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# ADENYL CYCLASE AND "ATPASE" IN RAT CORPUS LUTEUM

## I. SOME PROPERTIES OF THE ENZYMES

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SUMMARY

1. Adenyl cyclase activity is present mainly in the 600 × *g* pellet of rat corpus luteum. This tissue sub-fraction also contains "ATPase" activity, *i.e.* enzymes capable of hydrolysing ATP and releasing inorganic phosphate.

2. Adenyl cyclase of corpus luteum is activated by NaF at $1 \cdot 10^{-2}$ M but is inhibited by NaF at 0.1 M. This inhibition could not be reversed by adding further $Mg^{2+}$. "ATPase" is partially inhibited by NaF.

3. As the tissue concentration in the incubations was increased adenyl cyclase activity increased to maximum when 600 × *g* pellet derived from 75 mg fresh tissue was present, and declined thereafter. "ATPase" activity increased as the tissue concentration was increased, until at high tissue concentration the inhibition by NaF was not demonstrable.

4. The "ATPase" activity is a composite activity consisting of ATPase, ADPase and nucleotidase, and is capable of virtually complete hydrolysis of ATP to yield 3 moles inorganic phosphate per mole ATP.

5. Some properties of both systems are described, and the mechanism of the fluoride activation of adenyl cyclase is discussed.

## INTRODUCTION

Adenosine 3′, 5′-cyclic monophosphate (Ado-3′,5′-*P*) is produced from ATP by the enzyme adenyl cyclase, which has been shown to be present in a number of mammalian, avian, amphibian and invertebrate tissues[1], and in bacteria[2]. Many hormonal effects seem to be mediated *via* Ado-3′,5′-*P* and there is an increasing list of hormones which have the ability to increase the activity of adenyl cyclase in their target tissue[3–5]. The work reported here on the adenyl cyclase of corpus luteum[6] arose out of previous studies in this laboratory on the metabolism of glycogen and the acti-

Abbreviation: LH, luteinising hormone.

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vation by luteinising hormone (LH) of luteal phosphorylase[7], and the observations of others that LH increased the concentration of Ado-3',5'-*P* in luteal tissue slices, whilst Ado-3',5'-*P* was itself capable of stimulating progesterone synthesis in this tissue[8].

Despite the fundamental importance of adenyl cyclase in endocrine control systems it remains largely uncharacterised. This is due, primarily, to the insoluble nature of the enzyme which is membrane bound in all cases.

Our attention was necessarily focussed also on the activity of other ATP degrading enzyme systems which are present in the particulate tissue sub-fractions which bear the adenyl cyclase activity. The "ATPases"* compete with adenyl cyclase for the substrate, ATP, and one of the main aims of any study of an adenyl cyclase must be to selectively inhibit or other wise remove such competing systems. This paper describes experiments which demonstrate the presence of adenyl cyclase in rat corpora lutea, and which describe some properties of this enzyme and of the "ATPase" of luteal 600 × *g* pellet. Some preliminary reports of this work have already been published[9–11].

## MATERIALS AND METHODS

[8-$^{14}$C]ATP (specific activity 30–40 mC/mmole) and [8-$^{3}$H]Ado-3',5'-*P* (specific activity 2.34 C/mmole) were obtained from the Radiochemical Centre, Amersham, England. [8-$^{14}$C]Ado-3',5'-*P* (specific activity 22.2 mC/mmole) was purchased from New England Nuclear Corporation, Boston, Mass., U.S.A.

Dowex AG 2-X8-400 ion exchange resin (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) was purchased from Micro-Bio Laboratories, London, England. DEAE-cellulose anion exchanger (exchange capacity 0.8 mequiv/g; medium mesh) was purchased from Sigma (London) Chemical Co., England.

Silica gel $GF_{254}$ (Merck AG, Darmstadt, Germany) was purchased from Anderman and Co., London, England. $\beta,\gamma$-Methylene ATP was synthesised according to MYERS *et al.*[12] and also purchased from Miles Seravac, Maidenhead, England.

All other reagents were of Analar grade where possible, and were purchased from British Drug Houses, Poole, England. Rat Corpora lutea (superovulated rat ovaries) were prepared as previously described[13], the animals being of the Wistar strain supplied by Scientific Products Farm, Ash, Canterbury, England. The ovaries were placed immediately in ice cold homogenising medium.

### *Preparation of homogenates*

Superovulated rat ovaries were homogenised in motor driven glass/teflon pestle type homogenisers within 15 min of removal from the animals. The homogenising medium was either $2 \cdot 10^{-3}$ M diglycine buffer (pH 7.5), containing $1 \cdot 10^{-3}$ M $MgSO_4$, or, when differential centrifugation was to follow, 0.25 M sucrose solution containing $1 \cdot 10^{-3}$ M $MgSO_4$. Differential centrifugation was carried out according to HOGEBOOM[14] using International and M.S.E. Superspeed centrifuges.

* The term "ATPase" is used in this paper to denote the enzyme or enzymes present in a given tissue preparation which cause the release of inorganic phosphate from adenosine triphosphate.

*Assay of adenyl cyclase activity*

The final composition of the incubation medium was as follows: Tris buffer ($4 \cdot 10^{-2}$ M, pH 7.5), $MgSO_4$ ($3.5 \cdot 10^{-3}$ M), caffeine ($6.7 \cdot 10^{-3}$ M), ATP ($2 \cdot 10^{-3}$ M), [8-$^{14}$C]ATP (0.4 $\mu$C) and up to 1.0 ml tissue extract were present in a total volume of 2.2 ml. Some incubations also contained NaF ($1 \cdot 10^{-2}$ M unless otherwise stated in the text). The mixture is similar to that used by RALL AND SUTHERLAND[15]. Incubations were performed in 15-ml conical centrifuge tubes in a shaking metabolic bath at 37° for 40 min, unless otherwise stated.

*Separation and estimation of [8-$^{14}$C]Ado-3',5'-P*

The incubations were terminated by placing the incubation tubes in a boiling water bath for 3 min, and, after centrifuging away denatured protein, the supernatant fluid was assayed for radioactive Ado-3',5'-*P* by a method developed from that described by JUNGAS[16]. An aliquot of the supernatant was applied to the top of an ion-exchange column containing Dowex AG2-X8-400 resin in the formate form. The column dimension was 4.0 cm × 0.4 cm. The sample was followed onto the column by distilled water (10 ml), 0.12 M formic acid (20 ml) and 0.2 M formic acid (30 ml). Ado-5'-*P* was eluted in the 0.12 M formic acid and Ado-3',5'-*P* in the 0.2 M formic acid. ATP and ADP remained on the column. The 0.2 M formic acid eluate was lyophillised, and after being taken up in 50% aqueous ethanol (3 times 0.2 ml) the residue was applied to a glass plate spread with a 0.25-mm thick layer of Silica Gel $GF_{254}$. Unlabelled Ado-3',5'-*P* was added to aid in detection of the spots under ultraviolet light after the incubation was complete. The thin-layer chromatography plate was developed in a solvent mixture composed of acetone–propan-2-ol–0.03 M $NH_4CO_3$ (pH 6.0)–butan-1-ol (2:2:2:3, by vol.). In this system Ado-3',5'-*P* had $R_F$ of 0.55; Ado-5'-*P*, the main contaminant in the 0.2 M formic acid eluate, remained at the origin. Other metabolites of ATP were well separated from Ado-3',5'-*P* by this method[6]. The Ado-3',5'-*P* was located by observation of the plate under ultraviolet light, and the position confirmed by locating the radioactivity using a Panax radiochromatogram scanner (Panax, Redhill, England). The appropriate zone was removed from the thin-layer chromatography plate and the silica gel was eluted with 50% aqueous ethanol to remove Ado-3',5'-*P*. The aqueous ethanol extract was evaporated to dryness in scintillation tubes and the radioactivity measured using a dioxane based phosphor mixture in either a Nuclear Enterprises NE 8305 or a Nuclear Chicago Unilux II liquid scintillation spectrometer. The recovery of Ado-3',5'-*P* was 50–55%.

*Estimation of non-radioactive Ado-3',5'-P*

When the ATP analogue $\beta,\gamma$-methylene adenosine triphosphate was used as substrate the Ado-3',5'-*P* was purified as above, but the Ado-3',5'-*P* zone of the thin-layer chromatography plate was eluted into 1.5 ml of 4 mM Tris buffer (pH 7.5) and the absorbance of this solution was read at 250 nm in a Unicam SP 500 spectrophotometer using 40-mm light path micro-cells. The method could detect as little as 3 nmoles of Ado-3',5'-*P*.

*Assay of "ATPase" activity*

"ATPase" activity was determined on the boiled incubation supernatant (see

above) by determining the amount of inorganic phosphate produced during the incubation[17]. Caffeine interferes with this assay, but care was taken that caffeine was present at concentrations below the interference level (0.05%) in the samples taken for assay. When such determinations were made in the absence of concurrent adenyl cyclase assays the incubation volume was 2.0 ml, and the medium lacked NaF and caffeine.

*Estimation of residual ATP in incubated mixtures*

An aliquot of the incubated mixture was boiled and applied to a column of DEAE-cellulose (4.0 cm × 0.4 cm). The column was eluted successively with water (5 ml), 0.01 M HBr (15 ml) and 0.1 M HBr (10 ml). Ado-5'-*P* and ADP were eluted by 0.01 M HBr and ATP by 0.1 M HBr. The ATP containing eluate was collected directly into a scintillation vial and dried in a vacuum dessicator before scintillation fluid was added, and the radioactivity measured as described above. Recovery of [8-$^{14}$C]ATP was 50%.

All estimations were carried out at least in duplicate.

## RESULTS

Adenyl cyclase activity was demonstrated in the 600 × *g* pellet from Rat corpus luteum homogenate, but the activity was only detectable in the presence of NaF ($1 \cdot 10^{-2}$ M). The apparent activity was much greater in the 600 × *g* pellet fraction than in the equivalent volume of whole homogenate, and the low speed pellet contained much more adenyl cyclase activity than either the 5 000 × *g* or the 50 000 × *g* pellets. No activity was detectable in the 50 000 × *g* supernatant fraction[6]. Fig. 1 shows that there is maximal production of Ado-3',5'-*P* when the incubates contain 600 × *g* pellet derived from 50–100 mg fresh tissue, the yield decreasing when the amount of tissue is raised, until with 600 × *g* pellet derived from 200 mg fresh tissue it was negligible. 600 × *g* pellet derived from 75 mg tissue was used routinely in subsequent incubations.

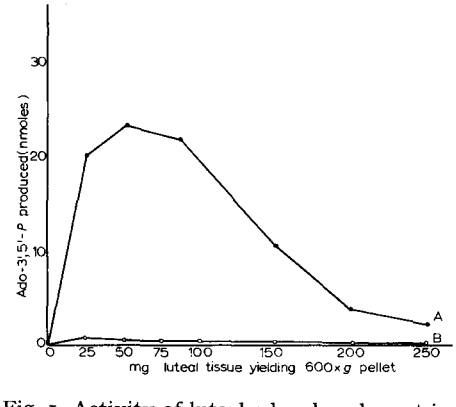


Fig. 1. Activity of luteal adenyl cyclase at increasing tissue concentration. A, incubations in the presence of NaF ($1 \cdot 10^{-2}$ M); B, incubations in the absence of NaF.

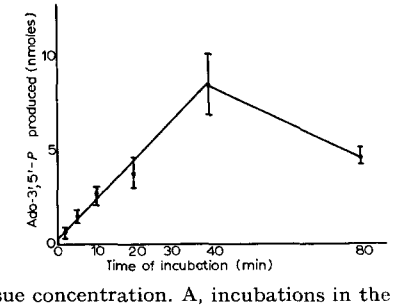


Fig. 2. Time course of formation of Ado-3',5'-*P* from ATP by 600 × *g* pellet of rat luteal tissue. Incubation conditions are as described in MATERIALS AND METHODS.

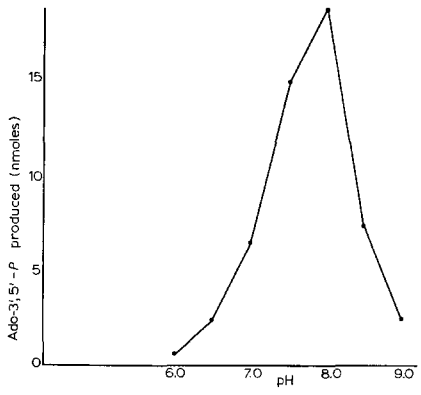


Fig. 3. pH *vs.* activity curve for luteal adenyl cyclase. All incubations contained NaF ($1 \cdot 10^{-2}$ M).

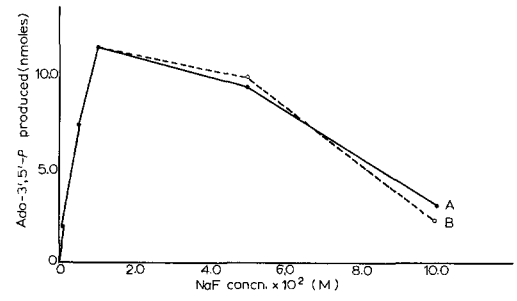


Fig. 4. Effect of increasing concentration of NaF on luteal adenyl cyclase activity. A, incubations with $Mg^{2+}$ concentration held at 3.5 mM; B, incubations with NaF/$Mg^{2+}$ ratio maintained at 2.85.

Maximal Ado-3',5'-*P* production was of the order of $6.6 \cdot 10^{-10}$ moles/min during 40 min incubation at 37° and pH 7.5 in the presence of $1 \cdot 10^{-2}$ M NaF; this represents 0.6% conversion of substrate. At the optimal tissue concentration Ado-3',5'-*P* production was linear with time for 40 min at 37°; if the incubation were prolonged for a further 40 min there was an apparent decrease in adenyl cyclase activity (Fig. 2). The optimum pH for luteal adenyl cyclase in rat luteal 600 × *g* pellet was 7.5–8.0 (Fig. 3). Adenyl cyclase activation by NaF was maximal at $1 \cdot 10^{-2}$ M; if the concentration of NaF were increased to 0.1 M adenyl cyclase activity was inhibited (Fig. 4). This inhibition was not relieved by simultaneously increasing the $Mg^{2+}$ concentration to maintain the $Mg^{2+}$/NaF ratio constant. When "ATPase" activity was measured in the presence of increasing amounts of luteal 600 × *g* pellet there was a linear increase in the amount of inorganic phosphate liberated, and a decrease in the ability of NaF to inhibit this activity: Table I shows that in the presence of 600 × *g* pellet derived from 75 mg luteal tissue "ATPase" was inhibited by some 50% in the presence of $1 \cdot 10^{-2}$ M NaF, whereas at higher tissue concentrations "ATPase" caused almost complete hydrolysis of substrate in the presence of this amount of NaF.

In the absence of NaF "ATPase" produced almost 3 moles of inorganic phos-

TABLE I

EFFECT OF NaF ON 'ATPase" AT VARYING TISSUE CONCENTRATIONS
Incubations were for 40 min at 37°.

| *Tissue concentration (mg) (as 600 × g pellet)* | *NaF (M)* | *$P_i$ released (moles/mole ATP)* |
|---|---|---|
| 75 | — | 2.75 |
| 50 | $1 \cdot 10^{-2}$ | 1.15 |
| 100 | $1 \cdot 10^{-2}$ | 1.60 |
| 200 | $1 \cdot 10^{-2}$ | 2.80 |

phate per mole of ATP. The system was also able to effect almost complete hydrolysis of GTP, ADP and Ado-5′-*P*. The 600 × *g* pellet could hydrolyse 5′-nucleotides with high efficiency, but was much less active towards Ado-3′-*P* and phenyl phosphate.

We are thus dealing with a composite system comprising true nucleoside triphosphatase, nucleoside diphosphatase and 5′-nucleotidase. The relatively low level of activity towards phenyl phosphate argues that non-specific phosphatase action is not significantly involved.

If the NaF concentration in the incubations is increased to 0.1 M the inhibition of "ATPase" increases to 80%. This increased inhibition is parallelled by the NaF inhibition of 600 × *g* pellet hydrolysis of ADP, but the hydrolysis of Ado-5′-*P* is inhibited maximally by $1 \cdot 10^{-2}$ M NaF (Fig. 5). The hydrolysis of IDP was not inhibited by NaF at up to 0.1 M. The "ATPase" of rat luteal 600 × *g* pellet is sufficiently active even in the presence of $1 \cdot 10^{-2}$ M NaF and at the optimum tissue con-

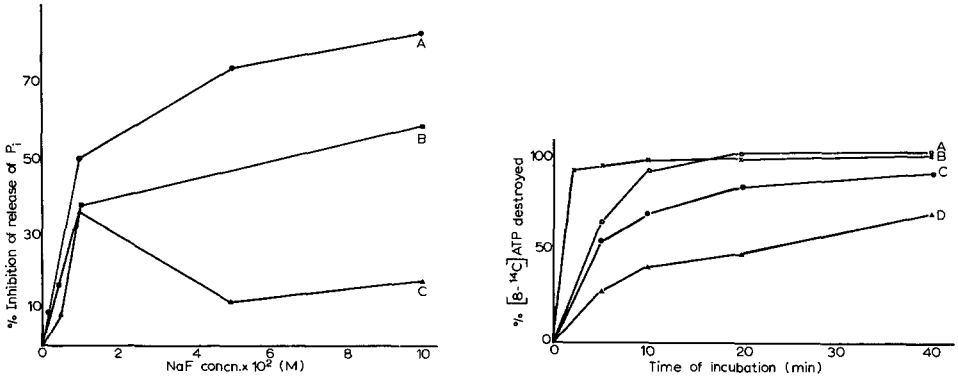


Fig. 5. Effect of increasing concentration of NaF on luteal hydrolysis of ATP, ADP and Ado-5′-*P*. A, with ATP as substrate; B, with ADP as substrate; C, with Ado-5′-*P* as substrate.

Fig. 6. Time course of destruction of ATP by luteal 600 × *g* pellet. A, *plus* ATP-regenerating system, *minus* NaF; B, *minus* ATP regenerating system and NaF; C, *plus* $1 \cdot 10^{-2}$ M NaF; D, *plus* $1 \cdot 10^{-2}$ M NaF and ATP regenerating system.

centration for Ado-3′,5′-*P* production to cause 90% hydrolysis of substrate ATP during the first 10 min of incubation at 37° (Fig. 6). The early removal of ATP can be corrected to some extent by including in the incubation medium an ATP regenerating system consisting of phosphocreatine and creatine phosphokinase, but NaF is necessary to ensure that ATP is available throughout the incubation. Under these conditions adenyl cyclase activity is detectable even in the absence of NaF, but there is also a great enhancement of cyclase activity when NaF ($1 \cdot 10^{-2}$ M) is also added (Table II). Adenyl cyclase of luteal 600 × *g* pellet has a strict requirement for $Mg^{2+}$, the activity decreasing by 52% if the $Mg^{2+}$ concentration is reduced from 4 to 2 mM (*i.e.* if the molar ratio of $Mg^{2+}$/ATP is reduced from 2:1 to 1:1). Maximum adenyl cyclase activity was seen in the presence of 8 mM $Mg^{2+}$ and $1 \cdot 10^{-2}$ M NaF. (Fig. 7). The activity of "ATPase" was not decreased by reducing the $Mg^{2+}$/ATP ratio over the same range, although the omission of $Mg^{2+}$ and the inclusion of $1 \cdot 10^{-2}$ M EDTA in the medium caused almost complete inhibition of phosphate release from ATP. "ATPase" showed no requirement for $Na^+$ or $K^+$. The $Mg^{2+}$ requirement of adenyl

TABLE II

ADENYL CYCLASE ACTIVITY IN THE PRESENT OF ATP REGENERATION SYSTEM AND NaF

| *Incubation time (min)* | *Regenerating system* | *NaF ($1 \cdot 10^{-2}$ M)* | *Cyclic AMP produced (nmoles)* |
|---|---|---|---|
| 5 | − | + | 3.4 |
| | + | − | 0.8 |
| | + | + | 6.2 |
| 10 | − | + | 6.7 |
| | + | − | 0.9 |
| | + | + | 9.1 |
| 20 | − | + | 11.4 |
| | + | − | 0.9 |
| | + | + | 24.2 |
| 40 | − | + | 15.1 |
| | + | − | 1.3 |
| | + | + | 39.5 |

cyclase could not be replaced by $Mn^{2+}$ (which caused 75% loss of activity), $Ca^{2+}$ or $Zn^{2+}$ (which both caused complete inhibition).

The possibility that the reaction products of "ATPase" activity might inhibit the hydrolysis of ATP by this system, but be unable to inhibit adenyl cyclase, was investigated. However, ADP was hydrolysed very rapidly by the luteal 600 × *g* pellet and phosphate (20 mM) inhibited adenyl cyclase by 50%. It was, however, possible to increase the detectable adenyl cyclase activity by including GTP in the medium at concentrations up to 4 mM, provided that the $Mg^{2+}$ concentration is maintained in excess of the combined concentration of ATP + GTP, and that NaF was present at $1 \cdot 10^{-2}$ M (Fig. 8).

At 10 mM GTP caused a pronounced decrease in the radioactive Ado-3′,5′-*P* produced. The effect of some other known inhibitors of ATPase was investigated in the luteal 600 × *g* system. Ouabain at concentrations up to $1 \cdot 10^{-2}$ M and *p*-hydroxymercuribenzoate at concentrations up to $5 \cdot 10^{-4}$ M were not inhibitory to "ATPase". $NaN_3$ inhibited phosphate release from ATP by some 56% at 50 mM, but was inhibitory to adenyl cyclase at this concentration, even when NaF was also present.

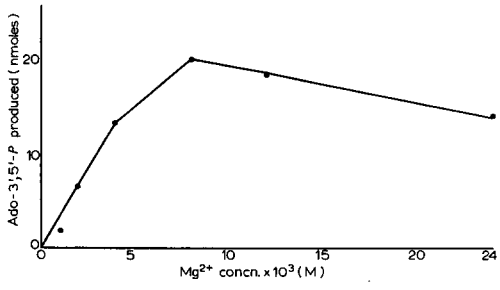


Fig. 7. Effect of increasing $Mg^{2+}$ concentration on luteal adenyl cyclase activity.

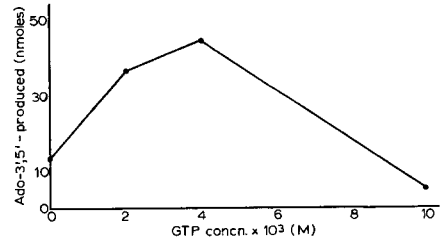


Fig. 8. Effect of increasing GTP concentration on NaF stimulated luteal adenyl cyclase. Incubations carried out in the presence of $1 \cdot 10^{-2}$ M NaF and $Mg^{2+}$ at concentrations equal to twice the combined concentrations of ATP and GTP.

The ATP analogue $\beta,\gamma$-methylene adenosine triphosphate (Ado-5'-*P*-*O*-*P*-*C*-*P*) was stable to attack by "ATPase" as evidenced by lack of phosphate release, but it was not a competitive inhibitor when "ATPase" was incubated with ATP at equimolar concentrations, and luteal adenyl cyclase was not able to convert it into Ado-3',5'-*P*. "ATPase" was in general much more stable than adenyl cyclase to preincubation procedures, freezing and thawing and dialysis.

## DISCUSSION

The studies reported here are an attempt to investigate an enzyme, adenyl cyclase, which is of importance in the response of the corpus luteum to gonadotrophins, and which, in other tissues, is involved in the actions of many hormones. The principal difficulty in working with adenyl cyclase is that it is found attached to insoluble cell fractions, usually those of the plasma membrane[18] but occasionally those of the microsomal fraction[19]. The problem of insolubility is further complicated by the presence, in the membranes which carry adenyl cyclase, of ATP hydrolysing systems. It is usual to find that an adenyl cyclase requires $F^-$ to be detectable in *in vitro* incubations, unless the tissue fraction is hormonally stimulated[15] or an ATP-regenerating system has been included in the incubations[20].

Our results suggest that the stimulatory effect of $F^-$ on luteal cyclase may be a result both of substrate sparing and of some degree of direct action of $F^-$ upon the adenyl cyclase. Thus Table II shows that adenyl cyclase activity is slightly stimulated in the absence of $F^-$ by an ATP-regenerating system, showing that availability of ATP is one factor which curtails cyclase activity. However, addition of $F^-$ to incubations which contain the ATP-regenerating system causes a further large increase in activity. A similar effect has been noted by MELSON *et al.*[21] when NaF was included in incubations of kidney tubule adenyl cyclase containing an ATP regenerating system. Fig. 5 shows that the production of $P_i$ from ATP and ADP is progressively inhibited by $F^-$ at concentrations up to 0.1 M, whereas inhibition of the nucleotidase component of "ATPase" and activation of adenyl cyclase are maximal at $1 \cdot 10^{-2}$ M NaF. Inhibition of adenyl cyclase by 0.1 M NaF is not due to removal of essential $Mg^{2+}$; the results presented in Table I indicate that 0.1 M NaF is still inhibitory even when the $Mg^{2+}$/NaF ratio is held constant throughout. This is not in agreement with the results of BIRNBAUMER *et al.*[22] in their study of the adenyl cyclase of fat cell ghosts. The following explanation is suggested by the work of REINER *et al.*[23] on the concentration dependent $F^-$ inhibition of acid phosphatase. It is our opinion that the stimulation of adenyl cyclase by NaF at $1 \cdot 10^{-2}$ M, and its inhibition at higher $F^-$ concentration is due to an activatory species of fluoride polyanion which exists at pH 7.5 only at certain NaF concentrations. The ion may include $Mg^{2+}$ or $P_i$ but is present in small concentration so that the effective $Mg^{2+}$ concentration is not disturbed. At higher NaF concentrations a further polyanion becomes of significant concentration; this latter species is either inhibitory to adenyl cyclase or else it prevents the activation of adenyl cyclase by the activatory species.

The graph of 600 $\times$ *g* pellet concentration *vs.* adenyl cyclase activity (Fig. 1) shows a maximum at the point where 600 $\times$ *g* pellet from 75 mg corpus luteum is incubated. Thereafter the activity of adenyl cyclase decreases as tissue concentration rises. This does not seem to be because an unfavourable tissue concentration/NaF

ratio has been reached, since increasing the amount of $F^-$ at the higher tissue concentration does not increase cyclase activity[6]. Similarly it is not possible for the decrease in adenyl cyclase activity with increasing tissue content to be because of the presence of Ado-3′,5′-*P* phosphodiesterase in amounts which begin to exceed the inhibitory capacity of the caffeine present in the incubations. STANSFIELD *et al.*[24], have shown that this enzyme is only a minor constituent of 600 × *g* pellet of corpus luteum, being found principally in the highspeed supernatant fraction. In addition, RALL AND SUTHERLAND[15] have also reported that adenyl cyclase activity could only be demonstrated with sufficiently diluted liver preparations. We believe that the shape of the tissue content/adenyl cyclase activity curve is dictated by the relative amounts of adenyl cyclase and "ATPase". There is also evidence that the $K_m$ of adenyl cyclase for ATP is much lower than that of "ATPase" for ATP[6]. As the tissue concentration is increased the rate at which "ATPase" removes the substrate for adenyl cyclase increases despite the presence of NaF (Table I).

If adenyl cyclase is to be studied it is essential that the effect of "ATPase" should be at least minimised, or preferably, removed so that substrate removal does not become a limiting factor in adenyl cyclase activity. Many workers in this field have used ATP-generating systems, and we have shown that such systems do slightly increase the adenyl cyclase activity of the luteal 600 × *g* pellet (Table II). However, the plasma membrane enzyme system is sufficiently complicated without introducing further enzymes, each with their own optima for pH, cation content and duration of incubation. We therefore have attempted to simplify the system rather than add more enzymes to it. Both adenyl cyclase and "ATPase" need $Mg^{2+}$; it seemed possible that the level of $Mg^{2+}$ requirement might differ. We have shown this is so, but unfortunately it is the adenyl cyclase which is susceptible to lowering of the $Mg^{2+}$ concentration. No other cation completely replaced $Mg^{2+}$ in the adenyl cyclase assay; $Mn^{2+}$ is 25% as effective as the equivalent $Mg^{2+}$ concentration and $Ca^{2+}$ and $Zn^{2+}$ allow no cyclase activity. Conflicting reports about $Ca^{2+}$ inhibiting or activating adenyl cyclase in other tissues have appeared; $Ca^{2+}$ have been reported to stimulate adenyl cyclase in fat cells[20,25] and calf brain[26], and to inhibit adenyl cyclase in fat cells[22], and dog brain[27]. We have also tried to inhibit the "ATPase" selectively, once again hoping that we would be left with unencumbered cyclase. Ouabain at concentrations up to $1 \cdot 10^{-2}$ M and *p*-chloromercuribenzoate at concentrations up to $5 \cdot 10^{-4}$ M did not inhibit the "ATPase", and "ATPase" activity did not require the presence of $Na^+$ or $K^+$. The only substance which was of comparable value to $F^-$ as an inhibitor of "ATPase" was $NaN_3$, but no adenyl cyclase activity could be detected in the presence of azide. Since adenyl cyclase catalyses the removal of pyrophosphate from ATP, whereas "ATPase" yields $P_i$ the possibility that "ATPase" might be selectively inhibited by excess $P_i$ was investigated. Once again, however, adenyl cyclase was itself inhibited. However GTP at up to 4 mM was shown to allow a further expression of the adenyl cyclase activity, in the presence of NaF and with $Mg^{2+}$ main tained in excess of the combined amounts of ATP and GTP (Fig. 8). This suggests that GTP, being hydrolysed by "ATPase", thus spares ATP for conversion to Ado-3′,5′-*P* by adenyl cyclase, and also that adenyl cyclase is specific for ATP. However, if the GTP concentration was raised to 10 mM, the adenyl cyclase activity decreased despite the presence of $F^-$ and excess of $Mg^{2+}$; this may be taken as indicative of a small degree of acceptance of GTP at the active centre of adenyl cyclase. Further

work is needed to see whether this represents an ability of the luteal adenyl cyclase to convert GTP to cyclic GMP or whether there is competitive inhibition by temporary occupation of the active centre by GTP. The ATP analogue Ado-5'-*P*-O-*P*-C-*P* was resistant to "ATPase" action as is to be expected of a compound with a methylene bridge in place of the hydrolysable oxygen bridge. We argued that adenyl cyclase might be able to hydrolyse off the terminal methylene diphosphonate moiety of Ado-5'-*P*-O-*P*-C-*P* and convert the nucleotide residue to Ado-3',5'-*P* in the same way that it normally hydrolyses off pyrophosphate from ATP. However, there was no detectable formation of Ado-3',5'-*P*. The reason for this is not immediately obvious. It may be that the presence of a methylene bridge in the polyphosphate side chain causes a large alteration in electron distribution, since this group lacks the lone pair of electrons of the oxygen atom. The oxygen lone pair are often involved in nucleophilic displacement reactions, the chief mechanism by which cleavage of a P–O bond occurs[28].

Ado-5'-*P*-O-*P*-C-*P* substitutes for ATP in the RNA polymerase reaction of azotobacter[29] and as an allosteric activator in adenylate deaminase[30]. The equivalent GTP analogue functions as an allosteric factor in the setting up of the initiator complex of protein synthesis[31].

It is also possible that methylene diphosphonate analogues of ATP do not bind $Mg^{2+}$ in the correct way to allow adenyl cyclase to act upon the molecule, or that the terminal oxygen bridge of ATP is involved in the reactions at the active centre. Our finding that Ado-5'-*P*-O-*P*-C-*P* does not inhibit "ATPase" is in agreement with the finding that methylene diphosphonic acid does not inhibit inorganic pyrophosphatase[32], but not with the finding that the myosin ATPase of muscle is inhibited by Ado-5'-*P*-O-*P*-C-*P*[12]. Our current work is directed towards attempts to selectively solubilise luteal adenyl cyclase and "ATPase" in order that they may be separated one from the other[9,11].

NOTE ADDED IN PROOF (Received May 14th, 1971)

After this paper was submitted for publication a series of 5 important papers has been published by RODBELL *et al.*[33–37]. It is shown[37] that the ATP analogue Ado-5'-*P*-O-*P*-N-*P* will, when incubated with liver plasma membrane preparations[33], act as a substrate for adenyl cyclase, whilst being resistant to "ATPase" activity. Thus what we were attempting unsuccessfully to perform with Ado-5'-*P*-O-*P*-C-*P* has been accomplished with the nitrogen containing analogue.

Using this substrate RODBELL *et al.* show that GTP or its analogue Guo-5'-*P*-O-*P*-C-*P* is essential for glucagon stimulation of liver adenyl cyclase. Moreover, these authors show that GTP at 0.2 mM inhibits $F^-$ activated adenyl cyclase ($1 \cdot 10^{-2}$ M $F^-$), whereas $F^-$ activates the cyclase both in the absence and the presence of 0.2 mM GTP. GTP inhibition of $F^-$ activated adenyl cyclase starts at about $1 \cdot 10^{-8}$ M and the enzyme is maximally inhibited at concentrations of GTP greater than $1 \cdot 10^{-5}$ M, the highest concentration used being $1 \cdot 10^{-3}$ M.

These results are not directly comparable to anything reported in this paper since we were using GTP at a concentration of 4 mM when we demonstrated activation, by GTP, of a system still subject to the depradations of "ATPase".

Thus our conclusion that GTP is sparing the substrate ATP for further meta-

bolism by cyclase is still valid. However, is it clear that for the system reported by RODBELL *et al.* (*i.e.* liver plasma membrane, acting upon Ado-5'-*P*-O-*P*-N-*P*) GTP is capable of exerting some regulatory influence upon adenyl cyclase. It will be of interest to determine whether this is also true for luteal adenyl cyclase.

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