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:

\[
\text{pregnenolone} \rightarrow \text{pregnenolone 20-} \text{(O-carboxymethyl)oxime} \\
\rightarrow \text{pregnenolone 20-} \text{(O-carboxy} \text{methyl)oxime methyl ester} \\
\rightarrow \text{progesterone 20-} \text{(O-carboxymethyl)oxime methyl ester} \\
\rightarrow \text{progesterone 20-} \text{(O-carboxymethyl)oxime (I)}
\]

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 sulhydryl 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

\textit{Pregnenolone 20-} \textit{(O-carboxymethyl)oxime—A solution of 10.45 gm. (0.033 mole) of pregnenolone and 10.45 gm. (0.082 mole) of (O-carboxy} \textit{methyl)hydroxylamine hydrochloride (6) 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); \(\text{n} \text{D}^0 - 3^0 \pm 3^0 \) (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).

\textit{Progesterone 20-} \textit{(O-carboxymethyl)oxime Methyl Ester—A solution of 0.68 gm. (1.8 mmoles) of pregnenolone 20-(O-carboxymethyl)oxime methyl ester 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\textdegree. Recrystallization from methanol yielded material of unchanged melting point; \(\text{n} \text{D}^0 - 3^0 \pm 2^0 \) (17.0 mg. in 2.0 ml. of ethanol). The infrared spectrum showed a band at 1750 cm\(^{-1}\) (C=O of an ester).

\textit{Conjugates of Conjugates with Progesterone, Deoxy-}

\textit{C_{21}H_{30}O_6N_2}

Calculated: \(C, 71.43; H, 9.24\)

Found: \(C, 71.14; H, 9.18\)

\textit{Progesterone 20-} \textit{(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-n-butanol yielded a semi-solid congruent with a sample of progesterone 20-(O-carboxymethyl)oxime (2).

\textit{Conjugates of Conjugates with Progesterone, Deoxy-}

\text{N}_{20}\text{H}_{30}

Calculated: \(C, 70.92; H, 9.05\)

Found: \(C, 70.96; H, 9.04\)

The infrared spectrum showed a band at 1685 cm\(^{-1}\) (20-keto C=O).
recrystallization gave a crystalline product, m.p. 138-140°; $[a]_D^2 = +123^\circ \pm 2^\circ$ (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 $\text{NaOH}$ (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 perchlorate method (6). After 3 hours, about 97 per cent of the ester was saponified. The reaction mixture was evaporated to a smaller volume, diluted with water, and extracted with two 50-ml portions of ether. The ether extract was found to contain about 0.04 gm. of progesterone. The aqueous layer was brought to pH 2 with 8.7 ml. of $\text{HCl}$. The resulting precipitate after standing over-night in the refrigerator, the solution was acidified to pH 2 with $\text{HCl}$, and then enough water was added to precipitate the product. The latter was extracted into ethyl acetate, which was washed three times with water, dried over sodium sulfate, and evaporated to dryness. The residue was recrystallized twice from acetone, yielding 1.12 gm. (86 per cent) of product, m.p. 113-125°. To complete isomerization the product was dissolved in 300 ml. of cold acetone the conjugate was reprecipitated by adjusting the pH to 5 with $\text{HCl}$, and the resulting precipitate collected by filtration, washed three times with water, dried over sodium sulfate, and redissolved in chloroform, washed three times with water, dried over sodium sulfate, and evaporated to dryness. The residue was reprecipitated twice from acetone, yielding 1.2 gm. (88 per cent) of product, m.p. 200-202°.

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 recrystallized twice from acetone, yielding 1.12 gm. (86 per cent) of product, m.p. 200-202°. Two further recrystallizations, one from benzene-hexane, and one from acetone gave pure compound, m.p. 213-214°; $[a]_D^2 = -75.8^\circ \pm 2^\circ$.

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

1 The abbreviations used are: Tris, tris(hydroxymethyl)aminomethane; $\epsilon$-DNP, $\epsilon$-dinitrophenyl.

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

Preparation of Progesterone 20-Albumin Conjugate—Progesterone 20-(O-carboxymethyl)oxime, 1.2 gm. (3.05 mmoles), and 0.75 ml. (0.80 gm., 3.05 mmoles) of tri-$n$-butylamine were dissolved 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 $\text{NaOH}$ (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 gm. of $\text{NaOH}$. Stirring was continued for another 30 minutes after which another 0.5 ml. of $\text{NaOH}$ 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 $\text{HCl}$, 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 $\text{NaOH}$. After the addition of 300 ml. of cold acetone the conjugate was precipitated by adjusting the pH to 5 with $\text{HCl}$. After 3 hours in the refrigerator, the conjugate was recovered by centrifugation and subjected to two similar precipitations, after which it was redissolved at pH 7, dialyzed against running water, and lyophilized: yield, 3.6 gm.; $[a]_D^{25} = -41.9^\circ \pm 2^\circ$ (15.5 gm. in 5.0 ml. of 0.05 M Tris buffer, brought to pH 11).”

Assumed molecular weight 77,400; 20 of 60 $\text{NH}_2$ groups substituted

Calculated: NH$_2$N 0.72, total N 14.89, 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

May 1959

B. F. Erlanger, F. Borek, S. M. Beiser, and S. Lieberman 1091
204-206°; [α]D^20 +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 249 nm (ε 16,610).

\[
C_{39}H_{42}O_6
\]

Calculated: Neutral equivalent 430, C 69.72, H 7.96
Found: Neutral equivalent 430, C 69.65, H 7.74

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 17.8 gm. (0.25 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 product precipitated and, after storage in the cold overnight, was collected by centrifugation. The solid was suspended in 200 ml. of water and redissolved by adjusting the pH to 5.5 with N NaOH. Reprecipitation was accomplished by adding 400 ml. of cold acetone and bringing the pH to 5.0 by the addition of N HCl. With volumes of acetone smaller than that previously reported (1) and by effecting precipitation by suitable adjustment of the pH, a more soluble product is obtained. The reprecipitation process was repeated twice after which the residue was redissolved in 200 ml. of water (pH adjusted to 7.0 with N NaOH), dialyzed against running water, and lyophilized. The product weighed 14.0 gm., [α]D^20 = -40.9° ± 2° (14.9 mg. in 5.0 ml. of 0.05 M Tris buffer, pH 11).

Assumed molecular weight 78,250; 20 of 60 NH₂ groups substituted
Calculated: NH₂-N 0.72, total N 14.35, ratio of NH₂-N to total N 0.050
Found: NH₂-N 0.67, total N 12.77, ratio of NH₂-N to total N 0.052, moisture 11.0

 Estrone 17-(O-carboxymethyl)oxime—This compound was prepared by the method of Huffman et al. (9), and also by the following method which was found to be more satisfactory for the preparation of moderate quantities of material. A solution of 5 gm. (18.5 mmoles) of estrone, 5 gm. (39.2 mmoles) of (O-carboxymethyl)hydroxylamine (5), 22.5 ml. of 2 N KOH, and 250 ml. of ethanol was refluxed for 3 hours. The alcohol was removed in a vacuum, 500 ml. of water were added, the pH was adjusted to 8.5, and the aqueous solution then extracted twice with volumes of 100 ml. of ethyl acetate. Acidification of the aqueous layer yielded a precipitate which was collected by filtration; yield, 6.5 gm. (97 per cent). Recrystallization from ethyl alcohol yielded 6.4 gm. (95 per cent, m.p. 188-189° (decomposed) (reported (8) m.p. 188°).

Preparation of Estrone 17-albumin Conjugate—Estrone 17-(O-carboxymethyl)oxime, 2.06 gm. (6.0 mmoles) and 2.86 ml. (2.28 gm., 12.0 mmoles) of tri-n-butylamine were dissolved in 90 ml. of dioxane. The solution was cooled and treated with 0.83 ml. (0.70 gm., 6.0 mmoles) of isobutylchlorocarbonate. Precipitation sometimes occurs after addition of tri-n-butylamine. This precipitate redissolves upon addition of isobutylchlorocarbonate. The reaction was allowed to proceed in the cold for 30 minutes and then the mixture was added in one portion to a stirred, cooled solution of 7.0 gm. (0.10 mmoles) of bovine serum albumin in 183.5 ml. of water, 123.5 ml. of dioxane, and 7.0 ml. of N NaOH. Stirring and cooling were continued for 4 hours, the pH remaining at 7 throughout the reaction. The solution was dialyzed against running water for 17 hours and brought to pH 4.6 with N HCl. The resulting precipitate was allowed to stand in the cold overnight and then was collected by centrifugation. The product was suspended in 200 ml. of water, dissolved by bringing the pH to 7.0 with a small amount of N NaOH, and reprecipitated by the addition of 300 ml. of cold acetone followed by adjustment of the pH to 4.5 with N HCl. The precipitate was collected by 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]D^20 = -40.8° ± 2° (24.5 mg. in 5.0 ml. of 0.05 M Tris buffer, pH 11.3).

Calculated: 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 0.068
Found: NH₂-N 0.69, total N 13.80, ratio of NH₂-N to total N 0.050, moisture 10.2

 Testosterone 3-(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 curves 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 273 μm, one would expect the curve to reflect a simple additive process but instead, its maximum occurs at 281 μm. 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
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.

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.

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.\(^{-1}\).

### Table I

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Ratio of NH(_2) to N</th>
<th>Ultraviolet spectra</th>
<th>Dinitrophenylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone 20-albumin</td>
<td>20</td>
<td>12</td>
<td>21</td>
</tr>
<tr>
<td>Deoxycorticosterone 21-albumin</td>
<td>20</td>
<td>23</td>
<td>19</td>
</tr>
<tr>
<td>Estrone 17-albumin</td>
<td>20</td>
<td>26</td>
<td>20</td>
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### Table II

<table>
<thead>
<tr>
<th>Protein or conjugate</th>
<th>Recovery</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>95</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>90</td>
</tr>
<tr>
<td>Deoxycorticosterone 21-albumin</td>
<td>72</td>
</tr>
<tr>
<td>Progesterone 20-albumin</td>
<td>68</td>
</tr>
<tr>
<td>Estrone 17-albumin</td>
<td>65</td>
</tr>
</tbody>
</table>

* A solution of 1 mg. of \(\epsilon\)-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 \(\epsilon\)-DNP-lysine was estimated spectrophotometrically as previously described (1).

### Table III

<table>
<thead>
<tr>
<th>Protein or conjugate</th>
<th>Mobility (\times 10^5) pH = 7.4, (\mu = 0.2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine serum albumin</td>
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</tr>
<tr>
<td>Progesterone 20-albumin</td>
<td>6.22</td>
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<td>Deoxycorticosterone 21-albumin</td>
<td>6.11</td>
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<td>Estrone 17-albumin</td>
<td>7.94</td>
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### Table IV

<table>
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<th>Conjugates</th>
<th>Related steroids</th>
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<tr>
<td>Progesterone 20-albumin</td>
<td>Progesterone 20-(O-carboxymethyl)oxime cm.(^{-1})</td>
</tr>
<tr>
<td>cm.(^{-1})</td>
<td>947 medium</td>
</tr>
<tr>
<td>940 weak</td>
<td>1090 medium</td>
</tr>
<tr>
<td>1080 weak</td>
<td>Deoxycorticosterone 21-hemisuccinate</td>
</tr>
<tr>
<td>940 weak</td>
<td>947 medium</td>
</tr>
<tr>
<td>Estrone 17-albumin</td>
<td>Estrone 17-(O-carboxymethyl)-oxime</td>
</tr>
<tr>
<td>1110 weak</td>
<td>1120 strong</td>
</tr>
<tr>
<td>1065 weak</td>
<td>1075 medium</td>
</tr>
<tr>
<td>935 weak</td>
<td>940 medium</td>
</tr>
<tr>
<td>840 weak</td>
<td>857 strong</td>
</tr>
<tr>
<td>820 weak</td>
<td>825 strong</td>
</tr>
</tbody>
</table>
values are listed in Table II. 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 pro-
gestrone 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 spec-
trophotometer.

SUMMARY
In an extension of a program designed to prepare steroidal sub-
stances which could elicit antibodies with antihormonal proper-
ties, three additional steroid-protein conjugates have been syn-
thetized. They are conjugates in which bovine serum albumin
is covalently linked to derivatives of progesterone, deoxycorti-
costerone, and estrone. These conjugates have been analyzed
and characterized by their ultraviolet and infrared spectra, elec-
trophoretic behavior, and by dinitrophenylation studies. It has
been estimated that at least 20 steroid residues have been co-
valently linked to each molecule of bovine serum albumin. Like
the previously prepared conjugates (1) these have also been found
to be antigenic in rabbits.

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