up in ether and washed with a 5% solution of sodium carbonate (no acidic material extracted), with water and finally with a saturated salt solution. The solvent was removed *in vacuo* and the residue, which partly crystallized on standing, was recrystallized from ether–petroleum ether to afford 0.035 g. of XII, m.p. 174–176.5°. A mixed m.p. with an authentic specimen was not depressed. The infrared spectra of the two samples were identical.

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RAHWAY, N. J.

[CONTRIBUTION FROM THE INSTITUTE FOR CANCER RESEARCH AND THE LANKENAU HOSPITAL RESEARCH INSTITUTE]

Preparation and Properties of Some Additional Carcinogen–Protein Conjugates[1]

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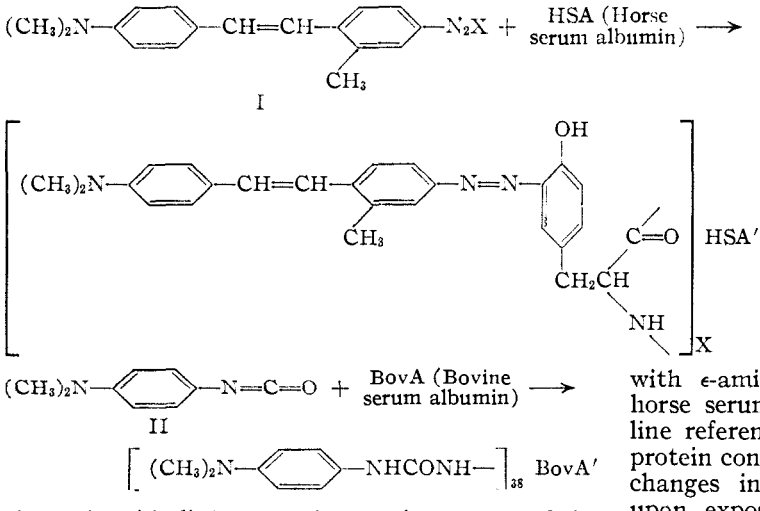
Several additional conjugates have been prepared from horse and bovine serum albumins by combination with isocyanates of 4-dimethylaminostilbene, its 2′-methyl analog, and 2-acetylaminofluorene. Electrophoretic studies on representative conjugates showed a range of mobility constants consistent with theoretical prediction. Studies in the ultracentrifuge were inconclusive because of aggregation. Stilbenyl-protein conjugates linked through the azo group have also been made and *p*-dimethylaminophenyl isocyanate and 9,10-dimethyl-1,2-benzanthryl-3-isocyanate have been combined with ϵ-aminocaproic acid and with serum albumins. The sensitivity of several of the conjugates to light has been studied.

In recent chemical and biological studies on an approach to an immunological defense against carcinogenesis[2–5] explorations were made of the serological specificity of antibodies elicited by conjugates prepared from proteins and carcinogenic stilbenes. An extension of this work has led to the preparation of conjugates in which the haptenic group was altered in two additional ways. In one, conjugation through an azo linkage between the stilbene molecule and the tyrosine molecules of the protein[6] was effected by the action of I on serum albumin. In the other, the styrene unit was eliminated from the stilbenyl component; reaction with the resultant isocyanate II gave a conjugate containing truncated haptenic groups attached through



the carbamido linkage to the ϵ-amino groups of the lysine molecules of the protein. In an attempt to obtain a reference compound for the colorimetric determination of the amount of stilbene introduced into the protein molecule through the azo linkage, the diazonium salt I was conjugated with N-acetyltyrosine. Although the reaction was found to occur, purification of the non-crystalline product has not been achieved. Combination of *p*-dimethylaminophenyl isocyanate II with ϵ-aminocaproic acid afforded a readily crystallizable spectrophotometric reference compound. With the use of this compound, it was found that 38 *p*-dimethylaminophenylcarbamido groups had been introduced into the bovine serum albumin molecule under the chosen experimental conditions.

Additional conjugates containing widely different amounts of 4-dimethylaminostilbene, its 2′-methyl analog and 2-acetylaminofluorene were prepared for further studies[5] of the optimum content of carcinogen needed to confer high prosthetic group activity upon the proteins.

To enable the continuation of investigations on the possibility of protecting animals against carcinogenesis due to the potent 9,10-dimethyl-1,2-benzanthracene,[7] several conjugates have been made by combining the 3-isocyanate[8] of this carcinogen with ϵ-aminocaproic acid and with bovine and horse serum albumins. Solutions of the crystalline reference compound as well as those of the protein conjugates were found to undergo extensive changes in their ultraviolet absorption spectra upon exposure to light. The behavior of these compounds appears to be analogous to that of the parent carcinogen which has been shown to be susceptible to photo-oxidation.[9,10] Satisfactory spectrophotometric analysis of the amount of 9,10-

(1) This research was supported in part by a Grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

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(3) R. M. Peck and H. J. Creech, *ibid.*, **74**, 468 (1952).

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(8) W. M. Smith, E. F. Pratt and H. J. Creech, THIS JOURNAL, **73**, 319 (1951).

(9) J. W. Cook and R. H. Martin, *J. Chem. Soc.*, 1125 (1940).

(10) J. Engelbreth-Holm and S. Iverson, *Cancer Research*, **7**, 372 (1947).

dimethyl-1,2-benzanthracene per molecule of protein was possible only when the conjugates were prepared in low actinic glassware and purified in the absence of light.

Preparations of 9,10-dimethyl-1,2-benzanthryl-3-carbamido horse serum albumin made several years ago, but not described until this time, have been found to be antigenic[11] and also to display pronounced physiological activity in arresting development and in producing chromosomal changes in frogs' eggs and embryos as shown by Green and Creech.[12] In this connection, preparations made, processed and tested in the absence of light did not behave identically with those which were exposed to light.[13] Recently, Dr. Albert Levan working in this Institute has tested some of the hydrocarbon–protein conjugates on Allium and has found that the prosthetic groups exhibit biological activity in this plant.[13]

Electrophoretic studies have demonstrated that representative stilbenyl protein conjugates were reasonably homogeneous since no abnormal spreading of the boundaries was evident. Conjugates containing increasingly greater amounts of carcinogen component exhibited significant increases in mobility in alkaline buffer. Sedimentation studies were inconclusive because of the high degree of aggregation shown by the conjugates.

Experimental

***p*-Dimethylaminophenyl Isocyanate.**[14]—To a vigorously stirred solution of about 60 g. of phosgene in 400 ml. of dry toluene cooled to −20° was added portion-wise a solution of 10.2 g. of *p*-dimethylaminoaniline in 130 ml. of dry toluene. The mixture was refluxed until nearly clear, filtered and concentrated. After distillation *in vacuo*, there was obtained 10.5 g. (86% yield) of low-melting crystalline product, b.p. 90–95° (1 mm.). Crystallization from benzene–petroleum ether gave 6.9 g. of isocyanate, m.p. 34.0–35.5°. Vacuum sublimation of a small sample raised the melting point to 34.5–35.5°.

ε-(4-Dimethylaminophenylcarbamido)-caproic Acid.—To a solution of the sodium salt from 0.65 g. of ε-aminocaproic acid in 15 ml. of water and 7.5 ml. of dioxane[15] was added a solution of 0.75 g. of *p*-dimethylaminophenyl isocyanate in 10 ml. of dioxane. After standing for ten minutes, the solution was filtered, diluted with water, and acidified with dilute sulfuric acid. Filtration gave 1.15 g. (84% yield) of crystals, m.p. 165–167°. Following reprecipitation from a solution of the sodium salt and recrystallization from 50 ml. of absolute ethanol, there was obtained 0.77 g. of an analytical sample, m.p. 166.2–167.2°. *Anal.* Calcd. for $C_{15}H_{23}N_3O_3$: N, 14.85. Found: N, 14.9, 15.0; $E^{1\%}_{1\ cm.}$ 581 at 253 mμ in an aqueous solution of the sodium salt (calculated for the free acid).

ε-(9,10-Dimethyl-1,2-benzanthryl-3-carbamido)-caproic Acid.—A solution of 0.0228 g. of freshly vacuum-sublimed 9,10-dimethyl-1,2-benzanthryl-3-isocyanate[8] in 10 ml. of dioxane was added dropwise to a stirred solution containing the sodium salt from 0.064 g. of ε-aminocaproic acid in 10 ml. of 1:1 dioxane:water. After standing for ten minutes, the solution was acidified and diluted with water to about 60 ml. Refrigeration and filtration gave 0.0264 g. (80% yield) of product, m.p. 164–166° d. with softening at 162° (vac.). Purification by precipitation from an aqueous dioxane solution of the sodium salt which had been clarified with Norit gave an analytical sample of melting point 168.5–170.5° d. with softening at 166° (vac.). *Anal.* Calcd. for $C_{27}H_{33}N_2O_3$: N, 6.54. Found: N, 6.93, 6.97. $E^{1\%}_{1\ cm.}$ 182 at 365 mμ in an aqueous solution of the sodium salt (calculated for the free acid). The same absorption curve was obtained after another recrystallization in low actinic glassware.

Preparation of 2′-Methyl-4-dimethylaminostilbenyl-azo-bovine Serum Albumin.—To a cold, stirred solution of 0.5 g. of 4-dimethylamino-2′-methyl-4′-aminostilbene[2] in 8 ml. of water containing 1.0 ml. of 12 *N* hydrochloric acid, was added dropwise a solution of 0.014 g. of sodium nitrite in about 1 ml. of water. After several minutes stirring at 0°, the solution was added dropwise to a stirred mixture containing 15 ml. of 1 *N* sodium carbonate, 1 g. of bovine serum albumin (Armour), 20 ml. of water, and ice. After 10 minutes additional stirring, the deep wine-colored mixture[16] was dialyzed against distilled water. The solution remained completely clear although the color changed to a dark brown (considerably lighter, however, than the original color). Ammonium sulfate was added through a rotating cellophane sac to give two products: (1) material precipitating up to 2.5 *M* and (2) material precipitating from 2.5–3.0 *M*. After dialysis, each of the two products was brought separately to 2.5 *M* ammonium sulfate and the centrifuged precipitates were combined as one fraction. The combined supernatant was taken to 2.78 *M* ammonium sulfate to give a second fraction of precipitate. After dialysis, the two fractions were brought separately to 2.3, 2.5 and 2.68 *M* ammonium sulfate and the precipitates obtained at each level were combined. Thoroughly dialyzed solutions of the three fractions were compared colorimetrically and by Kjeldahl nitrogen analysis. The color intensities for the three fractions were in the ratio 1:0.64:0.22, and the protein concentrations were 1:1.19:0.85, respectively. The prosthetic group concentrations per molecule of protein were thus in the ratio 1:0.54:0.26, indicating that fractionation had been achieved. It has not been possible to fractionate any conjugate in which the prosthetic group was joined to the protein by a carbamido linkage.

Other Conjugates.—Coupling of the aryl isocyanates with proteins was accomplished as described in the previous paper[2] to give products displaying characteristic absorption spectra. The experimental conditions are presented in Table I.

Electrophoretic and Ultracentrifuge Studies.—The electrophoretic studies were made at 2° in 0.1 *N* veronal buffer at *p*H 8.6. A Klett apparatus with the 92 mm. cell was used. The protein concentration was about 0.5% and the current was maintained at 17 ma. Mobility calculations were based on averages for the ascending and descending boundaries, measured in both the forward and reverse directions. The ultracentrifuge studies of the conjugates were carried out in a Spinco machine.

Results

Spectrophotometric Analysis.—The properties of the conjugates are described in Table I. The values in the last column were calculated by the method described previously.[2] The amounts of prosthetic group per 160 mg. of total nitrogen are about 4% less than these figures in the benzanthracene series and about 8% less with the other conjugates. The absorption spectra of ε-(4-dimethylaminophenylcarbamido)-caproic acid in *N* 0.001 sodium hydroxide and of *p*-dimethylaminophenylcarbamido-bovine serum albumin in water were smooth curves having a single maximum at 250 mμ. Although within the range of protein absorption, analyses of the content of this prosthetic group in the conjugated proteins were satisfactory because the absorptions of the amino acid conjugate and of serum albumin were found to be additive within experimental error.

Upon exposure to light, there was a progressive

(11) H. J. Creech, *Acta Unio int. contra cancrum*, **6**, 451 (1949).

(12) E. U. Green and E. M. H. Creech, *Cancer Research*, **11**, 252 (1951).

(13) Personal communication.

(14) Previously prepared from *p*-dimethylaminobenzazide by H. Staudinger and R. Endle, *Ber.*, **50**, 1042 (1917).

(15) All dioxane used was purified, dry and peroxide-free.

(16) Formation of the wine color and its subsequent change to brown occurred in this and other experiments with and without protection from light, and also in the reaction with N-acetyltyrosine from which no pure product has as yet been obtained.

TABLE I

PREPARATION AND PROPERTIES OF THE CONJUGATES

BovA = bovine serum albumin; HSA = horse serum albumin; S = 4-dimethylaminostilbenyl-4′-carbamido group; 2′-MeS = 2′-methyl-4-dimethylaminostilbenyl-4′-carbamido group; AAF = 2-acetylaminofluorenyl-7-carbamido group; P = *p*-dimethylaminophenylcarbamido group; 9,10-dime-3 = 9,10-dimethyl-1,2-benzanthryl-3-carbamido group; the figure following the abbreviations represents the number of prosthetic groups introduced per molecule of protein; in the 9,10-dime-3 preparations, the addition of the letter D indicates that the conjugates were prepared and processed in the absence of light.

Conjugate	Reaction mixture[a] Mg. Ar-NCO/g. protein	Buffer[b] ml./100 ml.	Properties of conjugates $E^{1\%}_{1\text{ cm.}}$	Abs. max., mμ	Mg. prosthetic group/g. protein
S-HSA-4[c,e]	70	6	22.8[i]	358	16
2′-MeS-BovA-37[c,f]	230	15	180[i]	357	149
AAF-BovA-17[d,e]	90	15	69[j,k]	300	64
AAF-BovA-32[d,g]	175	14	127[j,k]	298	121
P-BovA-38[d,g]	100	15	84.5[j,k]	250	87
9,10-dime-3-HSA-D27[d,g]	140	5	28.9[j]	373	115
9,10-dime-3-HSA-D37[d,g]	210	7	39.2[j]	373	157
9,10-dime-3-BovA-D42[d,g]	200	11	44.4[j]	373	179
9,10-dime-3-HSA-42[d,g,h]	200	11	26.5[j]	370	...

[a] The protein level was maintained at 1–2%. [b] 1:1 *N* $NaHCO_3$:Na_2CO_3. [c] Purified by three ammonium sulfate precipitations. [d] Purified by two ammonium sulfate precipitations and one cold acetone precipitation. [e] 36%. [f] 48% and [g] 40–44% dioxane in the reaction mixture. [h] Value for the number of groups estimated from experimental conditions. [i] Determined in 1:1 dioxane:water. [j] Determined in aqueous solution. [k] Value corrected for protein absorption at this wave length.

decrease in the intensity of absorption of an aqueous solution of the sodium salt of ε-(9,10-dimethyl-1,2-benzanthryl-3-carbamido)-caproic acid. When freshly dissolved in the dark, the free acid displayed a principal absorption maximum at 365 mμ ($E^{1\%}_{1\text{ cm.}}$ 182) and a minor maximum at 350 mμ ($E^{1\%}_{1\text{ cm.}}$ 142). The $E^{1\%}_{1\text{ cm.}}$ values were 148 at 365 mμ and 124 at 350 mμ after exposure to north window light for 25 minutes and 90 and 82 after 90 minutes. When the protein conjugates containing this carcinogen were prepared and processed in the absence of actinic light and absorption determinations were made on solutions obtained by dialysis in the dark from stock preparations kept under ammonium sulfate, the analyses for prosthetic group content were accurate. As was

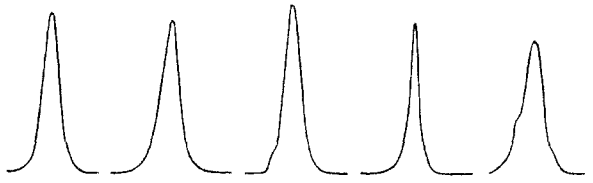

Fig. 1.—Tracings of descending boundaries of normal bovine serum albumin (a), stilbenylcarbamido conjugates of the albumin (b, c, and d) and a mixture of 10 parts of 2′-MeS-BovA-62 with 90 parts of 2′-MeS-BovA-37 (e). The tracings correspond to positions of the boundaries after they had migrated 5 cm. from the starting position. The direction of migration is from right to left.

the case with the carbamido caproic acid derivative a progressive irreversible flattening of the absorption maxima of the protein conjugates could be produced by intermittent exposure to light. The stilbenyl conjugates also required similar precautions[2] whereas, in general, the polycyclic aromatic hydrocarbon prosthetic groups[17] have not shown significant instability toward light.

Electrophoretic and Ultracentrifuge Studies.—These studies were conducted to test the homogeneity of representative protein conjugates in the stilbenylcarbamido series. It was expected that substitution in the ε-amino groups of the protein would increase the acidity of the molecule and consequently increase the mobility in alkaline buffer. Likewise, the weight of the albumin molecule is increased with the result that the sedimentation rate also should be increased.

Under similar experimental conditions, the values for the mobilities of normal bovine serum albumin and the conjugates S-BovA-20, 2′-MeS-BovA-37 and 2′-MeS-BovA-62[2] were found to be 6.7, 7.8, 9.7 and 10.4 × 10^{-5} cm./sec./volt/cm., respectively. It is apparent, therefore, that with an increase in the degree of substitution there was a significant increase in mobility. Furthermore, the mobility appeared to approach a limiting value with 2′-MeS-BovA-62 in which substitution had almost reached the theoretical limit of 66 groups. The electrophoretic homogeneity of the conjugates may be judged by the appearance of the boundaries as shown in Fig. 1. For comparative purposes, the boundary of normal bovine serum albumin (a) and that of a mixture of 10 parts of 2′-MeS-BovA-62 with 90 parts of 2′-MeS-BovA-37 (e) are also included in the figure. The boundaries of the S-BovA-20 and 2′-MeS-BovA-37 conjugates show a slightly greater spread than that of the normal albumin and suggest the presence in each preparation of small amounts of conjugates of higher degrees of substitution. The pronounced spread of the boundary of the artificially prepared mixture indicates, however, that the proportion of highly substituted conjugates occurring as impurities in the S-BovA-20 and 2′-MeS-BovA-37 preparations was less than 10%. The 2′-MeS-BovA-62 preparation revealed less boundary spread than the normal albumin, a property which may be related to the fact that complete coverage of amino groups was being approached. It may be concluded, therefore, that the conjugates were relatively homogeneous from an electrophoretic standpoint and that more than 90% of the molecules within any given preparation were substituted to essentially the same degree.

The sedimentation diagrams, however, revealed that each of the conjugates was very inhomogeneous with respect to particle size due to aggregation of the protein. Unfortunately, therefore, the use of sedimentation analyses was precluded as a test for differences between the sizes of the conjugates and those of the unsubstituted albumin.

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