Steroid-Protein Conjugates

II. PREPARATION AND CHARACTERIZATION OF CONJUGATES OF BOVINE SERUM ALBUMIN WITH PROGESTERONE, DEOXYCORTICOSTERONE, AND ESTRONE

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Conjugates of bovine serum albumin with progesterone, deoxycorticosterone, and estrone have now been synthesized as an extension of a program to prepare and characterize steroid-protein conjugates with the ultimate purpose of testing their ability to stimulate the formation of antihormonal antibodies (1).

The steroid derivatives which were coupled to albumin are shown in Fig. 1. The synthetic approach followed was similar to that described earlier (1), except for the preparation of Compound I which was synthesized via the analogous pregnenolone derivative, according to the following scheme:

pregnenolone → pregnenolone 20-(O-carboxymethyl)oxime
→ pregnenolone 20-(O-carboxymethyl)oxime methyl ester
→ progesterone 21-(O-carboxymethyl)oxime methyl ester
→ progesterone 20-(O-carboxymethyl)oxime (1)

The oxidation of pregnenolone 20-(O-carboxymethyl)oxime methyl ester was accomplished by two different techniques: the Oppenauer oxidation and chromic acid oxidation (Jones' solution) (2), the latter being the method of choice. A more satisfactory method for the preparation of testosterone 3-(O-carboxymethyl)oxime is included in the "Experimental" section of this paper.

All derivatives were coupled to the protein by means of the mixed aldehydure technique (1, 3).

The conjugates described herein were found to be antigenic in rabbits, the antibodies being specific for the steroid portion of the molecule. During the preparation of this paper, Sehon et al. (4) reported the synthesis of an estrone-protein conjugate by methods differing from those described here.

EXPERIMENTAL

Preparation of Steroid-Protein Conjugates

Pregnenolone 20-(O-carboxymethyl)oxime—A solution of 10.45 gm. (0.033 mole) of pregnenolone and 10.45 gm. (0.082 mole) of (O-carboxymethyl)hydroxyamine hydrochloride (5) in 617 ml. of ethanol containing 46.5 ml. of 2 N KOH was refluxed for 3 hours. The reaction mixture was reduced to a small volume in a vacuum, water was added (about 150 ml.), and the pH adjusted to 10 to 10.5 with 2 N KOH. After being extracted with ethyl acetate twice, the aqueous phase was acidified with concentrated hydrochloric acid to pH 2 and placed in the refrigerator for 24 hours. The precipitate was collected by filtration and washed with water: yield, 13 gm. (92 per cent); m.p. 191–192° with decomposition. It was recrystallized from ethyl acetate; yield 12 gm. (85 per cent); m.p. 204–205° with decomposition; [α]25° 0° ± 5° (2 per cent in methanol). The infrared spectrum (Nujol) showed a band at 1730 cm.⁻¹ (C=O of carboxyl) and no absorption at 1685 cm.⁻¹ (20-keto C=O).

C29H35O4N
Calculated: Neutral equivalent 389, C 70.92, H 9.05, N 3.60
Found: Neutral equivalent 389, C 70.96, H 9.04, N 3.80

Progesterone 20-(O-carboxymethyl)oxime Methyl Ester—A solution of 0.68 gm. (1.8 mmoles) of pregnenolone 20-(O-carboxymethyl)oxime in 50 ml. of ethyl acetate was treated with a solution of diazomethane in 10 ml. of ether prepared from 1.2 gm. (10.0 mmoles) of N-nitroso-N-methylurea. Gas evolution was apparent. The reaction mixture was allowed to stand overnight at room temperature. Excess diazomethane was decomposed by adding a small amount of glacial acetic acid. The reaction mixture was evaporated to dryness under reduced pressure, yielding 0.60 gm. (85 per cent) of crystalline product, m.p. 191–192°. Recrystallization from methanol yielded material of unchanged melting point; [α]25° 0° ± 2° (17.0 mg. in 2.0 ml. of ethanol). The infrared spectrum showed a band at 1750 cm.⁻¹ (C=O of an ester).

C30H37O4N
Calculated: C 71.43, H 9.24
Found: C 71.14, H 9.18

Progesterone 20-(O-carboxymethyl)oxime (by Oppenauer Oxidation)—A solution of 2.57 gm. (6.4 mmoles) of pregnenolone 20-(O-carboxymethyl)oxime methyl ester in 95 ml. of dry acetone and 25 ml. of dry benzene was heated to boiling and treated with 4.73 gm. (19.2 mmoles) of purified aluminum isopropoxide dissolved in 100 ml. of dry benzene. The reaction mixture became cloudy and, after heating under reflux for 8 hours, was transferred to a separatory funnel with an additional 25 ml. of benzene and washed with 10 ml. of 50 per cent Rochelle salt solution. The two-phase mixture was freed of the suspended solid by filtration through Celite.

The benzene layer was separated, washed three times with water, dried over sodium sulfate, and evaporated to dryness. The semisolid residue weighing 1.62 gm. was dissolved in 10 ml. of benzene and purified by passing through a Florisil column. Recrystallization of the purified material from acetone-skellysolve B yielded 1.43 gm. (56 per cent), m.p. 122–136°. Repeated
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recrystallization gave a crystalline product, m.p. 138-140°; 
\([\alpha]_D^{25} +123^\circ \pm 2^\circ \) (20.0 mg. in 2.0 ml. of ethanol). The infrared spectrum (Nujol) showed a band at 1670 cm.\(^{-1}\) (conjugated C=O).

A solution of 2.18 gm. (5.2 mmoles) of progesterone 20-(O-carboxymethyl)oxime methyl ester in 78.5 ml. of methanol was treated with 8.7 ml. of NaOH (final normality of base, 0.1 N) and allowed to stand at room temperature. Aliquots of 0.4 ml. were drawn at 1-hour intervals, diluted with water, and examined for ester content with a quantitative hydroxamic acid-ferric per- 

trum (Nujol) showed bands at 1725 cm.\(^{-1}\) (C=O of carboxyl) and a maximum at 249 μm (E 15,890). The infrared spec-

Fig. 1. Steroid derivatives used for the preparation of steroid-protein conjugates

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1 The abbreviations used are: Tris, tris(hydroxymethyl)amino-

methane; \(-\)DNP, \(-\)dinitrophenyl.

2 The specific rotation of bovine serum albumin at pH 11.1 is \([\alpha]_D^{25} -75.8^\circ \pm 2^\circ\).

Preparation of Progesterone 20-Albumin Conjugate—Proges-

terone 20-(O-carboxymethyl)oxime, 1.2 gm. (3.05 mmoles), and 0.75 ml. of 0.60 gm., 3.05 mmoles) of tri-n-butylamine were dis-

olved in 30 ml. of dioxane and, after the solution was cooled to 8°, 0.40 ml. (0.45 gm., 3.05 mmoles) of isobutylichlorocarbonate was added. The reaction was allowed to proceed for 35 minutes at 8°, after which the mixture was added in one portion to a well stirred, cooled solution of 4.2 gm. (0.06 mmole) of bovine serum albumin in a mixture of 110 ml. of water, 80 ml. of dioxane, and 4.8 ml. of 0.01 N NaOH (pH 9). The solution became turbid but after about 20 minutes it was completely clear. A check of the pH revealed that it had fallen to 6.8. It was brought back to 7.5 by the addition of 1.75 ml. of 0.1 N NaOH. Stirring was continued for another 30 minutes after which another 0.5 ml. of NaOH was added and the reaction allowed to proceed for a total of 4.5 hours. The solution was then dialyzed overnight against running water, the pH adjusted to 4.5 with NaHCO\(_3\), and the resulting precipitate collected by centrifugation after being stored in the cold for 24 hours. The conjugate was redissolved in 200 ml. of water and brought to pH 7 by the addition of 0.05 N NaOH. After the addition of 300 ml. of cold acetone the conjugate was reprecipitated by adjusting the pH to 5 with NaHCO\(_3\). After 3 hours in the refrigerator, the conjugate was recovered by centrifugation and subjected to two similar reprecipitations, after which it was redissolved at pH 7, dialyzed against running water, and lyophilized: yield, 3.6 gm.; \([\alpha]_D^{25} -41.9^\circ \pm 2^\circ\) (15.5 gm. in 5.0 ml. of 0.05 M Tris buffer, brought to pH 11).\(^{2}\)

Assumed molecular weight 77,400; 20 of 60 NH\(_2\) groups substituted

Calculated: NH\(_2\)-N 0.72, ratio of NH\(_2\)-N to total N 0.049

Found: NH\(_2\)-N 0.66, total N 13.87, ratio of NH\(_2\)-N to total N 0.048, moisture 5.8

Deoxycorticosterone 21-Hemisuccinate—A solution of 1.0 gm. (3.0 mmoles) of deoxycorticosterone and 1.0 gm. (10.0 mmoles) of succinic anhydride in 10 ml. of dry pyridine was refluxed for 4.5 hours. The reaction mixture was evaporated to dryness under reduced pressure, and the semisolid residue dissolved in chloroform, washed three times with water, dried over sodium sulfate, and evaporated to dryness. The residue was reprecipitated twice from acetone, yielding 1.12 gm. (86 per cent) of product, m.p. 200-202°. Two further recrystallizations, one from bene-

sene-hexane, and one from acetone gave pure compound, m.p.
Preparation of Deoxycorticosterone 21-albumin Conjugate—Deoxycorticosterone 21-hemisuccinate, 4.4 gm. (10.34 mmoles) and 2.40 ml. (1.91 gm., 10.34 mmoles) of tri-n-butylamine were dissolved in 100 ml. of dioxane and, after the solution was cooled to 11°, 1.27 ml. (1.41 gm., 10.34 mmoles) of isobutylchlorocarbonate were added. The reaction was allowed to proceed for 20 minutes at 4°, after which the mixture was added in one portion to a well stirred, cooled solution of 7.0 gm. (0.10 mmoles) of bovine serum albumin in 900 ml. of 1:1 water-dioxane and 17.8 ml. of n NaOH (resulting pH 9.5). Gas evolution and turbidity became apparent. The pH fell to 6.8 after 15 minutes and an additional 7.2 ml. of n NaOH were added to bring the pH back to 8.5. After 2 hours the pH was 7.0. Stirring and cooling were continued for a total of 4 hours. The solution was dialyzed overnight against running water and then brought to pH 4.5 with n HCl. The precipitate was collected by centrifugation and centrifugation and the acetone treatment was repeated twice. The third acetone extract was found to contain no detectable amount of estrone. The conjugate was taken up in 300 ml. of water and the pH was adjusted with n NaOH to 7.8. A small amount of solid which remained undissolved was removed by centrifugation and discarded. The supernatant liquid was dialyzed against running water for 8 hours and lyophilized, yielding 6.8 gm. of conjugate, [α][α]D -40.0° ± 2° (14.9 mg. in 5.0 ml. of 0.05 M Tris buffer, pH 11.3).

Assumed molecular weight 78,500; 20 of 60 NH2 groups substituted

Calculated: NH2-N 0.72, total N 14.95, ratio of NH2-N to total N 0.048

Found: NH2-N 0.69, total N 13.80, ratio of NH2-N to total N 0.050, moisture 10.2.

Preparation of Estrone 17-(O-carboxymethyl)oxime—A solution of 5 gm. (17.4 mmoles) of testosterone, 5 gm. (39.3 mmoles) of (O-carboxymethyl)hydroxylamine (5), 21 ml. of 2 N NaOH, and 250 ml. of ethanol was refluxed for 3 hours. It was reduced in volume in a vacuum, diluted with 3 times its volume of water, and adjusted to pH 8 with dilute NaOH. Unchanged testosterone was removed by ether extraction after which the aqueous layer was acidified and the oxime extracted into ethyl acetate. After drying over anhydrous sodium sulfate the ethyl acetate was removed in a vacuum leaving a crystalline residue which was recrystallized from a minimal quantity of ethyl acetate; yield, 4.7 gm. (75 per cent), m.p. 179-181° (reported (1) m.p. 179-181°).

Characterization of Steroid-Protein Conjugates

Ultraviolet Spectra—Fig. 2 shows the ultraviolet spectra of the three conjugates and the corresponding steroid derivatives, each accompanied by a spectrum of the albumin. The optical density values corresponding to the absorption maxima provided a basis for the estimation of the degree of steroid substitution in each conjugate with calculations and assumptions described in the previous paper (1). The results are listed in Table I.

The curve for the estrone conjugate deserves additional comment. Since the chromophoric groups of both the steroid and the protein moieties absorb in the same spectral region, i.e. with a maximum at 275 mμ, we would expect the curve to reflect a simple additive process but instead, its maximum occurs at 281 mμ. This bathochromic effect can be explained by an increased participation of the unshared electron pairs of the phenolic hydroxyl groups in the resonance of the aromatic systems of the chromophores. Why increased participation should occur in

204-206°; [α][α]D +158° ± 2° (13.0 mg. in 2 ml. of ethanol). The ultraviolet spectrum of the solution in 0.05 M Tris buffer (pH 8.5) showed a maximum at 210 mμ (e 16,610).

C23H32O6

Calculated: Neutral equivalent 430, C 69.72, H 7.96

Found: Neutral equivalent 430, C 69.65, H 7.74.
FIG. 2. Ultraviolet spectra of conjugates and related substances in 0.05 M Tris buffer, pH 8.5. The broken line curves represent values obtained by subtracting the absorption of bovine serum albumin from that of the conjugates. Concentrations: Progesterone conjugate (P-20-BSA), 6.4 mg. per 100 ml.; deoxycorticosterone conjugate (D-21-BSA), 4.2 mg. per 100 ml.; estrone conjugate (E-17-BSA), 46.7 mg. per 100 ml.; progesterone 20-(O-carboxymethyl)oxime, (P-20-CMO), 1 mg. per 100 ml.; deoxycorticosterone-21-hemisuccinate (D-21-HS), 1.4 mg. per 100 ml.; estrone 17-(O-carboxymethyl)oxime (E-17-CMO), 5.7 mg. per 100 ml. In each case the concentration of bovine serum albumin (BSA) equaled that of the conjugate.

To determine the degree of destruction of the respective DNP derivatives under the conditions of acid hydrolysis. Destruction of the DNP lysolec was considerably enhanced by the presence of the steroid-protein conjugates during acid hydrolysis, amounting to as much as 35 per cent, while only 10 to 15 per cent loss occurred when mixtures of DNP-lysine with the albumin were hydrolyzed under identical conditions. Some representative

| TABLE II |
| Recovery of ε-DNP-lysine after acid treatment in presence and absence of conjugates or bovine serum albumin* |
| Protein or conjugate | Recovery % |
| None | 95 |
| Bovine serum albumin | 90 |
| Deoxycorticosterone 21-albumin | 72 |
| Progesterone 20-albumin | 68 |
| Estrone 17-albumin | 65 |

* A solution of 1 mg. of ε-DNP-lysine (with or without conjugate or bovine serum albumin) was heated with 6 N HCl in a sealed tube at 118° for 18 hours. The recovered ε-DNP-lysine was estimated spectrophotometrically as previously described (1).

| TABLE III |
| Electrophoretic mobilities of conjugates |
| Protein | Mobility x 10^-5 |
| Bovine serum albumin | 7.46 |
| Progesterone 20-albumin | 6.65 |
| Deoxycorticosterone 21-albumin | 6.11 |
| Estrone 17-albumin | 7.94 |

| TABLE IV |
| Comparison of infrared spectral characteristics of conjugates with those of related steroids |

The absorption bands listed here are present only in the steroid albumin conjugates and do not appear in the infrared spectrum of bovine serum albumin. Additional bands common to both the conjugate and the albumin are present at 1175, 980, and 975 cm.⁻¹.

| Conjugates | Related steroids |
| Progesterone 20-albumin | Progesterone 20-(O-carboxymethyl)oxime |
| 940 weak | 947 medium |
| 1080 weak | 1090 medium |
| Deoxycorticosterone 21-albumin | Deoxycorticosterone 21-hemisuccinate |
| 940 weak | 947 medium |
| Estrone 17-albumin | Estrone 17-(O-carboxymethyl)-oxime |
| 1110 weak | 1120 strong |
| 1065 weak | 1075 medium |
| 953 weak | 940 medium |
| 840 weak | 857 strong |
| 820 weak | 823 strong |

The conjugate is not at all apparent. An attempt was made to relate this phenomenon to the lower net positive charge of the conjugate relative to that of unsubstituted albumin. Acetylated bovine albumin was prepared (9) and its ultraviolet spectrum compared with that of bovine albumin. They were found to be essentially identical (cf. 10). The charge effect is thus eliminated and the bathochromic shift observed in the spectrum of the estrone conjugate must, therefore, be due to an interaction between the steroid and the tyrosine and/or tryptophan residues of the protein, the nature of this interaction being unknown at this time.

Determination of Number of Steroid Residues by Dinitrophenylation—The conjugates and albumin were dinitrophenylated by the method of Sanger (11), hydrolyzed, and the amount of ε-DNP-lysine estimated spectrophotometrically. The procedure and calculations were the same as previously described (1), except that the recovery of lysine on acid hydrolysis was determined in a more rigorous manner. Mixtures of ε-DNP-lysine with albumin and conjugates of progesterone, deoxycorticosterone, and estrone were heated with 6 N HCl at 118° for 18 hours.
values are listed in Table I. As a result of these studies the estimates of steroid substitution by this technique agree more closely with data derived from other methods (Table I) whereas in Paper I of this series (1) the values were consistently higher.

Electrophoretic Mobilities—Table III lists the mobilities of the albumin and the conjugates in 0.02 M phosphate buffer, pH 7.4. Mixtures of each conjugate with albumin were easily resolved electrophoretically. The mobilities of the conjugates of progesterone and deoxycorticosterone were 1.2 times greater, and the mobility of the estrone conjugate 1.6 times greater than that of the albumin.

Infrared Spectra—The conjugates were also characterized by means of their infrared spectra since, as was shown in the earlier paper (1), some characteristic stretching frequencies of the steroid moieties are still apparent in the fingerprint region. The absorption bands are listed in Table IV along with those of some related steroid derivatives. The spectra of all conjugates were determined as Nujol mulls with a Perkin-Elmer model 21C spectrophotometer.

**REFERENCES**

9. **SANGER, F., Biochem. J., 45, 565 (1949).**