Acta vet. scand. 1973, *14*, 278—291.

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PREPARATION OF PROTEIN-STEROID CONJUGATES AND ANTISERA AGAINST OESTRADIOL-17β*

By

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MARTINSSON, KJELL, PAUL LINDBERG and ELOF D. B. JOHANSSON: *Preparation of protein-steroid conjugates and antisera against oestradiol-17β.* Acta vet. scand. 1973, *14*, 278—291. — A conjugate of oestradiol-17β and bovine serum albumin (BSA) has been prepared by the mixed anhydride method. The conjugate was characterized by u.v.-analyses and immunoelectrophoresis, and the number of moles of oestradiol-17β per mole of BSA was found to be 25. The conjugate was used for immunization of two sheep and one rabbit in order to elict antisera against oestradiol-17β. Antibodies could be detected in all three animals after 5—16 weeks depending on the route of immunization. The antiserum from one sheep could be used in a radioimmunoassay system for oestradiol-17β in a dilution of 1:5000 and was found to cross-react with oestrone and oestradiol-17α to a lesser extent than another antiserum against oestradiol-17β.

radioimmunoassay; oestrogens.

In the field of endocrinology there is a great need for rapid and sensitive methods to determine the levels of different hormones in biological fluids. Earlier methods including gas chromatography, spectrofluorometry or biological testing are time consuming, and the sensitivity is often not enough to make the method useful.

During the last decade it has become possible to detect minute quantities of both steroid and polypeptide hormones by the use of antibodies against the hormones in a competitive binding technique with labelled hormones based on the original report

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* This study was supported by the Swedish Medical Research Council (B 72-17x-3495-01), the Ford Foundation, the Population Council, and the John M. Olin Foundation.

of *Yalow & Berson* (1960). Several difficulties will arise in such a technique. Only polypeptide hormones as such are able to give rise to antibodies under certain conditions, after immunization of experimental animals. This may be due to the fact that there are some differences in the amino-acid sequence between such hormones in different species. However, no differences between species exist in the case of steroid hormones. Therefore it is impossible to elict antibodies against such hormones by immunizing experimental animals with the proper hormone.

In 1946 *Landsteiner* suggested that low molecular substances, which as such are not antigenic, may be rendered antigenic by coupling them to proteins. *Liebermann et al.* (1959), *Goodfriend & Sehon* (1961) and *Ferin et al.* (1968) have used this approach in order to elict antibodies against progesterone, testosterone and oestrogens. Several methods can be used for this purpose. If conjugation is desired at a position which contains a hydroxyl group, firstly either a hemisuccinate or a chlorcarbonate derivate is prepared. An oxime (o-carboxymethyl) derivate is prepared, if conjugation is desired through a ketone group. The techniques mostly used for these conjugations are those described by *Erlanger et al.* (1967).

In order to render these conjugates antigenic, several proteins can be coupled to them by the mixed anhydride method (hemisuccinate and oxime derivates) or by the Shotten-Bauman method (Chlorocarbonate derivates). For this purpose bovine serum albumin (BSA) has been most widely used. The hemisuccinate and oxime derivates are linked via an amide bond to ε-amino groups of the carrier protein (BSA) using a mixed anhydride reaction (*Erlanger et al.* 1967). In this way several conjugates have been prepared i.e. testosteron-17-BSA, progesteron-20-BSA, oestradiol-17β-BSA, and cortisol-21-BSA (*Liebermann et al.* 1959). After being characterized the steroid-protein conjugate may be used for immunization of experimental animals in order to elict antibodies.

Recently antibodies to oestradiol-17β have been produced by immunization of sheep with oestradiol-17β-hemisuccinate-BSA (*Ferin et al.* 1968). The biologic potency of these antibodies for neutralizing circulating oestrogens was demonstrated. The antisera were also used to determine the levels of oestrogens in serum by radioimmunological methods (*Abraham* 1969, *Hotchkiss et al.* 1971).

The aim of this investigation was to prepare succinate-BSA-conjugates of oestradiol-17β by conjugation of a hydroxyl group in 17-position and to study the antibody response in sheep and rabbits after immunization with the conjugate by different routes.

MATERIAL AND METHODS

Oestradiol-17β was obtained from Sigma Corp. All solvents used were of analytical grade.

Preparation of oestradiol-17β-hemisuccinate in 17-position

Eight hundred mg of oestradiol-17β and 1000 mg of succinic anhydride were dissolved in 10 ml dry pyridine. The solution was refluxed for 12 hrs., and the pyridine distilled off in vacuo. The dried product was recrystallized three times from a benzene-acetone solution, and then the crystals were isolated by filtering and dried in vacuo. The preparation was dissolved in 0.05 M tris buffer pH 8.4, and the u.v.-spectra were determined (Fig. 1).

Preparation of oestradiol-17β-hemisuccinate-BSA conjugate

Two hundred and twenty-five mg of oestradiol-17β-hemisuccinate and 286 mg of tri-n-butylamin were dissolved in 9 ml of dioxane. The solution was cooled in an ice bath during magnetic stirring, and then 63 μl of isobutylchloroformate was added. After 30 min. of stirring in the ice bath a solution of 700 mg bovine serum albumin (BSA) in 18.4 ml dioxane and 12.4 ml H_2O was added. The pH was adjusted to 8.5 by N-NaOH. The solution was stirred for 4 hrs. in the ice bath and was then dialyzed for 12 hrs. against tap water. After centrifugation the pH was adjusted to 4.6 with N- and 0.1 N-HCl during stirring. After centrifugation the precipitate was suspended in 30 ml of 60 % dioxane, and pH was adjusted to 8.5 with N-NaOH. Thereafter the same procedure was repeated twice. The supernatant was dialyzed against water for 12 hrs. and was centrifugated and filtered through Millipore filter, pore size 0.45 μ. The filtrate was freeze-dried and subjected to u.v.-absorption tests in 0.05 M tris buffer pH 8.4 (Fig. 2).

Experimental animals

a) Two ewes, 9 months old, were injected with 1.0 and 3.0 mg, respectively, of the oestradiol-17β-succinyl-albumin conjugate once a week for 1 month. Booster doses were given once a month. The conjugate was used in 0.1 per cent (w/v) solution of physiological saline and suspended in an equal volume of Freund complete adjuvant and injected i.m. into the right thigh. Blood samples were drawn 2 months after the first injection and thereafter 1 week after each booster dose for testing the antibody response.

b) One female rabbit was used for immunization with alun-precipitated conjugate. To 0.7 ml of 0.1 % solution of the conjugate,

one to two drops of a saturated solution of potassium-aluminium-sulphate were added. The pH of the solution was adjusted to 7.5—8.0 with 0.1 N-NaOH, and a heavy precipitate was formed. The precipitate (0.7—1.0 ml) was used as antigen and injected intraglandularly into the right popliteal lymph node. To enlarge the lymph node 0.5 ml of Freund complete adjuvant was injected twice into the foot pad 2 and 1 week before the immunization started. Then 0.7—1.0 ml of the precipitated antigen solution was injected once a day for 3 days and then once a week for 6 weeks. Blood samples for antibody determination were drawn 1 week after each booster dose.

Antisera analyses

Three methods were used to detect antibodies formed against the conjugate and oestradiol-17β.

a) *Immunodiffusion tests.* Serum from the immunized animals were tested by immunodiffusion in 1 % agar of veronal buffer pH 8.6, ionic strength 0.05 against the conjugate and BSA in 1 % solutions. The sera were used both unabsorbed and absorbed with BSA in proportion 15:1.

b) *Immunoelectrophoretic analyses.* Immunoelectrophoresis of the conjugate and BSA was performed according to the method of *Scheideggar & Zalund* (1957). Anti-bovine serum (Hyland Lab.) and sera from the immunized animals were used as antisera.

c) *Radioimmunological tests.* The untreated antiserum was diluted in a phosphate buffer (pH = 7) containing 0.1 % of gelatin. The first dilution was 1:100 and the highest dilution in each test run was usually 1:100,000. Oestradiol-17β-1,2,6,7-^{3}H (supplied by New England Nuclear Corporation) was used as the radioactive tracer. The screening of the potency of the antiserum was done in the following manner: Each dilution of the antiserum was tested in four tubes, two containing no oestradiol-17β and the other two containing 50 pg of oestradiol-17β. One hundred μl of the antiserum was added to each tube and mixed briefly and allowed to stand for 30 min. at room temperature. To each tube was then added 35 pg of oestradiol-1,2,6,7-^{3}H (100 μl in phosphate buffer containing 0.1 % of gelatin). After a brief mixing the solution was allowed to incubate overnight in the refrigerator. After incubation in the refrigerator, 100 μl of phosphate buffer containing 0.5 % of gelatin was added followed by 1 ml of dextrane-coated charcoal in phosphate buffer without gelatin. This solution was mixed and left for 15 min. in the refrigerator. The tubes were centrifugated for 5 min.s at 5000 r.p.m. at 0°C. After centrifugation the supernatant was decanted into counting vials and counted in a liquid scintillation spectrometer (Packard Tri-Carb, 3310) (*Hotchkiss et al.* 1971).

The results obtained with the unknown antiserum were compared to the performance of the antiserum received from Dr. Vande Wiele (*Ferin et al.* 1968). The dilution step that gave the

same response curve as the dilution 1:150,000 of the Vande Wiele antiserum was selected for further testing of specificity. Complete standard curves of oestradiol-17β, oestrone and oestriol were run together with plasma samples extracted with 10 volumes of diethyl ether. The results of these measurements of plasmas could then be compared to the results received from the same plasmas in the oestradiol method in which the Vande Wiele antiserum was used.

RESULTS

The u.v.-spectrophotometric analyses of the succinic anhydride and the oestradiol-17β succinate prepared are seen in Fig. 1. The absorption maximum of the oestradiol-17β succinate was found to be 279nm. No absorption of the succinic acid anhydride was found at this wave-length.

The u.v.-spectrophotometric analyses of the oestradiol-17β-succinate-BSA conjugate prepared are seen in Fig. 2. The absorption maximum was found to be 279nm for the conjugate and BSA.

The absorption curves were used to calculate the number of steroid residues in the conjugate. Assuming that the extinction coefficient of the conjugated oestradiol is identical with that of oestradiol-hemisuccinate it is possible to calculate, from the

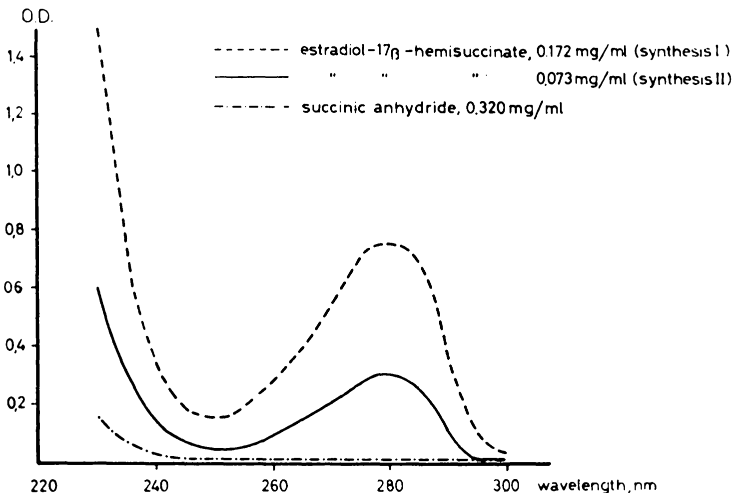
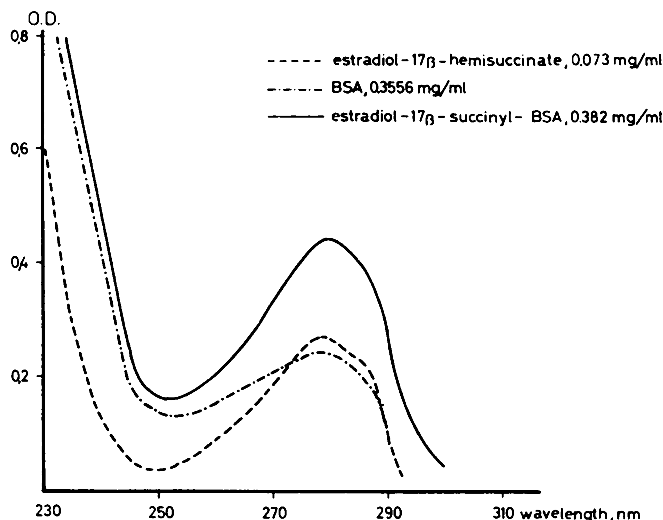


Figure 1. The u.v.-spectra of succinic anhydride and oestradiol-17β-hemisuccinate obtained by two different syntheses.



F i g u r e 2. The u.v.-spectra of oestradiol-17β-hemisuccinate, BSA and oestradiol-17β-succinyl-BSA.

absorbance of the conjugate at 279nm, the number of oestradiol residues in the conjugate (*Erlanger et al.* 1958). A simplified calculation follows:

The optical density of oestradiol-17β-BSA at 279nm at a concentration of 382 mg per l is 0.444. The absorbance of BSA at the same concentration and wave-length is $\frac{0.248 \times 0.382}{0.3556} = 0.266$.
The molar extinction coefficient of oestradiol-17β-succinate was found to be 1469. The difference attributed to oestradiol-17β-succinate therefore is $0.444 - 0.266 = 0.178$. Hence the concentration of steroid residues is $\frac{0.178}{1469} = 1.212 \cdot 10^{-4}$ mole per l.

Since the residue weight of oestradiol-17β-hemisuccinate is 373 g per mole its concentration is $1.212 \cdot 10^{-4} \times 373 = 45.2$ mg per l. By subtracting the concentration of the steroid residues from that of oestradiol-17β-BSA, the concentration of the BSA constituent is obtained ($382 - 45.2 = 336.8$ mg per l). If the molecular weight of BSA is assumed to be 70,000, the number of moles per l of BSA in the preparation is $\frac{336.8 \cdot 10^{-3}}{70{,}000} = 4.81 \cdot 10^{-6}$.
Therefore, the number of moles of oestradiol-17β per mole of

Table 1. The anti-oestradiol-titers of the sheep and the rabbit during the immunization.

Sheep				Rabbit		
date of immunization	date of sampling	dilution of antisera equivalent to antisera of Vande Wiele 1:150,000 — immunization dose 1 mg	dilution of antisera equivalent to antisera of Vande Wiele 1:150,000 — immunization dose 3 mg	date of immunization	date of sampling	dilution of antisera equivalent to antisera of Vande Wiele 1:150,000
Nov. 16, 23, 29				May 3, 4, 5		
Dec. 7				12, 18, 27		
Jan. 3				June 10	June 10	1: 100
Febr. 2, 24				17, 24	June 24	1:1,000
March 23	March 31	1:1,000	1:10,000	July 1, 26	July 26	1: 200
April 25	May 3	1:5,000	1:20,000	Aug. 14	Aug. 19	1:2,000
May 25	June 3	1:7,500	1:15,000			
	June 5	not tested	1: 5,000			
Aug. 11	Aug. 19	not tested	1: 5,000			

BSA is $\frac{1.212 \cdot 10^{-4}}{4.81 \cdot 10^{-6}} = 25.$

The titers of antibodies against oestradiol-17β after different samplings are seen in Table 1. The titers are expressed as dilutions equivalent to a dilution of 1:150,000 of an antiserum obtained by Vande Wiele.

The immunoelectrophoretic analyses of oestradiol-17β-BSA and BSA against anti-bovine serum and serum from the immunized sheep are seen in Figs. 3 and 4. It is observed that both antisera react with the conjugate and BSA. The BSA used for conjugation seems not to be quite pure since spur of a beta-globulin can be observed.

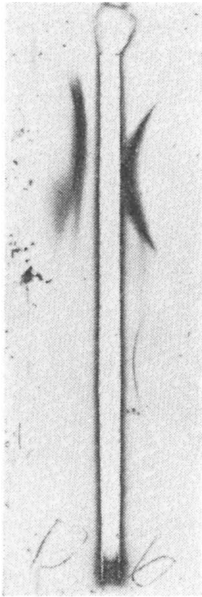


Fig. 3.

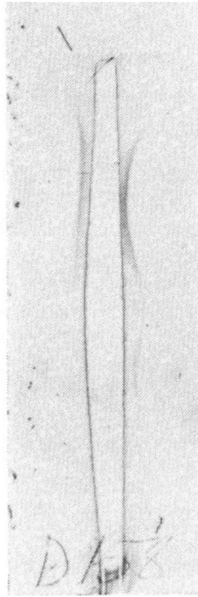


Fig. 4.

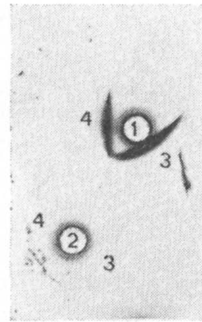


Fig. 5.

Figure 3. Immunoelectrophoresis of oestradiol-17β-BSA (to the left) and BSA (to the right) against anti-bovine serum.

Figure 4. Immunoelectrophoresis of oestradiol-17β-BSA (to the left) and BSA (to the right) against antiserum collected from a sheep immunized with 3 mg of the conjugate for 5 months.

Figure 5. Immunodiffusion tests of unabsorbed anti-oestradiol-17β-serum (1) and the same antiserum absorbed 15:1 with 0.1 % BSA (2) against BSA (3) and the conjugate (4).

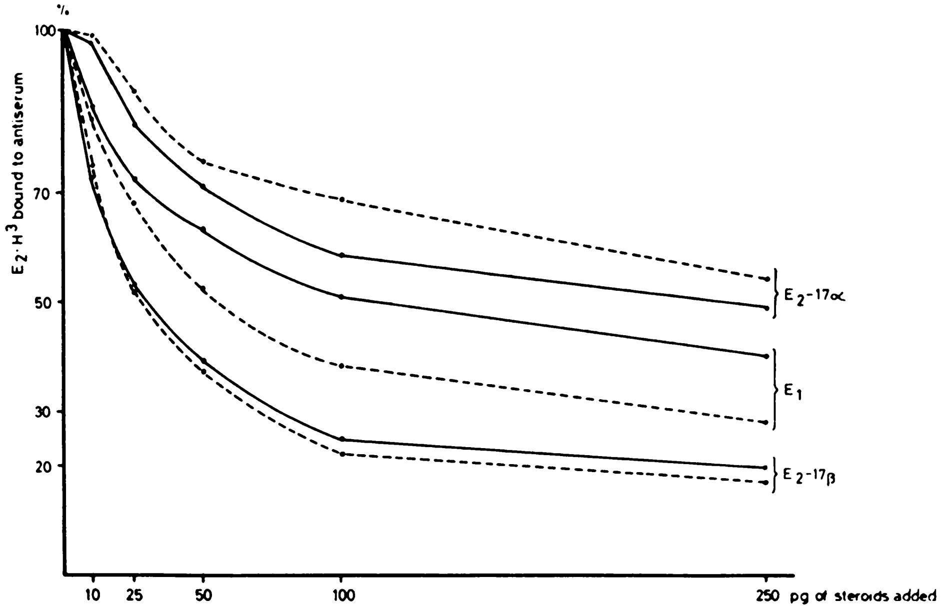
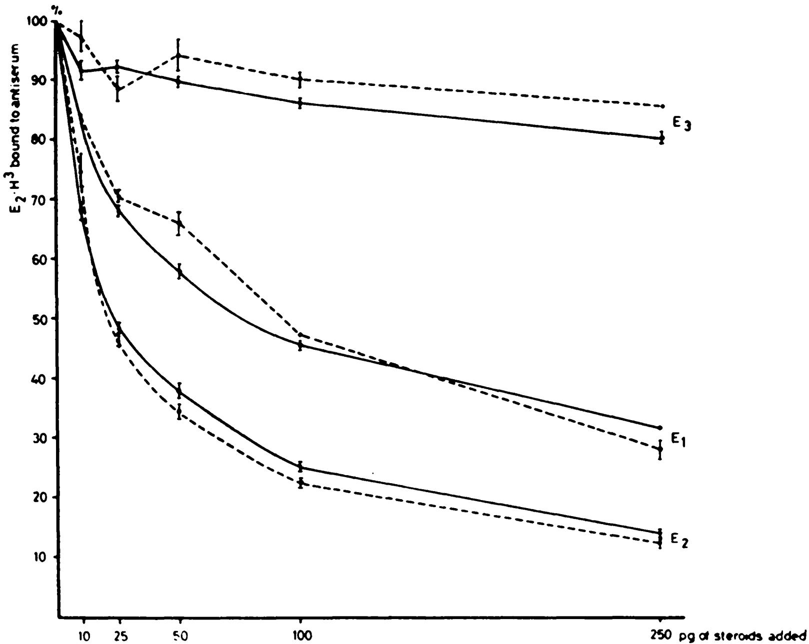


Figure 6. Binding percentage of tritiated oestradiol-17β of a sheep antiserum (collected June 5) diluted 1:5,000 after addition of increasing amounts of oestrone, oestradiol and oestriol (E_1, E_2 and E_3). Whole lines represent the antiserum unabsorbed and broken lines the antiserum absorbed 15:1 with BSA.

The immunodiffusion tests of oestradiol-17β-BSA against serum from the immunized sheep are seen in Fig. 5. The serum reacts with both the conjugate and BSA. However, after absorption of the serum with BSA no reactions can be observed either with BSA or the conjugate (Fig. 5). The radioimmunological test of the serum before and after absorption with BSA is seen in Fig. 6. It is observed that only slight differences exist between absorbed and unabsorbed antiserum to bind tritiated oestradiol-17β.

The specificity tests (Fig. 7) indicate that the antiserum against oestradiol cross-reacts with oestrone and to a lesser extent with oestradiol-17α. From Fig. 7 it can be seen that the cross-reaction with oestriol is very low.



F i g u r e 7. Binding percentage of tritiated oestradiol-17β (E_2-17β) of a sheep antiserum (collected June 3) diluted 1:5,000 after addition of increasing amounts of oestrone (E) and oestradiol-17α (E_2-17α) (whole lines). Broken lines indicate the performance of an antiserum obtained by Vande Wiele and used at a dilution of 1:150,000.

DISCUSSION

In the present investigations bovine serum albumin was coupled to oestradiol-17β-succinate by the mixed anhydride method. After purification the steroid-protein conjugate was characterized by u.v.-spectrophotometric assays. It was found that the absorption maximum of bovine serum albumin (BSA), oestradiol-17β-succinate and the steroid-protein conjugate was 279nm as was also found by *Liebermann et al.* (1959).

By taking advantage of the extinction and concentration of oestradiol-17β-succinate and the protein conjugate it was possible to determine the number of steroid moles coupled to each mole of BSA, which was found to be 25 in this investigation. Similar results have been reported also by other investigators (cit. *Peron & Caldwell* 1970). In earlier investigations (cit. *Peron & Caldwell*) it was shown that the results of the u.v.-analyses were in

agreement with other methods including dinitrophenylation techniques and radioactive analyses of 16-^{14}C-tagged oestrogen conjugates, in order to characterize the conjugates.

The immunoelectrophoretic analysis of the steroid-protein conjugate and BSA against anti-bovine serum indicates that both the conjugate and BSA reacts with anti-bovine serum. However, it is noticeable that the electrophoretic mobility of the conjugate is greater than that of BSA (Fig. 3). This may be due to the transformation of the cationic ε-amino groups of the lysine residues in the protein into relatively neutral functions, resulting in a greater anodic electrophoretic mobility of the conjugate than the carrier protein. BSA contains 59 lysine residues (*Erlanger et al.* 1958) and the more of them substituted with oestradiol-17β, the faster will the electrophoretic mobility of the conjugate be. Thus the electrophoretic mobility can be used for calculating the number of steroid residues in the conjugate (*Erlanger et al.* 1958). Such calculations, however, are not quite reliable since they rest upon the assumption that the frictional properties of the conjugate are the same as those of BSA.

Furthermore it is observed that the BSA for immunization is not quite pure. Therefore, it is possible that spur of impurities has also been bound to oestradiol-17β-succinate. Immunoelectrophoresis of the conjugate and BSA against serum from the sheep indicates that the serum contains antibodies against the impurities (Fig. 4). This finding is of no practical importance. Perhaps the impurities have influenced to a very low extent the calculations of the number of steroid residues in the conjugate. This may be the case, if the impurities have a molecular weight much greater than albumin.

The use of these methods of immunization of experimental animals is the most important and hazardous step in the production of anti-steroid antibodies. Concerning oestrogens some investigators have obtained antisera with greater titers in male individuals than in females. However, others have stated that no differences between the sexes exist (*Thorneycroft et al.* in *Peron & Caldwell*). It was postulated that endogenous oestrogens in the female lower the antibody titer by forming soluble complexes. However, it should be noted that one of the most potent anti-oestradiol sera produced was obtained from females (*Ferin et al.* 1968). Therefore, females of sheep and rabbits were used in the present investigation. The sheep were immunized by essentially

the same methods as described by *Ferin et al.* Immunizations were done at the same place every time in order to achieve a primary stimulation of the same lymph node at every immunization.

Antisera obtained from the sheep were shown to contain antibodies against the conjugate and BSA (Fig. 5). However, after absorption with BSA no reaction occurred either with the conjugate or BSA. But the radioimmunological tests indicate that the amount of antibodies was the same before and after absorption. This indicates either that the antibodies against the oestradiol residues are not precipitating or that the amount of such antibodies is too low to give a precipitine reaction. Previous investigations (*Liebermann et al., Gross et al.* 1968) have shown that precipitating antibodies are formed against both the hapten and the carrier protein. However, these investigations were performed as quantitative precipitations in tubes by calculating the amount of nitrogen in the precipitates. It may be possible that the diverging results obtained here are depending on the different methods used. This suggestion is supported by the fact that the antisera obtained in this study de facto have a high titer of specific steroid antibodies after absorption with BSA as shown by the radioimmunological tests (Fig. 6).

After immunization of the sheep it is observed that in general higher titers are obtained by immunization with 3 mg of the conjugate instead of 1 mg (Table 1). This is in accordance with the findings of *Ferin et al.* The time of sampling after each booster dose seems to be of great importance. From Table 1 it can be seen that the titer of one sheep decreased from 1:15,000 to 1:5,000 in 2 days. This finding indicates that it is necessary to take samples at frequent intervals after each booster dose to find out the most suitable time of collecting antiserum.

The immunization of the rabbit resulted in an antibody answer after only 1 month and in the sheep after 4 months. This difference may be due to the injection of the conjugate into a lymph node in the rabbit. The precipitation of the conjugate with potassium-aluminium-sulphate before injection may also have been responsible for the rapid antibody answer. However, it was also observed that the titer of the antiserum in the rabbit was lower than in the sheep.

The cross-reaction with oestrone complicates the use of the antiserum in radioimmunoassays of oestradiol-17β. However,

these difficulties can be avoided by purification of the extract before the final radioimmunoassay step. In some physiological situations where oestradiol-17β is the main oestrogen it is possible to obtain accurate measurements of oestradiol by radioimmunoassay without further purification of the diethylether extract.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Vande Wiele, for a generous gift of antiserum against oestradiol-17 and to Dr. Hotchkiss, who made details of her radioimmunoassay system for oestradiol known to us before its publication. The authors are indebted to the technical staff of the Royal Veterinary College and the Primate Laboratory for Reproduction Research for skilled assistance.

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SAMMANFATTNING

Framställning av protein-steroid konjugat och antisera mot 17β-estradiol.

Ett konjugat av 17β-estradiol och bovint serum albumin (BSA) har framställts med „mixed anhydride" metoden. Konjugatet karaktäriserades med u.v.-analys och immunoelektrofores och antalet mol av 17β-estradiol per mol BSA beräknades till 25. Konjugatet användes för immunisering av två får och en kanin för att framställa antisera mot 17β-estradiol. Antikroppar kunde påvisas hos alla tre djuren efter 5—16 veckor. Antiserum från ett får kunde användas i ett radioimmunologiskt system för 17β-estradiol i en spädning 1:5000 och korsreagerande med estrone och 17α-estradiol i mindre utsträckning i jämförelse med ett annat antiserum mot 17β-estradiol.

(Received March 29, 1972).

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