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

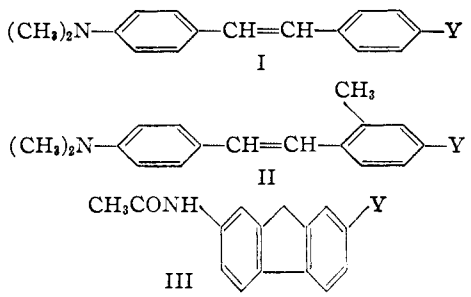
Conjugates Synthesized from Proteins and the Isocyanates of Certain Systemic Carcinogens[1]

BY HUGH J. CREECH AND RICHARD M. PECK

Isocyanates of the systemic carcinogens 4-dimethylaminostilbene, 2′-methyl-4-dimethylaminostilbene and 2-acetylaminofluorene have been conjugated with horse and bovine serum albumins in an aqueous dioxane medium. The numbers of carcinogen groups introduced into the proteins were determined spectrophotometrically using compounds obtained by coupling the aryl isocyanates with ε-aminocaproic acid as reference standards. Variations in experimental conditions produced conjugates containing from 13 to 62 carcinogen groups per molecule of protein.

Previous investigations[2,3] of the possibility of establishing an immunological defense against carcinogen-initiated tumors were conducted with polynuclear aromatic hydrocarbons which produce tumors at the site of application. In those experiments, it was shown that carcinogens conjugated with proteins through a carbamido linkage displayed haptenic activity, *i.e.*, antisera toward the conjugates were reactive *in vitro* toward both the protein and the hydrocarbon components. Theoretically, such antisera should be capable of preventing hydrocarbon carcinogenesis. Satisfactory immunological inhibition of carcinogenesis in experimental animals has not yet been demonstrated presumably because of the overwhelming and localized action of the subsequently applied carcinogens.[2]

In the present study, the emphasis has been shifted to systemic carcinogens which produce tumors at points distant from the site of application. The compounds selected for the first phase of this study were 4-dimethylaminostilbene, 2′-methyl-4-dimethylaminostilbene and 2-acetylaminofluorene (Ia–IIIa). The properties of these compounds have been investigated by Haddow, *et al.*,[4] by Bielschowsky[5] and others. In order to



I

II

III

(a) Y = H; (b) Y = NCO; (c) Y = $NHCONH(CH_2)_5COOH$

attach the carcinogen to the proteins, the isocyanates Ib–IIIb were synthesized.[6] These derivatives have been coupled with horse and bovine serum albumins. Following purification of the resultant conjugates, the numbers of carcinogen groups introduced into the protein were determined by the methods of Creech and Jones.[7] This necessitated the synthesis of the reference compounds Ic–IIIc, prepared by coupling the carcinogen isocyanates with ε-aminocaproic acid under conditions similar to those required for their conjugation with proteins, *i.e.*, in an aqueous dioxane medium at an alkaline *p*H. The compounds Ib–IIIb could not be used as standards for the ultraviolet absorption work because the additional double bond in the isocyanate group disappears upon conjugation with the proteins. The absorption spectra of solutions of reference compounds Ic–IIIc were shown to be unaffected by the addition of protein, thus supporting the validity of the methods of calculation.[8]

When water was used as the solvent for determinations of the absorption spectra of the stilbene–protein conjugates, it was found that, although the various conjugates had maxima often at different wave lengths (335–355 mμ), the wave length of the absorption maximum of any particular conjugate did not change upon exposure to light. In contrast, aqueous solutions of the stilbene amino acid conjugates displayed extremely rapid spectrophotometric changes upon exposure to light. The absorption of these compounds shifted within a few minutes to lower extinctions and to shorter wave lengths as the original *trans*-stilbene configuration changed to *trans–cis* equilibrium mixture.[4]

When 1:1 dioxane–water solutions of the stilbene protein conjugates were analyzed, all the absorption curves were found to be similar, with the wave lengths of the maxima at 355–358 mμ. Since freshly prepared 1:1 dioxane–water solutions of the amino acid reference conjugates also had maxima at 355–358 mμ, comparisons of their intensities of absorption with those of the carcinogen–protein conjugates in 1:1 dioxane–water solution permitted accurate calculations to be made of the amount of stilbene carcinogen combined with protein.

The amino acid and protein conjugates prepared from 2-acetylaminofluorenyl-7-isocyanate (IIIb) had absorption maxima at 294–298 mμ. The absorptions of the amino acid reference compound IIIc and albumin were found to be additive within experimental error. As a result, aqueous solutions of these conjugates were satisfactory for determinations of the absorption spectra and for the subsequent analysis of the amount of carcinogen combined with protein.

Experimental

Preparation of Amino Acid Conjugates. ε-(4-Dimethylaminostilbenyl-4′-carbamido)-caproic Acid and Sodium

(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.

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

(3) H. J. Creech, *et al.*, *Cancer Research*, **7**, 290, 297, 301 (1947).

(4) A. Haddow, R. J. C. Harris, G. A. R. Kon and E. M. F. Roe, *Phil. Trans. Roy. Soc. London*, **241**, 147 (1948).

(5) F. Bielschowsky, *Brit. Med. Bull.*, **4**, 382 (1947).

(6) R. M. Peck and H. J. Creech, THIS JOURNAL, **74**, 468 (1952).

(7) H. J. Creech and R. N. Jones, *ibid.*, **63**, 1661, 1670 (1941).

(8) R. N. Jones and H. J. Creech, *J. Optical Society*, **33**, 209 (1943).

Salt.—A solution of 0.66 g. of ε-aminocaproic acid in 50 ml. of water was neutralized with sodium hydroxide (slight excess) and 55 ml. of dioxane[9] was added. A solution of 0.50 g. (0.0019 mole) of 4-dimethylaminostilbenyl-4'-isocyanate in 60 ml. of dioxane was added gradually to the stirred solution of the amino acid. The mixture was stirred an extra ten minutes, acidified and cooled overnight. The product, removed by filtration, weighed 0.72 g. (96% yield). An analytical sample of the acid crystallized from alcohol melted at 280–285° (vac.). *Anal.* Calcd. for $C_{23}H_{29}N_3O_3$: N, 10.6. Found: N, 10.4, 10.5.

The acid was dissolved in 125 ml. of dilute sodium hydroxide and 75 ml. of dioxane. The hot solution was treated with Norit, filtered, and cooled slowly. The sodium salt was collected by filtration; the yield was 0.52 g. (66%), m.p. about 290°, with darkening near 240°. *Anal.* Calcd. for $C_{23}H_{28}N_3O_3Na$: N, 10.1. Found: N, 9.6, 9.8. $E^{1\%}_{1\,cm.}$ 982_{357} mμ in 1:1 dioxane–*N*/1000 sodium hydroxide (calculated for the free acid).

ε-(2'-Methyl-4-dimethylaminostilbenyl-4'-carbamido)-caproic Acid.—To a stirred solution of the sodium salt of 0.30 g. of ε-aminocaproic acid in 20 ml. of 1:1 water–dioxane there was added slowly a solution of 0.35 g. (0.00126 mole) of 2'-methyl-4-dimethylaminostilbenyl-4'-isocyanate in 15 ml. of dioxane. The product was precipitated by acidification, removed by filtration, redissolved in alkaline aqueous dioxane and again precipitated by acidification. The product weighed 0.43 g. (83.5% yield) and melted at 180.5–181°, resolidified near 200°, and remelted at 244–252° (vac.). *Anal.* Calcd. for $C_{24}H_{31}N_3O_3$: N, 10.3. Found: N, 10.3, 10.2. $E^{1\%}_{1\,cm.}$ 902_{355} mμ in 1:1 dioxane–*N*/1000 sodium hydroxide (calculated for the free acid).

ε-(2-Acetylaminofluorenyl-7-carbamido)-caproic Acid.—To a stirred solution of the sodium salt from 0.50 g. of ε-aminocaproic acid in 150 ml. of 2:1 dioxane–water, there was added dropwise a solution of 0.36 g. (0.00136 mole) of 2-acetylaminofluorenyl-7-isocyanate in 40 ml. of dioxane. The mixture was acidified, diluted, and centrifuged. The product weighed 0.44 g. (82% yield) after washing and drying. A solution of the sodium salt in water was clarified with Norit, an equal volume of dioxane was added, and the excess alkali was neutralized. After the removal of a small amount of precipitate, the filtrate was carefully acidified and cooled. The filtered product weighed 0.40 g. Another precipitation of the acid from an aqueous dioxane solution of the salt gave 0.23 g. (43% yield) of microcrystalline material, m.p. 275–281° dec. (vac.). *Anal.* Calcd. for $C_{22}H_{25}N_3O_4$: N, 10.6. Found: N, 10.3, 10.1. $E^{1\%}_{1\,cm.}$ 759_{294} mμ in *N*/1000 sodium hydroxide (calculated for the free acid).

Preparation of Protein Conjugates.—Crystallized bovine serum albumin (Armour) and crystalline horse serum albumin prepared according to the method of McMeekin[10] were used. The general procedure for the process of conjugation involved the gradual addition of the isocyanate in dioxane solution to a stirred aqueous solution of the albumin containing a quantity of buffer and of dioxane maintained at −5 to −10°. The amounts of the components of the reaction (after the addition of isocyanate) are recorded for each conjugate in Table II.

The reaction mixture was dialyzed against ice-water and then against cold distilled water to eliminate dioxane and buffer. After centrifugation to remove a small amount of insoluble material, the conjugate was precipitated with ammonium sulfate by the rotating cellophane membrane technique of McMeekin.[10] The conjugate was removed by centrifugation, dissolved in water, dialyzed, and centrifuged. The salting-out process was repeated several times. Samples of thoroughly dialyzed solution of the conjugates were analyzed for nitrogen content by the micro-Kjeldahl method and for prosthetic group content by the spectrophotometric procedure. Some of the preparations were purified further by precipitation with acetone as described previously.[7] Only slight changes in the prosthetic group content were noted following this treatment, thus indicating the presence of no more than traces of adsorbed stilbene derivatives on the conjugates. Even the most cautious treatment with acetone often caused moderate to extensive denaturation of the stilbene conjugates. As a result, this method of final purification could not be generally employed. Optically clear solutions containing 5–10 mg. of conjugate per ml. of water always were obtained readily by dialysis and centrifugation of samples of the final salted-out products which were stored routinely in the refrigerator under 2 *M* ammonium sulfate solution.

The conjugation of 2'-methyl-4-dimethylaminostilbenyl-4'-isocyanate with bovine serum albumin to give 2'-MeS-Bov A-62 is described as a representative procedure. A solution of 4.5 g. of bovine serum albumin in 140 ml. of water, 10 ml. each of 1 *N* sodium carbonate and 1 *N* sodium bicarbonate and 55 ml. of purified dioxane was cooled to −5°. To this carefully stirred solution, there was added dropwise over a 1.5-hour period a solution of 1.35 g. of 2'-methyl-4-dimethylaminostilbenyl-4'-isocyanate in 90 ml. of purified dioxane. During this period, the temperature of the reaction flask was reduced to −10°. A suspension appeared for about 15 minutes during the mid-course of the addition of isocyanate but was followed by complete solution of the components of the reaction mixture as the dioxane concentration was increased by addition of the remainder of the isocyanate. Twenty minutes after all the isocyanate had been introduced, the solution was transferred to cellophane sacs and dialyzed for 20 hours against ice-water and 20 hours against cold distilled water to remove the dioxane and buffer. Following removal of a small amount of residue by centrifugation, the conjugate was precipitated from solution in a 17° room by the addition through a rotating cellophane sac of enough ammonium sulfate to make the medium 2.5 molar. The precipitate was collected by centrifugation, dissolved in about 200 ml. of water containing 0.5 ml. of 1 *N* sodium hydroxide, dialyzed at 5° against tap water and then against distilled water. The solution was centrifuged to remove a trace of insoluble material and the conjugate was salted out by bringing the ammonium sulfate concentration to 2.35 molar. The salting-out process was repeated three times. Final precipitation was made at 2.05 molar ammonium sulfate concentration and the centrifuged conjugate was stored in a refrigerator as a suspension in a small amount of this medium. Approximately 4 g. of conjugate was obtained from 4.5 g. of bovine serum albumin.

The other conjugates were prepared in an essentially similar manner. The experimental conditions, properties of the conjugates and codes are presented in Table II.

Results

Spectrophotometric Analysis.—Although the basic principles and method of spectrophotometric analysis of hydrocarbon–protein conjugates developed by Jones and Creech[8] were again applicable, it was found that the procedure required some modifications in general experimental details before it could be used with the present conjugates. The existence of *cis* and *trans* forms of the stilbene molecule and the presence of two atoms of nitrogen in the isocyanates had to be taken

TABLE I

EFFECTS OF SOLVENTS AND TIME OF EXPOSURE TO LIGHT ON THE ABSORPTION SPECTRA OF THE STILBENYL AMINO ACID CONJUGATES

Compound, caproic acid	Solvent	Exposure to light[a]	$E^{1\%}_{1\,cm.}$	λ of Maximum mμ
ε-(4-Dimethylaminostilbenyl-4'-carbamido)-	1:1 Dioxane-*N*/1000 NaOH	0	982	358
	1:9 Dioxane-ethanol	0	1260	358
	3:1 Dioxane-buffer *p*H 10	0	1050	358
	3:1 Dioxane-buffer *p*H 10	30″	880	357
	3:1 Dioxane-buffer *p*H 10	140″	540	331
ε-(2'-Methyl-4-dimethylaminostilbenyl-4'-carbamido)-	1:1 Dioxane-*N*/1000 NaOH	0	902	355
	Dioxane	0	923	358
	N/1000 NaOH	0	872	342
	1:1 Ethanol-*N*/1000 NaOH	0	939	350
	Buffer *p*H 9	0	872	341
	Buffer *p*H 9	6′	588	334
	Buffer *p*H 9	10′	460	310

[a] No attempt was made to regulate the intensity of light.

(9) The dioxane used throughout the work was purified, dry and peroxide-free.

(10) T. L. McMeekin, THIS JOURNAL, **61**, 2884 (1939).

TABLE II

Bov A = 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; the figure following these abbreviations represents the number of prosthetic groups introduced per molecule of protein.

		Reaction mixture					Properties of conjugates					
Prep. no.	Conjugate	Protein, mg./ml.	Mg. ArNCO/ g. protein	Dioxane, %	N, Na_2CO_3, ml./100 ml.	N, $NaHCO_3$, ml./100 ml.	$E^{1\%}_{1\text{ cm.}}$ water	Abs. max. mμ, water	$E^{1\%}_{1\text{ cm.}}$ water–dioxane 1:1	Abs. max. mμ water–dioxane 1:1	Prosthetic group mg. per 160 mg. Of total N	Of protein N
1	2′-MeS-Bov A-13	10.5	250[a]	47	5.3	5.3	55	355	65	355	49	50.5
2	2′-MeS-Bov A-13[b]	19	65	35	3.2	3.2	...	...	66.5	355	50	51.7
3	2′-MeS-HSA-14[b]	19	72	35	3.2	3.2	49	352	71.9	355	53.5	55.3
4	S-HSA-15	11	200[a]	44	5.5	5.5	72	355	79	358	53.8	55.7
5	S-Bov A-20	12	200[a]	42	3	6	63	355	105	357	71.5	75
6	S-HSA-26	15	210	48	3	3	113	335	138	355	94	100
7	S-Bov A-31	15	210	48	3	3	133	340	160	355	109	116
8	S-HSA-34[b]	14.5	220	46	4.8	4.8	134	335	173	355	118	127
9	2′-MeS-HSA-55	15	300	48	3	3	210	345	256	355	194	221
10	2′-MeS-Bov A-62	15	300	48	3.3	3.3	207	350	284	355	215	248
11	AAF-HSA-32	14	175	44	6.8	6.8	128	298	...	...	113	122

[a] Impure isocyanates were used for the preparation of conjugates No. 1, 4 and 5. [b] Conjugates Nos. 2, 3 and 8 were prepared in low actinic glassware. Conjugate No. 11 was purified in addition by acetone precipitation; the values given are corrected for protein absorption at 298 mμ.

into consideration in the calculations. The method of analysis required determinations of the absorption spectra of the amino acid as well as the protein conjugates prepared from the aryl isocyanates. It was soon found that the amino acid conjugates from the stilbenyl isocyanates when dissolved in water, dioxane–water and other solvents rapidly changed from the *trans* to the *cis* form upon exposure to light as was shown by decreasing intensities of absorption and a shift of the maxima to shorter wave lengths (Table I).

The protein conjugates containing the substituted stilbenes were first examined spectrophotometrically in aqueous solution. Although there was no shift in the position of any specific absorption maximum with time, the absorption maxima of the various preparations were found to be located at widely different wave lengths (335–355 mμ). Even when the conjugates were prepared in low actinic glassware or in the dark room in attempts to decrease the *trans–cis* shift, the maxima were found to be at wave lengths similar to those of the corresponding preparations made in the presence of light (Table II). Spectra of the stilbene amino acid conjugates were determined in different solvents and after various times of exposure to light in order to obtain absorption curves with maxima corresponding in wave length to those of the protein conjugates. Calculations of the amount of carcinogen combined with protein were attempted by comparing the intensities of absorption obtained from these data. It was obvious that at least some of these values were incorrect.

It was decided that it would be of interest to conduct some spectrophotometric determinations with aqueous dioxane solutions of the protein conjugates. Since the absorption maxima under these conditions were found to be near 355 mμ, the entire series of protein and amino acid conjugates was analyzed in 1:1 dioxane–water solution. The spectra of all the protein conjugates then displayed maxima in the narrow region of 355–358 mμ. Variations in the *p*H of the solutions had no effect either on the intensity or wave length of the absorption maxima. Since there was no difficulty in determining the intensity of absorption maxima of the amino acid conjugates at these wave lengths, it was relatively simple to compare the spectra of both types of compound. Calculations of the number of prosthetic groups per molecule of protein thus became less complicated and appeared to have an error of less than 5%.

The absorption spectra of ε-(4-dimethylaminostilbenyl-4′-carbamido)-caproic acid and of ε-(2′-methyl - 4 - dimethylaminostilbenyl - 4′ - carbamido)-caproic acid in 1:1 dioxane–water solution are presented in Fig. 1.[11] The crystalline compounds were dissolved in aqueous dioxane in a darkroom and the spectra were determined immediately. The influence of solvents and exposure to light on the spectra of these compounds is given in Table I. The spectra of the protein conjugates containing the stilbene derivatives are given in Figs. 2 and 3. It will be noted that the absorption maxima are at 355–358 mμ indicating that the stilbene component is in a predominantly *trans* configuration. Stock solutions of the conjugates (5–10 mg. per ml. of water) were stable for more than four months under refrigeration since no change occurred in the wave lengths or intensities of their absorption maxima. Extremely dilute solutions decreased somewhat in intensity of absorption in about a month's time although there was no shift in the location of the maxima.

The method for the determination of the number of carcinogen groups per molecule of protein is illustrated below for the conjugate 2′-methyl-4-dimethylaminostilbenyl-4′-carbamido bovine serum albumin-62 (2′-MeS-Bov A-62).

$E^{1\%}_{1\text{ cm.}}$ for ε-(2′-methyl-4-dimethylaminostilbenyl-4′-carbamido)-caproic acid (IIc) (coded as 2′-MeS-NH-CO-NH$(CH_2)_5$COOH) was found to be 902.

$E^{1\%}_{1\text{ cm.}}$ for the prosthetic group (2′-MeS-NH-CO-) becomes $902 \times M_1/M_2$ (where M_1 is the

(11) The figures were kindly drawn by Mr. Reed F. Hankwitz, Jr.

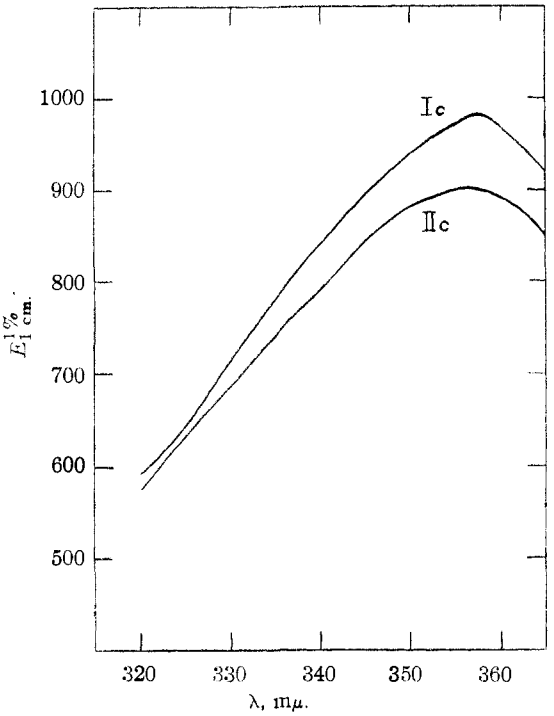


Fig. 1.—Absorption spectra (in 1:1 dioxane–N/1000 sodium hydroxide) of ε-(4-dimethylaminostilbenyl-4′-carbamido)-caproic acid (Ic) and of ε-(2′-methyl-4-dimethylaminostilbenyl-4′-carbamido)-caproic acid (IIc).

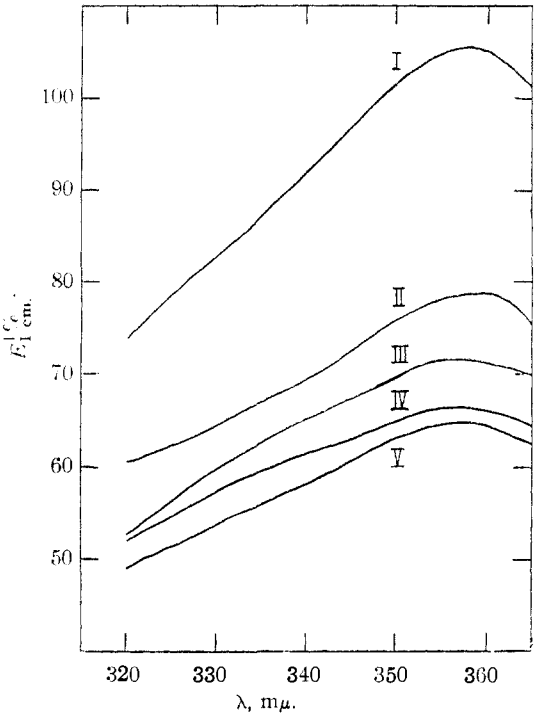


Fig. 2.—Absorption spectra (in 1:1 dioxane–water) of the conjugates S-Bov A-20 (I), S-HSA-15 (II), 2′-MeS-HSA-14 (III) and 2′-MeS-Bov A-13 (IV and V; two preparations).

molecular weight of 2′-MeS-NH-CO-NH$(CH_2)_5$-COOH and M_2 is that of 2′-MeS-NH-CO-) which equals 902 × 409/279 or 1320.

Determination of the number of milligrams of prosthetic group (2′-MeS-NH-CO-) per gram of protein in the conjugate 2′-MeS-Bov A-62 requires an initial calculation based on total nitrogen followed by a correction for the percentage of nitrogen in the introduced stilbene group.

The initial $E^{1\%}_{1\,cm.}$ (based on total nitrogen) for 2′-MeS-Bov A-62 from the curve in Fig. 3 was found to be 284. Thus, there are 284 × 1000/1320 or 215 mg. of 2′-MeS-NH-CO- per 100 ml. of a solution containing 160 mg. of *total* nitrogen (if there were no nitrogen in the prosthetic group, this would be equivalent to one gram of protein). Since there is 10.1% nitrogen in this prosthetic group, the values become 215 mg. of 2′-MeS-NH-CO- per (160 − 21.7) mg. of *protein* nitrogen. Thus, per 160 mg. of protein nitrogen or 1 g. of protein, there is present 215 × 160/138.3 or 248 mg. of 2′-MeS-NH-CO-.

The number of prosthetic groups per molecule of protein in 2′-MeS-Bov A-62 is

$$\frac{248}{1000} \times \frac{\text{molecular weight of bovine serum albumin}}{\text{molecular weight of 2′—MeS—NH—CO—}} = \frac{248 \times 70{,}000}{1000 \times 279} = 62.$$

Attention is drawn to the fact that with the present conjugates, calculations were made in terms of milligrams of prosthetic group (Ar-NH-CO-) per gram of protein whereas in our previous work[7,8] they were established as milligrams of radical (Ar-) per gram of protein. This does not make any difference in the final determination of the number of carcinogen groups per molecule of protein but does permit a significant correction to be made for the presence of two nitrogen atoms in the stilbene prosthetic groups.

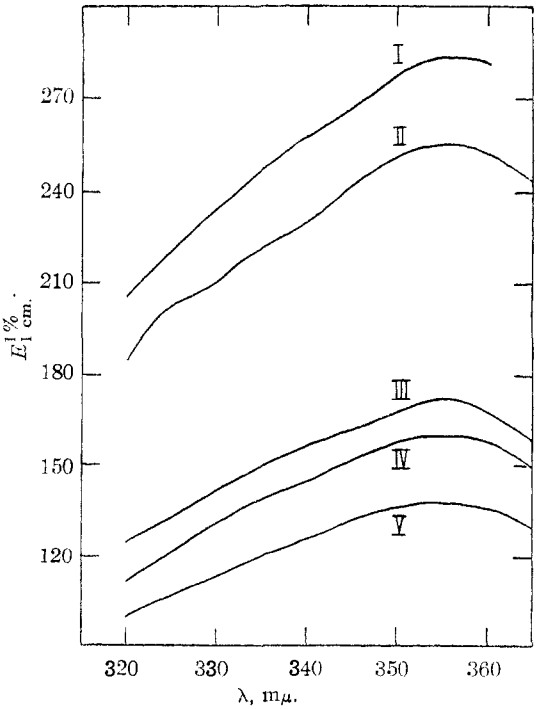


Fig. 3.—Absorption spectra (in 1:1 dioxane–water) of the conjugates 2′-MeS-Bov A-62 (I), 2′-MeS-HSA-55 (II), S-HSA-34 (III), S-Bov A-31 (IV) and S-HSA-26 (V).

The absorption spectrum of ε-(2-acetylaminofluorenyl-7-carbamido)-caproic acid (IIIc) is given in Fig. 4 and that of a conjugate of the isocyanate (IIIb) with horse serum albumin is given in Fig. 5.

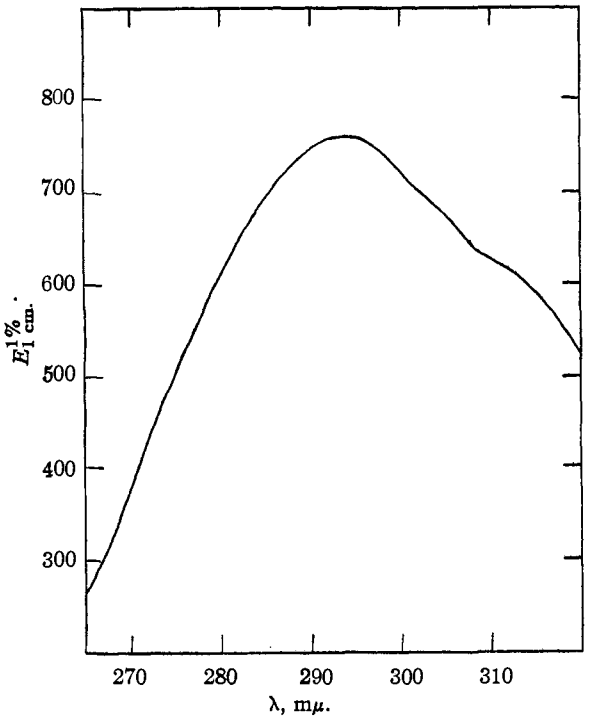


Fig. 4.—Absorption spectrum of ε-(2-acetylaminofluorenyl-7-carbamido)-caproic acid in *N*/1000 sodium hydroxide.

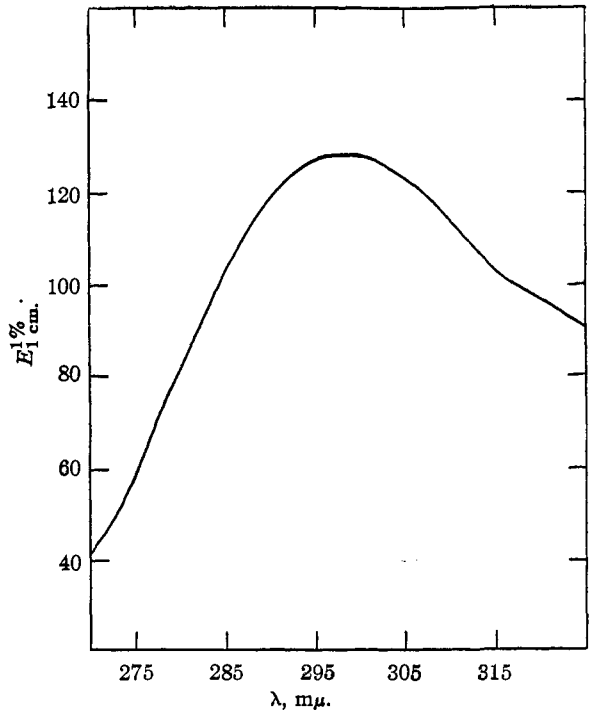


Fig. 5.—Absorption spectrum of the conjugate AAF-HSA-32 in water.

In both instances, the determinations were made on aqueous solutions of the compounds. Although the absorption maximum is below 300 mμ,[8] it was demonstrated that the analysis was satisfactory since the absorptions of the amino acid conjugate and albumin were additive within experimental error (Fig. 6) because of the high intensity of absorption of the fluorene nucleus. As was the case with the earlier work[7,8] with the polycyclic aromatic hydrocarbons, the spectrophotometric determinations on the fluorenyl conjugates were relatively uncomplicated and accurate.

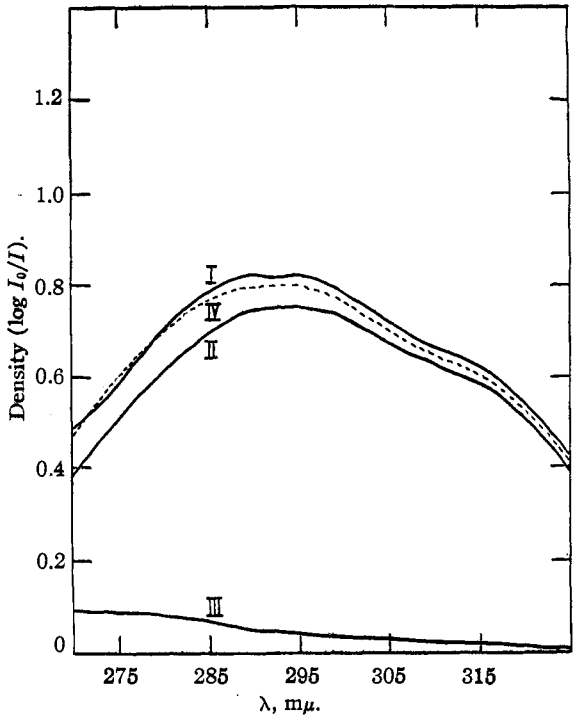


Fig. 6.—Absorption spectra of a solution containing 0.1 mg./ml. of horse serum albumin and 0.01 mg./ml. of ε-(2-acetylaminofluorenyl-7-carbamido)-caproic acid in *N*/1000 sodium hydroxide (I), a solution of ε-(2-acetylaminofluorenyl-7-carbamido)-caproic acid (0.01 mg./ml. in *N*/1000 sodium hydroxide) (II) and a solution of horse serum albumin (0.1 mg./ml.) (III). Arithmetic summation of (II) and (III) gives the broken curve (IV).

It seems desirable to mention several points of interest in Table II. Difficulties were encountered in the earlier part of the work with the purification and instability of the stilbenyl isocyanates. Three preparations (Nos. 1, 4 and 5) of the conjugates were made with impure isocyanates. In spite of a highly favorable dioxane content in the reaction mixture (prep. No. 1), the use of this isocyanate gave a 2'-MeS-Bov A conjugate containing the same number of milligrams of carcinogen per gram of protein as that obtained when about one-quarter the amount of pure isocyanate was used (prep. No. 2). Pure isocyanate as used for the preparation of conjugates Nos. 6 and 7 under essentially the same experimental conditions as those for preparations Nos. 4 and 5 with impure isocyanate resulted in the introduction of considerably more carcinogen groups into the proteins.

Comparison of preparations Nos. 6 with 7 and 9 with 10 indicates that, under similar experimental conditions, a slightly greater number of carcino-

gen groups was introduced into bovine serum albumin than into horse serum albumin. Increases both in the ratio of isocyanate to protein and in the percentage of dioxane in the reaction mixture caused the expected increase in the extent of conjugation. Paralleling previous experience with the polycyclic aromatic hydrocarbons,[7,8] the more dioxane-soluble 2'-methyl-4-dimethylaminostilbenyl-4'-isocyanate reacted more completely with the proteins than the less soluble 4-dimethylaminostilbenyl-4'-isocyanate. The greater reactivity of both stilbenyl isocyanates compared with those of the polycyclic aromatic hydrocarbons[7] led to a more complete reaction with the proteins. Preparations Nos. 9 and 10 contained 55 and 62 groups, respectively, whereas the greatest number of hydrocarbon groups introduced into the serum albumins was 38 by the reaction with 1,2-benzanthryl-10-isocyanate under equally favorable experimental conditions.

Horse and bovine serum albumin conjugates containing both high and low numbers of the two stilbenyl prosthetic groups were required for the immunological studies.[12] The blue fluorescence exhibited by solutions of these conjugates was applied advantageously in the serological work.

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(12) H. J. Creech and H. F. Havas, in preparation.

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Isocyanates of Dimethylaminostilbenes and Acetylaminofluorene[1]

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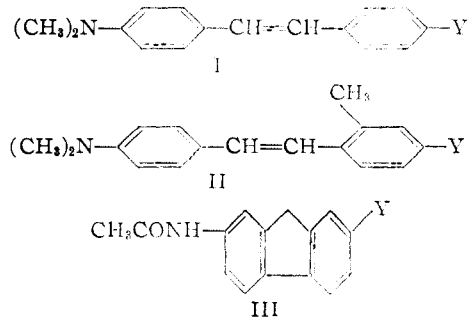
The synthesis of isocyanates of certain systemic carcinogens represents the initial step in an extension of a research program involving immunochemical studies of carcinogen–protein conjugates. 4-Dimethylaminostilbenyl-4'-isocyanate was prepared in 61% yield by the interaction of phosgene with 4-dimethylamino-4'-aminostilbene. 4-Dimethylamino-4'-amino-2'-methylstilbene, which had been prepared in 36% yield by a two-step process from 4-nitro-*o*-xylene and *p*-dimethylaminobenzaldehyde, was converted into the corresponding isocyanate in 79% yield. 2-Acetylaminofluorenyl-7-isocyanate was obtained from the amine in 63% yield. The isocyanates were characterized by conversion into their ethyl urethans.

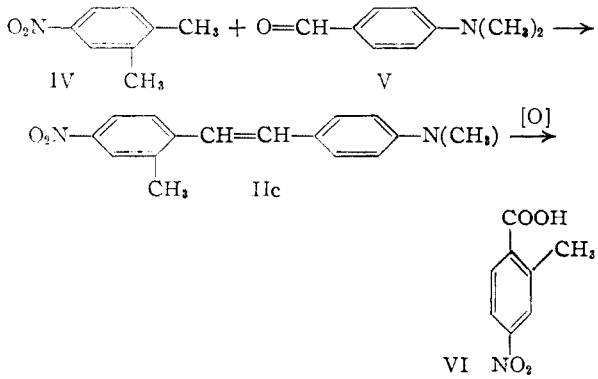
From studies of the potentialities of carcinogen–protein conjugates in the protection of animals against localized carcinogenesis due to polycyclic aromatic hydrocarbons,[2] it was thought desirable to investigate conjugates containing as prosthetic groups the systemic carcinogens 4-dimethylaminostilbene (Ia), 2'-methyl-4-dimethylaminostilbene (IIa) and 2-acetylaminofluorene (IIIa).[3]



(a) Y = H; (b) Y = NCO; (c) Y = NO_2; (d) Y = NH_2

Because of the advantages shown by the carbamido linkage in effecting conjugation of carcinogens with proteins,[4] the synthesis of isocyanates (Ib–IIIb) of these carcinogens was undertaken. In this series, the isocyanate group, obtained in the standard manner by the reaction of the appropriate amine with phosgene,[5] was introduced at a location distant from the biologically important substituted amino group. The conjugation of these isocyanates with amino acids and proteins is described in the accompanying paper[6]; the immunochemical properties of the conjugates are described elsewhere.[7]

The preparation of the compound IIc was carried out in the following manner



That the condensation of *p*-dimethylaminobenzaldehyde (V) had occurred with the methyl group para to the nitro group of 4-nitro-*o*-xylene (IV) to give IIc was demonstrated by oxidation of the

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

(3) A. Haddow, R. J. C. Harris, G. A. R. Kon and E. M. F. Roe, *Phil. Trans. Royal Soc. of London*, **241**, 147 (1948); F. Bielschowsky, *Brit. Med. Bull.*, **4**, 382 (1947).

(4) H. J. Creech and R. N. Jones, THIS JOURNAL, **63**, 1661, 1670 (1941); H. J. Creech, E. L. Oginsky and F. S. Cheever, *Cancer Research*, **7**, 290 (1947).

(5) H. J. Creech, THIS JOURNAL, **63**, 576 (1941).

(6) H. J. Creech and R. M. Peck, *ibid.*, **74**, 463 (1952).

(7) H. J. Creech and H. F. Havas, in preparation.