physical environments. It is also possible that the non-linearity of the Lineweaver-Burk plot, which is responsible for the discrepancy, is due to some kinetic property of this enzyme.

A variety of substances have been shown to alter the activity of phosphodiesterases. The reducing agents 2-mercaptoethanol and dithioerythritol at 5mM concentrations double the activity of dialysed rat brain enzyme. Their action is distinct from that of imidazole. The effect of imidazole, which is maximal at 15mM, is primarily to elevate $V_{\max}$, though at higher concentrations K_m is also increased.

The nucleosides and nucleotides of guanine, hypoxanthine and adenine inhibit rat brain phosphodiesterase with potencies comparable with those of the methylxanthines. Since the inhibitions are strictly competitive (with cyclic AMP concentrations about 0.1–1.0mM) it appears that their actions reflect a relatively broad specificity in the active site of phosphodiesterase. Although Cheung (1967) found that a variety of nucleoside triphosphates, inorganic pyrophosphate and citrate were also inhibitory, it appears likely that their Mg^{2+} -sequestering properties were at least partially responsible.

The primary value of investigating the properties of isolated preparations of phosphodiesterase is to provide an understanding of its function in the intact cell. Emphasis is placed here on the situation that exists in adipose tissue, where a clear relationship has been established between cyclic AMP concentrations and the rate of lipolysis. In this system it appears that cyclic AMP concentrations can be adequately interpreted in terms of the relative activities of adenylate cyclase and phosphodiesterase. Intracellular compartmentation of cyclic AMP has been suggested as a complicating factor by Kuo & De Renzo (1969), but, though it seems likely to have some influence, there is no convincing evidence of its importance.

In an attempt to quantify the relationship between cyclic AMP and the activities of adenylate cyclase and phosphodiesterase, Davies & Williams (1970) derived the relationship:

$$[\text{Cyclic AMP}] = \frac{K_2 \mathcal{V}_{11}}{V_2 - \mathcal{V}_{11}} + \frac{K_2 V_2 \left(\frac{\mathcal{V}_{12} - \mathcal{V}_{11}}{V_2 - \mathcal{V}_{11}} \right) [H]}{cK_H(V_2 - \mathcal{V}_{11}) - (\mathcal{V}_{12} - V_2)[H]}$$

K_2 and V_2 are the K_m and $V_{\max}$ values for phosphodiesterase. $\mathcal{V}_{11}$ and $\mathcal{V}_{12}$ represent the maximum rates of cyclic AMP formation at any specified ATP concentration when adenylate cyclase is in the non-activated and the hormone-activated form respectively. $[H]$ is the hormone concentration and

K_H is the dissociation constant of the hormone-adenylate cyclase interaction. The constant c differs from unity only if the interaction of hormone with adenylate cyclase alters the affinity of the latter for ATP.

The relationship between [cyclic AMP] and $[H]$ predicted takes the form of a rectangular hyperbola with the expected maximum in cyclic AMP if $V_2 > \mathcal{V}_{12}$, i.e. if the capacity of phosphodiesterase to destroy cyclic AMP exceeds the capacity of adenylate cyclase to bring about its synthesis. Efforts to demonstrate the validity of the relationship are in a preliminary stage. For these purposes the crude approximation has been made that over a limited range cyclic AMP concentrations in adipose tissue can be equated to the rate of lipolysis. The predicted effects of manipulating K_2 (with methylxanthines) and K_H (with catecholamine analogues and β -adrenergic blocking agents) are in good agreement with experimental findings.

On the basis of the above equation it is possible to predict the effect of changing the parameters of phosphodiesterase. The results serve to emphasize the profound effect on cyclic AMP concentrations made possible by altering the activity of this enzyme.

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Adenylate Cyclase and 'Adenosine Triphosphatase' in Corpus Luteum

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Cyclic AMP (adenosine 3':5'-cyclic monophosphate) is believed to be the mediator of many hormonal effects (see Robison, Butcher & Sutherland, 1968; Sutherland, Hardman, Butcher & Broadus, 1969). The formation of cyclic AMP is catalysed by adenylate cyclase, an enzyme present in

many mammalian and other tissues (see Robison, Butcher & Sutherland, 1968, 1970). Our work on luteal adenylate cyclase arose out of studies on the effect of luteinizing hormone on the glycogen content and phosphorylase activity of corpora lutea (Stansfield & Robinson, 1965), and the observations of others that luteinizing hormone increases the concentration of cyclic AMP in luteal slices and that cyclic AMP stimulates progesterone production in similar preparations (see Marsh, 1969).

Adenylate cyclase is usually associated with membranous components of the cell, and its insoluble nature has restricted attempts to study its characteristics. The aim of our research programme has been to obtain a preparation of luteal adenylate cyclase that was suitable for kinetic and endocrine studies with gonadotrophins.

In this work adenylate cyclase has been assayed by determining the cyclic [^{14}C]AMP produced from [^{14}C]ATP during incubations of rat luteal tissue (Parlow, 1958) at 37°C, the reaction mixture being similar to that of Rall & Sutherland (1958). The cyclic AMP produced was purified by a method based on that of Jungas (1966) before measurement by scintillation counting. Recovery of cyclic AMP was 55–60%.

Rat luteal adenylate cyclase is mainly found in the material sedimenting from tissue homogenate at 600g for 10 min. Although this fraction is free from cyclic AMP phosphodiesterase (D. A. Stansfield, J. Horne & G. Wilkinson, unpublished work), there is in this fraction a potent 'adenosine triphosphatase'. This appears to be a complex of adenosine triphosphatase(s), adenosine diphosphatase and 5'-nucleotidase, which hydrolyses up to 90% of the 4.4 μmol of ATP present within the first 5 min of incubation even at optimum tissue concentration for the production of cyclic AMP (600g pellet equivalent to 75 mg of fresh tissue) and in the presence of 10 mM-sodium fluoride. Under these conditions production of cyclic AMP was maximal at 0.66 nmol/min during 40 min incubations.

In the presence of 10 mM-sodium fluoride P_i release from ATP (i.e. 'adenosine triphosphatase' activity) was 50% inhibited. Increasing the sodium fluoride concentration to 100 mM further increased the inhibition to 80%, but this concentration of sodium fluoride inhibited adenylate cyclase. Hydrolysis of ADP by the 600g pellet was progressively inhibited as the sodium fluoride concentration was raised to 100 mM, whereas that of AMP was maximally inhibited at 10 mM-sodium fluoride. Adenylate cyclase activity was only detected in the absence of sodium fluoride when an ATP-regenerating system was included in the incubation mixtures, but there was still a marked activation of cyclic AMP production when sodium fluoride (10 mM) was also

added. This suggests that, although a part of the stimulation of the production of cyclic AMP by added sodium fluoride is due to inhibition of 'adenosine triphosphatase' and sparing of substrate for adenylate cyclase, there is also a direct effect of sodium fluoride on the cyclase system itself. We infer that sodium fluoride may be able to form at certain F^- concentrations a particular complex anion that activates adenylate cyclase. At concentrations of sodium fluoride higher than 10 mM a new ion species appears that is inhibitory. A similar suggestion has been made by Reiner, Tsuboi & Hudson (1955) to explain F^- inhibition of acid phosphatase.

Luteal adenylate cyclase requires Mg^{2+} in excess over ATP for maximal activity, but removal of Mg^{2+} as, e.g., a magnesium phosphorofluoridate complex cannot explain the swing-over from activation to inhibition as the sodium fluoride concentration is increased from 10 mM to 100 mM.

Attempts were made to inhibit the 'adenosine triphosphatase' by substituting other bivalent cations for Mg^{2+} , but the enzyme complex had a low requirement for Mg^{2+} , being fully active in the presence of ATP/ Mg^{2+} ratios of 4:1. 'Adenosine triphosphatase' was 80% inhibited by 10 mM-EDTA. Substitution of Mn^{2+} or Ca^{2+} for Mg^{2+} decreased the detectable adenylate cyclase activity by 75% and 100% respectively. 'Adenosine triphosphatase' was not inhibited by either ouabain at up to 10 mM or *p*-chloromercuribenzoate at up to 0.5 mM, and it had no requirement for Na^+ or K^+ . Sodium azide was the only substance that inhibited 'adenosine triphosphatase' as effectively as sodium fluoride, but sodium azide completely inhibited adenylate cyclase.

We have investigated the use of $\beta\gamma$ -methylene-ATP, an analogue of ATP that is resistant to adenosine triphosphatase hydrolysis. Adenylate cyclase catalyses the removal of the terminal pyrophosphate group of ATP, and it was reasoned that it might also be able to remove the terminal methylenediphosphonate group of $\beta\gamma$ -methylene-ATP. However, no detectable production of cyclic AMP occurred and, even though the analogue was resistant to 'adenosine triphosphatase' hydrolysis, it did not function as a competitive inhibitor when present in amount of equimolar with [^{14}C]ATP. GTP (4 mM) stimulated the production of cyclic AMP provided that excess of Mg^{2+} relative to nucleoside triphosphate was maintained. This suggests that luteal adenylate cyclase is specific for the entire ATP molecule and that alterations of the purine or the triphosphate side chain lead to rejection at the active centre. 'Adenosine triphosphatase' appears to hydrolyse GTP, thus sparing substrate ATP for attack by adenylate cyclase. With high GTP concentrations (10 mM) adenylate

cyclase activity decreased, even though Mg^{2+} was maintained in excess and sodium fluoride was present at optimum concentration.

Differential-centrifugation studies have shown that all particulate fractions of rat corpus-luteum homogenate contain adenylate cyclase and 'adenosine triphosphatase'. The cyclases of 5000g and 54000g pellets show a linear increase in the production of cyclic AMP with increasing tissue concentration in contrast with that of the 600g pellet.

Sonication increases adenylate cyclase and 'adenosine triphosphatase' activities in the 54000g pellet and high-speed supernatant at the expense of those in the 600g and 5000g pellets, but the solubilization was small and non-selective.

Triton X-100 (1%) increases the amount of adenylate cyclase in 54000g supernatant, but this is at the expense of a large overall inactivation of the enzyme. Lubrol-PX (1%) was somewhat less effective, and treatment of homogenate with deoxycholate (1%) resulted in elimination of adenylate cyclase activity.

Detection of adenylate cyclase activity in soluble fractions of luteal homogenate is complicated by the presence of 3':5'-cyclic nucleotide phospho-

diesterases, but, even after removal of cytosol before detergent treatment of the particulate fractions, the amount of adenylate cyclase solubilized was still small compared with the overall loss of activity.

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COMMUNICATIONS

A Modified Enzymic Assay for Adenosine 3':5'-Cyclic Monophosphate

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An understanding of the physiological role of cyclic AMP (adenosine 3':5'-cyclic monophosphate) depends on its successful assay. Here we outline a method based on the conversion of phosphorylase *b* into phosphorylase *a*, which indirectly requires cyclic AMP. This conversion is catalysed by phosphorylase *b* kinase, which is activated by phosphorylase *b* kinase kinase, the latter reaction having an absolute requirement for cyclic AMP (Walsh, Perkins & Krebs, 1968). Earlier assay methods that used phosphorylase *b* were only successful owing to variable amounts of phosphorylase *b* kinase found in inactive preparations of phosphorylase *b* kinase. In this method the optimum amount of purified phosphorylase *b* kinase kinase (Walsh *et al.* 1968) is added.

Tissues are rapidly frozen in isopentane or with tongs, cooled to -196°C . Samples (10–25mg) are weighted at -30°C and powdered while frozen. The powder is homogenized in 5 vol. of 10% (w/v) trichloroacetic acid at 0°C and after centrifugation

the supernatant is extracted four times with 5 vol. of water-saturated ether. Interfering nucleotides are removed by precipitation with zinc sulphate and barium hydroxide and centrifugation (G. Krishna, see Rodbell, 1967). The final supernatants are applied to cellulose plates with micro-syringes and chromatographed with propan-2-ol-water-35M-ammonia (14:3:3, by vol.) (Goldberg, Lerner, Sasko & O'Toole, 1968) with cyclic AMP markers. After the chromatogram has been dried, the areas containing cyclic AMP (R_f 0.45) are eluted with 0.5ml of water. This process gives recoveries of cyclic AMP of 90–95%.

The eluate (100 μl) containing cyclic AMP is added to 260 μl of a medium in a cuvette, containing 30mM-tris, 30mM-sodium glycerol 2-phosphate, 2.5mM-EDTA, 25mM-2-hydroxyethanethiol, 0.075mM-ATP, 0.25mM- Mg^{2+} , 2000 units of Norit A-treated crystalline rabbit muscle phosphorylase *b* (Fischer & Krebs, 1962), 12.5 μg of non-activated phosphorylase *b* kinase (Krebs *et al.* 1964) and 1.9 μg of phosphorylase *b* kinase kinase at pH 6.8 at 35°C . After 1 min the formation of phosphorylase *a* is terminated by the addition of 1.0ml of ice-cold 20mM-phosphate containing 0.5mM-NADP $^{+}$ and 0.1% of bovine serum albumin at pH 6.8. Phosphoglucomutase (1 U) and glucose 6-phosphate dehydro-