Journal of Steroid Biochemistry, 1975, Vol. 6, pp. 389–394. Pergamon Press. Printed in Great Britain

A COUNTER-STREAMING CENTRIFUGATION TECHNIQUE FOR THE SUPERFUSION OF ADRENOCORTICAL CELL SUSPENSIONS STIMULATED BY ACTH

D. SCHULSTER and CELIA JENNER
Biochemistry Group, School of Biological Sciences, University of Sussex, Brighton, England

SUMMARY

A system is described for the superfusion of isolated adrenocortical cells. Cells are retained in suspension in a small conical tube by balancing the viscous force of flowing medium against an opposing centrifugal force. Fractions of the effluent medium have been collected over short time periods, for examination of the dynamic responses of the isolated cells to single injections and to continuous infusions of ACTH. The effect of cycloheximide has been studied and an overshoot phenomenon observed following its withdrawal, similar to that found when ACTH alone was continuously infused. The characteristics of the output *rates* of cyclic AMP and of corticosterone were very much alike in response to different amounts of injected ACTH, and showed that the cells remained responsive in this system for several hours. Large increases in the *rate* of steroidogenesis were apparent in this sytem within 24 seconds of ACTH administration. The rapidity of this effect makes unlikely the direct induction by ACTH of the synthesis of new protein for regulation of steroidogenesis, unless this protein can be assembled in an exceptionally short time. The data are however consistent with a model proposing the post-translational activation by ACTH of a pre-formed, but labile, protein. The half-life of this regulatory protein was estimated at 3 min in this system.

1. INTRODUCTION

The superfusion system or *in vitro* continuous flow technique was developed independently by three different research groups. The potentialities of this system were elaborated in 1965 by Orti *et al.*[1], but it was not until 1967 that the experimental results of the technique, applied to the analysis of adrenal gland steroidogenesis, were simultaneously reported by Tait *et al.*[2] and Saffran *et al.*[3]. The rationale for this approach, methodology and results of studies utilizing bisected whole, decapsulated and capsular glands have been reviewed[4, 5], and the value of this technique has been established. This dynamic approach has allowed observation of variations in the output of different steroids by discrete zones of the adrenal cortex, during the course of the superfusion. In this way the pattern of the *in vitro* steroid response has been demonstrated to reflect the physiological status of the rat prior to adrenalectomy. Moreover the responses to stimulatory agents such as ACTH and cyclic AMP have been studied under conditions that eliminate factors complicating these responses in other *in vivo* and *in vitro* systems. Thus in the superfusion system flow-rates are precisely defined and effects of ACTH and cycloheximide on blood-flow rate[6, 7] eliminated, as are the feedback-inhibitory effects arising from accumulated products.

However previous superfusion studies [8–13] employing whole or sectioned glands have suffered from problems associated with the limited permeability of blocks of tissue. In the *in vivo* situation, each adrenocortical cell is well endowed with blood from a fine network of capillaries [14] providing for an efficient, plentiful supply of nutrients and the rapid removal of products. By contrast, in *in vitro* incubations of sectioned or whole glands, where medium merely bathes the outer surface of the tissue fragment, the permeabilities of added regulatory agents into the tissue and of metabolic products out of the tissue influence the characteristics of the response. These limitations may account for the relative sluggishness of the responses observed with superfused glands [9, 10] compared with those noted *in vivo*. Furthermore the comparative insensitivity of adrenal tissue *in vitro* implies that only the outer layer of cells in the tissue fragments are hormonally responsive, and that for example ACTH stimulates cells within the tissue mass, only with difficulty.

Recent developments for the disaggregation of adrenal tissue into suspensions of isolated cells [15–18] and their subsequent separation [19] into discrete cell types have provided an approach to overcome these *in vitro* permeability problems. In suspensions of isolated cells, each cell is adequately provided with its nutrient requirements, added regulatory agents may readily bind to or enter the cells, and cellular products are discharged directly into the medium. To investigate the dynamic control processes operating in the adrenal cell, the superfused isolated cell system thus offers a potentially valuable method for overcoming the limitations outlined above. To accomplish this, the counterstreaming centrifugation system, first described by Lindahl[20, 21] has been adopted, and superfused isolated adrenocortical cells have been examined for their ACTH responsiveness [22].

2. METHODOLOGY

Preparation of isolated adrenal cells

Collagenase (0·5 mg/ml; Boehringer Corp.) disaggregated cells were prepared [18] from decapsulated adrenal glands of intact female Sprague–Dawley rats (150–200 g). The cells were resuspended and washed in Krebs–Ringer bicarbonate buffer (KRBGA) containing glucose (0·2%, w/v) and albumin (0·5%, w/v; dialysed outdated human albumin, Lister Inst. Elstree), equilibrated at 37°C with O_2–CO_2 (95:5%) to maintain the pH at 7·4. KRBGA buffer was used in all superfusions as the flowing medium.

ACTH and cycloheximide solutions

Solutions of ACTH (porcine 1–39 ACTH; Armour Pharm. Co., Eastbourne, U.K.) were prepared in KRBGA buffer using all plastic apparatus to minimize adsorption. Single injections (0·1 ml) were made using a plastic syringe. Continuous infusions of ACTH were performed by changing to KRBGA medium containing appropriate concentrations of ACTH, or by infusing ACTH directly into the flowing medium *via* a separate pumping chamber (0·1 ml/min). Cycloheximide (Actidione; Koch–Light Labs. Colnbrook, Bucks., U.K.) was dissolved in saline and infused at a constant rate via a separate pumping chamber. These solutions were infused using LKB Varioperpex peristaltic pumps (Bromma, Sweden), via separate hypodermic needles inserted through the rubber cap sealing the entry port to the apparatus (see Fig. 1). Where concentrations of these agents are given (i.e. continuous infusions as opposed to single, instantaneous injections) these are always expressed in terms of the final concentration in the superfusate.

Superfusion system

The apparatus used has been previously described [22]. In recent experiments this has been slightly modified to that shown in Fig. 1. The modifications incorporate a zonal centrifuge feedhead assembly (Mark II; M.S.E. Scientific Inst., Crawley, U.K.) which has the advantage of eliminating leakages around the Rulon liquid seal. A further modification was a shortening of the side-arms of the Teflon rotor to an overall length of 2 cm., which facilitates the construction of this rotor out of a single cylindrical block of Teflon.

Isolated adrenal cells were spun in a siliconized glass conical tube (2·0 ml) at speeds between 300 and 500 rev./min (10–40 g) using the apparatus (Fig. 1) rotated about a vertical axis by means of a D.C. shunt-wound motor (Parvalux, Bournemouth, U.K.) with a constant speed control unit (D.C. Motor Controller Type 500; Eurotherm, Worthing, Sussex, U.K.). Incubation medium KRBGA, prewarmed to 37°C, was continuously infused into the conical glass tube, *via* a thin Teflon cannula fixed into the side-arm of the Teflon rotor. The medium was introduced directly at the apex of the conical glass tube; this is the region of maximal centrifugal force. Medium then flowed towards the neck of the tube, through the suspension of cells in a direction towards the axis of rotation. The behaviour of the cells in suspension was visualized using a synchronized stroboscope and the rotational speed and medium flow-rate adjusted so that the centrifugal force outwards on the cells was counterbalanced by the inward viscous force induced by the flowing medium.

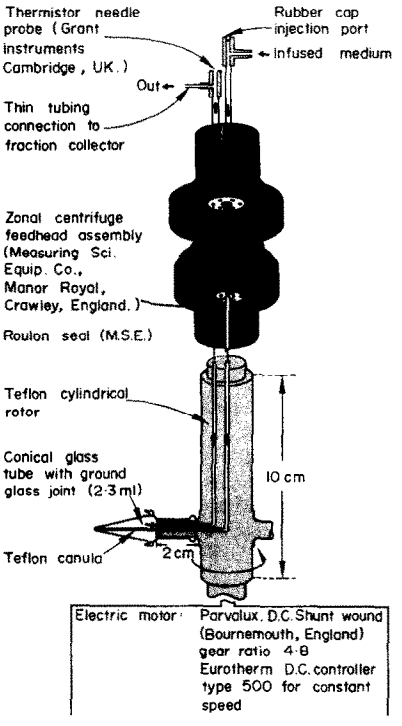

Fig. 1. Apparatus constructed for the superfusion of isolated adrenal cells in a continuously flowing medium. It comprises a zonal centrifuge feedhead (M.S.E. Sci. Instr.; Mark II) incorporating a Rulon liquid seal which remains stationary, and a rotating Teflon rotor with glass conical tubes attached to its side arms. Adrenal cells are placed in one of these glass tubes through which medium flows, while the other tube functions merely as a balance tube. A thin Teflon tube fixed into the side-arm of the Teflon rotor delivers medium directly at the apex of the glass conical tube. Medium then flows towards the neck of the tube (towards the centre of rotation), through the cell suspension and out through the upper orifice in the side arm. Short arrows indicate the direction of liquid flow. For further details see Methodology.

The chamber enclosing the apparatus was maintained at 37°C by means of a thermostatically regulated heating-coil and the temperature of the effluent was monitored using a thermistor thermometer with a needle probe (Model C; Edale Inst., Cambridge, U.K.). Further details of this apparatus have been published elsewhere [5, 22].

Samples were collected in a fraction collector (LKB Ltd.) over set time periods (usually 0·4, 1, 2 or 5 min depending upon the experiment) and assayed for corticosterone or cyclic AMP as previously described [23]. Cyclic AMP assays were performed by Dr. Caroline Mackie to whom grateful thanks are extended.

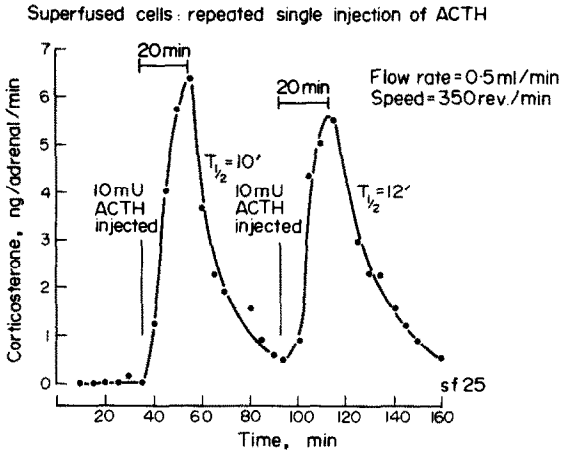

Fig. 2. Effect of a repeated single injection of ACTH on the superfused adrenocortical cells prepared from 5 rats. At 35 and 95 min after the start of the superfusion, ACTH (10 mU; 0·1 ml) was rapidly injected into the flowing stream of medium. The medium flow-rate was 0·5 ml/min and 5 min fractions were collected for fluorimetric steroid assay. The rotational speed was 350 rev./min.

3. RESULTS AND DISCUSSION

(i) *Steroid responses to single injections of ACTH*

To examine the characteristics of the dynamic response of the cells to repeated injections of ACTH, 10 mU of the hormone was added in a single instantaneous (less than 5 s) injection at 35 and 95 min after the start of the superfusion (Fig. 2). The responses at these different times were very similar, with maximal outputs (6·4 and 5·5 ng/adrenal/min respectively) achieved 20 min after the injections, and the subsequent decay in steroidogenesis having half-lives of 10 and 12 min respectively. In this experiment the relatively slow flow-rate of 0·5 ml/min was used. The cells clearly remain responsive during the course of a 2 h superfusion.

The effect of repeated single injections of increasing amounts of ACTH is shown in Fig. 3. Over the range

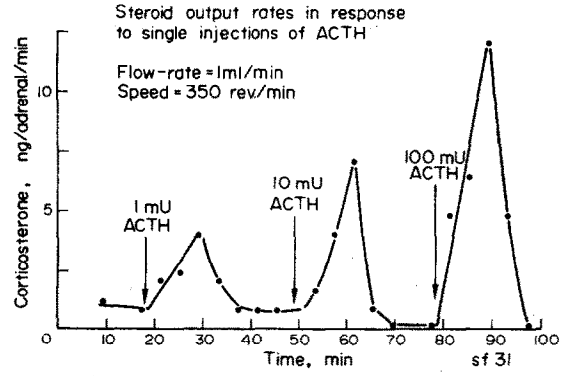

Fig. 3. Effect of single injections of ACTH in increasing amounts. At 18, 49 and 78 min after the start of the superfusion ACTH (1, 10 and 100 mU respectively) were injected into the flowing medium stream. The medium flow-rate used was 1·0 ml/min and 2 min fractions were collected; other details were as described in the legend to Fig. 2.

examined (1–100 mU ACTH) both the total amount of corticosteroid produced and the maximal rate of steroidogenesis, were proportional to the log of the *amount* of ACTH injected. Others have noted from superfusion studies using quartered glands [12] that the response to ACTH depends upon the total dose of hormone and not upon its concentration in the medium. It has recently become clear from binding studies utilizing ^{125}I-labelled ACTH [24] that under the superfusion conditions used in this experiment (Fig. 2) the vast majority of the hormone injected flows through the system without binding to the cells. Thus the concentration of ACTH that is actually effective in stimulating steroidogenesis must be very much less than that indicated by the injected amounts. Again it is evident that the cells remain responsive during this time period of superfusion and that 1 and 10 mU injections of ACTH are submaximal in this system.

(ii) *Cyclic AMP outputs*

The relationships between the output rate of cyclic AMP and of corticosteroid, in response to single injections of submaximal (1 and 10 mU) amounts of ACTH are depicted in Fig. 4. From these data it is evident that the characteristics of the rate of cyclic AMP output closely mimic those of the steroid output rate. The initial rate of increase of these two parameters is very similar following injections of both 1 and 10 mU ACTH. Moreover both product rates peak at very similar times for each of these two

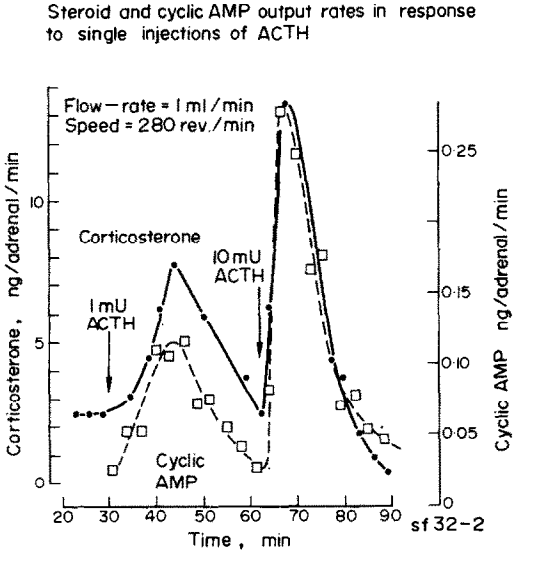

Fig. 4. Comparison of the corticosterone output rate (●——●) and cyclic AMP output rate (□----□) by superfused adrenocortical cells prepared from 5 rats. ACTH (1 and 10 mU) was administered as a single injection (0·1 ml) into the flowing medium stream at the times indicated by the arrows. Cyclic AMP and corticosterone were assayed [23] in 1 min. fractions of the superfusate. A flow-rate of 1 ml/min and rotational speed of 280 rev./min. were used. The half-lives for the decay in both the steroid and the cyclic AMP responses were the same ($T_{\frac{1}{2}}$ = 12 min after 1 mU ACTH; $T_{\frac{1}{2}}$ = 9 min after 10 mU ACTH).

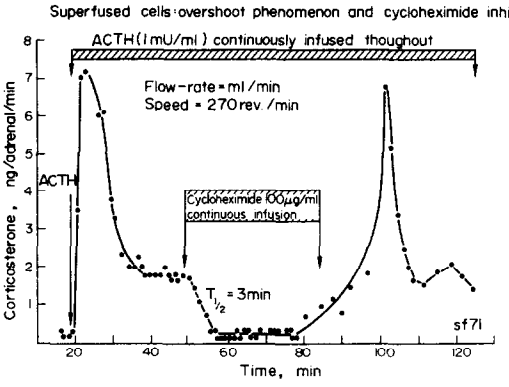

Fig. 5. Overshoot phenomenon and effect of cycloheximide. Superfused isolated adrenocortical cells prepared from six rats, continuously infused with ACTH (1 mU/ml) started 19 min after the incubation start and continued throughout. Cycloheximide (100 μg/ml) was continuously infused for 30 min only, over the time period indicated. Flow-rate of medium was 1 ml/min, rotational speed was 270 rev./min. and 1 min fractions were collected. For further details see text.

amounts of ACTH and the subsequent decay rates are similar for the two products. Finally there is the observation that the relative peak heights and the ratio of the total amounts produced in response to 1 mU ACTH compared with 10 mU ACTH, are very similar for both cyclic AMP and for corticosteroid. The striking resemblance between these responses on all these counts, strongly supports an intimate relationship between the steroidogenic action of ACTH and the involvement of cyclic AMP [25]. It is not of course possible to provide conclusive evidence for an obligatory, mediatory role for cyclic AMP in ACTH action, but nevertheless neither has it been possible using the technique described herein, to disprove this concept.

(iii) *Continuous infusions: the overshoot phenomenon and the effect of cycloheximide*

Urquhart and Li[26] using the perfused canine adrenal *in vivo* have established that when ACTH (2 μU/ml) was continuously infused, cortisol secretion rate rose rapidly, reaching a peak after 8–13 min that then gradually declined during the next 25–30 min to a secretion rate which was steady thereafter. Such an overshoot phenomenon has now been observed in the superfused isolated cell system, using continuous infusions of ACTH (both 100 and 1 mU/ml) with medium flow-rates of 1 ml/min. The characteristics of this overshoot response may be seen from the data in Fig. 5. In this experiment the continuous infusion of ACTH (1 mU/ml) elicited a rapid increase in corticosterone output rate, peaking within 4 min of the start of the ACTH infusion and declining over the next 15 min to a steady state level. When a continuous infusion of cycloheximide (100 μg/ml) was begun 30 min after the start of the ACTH infusion (which continued throughout), the steroidogenic response then decayed with a half-life of 3 min. This decay closely corresponds to that found following cycloheximide inhibition of isolated adrenal cells using static incubation procedures [27] but is less than the 7–10 min half-life observed for this decay *in vivo* for cycloheximide inhibition using the perfused, hypophysectomized rat [28], and much less than the 45–49 min half-life noted using superfused adrenals from hypophysectomized rats [10]. Moreover it is noteworthy that in the superfused isolated cell system, the decline in steroid output rate after cessation of ACTH infusion was surprisingly rapid (half-life 3–10 min; data not shown), as was that observed *in vivo* in the dog [26] with an approximate half-life of 2·5 min.

In isolated adrenocortical cells, cycloheximide (100 μg/ml) has been shown almost totally to inhibit protein synthesis and steroidogenesis [27]. That this inhibitory effect is reversible was demonstrated by the experiment shown in Fig. 5, wherein cessation of the cycloheximide infusion 30 min after it was started (but continued ACTH infusion), resulted in the gradual return of the ACTH response, with virtually the same peak steroid output rate that was observed prior to cycloheximide infusion. Again however, the overshoot phenomenon [26] was evident with a subsequent fall to the steady-state level observed in the earlier phase of this experiment in the absence of cycloheximide. The nature and reversibility of this inhibitory effect supports previous studies recently reviewed [29], implicating a requirement for continuing synthesis of a labile protein in the steroidogenic response to ACTH.

(iv) *The time-lag of the hormonal response*

A time-lag between ACTH addition and the discernible onset of steroidogenesis has been observed in previous studies *in vitro* [18] using static incubation procedures for isolated adrenal cells (of about 3 min), and *in vivo* [26] in the perfused canine adrenal (of about 2 min). However the former methodology measures only gross changes in accumulated product, and fine changes in steroid output rate are not possible to determine in this system; in the latter system the accuracy of this estimate was reported to be poor because of technical limitations. Extensive studies on this time-lag have been undertaken [27] using static incubations of isolated adrenal cells, and these studies indicated the necessity for an even greater critical evaluation of this time-interval between ACTH addition and the manifestations of its effect. The method applied in the presently reported study, lends itself to this objective, as shown by the experiment depicted in Fig. 6. The transit-time of the superfusion apparatus was evaluated by injection of [^{3}H]-corticosterone (0·8 μCi; 6·7 ng) simultaneously with the injection of ACTH (as a single dose of 10 mU). For this experiment the cells isolated from 6 rat adrenals were superfused at the relatively fast flow-rate of 1·5 ml/min, and 0·4 min fractions were collected. Effluent medium was assayed for both radioactivity and corticosterone. As shown in Fig. 6 the radioactivity began to emerge in the third fraction after the injection

(time period 0·8–1·2 min) and the transit-time of the apparatus under these conditions was thus evaluated at 0·8 min. The corticosterone output also first appeared in this same third fraction (0·8–1·2 min) and it should be noted that only 10% of the total weight of corticosterone produced in this fraction can derive from the injected radioactive material. Therefore allowing for the transit-time of the system (0·8 min) no time-lag between ACTH addition and response was detectable in this system, and this time-lag must certainly be less than 24 s. The same conclusion has derived from other similar repeat experiments. The implication of this observation is that there is insufficient time for ACTH to stimulate steroidogenesis by a mechanism involving direct induction of new protein, unless this protein contains few amino acid residues. The average time taken for ribosomes, after having attached to a messenger RNA, to complete translation and release a finished polypeptide, has been estimated in a variety of eukaryotic cells and found to be at least 1–2 min [30–32], even for relatively small polypeptides such as haemoglobin chains (mol. wt. 16000). Thus although a role for protein synthesis in the ACTH effect is clearly evident (as described previously), a translational control system for *de novo* protein synthesis by ACTH now appears most unlikely. An alternative scheme (Fig. 7) is proposed. In this concept ACTH (possibly *via* cyclic AMP) is suggested to activate a labile "precursor protein"; the activated "regulator protein" so formed is also labile and facilitates the conversion of cholesterol to corticosteroids (perhaps by regulating the intracellular pools of cholesterol [33]). Such an activation process would be rapid and consistent with the extreme rapidity of the ACTH response (Fig. 6). The effect of protein synthesis inhibitors would be to block the production of "precursor protein", and the ACTH response would then decay at a rate determined by the depletion from the cell of "regulator protein". This is assessed as having a half-life of three minutes from the studies reported here. This hypothetical model is proposed in the hope that it might provoke further experimentation into this recalcitrant aspect of the hormonal control system.

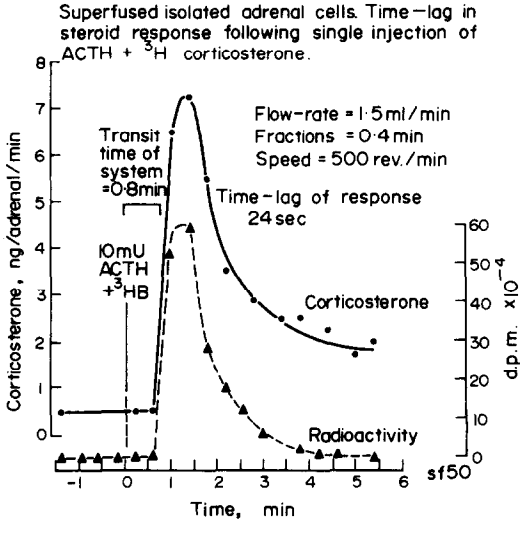

Fig. 6. Superfused isolated adrenocortical cells. Determinations of the transit-time of the system and the time-lag in steroid response following a single injection of ACTH (10 mU) plus [^{3}H]-corticosterone (0·8 μCi; 6·7 ng). Cells prepared from the adrenals of 3 rats were superfused with a flow-rate of 1·5 ml/min and a rotational speed of 500 rev./min. Fractions (0·4 min) were collected and assayed for radioactivity using a Packard liquid scintillation counter, and corticosterone [23]. The transit-time of the system was assessed at 0·8 min and the time-lag of the hormonal response at maximally 24 s.

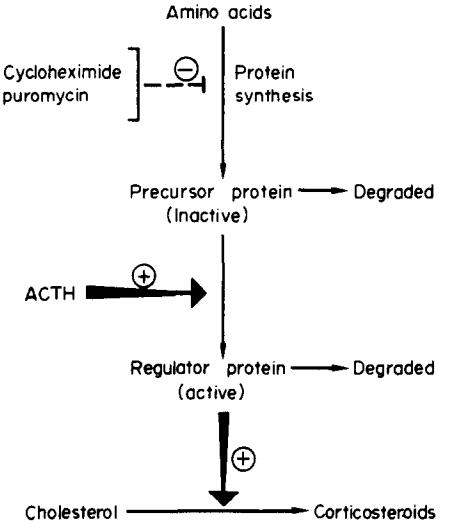

Fig. 7. Model for ACTH action via activation of preformed labile protein. For details see text.

Acknowledgements—The authors gratefully acknowledge the financial support of the M.R.C. and the Wellcome Trust. The Royal Society generously provided a capital equipment grant. We are very grateful to Dr. Caroline Mackie for performing the cyclic AMP assays, to Mr. Peter Lloyd of Armour Pharm. Co. for gifts of porcine ACTH and purified albumin, and to Dr. M. Richardson for much helpful discussion.

REFERENCES

1. Orti E., Baker R. K., Lanman J. T. and Brasch H.: *J. Lab. clin. Med.* **66** (1965) 973–9.
2. Tait S. A. S., Tait J. F., Okamato M. and Flood C.: *Endocrinology* **81** (1967) 1213–1225.
3. Saffran M., Ford P., Matthews E. K., Kraml M. and Garbaczewska L.: *Can. J. Biochem.* **45** (1967) 1901–1907.
4. Saffran M., Mathews E. K. and Pearlmutter F.: *Recent Prog. Horm. Res.* **27** (1971) 607–630.
5. Tait S. A. S. and Schulster D.: In *Methods in Enzymology* (Edited by O'Malley and Hardman) Academic Press, New York Vol. **39** (1975) in press.
6. Urquhart J.: *Am. J. Physiol.* **209** (1965) 1162–1168.
7. Stark E. and Varga B.: *Acta med. Hung.* **25** (1968) 367–381.
8. Saffran M. and Rowell P.: *Endocrinology* **85** (1969) 652–656.

9. Tait S. A. S., Schulster D., Okamoto M., Flood C. and Tait J. F.: *Endocrinology* **86** (1970) 360–382.
10. Schulster D., Tait S. A. S., Tait J. F. and Mrotek J.: *Endocrinology* **86** (1970) 487–502.
11. Huibregtse W. H. and Ungar F.: *Life Sci.* **9** Part 1 (1970) 349–359.
12. Pearlmutter A. F., Rapino E. and Saffran M.: *Endocrinology* **89** (1971) 963–968.
13. Mostafapour M. K. and Tchen T. T.: *Biochem. biophys. Res. Commun.* **48** (1972) 491–495.
14. Dobbie J. W., Mackay A. M. and Symington T.: In *Memoirs of Soc. Endocrinol.* (Edited by V. H. T. James and J. Landon). Cambridge University Press, London No. 1 (1968) p. 103.
15. Kloppenborg P. W. C., Island D. P., Liddle G. W., Michelakis A. M. and Nicholson W. E.: *Endocrinology* **82** (1968) 1053–1058.
16. Sayers G., Beall R. J., Seelig S. and Cummins K.: In *Brain-Pituitary-Adrenal Interrelationships* (Edited by A. Brodish and E. S. Redgate). Karger, Basel (1973) pp. 16–35.
17. Haning R., Tait S. A. S. and Tait J. F.: *Endocrinology* **87** (1970) 1147–1167.
18. Richardson M. C. and Schulster D.: *J. Endocr.* **55** (1972) 127–139.
19. Tait J. F., Tait S. A. S., Gould R. P. and Mee M. S. R.: *Proc. Roy. Soc. Lond. B.* **185** (1974) 375–407.
20. Lindahl P. E.: *Nature* **161** (1948) 648–649.
21. Lindahl P. E. and Nyberg E. I. V. A.: (Ingenioervetenskapsakad) *Tidskr. Tek.-Vetensk. Forsk* **26** (1955) 309–317.
22. Schulster D.: *Endocrinology* **93** (1973) 700–704.
23. Mackie C., Richardson M. C. and Schulster D.: *FEBS Lett.* **23** (1972) 345–348.
24. McIlhinney R. A. J. and Schulster D.: *Endocrinology* **94** (1974) 1259–1264.
25. Grahame-Smith D. G., Butcher R. W., Ney R. L. and Sutherland E. W.: *J. biol. Chem.* **242** (1967) 5535–5541.
26. Urquhart J. and Li C. C.: *Am. J. Physiol.* **214** (1968) 73–85.
27. Schulster D., Richardson M. C. and Palfreyman J. W.: *Molecular and Cellular Endocrinol.* **2** (1974) 17–29.
28. Garren L. D., Ney R. L. and Davis W. W.: *Proc. natn. Acad. Sci. U.S.A.* **53** (1965) 1443–1450.
29. Schulster D.: In *Advances in Steroid Biochem. and Pharmacol.* (Edited by M. H. Briggs and G. Christie). Academic Press, New York, Vol. 4 chapter 7, pp. 233–295.
30. Fan H. and Penman S.: *J. molec. Biol.* **50** (1970) 655–670.
31. Vaughan M. H., Pawlowski P. J. and Forshhammer J.: *Proc. natn. Acad. Sci. U.S.A.* **68** (1971) 2057–2061.
32. Palmiter R. D.: *J. biol. Chem.* **247** (1972) 6770–6780.
33. Mahaffee D., Reitz R. C. and Ney R. L.: *J. biol. Chem.* **249** (1974) 227–233.