[CONTRIBUTION FROM THE McARDLE MEMORIAL LABORATORY, THE MEDICAL SCHOOL, UNIVERSITY OF WISCONSIN]

The Metabolic Degradation in the Mouse of 1,2,5,6-Dibenzanthracene-9,10-C^{14}. III. Some Quinone Metabolites Retaining the Intact Ring System[1-3]

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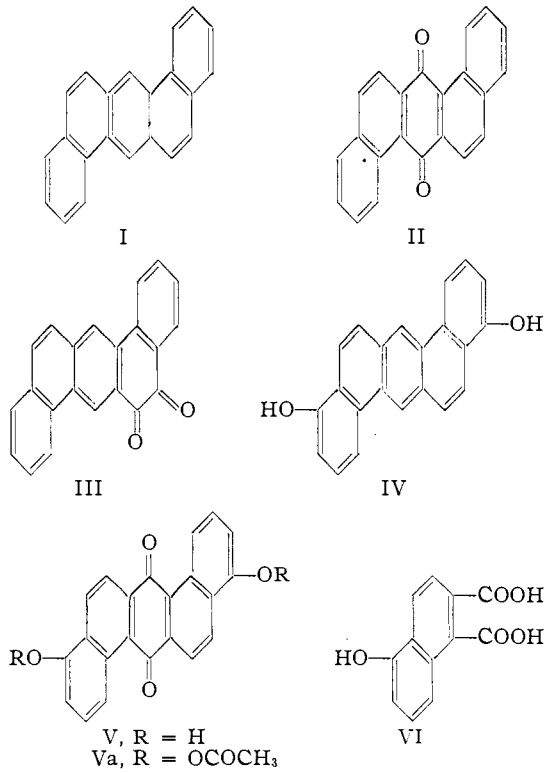
RECEIVED NOVEMBER 14, 1952

By means of the radioactive carrier technique, three new metabolites of 1,2,5,6-dibenzanthracene-9,10-C^{14} have been identified. They are quinones retaining the intact pentacyclic ring system and are dibenz-9,10-anthraquinone, dibenz-3,4-anthraquinone, and 4',8'-dihydroxydibenz-9,10-anthraquinone. The former has been detected in liver and skin, the latter two in liver only. These compounds are metabolites involving biological oxidation in the *meso* positions and phenanthrene double-bond; loci within the carcinogenic hydrocarbon molecule that have not previously been shown to be attacked. These metabolites are found quantitatively in only small amounts.

In the two decades that have elapsed since the classical experiments of Kennaway and Hieger[4] demonstrated for the first time that a pure compound, 1,2,5,6-dibenzanthracene, could induce cancer, some effort has been devoted to a study of the metabolism of this compound. By means of the usual techniques of organic and biochemistry three metabolites have been isolated from the excreta of animals given enormous doses of the carcinogen. One of these metabolities, isolated by Dobriner, Rhoads and Lavin[5] from urine of rats and mice, has been shown by the synthesis of Cason and Fieser[6] to be 4',8'-dihydroxydibenzanthracene. Another dihydroxy compound, as yet unidentified, was isolated from rabbit excreta by Levi and Boyland.[7] Cook and Schoental[8] have obtained an unidentified monohydroxydibenzanthracene from rabbit feces.

In 1947, 1,2,5,6-dibenzanthracene-9,10-C^{14} was synthesized,[9] and a comprehensive investigation of the distribution, metabolism and mechanism of carcinogenic action of this compound was initiated. It was possible, using minimal doses and individual mice, to trace quantitatively the anatomical distribution and pattern of excretion of radioactivity following administration by a variety of routes.[10] Furthermore, the rates of excretion of labeled dibenzanthracene and benzpyrene-5-C^{14} from the sites of subcutaneous injection and application to the skin have been determined and correlated with their carcinogenic potency.[11]

Concurrently, investigations on the metabolism of labeled dibenzanthracene were carried on, and it was demonstrated by chemical fractionation and radioactivity measurements[12] that the compound was degraded metabolically to a very large extent into carboxylic acids. One of these acids has been identified as 5-hydroxy-1,2-naphthalic acid, which results from cleavage of the pentacyclic ring system.[3] The identification was made possible by the use of carrier technique. In this method, a non-labeled sample of a compound suspected of being a metabolite is added to an appropriate radioactive fraction and is isolated and purified with great care. If radioactivity is present in the isolated carrier and is maintained constant through extensive and exhaustive purifications, it may be concluded that the compound added is indeed a metabolite. Moreover, the method furnishes a sensitive and accurate quantitative determination of the metabolite. This technique has been applied in the present study and the following three quinones have been shown to be metabolities: 1,2,5,6-dibenz-9,10-anthraquinone (II), 1,2,5,6-dibenz-3,4-anthraquinone (III) and 4',8'-dihydroxy-1,2,5,6-dibenz-9,10-anthraquinone (V).



The search for compounds II and III was initiated because quinones had been found as meta-

(1) This work was supported in part by a grant-in-aid from the National Cancer Institute, and in part by a grant from the Wisconsin Section of the American Cancer Society.

(2) An abstract of part of this work appears in *Cancer Research*, **11**, 290 (1951).

(3) For part II see C. Heidelberger and W. G. Wiest, *ibid.*, **11**, 511 (1951).

(4) E. L. Kennaway and I. Hieger, *Brit. Med. J.*, **1**, 1044 (1930).

(5) K. Dobriner, C. P. Rhoads and G. I. Lavin, *Proc. Soc. Exptl. Biol. Med.*, **41**, 67 (1939); *Cancer Research*, **2**, 95 (1940).

(6) J. Cason and L. F. Fieser, THIS JOURNAL, **62**, 2681 (1940).

(7) A. A. Levi and E. Boyland, *Chemistry and Industry*, **15**, 446 (1937).

(8) J. W. Cook and R. Schoental, *J. Chem. Soc.*, 9 (1952).

(9) C. Heidelberger, P. Brewer and W. G. Dauben, THIS JOURNAL, **69**, 1389 (1947).

(10) C. Heidelberger and H. B. Jones, *Cancer*, **1**, 252 (1948).

(11) C. Heidelberger and S. M. Weiss, *Cancer Research*, **11**, 885 (1951).

(12) C. Heidelberger, M. R. Kirk and M. S. Perkins, *Cancer*, **1**, 261 (1948).

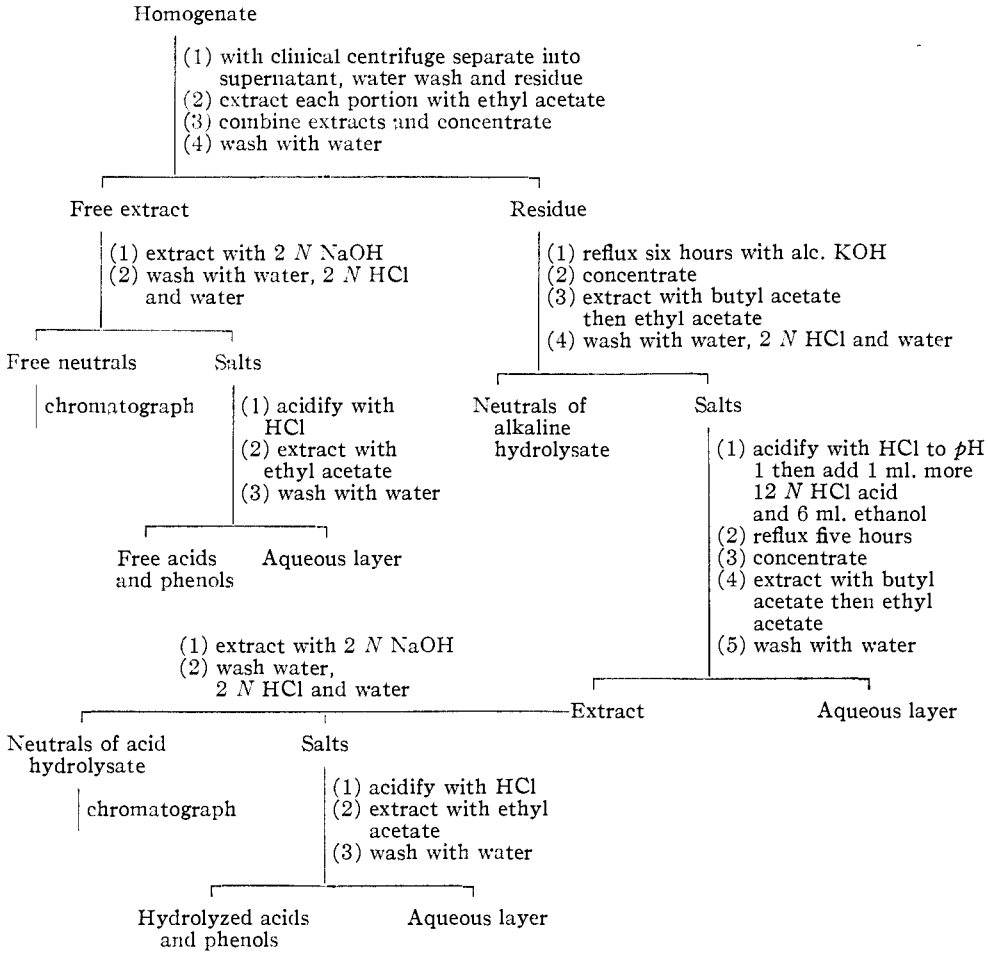
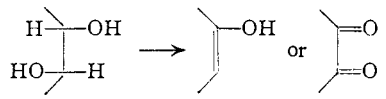


Fig. 1.—Tissue fractionation.

bolites of 3,4-benzpyrene,[13] and these compounds were known and could readily be synthesized. Moreover, it was believed that the phenolic metabolites of polynuclear hydrocarbons are derived by dehydration from *trans*-dihydrodiols. The latter substances have been shown to be produced



metabolically from naphthalene, anthracene and phenanthrene, as pointed out in reviews by Young[14] and Boyland.[15] Since it is known[14,16] that this type of compound is readily oxidized to quinones *in vitro*, it was felt that the identification of quinones might constitute indirect evidence for the presence of dihydrodiols as metabolic products of dibenzanthracene. Furthermore, since the identification of 5-hydroxy-1,2-naphthalic acid VI as a metabolite,[3] it became pertinent to search for the dihydroxyquinone V, which was postulated by Dauben and Tanabe[17] to be an intermediate. Dibenzanthracene-3,4,7,8-quinone was investigated, but has not yet been shown to be a metabolite.

(13) I. Berenblum, D. Crowfoot, E. K. Holiday and R. Schoental, *Cancer Research*, **3**, 151 (1943).
(14) L. Young, *Biochemical Society Symposia*, **5**, 27 (1950).
(15) E. Boyland, *ibid.*, **5**, 40 (1950).
(16) C. Heidelberger and G. Wolf, unpublished.
(17) W. G. Dauben and M. Tanabe, THIS JOURNAL, **71**, 2877 (1949).

In the present work the metabolism of dibenzanthracene was studied in liver and in skin. Liver was chosen, because it is an organ of high and diversified metabolism in which it has previously been shown[12] that the carcinogen is metabolized. By the use of liver, rather than urine or feces, the metabolic influence of the intestinal flora has been eliminated. Skin was chosen because it is a tissue susceptible to carcinogenesis, whereas liver is not. A complication in this type of work, not encountered in studies with excreta, is the time factor. It is almost certain that the quinones or their precursors we have studied are further metabolized, as has already been shown in the case of the conversion of V into VI. Hence the choice of a time when the quinone is present in greatest quantity is difficult to make, and in this case was done purely arbitrarily. Thus, larger amounts of metabolites might have been found if some other time were chosen.

In the experiments with liver the hydrocarbon was administered intravenously as an aqueous colloid; it was applied directly in benzene solution to a shaved area of skin on the back of mice. The fractionation scheme devised for the isolation of the metabolites is shown in Fig. 1. Each fraction was assayed for radioactivity. In practice it was found that most of the unchanged I was extracted from the homogenized tissue with ethyl acetate at

room temperature. Thus the possibility of the production of II and III from I as artifacts during the hydrolytic procedures was diminished by the initial ethyl acetate extraction. This procedure, employing simple chemical methods for hydrolyzing conjugated derivatives of metabolites consistently gave good recoveries of radioactivity.

Three methods were investigated for the separation of neutral extracts into fractions containing the quinones II and III. The quinone extraction procedure, shown in Fig. 2 failed to give reproducible results in the metabolic experiments. Paper

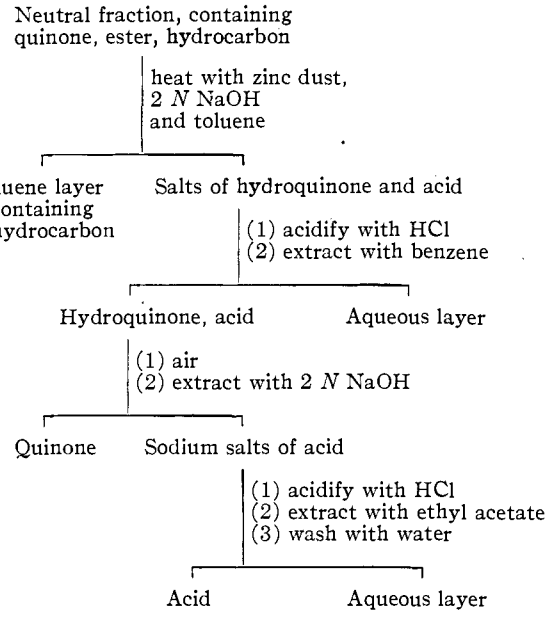


Fig. 2.—Quinone extraction.

chromatography in non-aqueous systems was studied extensively, but failed to give adequate separation of metabolites under the conditions of the experiments. The method of choice was chromatography on Florisil which, under the conditions indicated in Table I, gave a consistently good separation of I, II, III and Va. Once the meta-

TABLE I

CHROMATOGRAPHIC FRACTIONATION

Fraction	Eluent	Contents
1	11 ml. 90% Skelly B[a] + 10% benzene	Dibenzanthracene
2	2 ml. 80% Skelly B + 20% benzene	Wash
3	1 ml. 60% Skelly B + 40% benzene 5 ml. 100% benzene	Dibenz-9,10-anthraquinone
4	6 ml. 100% benzene	Wash
5	8 ml. 97.5% benzene + 2.5% acetone	4',8'-Diacetoxy-dibenz-9,10-anthraquinone
6	2 ml. 97.5% benzene + 2.5% acetone	Wash
7	6 ml. 90% benzene + 10% acetone	Dibenz-3,4-anthraquinone
8	1 ml. 80% benzene + 20% acetone 1 ml. 60% + 40% acetone 1 ml. 50% + 50% acetone 5 ml. 100% acetone	Wash

[a] B.p. 65–68°.

bolic fractions were separated in this way, the radioactivity data shown in Table II were obtained. The appropriate carriers were then added to the fractions and were rigorously purified by means of crystallization, vacuum sublimation and chromatography. The criterion of purity was the maintenance of constant specific activity after repetition of one or more of these varied processes.

Since it was known that the free phenolic quinone V is susceptible to air oxidation and unstable in the presence of acid,[6] the diacetoxy derivative Va was used as the carrier. Based on the likely assumption that V was present in the tissues as the glucuronide or sulfate ester, which could be cleaved by acidic hydrolysis, the acetylation procedure (Fig. 3) was carried out on alkali-soluble radioactive material subsequent to acid hydrolysis. The neutral material following acetylation was chromatographed on Florisil, and the carrier was added to the appropriate portion of the eluate. Purification was carried out as described above. The results of all carrier experiments are given in Table III.

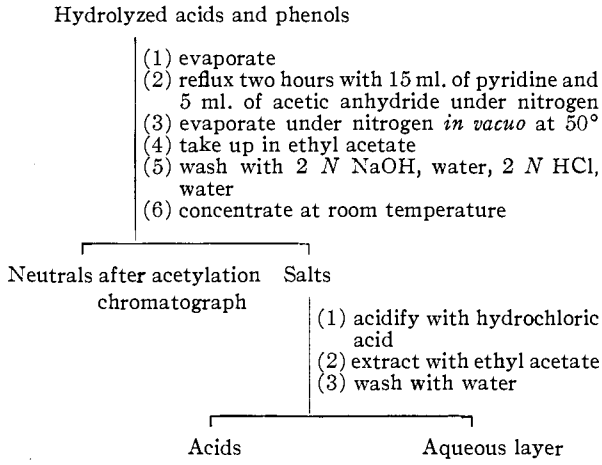


Fig. 3.—Phenol acetylation.

The 9,10-quinone has been found repeatedly in both liver and skin, and in appreciable quantities, as measured by the radioactivity. The 3,4-quinone has been obtained on several occasions in liver, but has not been detected in skin. In liver it is present in only minute amounts. The dihydroxy quinone has only been found in liver in intermediate quantity. Since VI has been detected in skin,[3] it was somewhat surprising that V was not found there. However, it may be that a compound as unstable as V could not exist very long in an exposed tissue such as skin. The amount of radioactivity assigned to the 9,10-quinone represents in a favorable instance 20% of the chromatographic fraction, 0.05% of the activity in the excised liver, and 1% of the activity due to all metabolites. The quantity is somewhat higher in skin. Although this is a very small quantity, it does represent an accurate quantitative determination of a metabolite in an individual animal given a small dose of carcinogen. In contrast, it should be recalled that little information is available about the quantities of metabolites previously isolated from excreta of animals given massive doses of dibenzanthracene. It is readily apparent that considerable effort will be required before the spectrum of radioactivity obtained by the chromatographic fractionation can be fully characterized and assigned to known metabolites. Nevertheless,

TABLE II

CHROMATOGRAMS OF NEUTRAL FRACTIONS

		Fraction number[a,b] Total c./min.							
	Source	1 (D.B.A.)	2 Wash	3 (9,10-Quinone)	4 Wash	5 (Di-acetoxy-quinone)	6 Wash	7 (3,4-Quinone)	8 Wash
1	Liver, 1 hr. free neutrals	383,000	460	800	110	580	40	410	900
2	Liver, 1 hr. free neutrals alkali modified[c]	331,000	570	630	67	950	98	300	340
3	Liver, 1 hr. neutrals, alkaline hydrolysate	2,500	430	160	85	67	13	96	72
4	Liver, 1 hr. free neutrals acid hydrolysate	650	23	260	150	400	66	630	780
5	Liver, 1 hr. neutrals after acetylation	.....	...	695	22	450	11	..	280
6	Liver, 3 hr. free neutrals[d]	150,000	140	2000	100	...	..	2200	470
7	Liver, 40 min. free neutrals	137,000	75	395	87	470	0	185	250
8	Skin, 1 day free neutrals	24,000	600	330	43	50	11	14	15
9	Skin, 1 day neutrals alkaline hydrolysate	115	8	16	12	33	0	0	0
10	Skin, 1 day neutrals acid hydrolysate	0	10	5	0	0	5	8	0
11	Skin, 5 days free neutrals	8,700	28	110	0	54	15	38	18
12	Skin, 5 days neutrals alkaline hydrolysate	110	0	7	0	16	0	0	0
13	Skin, 5 days neutrals acid hydrolysate	46	9	9	0	5	5	6	4
14	Skin, 5 days neutrals after acetylation	.....	...	15	17	20	8	..	29

[a] The fraction numbers refer to those given in Table I. The positions of the pure compounds on the chromatogram are indicated in parentheses. [b] The data of pooled samples have been reported on a per mouse basis. [c] An aliquot identical to the free extract of Source 1 was refluxed under nitrogen with 15 ml. of 10% alcoholic potassium hydroxide for six hours before fractionation in order to test for the presence of conjugated metabolites in the free, neutral fraction. [d] The free neutrals were first subjected to the quinone extraction, the various neutral fractions were combined and chromatographed.

TABLE III

TOTAL ACTIVITY IN CARRIERS OF QUINONE METABOLITES IN C./MIN.

	9,10-Quinone			3,4-Quinone			Diacetoxy quinone		
Source[a]	Fraction activity	Activity after purification[b]	Activity after repurification[b]	Fraction activity	Activity after purification[b]	Activity after repurification	Fraction activity	Activity after purification[b]	Activity after repurification
1	800	162	155[c]	410	9	0[c]			
2	630	30	27[c]	300	15	0[c]			
3	167	19	26[c]	96	0				
4	260	82	60[c], 70[c]	630	15	0[c]			
5							1800[f]	47	39[c], 36[c], 40[c]
6	2000	223	214[d]	2200	23	19[c], 17[e]			
7	395	18	15[e]	185	6	6[c], 7[c]			
8[g]	660	0		28[g]	7	0			
9[f]	63	0		0					
10[f]	20	6		30	0				
11[g]	220	62	63[c]	77	2	0			
12[f]	28	15	15[c]	0					
13[f]	36	0		25	0				
14[f]				0			80	0	0

[a] For description see Table II. [b] After two crystallizations, sublimation and chromatography. [c] After further chromatography. [d] After further sublimation. [e] After further crystallization, sublimation and chromatography. [f] Sample pooled from four mice. [g] Sample pooled from two mice.

TABLE IV

DISTRIBUTION OF RADIOACTIVITY[a,b]

				Total c./min. Neutrals			Acids and phenols	
Origin	No. of mice	Time	Homogenate	Free	Alkaline hydrolysate	Acidic hydrolysate	Free	Hydrolysate
Liver	4	1 hr.	291,000	297,000	7700	1480	1300	1100
Liver	1	3 hr.	267,000	153,000	..	..	2900	..
Skin	4	1 day	22,000	22,000	170	35	55	39
Skin	4	5 days	8,400	8,800	210	85	0	67

[a] The data of pooled samples has been reported on a per mouse basis. [b] The dose per mouse administered was 6.8×10^5 c./min. in the liver experiments, and 6.8×10^4 c./min. in the skin experiments.

in spite of the fact that these new metabolites occur only to a very small extent, we feel that there can be no doubt of their existence and importance in the metabolism of the carcinogen.

The possibility that the metabolites II and III were artifacts produced by the action of light or chemical manipulation on unchanged dibenzanthracene during the isolation procedure was eliminated in the following fashion. A colloidal suspension of dibenzanthracene was allowed to stand in light for several weeks, and was then extracted with benzene. This extract was then sub-

jected to the quinone extraction followed by chromatography, a procedure identical with that used in the early metabolic experiments. The quinones II and III were added as carriers to the proper fractions but did not carry radioactivity, thus proving their absence.

The detection of the metabolites II and III, together with the previously known metabolite IV clearly shows that primary biological oxidation may occur in any of the rings of dibenzanthracene. Heretofore the products isolated and characterized as metabolites of carcinogenic polycyclic aromatic hydrocarbons with the possible exception of the benzpyrene quinones[13] have demonstrated that primary biological oxidations occurred at peripheral positions, loci that are untouched by the known methods of chemical oxidation. Although many workers have discussed the possibility that ring scission might occur at the *meso* and phenanthrene double bond positions, and we have previously provided indirect evidence for this by the isolation of VI,[3] the present report constitutes the first direct demonstration of primary biological oxidation in these chemically reactive positions. The former concept has been summarized by Cook[18]: "Isolation of metabolic products of some of the polycyclic aromatic hydrocarbons, mainly by Young, Boyland, Berenblum and Schoental, revealed a surprising fact, namely, that biological oxidation did not attack the centers in the molecule which chemical reagents show to be the most active centers." Indeed, considerable effort has been expended to find chemical means of simulating the previously established course of biological oxidations.[19] These generalizations must now be reconsidered in light of the present findings.

A concept that attempts to correlate carcinogenicity and chemical structure has been that of the "K" region,[20] which is qualitatively equivalent to an activated phenanthrene double bond.[21] The quantitative calculations have recently been strongly criticized upon theoretical grounds,[22] and the correlation with carcinogenic potency of the rates of addition of osmium tetroxide to the phenanthrene double bonds of the carcinogens studied is far from satisfactory.[23] It has been postulated[24] that carcinogenesis may involve an interaction between a tissue component and this portion of the hydrocarbon molecule. Recently it has been established that dibenzanthracene is firmly bound to the proteins of skin and submaxillary gland in mice,[25] but in a manner as yet unknown. The present demonstration of the 3,4-quinone III as a metabolite constitutes the first evidence that biological oxidation can occur at the "K" region of dibenzanthracene. Because dibenzanthracene-3,4-quinone has a second phenanthrene double bond and this metabolite has not been demonstrated in skin, coupled with its ephemeral nature means that further investigation is required before its significance can be assessed.[26]

The structures of the various metabolites in conjunction with the previous observation that hydroxylation must precede ring cleavage[3] suggests two possible metabolic pathways from I to VI. One is I to IV to V to VI. The other is I to II to V to VI. The present evidence does not permit a decision between the two pathways.

Acknowledgment.—We wish to acknowledge the excellent technical assistance of Mrs. Edith Wallestad, Miss Nancy Lake and the late Kenneth Lipp in the radioactivity determinations.

Experimental

Radioactivity Assay.—All C^{14} samples were counted in internal flow counters and are statistically significant to at least 10%. Homogenates were combusted chemically[27]; the carbon dioxide was precipitated as barium carbonate and counted. Aliquots of extracts were plated as infinitely thin layers on aluminum discs,[28] and carriers were plated directly, weighed to 0.01 mg. and corrected for self-absorption.

Preparatory Treatment.—A colloidal suspension of 0.5 mg. of dibenzanthracene-9,10-C^{14}[9] (6.8×10^5 c.p.m.) in 1 ml. of 1% gelatin solution was injected into the tail vein of mice.[29] The preparation of the colloid has been described.[10] After the proper time interval the mouse was sacrificed with ether, the liver excised and homogenized as described below.

A solution of 0.05 mg. of dibenzanthracene-9,10-C^{14} (6.8×10^3 c.p.m.) in 0.05 ml. of benzene was applied with a micropipet to the shaved backs of mice. The animals were sacrificed after the proper time interval, the skin removed and treated by the liquid air technique,[25] which provides a skin preparation containing epidermis and that part of the dermis containing the sebaceous glands and hair follicles, but without the fatty and connective tissue of the dermis. This preparation was then homogenized as described below.

Tissue Fractionation.—The fractionation procedure is shown in Fig. 1. The liver or skin was homogenized in a few ml. of water with an all glass Potter–Elvehjem homogenizer. The homogenate was separated by centrifugation, the residue was washed with water and the residue, supernatant and wash were separately extracted with ethyl acetate. The extractions were all carried out in 15-ml. centrifuge tubes and the agitation and phase separation were accomplished entirely with dropping tubes. Each fraction was assayed for radioactivity. All reactions were carried out under nitrogen as were concentration of solutions. Data for a typical experiment are shown in Table IV.

Quinone Extraction.—The procedure is shown in Fig. 2. In a model experiment, a mixture of 3.7 mg. of dibenz-3,4-anthraquinone[30] (purified by chromatography on Florisil) in 1.5 ml. of hot toluene, 2 ml. of 2 *N* sodium hydroxide and 0.4 g. of zinc dust was boiled vigorously in an open 15-ml. centrifuge tube over a free flame for 45 seconds. The hot mixture was centrifuged for one minute and the aqueous layer was drawn off with a dropping tube. The heating and extraction was repeated twice with the addition of small quantities of zinc dust each time. The red alkaline solution was acidified with hydrochloric acid, the organic material was extracted with ethyl acetate and washed with water. A stream of air was then bubbled through the organic phase for one hour. On evaporation of the solvent there was obtained 3.5 mg. (94%) of a red substance, m.p. 326–329° (uncor.), which did not depress the melting point of the original quinone. When the above procedure was carried out on

(18) J. W. Cook, *Biochemical Society Symposia*, **5**, 1 (1950).
(19) R. Schoental, *ibid.*, **5**, 3 (1950).
(20) R. Daudel and C. R. Pullman, *Compt. rend.*, **220**, 888 (1945).
(21) R. Robinson, *Brit. Med. J.*, **1**, 945 (1946).
(22) H. H. Greenwood, *Brit. J. Cancer*, **5**, 441 (1951).
(23) G. M. Badger, *J. Chem. Soc.*, 456 (1950).
(24) E. Boyland, *Biochim. Biophys. Acta*, **4**, 293 (1950).
(25) W. G. Wiest and C. Heidelberger, *Cancer Research*, in press.
(26) In the case of phenanthrene, a non-carcinogenic hydrocarbon, the 9,10-dihydrodiol has been isolated and characterized from rabbit urine by E. Boyland and G. Wolf, *Biochem. J.*, **47**, 74 (1950).
(27) D. D. Van Slyke, J. Plazin and J. R. Weisiger, *J. Biol. Chem.*, **191**, 299 (1951).
(28) M. Calvin, C. Heidelberger, J. C. Reid, B. M. Tolbert and P. E. Yankwich, "Isotopic Carbon," John Wiley and Sons, Inc., New York, N. Y., 1949, p. 113.
(29) The mice used were adult female albino mice of the "Rockland" variety obtained from Arthur Sutter, Springfield, Mo.
(30) E. F. M. Stephenson, *J. Chem. Soc.*, 2620 (1949).

7.8 mg. of dibenz-9,10-anthraquinone,[6] the recovery was 68%.

Acetylation of Phenols.—The radioactive fraction containing hydrolyzed acids and phenols (Fig. 1) was acetylated as shown in Fig. 3.

Chromatographic Separation.—In model experiments, 20 μg. each of dibenzanthracene, dibenz-9,10-anthraquinone (II), dibenz-3,4-anthraquinone (III) and 4′,8′-diacetoxydibenz-9,10-anthraquinone (Va)[7] were separated chromatographically by the following procedure. The column, 3 × 180 mm., consisted of 0.7 g. of Florisil[31] (60/100 mesh) in a thistle tube. Gravity flow was used and the rate of elution was 0.25 ml. per minute. The dibenzanthracene was located by fluorescence and the three quinones were followed visually. The elution pattern is given in Table I.

In order to "clean up" the various radioactive neutral fractions, the solutions were concentrated and washed through the Florisil column with 25 ml. of ethyl acetate. This eluate was evaporated, Skelly B added and evaporated several times to remove traces of ethyl acetate. The residue was dissolved in 0.1 ml. of benzene diluted to 1 ml. with Skelly B and placed on the column. The elution was conducted as shown in Table I. Each chromatographic fraction was plated and counted. The results are given in Table II.

Purification of Carriers.—The appropriate quinone carrier (0.8–2 mg.) was added to the radioactive fractions obtained from chromatography. Each carrier was crystallized twice from butyl acetate and sublimed at 0.05 mm. The temperatures required were 180° for dibenz-9,10-anthraquinone, 250° for dibenz-3,4-anthraquinone and 280° for 4′,8′-dihydroxydibenz-9,10-anthraquinone. The carriers were then chromatographed on Florisil as shown in Table I and counted. One or more of the above operations was repeated until constant specific activity was achieved. The radioactivity of the carriers is reported as c.p.m. per total weight of carrier. The data are shown in Table III.

MADISON, WISCONSIN

(31) Obtained from the Floridin Co., Warren, Pa.

[CONTRIBUTION FROM THE LABORATORY OF CHEMICAL PHARMACOLOGY, NATIONAL CANCER INSTITUTE[1]]

Components of Podophyllin. XI. Isolation of Two New Compounds from *Podophyllum emodi* Wall.[2]

BY MORESHWAR V. NADKARNI,[3] JONATHAN L. HARTWELL, PRISCILLA B. MAURY AND J. LEITER

RECEIVED SEPTEMBER 13, 1952

Fractionation of the resin of *Podophyllum emodi* Wall., employing chromatographic adsorption on activated alumina yielded, beside podophyllotoxin, the new compounds 4′-demethylpodophyllotoxin and 1-O-(β-D-glucopyranosyl)-picropodophyllin, hereafter called demethylpodophyllotoxin and picropodophyllin glucoside. No α-peltatin or β-peltatin was found. Demethylpodophyllotoxin was active in causing damage to Sarcoma 37 in mice.

The isolation of α- and β-peltatin,[4] both active in producing damage to tumors in mice, from the resin of *Podophyllum peltatum* L. (American podophyllum), prompted an inquiry into the composition of the resin of *P. emodi* (Indian podophyllum, *Podophyllum indicum*).[5] Although the resins from both species of *Podophyllum* are official in the British Pharmacopoeia[6] and are used for the same purpose, and while earlier studies[7] had shown that only two components (podophyllotoxin and quercetin) were present in both species, the finding of α- and β-peltatin in the American resin[4a] and certain differences in the color reactions of the two resins[6,8] warranted the reinvestigation of the resin of *P. emodi* by the employment of chromatographic adsorption on alumina.

A modified alcohol–benzene process[4a] was used for the chromatographic fractionation. Unlike the American resin, when a solution of the Indian resin in alcohol was treated with 9 volumes of benzene, a flocculent suspension of tarry material was produced which could not be cleanly separated; the tar was always found to retain a substantial amount of tumor-damaging activity against Sarcoma 37 in mice. Consequently, this preliminary treatment was omitted and a solution of the resin in equal volumes of alcohol and benzene was used directly. It was also found that the cuts had to be taken at different points in the chromatogram from those in the *P. peltatum* process. The following substances were isolated, in the order of their appearance through the chromatogram: (1) podophyllotoxin, 45–48% yield, identified by m.p., m.p. of anhydrous form and acetyl derivative (m.p. and mixed m.p.)[9]; (2) a phenolic substance m.p. 250.0–251.6° cor., 1.7% yield, crystallizing in colorless, transparent prisms from ethanol, or as plates from 50% ethanol; and (3) a glycoside, m.p. 237.0–238.2° cor., 1.8% yield, crystallizing

(1) National Institutes of Health, Public Health Service, Federal Security Agency.

(2) (a) First reported as a Communication to the Editor by M. V. Nadkarni, P. B. Maury and J. L. Hartwell, THIS JOURNAL, **74**, 280 (1952); (b) for paper X in this series, see J. L. Hartwell, A. W. Schrecker and G. Y. Greenberg, THIS JOURNAL, **74**, 6285 (1952).

(3) Post-doctorate Research Fellow of the National Cancer Institute.

(4) (a) J. L. Hartwell and W. E. Detty, THIS JOURNAL, **72**, 246 (1950). For papers on the tumor-damaging action, *cf.* (b) J. Leiter, V. Downing, J. L. Hartwell and M. J. Shear, *J. Nat. Cancer Inst.*, **10**, 1273 (1950), and (c) E. M. Greenspan, J. Leiter and M. J. Shear, *ibid.*, **10**, 1295 (1950).

(5) We wish to thank Dr. W. G. Bywater of S. B. Penick and Co. for the procurement of the roots of this plant from India and the extraction of the resin therefrom by the procedure given in "National Formulary," Vol. IX (1950).

(6) "British Pharmacopoeia," 1948.

(7) (a) F. A. Thompson, *Am. J. Pharm.*, **62**, 245 (1890); (b) J. C. Umney, *Pharm. J.*, **23**, 207 (1892); (c) W. R. Dunstan and T. A. Henry, *J. Chem. Soc.*, **73**, 209 (1898); (d) W. Borsche and J. Niemann, *Ann.*, **494**, 126 (1932); (e) E. Späth, F. Wessely and L. Kornfeld, *Ber.*, **65**, 1536 (1932).

(8) "National Formulary," ninth ed., American Pharmaceutical Association, Washington, D. C., 1950.

(9) From the mother liquor of the podophyllotoxin a small amount (0.4%) of picropodophyllin sometimes separated; it was identified by m.p. and acetyl derivative (m.p. and mixed m.p.). The small amount obtained (less than 1% of the podophyllotoxin) makes it uncertain whether this compound occurs as such in the resin or is an artifact produced during the chromatography, inasmuch as traces of weak alkali are known to epimerize podophyllotoxin to picropodophyllin. In an unpublished experiment, Mr. W. E. Detty was able to recover 0.49 g. from 0.50 g. (98%) of pure podophyllotoxin after passage through an alumina column using a mixture of 1:9 ethanol–benzene, indicating that less than 2%, if any, is converted into other products.