Conclusions.—The examples discussed suggest that geometrical factors may play a greater part in determining the absorption spectra of substances containing aryl substituents than has been realized heretofore. In solution, the molecules are oriented randomly toward the electric vector of the light wave and the study of absorption spectra of oriented layers of molecules would help considerably to clarify this subject if suitable methods of measurement could be devised.

Acknowledgments.—The author wishes to express his appreciation of the encouragement given by Professor L. F. Fieser, and to thank the International Cancer Research Foundation for financial support. Thanks are also due to Dr. D. M. Bowen who measured the spectra of the dihydrophenanthrene compounds and to Dr. R. B. Woodward who has checked the calculations of the 9,10-dihydrophenanthrene rotation.

Summary

Ultraviolet absorption spectra measurements on derivatives of biphenyl in which the rings are constrained in a coplanar form by alicyclic bridge linkages between the 2,2′ and 6,6′ positions support the argument that a coplanar structure is necessary for the development of the typical biphenyl spectrum. From this it may be inferred that conjugated aromatic chromophores contribute additively to the total absorption when prevented from acquiring a coplanar configuration.

On the basis of this assumption, several examples of anomalous absorption spectra in the *meso*-arylanthracene series can be explained satisfactorily.

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The Conjugation of Horse Serum Albumin with Isocyanates of Certain Polynuclear Aromatic Hydrocarbons

BY HUGH J. CREECH AND R. NORMAN JONES

In an earlier paper,[1] a description was given of four conjugates prepared from horse serum albumin and 1,2-benzanthryl-3-isocyanate (I) and 1,2-benzanthryl-10-isocyanate (II). It was demonstrated that chemical combination of the components had occurred in an aqueous dioxane medium, and the extent of conjugation was determined by ultraviolet spectrophotometry. As prepared under the experimental conditions specified, the 1,2-benzanthryl-10-carbamido horse serum albumins contained twelve hydrocarbon prosthetic groups per molecule, and one preparation of the 1,2-benzanthryl-3-conjugate contained eighteen hydrocarbon groups per molecule. After preliminary removal of adsorbed isocyanate, the conjugates retained a constant benzanthryl content following repeated and different chemical treatments. By adding unreactive aminobenzanthracene to the albumin solution under the conditions used in the formation of the conjugates, it was shown that the methods of purification completely removed the adsorbed hydrocarbon derivative from the protein.

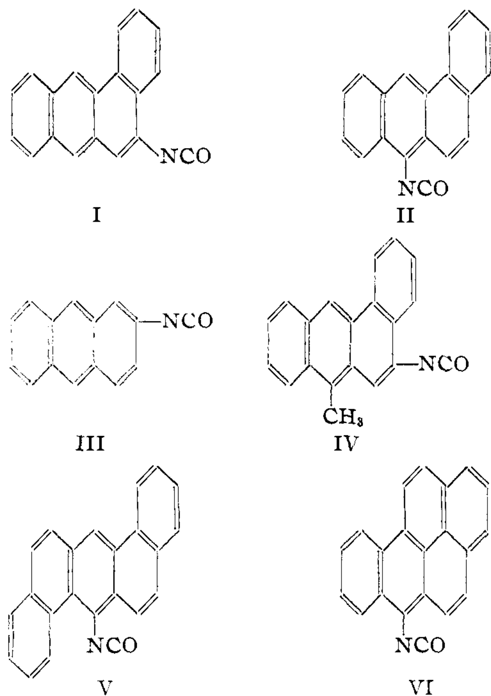
This work has been repeated and extended to the preparation of horse serum albumin conjugates formed from β-anthryl isocyanate (III),[2] 10-methyl-1,2-benzanthryl-3-isocyanate (IV),[3] 1,2,5,6-dibenzanthryl-9-isocyanate (V)[4] and 3,4-benzpyrenyl-5-isocyanate (VI).[3] It appears that the extent of introduction of hydrocarbon radical into the protein molecule at a given *p*H is dependent upon the amount of dioxane used in the reaction mixture and the solubility of the particular hydrocarbon isocyanate in dioxane. Under practically identical conditions, it was found that the more soluble isocyanates I, II and IV were coupled with serum albumin to a greater extent than the less soluble isocyanates III, V and VI. It was also found that the process of conjugation under a given set of experimental conditions could be repeated readily without any appreciable variation in the degree of coupling. The conjugates are soluble in distilled water and the solutions usually are optically clear at a protein concentration of 2%. The conjugates, however, are

(1) Creech and Jones, THIS JOURNAL, **62**, 1970 (1940).

(2) Fieser and Creech, *ibid.*, **61**, 3502 (1939).

(3) Creech, *ibid.*, **63**, 576 (1941).

(4) Creech and Franks. *ibid.*, **60**, 127 (1938).



less soluble than serum albumin and are precipitated at lower levels of ammonium sulfate concentration. Minor changes, in addition to the alteration caused by the introduction of the hydrophobic hydrocarbon groups, undoubtedly occur under the preparative conditions, but there is no gross evidence that the protein component of the conjugates has undergone any significant degree of denaturation. Horse serum albumin which had been treated with unreactive hydrocarbon amines under the same conditions used in the preparation of the conjugates did not seem to have suffered any obvious change in properties.

Since one object behind the synthesis of the conjugates was a study of their immunological behavior,[5] the coupling process was carried out under the mildest possible experimental conditions consistent with the introduction of a sufficient number of prosthetic groups to form conjugates capable of producing specific serological responses. The isocyanates and the conjugates are being tested for carcinogenic activity.[6] Because of the pronounced fluorescence in ultraviolet light exhibited by the conjugates, the procedure of coupling polynuclear hydrocarbon derivatives with proteins may provide a new type of "labeling" useful in immunological and various other investigations.

Experimental Procedure[7]

Preparation of the Conjugates.—Crystalline horse serum albumin prepared according to the method of McMeekin[8] was used in the experiments. The general procedure for the process of conjugation involved the gradual addition of the isocyanate in cold dioxane solution to a well-stirred solution of horse serum albumin containing a quantity of buffer and of dioxane maintained at 0 to 2°. The reaction mixture was diluted with water after two hours and again after four hours of stirring.

The relative proportions of reagents employed in the coupling process are recorded in Table III; the composition of the reaction mixture given in the table is that at the time when all of the isocyanate had been added. In the case of the mixtures containing less than 31% dioxane, the product was set in a refrigerator for twelve to twenty hours, shaken occasionally and then dialyzed. Mixtures containing more than 31% dioxane were dialyzed immediately after the first dilution following two hours of stirring.

The following detailed description is given of the preparation of 1,2-benzanthryl-10-carbamido horse serum albumin no. 5. Purified dioxane[9] (45 cc.) and sodium phosphate buffer (36 cc., 0.1 molar) of *p*H 8.2 to 8.3 were added to a thoroughly dialyzed horse serum albumin solution containing 4.5 g. of protein in 225 cc. of distilled water maintained at 0 to 2°. The 1,2-benzanthryl-10-isocyanate (1.08 g.) in 54 cc. of cold dioxane (colorless solution) was added with continuous stirring over a one and one-half hour period. During the early part of the reaction, the components remained in solution but as the addition of isocyanate was continued a slightly yellow suspension was formed and persisted throughout the coupling process. The reaction mixture was diluted with 120 cc. of water after thirty minutes, and two hours later another portion (240 cc.) was added. The material was set in a refrigerator at 5° for about twenty hours with occasional shaking and then dialyzed at 5° against running tap water for twenty hours and against slowly flowing distilled water for twenty hours to remove the dioxane and phosphate. During the period of dialysis, the suspended material settled leaving a slightly opalescent solution of the conjugate. The preparation was centrifuged and filtered at 2 to 5°. The precipitate after being dried in a desiccator went almost completely into solution in benzene. After two crystallizations from benzene–ligroin, a considerable amount (about 60% of that originally added) of unaltered isocyanate was obtained from the precipitate.

The other preparations were conducted in a similar manner; the specific experimental conditions for the various preparations may be calculated readily from the data in Table III. Experimentally satisfactory solutions of the 3- and 10-isocyanates of 1,2-benzanthracene and of 10-

(5) In coöperation with Dr. F. S. Cheever of the Department of Bacteriology, Harvard Medical School.

(6) Being conducted by Dr. Shields Warren and Dr. Charles E. Dunlap of the Huntington Memorial Hospital, Boston. The 3- and 10-isocyanates of 1,2-benzanthracene have not shown any significant carcinogenic activity whereas 1,2,5,6-dibenzanthryl-9-isocyanate has been found to be slightly more active than the parent hydrocarbon (see reference 3).

(7) Micro Kjeldahl analyses by Lyon Southworth, Miss E. Werble and one of us (H. J. C.).

(8) McMeekin, THIS JOURNAL, **61**, 2884 (1939); **62**, 3393 (1940).

(9) The dioxane was purified by refluxing with sodium for thirty hours followed by distillation.

methyl-1,2-benzanthryl-3-isocyanate were obtained by dissolving 100 mg. of the isocyanate in 5–8 cc. of dioxane whereas the other isocyanates required about 8–12 cc. of dioxane to dissolve the same quantity. In certain cases, this necessitates the use of an initially low concentration of dioxane in the protein solutions which is not a favorable condition. Unfortunately, the simple remedy of diluting the protein solution with water is not expedient because much more satisfactory results are obtained if the protein concentration at the time of maximum dioxane content is maintained in the region of 1% or greater. The subsequent dilutions at the two and four hour period before storage have been found to cause minimum denaturation of the protein component.

With the 45% level of dioxane, more dioxane was used for the solution of the isocyanates but a much greater proportion of dioxane was added initially to the protein solutions. In these instances, it was observed during the addition of the isocyanate that a suspension was formed temporarily between about 25–30% dioxane concentration followed by complete solution of the components as the dioxane concentration was increased to the maximum by addition of the solution of isocyanate. This appears to be brought about by the rapid utilization of the isocyanate in the formation of the dioxane–water soluble conjugate since in the case of the addition of the unreactive benzpyrenylamine under identical conditions, a yellow suspension appeared in the early stages and persisted throughout, presumably because of the insolubility of the amine in the dioxane–water medium.

The conjugation described in detail in the preceding paragraphs represents the largest run made to date. Usually, the experiments were done with 1–3 g. of protein. The buffer of *p*H 10 was prepared from 0.1 *N* sodium carbonate and bicarbonate solutions.

Purification of the Conjugates.—The specific preparation discussed in detail in the preceding section will be used for the description of the method of purification. To the chilled filtrate containing about 4.5 g. of conjugated protein in 850 cc. at *p*H 6.5, there was added with stirring 120 g. of solid ammonium sulfate. Sufficient ammonium sulfate to make the medium 2.8 molar was added over a thirty-hour period to the solution (kept at 0–5°) by the rotating cellophane membrane technique described by McMeekin.[8] The granular precipitate was collected by centrifugation and filtration at a low temperature and was washed with 2.8 *M* ammonium sulfate. It was dissolved in 200 cc. of water and dialyzed at 0–5° for thirty hours against tap water and then against distilled water. The solution was centrifuged and filtered to remove a small amount of insoluble material. The salting-out process was repeated twice; each time, the protein was precipitated from a smaller volume of solution, the final concentration of ammonium sulfate being brought to 2.5 molar. The solution of the conjugate had a faint yellow color and exhibited a pronounced blue fluorescence in ultraviolet light. A sample of the solution was dialyzed thoroughly, the amount of protein per cc. was calculated from micro Kjeldahl analyses and the content of 1,2-benzanthryl radical was determined by ultraviolet absorption spectrophotometry by the described method.[1]

Five volumes of acetone cooled to −15° was added to a dialyzed 2% solution of the conjugate chilled to 0°. After a ten-minute period of centrifugation at 2000 r. p. m. and at 5°, the faintly yellow supernatant was discarded and the colorless precipitate was washed rapidly with acetone. The conjugate was dissolved in about 200 cc. of cold water and dialyzed immediately against distilled water. After twenty hours, the conjugate was precipitated with ammonium sulfate by the described procedure and stored at 5° in this medium. Dialysis of a sample of the conjugate gave an optically clear solution; analyses for the amount of protein and prosthetic group were performed. The amount of conjugate after the above series of purifications was found to be 3.25 g.

With the exception of some of the preparations of 1,2-benzanthryl-3- and 10-methyl-1,2-benzanthryl-3-carbamido serum albumin, adsorbed material was almost completely removed from the conjugates before the acetone treatment. The only precipitates not decolorized by acetone treatment were the 3,4-benzpyrenyl conjugates which remained yellow.[10]

The adsorption of amines on proteins was investigated again with particular note being taken of the characteristics of the preparation from benzpyrenylamine and albumin made at a dioxane concentration of 45%. Dialysis of the deep yellow suspension led to the deposition of about 90% of the amine. After purification by means of ammonium sulfate precipitations, the solutions and filter cakes were only slightly colored; subsequent precipitation with acetone removed the color completely.

Fractionation Experiments.—Part of the 1,2-benzanthryl-10-carbamido horse serum albumin preparation no. 5 which had been obtained within the range of 1.5–2.6 *M* ammonium sulfate was fractionated carefully at 5° and at *p*H 6 by the gradual addition of the salt through a revolving cellophane membrane. Only traces of conjugate were precipitated below 1.7 molar and above 2.4 *M* ammonium sulfate. Approximately three-fifths of the conjugate was precipitated at the 2.0 molar level and about two-fifths of this fraction was reprecipitated at the 1.85 molar level. Observations on the benzanthryl content of the conjugate separating at the various levels are recorded in Table IV.

A preparation of horse serum albumin (235 mg.) soluble within the range 2.15–2.4 *M* ammonium sulfate was mixed with 160 mg. of the fraction of the benzanthryl conjugate soluble within the 1.7 to 1.85 molar range. A sample of the mixture was taken for analysis and the remaining 360 mg. was brought to a volume of 50 cc. in 1.4 *M* ammonium sulfate solution. The mixture was equilibrated at the 1.8, 2.1 and 2.4 molar levels by the usual method and the precipitated material at each level was removed by centrifugation at 3,000 r. p. m. with usual precautions being taken to obtain the most quantitative separation possible. The results (Table V) indicate that reasonably good fractionation was obtained by a single process.

Fractionations of solutions of 1,2-benzanthryl-10-carbamido albumin no. 6 and of 3,4-benzpyrenyl-5-carbamido albumin no. 5 prior to acetone treatment were

(10) The retention of color in this conjugate is not surprising since the parent hydrocarbon and its derivatives, including the isocyanate, are yellow whereas the other isocyanates and parent hydrocarbons are either colorless or practically so; see references 2 and 3.

attempted also. It was observed (Table VI) that the major portion of each preparation was precipitated at 1.1 *M* ammonium sulfate concentration.

Results

Spectrophotometric Analysis.—The basic principles and the method of spectrophotometric analysis were discussed in the earlier publication.[1] The spectra of suitable carbamido derivatives of the respective hydrocarbons were determined; the data on the 1,2-benzanthryl-3- and 10- amino acid conjugates were recorded previously[1,11] and data on the carbamido derivatives of several other hydrocarbons are given in Figs. 1 and 2 and in Table I. It is assumed that the

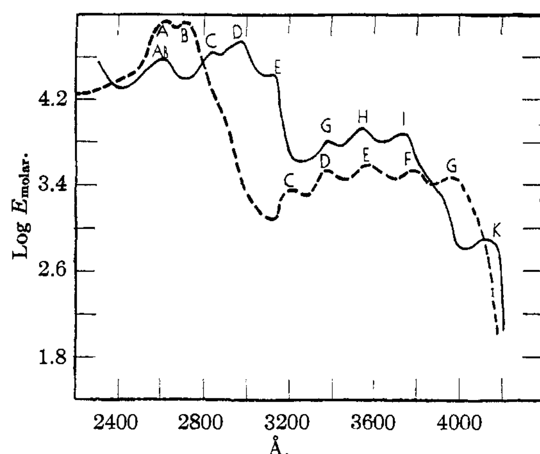


Fig. 1.——— Ethyl ester of 10-methyl-1,2-benzanthryl-3-carbamidoacetic acid (solvent aqueous ethanol); - - - - β-anthrylcarbamidoacetic acid (solvent aqueous ethanol + dioxane).

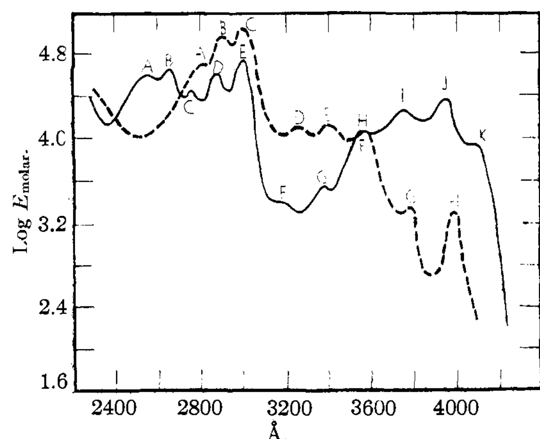


Fig. 2.— —— Ethyl ester of 3,4-benzpyrenyl-5-carbamidoacetic acid (solvent ethanol + dioxane); - - - - ε-(1,2,5,6-dibenzanthryl-9-carbamido)-caproic acid (solvent aqueous ethanol + dioxane).

(11) Jones, THIS JOURNAL, 63, 151 (1941).

factors influencing the intensity of absorption of the hydrocarbon-carbamido grouping are unchanged when this grouping is attached to the protein by an aliphatic linkage. For an accurate interpretation, it is necessary that the solutions of the conjugates be optically clear and that the protein component of the conjugate be transparent at wave lengths greater than 3250 Å.

TABLE I

POSITION (Å.) AND INTENSITY (LOG E_m. AND $E^{1\%}_{1\ cm.}$) OF THE ABSORPTION MAXIMA OF CARBAMIDO DERIVATIVES OF POLYNUCLEAR HYDROCARBONS

Substance	Solvent	Max.	λÅ.	Log E_{molar}	$E^{1\%}_{1\ cm.}$
β-Anthrylcarb-amidoacetic acid	Aqueous ethanol	A	2625	4.91	...
		B	2740	4.89	...
		C	3215	3.37	...
		D	3370	3.59	132
		E	3550	3.61	139
		F	3785	3.58	129
		G	3960	3.46	...
Ethyl ester of 10-methyl-1,2-benz-anthryl-3-carb-amidoacetic acid	Aqueous ethanol + dioxane[b]	AB[a]	2615	4.59	...
		C	2850	4.65	...
		D	2965	4.73	...
		E	3115	4.43	...
		G	3385	3.82	...
		H	3545	3.93	226
		I	3735	3.87	192
		K	4135	2.90	...
ε-(1,2,5,6-Dibenzan-thryl-9-carb-amido)-caproic acid	Aqueous ethanol + dioxane	A	2795	4.69	...
		B	2890	4.96	...
		C	3005	5.03	...
		D	3270	4.13	...
		E	3420	4.15	314
		F	3570	4.09	273
		G	3770	3.34	...
		H	3985	3.30	45
Ethyl ester of 3,4-benzpyrenyl-5-carbamidoacetic acid	Ethanol + dioxane	A	2560	4.61	...
		B	2670	4.65	...
		C	2760	4.43	...
		D	2875	4.60	...
		E	3000	4.75	...
		F	3175	3.39	...
		G	3365	3.52	...
		H	3540	4.05	...
		I	3745	4.35	565
		J	3940	4.39	619
		K	4080	3.94	...

[a] Lettering of maxima as used for 1,2-benzanthracene (see Jones, THIS JOURNAL, **62**, 148 (1940)). [b] The dioxane was purified by the method of Hess and Frahm, *Ber.*, **71**, 2627 (1938).

As in the earlier work with 1,2-benzanthryl-10-carbamido horse serum albumin, the spectra of the protein conjugates showed the characteristic absorption of the hydrocarbon-carbamido group in the near ultraviolet. Representative curves of

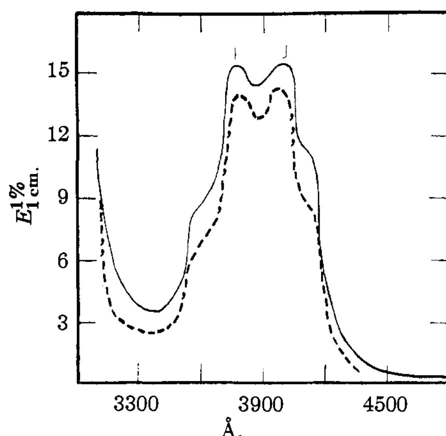


Fig. 3.—3,4-Benzpyrenyl-5-carbamido horse serum albumin no. 1: —— before acetone treatment; - - - - after acetone treatment.

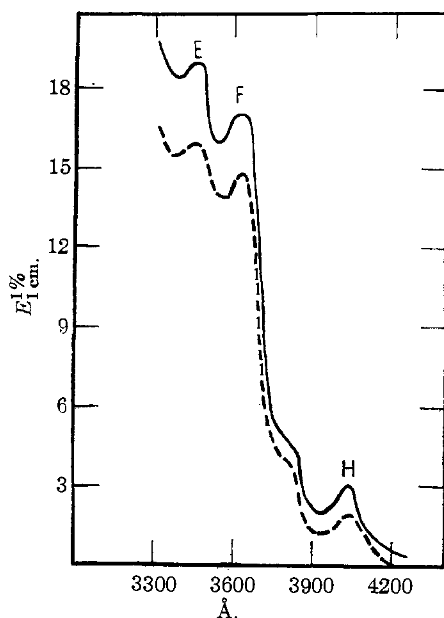


Fig. 4.—1,2,5,6-Dibenzanthryl-9-carbamido horse serum albumin: —— before acetone treatment; - - - - after acetone treatment.

the various conjugated proteins both before and after treatment with acetone are given in Figs. 3–6, the lettered maxima being those chosen as a basis for the calculation of the radical concentration.

In the previous publication[1] the equation[11a]

$$c_2 = \frac{10(\log I_0/I)_{\max.}\, d}{\left(E^{1\%}_{1\text{ cm.}}\right)_{\max.}\, l}\,\frac{1000}{c_1}$$

was derived, wherein c_1 is the concentration of the

(11a) In the equations, 1 to 5, developed in ref. 1, l, the cell length in cm., should have been in the denominator. The calculations given in Table IV of that paper are correct, however, because all measurements were made with a 1-cm. cell.

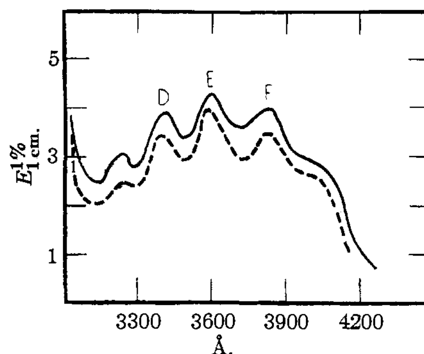


Fig. 5.—β-Anthrylcarbamido horse serum albumin no. 1: —— before acetone treatment; - - - - after acetone treatment.

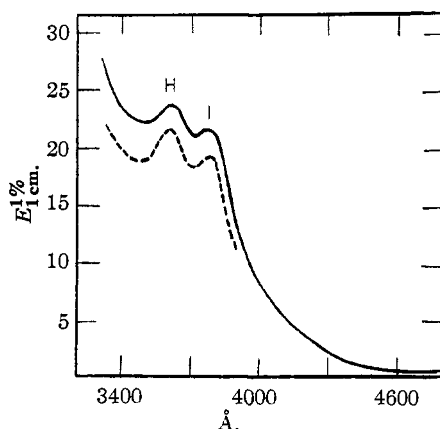


Fig. 6.—10-Methyl-1,2-benzanthryl-3-carbamido horse serum albumin: —— before acetone treatment; - - - - after acetone treatment.

protein in the initial solution in mg. per cc., $(\log I_0/I)_{\max.}$ the absorption coefficient at a maximum, c_2 the concentration of the absorbing group calculated as mg. of 1,2-benzanthryl-carbamido acetic acid per g. of protein, d the dilution factor, l the cell length in cm., and $(E^{1\%}_{1\text{ cm.}})_{\max.}$ the value of $E^{1\%}_{1\text{ cm.}}$ at the corresponding maximum of the spectrum of 1,2-benzanthryl-10-carbamidoacetic acid.

This equation may be generalized conveniently as follows. Let c_3 be the concentration of the absorbing group calculated as hydrocarbon radical, m_1 the molecular weight of the radical and m_2 the molecular weight of the carbamido reference compound.

$$c_3 = \frac{m_1}{m_2}\,c_2$$

and on substituting for c_2

$$c_3 = \frac{10(\log I_0/I)_{\max.}\, d}{\left(E^{1\%}_{1\text{ cm.}}\right)_{\max.}\, l}\,\frac{1000}{c_1}\,\frac{m_1}{m_2}$$

Substituting $100N/16$ for c_1 (where N is the nitro-

TABLE II

Radical	Carbamido derivative	m_1	m_2	Max.	$E'_{max.}$
β-Anthryl-	β-Anthryl-carbamidoacetic acid	177	294	D	219
				E	230
				F	215
10-Methyl-1,2-benzanthryl-3-	Ethyl ester of 10-methyl-1,2-benzanthryl-3-carbamido-acetic acid	241	386	H	362
				I	308
3,4-Benzpyrenyl-5-	Ethyl ester of 3,4-benzpyrenyl-5-carbamidoacetic acid	251	396	I	890
				J	975
1,2,5,6-Dibenzanthryl-9-	ε-(1,2,5,6-Dibenzanthryl-9-carbamido)-caproic acid	277	450	E	510
				F	443
				H	74
1,2-Benzanthryl-10-	1,2-Benzanthryl-10-carbamidoacetic acid	227	344	G	265
				H	303
				I	268
1,2-Benzanthryl-3-	1,2-Benzanthryl-3-carbamidoacetic acid	227	344	H	321
				I	258

gen content of the initial solution in mg. per cc. and 16% is the figure for the nitrogen content of horse serum albumin), replacing $(E^{1\%}_{1\text{ cm.}})_{max.}$ (m_2/m_1) by $E'_{max.}$ and collecting terms

$$c_3 = \frac{1600\ (\log I_0/I)_{max.}\ d}{E'_{max.} N\ l} \text{ mg. of hydrocarbon radical per g. of protein.}$$

The values of m_1, m_2 and $E'_{max.}$ for the various series of conjugates are summarized in Table II.

In the determination of the absorption coefficients, it was necessary in certain cases to dilute the solutions five to ten times and it was assumed in the calculations that the solutions obeyed Beer's law. To check this, representative solutions of 3,4-benzpyrenyl-5-carbamido albumin and 10-methyl-1,2-benzanthryl-3-carbamido albumin were measured in a 1-cm. cell and again, after fourfold dilution, in a 4-cm. cell. The curves fit closely and there is no reason to believe that any significant errors are introduced on account of deviations from Beer's law.

The values for the amount of hydrocarbon radical in the conjugates also may be checked rapidly from the graphs; for example, from Fig. 5, the $E^{1\%}_{1\text{ cm.}}$ values for β-anthrylcarbamido horse serum albumin at maximum D are 3.9 and 3.5 (before and after acetone treatment) which when divided by the $E'_{max.}$ value (219) for the β-anthryl radical at maximum D (Table II) gives values of 0.178 and 0.16 g. of radical per g. of protein. In the cases of 1,2-benzanthryl-3-carbamido albumin nos. 1 and 2 and 10-methyl-1,2-benzanthryl-3-carbamido albumin, the spectrophotometric measurements were made on slightly opalescent solutions and thus required the application of a small correction factor due to the scattering of incident light which reduced the hydrocarbon radical content by 5–8%. Corrected values for the content of hydrocarbon radical in these three conjugates are given in Table III. A discussion of the determination of the correction factor is given in another publication.[12]

Properties of the Conjugates.—The experimental conditions and observations on the hydrocarbon content of the conjugates are recorded in Table III. The values given for the amount of combined prosthetic groups in the conjugates were determined after two or three precipitations with ammonium sulfate and again after one precipitation with acetone. In the previous article,[1] it was demonstrated in the case of 1,2-benzanthryl-10-carbamido albumin that the benzanthryl content remained constant after one or two precipitations with ammonium sulfate and the consequent purification involved in the procedure. It was also shown when the ammonium sulfate precipitations were not sufficient for the removal of all of the adsorbed material, as was the case with the 1,2-benzanthryl-3-carbamido albumin, that only one precipitation with acetone was necessary for complete purification. It is evident that the acetone precipitations removed the last traces of adsorbed hydrocarbon derivatives but the only appreciable reductions in the values of the prosthetic group content occurred with some of the conjugates from the 3-substituted benzanthracenes in which instances the solutions after ammonium sulfate purifications were still slightly opalescent and brownish-yellow in color. It will be observed that although the major portion of the added isocyanate failed to react with the

(12) Creech and Jones, THIS JOURNAL, **63**, 1670 (1941).

TABLE III

No.	Conjugate	Compn. of reaction mixture: Mg. of isocyanate per g. of protein	% dioxane	pH	Protein, mg./cc.	Hycb.: protein ratio[a] $(NH_4)_2SO_4$ P. mg.	$(NH_4)_2SO_4$ P. groups	Acetone P. mg.	Acetone P. groups
1	10-HSA.	350	15	8.2	14	18	5–6	..	...
2	10-HSA.	230	25	8.2	18	39	12	36	11
3	10-HSA.	250	25	8.3	11	43	13	37	11–12
4	10-HSA.	250	25	8.0	11	35	11	31	9–10
5	10-HSA.	240	27.5	8.3	12	47	14–15	46	14
6	10-HSA.	200	45	10	16	98	30	98	30
1	3-HSA.	165	25	8.2	11	39	12	25	8
2	3-HSA.	220	30	8.2	14	63	19	55	17
3	3-HSA.	200	25	8.2	10	39	12	35	11
1	BP-HSA.	240	27.5	8.3	13	16	4–5	15	4
2	BP-HSA.	240	27.5	8.3	10	14	4	13	4
3	BP-HSA.	165[b]	30	8.3	9	22	6	..	...
4	BP-HSA.	65	45	10	25	26	7	24	6–7
5	BP-HSA.	200	45	10	16	68	19	67	19
	Db-HSA.	240	27.5	8.3	13	38	9–10	34	8–9
1	β-HSA.	220	26	8.3	12	19	7–8	17	7
2	β-HSA.	190	27.5	8.3	10	23	9	22	9
	10-Me-3-HSA.	240	27	8.3	10	61	17–18	56	16
	ads. 10-HSA.	125	25	8.3	11	0	0	0	0
	ads. BP-HSA.	100	45	10	15	0	0	0	0

[a] Calculated as mg. of hydrocarbon radical per g. of horse serum albumin and also as hydrocarbon groups per molecule of albumin after purification by two or three precipitations with ammonium sulfate and then by one precipitation with acetone, taking 70,000 as the molecular weight of horse serum albumin. The theoretical maximum, based on reaction with the ε-amino groups of lysine, would be 66 hydrocarbon groups per molecule of albumin. [b] In this case, benzpyrenyl-carbamido albumin no. 2 was used in the reaction mixture instead of albumin. The abbreviations used to designate the conjugates are as follows: 10-HSA. for 1,2-benzanthryl-10-carbamido horse serum albumin; 3-HSA. for the 1,2-benzanthryl-3-carbamido albumin; BP-HSA. for the 3,4-benzpyrenyl-5-carbamido albumin; Db-HSA. for 1,2,5,6-dibenzanthryl-9-carbamido albumin; β-HSA. for β-anthryl-carbamido albumin; 10-Me-3-HSA. for 10-methyl-1,2-benzanthryl-3-carbamido albumin; ads. 10-HSA. and ads. BP-HSA. for the adsorption of the respective amines on the proteins.

protein, a decrease in the isocyanate-protein ratio under otherwise the same experimental conditions lowered the extent of conjugation (3-HSA. nos. 1 and 3; BP-HSA. nos. 4 and 5). There is also a direct parallelism between the concentration of dioxane used in the reaction mixture and the degree of conjugation. The more soluble isocyanates I, II and IV underwent more complete reaction with the protein under approximately similar conditions (3-HSA no. 2, 10-HSA. no. 5 and 10-Me-3-HSA.) than did the more insoluble benzpyrenyl derivative (V) (BP-HSA. nos. 1 and 2) and the isocyanates III and IV which were of intermediate solubility (β-HSA. nos. 1 and 2, and Db-HSA.).[13] Although the benzanthrylamine and benzpyrenylamine were adsorbed loosely on the albumin to some extent in the presence of dioxane, the methods of ammonium sulfate and acetone purification entirely removed the characteristic yellow color and the sensitive absorption spectrophotometric determination did not reveal the presence of even a trace of the amines in the final preparations. It is interesting to note that polynuclear hydrocarbons are soluble to an extent of about 1–2 mg. per liter of horse serum[14] whereas the amount of combined benzpyrenyl radical in 3,4-benzpyrenyl-5-carbamido horse serum albumin no. 5 at a concentration of protein equal to that found in horse serum would be about 5 g. per liter.

The properties of 1,2-benzanthryl-10-carbamido albumin no. 5 were somewhat different from those of both the untreated serum albumin and the albumin obtained after removal of adsorbed amine in that the addition to a dialyzed solution containing 125 mg. of conjugate in 20 cc. of enough hydrochloric acid to bring the pH to 4.5 brought about the precipitation of about one-third of the conju-

(13) The above explanation based on solubility relationships appears to be more satisfactory than one involving any specific reactivities of the isocyanates. All of the isocyanates react almost instantaneously with free amino groupings (see ref. 2, 3 and 4) although they are not hydrolyzed to any appreciable extent in aqueous dioxane at pH 9 and at 20°. With the exception of β-anthryl isocyanate, they may be stored in the solid state without decomposition for a period of a year at least. Crystalline β-anthryl isocyanate undergoes considerable decomposition within six months. Although 1,2,5,6-dibenzanthryl-9-isocyanate reacts slowly with certain polynuclear hydrocarbon derivatives because of steric hindrance caused by the angular benzene ring, such an explanation does not seem applicable to the case of benzpyrenyl isocyanate.

(14) Lorenz and Andervont, *Am. J. Cancer*, **26**, 783 (1936).

gate. Spectrophotometric determinations on the supernatant and on the dialyzed solution of the precipitate showed that the benzanthryl contents of the total, the supernatant and the precipitate were the same (48 mg. per g.). The precipitation was repeated with similar results. No precipitate was observed when acid was added to the amine treated albumin prepared at either 25 or 45% dioxane concentration. It may also be noted that whereas the above conjugate was precipitated with ammonium sulfate in the 1.7–2.4 molar range, the amine treated albumin did not begin to precipitate until the 2.0 molar level was reached.

In the preceding article,[1] it was reported when coupling was attempted at dioxane concentrations greater than 30% and at a *p*H greater than 8.5 that extensive denaturation of the protein component occurred, even rendering a considerable portion of the product insoluble in distilled water. It has been found by using a concentration of protein above 1.5% (at the maximum dioxane concentration), by dialyzing the product immediately and by keeping it ice-cold during purification that satisfactory conjugates may be prepared at a 45% dioxane concentration and at *p*H 10. Such conjugates are completely soluble in distilled water but are almost entirely precipitated by acid in the region of *p*H 4–5. By the use of similar precautions, the treatment of the conjugate BP-HSA. no. 2 with isocyanate increased the benzpyrenyl content somewhat (BP-HSA. no. 3).

The results of attempted fractionation of 1,2-benzanthryl-10-carbamido albumin no. 5 are recorded in Table IV. Although there seemed to be a slight fractionation at 2.0 *M* ammonium sulfate concentration, no further fractionation was evident with the portion of conjugate soluble in the 1.7–2.0 molar range at the 1.85 molar level. Small amounts of conjugate were discarded in each case at the 1.7 molar level and it may well be that the benzanthryl content was somewhat higher in these rejected fractions particularly in the second fractionation where the percentage of radical lost exceeded that of the protein lost. The lack of any distinct fractionation, however, tends to indicate the occurrence of rather uniform coupling and the consequent formation of conjugates in which the majority of the protein molecules contain close to the average of fourteen hydrocarbon groups.

TABLE IV

FRACTIONATION OF 10-HSA. NO. 5

Molar concn. of $(NH_4)_2SO_4$	Starting volume, cc.	Mg. of conjugate pptd.	% of total conjugate ppt.	Mg. radical per g. of protein in ppt.	Total mg. of radical in ppt.
1.5–2.6	...	1170	100	48	56
1.2–1.7	120	trace	...	..	..
1.7–2.0	90	670	57	52.5	35
2.0–2.4	70	355	30.5	45	16
2.4–2.6	60	trace	...	..	..
(Losses: protein 12.5%, radical 9%)					
1.7–2.0	...	560	100	52	29
1.4–1.7	75	trace	...	..	..
1.7–1.85	65	200	36	50	10
1.85–2.0	50	260	46.5	48.5	12.5
2.0–2.4	40	sl. tr.	...	..	..
(Losses: protein 18%, radical 22%)					

That the method of fractionation is capable of effecting separation was demonstrated in the case of a mixture of albumin and benzanthryl conjugate. Admittedly the choice of constituents for the mixture from the standpoint of solubilities probably provided a more favorable opportunity for separation than was the case with the fractionation of the conjugate alone. It will be observed from Table V that a reasonably sharp fractionation of the components was achieved by one process. In fact, by including the usually discarded 1.4–1.7 molar fraction in the precipitate removed at the 1.8 molar level, it was found that a significantly greater content of benzanthryl radical was present in this small fraction of conjugate than was present in any previously isolated fraction. Approximately one-third of the total recovered protein was precipitated at the 2.1 molar level and this contained about 70% of the total amount of benzanthryl radical. By performing analyses before and after mixing the components, it was possible to check the accuracy of the Kjeldahl and spectrophotometric techniques. Calculated from the components, the

TABLE V

FRACTIONATION OF A MIXTURE OF ALBUMIN AND 10-HSA

Components of mixture: 235 mg. of albumin (2.15 to 2.4 *M* fraction). 160 mg. of 10-HSA. (1.7 to 1.85 *M*) (benzanthryl content 50 mg. per g.)

Molar concn. of $(NH_4)_2SO_4$	Starting volume, cc.	Mg. of protein pptd.	% of total protein pptd.	Mg. radical per g. of protein in ppt.	Total mg. of radical in ppt.
.....	..	360	100	20.5	7.2
1.4–1.8	50	28	8	59	1.6
1.8–2.1	45	69	19	44	3.0
2.1–2.5	45	195	54	9	1.8
2.5–2.8	40	Trace	...	..	...

(Losses: protein 19%, radical 10%)

TABLE VI

FRACTIONATION OF 10-HSA. No. 6 AND BP-HSA. No. 5

Molar concn. of $(NH_4)_2SO_4$	Starting volume, cc.	Mg. of conjugate pptd.	% of total conjugate pptd.	Mg. of radical per g. of protein in ppt.
10-HSA.				
0.8–2.0	...	650	100	98
0.8–1.1	100	450	70	96
1.1–1.5	50	25	4	100
BP-HSA.				
0.8–2.0	...	650	100	68
0.8–1.1	90	430	66	62
1.1–1.5	50	90	14	69

TABLE VII

OPTICAL CHARACTERISTICS OF SOLUTIONS OF THE CONJUGATES

Conjugate	$(NH_4)_2SO_4$ purif. Solution	Fluorescence	Acetone purif. Solution	Fluorescence
10-HSA.	Pale yellow, clear	Blue-green +++	Colorless, clear	Blue ++++
3-HSA.	Yellow-brown, sl. opal.	Blue-green +++	Colorless, v. sl. opal.	Blue ++++
10-Me-3-HSA.	Yellow-brown, sl. opal.	Green +++	Pale yellow, v. sl. opal.	Blue-green ++++
β-HSA.	Colorless, clear	Blue ++++	Colorless, clear	Blue ++++
Db-HSA.	Pale yellow, clear	Purple-blue ++	Colorless, clear	Purple-blue +++
BP-HSA.	Yellow, clear	Green +++	Yellow, clear	Green ++++

(Concentration of solutions was approximately 6 mg. conjugate per cc.)

mixture should have contained 395 mg. of protein of benzanthryl content 20.3; the analyses showed the presence in the mixture of 390 mg. of protein of benzanthryl content 20.5.

It is shown in Table VI that the benzanthryl and benzpyrenyl conjugates prepared at 45% dioxane concentration were not separated at 1.1 *M* ammonium sulfate concentration into fractions possessing significantly different contents of hydrocarbon radical. The characteristics of solutions of the various conjugates containing approximately 6 mg. of protein per cc. are recorded in Table VII. Qualitative observations on the fluorescence are also listed.

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Summary

A number of conjugated proteins having various polynuclear aromatic hydrocarbons as prosthetic groups have been prepared for studies of their possible immunological and carcinogenic properties. Coupling was effected in an aqueous dioxane medium, the conjugates were purified by ammonium sulfate and acetone precipitations and the degree of coupling was determined by ultraviolet spectrophotometry. Marked differences in the extent of conjugation with protein were observed with the various isocyanates under a given set of experimental conditions; those which were more soluble in dioxane (the 3- and 10-isocyanates of 1,2-benzanthracene and 10-methyl-1,2-benzanthryl-3-isocyanate) underwent reaction with serum albumin to the extent of introducing about sixteen groups per molecule of protein, the less soluble β-anthryl- and 1,2,5,6-dibenzanthryl-9-isocyanates formed conjugates containing nine groups per molecule and the still less soluble 3,4-benzpyrenyl-5-isocyanate formed conjugates containing only four hydrocarbon groups per molecule. By increasing the dioxane concentration of the reaction medium, preparations of 1,2-benzanthryl-10-carbamido albumin and 3,4-benzpyrenyl-5-carbamido albumin with, respectively, thirty and nineteen hydrocarbon groups per molecule were obtained. Fractionation experiments indicated that the major portion of the conjugate contains close to the average number of prosthetic groups. The characteristic fluorescence exhibited by the conjugates in ultraviolet light is recorded.

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