J. steroid Biochem. Vol. 17, pp. 11 to 16, 1982
Printed in Great Britain.

0022-4731/82/010011-06$03.00/0

EFFECT OF CORTICOSTEROID BINDING PROTEINS ON THE STEROIDOGENIC ACTIVITY OF BOVINE ADRENOCORTICAL CELL SUSPENSIONS

MICHÈLE BASSET, BÉATRICE ROSTAING-METZ and EDMOND M. CHAMBAZ
Laboratoire de Biochimie Endocrinienne (INSERM U-244, CNRS ERA 942), CERMO, Université Scientifique et Médicale, 53 X, 38041 Grenoble Cedex, France

(*Received* 16 *August* 1981)

SUMMARY

The possible role of steroid binding proteins in the hormonal secretion process of a steroidogenic tissue was examined using bovine adrenocortical cell suspensions, either under basal conditions or in the presence of half-maximally active concentration (1×10^{-9} M) of synthetic adrenocorticotropic hormone (ACTH). Three types of plasma cortisol binding proteins were used, namely bovine serum albumine (BSA), purified transcortin (CBG) and purified anticortisol immunoglobulins (IgG). When added to the incubation medium, CBG (at 1×10^{-10} to 2×10^{-9} M cortisol binding sites) and anticortisol IgG (at 4.8×10^{-10} to 3×10^{-9} M cortisol binding sites) did not influence either the basal nor the ACTH-stimulated net cortisol production of the cell preparations. Whereas crystallized and delipidated BSA showed also no effect, crude commercial BSA preparation (Cohn fraction V) exhibited an ACTH-like cofactor effect which resulted in a marked increase in the net cortisol production by stimulated cells. These observations might be explained by the presence in crude BSA of lipoprotein–cholesterol complexes, possibly acting as an extracellular source of cholesterol available for corticosteroidogenesis.

It may be concluded that specific high affinity cortisol binding systems present outside adrenocortical steroidogenic cells do not influence their secretory activity under short term *in vitro* condition. In addition, it can be stressed that use of ill defined protein preparations (e.g. crude BSA) may lead to artifactual observations in the study of the differentiated functions of isolated steroidogenic cells.

Biologically active steroidal hormones are found in mammalian blood mostly bound to circulating serum proteins [1, 2]. In addition to serum albumin which is able to bind with a relatively low affinity ($K_a < 10^6\ M^{-1}$, 4°C) a number of small molecules including steroids (unspecific binding), several high affinity serum steroid binding proteins have been identified, which exhibit higher binding affinity (i.e. $K_a > 10^6\ M^{-1}$) toward a limited number of structurally related steroidal structures (specific binding). Such is the case of corticosteroid binding globulin (CBG, transcortin) and testosterone-estradiol or sex steroid binding globulin (SBG) [1, 2]. Although often considered as a reservoir of active hormones representing a buffer system, the biological role of these serum steroid binding proteins is not clearly understood [1]. A possible role in the steroid transfer into target tissue has been suggested in the case of SBG and testosterone [3]. On the other hand, steroid secretion is usually considered to involve a passive diffusion process through plasma membrane; however, the presence of avidly binding system in the vascular compartment might suggest a facilitation in this directed traffic [1, 2]. Recently, this hypothesis has found support with the report that testosterone production by gonadotropin stimulated testis was greatly enhanced when serum albumin was added to the perfusion medium [4]. On the other hand, binding macromolecules were found to be secreted simultaneously with corticosterone by rat adrenal quarters, after ACTH stimulation *in vivo*, although restrictive incubation conditions were required to observe this phenomenon [5].

The present work was undertaken in order to examine the possible action of individual corticosteroid binding proteins on the steroidogenic activity of adrenocortical cell suspensions *in vitro*. It was hoped that clear cut effects might be visualized using highly sensitive cells devoid of surrounding interstitial tissue and purified proteins with well characterized steroid binding properties. Serum albumin was selected as a typical low affinity, large capacity (unspecific) binding system and purified transcortin (CBG) was taken as a model of a naturally occurring high affinity (specific) corticosteroid binder. In an attempt to distinguish between an effect involving molecular specificity and an effect solely due to binding properties, an artificial high affinity cortisol binding system (namely purified anticortisol immunoglobulin IgG), was also used.

MATERIALS AND METHODS

Chemicals

[1,2,6,7 ^{3}H]-cortisol (91 Ci/mmol) was purchased from New England Nuclear and unlabeled steroids were from Sigma. β 1–24 synthetic ACTH was supplied by CIBA. Trypsin (TRL) and lima bean trypsin inhibitor were from Worthington and minimum

essential Eagle Medium (MEM) was supplied by GIBCO.

Binding proteins

Bovine serum albumin (BSA) either Cohn fraction V, fatty acid free fraction V and crystallized BSA were from Miles Laboratories. Human transcortin (HCBG) was purified from human serum using the affinity chromatography technique of Le Gaillard *et al.*[6]. Purified IgG were prepared following the sodium sulfate precipitation technique [7] either from normal rabbit serum or from a cortisol antiserum obtained from a rabbit immunized with a cortisol-BSA antigen made by coupling cortisol to BSA using the cortisol 3-carboxymethyloxime derivative [8] and the mixed anhydride technique of Erlanger *et al.*[9].

All proteins were checked for homogeneity before use by 7.5% polyacrylamide gel electrophoresis [10]. Protein concentrations were measured according to Lowry *et al.*[11] as modified by Zack and Cohen[12] using BSA as the standard.

Binding assays

Binding properties of steroid binding proteins were determined after incubation (18 h at 4°C) with various concentrations of [^{3}H]-cortisol and isolation of the bound steroid species by the charcoal-dextran procedure, as previously described [13]. The data were treated by the graphical method of Scatchard[14] for the calculation of binding affinity (K_a) and binding capacity.

Adrenocortical cell preparations

Bovine adrenal glands were obtained from the local slaughterhouse, kept in ice-cold MEM before processing within 1 h after slaughter. Fasciculo-reticulata cell suspensions were prepared essentially following the method of Kloppenborg *et al.*[15] slightly modified as previously described [16]. The final cell pellet was suspended in pH 7.4 Krebs Ringer bicarbonate buffer containing 0.2% glucose (KRBG). Cell incubations were routinely performed using 0.5 ml aliquots (2×10^6 cells) in capped polyethylene tubes maintained at 37°C in a stirring water bath, under an O_2–CO_2 (95:5) atmosphere. Incubations were started after 1 h standing (preincubation), upon addition of test substances in 0.6 ml KRBG without or with addition of β 1–24 adrenocorticotropin (ACTH) in 0.1 ml KRGB as indicated. The incubations were stopped upon addition of 5 ml methylene chloride. The organic extract was then used for cortisol measurement by radioimmunoassay as described previously [16]. All test points were run in triplicate.

Other techniques

Low density lipoproteins were isolated from bovine serum by ultracentrifugation according to [17] and analysed by cellulose acetate and/or polyacrylamide gel electrophoresis using Sudan Black B as the staining agent [18]. Cholesterol determination was carried out using the acetic anhydride–sulfuric acid procedure with the corresponding assay kit supplied by Boehringer Manheim.

RESULTS

1. *Steroidogenic properties of bovine adrenocortical cell suspensions*

Steroidogenic activity of our adrenocortical cell suspensions was characterized in some detail in order

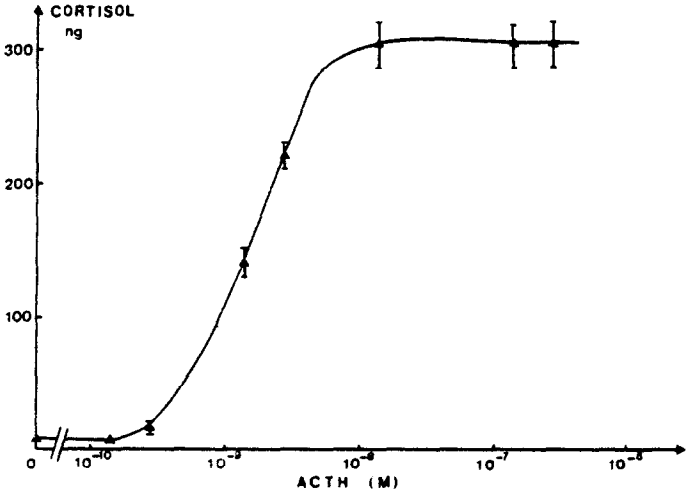

Fig. 1. Dose–response curve of ACTH-stimulated steroidogenesis by bovine adrenocortical cell suspension. Cell suspensions were incubated as described under materials and methods in the presence of various concentrations of β 1–24 ACTH. Cortisol is expressed as ACTH-elicited net steroid production (cortisol concentration after 1 h incubation in the presence of ACTH minus cortisol concentration in the control, in the absence of ACTH) per 2×10^6 cells, per h. Each point is the mean of three independent replicates; vertical bars represent the standard error of the mean (SEM).

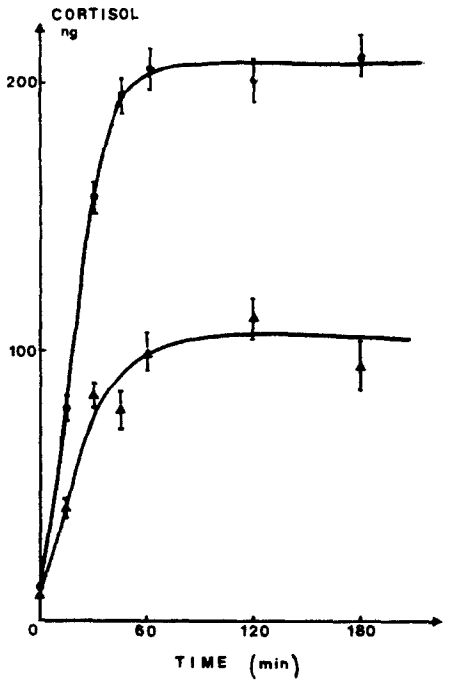

Fig. 2. Time-course of cortisol accumulation in the incubation mixture containing 2×10^6 adrenocortical cells in the presence of ACTH (1.4×10^{-9} M) (▲——▲) and in the presence of ACTH (1.4×10^{-9} M) plus Cohn fraction V bovine serum albumin (7.5×10^{-4} M) (●——●). Net cortisol production was calculated as indicated in the legend of Fig. 1. Each point is the mean of triplicate experiment; vertical bars give the corresponding standard error of the mean.

to study the possible effect of steroid binding proteins under well standardized conditions. Using 2×10^6 cells per individual incubation tube and 1 h incubation time, the dose response curve shown in Fig. 1 was obtained when an increasing amount of ACTH was added to the medium bathing the cells. The net production of cortisol (calculated by subtracting the value measured in control incubation without ACTH from the value found when ACTH was present) was clearly stimulated for ACTH concentrations below 5×10^{-9} M and reached a plateau (average of 300 ng cortisol produced per 2×10^6 cells per h) for ACTH concentrations higher than 10^{-8} M. For the study of possible binding protein effects, it was chosen to routinely use ACTH concentration (i.e. 1.4×10^{-9} M) giving a half-maximum steroidogenic response. Using this dosage, a time course study was conducted and showed that cortisol production increased rapidly with time to plateau after 1 h (Fig. 2). This incubation time was thus routinely employed in further experiments.

2. Effect of anticortisol IgG on adrenocortical cell steroidogenesis

Purified IgG isolated from a rabbit antiserum raised against cortisol as well as IgG from normal rabbit serum were used after extensive dialysis, at 4°C, against 50 vol. of 1 mM, pH 7.4 sodium phosphate buffer. Binding affinity and capacity for cortisol were determined and yielded a K_a of 10^9 M^{-1} for anticortisol IgG with a binding capacity of 1.1×10^{-10} mol cortisol per mg IgG protein, whereas no cortisol binding could be detected with normal rabbit serum IgG (Table 1).

The possible effect of anticortisol IgG preparation on adrenocortical cell steroidogenesis was investigated using increasing high affinity cortisol binding site concentrations in the medium, with and without the presence of 1.4×10^{-9} M ACTH. Normal rabbit serum IgG preparation was used as the control. The results obtained under these conditions show that no effect of anticortisol IgG could be detected either on the net cortisol production under stimulation by ACTH (Table 1), nor on the basal steroidogenic activity in the absence of ACTH (not shown). In addition, stimulated and unstimulated cortisol productions were similar whether the incubation medium

Table 1. Effect of purified anticortisol IgG and purified human transcortin (CBG) on the ACTH-elicited cortisol production by adrenocortical cell suspensions during 1 h incubations

Protein added (mg/ml medium)	Cortisol binding sites (M)	Net cortisol production (ng/2 × 10^6 cells/h)
None (control)	0	68 ± 3.0
Anticortisol IgG		
4.2	4.8×10^{-10}	70 ± 3.8
2.1	2.4×10^{-10}	69.5 ± 5
1.05	1.2×10^{-10}	78 ± 5
0.52	6×10^{-9}	74 ± 4.5
0.26	3×10^{-9}	74 ± 7
Normal rabbit serum IgG		
1.8	0	69 ± 9
CBG 10^{-3}	2×10^{-9}	73 ± 5
CBG 2×10^{-4}	1×10^{-10}	72 ± 9

Net cortisol production was calculated as indicated in the legend of Fig. 1. ACTH was used at 1.4×10^{-9} M concentration in the incubation medium. Each figure is the mean of replicate experiment ± SEM.

contained normal rabbit serum IgG or no added protein.

3. *Effect of purified transcortin*

Purified human transcortin (HCBG) preparation as obtained following the affinity chromatography preparation procedure contained cortisol. As emphasized by several research groups [1], purified transcortin is highly unstable when stripped of its bound ligand. However, after treatment by a charcoal-dextran suspension, it was possible to obtain a transcortin preparation partly devoid of its endogenous cortisol. The preparation still exhibited satisfactory binding properties when its interaction with cortisol was analysed by the Scatchard method ($K_a \sim 10^8\ M^{-1}$). When this charcoal-treated transcortin preparation was added to adrenocortical cell suspensions, this resulted in the introduction of cortisol in the incubation medium (Table 1). Taking into account this basal cortisol level, no effect of the added CBG (representing 1×10^{-10} to 2×10^{-9} M cortisol binding sites) could be detected on the net stimulated cortisol production in the presence of ACTH (Table 1). In addition, control experiments were run in the presence of equivalent amounts of cortisol added to the medium, in the absence of binding protein; the ACTH-elicited steroidogenic activity of the cells was not modified under these conditions (not shown).

4. *Effect of bovine serum albumin*

Whereas the presence of BSA (Cohn fraction V) in a wide concentration range (0.5 to 7.5×10^{-4} M) had no effect on basal cortisol production by the adrenocortical cell suspensions, a striking enhancement of ACTH-stimulated cortisol production was observed under these conditions. As illustrated in Fig. 2, the time course of steroidogenesis under ACTH stimulation was similar whether BSA (7.5×10^{-4} M) was present or not but the final plateau observed after 1 h incubation in the presence of BSA represented a net cortisol production about twice that seen in the absence of BSA. This enhancing effect of BSA on ACTH stimulated steroidogenesis exhibited a concentration dependent effect, as shown in Fig. 3. In addition, when ACTH was increased to its maximally active concentration (see Fig. 1), an enhancing effect of similar magnitude was still observed when BSA was present in the medium.

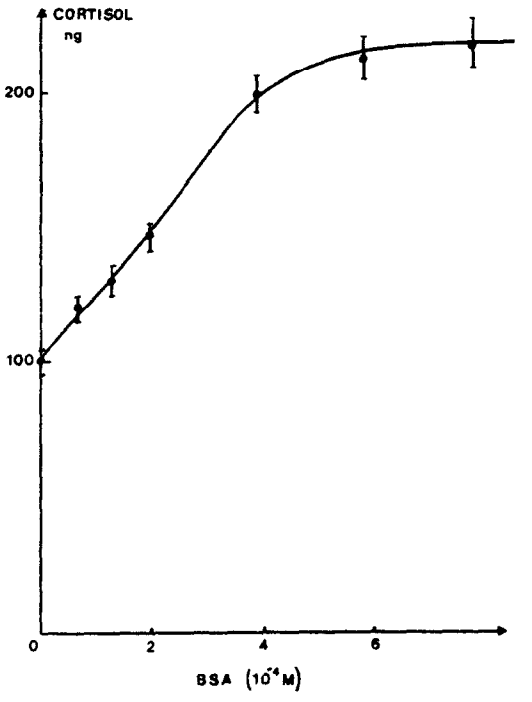

Fig. 3. Effect of BSA concentration upon ACTH-stimulated cortisol production by bovine adrenocortical cell suspensions. Cohn fraction V (BSA) was present at different concentrations in the incubation medium, with or without addition of 1.4×10^{-9} M β 1–24 ACTH. Net cortisol production was calculated as in Fig. 1. Each point is the mean of triplicate experiment; vertical bars represent the standard error of the mean.

When the BSA preparation was treated at 100°C (5 min) to purposely denature the protein structure, the enhancement of ACTH stimulation persisted (Table 2), suggesting that the observed steroidogenic effect may be due to a contaminant of the Cohn fraction V preparation rather than to albumin itself. This hypothesis was confirmed by the fact that commer-

Table 2. Effect of various preparations of bovine serum albumin (BSA) on ACTH-stimulated net cortisol production by bovine adrenocortical cell suspensions, over 1 h incubation

Protein added (7.5×10^{-4} M)	Net cortisol production (ng/2 × 10^6 cells/h)
None (control)	100 ± 10
Crude BSA	194 ± 12
Heat-treated crude BSA	202 ± 14
Charcoal-treated crude BSA	104 ± 9
Fatty acid free BSA	112 ± 11
Cristallized BSA	91 ± 8

ACTH was at 1.4×10^{-9} M concentration in the incubation medium. Each figure is the mean of triplicate experiment ±SEM.

cially available fatty acid free as well as cristallized BSA preparations had no effect on the basal or ACTH stimulated steroidogenesis of adrenocortical cell suspensions (Table 2). Similar negative result was obtained with Cohn fraction V after charcoal treatment according to Chen *et al.*[19]. It was then thought that the steroidogenic effect of BSA (Cohn fraction V) might be due to the presence of a lipidic contaminant, possibly functioning as an additional, extracellular supply of cholesterol, such as low density lipoprotein (LDL)-cholesterol complex which has been shown to support steroidogenesis in bovine adrenocortical cells in culture [20, 21]. Analysis of the Cohn fraction V (5 mg protein) by electrophoresis on cellulose acetate followed by Sudan blue staining did not disclosed any detectable LDL-like component. However, it may be pointed out that the sensitivity of the method would have required about 40 μg lipoprotein in the sample to be detectable by the staining procedure. When total cholesterol content of the BSA preparations was measured, Cohn fraction V yielded an average of 1.0 μg cholesterol/mg protein. This led to the calculation that at maximally effective steroidogenic concentration, this BSA preparation introduced about 50 μg cholesterol in the incubation medium, containing 2×10^6 cells. A LDL bovine serum fraction was then prepared by ultracentrifugation. The preparation contained 0.82 mg cholesterol/mg protein. When 100 μg of this LDL preparation (i.e. 80 μg cholesterol) was added to the adrenocortical cell suspensions under the aforementioned standard incubation conditions, the steroidogenic effect of ACTH (1.4×10^{-9} M) was increased by an average of 2-fold (not shown). It was thus concluded that, although not detected by electrophoretic analysis, a LDL-cholesterol contamination was most likely to be the explanation for the steroidogenic enhancing effect exhibited by Cohn fraction V commercial BSA preparations. This contaminant is not present in fatty acid free nor in crystallized BSA.

DISCUSSION

In a search for a possible role of plasma steroid binding proteins in the regulation of corticosteroid secretion, highly sensitive bovine adrenocortical cell suspensions were used and their basal and ACTH-stimulated cortisol producing activity examined with and without the presence of corticosteroid binding proteins. Three binding systems with different binding affinity and specificity were used, namely bovine serum albumin, purified serum transcortin and purified anticortisol IgG. The presence of high affinity binding sites in the incubation medium (anticortisol IgG, transcortin) did not influence the basal adrenocortical cell steroidogenic activity, nor the ACTH-stimulated corticosteroid production. These data do not support a role of high affinity plasma steroid binding proteins in the regulation of the steroidogenic tissue secreting activity, at least under short term *in vitro* conditions as used in this study.

Although these observations do not rule out the possible involvement of yet undefined endogenous binding macromolecular systems in the outward movement of hormonal steroids from the secretory cells, as suggested by others [5] plasma steroid binders do not appear to facilitate cellular steroid exit by acting as a pumping system outside the cell.

Crude serum albumin (Cohn fraction V) was the only protein preparation, which exhibited a potent enhancement of the steroidogenic action of ACTH when added to adrenocortical cell incubation medium. On the other hand, the preparation showed no detectable steroidogenic effect in the absence of ACTH. This ruled out the possibility that Cohn fraction V activity may contain ACTH or ACTH-like substance(s). In addition, this activity was relatively thermostable whereas authentic ACTH was totally inactivated by similar heat treatment (100°C, 5 min). Thus, crude BSA preparation contains a component(s) which acts as a co-factor in enhancing ACTH-elicited steroidogenesis of adrenocortical dispersed cells. This property of the Cohn fraction V preparation was lost upon treatment with dextran coated charcoal and was not observed with crystallized BSA nor with fatty acid free BSA from commercial sources. These findings strongly suggest that a lipidic component(s) is present in crude BSA and may account for the ACTH co-factor effect. Serum low density lipoproteins (LDL) have been shown to represent a source of extracellular cholesterol, able to support steroidogenesis by adrenocortical cells in culture [20, 21]. The fact that a bovine serum LDL preparation was able to increase cortisol production by our ACTH-stimulated adrenocortical cell suspensions under our incubation conditions (1 h) would support the hypothesis that an LDL contaminent may explain the similar effect of crude BSA. Due to the lack of sensitivity of the detection methods used, it was not possible to assess on a quantitative basis whether the ACTH co-factor effect could be entirely explained by LDL. However, total cholesterol content of Cohn fraction V as compared to that of equally effective LDL fraction are quite similar. This would be in agreement with an LDL–cholesterol complex participation in the crude BSA ACTH-enhancing effect. Assuming that the total amount of cholesterol measured in crude BSA (average of 1 μg/mg protein) is in LDL-cholesterol form, and assuming that the cholesterol–protein mass ratio in bovine LDL is about 1:1 [20] as found with our bovine serum LDL fraction, this would mean that crude BSA contains about 1 μg LDL protein per mg albumin.

It may thus be concluded that steroid binding proteins, including bovine serum albumin do not influence the steroidogenic properties of bovine adrenocortical cell suspensions. This is in contrast to the report that crystallized BSA enhanced the gonadotropin-stimulated testosterone production by perfused

dog testis [4]. However, it may be that steroid binding proteins play a role in the diffusion and/or transport of hormonal steroids after their excretion step from steroidogenic cells as for example through intertitial tissue and capillary walls.

The findings reported herein point to the fact that bovine serum albumin, which has often been used in steroidogenic cell suspension media [22] should be carefully selected with regards to the presence of contaminants, possibly giving rise to artifactual results in the study of isolated steroidogenic cell functions.

Acknowledgements—This work was supported by the INSERM (U-244, Contrat libre 78.1.214.4), the CNRS (ERA 942) and the Fondation pour la Recherche Médicale Française.

REFERENCES

1. Westphal U.: *Steroid–Protein Interactions.* Vol. 1. Springer Verlag, New York (1971).
2. Baulieu E. E., Raynaud J. P. and Milgrom C.: Measurement of steroid binding proteins. *Acta endocr., Copenh.* **64** suppl. **147** (1970) 104–121.
3. Rosen V., Jung I., Baulieu E. E. and Robel P.: Androgen-binding proteins in human benign prostatic hypertrophy. *J. clin. Endocr. Metab.* **41** (1977) 761–770.
4. Ewing L. L., Chubb C. E. and Robaire B.: Macromolecules, steroid binding and testosterone secretion by rabbit testes. *Nature* **264** (1976) 84–86.
5. Pritchett J. F., Harper W. L., Marple D. N., Bradley J. T. and Till M. L.: [^{3}H]-Corticosterone binding activity of adrenal incubation media in response to *in vivo* and *vitro* ACTH stimulation. *Biochem. biophys. Res. Commun.* **90** (1979) 1355–1363.
6. Le Gaillard F., Racadot A., Racadot-Leroy N. and Dautrevaux M.: Isolement de la transcortine humaine par chromatographie d'affinité. *Biochimie* **56** (1974) 99–108.
7. Heide K. and Schwick H. G.: Salt fractionation of immunoglobulins. In *Immunochemistry* (Edited by Weir D. M.). Blackwell, London, Vol. 1 (1978) pp. 7.1–7.11.
8. Janoski A. H., Shulman F. C. and Wright G. E.: Selective 3-(O-Carboxymethyl) oxime formation in steroidal 3,20-diones for hapten immunospecificity. *Steroids* **23** (1973) 49–64.
9. Erlanger B. F., Borek F., Beiser A. M. and Lieberman S.: Steroid–protein conjugates. I. Preparation and characterisation of conjugates of bovine serum albumin with testosterone and with cortisone. *J. biol. Chem.* **228** (1957) 713–727.
10. Davis B. J.: Disc electrophoresis II: Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121** (1964) 404–427.
11. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193** (1951) 265–275.
12. Zack B. and Cohen J.: Automatic analysis of tissue culture proteins with stable Folin reagents. *Clin. chim. Acta* **6** (1961) 665–670.
13. Hadjian A. J., Chedin M., Cochet C. and Chambaz E. M.: Cortisol binding to proteins in plasma in the human neonate and infant. *Pediat. Res.* **9** (1975) 40–45.
14. Scatchard G.: The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51** (1949) 660–672.
15. Kloppenborg P. W. C., Island D. P., Michelakis A. M. and Nicholson W. E.: A method of preparing adrenal cell suspensions and its applicability to the *in vitro* study of adrenal metabolism. *Endocrinology* **82** (1968) 1053–1058.
16. Duperray A. and Chambaz E. M.: Prostaglandin synthetase activity in bovine adrenocortical cells and subcellular preparations: effect of ACTH. *Mol. Cell. Endocr.* **18** (1980) 249–260.
17. Havel R. J., Eder H. A. and Bragdon J. H.: The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. clin. Invest.* **34** (1955) 1345–1353.
18. Narayan K. A.: Disc electrophoresis of human and animal serum lipoproteins. *Lipids* **2** (1967) 282–284.
19. Chen R. F.: Removal of fatty acids from serum albumin by charcoal treatment. *J. biol. Chem.* **242** (1967) 173–181.
20. Kovanen P. T., Faust J. R., Brown M. S. and Goldstein J. L.: Low-density lipoprotein receptors in bovine adrenal cortex. I. Receptor-mediated uptake of low density lipoprotein and utilization of its cholesterol for steroid synthesis in adrenocortical cells. *Endocrinology* **104** (1979) 599–609.
21. Brown M. S., Kovanen P. T. and Goldstein J. L.: Receptor-mediated uptake of lipoprotein-cholesterol and its utilization for steroid synthesis in the adrenal cortex. In *Recent Progress in Hormone Research* (Edited by R. O. Greep). Academic Press, New York, Vol. XXXV (1979) pp. 215–258.
22. Sayers G., Beall R. J., Seelig S. and Cummins K.: Isolation of adrenal cortex cells. Hormone responses. In *Methods in Enzymology* (Edited by Fleischer S. and Packer L.). Academic Press, New York, Vol. 32 (1974) pp. 673–693.