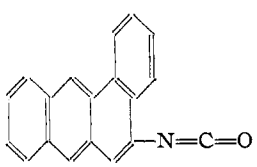
[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF HARVARD UNIVERSITY]

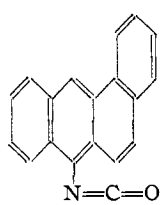
The Conjugation of Horse Serum Albumin with 1,2-Benzanthryl Isocyanates[1]

BY HUGH J. CREECH AND R. NORMAN JONES

This paper describes the coupling of 1,2-benzanthryl-3-isocyanate (I) and 1,2-benzanthryl-10-isocyanate (II)[2] with horse serum albumin. The reaction is considered to take place with the free amino groups of the protein forming a disubstituted urea derivative. Investigations are in progress to determine the carcinogenic[3] and serological[4] properties of the conjugates and their influence in protecting mice against hydrocarbon carcinogenesis.[5,6]



I



II

Although it is possible that other derivatives[7] of the polycyclic aromatic hydrocarbons may be sufficiently reactive for conjugation with proteins, the utilization of the isocyanate grouping originally adopted[8] has been continued because of the ease of reaction noted with the amino acids.[2,8] It was shown qualitatively in earlier experiments,[8] that chemical combination had occurred between the proteins and the *meso*-isocyanates[9] of anthracene and of 1,2,5,6-dibenzanthracene. In the present work, an accurate method involving ultraviolet absorption spectrophotometry has been developed for the determination of the degree of conjugation.

The conjugates were formed by the addition of the isocyanate in dioxane solution to a stirred chilled aqueous dioxane solution of the protein buffered at *p*H 8.2 to 8.3. They were purified by dialysis, centrifugation, filtration and precipitation with ammonium sulfate and acetone. After preliminary removal of adsorbed material, the benzanthryl content of the conjugates remained constant. Twelve prosthetic benzanthryl groups were found per molecule of 1,2-benzanthryl-10-carbamido horse serum albumin. Treatment of the albumin with 1,2-benzanthryl-3-isocyanate in one instance introduced eighteen prosthetic groups without denaturing the protein component.

The Absorption Spectra of 1,2-Benzanthryl-carbamido Proteins.—In a recent paper,[10] the ultraviolet absorption spectra of several alkyl-1,2-benzanthracenes were recorded and it was observed that although the positions of the numerous maxima varied from one derivative to another, the intensities remained constant and the curves were of the same general shape. If it can be shown that the benzanthryl radical when bound into the protein exhibits the same type of absorption spectrum as the free hydrocarbon, it should be possible to use spectrographic analysis as a means of determining the extent of conjugation.

As this involves a number of suppositions, a certain amount of preliminary investigation was necessary. In the first place, the radical added to the protein is not an alkyl-1,2-benzanthryl group but a 1,2-benzanthryl-carbamido group (Ar-NH-CO-NH-). Although the intensity of the absorption spectrum of 1,2-benzanthracene is not significantly altered by substitution of an alkyl group, it cannot be assumed that the same will apply to the substitution by a carbamido group in which considerable latent unsaturation is present, due to the carbonyl group and the unshared electrons of the imino nitrogen groups. The absorption spectra of 1,2-benzanthryl-10-carbamido-acetic acid, 1,2-benzanthryl-3-carbamido-acetic acid and of ϵ-(1,2-benzanthryl-3-carbamido)-caproic acid[2] were examined (Fig. 2) and it will be seen from a comparison with the absorption spectrum of the parent hydrocarbon (Fig. 1) that the intensities of the maxima in the spectra are comparable at wave lengths greater than 3250 Å. At shorter wave lengths differences are observed in the spectra of the 3-substituted derivatives, there being a fall in intensity and loss of fine structure. This would suggest that in using spectrographic data it will be

(1) Read in part before the American Association for Cancer Research, Pittsburgh, Pa., March 19, 1940.

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

(3) Conducted by Dr. Shields Warren and Dr. Charles Dunlap of the Huntington Memorial Hospital, Boston.

(4) In coöperation with Dr. F. S. Cheever of the Harvard Medical School.

(5) In coöperation with Dr. W. R. Franks of the Banting Institute, Toronto.

(6) Franks and Creech, *Am. J. Cancer*, **35**, 203 (1939).

(7) Bachmann and Cole, *J. Org. Chem.*, **4**, 60 (1939).

(8) Creech and Franks, *Am. J. Cancer*, **30**, 555 (1937).

(9) Creech and Franks, THIS JOURNAL, **60**, 127 (1938).

(10) Jones, *ibid.*, **62**, 148 (1940).

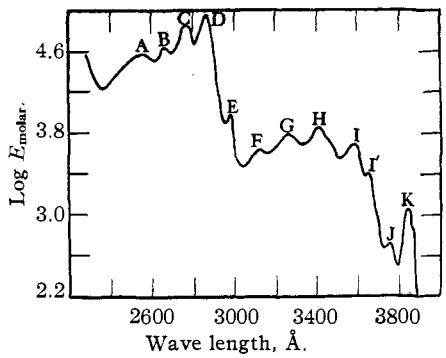


Fig. 1.—1,2-Benzanthracene (solvent ethanol).

necessary to employ the longer wave length region of the spectrum; as will appear later there are other reasons favoring this choice.

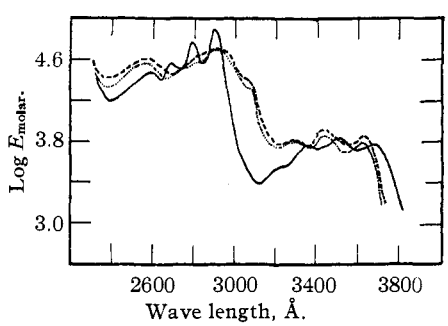


Fig. 2.— —— 1,2-Benzanthryl-10-carbamido-acetic acid (buffer *p*H 8.3); .... 1,2-benzanthryl-3-carbamido-acetic acid (buffer *p*H 8.3 + ethanol); - - - - ϵ-(1,2-benzanthryl-3-carbamido)-caproic acid (buffer *p*H 8.3 + ethanol).

The agreement of the absorption spectra of 1,2-benzanthryl-carbamido derivatives with that of 1,2-benzanthracene shows that the introduction of the carbamido linkage does not disturb the spectrum in the nearer ultraviolet but it remains to be shown that the intensity of the spectrum is not influenced by combination with the protein. It is not possible to give exact experimental proof of this; Ramart-Lucas[11] and others have made investigations of the mutual interaction of two or more chromophoric groups in the same molecule and have shown that, provided the two light-absorbing centers are separated from each other by two or more methylene groups, they behave independently and their contributions to the total light absorption of the molecule are additive. In the case of the 1,2-benzanthryl-carbamido proteins, if conjugation takes place through the ϵ-amino groups of the lysine side chains, the benzanthryl chromophore is separated from the rest of the protein molecule by four methylene groups and it may be assumed that no interaction occurs. A similar assumption has been made by Holiday[12] who has based a method of spectrographic analysis of tyrosine and tryptophan in proteins on the supposition that these aromatic chromophores have the same intensity of absorption when bound into the protein chain as they have in the free state.

Another factor to be considered is the absorption spectrum of the protein itself. The spectrum of horse serum albumin in distilled water is shown in Fig. 3; the maximum at 2770 Å. is attributed to the tyrosine, tryptophan and phenylalanine side chains. Although it is theoretically possible to deduce the amount of benzanthryl group present from an analysis of the spectrum in regions where the protein is also contributing materially to the total absorption,[13] it is much simpler to limit the analysis to the longer wave region of the spectrum where the protein absorption is very small and may be neglected (Fig. 3).

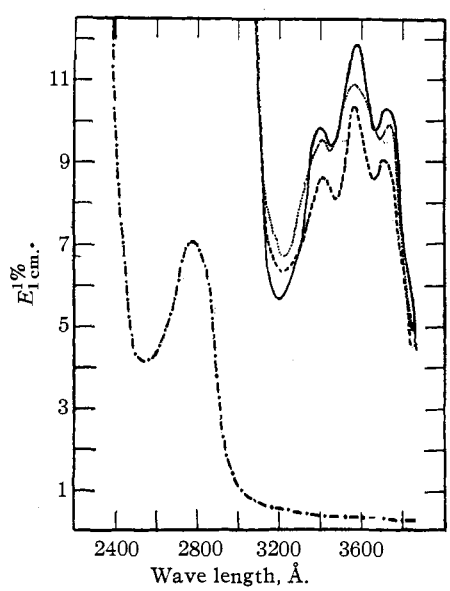


Fig. 3.—1,2-Benzanthryl-10-carbamido horse serum albumin (solvent distilled water): —— (Prepn. I, salting out no. 4); - - - - (Prepn. I, salting out no. 6); .... (Prepn. I, acetone treatment no. 1); - · - · horse serum albumin (solvent distilled water).

Experimental Procedure

Preparation of 1,2-Benzanthryl-10-carbamido Serum Albumin.—Purified dioxane (20 cc.) and sodium phosphate buffer (20 cc., 0.1 molar) of *p*H 8.2 to 8.3 were added to a thoroughly dialyzed cold aqueous solution (140 cc.) of horse serum albumin (2.5 g.) crystallized according to the

(11) Ramart-Lucas, *Bull. soc. chim.*, **51**, 289 (1932).

(12) Holiday, *Biochem. J.*, **30**, 1799 (1936).

(13) Twyman and Allsopp, "The Practice of Spectrophotometry," Adam Hilger, London, 1934, p. 43.

method of McMeekin.[14] To this solution, maintained at 2 to 5°, there was added with stirring over a one-hour period 0.6 g. of 1,2-benzanthryl-10-isocyanate in 30 cc. of dioxane. After four hours of stirring, the slightly yellowish suspension was diluted with 200 cc. of water and set in a refrigerator at 5° for an additional forty hours. The dioxane and phosphate were removed by dialysis at 5° against running tap water for forty-eight hours and against slowly flowing distilled water for twenty-four hours. Centrifugation and filtration of the protein solution removed a slightly yellow amorphous precipitate (0.8 g.) from which 0.35 g. of unaltered isocyanate was extracted with benzene.

Purification of the Conjugate.—Sufficient ammonium sulfate to make the solution 2.8 molar was added to the cold opalescent filtrate of *p*H 6.5 by the revolving cellophane membrane technique of McMeekin.[14] Forty-eight hours later, the granular precipitate was collected by suction filtration, washed with 2.8 *M* ammonium sulfate solution, dissolved in 100 cc. of water and dialyzed at 5° for fifty hours. The solution was centrifuged and filtered to remove a small amount of insoluble material. After two repetitions of the process, the salted-out protein was colorless and its solution was crystal clear. A dilute solution of the conjugate exhibited a blue fluorescence. After being salted out eight times the amount of conjugate was found to be 1.25 g.

To the dialyzed chilled solution of 1.25 g. of conjugate in 40 cc. of water there were added four volumes of acetone which had been cooled to −10°. The precipitate was separated by centrifugation and was washed three times with a total of 200 cc. of acetone.[15] The conjugate was dissolved in 50 cc. of water and dialyzed for forty hours. After being salted out and re-dialyzed, the amount of conjugated protein was 1.05 g. Fractionations of this material at concentrations of ammonium sulfate of 1.4, 1.7 and 2.0 molar were attended with about 40% loss. Approximately 0.3 g. of conjugate was obtained from each of the fractions precipitated between 1.4 and 1.7 molar and between 1.7 and 2.0 molar. Only very small amounts of protein were removed below 1.4 and above 2.0 molar. The conjugate has not been obtained in a crystalline condition. The content of 1,2-benzanthryl groups in the conjugate was determined at suitable intervals by means of absorption spectrophotometry and micro Kjeldahl determinations.

The 1,2-benzanthryl-3-carbamido serum albumin was prepared in a similar manner. Precipitation with ammonium sulfate failed to decolorize the conjugate which gave a somewhat turbid solution. In fact, it was noted that the filter cake became increasingly browner as the ammonium sulfate precipitations proceeded, suggesting the possibility of decomposition. A tendency toward decomposition was encountered with the conjugated amino acids.[2] Treatment with acetone, however, removed the color and the dialyzed solution possessed only a faint opalescence. Losses during both treatments were greater than those observed with 1,2-benzanthryl-10-carbamido serum albumin.

The adsorption of amines on proteins was investigated by adding the 3- and 10-amino-1,2-benzanthracenes in dioxane solution to the serum albumin under similar experimental conditions to those described with the reactive isocyanates. After dialysis, the yellow suspension deposited a voluminous yellow precipitate which was removed by centrifugation, dried and extracted with benzene. More than 85% of the amine was recovered. Further amounts were removed by two treatments with ammonium sulfate. The resultant slightly opalescent solution contained 90% of the protein originally used.

(14) McMeekin, THIS JOURNAL, **61**, 2884 (1939).

(15) The conjugate was left in contact with acetone for a period of not more than fifteen minutes.

Results

Spectrographical Analysis.—The spectrophotometric apparatus employed in this Laboratory has been described.[10] In a preliminary investigation, analyses were made of solutions containing known amounts of the 1,2-benzanthryl-carbamido acetic acids in a borate buffer of *p*H 8.3 and also in the presence of horse serum albumin (Table II). The concentrations were calculated from the intensities of the three prominent maxima G, H and I (see Fig. 1 and Table III); the arithmetical mean of the three results was accepted. In the 3-substituted series only the H and I maxima were used, as the shape of the curve made the intensity of the G maximum uncertain.

With solutions containing 1 g. of 1,2-benzanthryl-10-carbamido-acetic acid in 100 cc. and using a 1-cm. cell the values of the intensity of the various maxima, $(E^{1\%}_{1\ cm.})_{max.}$, obtained are given in Table I.

TABLE I

	$(E^{1\%}_{1\ cm.})_{max.\ G}$	$(E^{1\%}_{1\ cm.})_{max.\ H}$	$(E^{1\%}_{1\ cm.})_{max.\ I}$
1,2-Benzanthryl-10-carbamido-acetic acid	171	192	171
	174	198	181
	181	213	181
Mean	175	200	177
1,2-Benzanthryl-3-carbamido-acetic acid	...	212	170

TABLE II

ANALYSIS OF SOLUTIONS OF 1,2-BENZANTHRYL-CARBAMIDO-ACETIC ACIDS IN A BORATE BUFFER OF *p*H 8.3

Composition of solution: Benzanthracene derivative, γ per cc.	Protein, γ per cc.	Benzanthracene derivative found, γ per cc.	Error, %
10-Carbamido-acetic acid			
67.5	0	65.5	3.0
43.0	0	43.0	0.0
39.8	0	40.8	2.5
72.0	1025	66.4	7.8
29.0	1025	29.0	0.0
3-Carbamido-acetic acid			
79.0	0	82.6	4.5
			Mean error 2.9

If c_1 be the concentration in the given solution in grams per 100 cc.

$$c_1 = (\log I_0/I)_{\max.}\, d\, l/(E^{1\%}_{1\text{cm.}})_{\max.} \qquad (1)$$

where $(\log I_0/I)_{\max.}$ is the extinction coefficient observed at one of the maxima G, H or I, $(E^{1\%}_{1\text{cm.}})_{\max.}$ the corresponding value of $E^{1\%}_{1\text{cm.}}$, l the cell length in cm. and d the dilution factor of the solution measured.

In Fig. 3 are shown representative absorption curves of 1,2-benzanthryl-10-carbamido horse serum albumin solutions in distilled water. Similar curves for the 3-carbamido compound are shown in Fig. 4. In most of the experiments only the long wave length region of the spectrum was measured. The wave lengths of the maxima are shifted slightly toward longer wave lengths in comparison with the conjugated carbamido acids. Experiments made at *p*H 7 and *p*H 8.3 with the 10-carbamido protein and the serum albumin showed no difference in the intensity or the position of the curves.

TABLE III

WAVE LENGTHS OF THE G, H AND I MAXIMA

Substance	Solvent	Wave lengths, Å. G	H	I
1,2-Benzanthracene	Ethanol	3275	3410	3580
1,2-Benzanthryl-10-carbamido-acetic acid	Buffer *p*H 8.3	3360	3520	3680
10-carbamido-albumin	Distilled water	3395	3570	3730
3-carbamido-acetic acid	Buffer *p*H 8.3 + ethanol	3290	3435	3615
ε-(3-carbamido)-caproic acid	Buffer *p*H 8.3 + ethanol	3290	3435	3625
3-carbamido-albumin	Distilled water	3350	3490	3700

The intensities are calculated on a basis of $E^{1\%}_{1\text{cm.}}$, the 1% solution being regarded as unconjugated protein. This protein concentration was obtained from micro-Kjeldahl nitrogen analyses on the solutions examined spectrophotometrically. McMeekin's value[14] of 16% for the nitrogen content of horse serum albumin was used. The change in the nitrogen content of the protein resulting from the introduction of the nitrogen atom of the isocyanate was neglected because it is small in comparison with the probable errors in measurement suggested by the results in Table II (2.9%).

The calculation of the concentration of 1,2-benzanthryl groups in the protein was made as follows. Let N mg. per cc. be the nitrogen content of the solution analyzed. The protein concentration

$$(c_1) = (N\ 100/16) \text{ mg. per cc.} \qquad (2)$$

If $(\log I_0/I)_{\max.}$ be the absorption coefficient at a maximum, then c_2, the concentration of absorbing

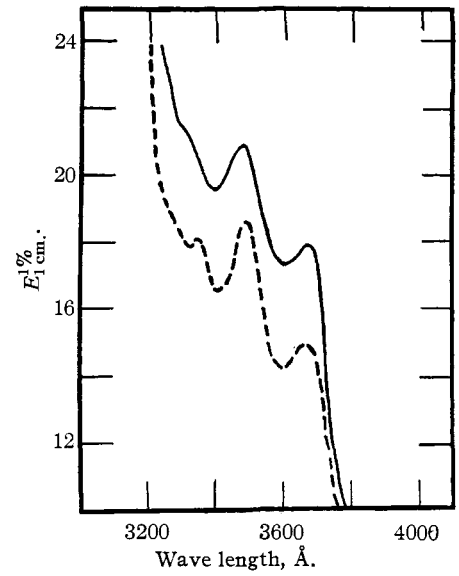


Fig. 4.—1,2-Benzanthryl-3-carbamido horse serum albumin (Prepn. I, salting out no. 6); ---- same (prepn. I, acetone treatment no. 1).

group calculated as 1,2-benzanthryl-carbamido-acetic acid, is

$$c_2 = 10(\log I_0/I)_{\max.}\, d\, l/(E^{1\%}_{1\text{cm.}})_{\max.} \text{ mg. per cc.} \qquad (3)$$

where d is the dilution factor and l the cell length in cm. Expressed as mg. per g. of protein this becomes

$$\frac{10\ (\log I_0/I)_{\max.}\, d\, l}{(E^{1\%}_{1\text{cm.}})_{\max.}} \cdot \frac{1000}{c_1} \qquad (4)$$

Converting from benzanthryl-carbamido-acetic acid to benzanthryl radical and substituting for c_1 (equation 2)

$$\frac{10\ (\log I_0/I)_{\max.}\, d\, l}{(E^{1\%}_{1\text{cm.}})_{\max.}} \cdot \frac{16}{100}\frac{1000}{N}\frac{227}{344}$$

which simplifies to

$$\frac{1056\ (\log I_0/I)_{\max.}\, d\, l}{(E^{1\%}_{1\text{cm.}})_{\max.} N} \qquad (5)$$

mg. benzanthryl radical per g. of protein. This equation (5) may be solved in terms of $\log I_0/I$ and $E^{1\%}_{1\text{cm.}}$ at the G, H and I maxima, the values of $(E^{1\%}_{1\text{cm.}})_{\max.}$ being taken from Table I.

As was the case with the calculations of the acetic acids, the mean of the results from the G, H and I maxima was taken for the 10-carbamido conjugated proteins; only the mean of the H and I maxima was used for the 3-carbamido series.

Observations on the four most satisfactory preparations of the conjugates are given in Table IV. The 1,2-benzanthryl-10-carbamido serum albumin was found to contain 36 mg. of the 1,2-benz-

TABLE IV

	10-Conjugate Prep. 1	10-Conjugate Prep. 2	3-Conjugate Prep. 1	3-Conjugate Prep. 2
Composition of the reaction mixture				
Mg. of isocyanate per g. of protein	230	250	220	165
Mg. of protein per cc. of solution	18	11	14	11
% dioxane	25	25	30	25
Conjugate				
Mg. of 1,2-benzanthryl radical per g. of protein				
After salting out no. 2	...	41	...	44
After salting out no. 4	39	43	74	39
After salting out no. 6	34	...	68	...
After salting out no. 8	36	...	70	...
After acetone treatment no. 1	36	37	55	24
After acetone treatment no. 2	37	...	...	26
Fractionation with ammonium sulfate				
Below 1.7 molar	37	...	...	...
From 1.7 to 2.0 molar	30	...	49	...
Above 2.0 molar	34	...	51	...
No. of benzanthryl groups per molecule of conjugate[a]	12	12	18	8
% conjugation (of the theoretical)[b]	18	18	27	12

[a] Calculated using a value of 73,000 for the molecular weight of horse serum albumin. [b] Based on a reaction with the ϵ-amino groups of the lysine; Cohn, Harvey Lecture 1939, gives a value of 66 lysine groups per molecule of horse serum albumin.

anthryl radical per gram of protein. This value was not affected to any extent by the ammonium sulfate and acetone treatments. Although the conjugate was less soluble than serum albumin, attempts to obtain a fraction containing a greater number of prosthetic groups per molecule of protein were not successful. This may be taken as an indication that the serum albumin has been coupled uniformly with the isocyanate although the difference in properties between the serum albumin and the conjugate may not be sufficient to allow a separation, particularly with such small amounts of material. Crystals of serum albumin were never observed in the preparation, however.

Precipitation with ammonium sulfate was not sufficient for the removal of adsorbed material from the 1,2-benzanthryl-3-carbamido albumin. One precipitation with acetone, however, gave a product of constant benzanthryl content.

The following additional evidence may be cited in support of the contention that the isocyanates were combined chemically with the protein and not adsorbed upon it. Although the 3- and 10-amino-1,2-benzanthracenes could be adsorbed loosely on the serum albumin, particularly in the presence of dioxane, ultraviolet absorption studies showed that after dialysis and two precipitations with ammonium sulfate the solutions contained no benzanthracene nucleus. The Sørensen method for the titration of free amino groups is not considered particularly reliable when applied to proteins, but determinations[15] indicated that the conjugates contained less free amino nitrogen than the serum albumin alone or the serum albumin which had been treated with the amino-1,2-benzanthracenes. The 10-conjugate had a ratio of free amino nitrogen to Kjeldahl nitrogen of 0.038, which is 40% lower than that (0.065) of the aminobenzanthracene-treated albumin. The value for the 3-conjugated preparation (No. 1) was 50% lower than the control. Some diminution in the number of free amino groups of the protein can be brought about by the medium and at the temperature employed in the process of conjugation. This evidence therefore must be regarded as interesting but far less conclusive than that derived from the spectrographical analysis which demonstrated that the conjugate, after preliminary purification, had a constant benzanthryl content following repeated and different chemical treatments. The shift in the wave length of the absorption maxima of the conjugates (Table III) is a further suggestion that coupling has occurred. A similar shift has been observed in the spectrographic studies on the yellow enzyme.[17]

Attempts to increase the degree of conjugation by using (a) an excess of isocyanate, (b) a greater proportion of dioxane in the reaction mixture, (c) buffers of *p*H greater than 8.5, (d) a higher concentration of protein (or by treating the conjugate with isocyanate), invariably led to denaturation of the protein component. The main factors preventing the introduction of more prosthetic groups are reduction in activity of the isocyanate grouping by virtue of its attachment to the benzanthracene nucleus and the impossibility of increasing the dioxane content and *p*H of the reaction mixture without harmful effects on the protein. More complete coupling has been reported when prosthetic groups of low molecular weight have been introduced into the protein molecule. Hop-

(16) Northrop, *J. Gen. Physiol.*, **3**, 715 (1920); **9**, 767 (1926); neutralized formaldehyde was used, however.

(17) Oppenheimer and Stern, "Biological Oxidation," Nordemann Publishing Co., New York, N. Y., 1939, p. 190.

kins and Wormall[18] were able to introduce 60–70% of the theoretical amount of phenyl isocyanate (based upon a reaction with lysine). Clutton, Harington, Mead and Yuill[19,20] found the glucose content of their preparation of O-β-glucosido-N-carbobenzyloxy-tyrosyl serum albumin to be 11.5%. Gurin and Clarke[21] demonstrated that the ε-amino groups of gelatin reacted with benzenesulfochloride at *p*H 10 to 11 by the isolation of ε-monobenzenesulfonyl-*d*-lysine upon hydrolysis of the conjugate.

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(18) Hopkins and Wormall, *Biochem. J.*, **27**, 740 (1933).
(19) Clutton, Harington and Mead, *ibid.*, **31**, 764 (1937).
(20) Clutton, Harington and Yuill, *ibid.*, **32**, 1111 (1938).
(21) Gurin and Clarke, *J. Biol. Chem.*, **107**, 395 (1934).

Summary

Conjugated proteins containing the 1,2-benzanthryl radical as the prosthetic group have been prepared in a condition suitable for studies of their possible carcinogenic and serological properties. Coupling was effected by interaction of the protein with the hydrocarbon isocyanate in an aqueous dioxane medium. By ultraviolet spectrophotometry, it has been possible to establish the fact that true conjugation has occurred and to determine the degree of conjugation. Purified samples of 1,2-benzanthryl-10-carbamido horse serum albumin contain approximately twelve hydrocarbon residues per molecule, while the best samples of 1,2-benzanthryl-3-carbamido horse serum albumin contain eighteen hydrocarbon prosthetic groups per molecule.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE UNIVERSITY OF ROCHESTER]

Photochemical Studies. XXXII. The Photochemical Reaction between Nitrous Oxide and Hydrogen

BY J. WILLIAM ZABOR[1] AND W. ALBERT NOYES, JR.

The photochemical decomposition of nitrous oxide gas has been studied for the general wave length region 1800–2000 Å.[2] Two mechanisms were proposed, either one of which would provide an adequate explanation for the data. In the first the primary process

$$N_2O + h\nu = N_2 + O \qquad (1)$$

was presumed to be followed by the secondary reactions

$$O + N_2O = 2NO \qquad (2)$$

and

$$O + O(+M) = O_2(+M) \qquad (3)$$

The products are known to be[2,3,4] NO, N_2 and O_2. If the sole primary process is assumed to be reaction (1), the production of nitric oxide can only be obtained through reaction (2).

The second mechanism assumes that both primary process (1) and primary process (4)

$$N_2O + h\nu = NO + N \qquad (4)$$

occur simultaneously. In order to obtain the correct quantum yields of products, it is necessary to assume further that these primary processes are followed by (3) plus (5) and (6)

$$N + N_2O = NO + N_2 \qquad (5)$$
$$N + N(+M) = N_2(+M) \qquad (6)$$

If this second mechanism is correct, the two primary processes must take place to approximately equal extents and (6) is relatively unimportant compared to (5).

A decision between these two mechanisms can be based on several types of investigation. The production of nitric oxide in the first mechanism would result from a reaction of oxygen atoms with nitrous oxide molecules. In a later investigation[5] an attempt was made to prove that this reaction

(1) Fellow for 1938–39 and for 1939–40 of the Sherman Clarke Fund in Research Chemistry of the University of Rochester.
(2) Noyes, *J. Chem. Phys.*, **5**, 807 (1937).
(3) Wulf and Melvin, *Phys. Rev.*, **39**, 180 (1932).
(4) Macdonald, *J. Chem. Soc.*, 1 (1928).
(5) Henriques, Duncan and Noyes, *J. Chem. Phys.*, **6**, 518 (1938).