Journal of Steroid Biochemistry, 1972, Vol. 3, pp. 643–653. Pergamon Press. Printed in Great Britain

STUDIES IN THE FORMATION AND DEGRADATION OF ADENOSINE 3′5′-CYCLIC MONOPHOSPHATE* IN CORPUS LUTEUM

D. A. STANSFIELD, D. J. FRANKS,† G. H. WILKINSON and JANET R. HORNE
Department of Biochemistry, Medical Sciences Institute, University of Dundee, Dundee, Scotland

SUMMARY

The possible role of cyclic AMP in increasing the rate of steroidogenesis, particularly in luteal tissue, is discussed.

The paper is concerned mainly with the enzyme systems responsible for the formation and the destruction of cyclic AMP in luteal tissue, i.e. adenyl cyclase and cyclic AMP phosphodiesterase; the results of enzymological studies upon adenyl cyclase, the complex "ATPase" of rat corpus luteum membranes, and cyclic AMP phosphodiesterase are reported, particularly in respect to attempts to "solubilise" the membrane-bound cyclase using detergents.

The results are discussed in relation to the findings of other workers.

INTRODUCTION

(a) *The involvement of cyclic AMP in steroidogenesis*

CYCLIC AMP was discovered in 1957 by Rall *et al.*[1, 2] as a heat-stable soluble factor causing the activation of liver phosphorylase which was able to replace the requirements for adrenaline and the homogenate fraction sedimenting at low speed. Sutherland and Rall[3] isolated this factor and proved it to be cyclic AMP. The chemical synthesis of cyclic AMP was achieved by Cook *et al.*[4, 5]. The role of cyclic AMP in steroidogenesis was suggested by Haynes[6] who showed that cyclic AMP concentration and phosphorylase activity of adrenal slices was increased by adding ACTH. Initially, this seemed to confirm the suggestion of Haynes and Berthet[7] that ACTH increased the rate of steroidogenesis by activating the metabolism of glycogen and consequently increasing the production of NADPH.

Bovine luteal phosphorylase was activated by LH *in vitro*[8] and Stansfield and Robinson[9] showed that LH *in vivo* activated rat luteal phosphorylase, and caused small decreases in glycogen content. These authors pointed out that the amount of luteal glycogen was probably too small to play a significant part in producing NADPH for steroidogenesis, and suggested that phosphorylase activation might be a misleading side effect of a substance, possibly cyclic AMP, which was performing a more fundamental role. Other observations have tended to support this belief: Armstrong[10] has shown that in corpus luteum glycolysis, rather than the hexose monophosphate shunt, is the predominant pathway of carbohydrate metabolism, and a number of observations have indicated that in the adrenal cortex both ACTH and cyclic AMP can bring about further increases in steroidogenesis even in the presence of maximally stimulating amounts of exogenous NADPH[11–14], Bell *et al.*[15] reported that ACTH

*Abbreviation: adenosine 3′5′-cyclic monophosphate = cyclic AMP.

†Present Address: Division of Biology, National Research Council, Ottawa 7, Canada.

increases adrenal glycolysis, but not the hexose monophosphate shunt or the tricarboxyllic acid cycle.

It thus seems that increased production of NADPH is but one facet of the control exerted by trophic hormones upon steroid hormone formation. The mediating role of cyclic AMP in the actions of ACTH[16] and LH[17–19] has been clearly shown, and Hirshfield and Koritz[20] have shown that cyclic AMP influences the rate of luteal steroidogenesis at a site between cholesterol and progesterone.

(b) *The mode of action of cyclic AMP in promoting luteal steroidogenesis*

The rate-limiting step of steroidogenesis is now generally accepted as being the side chain cleavage of cholesterol[21–23] with the 20α-hydroxylation step being the likely candidate[24]. The reactions are mitochondrial, although there is an as yet uncalculated contribution from microsomal enzymes[25], but Sulimovici and Boyd[26] could not demonstrate a stimulation of cholesterol side chain cleavage when isolated mitochondria from superovulated rat ovaries were incubated with cyclic AMP. This is suggestive of the involvement of extramitochondrial factors with which cyclic AMP must react before the mitochondrial enzymes can be stimulated. It is likely that the extra-mitochondrial protein phosphokinases which have been demonstrated in many mammalian and bacterial cells may be of importance in the action of cyclic AMP on luteal steroid formation. Kuo and Greengard[27] have shown such an enzyme in bovine ovaries, but most evidence is from other tissues. Gill and Garren[28] have shown that the purified cyclic AMP requiring protein phosphokinase of bovine adrenal cortex can phosphorylate a range of proteins. The phosphokinase is activated when cyclic AMP attaches to a repressor subunit and causes it to detach from the catalytic subunit, bringing about the activation of the catalytic subunit. Its ability to phosphorylate ribosomal proteins may be a partial explanation of the ability of ACTH to stimulate some protein synthesis which is essential to stimulation of steroidogenesis (see [29]). Reddi *et al.*[30] have shown that rat testis is replete with cyclic AMP protein phosphokinases which can phosphorylate histone; this is considered to be a possible way of regulation of the translation of DNA[31]. The proteins which are formed might include the rapidly turning over protein which, according to Koritz and Kumar[32] can increase mitochondrial permeability to pregnenolone, releasing the feedback inhibition of pregnenolone on mitochondrial side chain cleavage enzymes. It thus seems likely that cyclic AMP acts as a mediator of hormone action by releasing protein phosphokinases from their inhibited complexes, and that the character of the response elicited in a particular tissue will depend upon the spectrum of phosphorylatable proteins in the tissue.

(c) *Enzymes controlling the production and destruction of cyclic AMP*

Cyclic AMP is formed from ATP under the action of adenyl cyclase, with the formation of equimolar amounts of inorganic pyrophosphate. There is an ever-increasing list of hormones which are capable of stimulating adenyl cyclase(s) [33, 34]. Hormone-sensitive adenyl cyclases are membrane-bound enzymes which are currently considered to consist of one or more regulatory subunits (hormone receptors) linked in some way to a catalytic subunit[35]. Despite the fundamental importance of adenyl cyclase in hormonal control systems their insolubility and extreme lability have prevented detailed examination of their

properties. Cyclic AMP is hydrolysed to 5′-AMP by cyclic AMP phosphodiesterase (*c*-PDE), the only enzyme known to terminate its biological activity.

We have been studying the properties of rat luteal adenyl cyclase, and of the complex ATP hydrolysing system ("ATPase") which in *in vitro* systems rapidly destroys the substrate for cyclase and interferes with attempts to study the cyclase [36–38], and also the cPDE of bovine luteal tissue[39] and herein review some findings.

MATERIALS AND METHODS

These have been previously described in detail[38, 39]. In one experiment cyclic AMP was measured by the method of Krishna *et al.*[40]. Experiments on the solubilisation of adenyl cyclase were based upon the detergent extraction procedures of Sutherland *et al.*[41] and Levey[42]. Whole homogenates of rat luteal tissue or isolated particulate fractions were treated with Triton X-100 or Lubrol-PX, either with or without exposure to ultra-sound (Dawe Soniprobe) before fractional centrifugation.

RESULTS

We have reported[38] that adenyl cyclase activity can be demonstrated in rat luteal homogenates if the 600 *g* pellet is incubated at 37°C in the presence of NaF (10^{-2}M), $MgSO_4$ ($3{\cdot}5 \times 10^{-3}$M) and caffeine ($6{\cdot}7 \times 10^{-3}$M). At the optimum pH (7·5) and with optimum tissue concentration (600 *g* pellet equivalent to 50–100 mg fresh tissue), linear production of cyclic AMP continues at a rate of about 7×10^{-10} mol/min for 40 min (Fig. 1).

Rat luteal 600 *g* pellet contains enzymes capable of rapid hydrolysis of ATP with the release of nearly 3 mol inorganic phosphate/mol ATP. Estimation of the inorganic phosphate (Pi) released indicates that NaF at 10^{-2}M inhibits this "ATPase" activity by 50 per cent, and that NaF at 10^{-1}M causes 80 per cent inhibition. Adenyl cyclase is, however, less active in the presence of the higher concentration of NaF even if the Mg^{2+} is increased at the same time. If "ATPase" activity is measured by isolation of the [^{14}C]ATP remaining after incubation it is seen that NaF (10^{-2}M) increases the residual ATP after 40 min incubation from about 1 to about 10 per cent of the zero-time content (Fig. 2). The presence of an

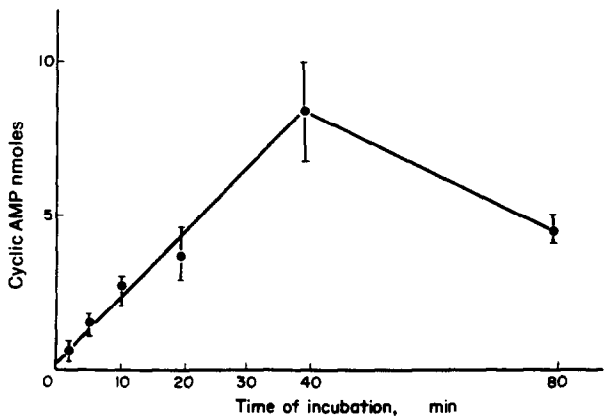

Fig. 1. Time course of cyclic AMP production.

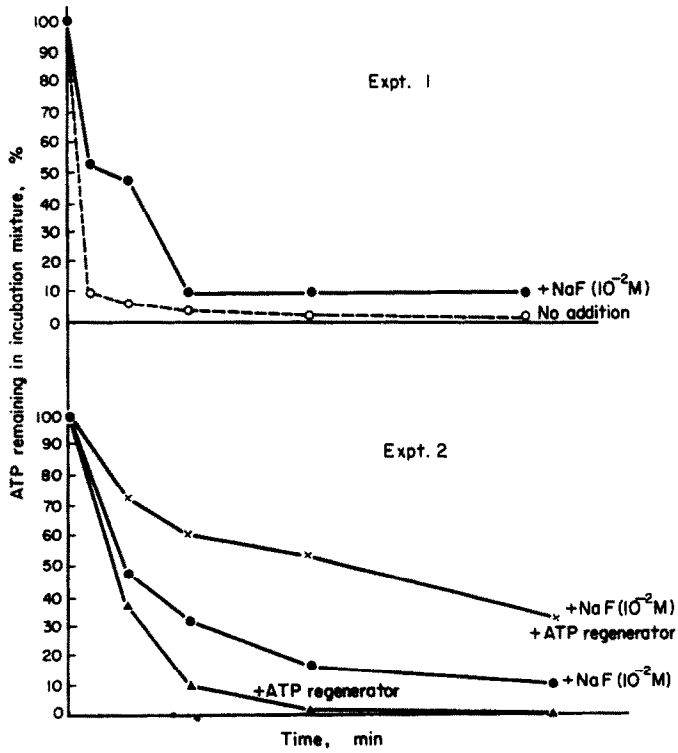

Fig. 2. Loss of ^{14}C-ATP during incubations of rat luteal 600g pellet.

ATP-regenerating system (creatine phosphate + creatine phosphokinase) did not prevent the almost complete loss of ATP during the incubation unless NaF (10^{-2}M) was also present, but it slowed down the initial rate of substrate loss and enabled low levels of adenyl cyclase activity to be demonstrated in the absence of NaF (Fig. 3). Maximum cyclic AMP production was observed with the ATP-regenerating system and NaF at 10^{-2}M.

Attempts to selectively inhibit "ATPase" by lowering Mg^{2+} levels, by replacing Mg^{2+} with Ca^{2+}, Mn^{2+} or Zn^{2+}, or by including NaN_3, led to decreases in adenyl cyclase activity. "ATPase" was not inhibited by ouabain, *p*-chloromercuribenzoate or the exclusion of Na^+ or K^+. GTP at concentrations of up to

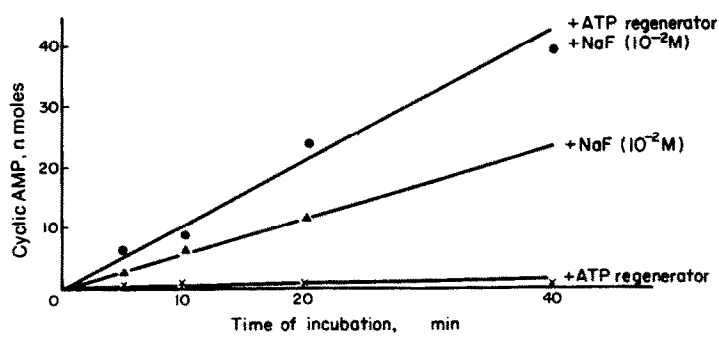

Fig. 3. Effect of an ATP-regenerating system and NaF upon adenyl cyclase activity.

4 mM increased cyclic AMP production, provided that the content of Mg^{2+} was maintained in excess of the combined ATP + GTP concentrations.

We have subsequently attempted to solubilise adenyl cyclase in order to separate it physically from "ATPase".

Figure 4 shows the distribution of adenyl cyclase in fractionated luteal

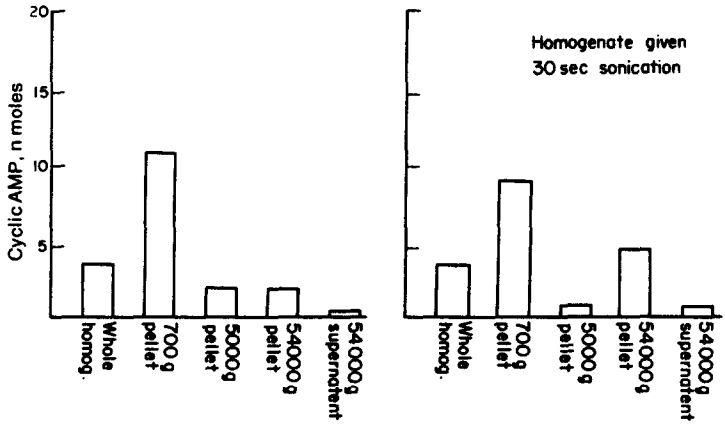

Fig. 4. Effect of sonication of sub-cellular distribution of adenyl cyclase.

homogenate before and after treatment with ultra-sound for 30 sec at 0°C. Ultra-sound lowers adenyl cyclase activity in the 700 *g* and 5000 *g* pellets, with a simultaneous increase in the 54,000 *g* pellet, and a very small activity appearing in the 54,000 *g* supernatant. 1 per cent Triton X-100 slightly lowered the adenyl cyclase activity of 700 *g* pellet when compared to homogenate exposed to ultra-sound for 5 min, and severely reduced the activity present in the 5000 *g* and 54,000 *g* pellets (Table 1). Ultra-sound treatment in the presence of Triton X-100 caused severe losses in all fractions. Triton X-100 caused only small increases in "soluble" cyclase.

Levey[42] claimed that 20 mM Lubrol PX successfully solubilised heart muscle adenyl cyclase. Table 2 shows that this concentration was inhibitory to luteal adenyl cyclase, but that 2 mM Lubrol increased the adenyl cyclase activity of 700 *g* pellet and 54,000 *g* pellet, while it decreased the activity in the 5000 *g* pellet. In a second experiment (Table 3) Lubrol alone or with ultra-sound treatment again increased the adenyl cyclase activity of the 54,000 *g* pellet, but did not increase the activity of 700 *g* pellet. There was a small increase in "soluble" cyclase following treatment with Lubrol PX and ultra-sound.

Table 1. nmoles cyclic AMP produced

	Sonication	1% Triton X-100	1% Triton X-100 + Sonication
Whole homogenate	1·8	1·0	1·0
700 *g* pellet	19·0	15·5	3·5
5000 *g* pellet	8·8	0·7	1·7
54,000 *g* pellet	16·6	0·8	4·3
54,000 *g* supernatant	0·8	1·4	1·8

Table 2. Effect of Lubrol PX on adenyl cyclase distribution in rat luteal homogenate

Fraction	Conc. Lubrol PX (mM)	nmoles Cyclic AMP produced
Whole homogenate	0	7·8
	2	8·0
	20	5·4
700 *g* pellet	0	37·0
	2	60·0
	20	4·0
5000 *g* pellet	0	12·6
	2	1·1
	20	0
5400 *g* pellet	0	6·7
	2	30·2
	20	0
54000 *g* supernatant	0	0·3
	2	0·5
	20	0·5

These surprising increases in adenyl cyclase activity in particulate fractions are being further investigated; they do not seem to be related to selective solubilisation of "ATPase" as judged by Pi release. To check whether we might be solubilising cyclase, but failing to detect the cyclic AMP produced in the soluble fraction because of the high activity of cPDE therein, we prepared a 600 *S* pellet free of soluble fractions, resuspended it in medium containing up to 20 mM Lubrol PX, and re-isolated the 600 *S* pellet and a 600 *S* supernatant. Table 4 shows none of the activity was solubilised.

In this experiment the method of Krishna *et al.*[40] was used to estimate cyclic

Table 3. Effect of Lubrol PX and ultra-sound on adenyl cyclase distribution in rat luteal homogenate

Fraction	Treatment of homogenate	nmoles Cyclic AMP produced
Whole homogenate	None	0·1
	2mM Lubrol PX	—
	2mM Lubrol PX + sonication	—
700 *g* pellet	None	36·0
	2mM Lubrol PX	34·0
	2mM Lubrol + sonication	30·0
5000 *g* pellet	None	9·5
	2mM Lubrol PX	6·2
	2mM Lubrol PX + sonication	4·9
54,000 *g* pellet	None	8·8
	2mM Lubrol PX	33·3
	2mM Lubrol PX + sonication	45·5
54,000 *g* supernatant	None	—
	2mM Lubrol PX	—
	2mM Lubrol PX + sonication	2·5

Table 4. Effect of Lubrol PX treatment on isolated 600 *S* pellet of rat luteal homogenate

Tissue fraction	Lubrol-PX treatment	NaF(10^{-2}M)	Cyclic AMP produced (nmoles)
600 *g* pellet	−	+	36·0
600 *g* pellet	−	−	5·0
600 *g* supernatant	−	+	3·0
600 *g* supernatant	−	−	4·0
600 *g* pellet	+	+	30·0
600 *g* pellet	+	−	6·0
600 *g* supernatant	+	+	4·0
600 *g* supernatant	+	−	4·0

Luteal tissue homogenate (80 mg/ml) was centrifuged at 600 *g*, and the pellet was resuspended in diglycine/Mg^{2+} buffer (pH 7·4) which was either with or without 2 mM Lubrol PX. After 10 min gentle shaking at 0–5°C, the 600 *g* supernatant and pellet were separated by centrifugation and the activity of adenyl cyclase assayed by the method of Krishna *et al.*[40].

AMP production. We have reported[39] that cyclic AMP phosphodiesterase (cPDE) of bovine corpus luteum is a soluble enzyme with K_m of 0·25 mM. Our results indicate a continuous rise in the tissue content of the enzyme as the cyclic corpus luteum ages. Kinetic analyses show no change in K_m, but there seems to be an increase in V_{max} (Table 5.).

DISCUSSION

In this paper we have reviewed the evidence implicating cyclic AMP in the stimulation of steroidogenesis by trophic hormones. It is paradoxical that at this time there is more detailed information about how cyclic AMP works than about the mechanism of the enzyme which produces it. The results we have presented are part of an attempt to study luteal adenyl cyclase at the *in vitro* level. Our work has been entirely concerned with the catalytic part of the adenyl cyclase complex and we have not attempted to use hormones to stimulate our cyclase preparations. We think that studies with hormonally stimulated cyclase are, at this stage of our ignorance concerning the mechanism of action of adenyl cyclase, more difficult to evaluate because of the additional complications arising from

Table 5. Cyclic AMP phosphodiesterase (cPDE) activity in bovine corpus luteum taken at various stages of the estrous cycle

	Appearance of corpus luteum		
	Brown	Yellow-orange	Red-orange
Estimated day of cycle	5	15	20
cPDE/Corpus luteum	3·08	13·8	25·8
	3·95		27·0
cPDE/g luteal tissue	2·88	3·90	5·90
	2·87		5·88
cPDE V_{max}/mg Luteal tissue	0·0086	0·0113	0·0153
± S.D.	±0·0043	±0·0021	±0·0038

the unknown linkage mechanisms between the catalytic and the receptor sites. The use of fluoride as an activating agent for adenyl cyclase appears to be justified in this context since Birnbaumer *et al.*[35] have concluded that although both hormones and NaF activate the cyclase by increasing its affinity for Mg^{2+}, the two agents act at different sites. It is reasonable to believe that NaF acts at the catalytic site rather than at the hormone receptor site. Our results have led us to suggest[37, 38] that fluoride acts in two ways in *in vitro* incubations of luteal adenyl cyclase: by partially inhibiting the "ATPase" which competes with cyclase for substrate, and by some direct effect upon adenyl cyclase. It seems that the inhibitory effect of NaF on Pi release from ATP may be due to inhibition both of ADPase and nucleotidase activities and the true ATPase component since conditions which inhibit Pi release by 50 per cent only allow a conservation of 10 per cent of the ATP.

Birnbaumer *et al.*[43] have suggested that the inhibition of adenyl cyclase by NaF at 10^{-1}M is due to removal of essential Mg^{2+}, but we have shown that if the Mg^{2+} are also increased with the concentration of NaF inhibition still occurs. We have instead suggested that the activatory and inhibitory concentrations of NaF may allow the formation of differing fluoride polyanions which may interact in opposed ways with the active centre of the luteal cyclase[38]. We are of the opinion that it is essential to either remove the "ATPase" from adenyl cyclase, or to selectively inhibit the "ATPase" in order to obtain meaningful kinetic data concerning adenyl cyclase. Selective inhibition has so far been unsuccessful; the substrate analogue $\beta\gamma$ methylene ATP was resistant to "ATPase" hydrolysis but was not converted into cyclic AMP. We are currently preparing another ATP analogue $\beta\gamma$ imido ATP which Rodbell *et al.*[44] have demonstrated to act as a substrate for fat cell cyclase.

Our preliminary attempts at solubilisation of luteal cyclase have not been successful, but it seems apparent that the luteal enzyme is different from those of liver and heart muscle, both of which proved amenable to "solubilisation" by the detergents we have used. Our investigations of the bovine luteal cPDE have shown that it is an enzyme of the soluble fraction and that its K_m is 0·25 mM. We have not attempted to perform K_m determinations at very low substrate concentration (ca. 10^{-6}M) and have as yet no information about the occurrence in luteal tissue of the high and low K_m forms of the enzyme which have been claimed in other tissues (e.g. [45, 46]). We have suggested[39] that the increase in the amount of cPDE in luteal tissue as the cyclic corpus luteum approaches regression may be involved with the termination of the physiological effect of LH.

Hahn[47] has argued for control of cyclic AMP levels in prenatal adipose tissue by high levels of cPDE; during the post-natal period there is a decline in cPDE levels and a corresponding increase in the activity of hormone-sensitive lipase.

ACKNOWLEDGEMENTS

The financial support of the Medical Research Council and the Scottish Hospitals Endowments Research Trust is gratefully acknowledged.

REFERENCES

1. E. W. Sutherland and T. W. Rall: *J. Am. Chem. Soc.* **79** (1957) 3608.
2. T. W. Rall, E. W. Sutherland and L. Berthet: *J. biol. Chem.* **224** (1957) 463.
3. E. W. Sutherland and T. W. Rall: *J. biol. Chem.* **232** (1958) 1077.

4. W. H. Cook, D. Lipkin and R. Markham: *J. Am. Chem. Soc.* **79** (1957) 3607.
5. D. Lipkin, W. H. Cook and R. Markham: *J. Am. Chem. Soc.* **81** (1959) 6198.
6. R. C. Haynes, Jr.: *J. biol. Chem.* **233** (1958) 1220.
7. R. C. Haynes, Jr. and L. Berthet, *J. biol. Chem.* **225** (1957) 115.
8. J. M. Marsh and K. Savard: *J. biol. Chem.* **239** (1964) 1.
9. D. A. Stansfield and J. W. Robinson: *Endocrinology* **76** (1965) 390.
10. D. T. Armstrong: *Endocrinology* **72** (1963) 908.
11. S. B. Koritz and F. G. Peron: *J. biol. Chem.* **230** (1958) 343.
12. J. E. Creange and S. Roberts: *Biochem. Biophys. Res. Commun.* **19** (1965) 73.
13. J. E. Creange and S. Roberts: *Steroids*, Supp. **2** (1965) 13.
14. S. Roberts, R. W. McCune, J. E. Creange and P. L. Young: *Science* **158** (1967) 372.
15. J. Bell, G. Brooker and B. W. Harding: *Biochem. Biophys. Res. Commun.* **41** (1970) 938.
16. D. G. Grahame-Smith, R. W. Butcher, R. L. Ney and E. W. Sutherland: *J. biol. Chem.* **242** (1967) 5535.
17. J. M. Marsh and K. Savard: *Steroids* **8** (1966) 133.
18. K. Savard, J. M. Marsh and B. F. Rice: *Recent Prog. Hormone Res.* **21** (1965) 285.
19. J. Dorrington and R. Kilpatrick: *Biochem. J.* **104** (1967) 725.
20. I. N. Hirshfield and S. B. Koritz: *Endocrinology* **78** (1966) 165.
21. P. F. Hall and S. B. Koritz: *Biochemistry* **4** (1965) 1037.
22. S. Ichii, E. Forchielli and R. I. Dorfman: *Steroids* **2** (1963) 631.
23. G. C. Karaboyas and S. B. Koritz: *Biochemistry* **4** (1965) 462.
24. S. Burstein and M. Gut: *Steroids* **14** (1969) 207.
25. D. T. Armstrong and A. P. Flint: *Nature New Biol.* **231** (1971) 60.
26. S. Sulimovici and G. S. Boyd; *Eur. J. Biochem.* **3** (1968) 332.
27. S. Kuo and P. Greengard: *Proc. natn. Acad. Sci. (U.S.A.)* **64** (1969) 1349.
28. G. N. Gill and L. D. Garren: *Proc. natn. Acad. Sci. (U.S.A.)* **68** (1971) 786.
29. D. G. Grahame-Smith: *Biochem. J.* **120** (1970) 3P.
30. A. H. Reddi, I. L. Ewing and H. G. Williams-Ashman: *Biochem. J.* **122** (1971) 333.
31. T. A. Langan: *Proc. natn. Acad. Sci. (U.S.A.)* **64** (1969) 1276.
32. S. B. Koritz and A. M. Kumar: *J. biol. Chem.* **245** (1970) 152.
33. E. W. Sutherland, J. G. Hardman, R. W. Butcher and A. E. Broadus: In *Progress in Endocrinology* (Edited by C. Gual). North Holland, Amsterdam, *Exerpta Med. Found. Inter. Cong. Ser* **184** (1969) 26.
34. G. A. Robison, R. W. Butcher and E. W. Sutherland: In *Fundamental Concepts of Drug-Receptor Interactions* (Edited by J. F. Dannielli, J. F. Moran and D. J. Triggle). Academic Press, London (1970) p. 59.
35. L. Birnbaumer, S. L. Pohl, H. Michiel, J. Krans and M. Rodbell: In *Role of Cyclic AMP in Cell Function* (Adv. in Biochemical Psychopharmacology, Vol. 3, P. Greengard and E. Costa, eds.). Raven Press, New York (1970) p. 185.
36. D. J. Franks and D. A. Stansfield: *Biochem. J.* **117** (1970) 25p.
37. D. A. Stansfield and D. J. Franks: *Biochem. J.* **120** (1970) 5p.
38. D. A. Stansfield and D. J. Franks: *Biochim. biophys. Acta* **242** (1971) **606**.
39. D. A. Stansfield, J. R. Horne and G. H. Wilkinson: *Biochim. biophys. Acta* **227** (1971) 413.
40. G. Krishna, B. Weiss and B. B. Brodie: *J. Pharm. Exp. Therap.* **163** (1968) 379.
41. E. W. Sutherland, T. W. Rall and T. J. Menon: *J. biol. Chem.* **237** (1962) 1220.
42. G. S. Levey: *Biochem. biophys. Res. Commun.* **38** (1970) 86.
43. L. Birnbaumer, S. L. Pohl and M. Rodbell: *J. biol. Chem.* **244** (1969) 3468.
44. M. Rodbell, L. Birnbaumer, S. L. Pohl and H. M. J. Krans: *J. biol. Chem.* **246** (1971) 1877.
45. E. G. Loten and J. G. T. Sneyd: *Biochem. J.* **120** (1970) 187.
46. W. J. Thompson and M. M. Appleman: *Biochemistry* **10** (1971) 311.
47. P. Hahn: *Experientia* **26** (1970) 1300.

DISCUSSION

Munck: How many binding sites do you think there are per adenyl cyclase: how many hormone binding sites, and how many fluoride binding sites?

Stansfield: This I would say is impossible to ascertain from what we've done so far; as I said, we've never in fact used any hormonally activated cyclase. We haven't as yet tried to ascertain how many fluorides might be picked up by the

protein we're using, mainly because it's such a heterogeneous protein system, consisting of a 600 *g* pellet composed of bits of plasma membrane and presumably nuclei and other subcellular particles. Until we've actually made a soluble purified preparation we would not be any wiser in measuring the number of binding sites.

Munck: I was wondering if from an analysis of the fluoride activation as a function of concentration you could tell whether there was one or two sites?

Stansfield: This may be possible, but we haven't attempted it.

Van der Molen: Can you formally argue that the V_{max} of your phosphodiesterase is increasing? Isn't it, at least theoretically, necessary to isolate the purified enzymes first?

Stansfield: One of the difficulties with luteal enzymes is that the homogenate contains so much lipid material that it is quite problematical to purify the proteins by conventional techniques. If you try to take out the lipid, e.g. by butanol extraction, then you denature the enzymes quite markedly. Straight salt precipitations on the homogenate result in a precipitated protein which doesn't spin down, apparently, because there's so much lipid there buoying it up all the time. We have achieved a very small partial purification starting from acetone dried material, and by ammonium sulphate fractionation we've been able to increase the specific activity by a matter of 10 or 15 times, but this is nowhere near enough.

Spät: Have you any personal experience about the involvement of prostaglandins in the control of adenyl cyclase activity? Contaminations with prostaglandins could occur in these experiments which could modify the results in some instances.

Stansfield: Yes, I'm aware of the possible involvement of prostaglandins here, but we haven't investigated them at all.

Cooke: Have you attempted to characterize further any of your cell fractions, because the distribution of the adenyl cyclase that you've found seems to be somewhat different from the adrenal cortex and also from some studies we're carrying out on the rat testis. In these two steroid hormone-producing tissues, it appears that the adenyl cyclase may also be located in the mitochondria. However, we have found it rather difficult to use the conventional enzyme markers to characterize our subcellular fractions. For example, the 5′-nucleotidase, a plasma membrane marker in the liver, doesn't seem to stay with the plasma membrane at all in the testis and seems to go into the other fractions.

Stansfield: The 600 *g* pellet is very heterogeneous. We have tried to make plasma membrane preparations by the method of Neville, and we certainly got a nice-looking plasma membrane preparation, but when we tried to assay for cyclase, we couldn't find any in it at that time. I think myself that the cyclase activities in the lower size particulate range are probably just because of a very heterogeneous distribution of particle break-up during the homogenization. We haven't tried to fit any marker enzymes into this scheme to see how pure these other fractions are. All we were interested in basically was merely to determine whether or not we could further reduce the size of the particles with an ultimate aim of getting them so small that they would appear to be in the soluble fraction.

Adlercreutz: I saw in one of your slides you had LH indicated as stimulator of the adenyl cyclase. Have you done any experiments yourself with this compound?

Stansfield: No, here we rest upon the word of Marsh and Savard and of Kilpatrick and Dorrington in the pharmacology department in Sheffield. As I said, I thought it much more needful at this stage to try and look at the activity of the enzyme itself rather than to get involved in the hormonal activation. I think that side of it is reasonably clear. What is unclear is the catalytic nature of adenyl cyclase, and what is even less clear is the way in which the receptor sub-unit interacts with the catalytic subunit, presumably through the membrane in some way, in order to bring these effects about.

Liao: We are very much interested in the amount of cyclic AMP inside the nucleus. We think nuclear membranes contain adenyl cyclase, and a similar report has been published by Oscar Hechter. Have you tried to purify nuclei?

Stansfield: No.

Liao: How stable is cyclic AMP in the nuclei? How active is phosphodiesterase in the nucleus?

Stansfield: Very little, probably less than 3–5% of the total phosphodiesterase activity of the homogenate. Most of it is in the soluble supernatant.

Siiteri: You say you haven't done experiments with the hormones, but I was wondering whether you know if people have attempted to look at the levels of activity of the cyclase in the corpora luteal system and the response to antisera, say of LH or prolactin. Is there a basal level of cyclase that's significant is really the question.

Stansfield: I'm not aware of any work which has been done with anti-LH sera in this respect. I think the present state of knowledge is that LH obviously will increase steroidogenesis in tissue slices. But there has been rather little work carried out to tie this in with the physiological happenings in the ovary.

Crabbé: Dr. Stansfield, I was surprised by the statement that you can't inhibit, to a significant extent ATPase activity in your pellet with ouabain. Would you have any idea about why this is so? Another point I can certainly see why you want to spare ATP for adenyl cyclase activity measurements, and I wondered whether the choleratoxin might offer you a way out here, because from work recently carried out it seems that toxin activates adenyl cyclase in several tissues and that it binds to the membrane in a rather firm way.

Stansfield: This could be quite true. As regards the first part of your question. the ATPase is not inhibited by ouabain. This fits in with the fact that it doesn't seem to be a sodium-potassium ATPase, it doesn't seem to be one of the pump ATPases in that sense. We do of course have nuclei and nuclear fragments present in our 600 *g* pellet. It could be something to do with nuclear nucleic acid synthesis that is going on, and utilizing ATP. There is also, of course, in corpus luteum the active transport system for ascorbic acid, pumping ascorbic acid into the tissue, and the ATPase could be involved with this as well.