

Two lines of inquiry concerning the function of exocrine glands, especially the salivary glands, have recently come into focus. The first deals with the problem of how these glands produce hypotonic secretions whose electrolyte and osmolal composition is flow dependent. By indirect methods, several workers have attempted to dissect the role of acinar from tubular components to understand this phenomenon. Their conclusions, however, are contradictory(4,5). By direct methods of micropuncture and microcatheterization other workers have clarified some aspects of this problem, at least in the rat submaxillary gland(6,7). The tubular system buried deep in the gland, however, remains invulnerable to micropuncture. Until this system can be made accessible, an understanding of water and solute movements between cell, interstitium and lumen will not likely be achieved.

The second area of inquiry relates to the questions of the metabolic substrates for secretion and the controls of secretory events. This area is relatively unexplored in these glands(8), owing in part to the complexity of their blood supply and the difficulty of critically manipulating their milieu *in vivo*.

An *in vitro* preparation of these tissues could allow exposure of the tubular system, critical adjustment of the cellular environment, and convenient detection of cellular

functional changes. It is felt that the collagenase preparation described here provides the opportunity for fulfilling these requirements.

*Conclusions.* This paper has presented a method of preparation of acini and tubuli from the parotid gland of the dog. The viability of the system is evidenced by its capacity to accumulate  $I^{131}$  by a metabolically dependent process. Free inulin distribution is restricted. It is suggested that this *in vitro* preparation might prove useful in the solution of two of the major issues in exocrine gland physiology.

1. Burg, M. B., Orloff, J., Am. J. Physiol., 1962, v203, 327.
2. Walser M., Davidson, D. G., Orloff, J., J. Clin. Invest., 1955, v34, 1520.
3. Cohen, B., Myant, N. B., J. Physiol., 1959, v145, 595.
4. Henriques, B. L., Am. J. Physiol., 1961, v201, 935.
5. Burgen, A. S. V., Terroux, K. G., J. Physiol., 1963, v169, 663.
6. von Martinez, J. R., Holzgrev, H., Vogel, A., Pflüg Arch. f.d.g. Physiol., 1965, v283, R1.
7. Schogel, E., Young, J. A., J. Physiol., 1966, v183, 73P.
8. Schneyer, L. H., Schneyer, C. A., Advances in Oral Biol., 1963, v1, 1.

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### Low Carcinogenicity of the K-Region Epoxides of 7-Methylbenz(a)-anthracene and Benz(a)anthracene in the Mouse and Rat.\* (31885)

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The induction of tumors at sites of application of sub-milligram quantities of the carcinogenic polycyclic aromatic hydrocarbons and the very low carcinogenic activity of the phenolic or quinone metabolites which have been tested(1) have led to the idea that the hydrocarbons may be carcinogenic without metabolic conversion. On the other hand, correlations have been obtained between the covalent binding of various hydrocarbons to

proteins and nucleic acids in mouse skin *in vivo* and the carcinogenic activities of the

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hydrocarbons in this tissue(2-5). These data may indicate that the hydrocarbons are converted *in vivo* to reactive metabolites with carcinogenic activities comparable to or greater than those of the parent hydrocarbons. Furthermore, recent studies of Boyland and his colleagues(6-9) strongly indicate that an epoxidative attack occurs at the phenanthrene-like double bond or K-region, as well as at other sites, of several polycyclic aromatic hydrocarbons. The reactivity of these epoxides (8,9) and the carcinogenic activity of a number of aliphatic epoxides(10-13) suggested that the K-region epoxides might be proximate carcinogenic metabolites of the polycyclic aromatic hydrocarbons. A test of this hypothesis was made possible by the synthesis of the K-region epoxides of benz(a)anthracene and 7-methylbenz(a)anthracene by Newman and Blum(14). The data in this report show that these compounds have very little carcinogenic activity for mouse skin and the subcutaneous tissues of the rat and mouse.

*Materials and methods.* Young adult female albino mice of the ICR/HA and STS strains (A. R. Schmidt Co., Madison, Wisc.), 25-30 g in weight, and young adult male CD non-inbred albino rats (Charles River Breeding Laboratories, North Wilmington, Mass.), 180-200 g in weight, were employed. Both species were fed Wayne Breeder Chow and water *ad libitum*. The mice were kept in groups of 5 and the rats in groups of 2 in raised-bottom screen cages. 7-Methylbenz(a)anthracene, 5,6-epoxy-5,6-dihydro-7-methylbenz(a)anthracene, and 5,6-epoxy-5,6-dihydrobenz(a)anthracene were kindly supplied by Drs. M. S. Newman and S. Blum, Department of Chemistry, Ohio State University, Columbus, Ohio. Benz(a)anthracene was obtained from Eastman Organic Chemicals and recrystallized from benzene-hexane to a m.p. of 159-160°, uncorr.

7-Methylbenz(a)anthracene and its epoxide were tested in rats and mice for their ability to induce the formation of sarcomas following subcutaneous injection of single doses, in mice for their ability to produce skin tumors following repeated topical applications, and in mice for their capacity to initiate papilloma formation by limited topical doses followed

by repeated topical doses of croton oil. Benz(a)anthracene and its epoxide were compared only in the latter test. In all cases the compounds were dissolved without heat immediately prior to administration of the solution. To minimize possible decomposition the trioctanoin solutions of 5,6-epoxy-5,6-dihydro-7-methylbenz(a)anthracene were prepared with nitrogen bubbling through the oil. The details of these tests are given in Table I-III. Tumor counts were made at biweekly intervals. At autopsy the tumors were fixed in 10% neutral formalin, sectioned at 6  $\mu$ , and stained with hematoxylin and eosin. We are indebted to Dr. Henry Pitot of the McArdle Laboratory for histological examination of these tissues.

*Results. Sarcomas.* Table I presents the incidences of sarcomas in rats and mice following single subcutaneous injections of 7-methylbenz(a)anthracene, a moderately active carcinogenic hydrocarbon, or its K-region epoxide. The hydrocarbon induced sarcomas at the site of injection in 75 and 63% of the rats and mice, respectively. Only 1 of 20 rats and 7 of 40 mice which received injections of the epoxide developed sarcomas. No sarcomas were found in the control animals which received injections of the solvent trioctanoin.

*Skin tumors.* As shown in Table II only 1 mouse developed a skin tumor after repeated topical applications of the epoxide of 7-methylbenz(a)anthracene, while 53% of the mice treated in the same way with the parent hydrocarbon developed carcinomas of the skin.

When an initiating dose of 7-methylbenz(a)anthracene was applied once to the skin of mice and followed by repeated applications of a solution of croton oil, moderate incidences of benign and malignant skin tumors were obtained (Table III). The epoxide of 7-methylbenz(a)anthracene did not cause a significant increase in the number of skin tumors as compared to the control group which received only the applications of croton oil. Furthermore, under the same conditions the very weak carcinogen benz(a)anthracene and its K-region epoxide, though applied at 4 times the dose of 7-methylbenz(a)anthracene, did not induce significantly more skin tumors

TABLE I. Incidence of Sarcomas in Rats and Mice Given Single Subcutaneous Injections of 7-Methylbenz(a)anthracene or 5,6-Epoxy-5,6-dihydro-7-methylbenz(a)anthracene.\*

Group	Compound	No. and species	Cumulative No. of animals with sarcomas at injection site†				No. killed without sarcomas at 15 mo
			4 mo	6 mo	9 mo	15 mo	
1	7-Methylbenz(a)anthracene	20 rats	1	4	11	15	2
2	5,6-Epoxy-5,6-dihydro-7-methylbenz(a)anthracene	" "	0	0	0	1	13
3	None	" "	0	0	0	0	16
4	7-Methylbenz(a)anthracene	40 mice	14	22	25	25	4
5	5,6-Epoxy-5,6-dihydro-7-methylbenz(a)anthracene	" "	0	2	3	7	16
6	None	" "	0	0	0	0	18

\* The rats each received 1 subcutaneous injection of 0.25 ml of trioctanoin which contained 2.0 mg of 7-methylbenz(a)anthracene, 2.14 mg of the epoxide, or no addition. The mice (ICR/HA strain) each received 1 subcutaneous injection of 0.1 ml of trioctanoin which contained 1.0 mg of 7-methylbenz(a)anthracene, 1.07 mg of its epoxide, or no addition. In each case the solution of the hydrocarbons was facilitated by stirring with a magnetic stirrer; the trioctanoin was flushed with nitrogen prior to addition of the epoxide and during its solution. All injections were made in the right hind leg.

† In addition to sarcomas the following tumors were found: Group 1—1 lymphosarcoma in the spleen; Group 2—2 rats with mammary adenomas and 1 with a mammary adenocarcinoma; Group 5—4 mice with mammary carcinomas, 1 with a thymoma, and 1 with a lung adenoma; Group 6—6 mice with mammary carcinomas, 2 with lymphoma, 1 with a thymoma, and 2 with lung adenomas.

TABLE II. Incidence of Skin Tumors in Mice Given Repeated Topical Applications of 7-Methylbenz(a)anthracene or 5,6-Epoxy-5,6-dihydro-7-methylbenz(a)anthracene.\*

Group	Compound	Cumulative No. of mice with skin tumors†					No. killed without skin tumors at 15 mo
		Malignant				Benign	
		7 mo	9 mo	12 mo	15 mo		
1	7-Methylbenz(a)anthracene	4	9	17	21	4	5
2	5,6-Epoxy-5,6-dihydro-7-methylbenz(a)anthracene	1	1	1	1	0	28
3	None	0	0	0	0	0	28

\* Groups of 40 ICR/HA female mice were administered topically 0.05 ml of redistilled acetone alone or containing 70  $\mu$ g of 7-methylbenz(a)anthracene or an equimolar amount of the epoxide twice weekly for 20 wk.

† Mice with both malignant and benign skin tumors were tabulated only in the group with malignant tumors. In addition to skin tumors the following tumors were found: Group 1—1 mouse with lymphoma; Group 2—1 mouse with lymphoma, 2 with thymoma, 2 with pulmonary adenomas, and 1 with mammary carcinoma; Group 3—1 mouse with lymphoma, 2 with pulmonary adenomas, and 2 with mammary carcinomas.

than the croton oil treatments alone.

*Discussion.* The data in this paper do not appear to support the idea that metabolically derived epoxides in the K-region are proximate carcinogenic metabolites of the polycyclic aromatic hydrocarbons. However, two qualifying factors must be recognized. First, the steric configuration of the synthetic epoxides may not be the same as that of the possible metabolites. The synthetic epoxides have the *cis* configuration (personal com-

munication from Dr. Newman). Second, for a meaningful test the epoxide would have to penetrate the cells of the skin and subcutaneous tissue; the ready solubility of the compound in lipids and lipid solvents makes such penetration a reasonable assumption.

Two other types of possible proximate carcinogenic metabolites of the polycyclic aromatic hydrocarbons are worthy of exploration. Thus, addition of molecular oxygen to the hydrocarbons might yield reactive

TABLE III. Incidence of Skin Tumors in Mice Given Initiating Doses of Benz(a)anthracene, 7-Methylbenz(a)anthracene, or the 5,6-Epoxy-5,6-dihydro Derivatives of These Hydrocarbons and Repeated Applications of Croton Oil.\*

Compound	Dose	No. of mice alive at		Incidence of benign skin tumors†				No. of mice with skin carcinomas by 38 wk
		20 wk	29 wk	13 wk	16 wk	20 wk	29 wk	
7-Methylbenz(a)anthracene	1 × 300 μg	24	18	14 (23)	15 (37)	16 (49)	11 (41)	9
5,6-Epoxy-5,6-dihydro-7-methylbenz(a)-anthracene	1 × 322 μg	22	20	3 (3)	4 (5)	9 (12)	10 (25)	3
Benz(a)anthracene	4 × 300 μg	24	23	2 (2)	6 (11)	5 (11)	6 (15)	1
5,6-Epoxy-5,6-dihydro-benz(a)anthracene	4 × 320 μg	26	26	1 (1)	2 (2)	10 (16)	15 (21)	1
None (solvent only)	4	24	21	0	3 (5)	6 (9)	5 (15)	0

\* Groups of 26 female mice of the STS (skin tumor-sensitive) strain were administered topically 0.1 ml of dimethylsulfoxide (spectroquality reagent, Matheson, Coleman and Bell) containing 7-methylbenz(a)anthracene or its epoxide once or four 0.1-ml doses of benz(a)anthracene or its epoxide at 3-4 day intervals. The mice in all groups received twice weekly applications of 0.05 ml of a 0.3% solution of croton oil in redistilled acetone starting 10 days after the last initiating dose; concentration of croton oil was reduced to 0.2% at 10 wk. Croton oil applications were continued until experiment was terminated at 38 wk.

† In each case the first number is the number of mice bearing one or more benign skin tumors; number in parentheses is total number of benign skin tumors in the group.

hydroperoxides. These compounds are apparently not yet known in the aromatic hydrocarbon series, but can not be excluded as possible transient metabolic intermediates. A second possibility is suggested by the metabolism of benzene to phenol by guinea pig liver microsomes in which benzyl alcohol occurs as an intermediate(15). According to this model hydroxymethyl derivatives of the polycyclic aromatic hydrocarbons might be precursors of one or more of the observed non-carcinogenic phenolic metabolites. Furthermore, esterification of such benzylic alcohols to acyloxymethyl derivatives, if it occurred *in vivo*, might yield metabolites with reactivities for cellular constituents similar to those noted recently for N-acyloxy metabolites of carcinogenic aromatic amines(16,17). Either of these types of possible metabolic activation of the hydrocarbons might occur most reasonably at the K-region, especially at carbon-5(18,19), for example, in 7-methylbenz(a)anthracene.

**Summary.** 5,6-Epoxy-5,6-dihydro-7-methylbenz(a)anthracene, a K-region epoxide of the carcinogenic hydrocarbon 7-methylbenz(a)-anthracene, had only low activity as a carcinogen in the subcutaneous tissues of the rat and mouse or in the skin of the mouse under

conditions which permitted the parent hydrocarbon to exhibit moderate to high carcinogenicity in these tissues. Likewise, 5,6-epoxy-5,6-dihydrobenz(a)anthracene or its 7-methyl derivative showed little or no activity as initiators of papilloma formation in mouse skin.

1. Clayson, D. B., *Chemical Carcinogenesis*, Little, Brown & Co., 1962, p467.
2. Abell, C. W., Heidelberg, C., *Cancer Research*, 1962, v22, 931.
3. Heidelberg, C., *J. Cell. Comp. Physiol.*, 1964, v64, suppl. 1, 129.
4. Brookes, P., Lawley, P. D., *Nature*, 1964, v202, 781.
5. ———, *J. Cell. Comp. Physiol.*, 1964, v64, suppl. 1, 111.
6. Boyland, E., *Brit. Med. Bull.*, 1964, v20, 121.
7. Boyland, E., Sims, P., *Biochem. J.*, 1965, v95, 788.
8. ———, *ibid.*, 1965, v97, 7.
9. Sims, P., *ibid.*, 1966, v98, 215.
10. Hendry, J. A., Homer, R. F., Rose, F. L., Walpole, A. L., *Brit. J. Pharmacol.*, 1951, v6, 235.
11. Walpole, A. L., *Ann. N. Y. Acad. Sci.*, 1958, v68, 750.
12. Van Duuren, B. L., Nelson, N., Orris, L., Palmes, E. D., Schmitt, F. L., *J. Nat. Cancer Inst.*, 1963, v31, 41.
13. Van Duuren, B. L., Orris, L., Nelson, N., *ibid.*, 1965, v35, 707.

14. Newman, M. S., Blum, S., J. Am. Chem. Soc., 1964, v86, 5598.
15. Sloane, N. H., Biochem. Biophys. Acta, 1965, v107, 599.
16. Lotlikar, P. D., Scribner, J. D., Miller, J. A., Miller, E. C., Life Sci., 1966, v5, 1263.
17. Miller, E. C., Juhl, U., Miller, J. A., Science, 1966, v153, 1125.
18. Miller, E. C., Miller, J. A., Cancer Research, 1960, v20, 133.
19. Miller, J. A., Miller, E. C., *ibid.*, 1963, v23, 229.

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### Immunosuppression by Endotoxin and its Lipoid A Component.\* (31886)

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An unusual type of immunosuppression was identified on the basis of studies dealing with the seeming lack of immunogenicity of the common antigen (CA) present in cultures of enterobacteriaceae. This antigen was first recognized by Kunin *et al*(1,2) by means of the hemagglutination test and *E. coli* O14 antiserum. Although *Escherichia*, *Aerobacter*, *Salmonella*, *Shigella*, and *Proteus* all contain CA, only *E. coli* O14 upon intravenous injection into rabbits elicits the formation of CA antibodies in high titers. Otherwise, diagnostic antisera used for serotyping of enteric bacteria would contain CA antibodies in significant amounts, and they do not. The lack of immunogenicity of organisms other than *E. coli* O14 is not due to the fact that they produce less CA than the latter, for immunization with equivalent amounts of CA results in the formation of antibodies only with *E. coli* O14(3). Nor can the lack of immunogenicity of the bacteria be explained on the basis of its absence on the cell surface, for CA has been detected there by immunofluorescence(4). CA antibodies in moderate titers can be produced if supernates of cultures of enteric bacteria are injected together with Freund's adjuvant or in the cell-attached rather than in the soluble state(3,5,6). Subsequent investigations in our laboratories have revealed that CA, after separation from

the simultaneously present O antigen, is highly immunogenic(7). The surprising observation was then made that the injection of mixtures of CA and O antigen (lipopolysaccharide) failed to result in the formation of CA antibodies in high titer(8). Lipoid A produced the same immunosuppressive effect(9). This effect does not take place, if the inhibitors and CA are injected separately, although simultaneously(8). Even though both these inhibitors interfere with the production of circulating CA antibodies, they do not abolish immunologic priming, for animals thus immunized respond with the rapid formation of CA antibodies in significant titers upon the injection of subeffective doses of isolated CA (10). It would be surprising, indeed, if this type of immunosuppression by lipopolysaccharide and its lipoid A component were operative only with a single antigen. The present investigation has revealed that both lipopolysaccharide and lipoid A also interfere with the formation of circulating antibodies against a common antigen of gram-positive bacteria, first described by Rantz *et al*(11).

*Materials and methods.* Antigens were prepared as follows. Strains of *Staphylococcus aureus* (coagulase-positive; Difco) and *Bacillus subtilis* were grown on brain veal agar in Kolle flasks at 37°C for 18 hours. The growth was suspended in 25 ml of phosphate hemagglutination buffer (pH 7.3; Difco) per Kolle flask. The suspension was centrifuged at

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