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The Structure of the Enol Acetate Derivatives of Steroid 7- and 11-Ketones

BY RALPH HIRSCHMANN AND N. L. WENDLER

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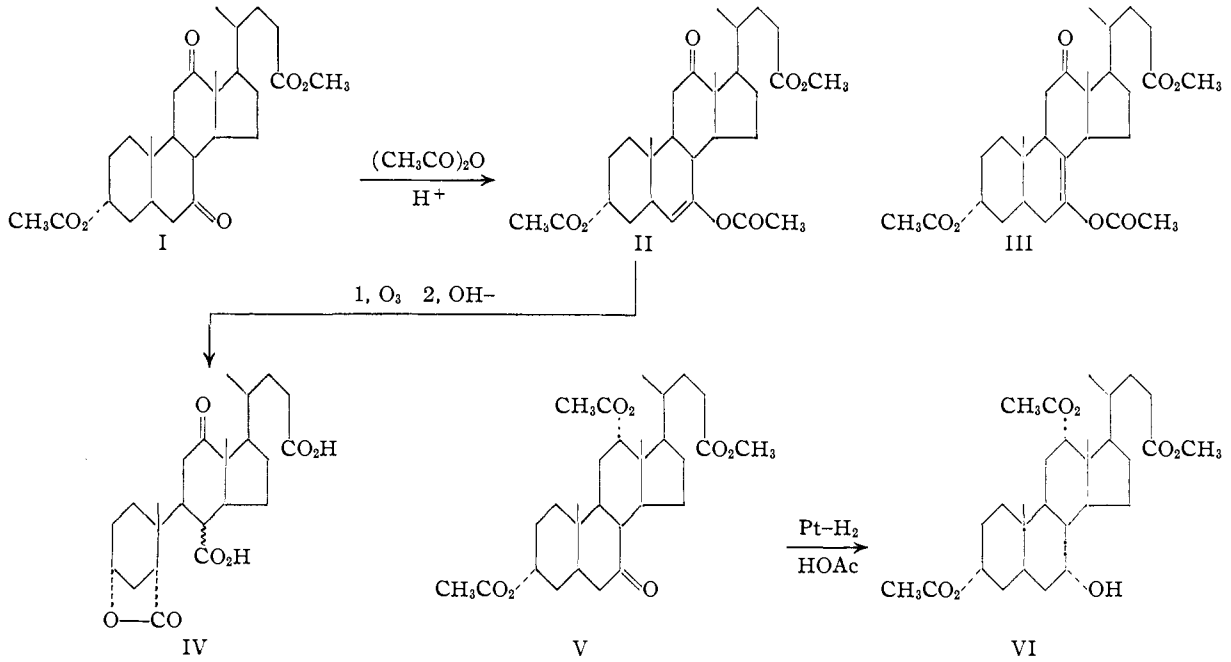
The center of unsaturation in the enol acetate derivatives of normal steroid 7- and 11-ketones has been demonstrated to be positionally Δ^6 and Δ^9, respectively.

A novel synthetic route to desoxycholic acid *via* hydrogenation of the enol acetate derivative of methyl 3α-acetoxy-7,12-diketocholanate (I) was reported recently.[1] In connection with this work it became of interest to ascertain the position of the carbon–carbon double bond in this substance.

Apart from the indeterminate influence of the geometry of the system on the position assumed by the double bond, the conjecture appeared reasonable that second-order conjugation effects would favor the Δ^7-structure III over the Δ^6-structure II.[2] This premise seemed to be supported by the original observation that 20-keto steroids with acetic anhydride and *p*-toluenesulfonic acid afford the Δ^{17}-acylate derivative.[3] Recently, however, it has been found that under certain conditions 20-keto steroids can also form the Δ^{20}-derivatives.[4] that Δ^7-steroids lacking the 7-acetoxy group are generally not prone to reduction.[5] Furthermore, in certain instances of hydrogenation of a double bond involving C_8, rear attack has occurred, affording products having the C_8-hydrogen α-oriented.[6] While such attack from the rear appears to be favored in most hydrogenations of steroid double bonds, it is not favored in the reduction of normal 7-keto steroids (*cis* A/B), as evidenced by the hydrogenation of methyl 3α,12α-diacetoxy-7-ketocholanate (V) to give methyl 3α,12α-diacetoxy-7α-hydroxycholanate (VI).

In view of these considerations the futility of an *a priori* structure assignment to the enol acetate derivatives of unsymmetrically substituted ketones in the absence of definitive experimental proof was apparent.[7]



The steric course of the reduction of the 7-enol acetate derivative which yields methyl 3α,12α-diacetoxycholanate with a β-hydrogen at C_8 also does not provide unequivocal evidence for the position of the double bond. It is well recognized

For the purpose of establishing the double bond position in the enol acetate derivative of a 7-keto steroid, methyl 3α-acetoxy-7,12-diketocholanate (I) was chosen for reasons of the excellent crystallizing properties of its 7-acylate derivative. The failure, moreover, of 12-keto functions to partici-

(1) R. Hirschmann, M. Brown and N. L. Wendler, THIS JOURNAL, **73**, 5373 (1951).

(2) For a discussion and application of this concept to triterpenoid olefins see: D. H. R. Barton and C. J. W. Brooks, *J. Chem. Soc.*, 257 (1951); see also: P. B. D. De La Mare, E. D. Hughes and C. K. Ingold, *ibid.*, 17 (1948).

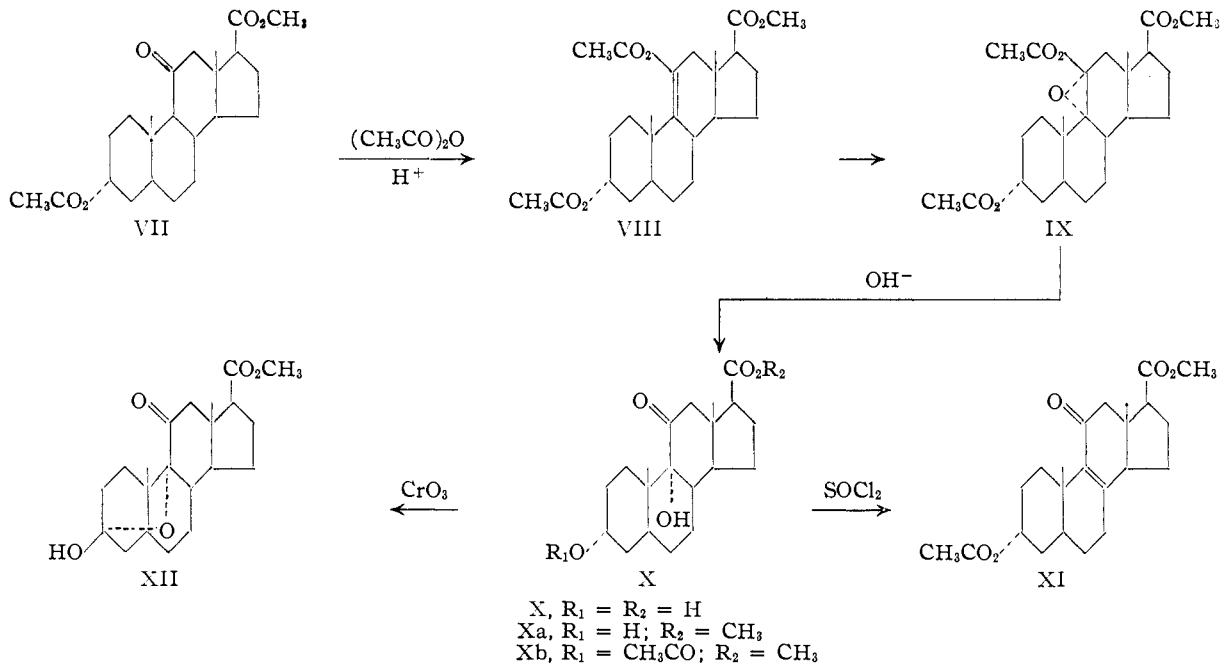
(3) C. W. Marshall, T. H. Kritchevsky, S. Lieberman and T. F. Gallagher, THIS JOURNAL, **70**, 1837 (1948).

(4) R. B. Moffett and D. I. Weisblat, *ibid.*, **74**, 2183 (1952); see also, H. Vanderhaeghe, E. R. Katzenellenbogen, K. Dobriner and T. F. Gallagher, *ibid.*, **74**, 2810 (1952).

(5) See for example: M. Fieser and L. Fieser, "Natural Products Related to Phenanthrene," 3rd Edition, Reinhold Publ. Corp., New York, N. Y., 1949, p. 243.

(6) A. Serini and W. Logemann, *Ber.*, **71**, 186 (1938); F. Sondheimer, R. Yashin, G. Rosenkranz and C. Djerassi, THIS JOURNAL, **74**, 2696 (1952).

(7) In this connection see: E. H. Man, F. C. Frostick, Jr., and C. Hauser, *ibid.*, **74**, 3228 (1952). Also W. G. Dauben, J. F. Eastham and R. A. Micheli, *ibid.*, **73**, 4496 (1951).



X, $R_1 = R_2 = H$
Xa, $R_1 = H$; $R_2 = CH_3$
Xb, $R_1 = CH_3CO$; $R_2 = CH_3$

pate in the enol acetylation reaction had previously been established.[1] Methyl 3α,7-diacetoxy-12-ketocholenate, derived from the parent diketone I with either acetic anhydride or isopropenyl acetate in the presence of *p*-toluenesulfonic acid, was ozonized at −70° in chloroform solution. Saponification followed by acidification of the decomposed ozonide produced the lactonic diacid IV possessing two carboxyl functions by direct titration and exhibiting a well-defined carbonyl band in the infrared spectrum at 5.67 mμ characteristic of 5-membered ring lactones.[8] The structure of the enol acetate derivative of I is therefore established as II, possessing a double bond between carbon atoms 6 and 7. It is to be presumed, moreover, that other related 7-keto-5β-steroids would similarly give rise to Δ^6-enol acetate derivative.

In view of the directional course of enol acetylation of the 7-keto function, it was of additional interest to ascertain the course of this reaction as applied to an 11-keto system. The formation of the enol acetate of an 11-keto steroid was first reported by Koechlin, Garmaise, Kritchevsky and Gallagher.[9] This derivative was subsequently assigned a Δ^9-structure on the basis of the non-reactivity of the 11-acylate grouping with perbenzoic acid under the conditions whereby the corresponding Δ^{17}-acylate function was readily epoxidized.[10] These authors, however, did not provide experimental confirmation for their provisional assignment. The desirability of obtaining experimental proof for the structure of the 11-enol acetate grouping was re-emphasized by the observed directional course taken by the enol-acetylation at 7 together with our own experience that enol acetates at 11 can indeed be smoothly epoxidized under more rigorous conditions.

Methyl 3α-acetoxy-11-ketoetiocholanate (VII) was readily converted to its 11-enol acetate derivative VIII and the latter in turn quantitatively epoxidized with perbenzoic acid in benzene solution at 40° to give the crystalline oxido acetate IX. This oxide on alkaline hydrolysis and subsequent esterification yielded methyl 3α-acetoxy-9α-hydroxy-11-ketoetiocholanate (Xb). The ketol Xb on treatment with thionyl chloride in pyridine[11] was transformed to the α,β-unsaturated ketone, methyl 3α-acetoxy-11-keto-Δ^8-etiocholenate (XI), λ_{max} 253 mμ, log ϵ 3.93. This sequence of transformations unequivocally establishes the Δ^9-structure VIII for the enol acetate derivative of the 11-keto steroid in question. It is to be presumed, moreover, that related 11-keto steroids would behave in the same directional sense to give similarly constituted Δ^9-enol acylate derivatives.

The structure of the ketol Xa as well as its configuration at C_9 were conclusively established by oxidation with chromic acid to give methyl 3β-hydroxy-3α,9α-oxido-11-ketoetiocholanate (XII), m.p. 175–176°, found to be identical with the same compound prepared by Heymann and Fieser[12] by another route.

The conversion of Xb to XII constitutes a novel variation in reaction sequence leading to 3α,9α-oxide bridging, a phenomenon characteristic of the normal steroid series.

The structure of the 9α,11α-oxido acetate (IX) follows, *inter alia*, from the established configuration at C_9 (see above) and the mechanics for the directional opening with alkali of oxido ester systems. It is interesting to note in this connection, moreover, that if perchance the mechanics of the oxide scission

(8) R. Rasmussen and R. Brattain, THIS JOURNAL, **71**, 1073 (1949).

(9) B. A. Koechlin, D. L. Garmaise, T. H. Kritchevsky and T. F. Gallagher, *ibid.*, **71**, 3262 (1949).

(10) T. H. Kritchevsky, D. L. Garmaise and T. F. Gallagher, *ibid.*, **74**, 483 (1952).

(11) This reagent has been recently employed by H. Heymann and L. F. Fieser (*ibid.*, **73**, 5252 (1951)), for the conversion of methyl 3α-acetoxy-9α-hydroxy-11-ketocholanate to 3α-acetoxy-11-keto-Δ^8-cholenate.

(12) H. Heymann and L. F. Fieser, *ibid.*, **73**, 4054 (1951). We are grateful to Drs. Heymann and Fieser for providing us with a sample of this compound for comparison purposes.

had uniquely involved displacement at C_9 of a 9β,-11β-oxide structure that 3α,9α-oxido-11-ketoetiocholanic acid would have been the expected product rather than X.[13]

Experimental[14]

Ozonolysis of Methyl 3α,7-Diacetoxy-12-keto-Δ⁸-cholenate.—A 1.50-g. sample of II,[1] m.p. 120–122°, in 100 cc. of chloroform was ozonized at −70° and the ozonide decomposed with peroxide as described by Wilms.[15] After removal of the solvent the oily product was dissolved in ethyl acetate and the filtered solution extracted with a 5% aqueous solution of sodium bicarbonate. The alkaline extracts were treated with an excess of hydrochloric acid and after addition of salt the acidic material was extracted with ethyl acetate. The washed organic layer afforded 0.59 g. of amorphous material which could not be crystallized and was consequently saponified. The hydrolysis mixture was acidified to congo red with dilute aqueous hydrochloric acid and refrigerated for two days to allow separation of the acidic reaction product. After separation of the aqueous supernatant layer (fraction A) by decantation, the oily residue was washed free of mineral acid and digested with ether. From the filtered ethereal solution 0.120 g. of a solid which melted at *ca.* 145° could be crystallized by the addition of petroleum ether; λ_{max}^{chf}, 3–4, 5.67 and 5.86 mμ.

Anal. Calcd. for $C_{24}H_{34}O_7$ (IV): C, 66.34; H, 7.89; neut. equiv., 217.2; sapon. equiv., 144.8. Found: C, 66.57; H, 8.03; neut. equiv., 210; sapon. equiv., 145.

From the aqueous layer (fraction A) an additional 0.130 g. of the solid acid was obtained.

Treatment of IV with an excess of an ethereal solution of diazomethane gave an oil having $\lambda_{max}^{CCl_4}$ 5.65, 5.75 and 5.85 μ and only a *weak* carbonyl harmonic at 3.0 μ.

Methyl 3α,12α-Diacetoxy-7α-hydroxycholanate (VI).—Hydrogenation of 1.79 g. of methyl 3α,12α-diacetoxy-7-ketocholanate (V), m.p. 114.5–115.5°, was carried out in 75 cc. of acetic acid over 0.4 g. of platinum oxide at atmospheric pressure and room temperature. After 1 hour and 20 minutes 94% of the theoretical amount of hydrogen had been absorbed. Concentration of the filtered solution followed by crystallization from methanol–water gave 1.5 g. of the ester, m.p. 137–141°. One further recrystallization from the same solvent pair raised the m.p. to 143.5–145.5°. The mother liquor from the initial crystallization yielded an additional 0.1 g. of the triol diacetate, m.p. 140–142°. An analytical sample, m.p. 143–145°, was prepared from the latter by repeated recrystallization from methanol–water.

Anal. Calcd. for $C_{29}H_{46}O_7$: C, 68.75; H, 9.15. Found: C, 68.99; H, 9.20.

A 0.1-g. sample of the above diacetate, m.p. 143.5–145.5°, was saponified with an excess of 5% methanolic potassium hydroxide and the free acid precipitated with dilute hydrochloric acid. Recrystallization from acetone–water gave cholic acid, m.p. 202–204°, identified by mixed m.p. determination and examination in the infrared spectrum.

Methyl 3α,11-Diacetoxy-Δ⁹⁽¹¹⁾-etiocholenate (VIII).—The enol acetate was prepared from the keto ester VII[16] with acetic anhydride essentially as described previously for the preparation of II. In this manner 5.0 g. of VII, m.p. 151–152°, afforded after chromatographic purification of the crude reaction product 5.1 g. of amorphous and oily eluates. The fractions eluted with petroleum ether (2.8 g.) were crystallized from ether–petroleum ether and then recrystallized twice from methanol to afford the enol acetate as large prisms, m.p. 168–171°; λ_{max}^{chf} 5.73, 7.78 and 6.07 mμ.[17] An analytical sample melted at 169–172°, $[\alpha]^{25}D$ +112° (chf.).

Anal. Calcd. for $C_{25}H_{36}O_6$ (VIII): C, 69.42; H, 8.39. Found: C, 69.66; H, 8.21.

The mother liquors from the above recrystallizations as well as the fractions obtained from the benzene eluates were combined and retreated with acetic anhydride to give additional amounts of the enol acetate.

Methyl 3α,11β-Diacetoxy-9α,11α-oxidoetiocholanate (IX).—The oxide compound was prepared from 0.924 g. of the enol acetate VIII, m.p. 164–170°, by treatment with a benzene solution containing a fourfold excess of perbenzoic acid at 40° for about 30 hours. The benzene solution was diluted with ether and gave after removal of acidic components with alkali 0.940 g. (98% yield) of IX, m.p. 197–201° (capillary m.p.). The compound crystallized from acetone–petroleum ether as plates, capillary m.p. 201.5–203°, $[\alpha]^{27}D$ +88.8° (chf.).

Anal. Calcd. for $C_{25}H_{36}O_7$: C, 66.94; H, 8.09. Found: C, 67.10; H, 8.09.

Methyl 3α,9α-Dihydroxy-11-ketoetiocholanate (Xa).—A suspension of 0.576 g. of the above oxido acetate in 25 cc. of methanol was refluxed with a solution of 2.7 g. of potassium hydroxide in 66 cc. of water for 3.5 hours; within five minutes all of the starting material had gone into solution. Most of the methanol was removed *in vacuo* and the solution—diluted with water—was added dropwise to 25 cc. of cold dilute hydrochloric acid to precipitate the crude acid X.

An ether–methanol solution of the acid was treated directly with an ethereal solution of *diazomethane* to afford Xa (0.462 g., 89% yield from IX) as an amorphous solid which could not be crystallized from methanol but which readily crystallized on trituration with hot petroleum ether to give 0.421 g. (81% yield) of the crystalline methyl ester, m.p. 163–166°. An analytical sample melted at 166–167.5°, $[\alpha]^{23}D$ +91.9° (chf).

Anal. Calcd. for $C_{21}H_{32}O_5$: C, 69.20; H, 8.85. Found: C, 69.18; H, 8.62.

Methyl 3α-Acetoxy-9α-hydroxy-11-ketoetiocholanate (Xb).—The pure ketol methyl ester Xa (0.390 g.) was treated with 4 cc. each of pyridine and acetic anhydride at room temperature overnight. After removal of the solvents *in vacuo* the product was dissolved in ether and washed successively with dilute aqueous hydrochloric acid, water, a 5% aqueous sodium carbonate solution, water, and finally with a saturated solution of sodium chloride. The residue obtained after evaporation of the solvent, (0.40 g.) was readily crystallized from ether–petroleum ether to yield 0.245 g. of transparent plates, m.p. 156–158°, λ_{max}^{nu} 2.82, 2.93, 5.75, 5.79 and 5.86 mμ. A second crop 0.091 g., m.p. 157–158°, raised the yield to 77%. The analytical sample was prepared by an additional recrystallization from the same solvent pair yielding Xb, m.p. 157.5–158°, $[\alpha]^{23}D$ +108° (chf.).

Anal. Calcd. for $C_{23}H_{34}O_6$: C, 67.95; H, 8.43. Found: C, 68.36; H, 8.27.

Methyl 3α-Acetoxy-11-keto-Δ⁸⁽⁹⁾-etiocholenate (XI).—To a chilled solution of 0.093 (0.23 millimole) of the ketol Xb in 0.6 cc. of anhydrous pyridine was added 0.1 cc. (1.53 millimoles) of thionyl chloride and the mixture was allowed to stand at room temperature for 45 minutes. The mixture was chilled, treated with ice, and the solid which formed on scratching was collected yielding 0.082 g. (91% yield) of XI, $\lambda_{max}^{CH_3OH}$ 253 mμ, log ε 3.91. The product was recrystallized from ether–petroleum ether until m.p. and extinction remained constant. The analytical sample thus obtained as long slender prisms melted at 148–150°, $\lambda_{max}^{CH_3OH}$ 254 mμ (3.93,[18] $[\alpha]^{23}D$ +187.5° (chf).

Anal. Calcd. for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30. Found: C, 70.82; H, 8.40.

Methyl 3β-Hydroxy-3α,9α-oxido-11-ketoetiocholanate (XII).—To a chilled solution of 0.075 g. (0.206 millimole) of Xa in 1.1 cc. of glacial acetic acid was added 0.1 cc. of water and 0.84 cc. of a 0.348 molar solution of chromium trioxide in 95% acetic acid. After 17 hours at about 5° the mixture was kept at room temperature for 4 hours. The mixture was diluted and 0.050 g. of XII (67% yield), m.p. 165–171°, was removed by filtration. The crude product was taken

(13) Compare the conversion of scopolamine to scopoline with alkali: J. Gadamer, *Arch. Pharm.*, **239**, 294 (1901); R. Willstätter and E. Berner, *Ber.*, **56**, 1079 (1923).

(14) All m.ps. were taken on a micro hot-stage and corrected unless otherwise indicated.

(15) H. Wilms, *Ann.*, **567**, 96 (1950).

(16) J. von Euw, A. Lardon and T. Reichstein, *Helv. Chim. Acta*, **27**, 1287 (1944).

(17) It is noteworthy that the di- secondary double bond in methyl 3α,7-diacetoxy-12-keto-Δ⁸-cholenate shows a λ_{max} at 5.95 mμ in contrast to the 6.07 mμ band in the Δ⁹⁽¹¹⁾-enol acetate (*cf.* also ref. 5b).

(18) Heymann and Fieser (ref. 11) took note of the fact that the 3α-acetoxy-11-keto-Δ⁸⁽⁹⁾-cholenates also showed an unusually low extinction coefficient (log ε 3.9).

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.

Acknowledgment.—We are indebted to Mrs. H. Gager and Mr. E. Ball of our Physical Measurement Division for the titrations reported herein and to Mr. R. Miller for technical assistance.

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[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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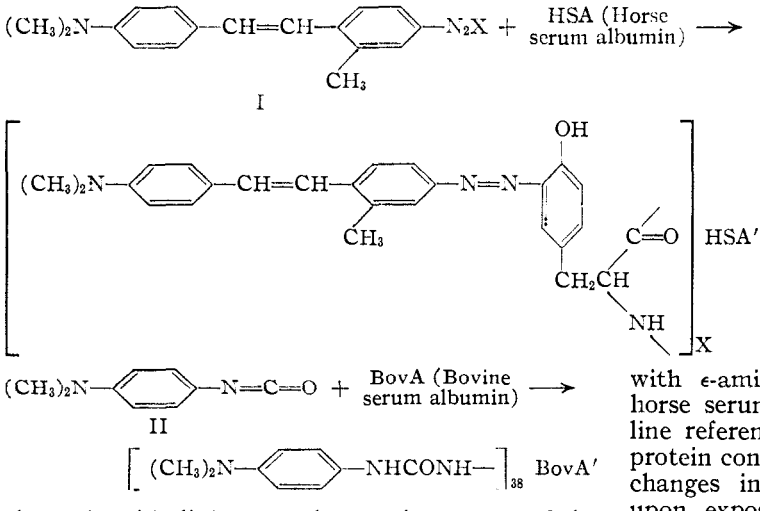
Several additional conjugates have been prepared from horse and bovine serum albumins by combination with isocyanates of 4-dimethylaminostilbene, its 2′-methyl analog, and 2-acetylaminofluorene. Electrophoretic studies on representative conjugates showed a range of mobility constants consistent with 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.

(2) H. J. Creech and R. M. Peck, THIS JOURNAL, **74**, 463 (1952).

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

(4) H. J. Creech, *Cancer Research*, **12**, 557 (1952).

(5) H. J. Creech, H. F. Havas and J. Andre, *ibid.*, in press.

(6) M. Heidelberger and F. E. Kendall, *J. Exper. Med.*, **62**, 467 (1935).

(7) M. J. Shear, *Am. J. Cancer*, **33**, 499 (1938).

(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).