STEROID-PROTEIN CONJUGATES. I

Preparation and Characterization of Conjugates of Bovine Serum Albumin with Aldosterone, Hydrocortisone, Progesterone, Testosterone, Oestradiol and Oestriol

By

B. PENKE, P. PALLAI and K. KOVÁCS

Institute of Organic Chemistry, Attila József University, Szeged, Hungary

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BSA conjugates with aldosterone, hydrocortisone, progesterone, testosterone, oestradiol and oestriol were synthesized for radioimmunoassay studies.

The most useful and most sensitive method for determining small quantities of polypeptide and steroid hormones is the radioimmunoassay. Usually, these hormonal compounds with molecular weight below 1000 are not immunogenic *per se*. (The smallest peptide hormone which has been shown to be immunogenic is vasopressin, MW=1080.) However, LANDSTEINER showed that certain small molecules (haptens), when covalently linked to macromolecules (such as bovine serum albumin, BSA) become antigenic, provoking the production of antibodies. Thus steroid haptens bound covalently to proteins may be used as artificial antigens which are able to elicit the formation of antibodies with specificity not only for the protein but also for the particular steroid. The determination of the steroid hormones by radioimmunoassay is based on the reversible bonding of these specific antibodies with the steroids.

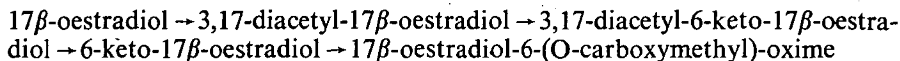
For the synthesis of steroid-BSA antigen conjugates, steroid derivatives suitable for covalent bonding with the proteins were synthesized. (The formation of stable peptide bonds is the most suitable, as these are not subject to decomposition even *in vivo* under the conditions of immunization.) To this effect, steroid derivatives containing a carboxyl group had to be synthesized.

The steroid derivatives coupled to BSA are shown in Fig. 1.

The synthesis of aldosterone-21-hemisuccinate (**I**) and hydrocortisone-21-hemisuccinate (**II**) was referred to in our earlier papers [1, 2]. Both derivatives can be obtained with good yield by the methods of ERLANGER [3] and BUZBY [4], respectively, by reacting succinic anhydride and the corresponding steroid. (The synthesis of **I** is only mentioned in literature; to our knowledge, no detailed description has been published.) The secondary and tertiary hydroxyl groups in position 11 and 17 are not susceptible of acylation under the above conditions [5].

III and **IV** were obtained by reacting phosgene with 11-hydroxy-progesterone and testosterone, respectively, in toluene or dioxane. Testosterone-17-chloroformate formed quantitatively in some hours, whereas the progesterone derivative (**IV**) could not be obtained with good yield in a reaction time shorter then 24—36 hours. Both chloroformates are fairly stable crystalline compounds.

V was synthesized with the methods of ERLANGER [3] and ABRAHAM [5], respectively. To obtain better yields, in this case, as well as for the synthesis of **VI** and **VII**, (O-carboxymethyl)-hydroxylamine was used in 6-fold excess; thus we succeeded in producing **V** with 80% yield. This is in good accordance with ABRAHAM's results [5]. The synthesis of **VI** was based on the following reaction scheme:



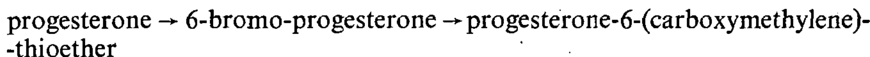
17β-oestradiol → 3,17-diacetyl-17β-oestradiol → 3,17-diacetyl-6-keto-17β-oestradiol → 6-keto-17β-oestradiol → 17β-oestradiol-6-(O-carboxymethyl)-oxime

VII was synthesized in an analogous way, starting from oestriol-triacetate. The step of poorest yield in both syntheses is the formation of the 6-keto group.

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

This is carried out by CrO_3 oxidation in acetic acid [6, 7]; the reaction proceeds with the formation of great quantities of by-products. For the purification of the rough product, DEAN *et al.* [8] used silicagel column chromatography. This method was found to be the most suitable for the purification of **VI** and **VII** in our experiments, too. Unfortunately, yields of the pure products are not given in the papers of either DEAN [8] or LINDNER *et al.* [9]. KUSS and GOEBEL [10] reacted 6-oxo-oestriol with (O-carboxymethyl)-hidroxylamine without purification, and purified only the end-product **VII** on Sephadex G-25 column, without giving the yield of the pure product. According to our experience, **VI** and **VII** were obtained with 7—8% yield, calculated for the starting quantities of oestradiol and oestriol. This rather poor yield may be explained by the by-products formed in the CrO_3-oxidation step.

VIII was obtained by the following reaction scheme:



6-bromo-progesterone was isolated with a comparatively poor yield, whereas the formation of the thioether resulted to be better than in the literature [9].

The chloroformates **III** and **IV** were coupled to BSA by the Schotten-Baumann method [3]; for the other steroid derivatives the mixed anhydride method was used. The hapten/protein molar ratio was 1:1 in each case. To obtain mixed anhydride, isobutyl-chloroformate and N-methyl-morpholine were used; the formation of the mixed anhydride proceeds in dioxane at 10 °C in 30—60 sec. This reaction time,

Fig. 2. a, b

much shorter than that found in literature, has the advantage of avoiding hydrolysis of the mixed anhydride and occasional racemization [11].

The by-products of low molecular weight were separated from the reaction mixture by dialysis; the antigens were lyophilized, the quantity of the coupled steroids was determined by Van Slyke N-analysis. The purity of antigens was tested by UV spectrophotometry and electrophoresis. The UV-spectra are to be seen in Figs. *2a*, *2b* and *2c*.

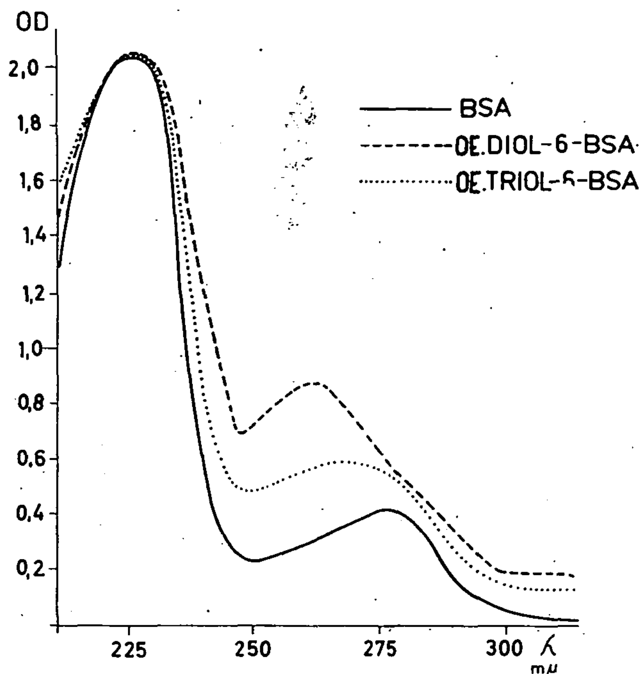


Fig. 2a—c. UV-spectra of BSA-steroid conjugates in 0.05 M Tris buffer. BSA = bovine serum albumin; P-6-BSA = progesterone-6-BSA, P-11-BSA = progesterone-11-BSA, T-3-BSA = = testosterone-3-BSA, T-17-BSA = testosterone-17-BSA, OE.DIOL-BSA = oestradiol-6-BSA, OE.TRIOL-BSA = oestriol-6-BSA. Concentrations: in Fig. *2a—b.* 10^{-6} M; in Fig. *2c* 5×10^{-7} M.

According to our calculations, the conjugates contain 20—30 steroid haptens in a BSA molecule; their isoelectric points are in the pH range 4.5—4.6. (The isoelectric point of the starting BSA is at pH 4.8).

The biological investigations are still in course; it can be stated, however, that all steroid conjugates exert a good antigen effect. (The BSA-conjugates of **I** and **II** are already used in medical practice for determination of aldosterone and cortisol.) As the functional groups of the rings A and D in the BSA-conjugates of the compounds **III** and **VI** to **VIII** are unchanged, on the base of the theory of MIDGLEY and NISWENDER [12], their antigenic properties can be expected to be better than those of the aldosterone- and cortisol-antigens [1, 2] already investigated in detail.

Experimental

UV-spectra were measured with an UNICAM-800 spectrophotometer. The solvent mixtures used for thin layer chromatography on silicagel were as follows:

1. $CHCl_3$—methanol—acetic acid 85:10:5
2. $CHCl_3$—ethanol 9. 5
3. $CHCl_3$—acetone 9:.
4. $CHCl_3$—acetone 7:3
5. $CHCl_3$—methanol—acetic acid 70:30:2
6. $CHCl_3$—methanol—acetic acid 80:20:2
7. $CHCl_3$—acetone—acetic acid 90:10:1
8. ethyl acetate—hexane 1:4

(The mixture used is denoted by a superscript in the respective R_f values). For electrophoresis, acetate buffer pH 4.5 (0.1 M) and phosphate buffer pH 7.4 (0.05 M) were used.

Aldosterone-21-hemisuccinate (**I**)

190 mg aldosterone and 190 mg succinic anhydride was reacted in 3 ml pyridine at room temperature for 24 hrs. The reaction mixture was poured into 50 ml 2*N* HCl and stirred for 1 hour; **I** separated as crystalline product (182 mg). Recrystallization from methanol—ether yielded 150 mg (60%) pure **I**, mp. 194—196 °C. $R_f^1=$ $=0.80$, $R_f^2=0.15$. $C_{25}H_{32}O_8$ (460.52). Calc.: C 65.18; H 6.91. Found: C 64.94; H 5.68%.

Hydrocortisone-21-hemisuccinate (**II**)

was synthesized in a similar way; on acidification **II** immediately separated in amorphous form. Solving the rough product in ethyl acetate, **II** was extracted with $NaHCO_3$ soln.; acidification of the $NaHCO_3$ phase (pH $\sim$3) yielded 75% pure **II**, m.p. 171—172 °C. $R_f^1=0.75$, $R_f^2=0.20$. $C_{25}H_{34}O_8$ (462.53) Calc.: C 65.03; H 7.01. Found: C 64.78; H 6.89%.

Progesterone-11-chloroformate (**III**)

500 mg 11-α-hydroxy-progesterone was dissolved in 5 ml $CHCl_3$, then phosgene was led into this solution for 1 hour. After 24 hours standing N_2 was bubbled through the mixture for 30 min and the solvent evaporated *in vacuo*. The crystalline residue was recrystallized from acetone; yield 300 mg (59%), $R_f^3=0.65$. $C_{22}H_{28}O_4Cl$ (390.9) Calc.: C 67.43; H 7.2. Found: C 67.70; H 6.95%.

Testosterone-17-chloroformate (**IV**)

was synthesized from 500 mg testosterone like **III**. The product obtained was a semi-crystalline solid; yield 90%; m.p. about 55 °C. $R_f^3=0.60$; $R_f^4=0.75$. $C_{20}H_{27}O_3Cl$ (350.9) Calc.: C 68.46; H 7.76. Found: C 68.15; H 7.38%.

Testosterone-3-(carboxymethyl)-oxime (**V**)

500 mg (1.75 mmole) testosterone was reacted with 1.5 g (12 mmole) (O-carboxymethyl)-hydroxylamine in a mixture of 25 ml ethanol and 6 ml 2*N* NaOH under reflux for 2 hours. After cooling, the solution was diluted with 2 vol. water and acidified at 0 °C with 3*N* HCl to pH 3.0, and the product separated was extracted three times with ethylacetate. **V** was washed out from the combined ethyl acetate phases with 5% $NaHCO_3$ solution. Acidifying the $NaHCO_3$ phases (pH ~2) at 0 °C, **V** was extracted with ethylacetate. The organic phase was washed with brine, dried, and on evaporation gave 480 mg (75%) colorless crystals of **V**; m.p. 178—180 °C (Lit [3] 179—181 °C) $R_f^5=0.95$. $C_{21}H_{31}O_4N$ (321.2). Calc.: C 69.81; H 8.59. Found: C 69.95; H 8.70%.

6-Keto-17β-oestradiol-diacetate

was prepared by the method of SNEDDON [8]. From 6 g 17β-oestradiol-diacetate 6.5 g rough product was obtained and purified on silicagel column, using the following solvent gradient: $CHCl_3$-petroleum ether 1:4, $CHCl_3$-ethylacetate-petroleum ether 1:1:8 and ethyl acetate-petroleum ether 1:4, to yield 840 mg (13%) pure 6-keto-17β-oestradiol-diacetate, m.p. 174—175 °C (Lit [8] 173.5—175 °C) $R_f^6=0.5$.

17β-Oestradiol-6-(carboxymethyl)-oxime (**VI**)

a) 6-Keto-oestradiol was prepared by the method of DEAN and EXLEY [10]. From 840 mg 6-keto-17β-oestradiol-diacetate 360 mg (55%) pure product was obtained, m.p. 280—82 °C; $R_f^6=0.1$; $R_f^7=0.9$.

b) 314 mg (1.2 mmole) 6-keto-oestradiol dissolved in a mixture of 10 ml ethanol, and 3 ml 2*N* NaOH was refluxed with 750 mg (6 mmole) (O-carboxymethyl)-hydroxylamine-hydrochloride. After cooling, 50 ml ether was added and the solution extracted with 3×50 ml 5% Na_2CO_3. The collected aqueous phase was acidified and extracted with 3×50 ml ethyl acetete. The product obtained by drying and evaporating the combined extracts was recrystallized from acetone, giving 250 mg (58%) pure **VI**; m.p. 199—200 °C. $C_{20}H_{25}O_5N$ (359.4). Calc.: C 66.83; H 7.01. Found: C 66.50; H 7.11%.

6-Keto-oestriol-triacatete

was synthesized from 3 g oestriol-triacetate as described 6-keto-oestradiol-diacetate, giving 3.5 g rough product. Purification by column chromatography on silicagel yielded 440 mg (14.5%) pure product, m.p. 137—139 °C, $R_f^3=0.55$.

Oestriol-6-(carboxymethyl)-oxime (**VII**)

a) 6-Keto-oestriol was obtained in a similar way as 6-keto-oestradiol. Hydrolysis of 440 mg 6-keto-oestriol-triacetate gave 230 mg (71%) 6-keto-oestriol, m.p. 238—240 °C; $R_f^3=0.35$.

b) **VII** was prepared from 230 mg 6-keto-oestriol to the analogy of **VI**; yielded 160 mg pure **VII**, m.p. 220—222 °C, $R_f^7=0.50$. $C_{20}H_{25}O_6N$ (375.4). Calc.: C 63.98; H 6.71. Found: C 64.20; H 6.85%.

Progesterone-6-(carboxymethylene)-thioether (**VIII**)

a) 6-Bromo-progesterone was prepared according to [13]. From 1 g progesterone 0.47 g rough product was obtained and recrystallized from a mixture of acetone and hexane, giving 0.356 g pure 6-bromo-progesterone (30%), m.p. 143—145 °C, $R_f^3=0.7$.

b) **VIII** was prepared by the method of LINDNER and PEREL [11], refluxing the mixture of 314 mg 6-bromo-progesterone and 100 mg anhydrous thioglycolic acid in a solution of 1.2 ml methanol and 120 mg KOH for 2 hours. Working up the reaction mixture gave 168 mg (52%) pure **VIII**, m.p. 148—150 °C, $R_f^9=0.12$. $C_{23}H_{32}O_4S$ (404.58). Calc.: C 68.22; H 7.97. Found: C 67.93; H 8.09%.

Preparation of steroid-protein conjugates

a) To a solution of 1.40 g (0.02 mmole) of bovine serum albumin in 30 ml water, 20 ml dioxane and 1.4 ml *N* NaOH solution of 1 mmole of testosterone 17-chloroformate (**IV**) or progesterone-11-chloroformate (**III**) in 15 ml dioxane was added, with stirring at 0 °C in 30 minutes. During the reaction an additional 0.8 ml *N* NaOH was added dropwise, to maintain the pH above 7.0. Stirring and cooling were continued for 3 hours. The solution was dialyzed overnight against running water and brought to pH 4.6 with *N* HCl. The conjugate precipitated and, after storage in a refrigerator for 24 hours, was collected by centrifugation. After washing with water, it was dissolved in water by adding a small quantity of $NaHCO_3$. The clear solution was lyophilized, yielding 1.30—1.40 g of conjugate. Analysis see Table I.

Table I

N-analysis values of the steroid BSA-conjugates

Conjugate	Assumed mol. weight (20 to 30 of the 60 NH_2 groups substituted)		Calculated (with 10% H_2O cont.)		Found	
			NH_2—N	total N	NH_2—N	total N
Aldosterone-21-BSA	80 330	(30)	0.47	12.23	0.46	11.85
Cortisol-21-BSA	80 350	(30)	0.47	12.48	0.47	12.13
Progesterone-11-BSA	78 100	(25)	0.64	12.65	0.68	12.82
Testosterone-17-BSA	76 900	(25)	0.68	12.80	0.71	13.01
Testosterone-3-BSA	80 350	(30)	0.47	13.02	0.48	12.85
Oestradiol-6-BSA	81 550	(30)	0.45	12.86	0.47	12.93
Oestriol-6-BSA	82 000	(30)	0.44	12.73	0.42	12.68
Progesterone-6-BSA	77 100	(20)	0.71	12.91	0.69	13.02

b) 1 mmole of steroid derivative containing a free carboxyl group (as hemisuccinate **I** and **II**, as (O-carboxymethyl)-oxime **V** to **VII** and as carboxymethylene-thioether **VIII**, respectively) and 1 mmole N-methyl-morpholine were dissolved in 10 ml dioxane. This solution was cooled, and reacted with 1 mmole (0.13 ml) isobutyl-chlorocarbonate for 30—60 sec. Then the whole mixture was added in a single portion to a stirred, cooled solution of 1.40 g (0.02 mmole) bovine serum albumin dissolved in a mixture of 40 ml water, 30 ml dioxane and 1.5 *N* NaOH (pH 8.5). Stir-

ring and cooling were continued for 3 hours keeping the pH at 7.5 by the addition of *N* NaOH. The solution was then dialyzed against running water for 12 hours, the pH adjusted to 4.5 with *N* HCl, the resulting precipitate collected by centrifugation after storing in a refrigerator overnight. The steroid-BSA conjugate was redissolved in 50 ml water and the pH was adjusted to 7.5 with solid $NaHCO_3$, dialyzed against distilled water and lyophilized, yielding about 1.3—1.4 g of conjugate. Analysis see Table I.

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СТЕРОИД — ПРОТЕИН КОНЪЮГАТЫ. I.
СИНТЕЗ И ХАРАКТЕРИСТИКА BSA — КОНЪЮГАТОВ АЛЬДОСТЕРОНА, ГИДРОКОРТИЗОНА, ПРОГЕСТЕРОНА, ТЕСТОСТЕРОНА, ЭСТРАДИОЛА И ЭСТРИОЛА

Б. Пэнкэ, П. Паллаи, К. Ковач

Синтезированы и исследованы с целью радиоиммуноэссэйа BSA — конъюгаты стероидов, перечисленные в названии статьи.