMBA, 20 mg, at day 0, there ensues a decline in the adrenals of the concentration of G-6-PD, 6-PGD, and ICD. A subsequent rise in these enzymes as adrenal regeneration occurs begins after day 3.

Dawley rats was 4.75 mg/100 gm. The optimal dose for induction of adrenal necrosis under these conditions was 5 mg (3 mg/100 gm); no rat injected with this amount died from the treatment and all developed adrenal hemorrhage and necrosis. Rats injected with the optimal dose usually developed a mild diarrhea on day 2. On average, the rats on day 3 had gained about 1.2 gm above the preinjection weight. The mean leukocyte count of the blood was 5,700/mm^3, *ca.* 71 per cent of the control values of injected rats.

Adrenal necrosis also developed in male rats following an intravenous injection of DMBA but a slightly higher amount (Table III) of the compound was necessary in the males than in females to demonstrate the effect.

Ten female rats of Long-Evans strain were injected intravenously with a single dose of DMBA (3.2 mg/100 gm); 4 of the rats died within 3 days and all rats developed leukopenia. 8 of the 10 rats developed adrenal hemorrhage following the injection; the adrenals of 2 rats apparently were undamaged.

Manifestation of Adrenal Damage.—

(*a*) *Morphologic changes:* The optimal doses fed or injected in a vein always led to adrenal hemorrhage and necrosis in intact Sprague-Dawley rats.

Day 1.—The adrenals *in situ* were yellow or tan in color and not enlarged. The surrounding retroperitoneal tissues were hyperemic. On microscopy, many dark shrunken cells (Fig. 4) were found in zona reticularis. In adrenal medulla the blood vessels were dilated.

Day 2.—The adrenals had a remarkable appearance; they were dark red in color (Fig. 1), turgid with blood, and their weight was double that of the adrenals of control rats. The

TABLE III

Adrenal Necrosis Related to Solitary Dose of DMBA

Necropsy was performed at age 53 days, 3 days after administration of the compound.

Dose of DMBA		Route	No. of rats	Adrenal necrosis		
				No.	Per cent	Deaths
mg	*mg/100 gm*					
Females; normal						
20	12	Oral	14	11	79	0
30	18	"	45	45	100	0
5	3	IV	10	10	100	0
2.5	1.6	IV	10	10	100	0
1	0.7	IV	10	0	0	0
Males; normal						
10	5.4	IV	19	19	100	9
10	3.7	IV	10	6	60	1
5	2	IV	10	0	0	0

IV. Compound was administered by intravenous injection.

periadrenal tissues were edematous with serous fluid. Microscopy revealed complete destruction of zona fasciculata and zona reticularis, *except* for a few islands of cells around the principal adrenal artery and vein (Fig. 7). Much hemorrhage was evident around the necrotic adrenal cortex both beneath zona glomerulosa and above the medulla. Zona glomerulosa and adrenal medulla appeared undamaged.

Day 3.—The hemorrhage and necrosis were more pronounced than on day 2. Necrosis of the vulnerable areas of adrenal cortex was maximal at this time (Fig. 5). But the glomerular and medullary zones of the adrenal were not abnormal.

Day 6.—The adrenals were dark brown in color and still swollen. Section of these glands revealed a horseshoe shaped area of necrosis in the inner adrenal cortex (Fig. 2). Microscopically, extensive necrosis and hemorrhage were seen in the inner half of the adrenal cortex. There had been a considerable increase in thickness of living cells in the outer half of the cortex, since regeneration had begun (Fig. 6) originating from zona glomerulosa.

Day 14.—The adrenals were brown and a few small patches of hemorrhage were still evident. On microscopic examination a zone of regeneration and a zone of dead cells were found, but these zones were not in disarray. Proceeding from the periphery, in most of the adrenals one observed (Fig. 8): (*a*) normal zona glomerulosa; (*b*) partially regenerated zona

fasciculata; (*c*) a narrow band of connective tissue; (*d*) calcification—evident for the first time since the compound was given; (*e*) necrosis; (*f*) apparently normal adrenal medulla.

Day 69.—The adrenals were yellow and slightly pitted on their surface. Section of the adrenals disclosed extensive calcification in many of the glands. On microscopy, much regeneration of the zona fasciculata was apparent. In many of the adrenals a zone of calcification, most commonly in the form of a horseshoe was present at the corticomedullary junction (Fig. 9). Likewise, the horseshoe pattern of calcification was evident in X-rays of the adrenal (Fig. 3). No necrotic cells were evident. As at all of the earlier stages, zona glomerulosa and adrenal medulla appeared undamaged.

Whereas necrosis was produced regularly and selectively in the adrenal cortex, calcification did not occur subsequently in every adrenal. When calcification was present it was always bilateral. On section of the adrenals, calcification was evident in the gross in 53 of 129 rats (48 per cent) which had been given a single feeding of DMBA, 20 mg. Bone was not found in histological section.

(*b*) *Blood pigments:* When the adrenal of a normal untreated rat which had been exsanguinated by decapitation was homogenized in saline, 3 ml, the supernatant after centrifugation was water-clear or, less often, opalescent with fat. The corresponding supernatant of hemorrhagic adrenals was brown. The amounts of blood pigments were estimated by two methods which have been described above.

A normal adrenal contained 0.01 to 0.05 mg of pigment expressed as hemoglobin. The apoplectic adrenals on day 3 after DMBA contained much more—on the order of 0.2 to 1.1 mg of pigment expressed as hemoglobin.

DMBA and Hypophysectomy.—

DMBA, 3 to 3.7 mg/100 gm, was injected once intravenously in rats in an experiment in which hypophysectomy was performed; necropsy was always performed 3 days after the injection. In addition there were 12 hypophysectomized rats which did not receive DMBA; neither hemorrhage or necrosis was observed in adrenals or elsewhere in these control animals.

Groups of rats were hypophysectomized within 1 hour of the injection of DMBA in a vein—one group just before, the other just after the injection. The results were similar in both groups (Table IV). In both groups severe bleeding and necrosis were found in the adrenal cortices of all of the rats.

In a group of 9 rats injected with DMBA 3 to 7 days after hypophysectomy, the adrenals contained hemorrhage and there was necrosis, but only in 6 of the animals (Table IV); degenerative lesions of this sort were not found in the adrenals of 3 members of this group injected with DMBA.

Six rats were injected with DMBA 2 weeks after hypophysectomy. At necropsy the adrenal glands were free from necrosis and hemorrhage in the gross but microscopic examination disclosed hemorrhage (which was slight) in the adrenals of 2 of these animals. There was no evidence of hemorrhage or necrosis in adrenals of 4 members of this group.

Twelve uninjected hypophysectomized rats serving as controls developed leuko-

cytosis in the blood, on average 16,700 white blood cells/mm³. The injection of DMBA in hypophysectomized rats lowered the leukocyte count to about one-half the number (Table IV) found in the blood of the controls. The leukopenia of rats injected with DMBA is not caused by effects resulting from the excessive secretion of pituitary or adrenal hormones.

DMBA and Adrenal Enucleation.—Will DMBA induce cortical necrosis of adrenals from which the medulla has been removed?

TABLE IV

DMBA in Hypophysectomized Rats

The compound was administered intravenously before or after hypophysectomy with necropsy 3 days later. Mean values are given.

No. of rats	DMBA: interval from hypophysectomy	Dose	Died	Adrenal				
				Weight	Hemoglobin	ICD units	Necrosis	Leukocytes / 1 mm³
		mg/100 gm		*mg*	*mg*			
8	Just before	3.1	0	30.6	0.22	0.93	8/8	8,097
10	Just after	3.2	0	28.6	0.25	1.08	10/10	6,620
4	3 days after	3.7	1	22.9	0.34	3.04	3/4	7,242
7	7 days after	3.5	4	16.4	0.47	3.87	3/5	—
6	14 days after	3.0	0	7.0	0.05	4.54	None	8,700

Hemoglobin, mg per adrenal
ICD, units per 1 gm of adrenal

The adrenal glands of 23 rats were enucleated by a standard procedure: a small incision was made in the gland and the contents expressed leaving a rim of cortex. Both adrenals were enucleated at the same sitting. No special treatments or hormones were provided or required after the operation to preserve life.

Seven of these rats were subjected to enucleation only and were sacrificed at the time of their mates which received DMBA. 1 month after the procedure, in these rats there was regeneration of the adrenal cortex with a small amount of scar tissue, containing dilated capillaries, in the center of the gland. There was no hemorrhage or necrosis in the regenerated adrenals; the mean hemoglobin content was 0.06 mg and mean ICD concentration was 3 units/gm.

Sixteen rats were injected intravenously with DMBA, 3.3 to 4.2 mg/100 gm, 31 to 41 days after adrenal enucleation and the rats were sacrificed 3 days later. No adrenal damage was detected in 2 rats. Rather small amounts of adrenal necrosis and hemorrhage were present in 14 rats; the mean hemoglobin content was 0.16 mg per gland and the mean ICD concentration was 2 units/gm.

In sum, DMBA induced hemorrhage and necrosis in the cortex of many, but not all, of the adrenal glands which had regenerated after enucleation and in which the adrenal medulla was absent. The extent of the adrenal cortical

damage was considerably less than that produced in comparison to rats with intact adrenals, treated concurrently with the same dosage of DMBA.

Molecular Structure Related to Adrenal Damage.—

A single large dose of 12 polynuclear aromatic hydrocarbons (Table V) dissolved in sesame oil was fed by gastric tube to normal female Sprague-Dawley rats, age 50 days; the animals were sacrificed 3 days later. The effects of each individual compound (I-XIII) on adrenal cortex and the leukocyte count were compared with those induced by DMBA.

7,12-Dimethylbenz(a)anthracene-16d (II) had more than 95 per cent of its hydrogen atoms replaced by deuterium in both the aromatic and aliphatic sites.[3] This compound, 50 mg, induced adrenal necrosis in 4 members of a series of 10 rats. The leukocyte count was not abnormal. 7,12-diethylbenz(a)anthracene (III), 100 mg, did not induce pathologic changes in the adrenals.

Four compounds (IV to VII) which are constituents of the DMBA molecule did not cause any abnormalities in the adrenal glands or any significant effect on the leukocyte count (Table V). The compounds, ineffective in this regard, are, 7-methylbenz(a)anthracene (IV); benz(a)anthracene (V); 9,10-dimethylanthracene (VI); 1,4-dimethylphenanthrene (VII).

Five compounds which are strong carcinogenic hydrocarbons failed to induce changes in the adrenal glands. These hydrocarbons are 3-methylcholanthrene (VIII); benzo(a)pyrene (IX); 4-dimethylaminostilbene (X)[2]; 4-nitroquinoline-*N*-oxide (XI); 2-acetylaminofluorene (XII). All of these compounds evoke cancer locally when painted on the skin or injected in the muscle of certain rodents. All of these hydrocarbons, when fed once only in the stated doses (Table V) to Sprague-Dawley female rats, ages 50 to 65 days, have the remarkable property of inducing mammary cancer selectively and within a few weeks (1, 2); neoplasms have not been observed in the gastrointestinal tract although this region was exposed in the feeding to the brunt of the assault of the hydrocarbon. None of these polynuclear aromatic hydrocarbons caused hemorrhage or necrosis in the adrenal glands; the concentration of ICD in adrenals lay within normal limits (Table V). Of these compounds, 4-dimethylaminostilbene (X) alone caused leukopenia under the stated conditions.

o,p'-DDD (XIII), 200 mg/100 gm, was fed once only to 15 rats. All of them became drowsy and developed diarrhea; these conditions lasted 1 to 2 days. 5 of the rats died, but a group of 10 rats survived this large dose despite a considerable loss of weight. On day 3, at necropsy, the adrenals were big (Table V) and yellow in color. On microscopic examination, the adrenocortical cells were foamy and crammed with lipids in contrast to the cortical cells of rats which had received DMBA. Small patches of necrosis and lymphocytic infiltration in zona reticularis were seen in the adrenals of 1 rat and hemorrhage in the adrenals occurred in 2 rats. Otherwise the adrenals of rats fed a single but large dose of *o,p'*-DDD were not remarkable.

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[3] Dr. A. T. Morse of Merck, Sharp and Dohme of Canada, Ltd., Montreal, generously prepared perdeuterated DMBA by isotope exchange with DMBA.

In quantitative and qualitative aspects the effect of a single dose of *o,p'*-DDD on the adrenal cortex was somewhat different and less in extent than that of DMBA but the lesions were in the same adrenal zones with each compound. Both hydrocarbons inflicted lesions in zona fasciculata and in the reticularis.

TABLE V

Effect of Polynuclear Hydrocarbons on Adrenal Glands and Leukocyte Count

A single dose of hydrocarbon was fed with 10 rats in each group and necropsy 3 days later. Mean values are given. The right adrenal (R) was studied for hemoglobin and ICD content. The left adrenal (L) was used for histological study.

Compound	Dose	Weight	Adrenal (R)		Adrenal (L)	
			Hemoglobin	ICD units	Necrosis	Leukocytes / 1 mm³
	mg/100 gm	*mg*	*mg*			
None: controls	—	29.5	0.04	4.4	0	7,836
I. 7,12-Dimethylbenz(a)anthracene	18.5	42.5	0.40	0.7	10/10	4,873
II. 7,12-Dimethylbenz(a)anthracene-16d	29.2	24.8	0.26	4.1*	4/10	7,285
III. 7,12-Diethylbenz(a)anthracene	49.8	33.2	0.06	4.8	0	9,230
IV. 7-Methylbenz(a)anthracene	77.6	25.3	0.02	6.8	0	9,035
V. Benz(a)anthracene	78.6	23.5	0.02	6.3	0	7,555
VI. 9,10-Dimethylanthracene	51.4	27.9	0.03	4.9	0	9,000
VII. 1,4-Dimethylphenanthrene	158.2	24.0	0.02	6.7	0	6,606
VIII. 3-Methylcholanthrene	62.9	23.6	0.03	5.6	0	8,059
IX. Benzo(a)pyrene	62.1	28.7	0.05	6.0	0	10,378
X. 4-Dimethylaminostilbene	11.5	37.8	0.08	5.4	0	4,389
XI. 4-Nitroquinoline-*N*-oxide	11.9	32.8	0.03	4.8	0	8,553
XII. 2-Acetylaminofluorene	55.9	36.5	0.07	3.7	0	8,418
XIII. *o,p'*-DDD	202	51.0	0.06	3.2	2/10	8,263

Hemoglobin, mg per adrenal; ICD, units per gram.

* 4 adrenals with necrosis had ICD values, 1.1 to 2 units.

DISCUSSION

The most interesting findings to emerge from these experiments were the high selectivity of lesions caused by DMBA and the molecular specificity involved in the adrenocorticolytic effect.

A single feeding of DMBA resulted in a mild diarrhea and loss of weight. There was, in addition, a gradual decline in the level of alkaline phosphatase in the plasma to very low levels. There was a decrease in the number of circulating leukocytes with degenerative changes in spleen, thymus, lymph nodes, and bone marrow; the damaging effect of a carcinogenic hydrocarbon on

blood-forming organs has been described earlier (22). Moreover, there were the vivid changes in the adrenals first observed 2 days after the administration of the compound. Yet there were no changes in the estrus cycle of the rats proving that pituitary, ovary, and vagina had not been damaged significantly, if at all, by the hydrocarbon.

Visible hemorrhage caused by DMBA was found in the adrenal glands and in no other organ of the rats. Hemorrhage and necrosis induced by this compound in adrenals did not result in complete and permanent destruction of the glands; regeneration always occurred.

Adrenal hemorrhage and necrosis were caused equally as readily when DMBA was injected intravenously with no resultant loss of weight as when the compound was fed causing a moderate decline in body weight. The damage to the adrenals did not require the presence of the adrenal medulla—it occurred in enucleated adrenals whose cortex had regenerated subsequently. True, adrenal damage was often somewhat less in regenerated adrenals than in adrenal glands of rats which had not been operated upon but this might be attributed to a decreased blood supply in the enucleated adrenals resulting in a less effective exposure of the glands to DMBA.

Adrenal damage caused by DMBA is not a secondary effect resulting from unusually heavy stimulation of the adrenal cortex by ACTH or other pituitary hormones as a result of the stress of exposure to the compound. When DMBA was injected in rats which had been hypophysectomized just before the injection, the extent of the damage was as severe as in intact mates similarly treated. As the adrenals became increasingly atrophic following hypophysectomy (3 to 14 days after the operation) the extent of DMBA-induced adrenal necrosis decreased in parallel. In rats from which the pituitary had been removed 2 weeks or more before administration of DMBA, adrenal hemorrhage occurred in only a small number and the extent of the lesions in these was small; most of the rats in this category suffered no adrenal damage from DMBA. Necrosis did not occur in adrenals which were profoundly atrophic after hypophysectomy.

There is a topographic factor in adrenal damage from DMBA. Despite massive necrosis of most of zona fasciculata and zona reticularis by DMBA, living cortical cells were always found around the principal adrenal blood vessels.

A solitary feeding of *o,p'*-DDD resulted in large yellow fat-filled adrenal glands, with, in a small number of the recipients, hemorrhage which was of only slight degree and small areas of patchy necrosis in some of them. By contrast, DMBA resulted in adrenals depleted of fat with extensive hemorrhage and massive necrosis. Whereas both *o,p'*-DDD and DMBA induced pathological changes in rat adrenals which were distinctive with each compound, the *site* of the damage was the same with both compounds, namely the zona

fasciculata and zona reticularis, while zona glomerulosa and medulla were spared from damage.

The induction of selective and massive adrenal necrosis was not a property possessed in common by all carcinogenic hydrocarbons. Among the most powerful polynuclear aromatic hydrocarbons in evoking cancer are 3-methylcholanthrene, benzo(a)pyrene, 4-dimethylaminostilbene, 4-nitroquinoline-*N*-oxide, and 2-acetylaminofluorene; none of these induced changes in adrenals under the stated conditions. DMBA had unusual properties in this regard and these were related to the molecular structure of the compound.

DMBA was a highly effective corticolytic agent, yet its 9, 10-dimethylanthracene moiety was ineffective in this regard. The extra ring on the (a) face of 9, 10-dimethylanthracene (forming DMBA) was necessary to induce adrenal damage. 1,4-Dimethylphenanthrene did not damage the adrenals; the extra ring on the (b) face of this molecule (forming DMBA) was necessary to elicit the phenomenon. Therefore, the full complement of 4 rings in DMBA is necessary in order to have damage of the adrenal cortex.

Neither benz(a)anthracene nor 7-methylbenz(a)anthracene inflicted damage on the adrenals. Moreover 7,12-diethylbenz(a)anthracene did not injure the adrenal cortex. Therefore, the presence of the 2 methyl groups in the *meso* positions of benz(a)anthracene (forming DMBA) are necessary for adrenal damage to result from the latter compound.

In DMBA, hydrogen atoms are not necessary for the induction of adrenal damage by this compound. Dimethylbenz(a)anthracene-16d, having more than 95 per cent of the hydrogen atoms replaced by deuterium atoms, caused the corticolytic effect. Therefore, the nuclei of the hydrogen atoms in DMBA are not of cardinal importance in adrenal damage. The hydrogen atoms can be replaced by deuterium and still adrenal damage results. By exclusion we are left with the electronic status of hydrogen (or deuterium) atoms in DMBA or its perdeuterated isomer.

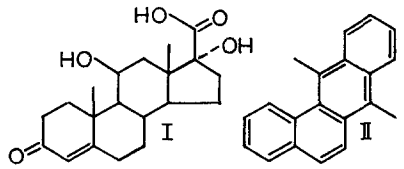
An outstanding property of DMBA is its ability to donate electrons to appropriate electron acceptors and so to form charge-transfer complexes. In this regard charge-transfer may comprise the formation of a donor-acceptor complex of DMBA with a large aromatic molecular acceptor (23) and may involve a strong local donating property of certain C atoms to small molecules as Szent-Györgyi (24) has discovered with I:I. We find in qualitative tests that both 9,10-dimethylanthracene and DMBA as well form strong charge-transfer complexes with 1,3,5-trinitrobenzene or 2,4,7-trinitro-9-fluorenone and also with iodine molecules (24). 1,4-Dimethylphenanthrene did not give these reactions. Both 9,10-dimethylanthracene and DMBA participate very well in local and also the more general type of charge-transfer; yet only DMBA causes adrenal necrosis.

In examination of the structural formulae of DMBA and of hydrocortisone

(Text-fig. 4) and also of Stuart-Briegleb molecular models of DMBA and cortisone (Fig. 10), the similarity of the 2 compounds from a steric standpoint is apparent. It is well established that hydroxycorticosteroids are synthesized in zona fasciculata and the reticularis of the adrenal cortex which are the zones damaged by DMBA.

We postulate that there are two considerations of prime importance in the induction of adrenal necrosis by DMBA: (*a*) steric factor; (*b*) electronic factor. The structural similarity of cortisone and DMBA permits entry of the latter into the cortex and a steric fit into molecular sites of the adrenal cortex where hydrocortisone and cortisone are synthesized. Once DMBA is localized in a closely fitted site charge-transfer sets events in motion which result in death of the cell and adrenal apoplexy results around the damaged cells.

In regard to the mechanism of production of cancer by polynuclear hydro-



TEXT-FIG. 4. Hydrocortisone (I) and DMBA (II).

carbons, Szent-Györgyi has demonstrated that "carcinogenicity of these substances is connected with their ability to form strong charge-transfer complexes with local acceptors and (to) give off an electron" (24). In addition, our colleague, Dr. Yang (25), has demonstrated that there is a steric factor responsible for the carcinogenicity of polynuclear aromatic hydrocarbons. "A carcinogenic polynuclear aromatic hydrocarbon must bear steric resemblance to steroids" (25).

It would appear that factors similar to those of importance in carcinogenesis by polynuclear aromatic hydrocarbons operate in the production of adrenal necrosis by DMBA. DMBA shares with other powerful carcinogens such as 3-methylcholanthrene and 4-dimethylaminostilbene among others the ability to form strong charge-transfer complexes with appropriate electron acceptors but adrenal necrosis was not caused by the other hydrocarbons. The unique property of DMBA in damaging adrenal is due to the fact that it alone among the hydrocarbons tested possesses the necessary geometry *vis-à-vis* the inner adrenal cortex. The steric factor assumes primary importance in DMBA-induced adrenal damage.

It has been demonstrated (1) that mammary cancer arises in a few weeks in every Sprague-Dawley female rat that has received a single dose of DMBA at age 50 days in dosage which was found in the present experiments to be optimal in inducing adrenal necrosis. In the mammary glands of these animals, DMBA induces cancer but not necrosis; in the adrenal glands of the same

animals extensive necrosis occurred but cancer of the adrenal was not observed after prolonged observation of the animals. Cancer cannot arise in dead cells.

But adrenal damage is not a prerequisite to mammary cancer. A single feeding of many hydrocarbons (1, 2) which do not damage the adrenal glands induces mammary cancer. Moreover, doses of DMBA insufficient to induce adrenal damage cause cancer of the breast. Further mammary cancer arises readily in adrenalectomized rats given DMBA.

The technical procedures of detection and measurement of the happenings in the adrenal cortex after a dose of DMBA by enzyme methods are simple and useful. Serial determination of the pyridine nucleotide–linked dehydrogenases provides a quantitative measure of intra-adrenal damage and repair and permits a graphic representation of the biochemical events in the adrenal cortical cell. Since all of these enzymes decline and subsequently rise in parallel with each other and in response to necrosis and repair, respectively, it is not necessary to measure many of these enzymes to obtain informative results. The measurement of ICD alone was found to be simple and meaningful but care must be exercised in interpreting ICD values since this enzyme reflects estradiol-17β status as well as the growth or degeneration of the adrenal cortex.

An incidental finding in the present work was that the level of ICD in adrenal reflects the presence of estradiol-17β in the body. It is known that in the rat the adrenals of females are larger than those of the males (26). The effect of estradiol-17β on ICD concentration is the first indication that sex hormones can exert a specific influence on the enzyme status of adrenals.

CONCLUSIONS

Invariably in every normal rat a single dose of 7,12-dimethylbenz(a)anthracene, by mouth or injected in a vein, was found to cause apoplexy and massive necrosis in the inner zones of the adrenal cortex; the zona glomerulosa, the adrenal medulla, and a small region of cortex adjacent to the great adrenal vessels were spared from damage. DMBA caused these selective lesions in females and in males of 2 strains of rats. Hemorrhage and necrosis were observed in no organ other than the adrenal gland. Whereas adrenal glands were heavily damaged by DMBA, pituitary and ovary escaped injury by the compound.

A single huge but sublethal feeding of *o,p'*-DDD caused degenerative changes of minor magnitude in the adrenals and only in a small percentage of rats; the property of inducing adrenal damage was not shared by other polynuclear aromatic hydrocarbons which were investigated, including strong carcinogens.

Presence of adrenal medulla is not a prerequisite to damage of the adrenal cortex by DMBA. The adrenal damage occurred in rats, given DMBA, from which the pituitary had been removed but the lesions were smaller in extent and less in incidence as post-hypophysectomy atrophy of the adrenal cortex progressed.

The entire DMBA molecule was necessary to induce adrenal damage;

fragments of this molecule did not induce adrenal lesions. Two components which are of cardinal importance in this specific damaging effect are: (*a*) electronic factor; (*b*) steric factor.

The level of isocitric dehydrogenase in adrenal is modified considerably by presence or absence of estradiol-17β.

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EXPLANATION OF PLATES

Figs. 4 to 9 are photomicrographs of paraffin sections stained with hematoxylin and eosin.

PLATE 74

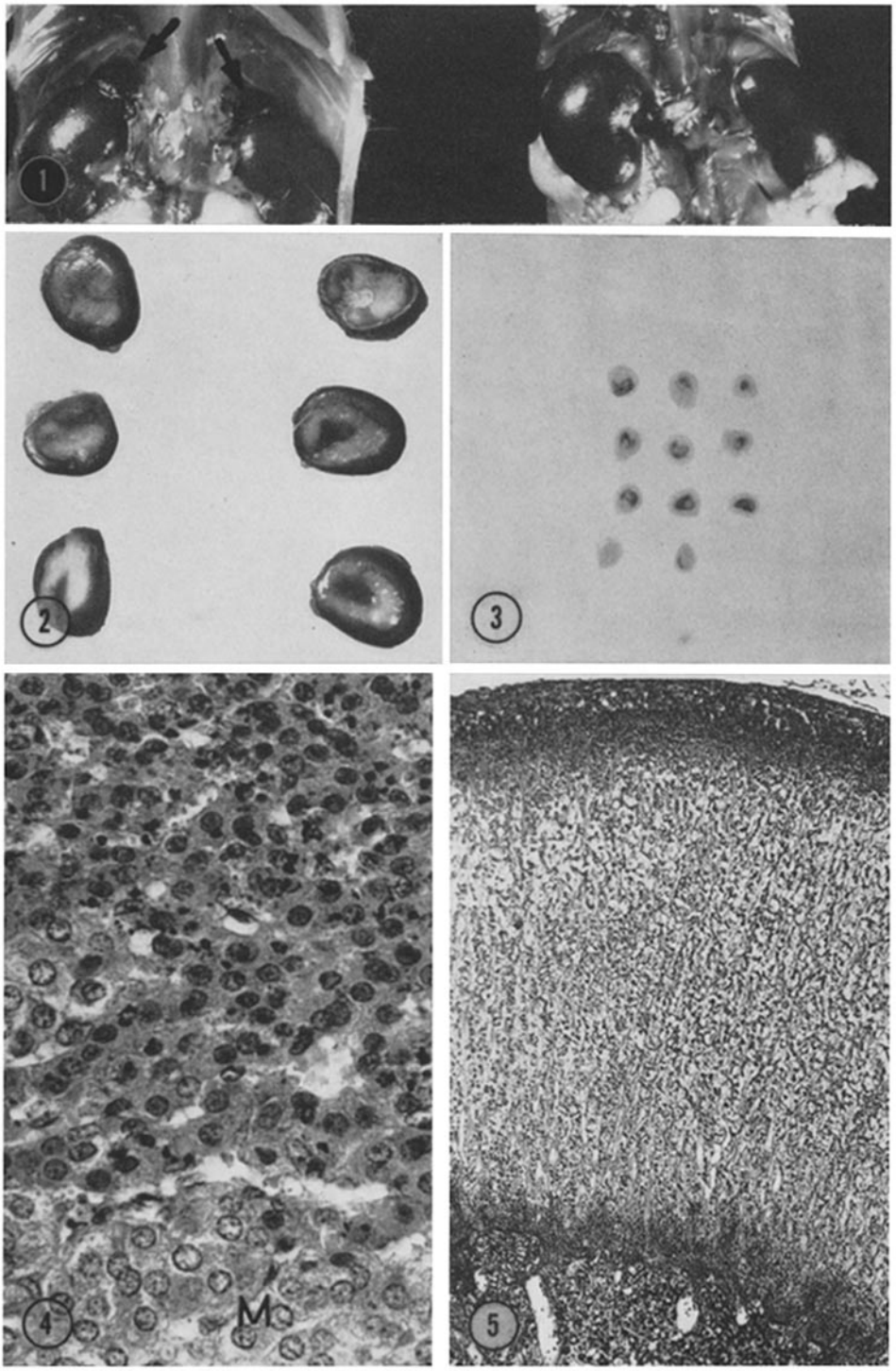
FIG. 1. On the left bilateral adrenal apoplexy (arrows), on day 3 following a single feeding of DMBA, 30 mg, and on the right normal adrenal glands of an untreated sister are shown.

FIG. 2. 6 adrenal glands from 3 rats, on day 7 after a single feeding of DMBA, 30 mg. There is no necrosis in the adrenal medulla. Partial regeneration of the adrenal cortex surrounds the white necrotic areas.

FIG. 3. Radiographs of 11 adrenal glands on day 69 after a single feeding of DMBA, 30 mg. Calcification in horseshoe pattern is evident in 10 glands; 1 adrenal is not calcified.

FIG. 4. Many shrunken pycnotic cells are demonstrated in zona reticularis on day 1 after feeding DMBA, 30 mg. The adrenal medulla (*M*) is undamaged. × 500.

FIG. 5. On day 3 after a single feeding of DMBA, 30 mg, complete necrosis of the inner zones of adrenal cortex is found. Necrotic areas are surrounded with hemorrhage beneath zona glomerulosa and above medulla. Compare with Fig. 1. × 60.

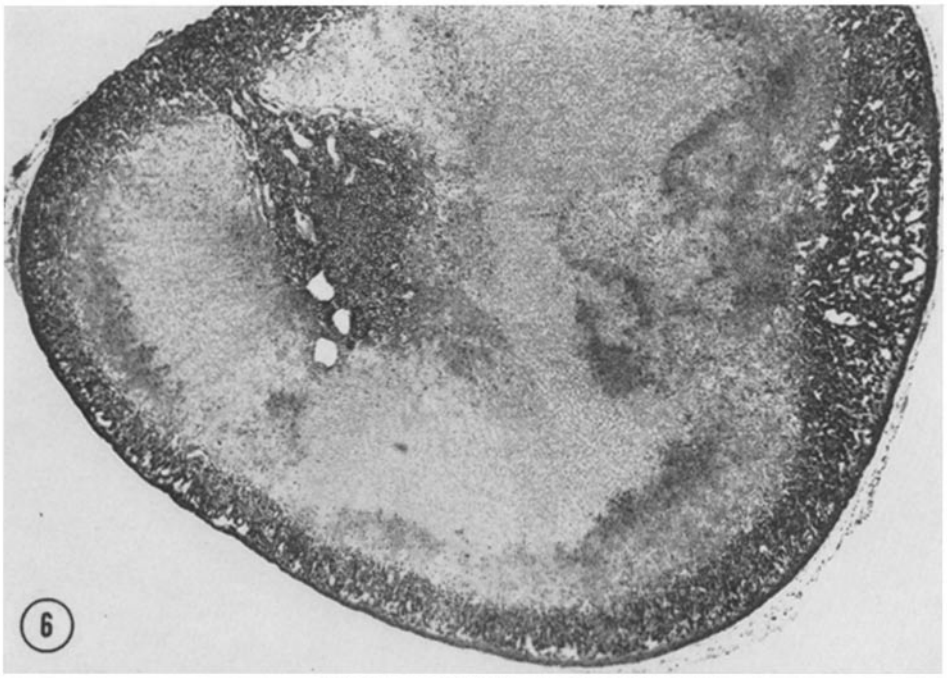


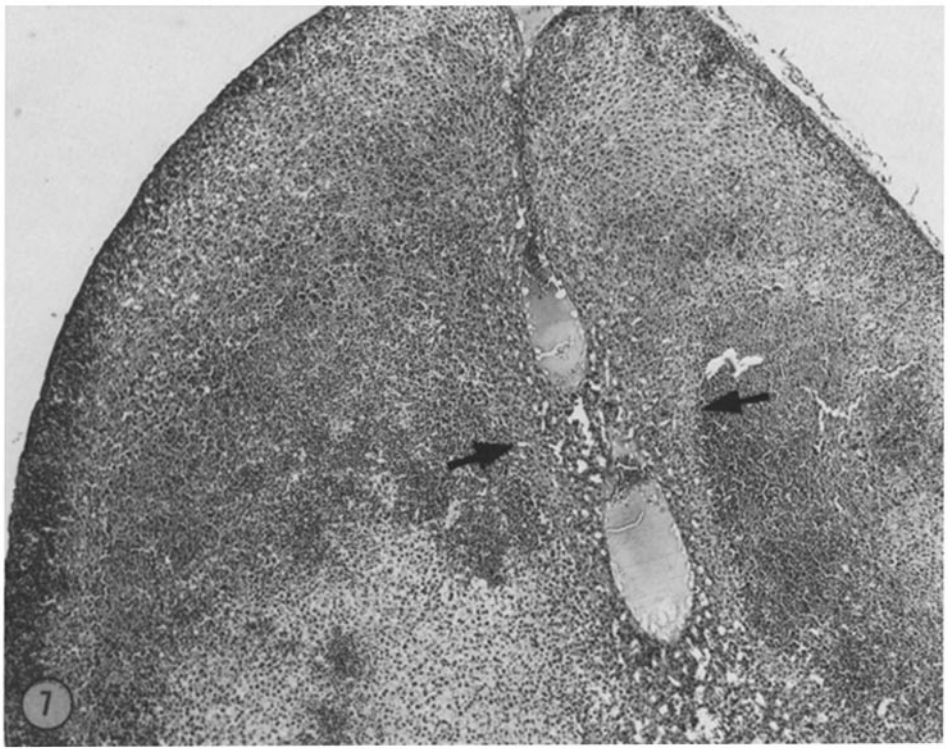
(Huggins and Morii: Selective adrenal necrosis)

PLATE 75

FIG. 6. Partial regeneration of adrenal cortex from zona glomerulosa on day 6 after a single feeding of DMBA, 30 mg. Massive necrosis, with hemorrhage, of the inner cortex is still present. Compare with Fig. 2. × 33.

FIG. 7. On day 4 after feeding DMBA, 30 mg, much adrenal cortical necrosis and hemorrhage are found but there is a topographic influence—cortical cells (arrows) near the great adrenal vessels are uninjured. × 60.



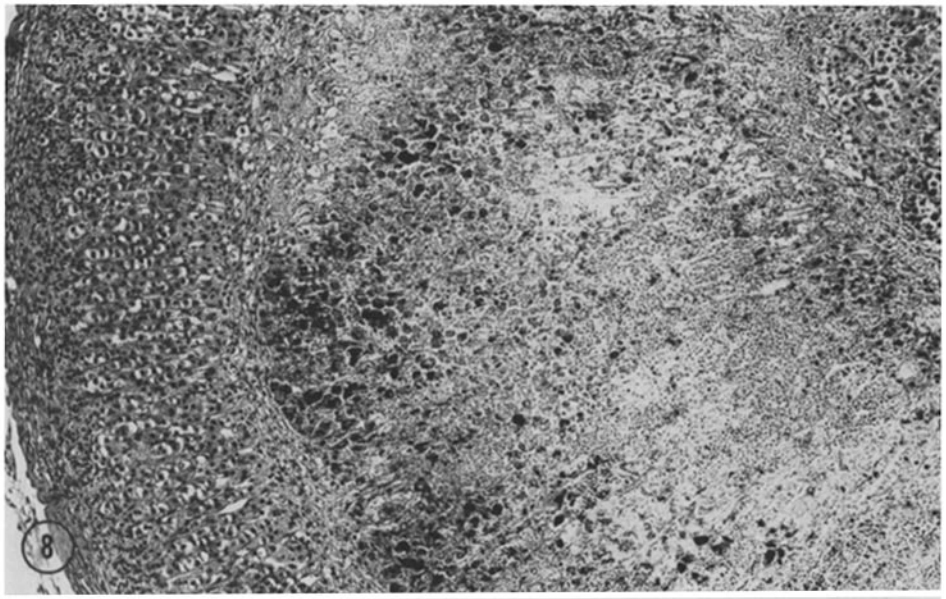


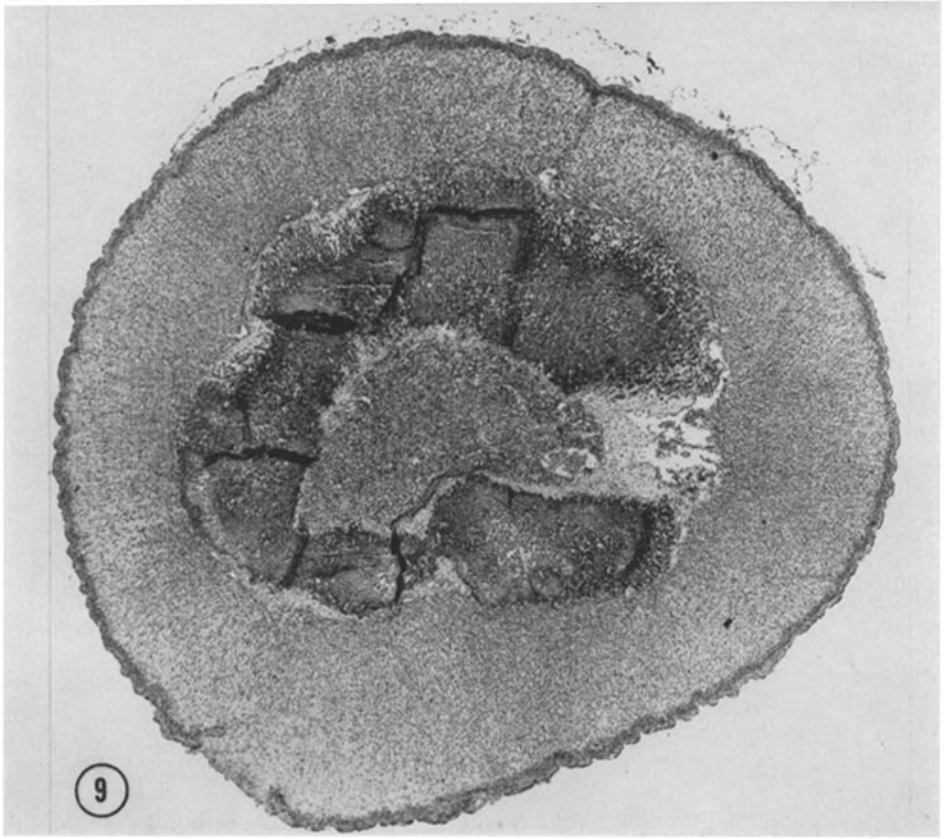
(Huggins and Morii: Selective adrenal necrosis)

PLATE 76

FIG. 8. On day 14 after a single feeding of DMBA, 30 mg, there is seen in the adrenal cortex, from left to right—zona glomerulosa, regenerating adrenal cortex, a narrow band of fibroblasts, calcification, and necrosis. × 100.

FIG. 9. On day 69 after a single feeding of DMBA, 30 mg, a horseshoe-shaped zone of calcification is evident surrounding adrenal medulla. Necrosis has disappeared. × 35.

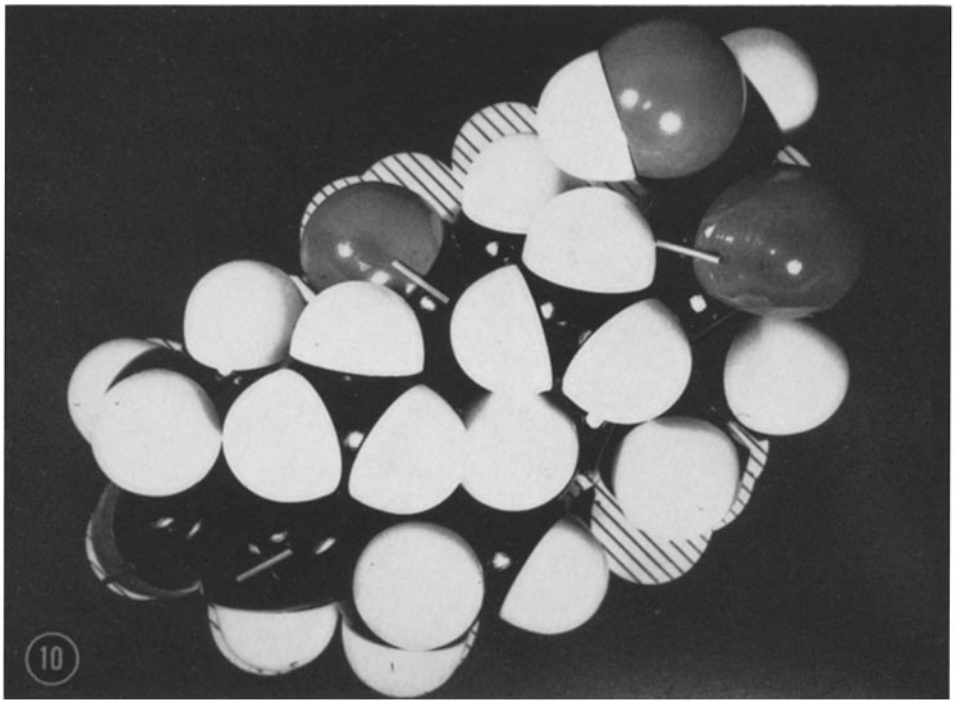




(Huggins and Morii: Selective adrenal necrosis)

PLATE 77

FIG. 10. This figure demonstrates the close steric similarity between DMBA and cortisone. A Stuart-Briegleb model of DMBA was constructed and its impression (cross-hatched) was photographed in natural size. A similar model of cortisone was then superimposed on the photographed impression of DMBA. The overlap of the molecular models is nearly complete. Dr. N. C. Yang kindly constructed the models.



(Huggins and Morii: Selective adrenal necrosis)