crops of colorless crystals weighing a total of 12.5 g. (70%) were obtained; m.p. 126–128°. Samples melting at 116–117° were also obtained by recrystallization from ethyl acetate. Late samples gave only the higher m.p.

Anal. Calcd. for $C_{12}H_{14}ON_2$: C, 71.26; H, 6.98; N, 13.85. Found: C, 71.07; H, 6.82; N, 13.37.

The infrared spectra in chloroform of the two samples with different m.p. were identical, with a strong amide CO band at 6.10 μ.

3-(2-Dimethylaminoethyl)-indole (N,N-Dimethyltryptamine).—A suspension of finely-divided amide (2.1 g.) in 100 ml. of ether was added to a slurry of 0.8 g. of lithium aluminum hydride in 50 ml. of ether, and the mixture was heated under reflux for 4 hr. The mixture was treated in the usual way, and the final organic extract of the amine gave, after recrystallization from hexane, 1.6 g. (85%) of material melting at 47–49°. This material was converted to a higher melting form (71–73°) by crystallization from hexane after seeding with an authentic specimen of m.p. 73–74°.[17] The infrared spectra in chloroform of the two samples were identical.

(17) We are indebted to Dr. M. E. Speeter of the Upjohn Co. for this sample.

BETHESDA 14, MARYLAND

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

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

BY P. M. BHARGAVA AND CHARLES HEIDELBERGER

RECEIVED NOVEMBER 21, 1955

We have previously shown that 2-phenylphenanthrene-3,2′-dicarboxylic acid (PDA) is obtained on the alkaline hydrolysis of the protein-bound compounds following the application of 1,2,5,6-dibenzanthracene-9,10-C^{14} to the skin of mice. By hydrazine treatment of the proteins and by carrier experiments, it has now been demonstrated that 25% of the protein-bound radioactivity involves the binding of PDA to the protein, partly through the diamide and partly through a monoamide of the acid.

In 1947, the Millers[3] demonstrated that following the feeding of the potent hepatic carcinogen, *p*-dimethylaminoazobenzene, to rats, some dye was bound to liver proteins. Subsequently, it has been found that several hydrocarbons and 2-acetylaminofluorene are also bound to the proteins of susceptible tissues.[4–9] These findings have led to the formulation of the "protein deletion" hypothesis of carcinogenesis, which states that as a result of the chemical interaction of the carcinogen with the proteins, an enzyme system important to the control of growth is deleted, thus initiating the production of a cancer cell.[5] In view of the probable importance of this chemical binding in the carcinogenic process, it is essential to determine the structure of the carcinogen–protein complex. Work along these lines, using 1,2,5,6-dibenzanthracene-9,10-C^{14} (DBA) has been in progress in this Laboratory for some time, and it has been reported[10] that 2-phenylphenanthrene-3,2′-dicarboxylic acid (PDA) is obtained following rather drastic hydrolysis of several fractions of skin proteins. It then became important to determine whether this acid was produced as an artifact during the isolation procedure or is bound directly to the proteins. The work reported here demonstrates that PDA is bound to the proteins through its carboxyl groups in amide linkage.

Results and Discussion

It is known that imides of certain dicarboxylic acids, such as phthalic acid,[11] give cyclic hydrazides on treatment with hydrazine. Sheehan and Frank[12] have further shown that treatment with hydrazine hydrate of phthalimides substituted on the nitrogen with peptide groups, gives the cyclic hydrazide of phthalic acid and the free peptide. Similarly, it is also known that amides (*e.g.*, benzamide[13,14]) and esters (*e.g.*, the dimethyl and diethyl esters of diphenic acid[15,16]) of aromatic acids give their hydrazides on treatment with hydrazine. If, therefore, PDA were bound to the protein(s) through its carboxyl groups as an ester, amide or imide, hydrazine treatment of the protein would result in cleavage of the peptide–metabolite bond and thus yield radioactivity extractable into organic solvents. According to Akabori,[17] hydrazine treatment of proteins splits the amino acid chain, giving rise to the hydrazides of free amino acids, although his conditions were somewhat different from those of Sheehan and Frank. The extent of degradation of the protein was, however, immaterial to us, as we are concerned only with the structure of the DBA metabolite

(1) This work was supported in part by a research grant, C-1132, from the National Cancer Institute, National Institutes of Health, Public Health Service, 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 *Proc. Am. Assoc. Cancer Research*, **2**, 5 (1955).

(3) E. C. Miller and J. A. Miller, *Cancer Research*, **7**, 468 (1947).

(4) E. C. Miller, *ibid.*, **11**, 100 (1951).

(5) E. C. Miller and J. A. Miller, *ibid.*, **12**, 547 (1952).

(6) W. G. Wiest and C. Heidelberger, *ibid.*, **13**, 246, 250, 255 (1953).

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

(8) 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.

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

(10) P. M. Bhargava, H. I. Hadler and C. Heidelberger, THIS JOURNAL, **77**, 2877 (1955).

(11) H. D. K. Drew and H. H. Hatt, *J. Chem. Soc.*, 16 (1937).

(12) J. C. Sheehan and V. S. Frank, THIS JOURNAL, **71**, 1855 (1949).

(13) G. Struve, *J. prakt. Chem.*, **50**, 295 (1894).

(14) W. J. Hickinbottom, "Reactions of Organic Compounds," Longmans, Green and Co., Ltd., London, 1948, pp. 230–231.

(15) L. Kalb and O. Gross, *Ber.*, **59**, 736 (1926).

(16) W. Borsche, W. Müller and C. A. Bodenstein, *Ann.*, **475**, 120 (1929).

(17) S. Akabori, K. Ohno and K. Narita, *Bull. Chem. Soc. Japan*, **25**, 214 (1952).

bound to the protein. The distribution of the radioactivity in the organic, aqueous and insoluble phases, following hydrazine treatment of the pepsin-insoluble (X) and pepsin-soluble (Y) large-granular protein fractions and the pepsin treated soluble protein (Z), is given in Table I. In each case, nearly 25% of the radioactivity in the peptide

TABLE I

HYDRAZINE TREATMENT OF PEPTIDE FRACTIONS

Peptide fraction[a]	Wt. or vol.	Total c.p.m. at start	C.p.m. in EtOAc	C.p.m. in aq. fraction	C.p.m. in insoluble residue
X	34.0 mg.	20,800	7300 (26%)	1010 (3.5%)	9080 (32%)
Y	1.0 ml.	3,300	750 (23%)	2210 (67%)	None
Z	1.0 ml.	3,380	810 (24%)	2680 (80%)	None

[a] X = pepsin-insoluble large-granular protein; Y = pepsin-soluble large-granular protein; Z = pepsin-treated soluble protein.

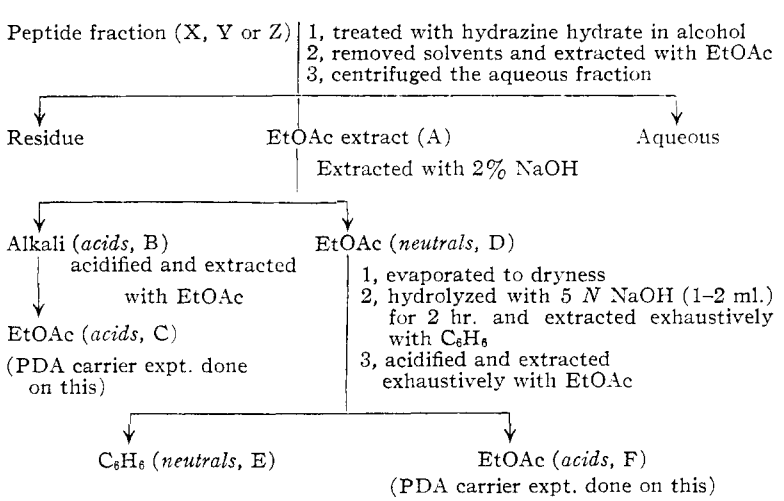


Fig. 1.—Degradation of the peptide fractions with hydrazine, followed with alkali. X, Y and Z denote the same peptide fractions as in Table I.

fraction became extractable with organic solvents (fraction A, Fig. 1 and Table II) after hydrazine treatment, and nearly half was neutral; the other

TABLE II

RADIOACTIVITIES IN VARIOUS FRACTIONS AFTER ALKALI TREATMENT FOLLOWING REACTION WITH HYDRAZINE[a]

Degraded fraction[b]	Peptide fraction: Pepsin-insoluble large-granular (X)	Pepsin-soluble large-granular (Y)	Pepsin-treated soluble (Z)
A	1050 c.p.m.	500 c.p.m.	660 c.p.m.
B	51	70	58.5
C	48	70	47
D	51	31	50
E	11	0	14
F	42	31	29

[a] All radioactivities for fractions B to F are expressed as percentage recoveries of the starting radioactivity in the corresponding parent fraction, A. [b] For explanation of the symbols, see Fig. 1.

half was acidic. A PDA carrier experiment was carried out directly on the acidic fraction. The neutral fraction was hydrolyzed with alkali and a PDA carrier experiment was done on the fraction of radioactivity rendered acidic by hydrolysis; in the case of peptide fractions X and Z, 20–30% of the radioactivity remained neutral after hydrolysis (Fig. 1 and Table II). The results of the PDA carrier experiments are described in Table III. Varying amounts of PDA were detected in both the acidic (B) and the hydrolyzed neutral (F) fractions derived from X, Y and Z. The PDA in the acidic fractions could have arisen due to a partial hydrolysis of a neutral precursor during extraction with alkali. The sum, PDA in B plus PDA in F, for the peptide fractions, X, Y and Z, was 3.2, 1.4 and 1.5% of the *total* bound readioactivity, in comparison to 3.8, 1.1 and 1.7% obtained on direct alkaline hydrolysis.[10]

These observations made it necessary to synthesize the dihydrazide and the cyclic hydrazide of PDA, in order to test them as carriers in fraction A and determine their behavior toward aqueous alkali. The most straightforward methods for the synthesis of these compounds (X and VI, Fig. 2), appeared to be the treatment of the dimethyl ester IX and the imide V of PDA, respectively, with hydrazine, by analogy to the preparation of the dihydrazide of diphenic acid[15] and the cyclic hydrazide of phthalic acid.[11] Attempts were unsuccessful to prepare V by treatment of PDA with methyl cyanide following the method of Mathews,[18] or by heating the ammonium salt of PDA according to the method of Wegerhoff,[19] for the preparation of diphenimide. Stephenson[20] had earlier obtained V by vacuum sublimation of a mixture of the isomeric monoacid-monoamides of PDA obtained as a by-product during the Schmidt reaction on 1,2,5,6-dibenz-3,4-anthraquinone. We obtained a similar mixture (III) by treating the anhydride II with alcoholic ammonia. Vacuum sublimation of III or the mixture of monomethyl ester-monoamides IV obtained by esterification of III, gave V in good yield. The treatment of the imide V with hydrazine, however, yielded the *dihydrazide* X, which was also obtained from the dimethyl ester IX and the diamide VIII by treatment with hydrazine and hydrazine hydrate, respectively. The dichloride VII gave VIII on treatment with anhydrous ammonia in ether. On the other hand, the *cyclic hydrazide* VI was produced from VII by treatment with hydrazine hydrate; under some conditions, X could also be obtained from VII.

The carrier experiments (Table III) with the hydrazides X and VI on fraction A from peptide fractions X, Y and Z, revealed that in each case, half the radioactivity in A was due to the dihydrazide X and the other half due to the cyclic hydrazide VI. Furthermore, X was found to be easily soluble in dilute sodium hydroxide (apparently accompanied by considerable decomposition, as indicated by the lack of purity of the product obtained on acidifica-

(18) J. A. Mathews, THIS JOURNAL, **20**, 648 (1898).
(19) P. Wegerhoff, *Ann.*, **252**, 1 (1889).
(20) E. A. M. Stephenson, *J. Chem. Soc.*, 2620 (1949).

TABLE III

CARRIER EXPERIMENTS

Starting peptide fraction[a]	Degraded fraction on which carrier expt. carried out,[b] c.p.m.	Carrier,[c] mg.	Specific activities recorded,[d] c.p.m./mg.	Mean constant sp. act., c.p.m./mg.	Carrier in the degraded fraction, %
X	C (373)	PDA (15.40)	12(1S), 8(1R), 5(1R), 2(1S)	0	0
X	F (331)	PDA (13.40)	13(1S), 12(1R), 12(1S), 12(1R)	12	49
Y	C (141)	PDA (5.40)	5(S), 5(1R), 5(1S)	5	19
Y	F (121)	PDA (11.72)	8(1S), 7.5(2R), 8(1S)	8	77
Z	C (211)	PDA (8.69)	8(1S), 5.5(1R), 7(1S)	7	28
Z	F (161)	PDA (14.34)	7(1S), 7(2R), 7(1S)	7	62
X	A (3290)	Di Hyd (14.53)	129(1R), 138(1R), 157(2R), 150(2R)	148	65
X	A (3290)	Cy Hyd (18.70)	94(1R), 87(2R), 83(2R), 81(1R)	83	47
Y	A (1360)	Di Hyd (16.26)	41(1R), 46(1R), 52(2R), 49(2R)	49	58
Y	A (1360)	Cy Hyd (16.40)	34(2R), 32(2R), 32(1R)	33	40
Z	A (1210)	Di Hyd (15.50)	40(1R), 40(1R), 41(2R), 42(2R)	42	52.5
Z	A (1210)	Cy Hyd (14.00)	35(2R), 33(2R), 34(1R)	34	39
X	G (1245)	PDA (20.0)	60(1R), 54(1S), 63(1R), 61(1R)	59.5	96

[a] X, Y and Z represent the same peptide fractions as in Table I. [b] C, F and A denote the same fractions as in Fig. 1; G denotes the acidic fraction obtained on treating X with PCl_5 followed by reduction and hydrolysis. [c] PDA = 2-phenylphenanthrene-3,2′-dicarboxylic acid; Di Hyd = dihydrazide of PDA; Cy Hyd = cyclic hydrazide of PDA. [d] The figures and the letters in parentheses denote the number of recrystallizations (R) or sublimations (S) after which the sp. act. was determined.

tion), which accounts for the extractability of half the radioactivity in A by alkali. The presence of a small amount of PDA in the acidic fractions C derived from X and Z, was probably due to a partial hydrolysis of the dihydrazide. By contrast, VI was found to be very resistant to hydrolysis (see

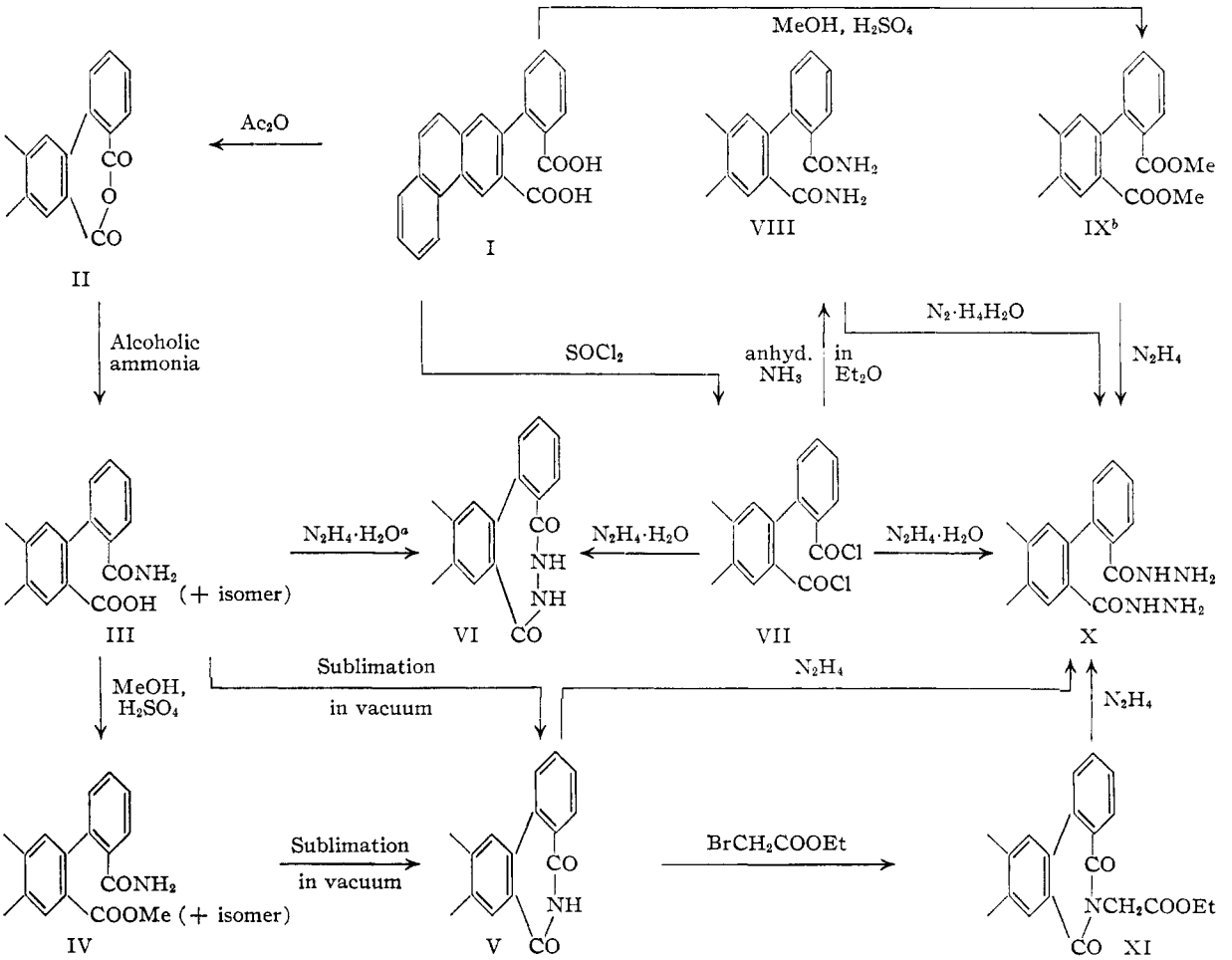
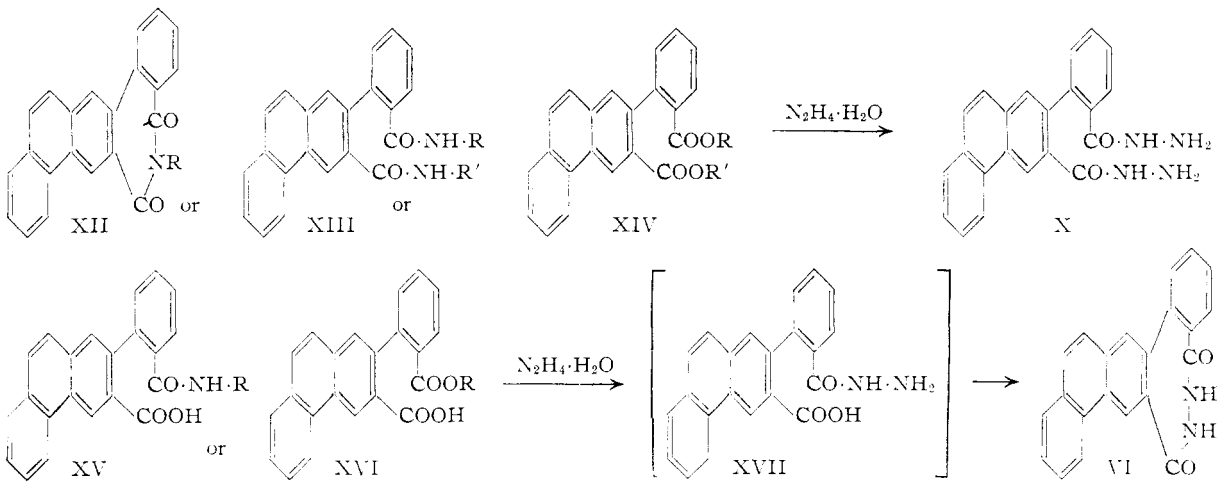


Fig. 2.—Preparation of the derivatives of 2-phenylphenanthracene-3,2′-dicarboxylic acid (PDA). [a] The major product in this reaction was a mixture of monohydrazides of PDA. [b] The dimethyl ester of PDA also gave a mixture of isomeric monoamides (III) on treatment with alcoholic ammonia.



R and R′ represent peptide chains; R may be same as R′. In structures XV and XVI, –COOH may be in the 2′-position, and –CO·NH·R or –COOR in the 3-position, in which case the positions of –COOH and –CO·NH·NH₂ in XVII will also be reversed.

Experimental section). This might account for the incomplete hydrolysis on alkali treatment of the neutral part of A (fraction D) derived from X or Z; moreover, the hydrolysis could have been accompanied by some decomposition, which would explain the non-quantitative yields of PDA in fraction F (see Fig. 1 and Tables II and III.)

In order to determine whether a N-substituted imide or amide of PDA would also give the dihydrazide X, N-carbethoxyethyl-2-phenylphenanthrene-3,2′-dicarboximide (XI) was synthesized; on treatment with hydrazine, it yielded X. Thus, it seemed probable that the formation of X by treatment of the various peptide fractions with hydrazine hydrate was due to binding of PDA to the protein(s) through an imide (XII) and/or a diamide (XIII) and/or a diester (XIV) type of linkage. The possibility of a thioester linkage appears to be ruled out by earlier, negative Raney nickel desulfuration reactions. The formation of the cyclic hydrazide VI could be due to a monoamide XV or monoester XVI linkage to the protein, with the intermediate formation of a monohydrazide, as shown below. Some evidence for this hypothesis is the analogous case of the conversion of the monohydrazide of diphenic acid to the cyclic hydrazide under very mild conditions.[15] To confirm this scheme, a mixture of the monoacid-monoamides III of PDA was treated with hydrazine hydrate, and a small amount of the cyclic hydrazide VI was obtained, presumably through the intermediate formation of a mixture of XVII and its 3′-carboxy isomer.

After thus having established that PDA is bound to the protein through a nitrogen (amide or imide) or oxygen (ester) bond, or both, we proceeded to determine which of these was involved and to what extent in the binding. If it were through an ester linkage, treatment of the peptide fractions with lithium aluminum hydride would result in the cleavage of the PDA—protein bond with the formation of an alcohol corresponding to PDA, which would be extractable in organic solvents. However, no radioactivity was extractable when the totally methylated, pepsin-insoluble large-granular peptide fraction was treated with lithium aluminum hydride according to the method of Bailey.[21]

The dimethyl ester IX of PDA on reduction under similar conditions yielded the corresponding dialcohol in good yield; all attempts to esterify PDA with N-acetylserine ethyl ester were unsuccessful. Positive evidence in favor of the amide linkage between PDA and the protein was obtained by carrying out a sequence of reactions indicated by the following scheme,[22] on the peptide fraction X (neither these reactions nor the lithium aluminum hydride reduction were run on the other two peptide fractions, as they had earlier consistently given results similar to those obtained with X)

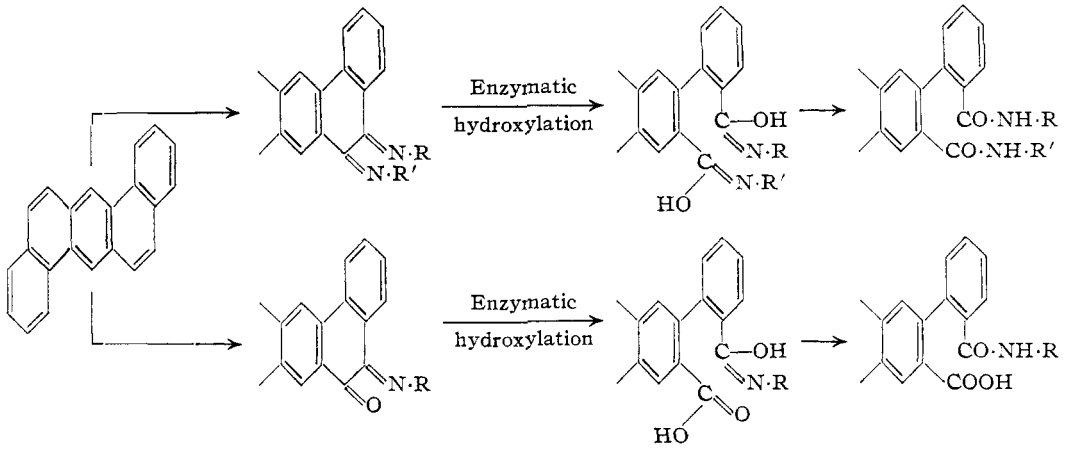
$$\text{Ar·CO·NH·R} \xrightarrow{PCl_5} \text{Ar·CCl=NR} \xrightarrow[HCl]{SnCl_2}$$

$$\text{Ar·CH=NR} \xrightarrow{H_2O} \text{Ar·CHO} + \text{R·NH}_2$$

If the binding involved an amide linkage, one would expect to obtain as the final product of these reactions, a mono- and/or dialdehyde of PDA, which would be extractable in organic solvents. If, however, the initial binding were through an imide or an ester linkage, cleavage of the PDA—protein bond would not be possible, and even though an extensive alteration might take place in the structure of the protein no radioactivity would be rendered soluble in organic solvents; 20% of the radioactivity became extractable following the above-mentioned treatment of the peptide fraction X. Half of this radioactivity was acidic and the other half neutral. The acidic radioactivity was shown by carrier technique to consist entirely of PDA, which was presumably obtained by the oxidation of a monoaldehyde corresponding to PDA, derived from a PDA–protein complex of structure XV. The neutral radioactivity was probably due to the dialdehyde, obtained from a PDA–protein complex of structure XIII. The dimethyl ester (IX) of PDA was recovered unchanged when subjected to successive treatments with phosphorus

(21) J. L. Bailey, *Biochem. J.*, **60**, 170 (1955).

(22) R. C. Fuson, "Advanced Organic Chemistry," John Wiley and Sons, Inc., New York, N. Y., 1951, p. 147.



pentachloride, stannous chloride in ethereal hydrochloric acid, and water.

On the basis of the above evidence, it appears that all radioactivity which is extractable with ethyl acetate after hydrazine treatment (nearly 25% for each of the peptide fractions, X, Y and Z, and therefore, about 25% of the *total* bound radioactivity), involves the binding of PDA to the mouse skin protein through an amide (both mono and di) linkage. It is possible that the initial binding *in vivo* might involve the intact DBA ring system as indicated below; no direct evidence for this is yet available.

Our scheme is in accordance with the theoretical speculations of the Pullmans[23] with respect to a quinonoid bond between the hydrocarbon and the tissue. It should be emphasized that the PDA–protein complex represents only about 25% of the total bound radioactivity. We are not now in the position of knowing whether this or the other 75% represents the structure of the complex directly concerned in the initiation of cancer. Work is continuing along these lines, aimed at the eventual elucidation of the chemistry and biochemistry of the carcinogenic process.

Experimental

All m.p.'s are uncorrected. Microanalyses are by Drs. Weiler and Strauss, Oxford, England. 2-Phenylphenanthrene-3,2′-dicarboxylic acid (I, PDA) and its dimethyl ester IX were prepared as described previously.[10]

Synthetic Work (Fig. 2)

2-Phenylphenanthrene-3,2′-dicarboxylic Anhydride (II).—The acid I (400 mg.) was refluxed for 8 hr. with acetic anhydride (4 ml.) and the excess anhydride was removed by distillation or by evaporation at 100° under nitrogen. The crude product was recrystallized once from acetone for subsequent use; yield 70–80%. Two more crystallizations gave colorless needles, m.p. 257–259°.

Mixture of the Isomeric Monoamides of 2-Phenylphenanthrene-3,2′-dicarboxylic Acid (III).—The anhydride of PDA (230 mg.) was covered with 25 ml. of either alcohol saturated with ammonia, or a mixture of equal volumes of absolute alcohol and concentrated ammonium hydroxide, and left at room temperature for 24 hr., when the anhydride dissolved to give a reddish-yellow solution. Alcohol and excess ammonia were removed by evaporation at 100°, and the residue was extracted with 10% sodium bicarbonate. Acidification yielded a white solid, m.p. 150–160°, yield 90–95%, which could not be further purified. *Anal.* Calcd. for $C_{22}H_{17}O_3N$: N, 4.05; neut. equiv., 344. Found: N, 3.84; neut. equiv., 360.

(23) A. Pullman and B. Pullman, *Advances in Cancer Research*, **3**, 117 (1955).

Mixture of the Isomeric Monomethyl Ester-monoamides of 2-Phenylphenanthrene-3,2′-dicarboxylic Acid (IV). (a).—The above mixture (III, 188 mg.) was esterified by refluxing with 20 ml. of methyl alcohol and 1 ml. of concentrated sulfuric acid, for 4 hr. Most of the alcohol was then distilled, and 5 ml. of water was added, followed by 10% sodium bicarbonate until the solution was alkaline. The mixture of monomethyl ester-monoamides of PDA was thus obtained as a white solid, m.p. 85–100°. It was not separated into its constituents.

(b) A mixture, IV, was also obtained by heating the dimethyl ester of PDA (IX, 94 mg.) with 5 ml. of 2:1 concentrated ammonia:alcohol in a sealed tube at 150° for 48 hr. The clear, yellow solution obtained after centrifugation and decantation was evaporated, and the resulting dark, viscous oil was crystallized with ether. Two precipitations with ether from an alcoholic solution gave m.p. 100–170°.

2-Phenylphenanthrene-3,2′-dicarboximide (V).—The mixture (100–200 mg.) of isomeric monoamides III or monomethyl ester-monoamides IV was sublimed at 0.3–0.4 mm., at 220–250° (sand-bath temperature). The sublimate was washed twice with 2 ml. of benzene. It was then recrystallized 1–3 times from *n*-butyl alcohol to give colorless needles, m.p. 244–244.5° (Stephenson[20] reported m.p. 239–240°).

N-Carbethoxyethyl-2-phenylphenanthrene-3,2′-dicarboximide (XI).—The imide of PDA (V, 90 mg.), anhydrous potassium carbonate (200 mg.) and ethyl bromoacetate (1.2 ml.) were refluxed for 8 hr. The mixture was evaporated to dryness and the product extracted with water. The insoluble residue was recrystallized twice from 90% ethanol to give colorless plates, m.p. 175.5°, yield 60 mg. *Anal.* Calcd. for $C_{26}H_{23}O_4N$: C, 76.1; H, 4.9; N, 3.4. Found: C, 76.1; H, 4.9; N, 3.3.

2-Phenylphenanthrene-3,2′-dicarboxyl Chloride (VII).—PDA (400 mg.) was refluxed with thionyl chloride (5 ml.) for 6 hr., and the excess thionyl chloride was removed first by distillation at room temperature and then in a vacuum desiccator. The crude product was used for all subsequent reactions. Treatment of the acid chloride with concentrated ammonium hydroxide at room temperature for 24 hr. yielded a yellow, crystalline compound, m.p. 352–353°, after two recrystallizations from dioxane. This on refluxing with an excess of hydrazine (95%) for 6 hr. and evaporation, gave colorless needles, m.p. 311–313°, after two recrystallizations from alcohol. Neither of these products was characterized.

2-Phenylphenanthrene-3,2′-dicarboxamide (VIII).—The acid chloride obtained from 400 mg. of PDA was suspended in 75 ml. of dry ether and stirred. Anhydrous ammonia was bubbled through the suspension for 3 hr., after which the mixture was left overnight. Following filtration and washing with water a colorless product, insoluble in sodium bicarbonate, was obtained; yield 80%. Three recrystallizations, the first from benzene and the others from benzene–alcohol, gave needles, m.p. 271.5–272.5°. *Anal.* Calcd. for $C_{22}H_{16}O_2N_2$: N, 8.2. Found: N, 8.0.

Dihydrazide of 2-Phenylphenanthrene-3,2′-dicarboxylic Acid (X). (a).—The dimethyl ester of PDA (IX, 410 mg.) was refluxed with hydrazine (95%, 4.5 ml.) for 6 hr. Most

of the hydrazine was then evaporated on a steam-bath under nitrogen. Water was added to the residue after cooling and the pale yellow solid obtained was filtered; m.p. 156–165°, yield 75–80%. Several (6–8) precipitations with petroleum ether from an ethyl acetate, benzene or alcohol solution (decolorized with Norit), and recrystallizations from hot benzene gave a white solid, m.p. 149–151°. *Anal.* Calcd. for $C_{22}H_{18}O_2N_4$: C, 71.3; H, 4.9. Found: C, 71.4; H, 5.1. This compound exists in another form, melting at 166–168°, which was sometimes obtained during purification and was converted to the lower melting form during subsequent steps.

(b)—The dihydrazide X was also obtained when V (67 mg.) or XI (15 mg.) was treated with excess hydrazine in the above manner, the yields being 75 and 90%, respectively; m.p. and mixed m.p. with the product obtained in a, 149–151°. The crude products were purer than in a.

(c).—The diamide VIII (156 mg.) was refluxed with 4 ml. of hydrazine hydrate (85% in water) for 3 hr. On cooling the yellow solution, a precipitate was obtained; most of the liquid was then evaporated under nitrogen and 10 ml. of 0.5 *N* hydrochloric acid was added. The solution was filtered, made alkaline with sodium bicarbonate and filtered again. Two precipitations with petroleum ether from a decolorized (Norit) ethyl acetate solution of the residue, gave the pure dihydrazide X, m.p. and mixed m.p. with the product obtained in a, 149–151°, yield 58%.

Cyclic Hydrazide of 2-Phenylphenanthrene-3,2′-dicarboxylic Acid (VI).—The acid chloride VII, prepared from 400 mg. of PDA, was warmed for 15 min. on a steam-bath with 15 ml. of hydrazine hydrate (85% in water), and then left overnight at room temperature. The yellow product was filtered and washed with water. Three recrystallizations from alcohol yielded yellow granules, m.p. 240–241°. *Anal.*[24] Calcd. for $C_{24}H_{14}O_2N_2$: 3, 78.1; H, 4.2; mol. wt., 338. Found: C, 78.3; H, 4.95; mol. wt. (Rast), 343. The compound is soluble in ethyl acetate and chloroform, and insoluble in ether and aqueous alkali. It was unaltered by heating with 5 *N* sodium hydroxide at 100° for 2 hr. Similar treatment with alcoholic sodium hydroxide, gave the sodium salt from which the cyclic hydrazide was regenerated after treatment with dilute hydrochloric acid.

If the acid chloride was heated with hydrazine hydrate for several hours at 100°, the dihydrazide X was obtained in a fairly pure form and in nearly quantitative yields.

Treatment of the Mixture of Isomeric Monoamides III with Hydrazine.—The mixture (65 mg.) was refluxed with hydrazine hydrate (85% in water, 4 ml.) for 3 hr., cooled, evaporated and dissolved in water; the mixture of the ammonium salts was then acidified with 2 *N* hydrochloric acid. The white precipitate became yellow when kept overnight. It was filtered and extracted with sodium bicarbonate. The bicarbonate soluble fraction yielded a white precipitate on acidification, which was probably a mixture of the two isomeric monohydrazides. The yellow, bicarbonate-insoluble fraction, gave 3 mg. of the cyclic hydrazide of PDA on two recrystallizations from alcohol, m.p. and mixed m.p. 241–242°.

Metabolic Studies

The radioactivity assay techniques and the liquid–liquid extractions were carried out as described previously.[10]

Each of the two major mouse-skin protein fractions, the "large-granular" and the "soluble," were treated with pepsin, the former to give the pepsin-insoluble large-granular (X) and the pepsin-soluble large-granular (Y), and the latter to give the pepsin-treated soluble (Z) peptide fractions.

Treatment of Peptide Fractions with Hydrazine.—The peptide fraction (X, Y or Z; 10,000–20,000 c.p.m.) was refluxed with hydrazine hydrate (85% in water; 4 ml. per 10,000 c.p.m. of Y or Z, 1 ml. per 10,000 c.p.m. of X) and alcohol (8–10 ml. per 10,000 c.p.m.) for 2 hr. The solution was cooled and the solvents were removed first by distillation at atmospheric pressure and then in a vacuum desiccator. The residue was warmed at 50–60° with 3–4 ml. of 2 *N* hydrochloric acid for 5–10 min. It was then extracted exhaustively with ethyl acetate. The solid residue, if any, was then separated from the aqueous phase by centrifugation and was plated. The radioactivities obtained in the various fractions in typical experiments, are given in Table I. The variation in different experiments using the same peptide fraction was negligible.

Alkali Treatment of the Ethyl Acetate Extractable Radioactivity after the Reaction of the Peptide Fractions with Hydrazine.—The radioactivity in the organic phase was extracted with 2% sodium hydroxide (the aqueous layer was checked for alkalinity). Carrier experiments with PDA were carried out on the acidic fraction, and on the neutral fraction after hydrolysis, as described in Fig. 1. The radioactivities obtained in the various fractions (indicated in Fig. 1) from the three peptide fractions, X, Y and Z, are given in Table II. The carrier experiments with PDA are described in Table III; the acid was purified by recrystallization from acetone and sublimation in vacuum.

Carrier Experiments with the Hydrazides of 2-Phenylphenanthrene-3,2′-dicarboxylic Acid.—These were carried out directly on the total radioactive fraction (A in Fig. 1 and Table I) extractable with ethyl acetate after treatment of the three peptide materials with hydrazine hydrate. The dihydrazide was purified by repeated recrystallization to constant specific activity, from benzene, benzene-alcohol or benzene-petroleum ether. The cyclic hydrazide was purified by recrystallization from alcohol, ethyl acetate, chloroform or a suitable mixture of two of these solvents. Attempts to sublime ether hydrazide under vacuum, resulted in decomposition.

Reduction of the Pepsin-soluble Large-granular Peptide Fraction with Lithium Aluminum Hydride.—The peptide fraction (2 ml., 10,700 c.p.m.) was evaporated to dryness in a centrifuge tube at room temperature. Methyl alcohol (5 ml.) and 0.08 ml. of concd. hydrochloric acid were added and a white fluffy precipitate was obtained. The mixture was left at room temperature for 48 hr., and the alcohol and hydrochloric acid were removed, first by evaporation at room temperature and pressure, and then in a vacuum desiccator. Water (2 ml.) was added and the mixture extracted exhaustively with ethyl acetate; 1,370 c.p.m. (13%) were extractable. The aqueous fraction, consisting of the esterified peptides, was evaporated to dryness at room temperature under nitrogen. Dry tetrahydrofuran (1 ml.) was added to the residue, followed by a solution of lithium aluminum hydride (0.41 g.) in ether (4 ml.). A vigorous reaction took place almost immediately, with separation of a white solid. The mixture was left at room temperature overnight and decomposed by the dropwise addition of water (3–4 ml.). The ether layer was removed and the aqueous layer was then extracted with ethyl acetate. Only 37 c.p.m. (0.3%) were found in the combined organic extracts. On acidification of the aqueous fraction and extraction with ethyl acetate, 685 c.p.m. (6.6% of the original activity in the peptide fraction) were extracted. No radioactivity was extracted by ethyl acetate or ether from the aqueous phase made strongly alkaline (so as to dissolve the precipitate first obtained).

Reduction of the Dimethyl Ester of 2-Phenylphenanthrene-3,2′-dicarboxylic Acid with Lithium Aluminum Hydride.—The ester IX (120 mg.) was dissolved in 1 ml. of dry tetrahydrofuran and 3 ml. of a solution of lithium aluminum hydride (75 mg.) in ether was added dropwise. The mixture was stirred overnight at room temperature, water (2 ml.) was added, followed by 1 ml. of 10% sulfuric acid. The mixture was extracted with ether and the ethereal extract washed once with 2 ml. of water. On concentration, white needles of 3,2′-bis-(hydroxymethyl)-2-phenylphenanthrene were deposited, m.p. 163–164°, yield 60 mg. (59%). Further recrystallization from ether did not raise the m.p. *Anal.* Calcd. for $C_{22}H_{18}O_2$: C, 84.03; H, 5.77. Found: C, 83.65; H, 5.74.

Treatment of the Pepsin-insoluble Large-granular Peptide Fraction with Phosphorus Pentachloride and Subsequent Reduction and Hydrolysis.—The peptide fraction (10.3 mg., 14,550 c.p.m.) was refluxed with an excess of phosphorus pentachloride in benzene, for 6 hr., in a 10-ml. centrifuge tube. After standing overnight at room temperature, the volatile components of the mixture were removed first under nitrogen, then in a vacuum desiccator. A solution (3 ml.) of stannous chloride in ethereal hydrochloric acid, prepared according to the method of Stephen,[25] was then added and the mixture let stand overnight with occasional shaking. The ether and hydrochloric acid were removed under nitrogen, and the residue was warmed with

(24) The carbon and hydrogen analyses are by Chemistry Department, University of Wisconsin. The molecular weight determination is by the authors.

(25) H. Stephen, *J. Chem. Soc.*, **127**, 1874 (1925).

3 ml. of water at 50–60° for 2 hr. The aqueous mixture was then extracted exhaustively with ethyl acetate, to give 2,900 c.p.m. (20%) in the organic phase. On extraction with 2% sodium hydroxide, half of this radioactivity was found to be acidic and the other half neutral. The acid fraction (G) was shown to consist almost entirely of PDA, by a carrier experiment (Table III).

The Above Treatment of the Dimethyl Ester of 2-Phenylphenanthrene-3,2′-dicarboxylic Acid.—The ester IX (50 mg.) was treated with phosphorus pentachloride (500 mg.), a solution (4 ml.) of stannous chloride in ethereal hydrochloric acid, and then water, as above. On filtration of the aqueous mixture, a yellowish-red solid was obtained. This was dried and extracted with alcohol. On evaporation of the alcoholic extract, 40 mg. (80%) of the unchanged ester was recovered.

MADISON, WISCONSIN

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

The Synthesis of Substituted Penicillins and Simpler Structural Analogs. IX. 4-Carboxy-5,5-dimethyl-α-phthalimido-2-thiazolidineacetic Acid Derivatives

BY JOHN C. SHEEHAN AND PHILIP A. CRUICKSHANK

RECEIVED JUNE 28, 1955

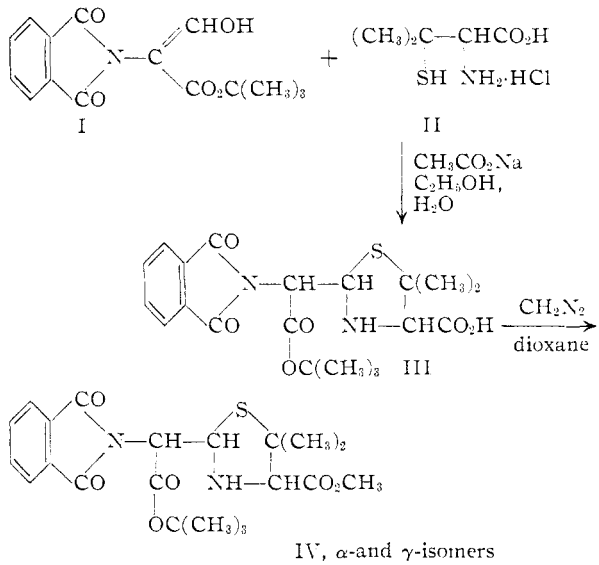
t-Butyl 4-carbomethoxy-5,5-dimethyl-α-phthalimido-2-thiazolidineacetate has been isolated in two stereoisomeric modifications (two racemates), one of which was shown to correspond in configuration to the natural penicilloates. Removal of the phthaloyl blocking afforded an aminothiazolidine valuable as an intermediate for the synthesis of a variety of penicilloate derivatives. Cleavage of the *t*-butyl ester group gave three isomeric thiazolidineacetic acids, of interest as precursors for fused thiazolidine-β-lactams closely related to the penicillins.

In a preceding article of this series[1] there was described the preparation of penicilloate derivatives, the structure of which precludes the possibility of azlactone formation under conditions designed to effect closure of the β-lactam ring. This was accomplished by the incorporation of the phthaloyl blocking group.

Among the compounds described was *t*-butyl 4-carbomethoxy-5,5-dimethyl-α-phthalimido-2-thiazolidineacetate (IV). This compound has now been isolated in two stereoisomeric modifications (racemates), one of which has been shown to correspond to the configuration of the natural penicilloates. By removal of the phthaloyl blocking group there has been obtained an intermediate, *t*-butyl 4-carbomethoxy-5,5-dimethyl-α-amino-2-thiazolidineacetate hydrochloride (V), invaluable for the preparation of many otherwise difficultly accessible penicilloate derivatives. Cleavage of the *t*-butyl ester group of IV has afforded three stereoisomeric modifications of 4-carbomethoxy-5,5-dimethyl-α-phthalimido-2-thiazolidineacetic acid hydrochloride (VI).

The interaction of DL-penicillamine hydrochloride (II) and *t*-butyl phthalimidomalonaldehydate (I) in sodium acetate buffered aqueous ethanol afforded directly the crystalline thiazolidine III, *t*-butyl 4-carboxy-5,5-dimethyl-α-phthalimido-2-thiazolidineacetate, in 75% yield. Treatment of this with diazomethane gave two methyl esters, m.p. 121–122° and m.p. 176–176.5°. These were shown to be stereoisomers of *t*-butyl 4-carbomethoxy-5,5-dimethyl-α-phthalimido-2-thiazolidineacetate (IV) by the identity of their infrared spectra in the region of 2–7 μ. The preponderant lower melting form, the sole product upon esterification of the first crop of III, was designated as the γ-isomer.[2] The higher melting form was shown to be a racemate corresponding in configuration to the natural penicilloates, and so was designated alpha. Of the four theoretically possible racemates of IV, apparently only these two were formed in significant amounts.

The more interesting α-isomer of IV was obtained in rather low yields from the products of the condensation of I and II. Additional quantities could



IV, α-and γ-isomers

be prepared, however, by heating a triethylamine solution of IVγ under reflux, setting up an equilibrium consisting of two parts in five of IVα. The latter crystallized directly in an essentially pure state upon cooling the mixture. The unchanged IVγ could then be isolated from the mother liquors, or the solution again heated under reflux to give additional α-isomer.

One of the principal difficulties encountered in the preparation of α-amino-2-thiazolidineacetate derivatives of penicillamine is the synthesis of the required derivatives of aminomalonaldehydic esters. The facile liberation of amino groups from

(1) J. C. Sheehan and D. A. Johnson, THIS JOURNAL, **76**, 158 (1954).

(2) In analogy to the designation gamma for the first isomer obtained in the condensation of benzylpenaldate and penicillamine; H. T. Clarke, J. R. Johnson and R. Robinson, editors, "The Chemistry of Penicillin," Princeton University Press, Princeton, N. J., 1949, p. 535.