[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF HARVARD UNIVERSITY]

Conjugates Synthesized from Various Proteins and the Isocyanates of Certain Aromatic Polynuclear Hydrocarbons

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In the preceding publication,[1] the synthesis of horse serum albumin conjugates containing various hydrocarbon radicals as the prosthetic groups was described. The present paper reports the preparation and properties of conjugates made from several of the same isocyanates and various other proteins. The most satisfactory preparations were those made from bovine serum albumin, which was found to undergo conjugation to the same extent as did horse serum albumin under similar conditions. Experiments with horse serum pseudoglobulin, bovine pseudoglobulin and egg albumin demonstrated that only a small amount of hydrocarbon radical could be introduced without causing pronounced denaturation of the protein. One particularly interesting conjugate made for some experiments of Dr. A. H. Coons of the Department of Bacteriology of the Harvard Medical School was prepared by the interaction of β-anthryl isocyanate with a concentrated Type III antipneumococcus rabbit serum. The resultant conjugate exhibits a brilliant blue fluorescence in ultraviolet light; no qualitative alteration in the immunological properties as a result of the introduction of "tracer groups" into the antibodies has been found to date.[2]

Experimental Procedure[3]

Bovine Serum Albumin Conjugates.—Bovine serum albumin was prepared according to the general procedure of McMeekin[4] for the isolation of horse serum albumin. Bovine serum was diluted with an equal volume of half-saturated ammonium sulfate solution, and then more ammonium sulfate was added to this solution through a rotating cellophane membrane. Fractions were removed at the 1.8, 2.8 and 3.4 molar levels. The precipitates obtained at the latter two levels were dissolved in a volume of water equal to that of the original serum and the portion precipitating within the range of 2.1–2.9 molar was collected. This was dialyzed against distilled water and the insoluble portion was discarded. The soluble portion was brought to a 2.2 *M* ammonium sulfate concentration, the precipitate was removed by centrifugation, and the supernatant was brought to the 2.8 molar level. The precipitate at this level was dialyzed and the soluble portion was again fractionated at the 2.2 and 2.8 molar levels. The slightly colored precipitate of albumin removed at the latter concentration of ammonium sulfate was collected and used for the conjugation experiments.[5]

3,4-Benzpyrenyl-5-isocyanate and 1,2-benzanthryl-10-isocyanate were allowed to react with bovine serum albumin in an aqueous dioxane medium. The methods of preparation and purification of these conjugates were identical with those described for the horse serum albumin conjugates in the preceding article,[1] with the exception that a somewhat greater ammonium sulfate concentration was necessary for the precipitation of the bovine serum albumin conjugates.[6]

Bovine Serum Pseudoglobulin Conjugate.—A portion of the bovine serum proteins precipitating at 1.8 *M* ammonium sulfate concentration was dialyzed against distilled water, the insoluble portion was removed by centrifugation and the supernatant was fractionated at the 1.4 and 1.7 molar levels. The protein soluble at 1.4 molar and insoluble at 1.7 *M* ammonium sulfate concentration was dialyzed thoroughly and the soluble portion was again fractionated at the 1.4 and 1.7 molar levels. The precipitate separating at the latter level was used in the conjugation experiments.

The reaction mixture was dialyzed immediately after the two hours of stirring following the addition of 1,2-benzanthryl-10-isocyanate. The portion soluble in distilled water was salted out at 2.2 *M* ammonium sulfate concentration and the precipitated protein was dialyzed against distilled water. The amount of 1,2-benzanthryl-10-carbamido bovine serum pseudoglobulin obtained by precipitation of the optically clear dialyzed solution at 2.0 *M* ammonium sulfate concentration was found to be half that of the globulin used for the conjugation process.

Horse Serum γ-Pseudoglobulin Conjugates.—Some coupling experiments were carried out with a preparation of γ-pseudoglobulin[7] kindly supplied by Professor E. J. Cohn. This protein was found to be an unsatisfactory one for use in the synthesis of conjugates by the usual procedure because of its instability. Only a few benzanthryl groups were introduced into the protein under the usual experimental conditions and even then marked denaturation occurred. About 20% of the total amount of conjugate was soluble in distilled water; part of the remainder was brought into solution by the addition of one-twentieth volume of dioxane and enough sodium carbonate

(1) Creech and Jones, THIS JOURNAL, **63**, 1661 (1941).

(2) Personal communication.

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

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

(5) A personal communication from Professor E. J. Cohn confirms the fact that the albumin fraction is precipitated from bovine serum within the range of ammonium sulfate concentrations specified.

(6) For the preparation of 3,4-benzpyrenyl-5-carbamido bovine serum albumin no. 2, there was used a mixture of 1 g. of albumin prepared by the above method and 1 g. of bovine serum albumin (supplied by Professor E. J. Cohn) which had been prepared by ethanol fractionation; see Cohn, Luetscher, Oncley, Armstrong and Davis, THIS JOURNAL, **62**, 3396 (1940).

(7) Cohn, McMeekin, Oncley, Newell and Hughes, *ibid.*, **62**, 3386 (1940).

to bring the pH to 8. The water soluble portion precipitated by the procedure described at 1.5 *M* ammonium sulfate concentration was purified in the usual manner. The final solutions after dialysis were still slightly opalescent. The portion soluble in aqueous dioxane was centrifuged and filtered through hardened paper and the conjugate was precipitated by the addition of dilute hydrochloric acid. The conjugate was dissolved and reprecipitated three times. Spectrophotometric analyses were performed on the opalescent solution and again after it had been purified by acetone precipitation.

Egg Albumin Conjugates.—Crystalline egg albumin prepared by standard methods was treated with 1,2-benzanthryl-3- and 10-isocyanates in the usual manner. It was found that this protein also was seriously denatured in the course of the preparation, although small quantities of undenatured conjugate usually could be obtained.

Antipneumococcus rabbit serum (Type III, concentrated) was allowed to react with β-anthryl isocyanate in an aqueous dioxane medium. The resultant conjugate after dialysis against 0.9% sodium chloride solution and subsequent removal of suspended material was precipitated with ammonium sulfate at the 1.8 molar level. Dialysis and salting-out were repeated. A solution of this conjugate in 0.9% sodium chloride solution was optically clear and exhibited a pronounced blue fluorescence in ultraviolet light.

A preparation of **zein**, kindly supplied by Professor H. B. Vickery, was dissolved in aqueous dioxane (80% dioxane, 20% aqueous buffer of pH 8.3) and treated at 2° with 1,2-benzanthryl-10-isocyanate in dioxane solution. After four hours, water was added gradually through a revolving cellophane membrane until the dioxane concentration had been reduced to about 65%. A small precipitate, consisting mainly of isocyanate, was removed at this stage and the filtrate was diluted until the protein was precipitated. After collection by centrifugation, the protein was dissolved in dioxane–water (8:1) and reprecipitated by the addition of water. The process was repeated using aqueous ethanol as the solvent and the final product was dried and extracted in a Soxhlet apparatus with acetone and then with ether. The protein was dissolved in 80% ethyl alcohol and examined spectrophotometrically.

Results

Spectrophotometric Analysis.—The method for the determination of combined hydrocarbon radical has been discussed in detail in previous publications.[1,8] In the case of the bovine serum albumin conjugates and the horse serum albumin conjugates,[1,8] the solutions for spectrophotometric examination were almost always optically clear. A considerable number of the solutions of the globulin conjugates were appreciably opalescent, however, and it was necessary to apply a correction to compensate for the loss of light caused by scattering, otherwise this loss would be attributed to absorption and the calculated hydrocarbon content would be too high.

Under certain conditions, the amount of light scattered by suspended particles may be calculated from the Rayleigh equation,[9] according to which the intensity of the scattered light varies inversely as the fourth power of the wave length of the incident radiation. Ginsel[10] has applied this equation to correct absorption spectra of solutions of thyroglobulin for light lost due to scattering by measuring the "absorption" at 380 mμ, where the total light loss may be attributed to scattering, and extrapolating further into the ultraviolet according to the inverse fourth power law.

In the derivation of the Rayleigh equation it is assumed that the scattering particles are small in comparison with the wave length of the light. In the protein solutions discussed here the opalescence varied considerably in solutions of the same total protein concentration and it seemed probable that the scattering was due to traces of non-filterable suspended material and not to dissolved protein, and therefore it could not be assumed *a priori* that the Rayleigh equation for scattering was applicable.

The measurement of the absorption spectra of these solutions was extended to 6000 Å. and the absorption coefficient ($E^{1\%}_{1\,\mathrm{cm.}}$) was plotted against $1/\lambda^4$. Several examples of such curves are given in Fig. 1. In the case of the opalescent solutions, it will be observed that at the longer wave lengths a linear relationship holds fairly well (curves Ia, Ib and Ic). The straight part of the curve may be extrapolated to a wave length corresponding to one of the absorption maxima and a correction applied to the calculated hydrocarbon radical concentration. Curve II is the absorption spectrum of 1,2-benzanthracene in chloroform at high concentration and the change in the slope of the conjugated protein curves occurs near this wave length which represents approximately the threshold of the 1,2-benzanthryl radical absorption. Curve III was obtained from an optically clear conjugated protein and curve IV from a solution of horse serum pseudoglobulin. It is probable that the residual "absorption" of non-conjugated protein solutions at wave lengths greater than 3250 Å. is also a minor scattering effect and the application of the scattering correction also corrects for this protein absorption (a small effect which has otherwise been neglected).

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

(9) Schmidt, "The Chemistry of the Amino Acids and Proteins," C. C. Thomas, Baltimore, 1938, p. 566.

(10) Ginsel, *Biochem. J.*, **33**, 428 (1939).

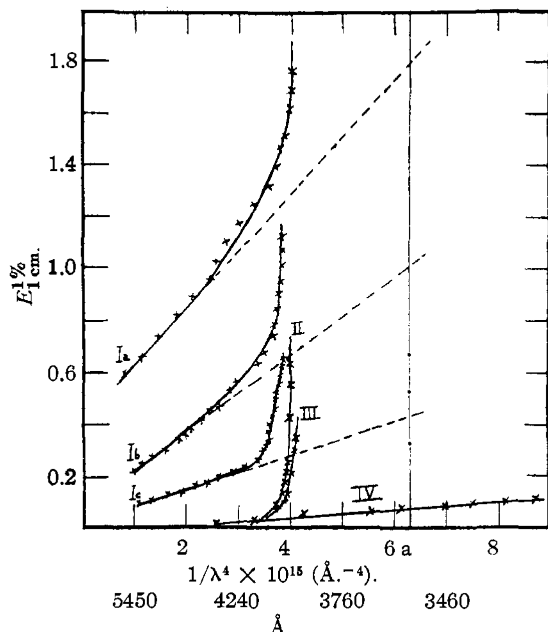

Fig. 1.—Scattering correction: Curve Ia, 1,2-benzanthryl-10-carbamido horse serum pseudoglobulin prepn. no. 3a before acetone treatment (very opalescent); Curve Ib, same, after acetone treatment (opalescent); Curve Ic, same, prepn. no. 3 (faintly opalescent); Curve III, same, prepn. no. 2 (clear); Curve II, 1,2-benzanthracene (solvent chloroform); Curve IV, horse serum pseudoglobulin (clear). a = wave length of H maximum of 1,2-benzanthryl-10-carbamido horse serum pseudoglobulin.

The correction is applied in the following manner: If m mg. per g. of protein be the calculated concentration of the hydrocarbon radical, E_1 the observed value of $E^{1\%}_{1\,\text{cm.}}$ at $\lambda_{\text{max.}}$ and E_s the intensity of the extrapolated linear portion of the scattering correction curve at $\lambda_{\text{max.}}$, then m_2 the corrected radical concentration is given by $m_2 = m[(E_1 - E_s)/E_1]$ mg. of hydrocarbon radical per g. of protein.

If the relation between the intensity of the scattered light and the wave length deviates from the Rayleigh equation under the conditions of extrapolation, such deviations would almost certainly reduce the amount of scattering loss. Thus the values calculated for the radical concentration before and after the application of the scattering correction can be regarded as maximal and minimal, respectively. The application of the correction factor usually reduced the content of hydrocarbon radical in the globulin conjugates by 5–12%.

Properties of the Conjugates.—The experimental conditions for the preparation of the conjugates and the content of hydrocarbon radical in the conjugates are recorded in Table I. Corrected values are given for the globulin conjugates. It will be noted that the extent of conjugation obtained with bovine serum albumin was very similar to that obtained with horse serum albumin,[1] whereas the number of hydrocarbon groups introduced into egg albumin and the various globulin preparations was quite low. The content of hydrocarbon in the water soluble portion of 1,2-benzanthryl-10-carbamido horse serum γ-pseudoglobulin (no. 3) was only half that contained in the portion soluble in aqueous dioxane (no. 3a). It is considered that the protein component in the latter preparation has been denatured to such an extent that water alone cannot bring the conjugate into solution whereas the presence of dioxane in the medium assists in the process by providing a solvent for the hydrophobic hydrocarbon component. This method of purification, however, cannot be considered to be as efficient as the usual procedure.

Hopkins and Wormall[11] stated that attempts to prepare *p*-bromophenyl isocyanate compounds of zein were unsuccessful; no experimental details were given, however. In view of the sensitivity of the spectrophotometric method, some experiments were conducted with zein, a protein which is deficient in lysine; the results recorded in the table show that the preparations, even after Soxhlet extraction, contained a small amount of 1,2-benzanthryl residue. The purification of these preparations, however, cannot be considered as extensive as those of the other preparations, in which the removal of adsorbed material was accomplished primarily by dialysis during which process the conjugate went into solution and the water insoluble hydrocarbon isocyanate was deposited. Precipitation of zein from aqueous dioxane (1:8) solution, in which the isocyanate is also slightly soluble, was carried out by dilution with water which would also cause the precipitation of any isocyanate present in the solution. Extraction of the dried product in a Soxhlet apparatus was not particularly efficient because the material tended to become somewhat gummy. It may be recorded, however, that unreactive aminobenzanthracene was removed to a considerably greater extent than the isocyanate by the same procedure. The important observation is that the possible introduction of two hydrocarbon groups per molecule of zein is insig-

(11) Hopkins and Wormall, *Biochem. J.*, **27**, 740 (1933).

TABLE I

No.	Conjugate	Compn. of reaction mixture: Mg. of isocyanate per g. of protein	% dioxane	*p*H	Protein, mg./cc.	Hycb.: protein ratio[a] (NH₄)₂SO₄ P. mg.	groups	Acetone P. mg.	groups
1	10-Bov. A.	250	25	8.3	12	...	...	49	15
2	10-Bov. A.	240	27.5	8.3	10	49	15	47	14–15
3	10-Bov. A.	145	40	10	18	103	32	99	31
1	BP-Bov. A.	240	27.5	8.3	10	15	4	14	4
2	BP-Bov. A.	145	45	10	17	73	20	68	19
1	10-HSG.	80	24	8.3	7	2	1	..	...
2	10-HSG.	110	25	8.3	14	8	5	..	...
3	10-HSG.	200	47	8.8	21	14	9	..	...
3a	10-HSG.					25	16	22	15
1	3-HSG.	80	24	8.3	8	1–2	1	..	...
2	3-HSG.	135	20	8.3	15	3	2	..	...
3	3-HSG.	190	46	8.8	27	19	13	18	13
	10-Bov. G.	220	25	8.3	11	5–6	3–4	..	...
	10-Egg A.	110	25	8.2	14	7	1	8	1
	3-Egg A.	100	25	8.2	12	5	1	..	...
1	10-Zein	50	80	8.2	50	...	..	11	2
2	10-Zein	125	80	8.2	17	...	..	15	2
	β-APS.	90	25	8.3	13	3	2	..	...

[a] Calculated as mg. of hydrocarbon radical per g. of protein and as hydrocarbon groups per molecule of protein after purification by two or three precipitations with ammonium sulfate and then by one precipitation with acetone. The following molecular weights were used for the calculations: bovine serum albumin 70,000; bovine serum pseudoglobulin 150,000; horse serum γ-pseudoglobulin 150,000; egg albumin 35,000; zein 39,000; antipneumococcus serum protein 150,000. The theoretical maximum, based on reaction with the ε-amino groups of lysine of the proteins, would be approximately the following number of groups: Bov. A. 68, Bov. G. 80; HSG. 80; Egg A. 12; Zein 0; APS. 80. The molecular weights of the hydrocarbon radicals are as follows: 10-(1,2-benzanthryl-10-) 227; 3-(1,2-benzanthryl-3-) 227; BP-(3,4-benzpyrenyl-5-) 251; β-(β-anthryl-) 177. Preparations 10-HSG. no. 3a and 3-HSG. no. 3 were soluble only in an aqueous medium of *p*H 8 containing 5% dioxane. The zein preparations were dried and then extracted in a Soxhlet apparatus.

nificant compared with the number of hydrocarbon groups present in the serum albumin conjugates which were prepared under less favorable conditions of dioxane concentration. It may be observed from the tables in this article and in the preceding publication,[1] that each additional increase of about 15% in the dioxane content of the reaction mixture above the 15% level led to three- and two-fold increases, respectively, in the extent of conjugation noted with the serum albumin. It is reasonably certain, therefore, that the conjugation of isocyanates with proteins occurs predominantly with the free amino groups of the proteins (as originally considered by Hopkins and Wormall and assumed by us) and that the phenolic hydroxyl, sulfhydryl and other groups, theoretically capable of reacting with isocyanates under certain conditions, are not involved in the coupling process under the experimental conditions specified.

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Summary

Conjugates prepared by the interaction of isocyanates of 1,2-benzanthracene and 3,4-benzpyrene with bovine serum albumin were found to possess the same hydrocarbon content as horse serum albumin conjugates prepared under identical conditions. Only a small number of hydrocarbon groups could be introduced into egg albumin and bovine and horse serum pseudoglobulins without causing marked denaturation of the protein component. β-Anthryl isocyanate was coupled with an antipneumococcus serum to give a new type of "labelled" antibody. No significant degree of coupling was observed with zein.

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