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Studies on the Structure of the Skin Protein-bound Compounds Following Topical Application of 1,2,5,6-Dibenzanthracene-9,10-C^{14}. I. 2-Phenylphenanthrene-3,2′-dicarboxylic Acid, a Degradation Product[1,2]

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Studies on the structure of the protein-bound compounds following the application of 1,2,5,6-dibenzanthrazene-9,10-C^{14} to the skin of mice have been carried out by the tracer technique. The binding, which is correlated with carcinogenesis, survives pepsin hydrolysis and must be covalent in nature. Alkaline hydrolysis liberates carboxylic acids and amino acid-bound metabolites. Carrier experiments have shown that 2-phenylphenanthrene-3,2′-dicarboxylic acid is present in the hydrolysis mixture; oxidative hydrolysis increases the amount of this compound to 23% of the total bound radioactivity. Some implications of these findings have been discussed.

In spite of the enormous amount of experimental work that has been carried out in the 24 years since the classical demonstration by Kennaway and Hieger[3] that a pure chemical compound, 1,2,5,6-dibenzanthracene, could induce tumors in experimental animals, only comparatively recently have significant advances in elucidating the biochemical mechanism of carcinogenesis been made. An important milestone along the road to the final understanding of the process is the discovery by the Millers,[4] of the combination between the carcinogenic aminoazo dyes and the proteins of susceptible liver tissue, and the correlation of this binding with the carcinogenic process. Although there have been suggestions in the literature[5,6] that carcinogenic hydrocarbons might interact with tissue constituents, the first clear demonstration of this phenomenon was made by Miller in 1951.[7] She observed that fluorescent substances were bound to epidermal protein following the application of benzpyrene to the skin of mice, and were liberated by strong alkaline reductive hydrolysis.

Since 1947, a continuing investigation of the distribution[8,9] and metabolism[10–12] of 1,2,5,6-dibenzanthracene-9,10-C^{14} [13] has been under way in these laboratories. It appeared desirable to extend the tracer technique to the quantitative study of the interaction of dibenzanthracene and other hydrocarbons with the proteins of cancer-susceptible and non-susceptible tissues. It already has been shown unequivocally[14] that this binding to protein in mouse skin and submaxillary gland is not due to absorption or occlusion, and does not involve the nucleic acids, contrary to the suggestion[15] that carcinogenesis results from the direct interaction between the hydrocarbons and desoxyribonucleic acid, thus producing a genetic mutation. Furthermore, an excellent correlation has been obtained[16] between the quantity of radioactivity bound to the skin proteins, following application of a series of carbon-14 labeled hydrocarbons to the skin of mice, and their carcinogenic potencies. All these studies, and others now in progress, make it abundantly clear that a chemical interaction between carcinogenic hydrocarbons and tissue proteins actually *does* take place, in contrast to a recent understatement[17] that "a recent review by Wolf indicates that it (the concept of a chemical interaction) is still entertained by some workers in the field."

In view of the fact that the phenomenon of protein binding has been demonstrated not only in the case of the azo dyes and carcinogenic hydrocarbons, but also for 2-acetylaminofluorene[4b,18] it is a strong possibility that this may be a mechanism of causal significance common to all types of chemical carcinogens. If this should be the case, it is urgently important that studies on the structure of the bound derivative(s) of these carcinogens be carried out. The present report describes the initial efforts toward the elucidation of the structure of the dibenzanthracene–protein complex using the tracer technique.

Results and Discussion

The use of the tracer technique for the elucidation of the structure of the bound derivatives of carcinogenic hydrocarbons provides a number of advantages over the fluorescence methods used heretofore.[7,19,20] The data obtained by the radioactivity measurements are truly quantitative and do not depend on arbitrary fluorescent units and unknown

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(2) Abstracts of part of this work appear in *Proc. Am. Assoc. Cancer Research*, **1**, 22 (1953); **1**, 5 (1954).

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

(4) (a) E. C. Miller and J. A. Miller, *Cancer Research*, **7**, 468 (1947); (b) **12**, 547 (1952); (c) *Advances in Cancer Research*, **1**, 340 (1953).

(5) L. F. Fieser, "Production of Cancer by Polynuclear Hydrocarbons," University of Pennsylvania Press, Philadelphia, Pa., 1941, pp. 1–27.

(6) E. Boyland, *Yale J. Biol. Med.*, **20**, 321 (1948).

(7) E. C. Miller, *Cancer Research*, **11**, 100 (1951).

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

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(12) C. Heidelberger, H. I. Hadler and G. Wolf, THIS JOURNAL, **75**, 1303 (1953).

(13) C. Heidelberger, P. Brewer and W. G. Dauben, *ibid.*, **69**, 1389 (1947).

(14) W. G. Wiest and C. Heidelberger, *Cancer Research*, **13**, (a) 246, (b) 250, (c) 255 (1953).

(15) E. Boyland, *ibid.*, **12**, 77 (1952).

(16) C. Heidelberger and M. G. Moldenhauer, *Proc. Am. Assoc. Cancer Research*, **2**, 24 (1955).

(17) L. F. Fieser, *Science*, **119**, 710 (1954).

(18) J. H. Weisburger, E. K. Weisburger, H. P. Morris and H. A. Sober, *Cancer Research*, **12**, 305 (1952).

(19) M. M. Moodie, C. Reid and C. A. Wallick, *ibid.*, **14**, 367 (1954).

(20) D. S. Tarbell, E. G. Brooker, P. Seifert, A. G. Fluka and T. J. Hall, Abstracts Am. Chem. Soc., 126th Meeting, Sept. 12–17, 1954, p. 5N.

TABLE I

PEPSIN DIGESTION OF SKIN PROTEINS

Mouse batch	Type	Starting protein Wt., mg.	Sp. act., c.p.m./mg.	Pepsin concn., %	Pepsin-insoluble fraction Wt., mg.	Sp. act., c.p.m./mg.	Total act., c.p.m.	Pepsin-soluble fraction Total act., c.p.m.	Extractable act. c.p.m.	Total recovery of radioactivity, %
I	Large-granular	100	438	0.05	48.1 (48%)	680	32,700 (75%)	11,000 (25%)	None	100
I	Soluble	50	434	.05	3.8 (7.6%)	Not detd.	....	19,700 (91%)	856 (3.9%)	91
II	Large-granular	50	708	.1	29.3 (59%)	417	12,300 (35%)	17,100 (48%)	1220 (3.4%)	83
II	Soluble	20	653	.5[a]	None	...	....	10,800 (83%)	160 (1.2%)	83

[a] In 0.2 *N* HCl.

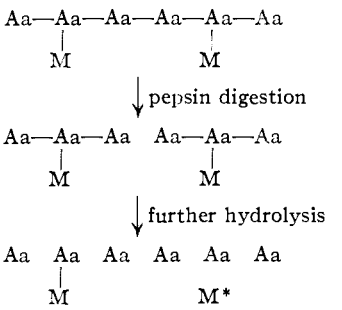
recoveries from extraction procedures which, as the present work shows, are far from complete. Furthermore, the use of labeled hydrocarbons has made it possible to make quantitatively valid comparisons of the binding of carcinogens of varying potency to skin proteins[16] and to identify and characterize metabolites by the carrier technique.[11,12] In the case of dibenzanthracene the fluorescence is so weak that metabolic studies by optical methods are impractical.

In the present work, the mice were sacrificed two days after application of dibenzanthracene-9,10-C^{14}, at which time the binding is at its peak.[14b] The backs of the mice were washed thoroughly with benzene to remove any free hydrocarbon and processed as described later. The liquid air treatment gives a preparation of skin including the epidermis and that part of the dermis containing the hair follicles and sebaceous glands.[14a] The washing procedures remove lipids, nucleic acids and free DBA, and the resulting proteins can be dissolved, extracted and reprecipitated without change in specific activity.[14b,c]

Some variation in the specific activities and yields of the proteins (and subsequently of the peptides) was observed in several experiments with different batches of mice, probably due to differences in the areas of skin excised. Because we have no information at present as to the relative importance in the carcinogenic process of the metabolites bound to the large-granular and soluble proteins, both fractions have been studied in parallel in this structure work.

As the first step in the degradation of the proteins mild conditions were sought, in contrast to the drastic hydrolytic procedures used for the liberation of the fluorescent derivatives of benzpyrene.[7,19,20] Accordingly, pepsin digestion, which degrades proteins to the polypeptide level,[21] was carried out on the large granular and soluble proteins. The results given in Table I show that almost all the precipitated soluble protein and about one-half the large-granular protein was solubilized and that, although the recovery of radioactivity was essentially quantitative, little if any labeled material could be extracted with organic solvents. Of the 1.2% of radioactivity extractable in the experiment on the soluble protein of batch II, approximately half was neutral. However, chromatography of the neutral material on Florisil[12] showed that no dibenzanthracene was present. These experiments demonstrate unequivocally in the case of the pepsin-soluble fractions that the binding is not due to adsorption or occlusion of DBA on the protein (which was highly unlikely because of the washing to which the protein had been subjected), nor could the binding be attributable to van der Waals forces because the protein was degraded by pepsin. Furthermore, since simple ionic bonds could not survive the treatment that the proteins were subjected to, we have been forced to the conclusion that the binding involves a covalent linkage between the DBA or a derivative and amino acid(s).

On this basis, a simplified diagrammatic representation of the situation may be made



The first line represents a polypeptide chain in the original protein, with DBA or a metabolite (M) attached to amino acids (Aa) by covalent bonds. Pepsin treatment degrades the protein to polypeptides, indicated in the second line, without affecting the linkages between the metabolite and amino acids. It would then appear reasonable that on further hydrolysis of the peptides to the free amino acid level (bottom line), some metabolite might remain bound to amino acid, and that some might be liberated, probably in chemically altered form (M*). It will be demonstrated below that this formulation is probably correct.

One consequence of this hypothesis is that on further hydrolysis of the pepsin-treated proteins, radioactivity should become extractable in organic solvents (M*). The initial experiments, described in Table II, were carried out under acidic conditions of hydrolysis. Varying amounts of C^{14} were recovered in the organic solvent extracts, and this radioactivity was present in much larger quantities in acidic than in neutral form.

(21) *Cf.* J. S. Fruton and S. Simmonds, "General Biochemistry," John Wiley and Sons, Inc., New York, N. Y., 1953, pp. 596–600.

TABLE II

ACID HYDROLYSES OF PEPTIDES[a]

Type of protein[b]	Starting material Wt., mg.	Starting material Total act.	Conditions[c]	Acid-insoluble fraction Wt., mg.	Acid-insoluble fraction Sp. act.	Acid-insoluble fraction Total act.	Acid-soluble fraction Aqueous	Acid-soluble fraction Neutral	Acid-soluble fraction Acidic	Total recovery of radioactivity, %
A	12.9	8,770	1	2.5	1850	4640	3140	101	294	94
	(sp. act. 680)			(19%)		(53%)	(36%)	(1.2%)	(3.4%)	
A	19.2	13,000	2	4.3	1960	8430	968	Neutrals and acids		
	(sp. act. 680)			(22%)		(65%)	(7.4%)	258(2%)		74
B[d]	....	2,780	1	...	..	..	820	131	446	
							(29%)	(4.7%)	(16%)	50
B[d]	....	1,550	2	...	..	..	884	79	320	
							(57%)	(5.1%)	(2.1%)	64
C[d]	....	2,650	1	...	..	..	905	234	598	
							(34%)	(8.8%)	(23%)	66
C[d]	....	4,530	2	...	..	..	3590	97	237	
							(79%)	(2.1%)	(5.2%)	86

[a] All radioactivities are in c.p.m. and specific activities in c.p.m./mg. [b] A = pepsin-insoluble fraction of large-granular protein; B = pepsin-soluble fraction of the same protein; and C = pepsin treated soluble protein. [c] 1 = Boiling 2 *N* HCl; 2 = autoclaving with 6 *N* HCl; both for 16 hr. at 15 p.s.i. [d] These proteins were obtained from batch II (Table I).

TABLE III[a]

ALKALINE HYDROLYSES OF PEPTIDES AND LABELED DBA

Expt.	Starting material Type[b]	Starting material Total act.	Conditions[c]	Radioactivity in neut. fract.	Stable radioactivity in acid fract.	Radioactivity in aqueous fract.
1	A	30,200	e	0	19,300 (64%)	Not assayed
2	A	5,320	g	45 (0.8%)[d]	2,920 (55%)	Not assayed
3	A	11,700	h	0	6,050 (52%)[e]	Not assayed
4	A	10,100	i	327 (3.2%)	1,680 (17%)	Not assayed
5	A	17,500	j	964 (5.5%)	6,860 (39%)[f]	693 (4%)
6	B	5,340	c	0	921 (17%)	2880 (54%)
7	D	2,880	b	42 (0.8% of 5340)	1,090 (21% of 5340)	Not assayed
8	E	....	d	0	435 (8% of 5340)	Not assayed
9	B	11,000	e	38 (0.3%)	2,860 (26%)	4310 (39%)
10	B	10,600	h	0	5,150 (49%)[e]	Not assayed
11	C	18,200	e	139 (0.8%)	7,060 (39%)	7060 (39%)
12	C	17,500	h	0	6,450 (37%)[e]	Not assayed
13	F	10,500	j	623 (5.9%)	5,810 (55%)	0
14	G	38,500	e	10,700 (28%)	1,830 (4.8%)	0
15	G	18,400	f	40 (0.2%)	604 (3.3%)	372 (2%)
16	G	26,500	j	18,500 (70%)	194 (0.7%)	0

[a] All radioactivities are expressed as c.p.m. [b] A, B and C represent the same peptide fractions as in Table II, footnote *b*; D = the aqueous fraction obtained in expt. 6 (a part of this was assayed for radioactivity; figures in the last three columns have been computed for the radioactivity given in column 3); E = the aqueous fraction obtained in expt. 7, the whole of which was used for further hydrolysis (Expt. 8); F = large-granular protein; G = 1,2,5,6-dibenzanthracene-9,10-C^{14}. [c] (a) and (b) with 3 *N* NaOH at 38° for 18 and 144 hr., respectively; (c) and (d) with 6 *N* NaOH for 18 hr. at 38 and 100°, respectively; (e) with 6 *N* NaOH at 100° for 40–48 hr.; (f) as in (e), but with addition of 2% potassium permanganate; (g) as in (e), but with the addition of chromatographically pure, non-radioactive dibenzanthracene (5–6 mg.); (h) as in (f), but with the addition of 15–20 mg. of 2-phenylphenanthrene-3,2′-dicarboxylic acid (PDA); (i) with 6 *N* NaOH under reflux (on a sand-bath) for 40–48 hr., with the addition of zinc dust (approximately 0.1 g. per mg. of the protein); and (j) with 4 *N* NaOH, toluene, alcohol and zinc dust under reflux, for 2 hr.[7] [d] 5.80 mg. of DBA was added initially and 4.50 mg. recovered in the neutral fraction after hydrolysis. [e] These figures are the totals of the radioactivity obtained in PDA recovered after hydrolysis, and that which could be extracted with ethyl acetate from the aqueous solution after removal of PDA. [f] Only 12.7% (2230 c.p.m.) could be extracted by benzene; rest was extracted by ethyl acetate.

Because of the relatively small amount of metabolites recovered in the extractable fractions following acidic hydrolysis, a number of alkaline hydrolyses were carried out in which considerably more organic solvent-extractable radioactivity was obtained from all the peptide fractions (Table III). In all cases of simple alkaline hydrolyses (expts. 1, 6, 7, 9 and 11) the organic extracts contained little or no neutral metabolites, the bulk being present as acids or phenols. Stepwise alkaline hydrolysis of a single fraction, with concomitant extractions (expts. 6, 7, 8), liberated increased amounts of acidic extractable metabolites at each stage.

It appeared necessary to investigate the behavior of minute quantities of DBA under these rather drastic conditions and, in expt. 14, 3 μg. of the labeled hydrocarbon was hydrolyzed with alkali. A recovery of only 33% was obtained, 2 μg. being lost, presumably by volatility (which is known to be appreciable when dealing with such small quantities). The radioactivity recovered was largely neutral; only a small amount of acidic compounds were produced. On the basis of this information, the high total recoveries of radioactivity in the hydrolysates of all peptide materials, together with the fact that the extractable labeled metabolites

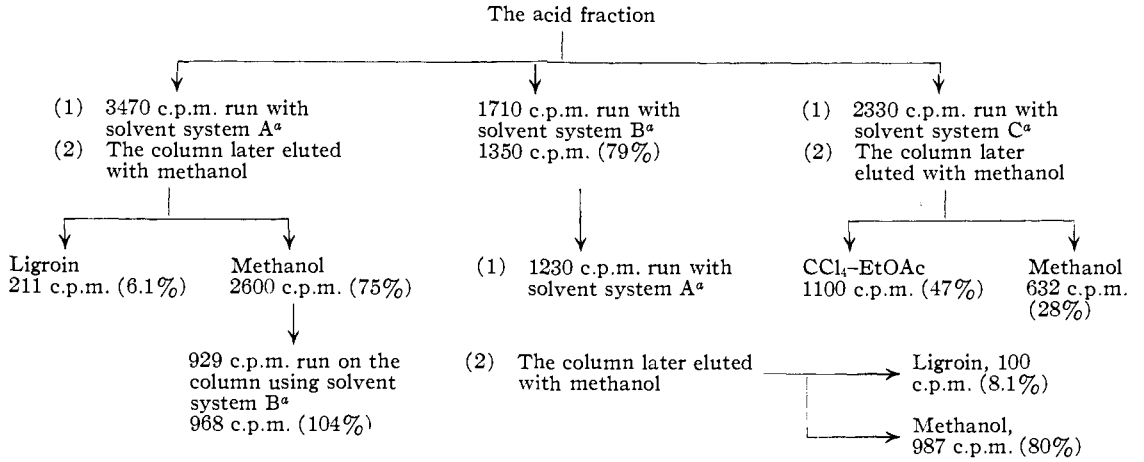


Fig. 1.—Partition chromatography on silicic acid columns of the acid fraction from the pepsin-insoluble large-granular protein hydrolysate. [a] The three solvent systems used are (mobile phase–stationary phase, both phases equilibrated with each other): A = ligroin/9:1 aqueous methanol (90%) + sulfuric acid (0.5 *N*); B = 35:65 butanol + chloroform/0.5 *N* sulfuric acid; C = 1:1 ethyl acetate + chloroform/0.5 *N* sulfuric acid.

were predominantly acidic and not neutral, preclude the possibility that any dibenzanthracene was bound as such or generated during the course of the hydrolysis. Thus the conclusions reached earlier about the covalent nature of the linkage in the pepsin-soluble large-granular (B) and in the soluble fractions (C) can now be extended to include the large-granular pepsin-insoluble fraction (A) as well.

In order to determine whether appreciable amounts of neutral DBA derivatives could be obtained under conditions by which such substances have been liberated from protein-bound benzpyrene,[7,19,20] alkaline hydrolyses in the presence of zinc dust were carried out on protein and peptide fractions (Table III, expts. 5 and 13). Only slightly greater recoveries were obtained in the neutral fractions by this treatment; the C^{14} content of the acidic fraction was virtually unaffected. A carrier experiment with DBA on the neutral fraction from expt. 5 showed that in the 5.5% of neutral substances obtained, no dibenzanthracene was present.

The acidic metabolites extractable with organic solvents after alkaline hydrolysis appeared to be the substances of choice with which to carry out further characterization studies. The solubility tests in various organic solvents described in the Experimental section made it clear that this fraction contained a mixture of substances. Attempts to effect a separation by paper chromatography[22] were unsuccessful because of the presence of salts and amino acids derived from the protein in quantities enormous compared with those of the metabolites. Hence, it was decided to attempt the separation by means of partition chromatography on silicic acid, which we had found to be successful in resolving mixtures containing a variety of aromatic acids.[23] Three solvent systems were tried on the radioactive metabolites with the results illustrated in Fig. 1. Although it was clear that only partial separations were accomplished with this technique, a considerable degree of purification was achieved, since the radioactive metabolites were separated cleanly from the large amounts of extraneous substances.

Because satisfactory resolution of the mixtures was not realized, characterization of the components was undertaken by carrying out qualitative organic characterization reactions and measuring

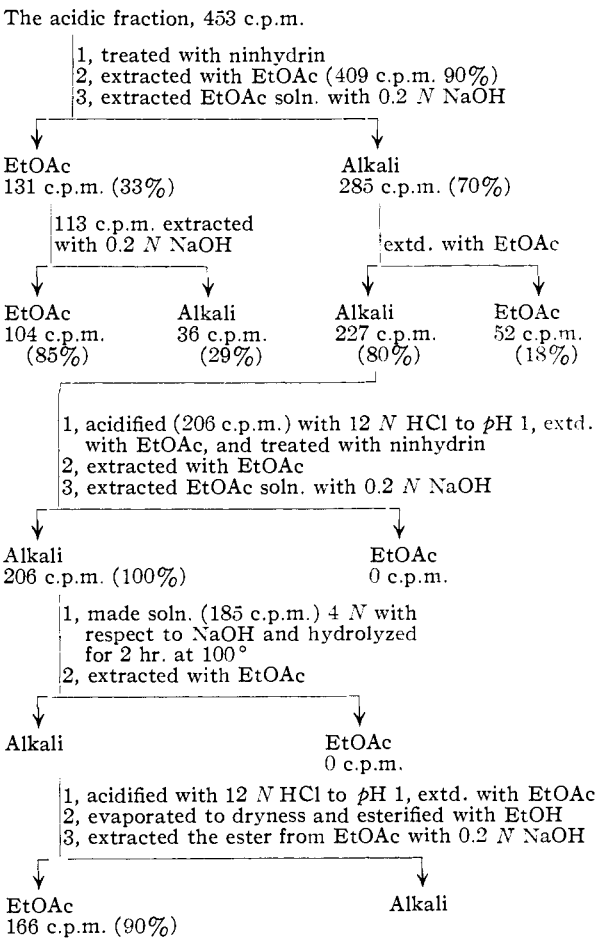


Fig. 2.—Ninhydrin treatment of the acid fraction from the pepsin-insoluble large-granular protein hydrolysate, after preliminary purification on a silicic acid column.

(22) M. E. Fewster and D. A. Hall, *Nature*, **168**, 78 (1951).

(23) P. M. Bhargava and C. Heidelberger, THIS JOURNAL, **77**, 166 (1955).

TABLE IV

NINHYDRIN TREATMENT OF ACID FRACTIONS OBTAINED ON HYDROLYSIS OF PEPTIDES

Starting peptide fraction[a]	Solvent system for chromatography[b]	Eluted with	Radioactivity at start, c.p.m.	Recovery of activity in EtOAc, %	Activity extractable in alkali, % (acids and phenols)	Activity remaining in EtOAc, % (amino acid-bound)
A	a	Methanol	453	90	70	33
A[c]	b	Solvent front	629	75	69	39
A	c	Solvent front	841	100	52	45
A	c	Methanol	351	44	83	17
B	a	Methanol	1610	77	89	20
C	a	Methanol	732	63	88	31

[a] A, B and C represent the same peptide fractions as in Table II, footnote *b*. [b] See text (section on chromatography). [c] The acid fraction obtained on hydrolysis was first run on a ligroin column, and the fraction *eluted with methanol from this column* was used.

the radioactivities distributed between immiscible solvents at each step. In an attempt to verify the prediction that some metabolite(s) would remain bound to amino acid(s), experiments with ninhydrin were carried out. If indeed the metabolites in the acidic fraction were bound to an amino acid, ninhydrin treatment, which decarboxylates and deaminates amino acids, would result in the production of a neutral compound. The experiments were carried out as shown in Fig. 2, and the results demonstrated that one-third of the radioactivity in the acidic mixture from large-granular pepsin-insoluble fraction was present as metabolite(s) bound to amino acid.[24] As shown in Table IV, amino acid-bound metabolites were also found in the other peptide hydrolysates.

In an attempt to determine whether the non-amino acid-bound radioactivity in acidic fraction from A was present as phenols, carboxylic acids or phenolic acids, a Fischer esterification reaction was performed as outlined in Fig. 3. The production of a neutral ester, amounting to approximately one-half of the radioactivity present, which was hydrolyzed back to an acid, showed the presence of carboxylic acid(s). Phenols or phenolic acids were not present in more than 8.5% amounts. It is of interest that the radioactivity insoluble in ethyl acetate after esterification was shown by ninhydrin treatment to be bound to amino acids, and the quantity corresponded well with that obtained in the other ninhydrin experiment (Fig. 2). The insolubility was probably due to the formation of the amino acid hydrochlorides under the esterification conditions.

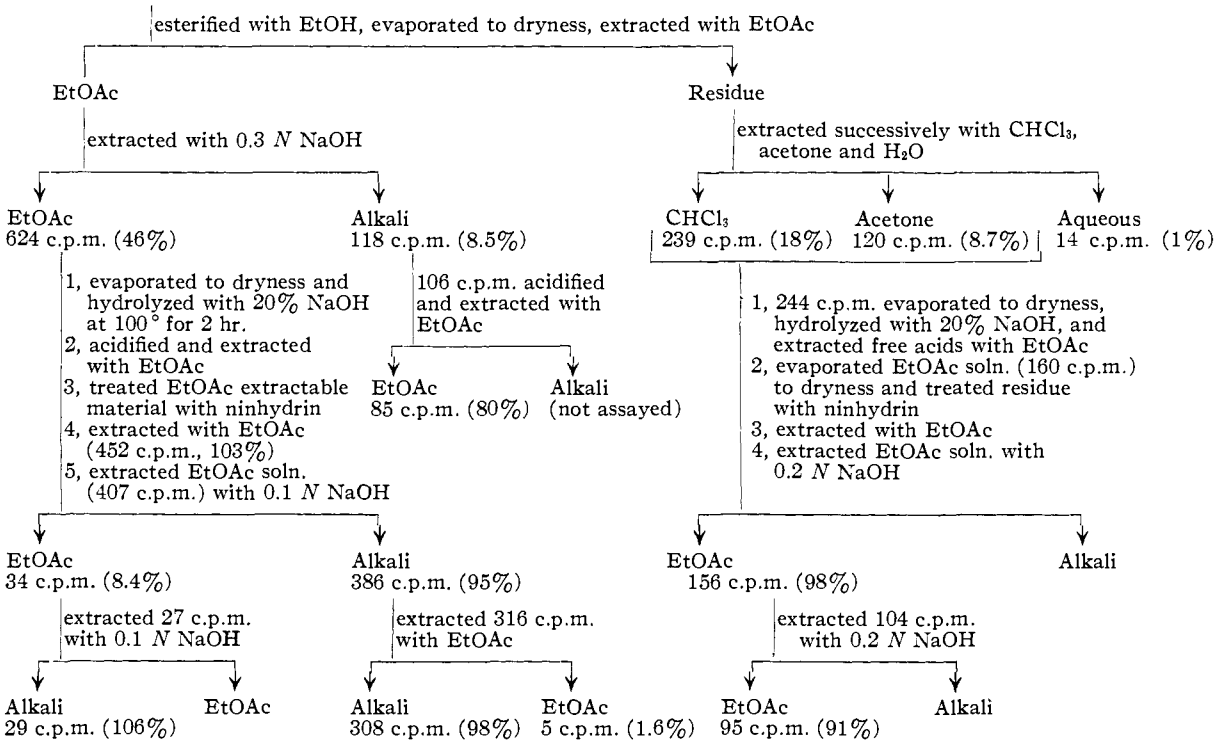


Fig. 3.—Esterification of the acid fraction from the pepsin-insoluble large-granular protein hydrolysate, after preliminary purification on a silicic acid column.

The next step in the characterization of the acidic

(24) The possibility existed that the ninhydrin treatment might have produced compounds such as β-aldehydo-acids. Hence the alkaline hydrolysis after ninhydrin treatment was carried out to cleave such compounds and produce neutral labeled substances.

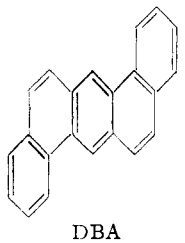
TABLE V

CARRIER EXPERIMENTS

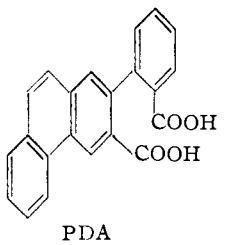
Expt.	Starting material Type[a]	Radioactivity, c.p.m.	Method[b]	Recov. of radioact. in acid fract. from peptide source, %	Carrier[c] added, mg.	Sp. act. at the first stage of purifcn.	Constant sp. act.[d]	Percentage of the carrier in acid fract.
1	D	4,420	b	48	19.05[e]	28	30	9.7
2	E	2,800	b	26	15.65[e]	41	43	19
3	F	6,840	b	32	69.63[e]	34	27	22
4	A	11,700	c	52	25.00	172	152	63
5	B	10,600	c	49	20.10	171	32	12
6	C	17,500	c	37	20.30	358	116	36
7	G	4,030	d	39	16.60	44	36	15

[a] A, B and C represent the same peptide fraction as in Table II, footnote *b*; D, E and F = the acidic fractions obtained by the hydrolysis of A, B and C, respectively, following method *e* (radioactivities calculated for the original peptide fraction); G = acidic fraction obtained on hydrolysis of A, following method *j*. [b] See text (section on carrier Experiments). [c] Unless otherwise mentioned, PDA was added as the carrier. [d] The specific activities given are the mean of several values obtained (within 10% of each other). [e] An impure sample of acid was used for these experiments (see text); the percentages in the last two columns have been calculated on the basis of 80% PDA content of the sample used.

fractions was accomplished by the stochastic process of selecting the proper compound to use as a carrier and determining whether it would become radioactive and maintain a constant specific activity under rigorous conditions of purification. The acid in question would have to be non-phenolic, and because of the solubility characteristics of the radioactive materials would also have to be relatively polar. On theoretical grounds it appeared likely that 2-phenylphenanthrene-3,2′-dicarboxylic acid (PDA)[25,26] might be produced from dibenzanthracene by oxidative cleavage of the phenanthrene double-bond. The compound was synthesized and found to behave identically to the major radioactive fractions in all three silicic acid partition chromatographic systems. This result encouraged us to perform the carrier experiments, which proved that this compound accounts for an appreciable portion of the carboxylic acid fraction.



DBA



PDA

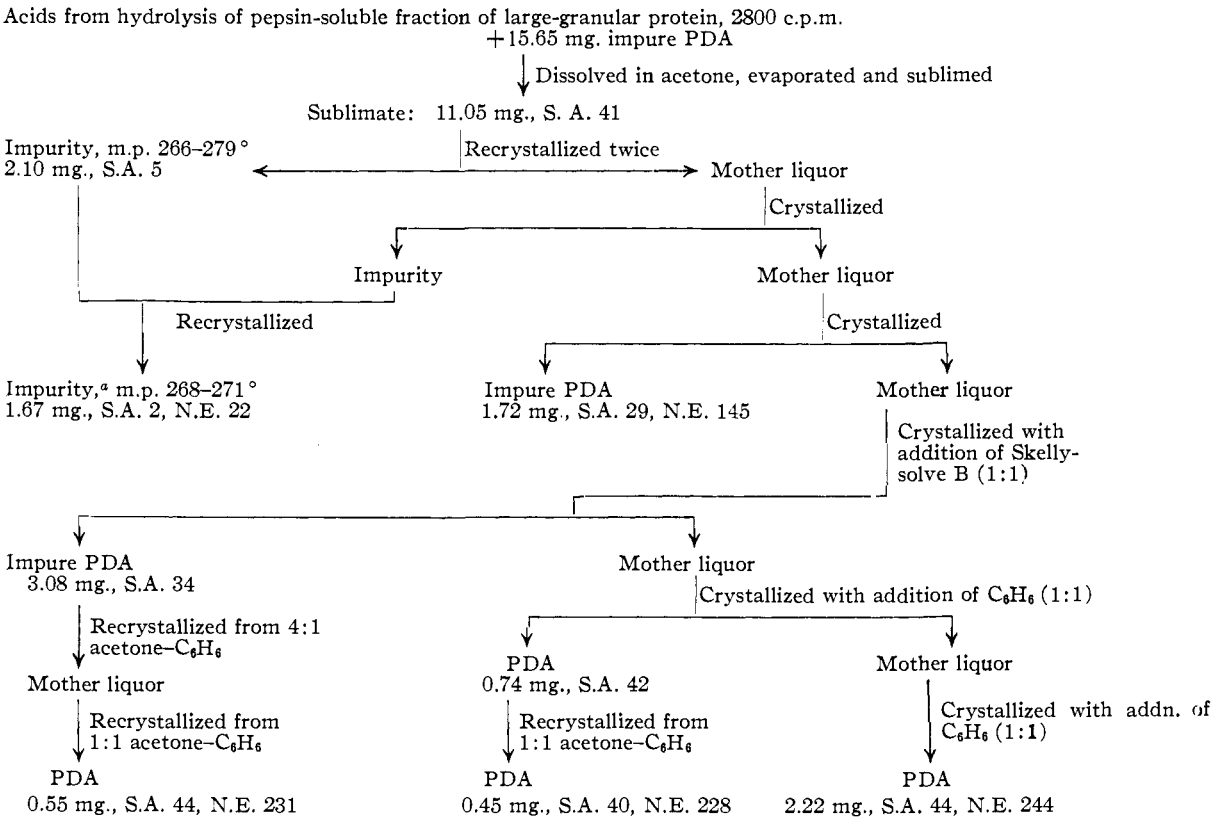
In the initial carrier experiments (Table V, expts. 1, 2 and 3), although the melting point of the phenylphenanthrenedicarboxylic acid used corresponded with that reported,[25] a considerable amount of a neutral impurity was present, which was removed during the course of recrystallizations. The results of a typical purification are shown in Fig. 4, and it will be noted that a linear relationship between the specific activity and the neutral equivalent of the carrier acid was obtained. As a result of this scheme, three different samples of purified carrier were obtained, all having identical specific activities and the proper neutral equivalents. Thus, paradoxically, as a consequence of the presence of an impurity, added confidence in the validity of the results was achieved in these experiments. As an additional criterion of purity, the acid was converted into the dimethyl ester, which was purified and had the theoretical specific activity. Therefore, we believe that these data constitute proof that this acid is produced by hydrolysis of the protein or peptide materials containing the bound hydrocarbon or its derivative(s), and that the results obtained by the carrier technique represent a valid quantitative estimation of its presence. This compound and dibenzanthracene-3,4-dihydrodiol are being tested for carcinogenic activity.

The data from experiments 1, 2, and 3 in Table V show that a small, but nevertheless appreciable, amount of the radioactivity in the acid fractions derived from all three protein fractions was present as PDA. Because of the structure of this compound it seemed possible that under the conditions of alkaline hydrolysis it might have been produced by the oxidation of some precursor. In order to gain further information about this point, alkaline hydrolyses of the peptide samples were carried out, after the initial addition of carrier PDA, under oxidative and reducing conditions.

In experiments 3, 10 and 12 of Table III, which were conducted as before but with the addition of permanganate, only minor differences were encountered in the amount of acidic extractable radioactivity as compared with the non-oxidative hydrolyses except in experiment 10. When a minute amount of labeled dibenzanthracene was treated under the oxidative conditions (expt. 15), only 5% of the radioactivity could be recovered, and this was primarily in the acidic fraction. In view of the high recoveries obtained in the protein hydrolyses, this fact offers additional support to the contention that dibenzanthracene was not present as such, or produced during the course of the hydrolytic treatment.

When the carrier was isolated from the oxidative hydrolysis of the large-granular pepsin-insoluble fraction (expt. 4, Table V), the radioactivity showed that a sixfold increase in the quantity of PDA resulted from this treatment. No increase of PDA was observed in the large-granular pepsin-soluble fraction (expt. 5), but a doubling of the amount of this acid was obtained from the soluble protein (expt. 6). From these experiments it is evident that

(25) E. A. M. Stephenson, *J. Chem. Soc.*, 2620 (1949).
(26) J. W. Cook, *ibid.*, 1594 (1933).



[a] NOTE ADDED IN PROOF.—This impurity has been identified as the anhydride of PDA.

Fig. 4.—A typical carrier experiment using an impure sample of PDA. Unless otherwise mentioned all crystallizations were from acetone; S.A. = specific activity expressed in c.p.m./mg.; N.E. = neutral equivalent.

the majority of the acidic compounds in the alkaline protein hydrolysates were converted by oxidation to the phenylphenanthrenedicarboxylic acid. In the absence of additional data, it would be fruitless to speculate about the structure of these precursors. It is worth mentioning that under similar conditions on a semi-micro scale no acidic products were obtained from the treatment of unlabeled 3,4-dihydro-3,4-dihydroxydibenzanthracene or dibenz-3,4-anthraquinone. However, this need not necessarily exclude these compounds as precursors because of altered solubilities in the ultramicro quantities encountered in these experiments.

Because it had been reported[7,19] that the amount of neutral fluorescent material derived from protein-bound benzpyrene was increased by carrying out the alkaline hydrolyses in the presence of zinc dust, such experiments were tried here. As shown in Table III (expts. 5 and 13), there was only a very slight increase in the amount of radioactivity in the neutral fraction under these reducing conditions. However, a 70% recovery of radioactivity in the neutral fraction was obtained in expt. 16 with DBA. This, therefore, represents further evidence against the presence in or production of dibenzanthracene from this peptide material. In the PDA carrier experiment following reductive hydrolysis (Table V, expt. 7), there was a slight decrease in radioactivity in PDA as compared with the same fraction under ordinary alkaline hydrolysis.

In order to assess the quantitative significance of the data presented here, the losses and recoveries of radioactivity at various stages are calculated in Table VI as percentages of the total radioactivity obtained in the original, unfractionated, protein preparation. Each value given is the mean of several experiments.

It is evident from line 3 that the largest single fraction is not extractable by ethyl acetate following alkaline hydrolysis, and is appreciable in all three peptide sources. This fraction, which very likely contains metabolites bound to polar amino acids, was not characterized further in the present study, but is currently under investigation.

In the ethyl acetate fraction there was a small loss of acidic, uncharacterized, radioactivity by volatility (line 4), and the loss of substances on the ligroin silicic acid partition column is shown in line 5. The compounds eluted from this chromatographic system with ligroin (line 6) represent only a very small amount, and have not been studied further.

The quantity of extractable amino acid-bound metabolites (line 7) was equal to 9% of the original radioactivity, as determined by the ninhydrin experiments. Since it is reasonable to suppose that the non-extractable fraction (line 3) contains the majority of the amino acid-bound substances, it appears likely that this extractable fraction contains the metabolites bound to the less polar amino acids.

Although the carrier experiments with the phenylphenanthrenedicarboxylic acid were performed on the crude ethyl acetate extracts, it is possible to

TABLE VI

PERCENTAGE RECOVERIES REFERRED TO UNFRACTIONATED PROTEIN[a]

		Large-granular pepsin-insoluble	Large-granular pepsin-soluble	Soluble protein	Total
1	Loss on pepsin digestion	0	0	1.0	1.0
2	Neut. radioactivity after alk. hydrolysis	0.6	0	0.2	0.8
3	Not extractable after alkaline hydrolysis	21.3	13.1	12.5	46.9
	All subsequent items derived from EtOAc extractable substances following alkaline hydrolysis				
4	Loss of acids by volatility	5.5	0	0	5.5
5	Acids remaining on ligroin column	6.4	0	2.1	8.5
6	Acids eluted with ligroin	2.0	0.3	0.4	2.7
7	Amino acids eluted with methanol	7.5	0.7	0.8	9.0
8	Carboxylic acids (and phenols) eluted with methanol	13.7	3.2	2.1	19.0
9	Losses of acids during ninhydrin treatment and esterifcn.	3.8	1.1	1.7	6.6
10	Totals (1 to 9)	60.8	18.4	19.8	100.0
11	PDA alkaline hydrolysis	3.2	1.0	1.6	5.8
12	PDA oxidative hydrolysis	19.5	1.0	2.7	22.7
13	PDA reductive hydrolysis	3.6			

[a] Percentages are based on the *mean* values obtained in several experiments; the mean deviation in no case exceeded 8% and generally was of the order of 2% of the percentages reported in the various tables.

calculate the amount of the acidic fraction that can be accounted for by this compound. It is only possible for the PDA to be present in the fraction indicated in line 8, and thus it accounts for 30% of these compounds under ordinary alkaline hydrolysis. In the oxidative experiments it is conceivable that the precursors could be present in the fractions described in lines 5, 8 and 9. Hence, the PDA produced under oxidative hydrolysis accounts for 67% of the substances that could be present in these fractions.

It is not possible to determine, with the information currently available, whether the precursors of PDA that are *actually* bound to the protein retain the dibenzanthracene ring system or have been opened. Furthermore, the nature of the covalent bond between the hydrocarbon derivative and the protein, whether carbon–carbon, carbon–sulfur, carbon–nitrogen or some other, is not now known. Nor is the relation of the binding of the extractable substances to that of the amino acid-bound substances understood at present. All these points are currently under study, and further speculations, although tempting, would be unjustified.

It is, however, of interest to examine the demonstration of PDA as a degradation product of the bound hydrocarbon derivative in relation to the so-called "K" region theory of carcinogenesis. This theory, which has been discussed recently in two reviews,[27,28] postulates the necessity of a rather high and critical electron density in the "K" region, or phenanthrene double bond, for carcinogenic activity. As a result of this, it has been predicted[6,27] that interaction of the carcinogen and tissues might occur in this region of the molecule. If, as we now firmly believe, protein binding is of importance in the carcinogenic process, the identification of PDA as a degradation product of the bound metabolite of dibenzanthracene represents the first *direct* experimental demonstration of the involvement of the "K" region in the carcinogenic process.

When the structure of the bound metabolite has been determined, the first step in the chemical definition of the process of hydrocarbon carcinogenesis may well have been achieved. At the same time, work on the characterization of the protein(s) involved is not being neglected. Much work remains to be done, however, before the crucial investigation of the biological or enzymatic function[29] of the key proteins can be launched. If this function can ever be determined, the problem of the mechanism of chemical carcinogenesis may well have been solved.

Experimental

All m.p.'s are uncorrected.

Solvents.—Redistilled, analytical reagent grade solvents were used.

Radioactivity Assay Techniques.[30]—Aliquots of organic solutions and small volumes (0.1 ml. or less, containing less than 20–25 mg. of dissolved solid) of neutral or weakly acidic or alkaline aqueous solutions were pipetted onto aluminum discs on a turntable in a current of air. The proteins were plated as a well-ground slurry in 30% alcohol (0.1–0.3 ml.) which was spread with a pipet on an aluminum disc and dried at room temperature.

With strongly acidic or alkaline aqueous samples or aqueous samples containing a large amount of dissolved solids, an aliquot was combusted by the Van Slyke–Folch or the persulfate oxidation procedures. All samples were counted in internal flow counters, and corrected for self absorption. All radioactivities are given as counts per minute (c.p.m.), the specific activities as c.p.m./mg., and are statistically significant to at least 10%.

Liquid–Liquid Extraction Techniques.—The extractions were carried out in centrifuge tubes (15 or 40 ml.) whenever feasible. It was essential in all cases to keep the volumes as small as possible.

Application of Dibenzanthracene-9,10-C^{14} to Skin and Isolation of the Proteins.—The general technique has been described earlier.[14a] Seventy-two female mice[31] were shaved on the back, and two days later, 0.1 ml. of a 0.2% solution of labeled dibenzanthracene (sp. act., 1.2×10^7 c.p.m./mg.) in benzene was applied uniformly to the shaved portion of the skin of each mouse with a micro-pipet. Two days later, the mice were killed individually with ether. The skin was re-shaved closely, washed with benzene, and quickly excised. Fat and connective tissue were removed by the liquid air scraping technique[14a]; the total wet weight of the combined samples was 9.2 g. The skin preparations, 2–4 at a time, were then homogenized at 0° with a Potter–Elvehjem homogenizer in 0.154 *M* KCl. The homogenate was centrifuged at 600 × g. for 10 min. to give the large-granular (including nuclear) and the soluble proteins. Both crude protein fractions then were denatured and precipitated by treatment with 5% trichloroacetic acid (TCA) at 0°. The proteins then were centrifuged, washed at 0° twice with 5% TCA and then with 5% NaCl until neutral. The nucleic acids were extracted by treating with 10% NaCl at 100° for 1 hour, decanting the aqueous layer and repeat-

(27) C. A. Coulson, *Adv. in Cancer Res.*, **1**, 1 (1953).

(28) G. M. Badger, *ibid.*, **2**, 73 (1954).

(29) H. P. Rusch, *Cancer Research*, **14**, 407 (1954).

(30) 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, pp. 92–96, 104–121.

(31) "Rockland" strain, obtained from Arthur Sutter, Springfield, Mo.

ing the treatment for another half-hour. The proteins then were washed 3 times each with 15% ethanol, 95% ethanol, 3:1 ethanol–ether at 50°, dioxane at 50°, benzene at 50°, and finally with ether. The yields of the large-granular and soluble proteins were 942 and 247 mg., and their specific activities, 438 and 434 c.p.m./mg., respectively (batch I). Thus the total recovery of radioactivity in the bound protein was 0.3% of that applied to the skin (0.24% in the large granular and 0.06% in the soluble fraction); 1 mg. of total dry protein contained radioactivity equivalent to 0.0375 μg. of dibenzanthracene. Unless otherwise specified, all experiments subsequently described were carried out on proteins from batch I.

Degradation of the Proteins to Small Polypeptides with Pepsin.—Twenty-five to one hundred mg. of the protein was digested with 1 ml. of a 0.05% solution of pepsin in 0.01 *N* HCl (or 0.5 ml. of a 0.1% solution) per 25 mg. of protein in a 15-ml. centrifuge tube at 38° for 18–24 hr. with continuous shaking. The insoluble fraction (negligible from the soluble protein) was separated by centrifugation and washed once with 1–2 ml. of water. The pepsin-soluble fractions were extracted exhaustively with ethyl acetate and/or benzene and/or ether. The yields and radioactivities of the various fractions in typical experiments using two different batches of proteins are shown in Table I. The pepsin-insoluble fractions were not affected by further treatment with pepsin. Increasing the amount of the pepsin solution or the concentration of pepsin, or both, did not affect the results.

Acidic Hydrolysis of the Pepsin-treated Materials.—All three peptide fractions, the pepsin-insoluble large-granular, the pepsin-soluble large-granular, and the pepsin-treated soluble fraction, were hydrolyzed as indicated in Table II. The hydrolysate then was extracted with ethyl acetate. The ethyl acetate solution was extracted with alkali leaving neutral material; the alkaline solution then was acidified with HCl and the free labeled acids extracted with ethyl acetate. In the case of the pepsin-insoluble large-granular fraction, a small amount of residue, insoluble in acid and with a specific activity higher than that of the original protein, was obtained; this was removed by centrifugation, dried, weighed, and plated. The supernatant was worked up as described above.

The recoveries of radioactivity in the various fractions in different experiments are given in Table II. The gases evolved during hydrolysis by condition 1 were combusted both in a combustion furnace and by chemical oxidation; no radioactive CO_2 was obtained.

Alkaline Hydrolysis of the Protein Materials.—The original large-granular protein, the two fractions (soluble and insoluble) obtained on pepsin treatment of this protein, the pepsin-treated soluble protein, and dibenzanthracene-9,10-C^{14} were hydrolyzed as shown in Table III.

All hydrolyses at 38° were carried out in small conical flasks; the hydrolyses at higher temperatures, with 6 *N* alkali, were conducted in a cylindrical stainless steel bomb (capacity approx. 13 ml.), the neck of which was machined to fit a standard 14/20 joint. When a glass vessel was used with 6 *N* alkali at elevated temperatures for prolonged periods, a considerable amount of sodium silicate was obtained which interfered with the subsequent working-up of the hydrolysate. Approximately 1 ml. of the alkali was used for every 2,000–4,000 c.p.m. in the pepsin-insoluble fraction of large-granular protein or 5,000–10,000 c.p.m. of labeled dibenzanthracene.

After the hydrolyses, the contents of the reaction vessel were transferred to a centrifuge tube with a dropping pipet; the condenser and the reaction vessel were rinsed with distilled water (1.5–2 ml., except in case of oxidative and reductive hydrolysis when more water was required to complete the transfer) and then 2–3 times with 1–2 ml. of benzene, ether or ethyl acetate. The hydrolysate was then extracted exhaustively with benzene and/or ether and/or ethyl acetate to remove the neutral materials; usually 3–4 extractions with 5–10 ml. of the solvent(s) sufficed. Ethyl acetate was not used when the alkali concentration was above 3 *N* because it generated appreciable amounts of acetic acid, which interfered at later stages. In the case of oxidative and reductive hydrolyses, the MnO_2 or the $Zn–Zn(OH)_2$ mixture was removed by centrifugation prior to extraction of the neutral material. Exhaustive extraction of the MnO_2 or $Zn–Zn(OH)_2$ mixture with benzene, ether and ethyl acetate, gave no recovery of radioactivity; nor was any recovered from the zinc on extraction of its solution in dil. HCl by the above solvent.

After the removal of the neutral material, the alkaline solution was acidified at 0° with concd. HCl to *p*H 1. It then was extracted thoroughly with ethyl acetate. Ether removed only 60–70% of the radioactivity extractable by ethyl acetate. In the case of oxidative hydrolyses carried out with the addition of PDA, the precipitated dicarboxylic acid was filtered before extraction and purified as described later.

It was not found necessary to wash the organic extracts free of alkali or acid for subsequent operations. All extracts were evaporated to a small volume (usually less than 15 ml.) under a current of nitrogen, at temperatures slightly below the boiling point of the solvent, before being assayed for radioactivity; any solid material was removed by centrifugation. In some cases a small fraction (up to 10%) of the radioactive acidic material was found to be volatile or unstable. The radioactivity was "stabilized" by evaporation of the solution to dryness, dissolution, and reevaporation; usually 1–2 repetitions of this process gave "stable" activity.

Representative results of various alkaline hydrolyses are shown in Table III. Some of these experiments were repeated and essentially the same results were obtained.

Solubility in Organic Solvents of the Acidic Fraction Obtained on Alkaline Hydrolysis of Pepsin-insoluble Large-granular Protein.—When material containing 106 c.p.m. of the acidic fraction in the dry state was extracted exhaustively with the following solvents, the percentage of the radioactivity shown went into solution; the remaining radioactivity in each case was extractable with ethyl acetate, which was found to be a suitable water-immiscible solvent for the acidic material: carbon tetrachloride, 19%; 1:2 ether–ligroin, 30%; 1:19 propanol–cyclohexane, 44%; benzene, 37%; 1:9 ethyl acetate–carbon tetrachloride, 50%.

Chromatography of the Acid Fractions Obtained on Alkaline Hydrolysis of the Various Peptide Materials.—Partition chromatography on a silicic acid column[23] was carried out on the acid fraction derived from pepsin-insoluble large-granular protein by hydrolysis method (e); three solvent systems described in Fig. 1 were used. Varying amounts of radioactivity were eluted with the solvent front in the different systems; no more radioactivity could be eluted with 400 ml. of the external phase. In case of the systems employing ligroin or an ethyl acetate–carbon tetrachloride mixture, when less than 50% of the radioactivity was eluted with the solvent front, the columns subsequently were eluted with methanol, which removed more radioactivity with the methanol front. However, in no case could more than 75–80% of radioactivity applied to the column be eluted in the combined fractions; attempts to elute the remainder with ethyl acetate, chloroform or benzene, failed. When, however, the material eluted from the ligroin column was run on the butanol–chloroform column, or *vice versa*, the recoveries were virtually quantitative. The results obtained are described in Fig. 1 and were found to be reproducible to within ±2%.

When the acidic compounds obtained by hydrolysis of the pepsin-soluble fractions of large-granular and soluble proteins by method (e) (1710 and 1250 c.p.m., respectively) were run on the ligroin column, 6 and 5.3%, respectively, of the radioactivities were eluted with the solvent front; 94 and 65% were eluted later with methanol.

Chromatography of 2-Phenylphenanthrene-3,2′-dicarboxylic Acid.—This acid was chromatographed on a silicic acid column using all three systems mentioned before. From the butanol–chloroform and ethyl acetate–carbon tetrachloride columns, the acid was eluted with the solvent front. From the ligroin column, the acid could be eluted only with methanol.

Ninhydrin Treatment and Esterification.—The mixture of acidic compounds obtained by hydrolysis of the various pepsin treated protein fractions was partially purified on silicic acid partition columns using one or more of the three systems described above. The ninhydrin reactions were carried out as illustrated in Fig. 2 in a centrifuge tube on a steam-bath for one hour, with 1–2 ml. of a 0.1% aqueous solution of ninhydrin, and solid KH_2PO_4 sufficient to bring the *p*H to 3–6. The results obtained with the various purified acidic fractions are given in Table IV.

The acidic substances from the hydrolysis of pepsin-insoluble large-granular protein, purified on a ligroin column (methanol elution), were esterified by refluxing for two

hours with absolute alcohol saturated with dry HCl gas, the alcohol was evaporated under nitrogen, and the residue worked up as in Fig. 3.

All the liquid–liquid extractions were carried out with equal volumes of ethyl acetate or alkali.

Preparation of 1,2,5,6-Dibenz-3,4-anthraquinone.—Crude dibenzanthracene-3,4-dihydrodiol, obtained from 9.4 g. of dibenzanthracene through its osmium tetroxide adduct by the method of Cook and Schoental,[32] was oxidized with sodium dichromate (9.2 g. in 27 ml. of water) and acetic acid (800 ml.) by modification of the method of Stephenson.[25] The reactants were mixed and slowly (1 hour) brought to reflux; the mixture turned red. It was refluxed for 30 min. and left at room temperature overnight. The red solid was filtered, washed with water and dried, yield 5.4 g. (53% based on DBA). The m.p. was 348–350° in contrast to the literature reports of 327–329°,[24,25] and 325°.[33] One recrystallization from ethyl acetate (not necessary for oxidation to the dicarboxylic acid) gave a m.p. of 349.5–350°. *Anal.*[34] Calcd. for $C_{22}H_{12}O_2$: C, 85.67; H, 3.92. Found: C, 85.22; H, 4.12. The mother liquor when poured into water gave a yellow crystalline solid, m.p. 208–210°, yield 0.46 g., which was not characterized further. It was not reported previously.[25]

Preparation of 2-Phenylphenanthrene-3,2′-dicarboxylic Acid (PDA).—This acid[25,33] was prepared from 1,2,5,6-dibenz-3,4-anthraquinone by a modification of the method used by Collins, *et al.*,[35] for the preparation of 2-phenylnaphthalene-1,2′-dicarboxylic acid. The quinone, 1.5 g., 900 ml. of *sym*-tetrachloroethane, 300 ml. of acetic acid and 420 ml. of 30% H_2O_2 were mixed in a 3-l. flask, heated for 18 hr. at 80–90° (when the organic layer turned yellow) and left overnight. The solvents were removed *in vacuo* when a yellow solid separated. The flask was cooled, the contents left for several hours, covered with an excess of 10% $NaHCO_3$ and then filtered. The crude dicarboxylic acid was precipitated from the filtrate by acidification with concd. HCl, and was cooled and filtered, yield 1.1 g. (67%), m.p. 300–309°. One recrystallization from acetone gave pure pale-yellow acid, m.p. 315–317°, yield 0.48 g., neutral equivalent 224 (calcd. 233).

In some of the carrier experiments, a sample of acid was used which, although its m.p. was 309–312° in accordance with the literature,[25] was shown later by neutral equivalent determinations to be only 80% pure.

(32) J. W. Cook and R. Schoental, *J. Chem. Soc.*, 170 (1948). After the decomposition of the adduct, only the diol (almost colorless) obtained on filtration and washed with water was used.

(33) J. W. Cook and R. Schoental, *J. Chem. Soc.*, 47 (1950).

(34) Microanalysis carried out by Clark Microanalytical Laboratory, Urbana, Ill.

(35) C. J. Collins, D. N. Hess, R. H. Mayor, G. M. Toffel and A. R. Jones, THIS JOURNAL, **75**, 397 (1953).

Carrier Experiments.—(a) General techniques: The nonradioactive phenylphenanthrenedicarboxylic acid (PDA), mixed with the radioactive material was purified by repeated crystallizations from acetone and by sublimation. Less than 3 mg. of the acid was recrystallized from a 4:1 or 1:1 mixture of acetone with benzene or ligroin. Sublimations were carried out at less than 0.1 mm. and 280–320° (bath temperature). The acid was assayed for radioactivity by direct plating from acetone solution.

Dibenzanthracene carrier was purified by recrystallization from benzene and acetic acid and by chromatography on Florisil.[12] It was plated from benzene solution.

Carrier experiments which were repeated were reproducible.

(b) Carrier experiments on acidic materials obtained under hydrolysis condition (e): The radioactive material (2500–7000 c.p.m.) was mixed in the dry state with a known amount (15–70 mg.) of the *impure* PDA carrier. The mixture was dissolved in acetone, and sublimed 1–3 times. The non-acidic, more soluble impurity was removed and the dicarboxylic acid recrystallized to constant specific activity as illustrated in Fig. 4. Experiments 1–3 in Table V summarize the results obtained. In these three experiments, when neutralization equivalents of the samples of carrier acid obtained at various stages of purification after the initial sublimation(s) were plotted against their specific activities, a straight line was obtained.

The samples of PDA that had been purified to constant specific activity in experiments 1–3 (Table V), were combined and esterified with methanol as already described. The ester was isolated in the usual way. Two recrystallizations gave specific activities of 26 and 27 c.p.m./mg., respectively (calculated value 26); m.p. 142° (Cook and Schoental[33] give m.p. 144–145°).

(c) Carrier experiments on materials obtained by oxidative hydrolysis: The pepsin-soluble and pepsin-insoluble fractions derived from large-granular protein and the pepsin treated soluble protein (10,000–20,000 c.p.m.) were hydrolyzed according to method (h), using the pure carrier. The dicarboxylic acid was precipitated from the alkaline solution (after removal of MnO_2 and neutral substances) as already described. Three to six recrystallizations interspersed with two to three sublimations sufficed to achieve radiochemical purity.

(d) Carrier experiments on acidic and neutral materials obtained from pepsin-insoluble large-granular protein by hydrolysis method (j): The experiment using PDA as the carrier on the acidic material was done as in (a), except that the pure acid was used. The neutral fraction was mixed with carrier DBA, which was then purified to constant specific activity as already described.

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NOTES

The Infrared Intensity of the C–D Stretching Vibration in Deuterobenzene, *m*-Deuteronitrobenzene and *m*-Deuteroaniline

BY WILLIAM G. DAUBEN, GEORGE C. PIMENTEL AND C. WHEATON VAUGHAN, JR.[1]

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It has recently become of interest in this Laboratory to employ infrared spectroscopy to determine the percentage of deuterium in certain aromatic compounds. In this regard it has been reported by Alexander and Burge[2] that "with nitrobenzene and aniline hydrochloride, the absence of a characteristic infrared absorption in the C–D stretching frequency cannot be taken as evidence for the absence of small amounts of deuterium in the *meta* position." Such a conclusion was based upon a comparison of the infrared spectrum of deuterobenzene in benzene solution with the spectrum of a sample of *m*-deuteronitrobenzene containing 2.55 atom per cent. excess deuterium. These observations cast doubt upon the utility of

(1) Du Pont Postgraduate Teaching Fellow in Chemistry, University of California (Berkeley), 1953–1954.

(2) B. R. Alexander and R. E. Burge, Jr., THIS JOURNAL, **72**, 3100 (1950).