Endogenous Phosphorylation of Microsomal Proteins in Bovine Corpus Luteum

TENFOLD ACTIVATION BY ADENOSINE 3':5'-CYCLIC MONOPHOSPHATE

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Free ribosomes and a smooth-microsomal fraction were prepared from bovine corpus luteum. Both preparations will self-phosphorylate when incubated with Mg^{2+} and ATP, but at low concentrations of Mg^{2+} and ATP the self-phosphorylation of the smooth-microsomal fraction was much more dependent on cyclic AMP than was that of free ribosomes, stimulation by the nucleotide being up to 10-fold in the former case. The self-phosphorylation of the smooth-microsomal fraction was studied further. The reaction bears similarities to that brought about by soluble cyclic AMP-dependent protein kinase, being inhibited by Ca^{2+} and the heat-stable inhibitor protein from skeletal muscle. Cyclic GMP will activate the reaction at concentrations higher than those required for full activation by cyclic AMP. In the presence of cyclic AMP, phosphate bound to protein is found almost exclusively as phosphoserine. Several proteins are phosphorylated, as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and the phosphorylation of all of them is markedly stimulated by cyclic AMP. If the reaction is carried out at high concentrations of Mg^{2+} and ATP, a distinct cyclic AMP-independent phosphorylation is observed. This activity is not inhibited by the heat-stable inhibitor protein, and phosphate is found esterified with both threonine and serine residues.

Steroidogenesis in the corpus luteum and other ovarian tissues is regulated by lutropin (luteinizing hormone), and there is good evidence that this action of the hormone is mediated by cyclic AMP (Marsh, 1975). Lutropin increases the endogenous concentration of the nucleotide when incubated with slices of bovine corpus luteum (Marsh *et al.*, 1966), and exogenous cyclic AMP can reproduce the action of the hormone by increasing progesterone output (Marsh & Savard, 1966).

At present the only mechanism by which cyclic AMP is known to produce its biological effects is through its activation of cyclic AMP-dependent protein kinase, which is present in all mammalian tissues examined, including corpus luteum (Menon, 1973; Webb & Stansfield, 1974). However, although Clark *et al.* (1976) have concluded that activation of cyclic AMP-dependent protein kinase is a part of the physiological function of lutropin and chorionadotropin in corpus luteum, very little is known of the natural substrates whose phosphorylation is presumed to bring about the steroidogenic effects of cyclic AMP. Both cholesterol esterase (Beckett & Boyd, 1975) and a reconstituted side-chain-cleavage system (Caron *et al.*, 1975) are slightly stimulated by cyclic AMP in the presence of cyclic AMP-dependent protein kinase *in vitro*, but on present evidence these effects are not sufficient to explain all the actions of the

hormone. The stimulation of luteal progesterone synthesis by lutropin or cyclic AMP is inhibited by cycloheximide (Marsh *et al.*, 1966; Hermier *et al.*, 1971), leading to speculation that protein synthesis is a necessary adjunct of the increased steroidogenesis. Ribosomes from corpus luteum can be phosphorylated by cyclic AMP-dependent protein kinase *in vitro* (Azhar & Menon, 1975a), but as yet phosphorylation of free ribosomes has not been shown to produce any functional change *in vitro* (Eil & Wool, 1973). We are interested in the possibility that phosphorylation of rough-endoplasmic-reticulum proteins could produce a change in the activity of membrane-bound ribosomes. As a preliminary study we have characterized the phosphorylation of proteins in smooth microsomes (i.e. a microsomal fraction lacking ribosomes, prepared by isopycnic centrifugation). These results will serve as a standard against which future studies of rough endoplasmic reticulum may be compared.

Experimental

Materials

[γ - ^{32}P]ATP (sodium salt) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and was diluted with unlabelled ATP as required. Marker proteins for gel electrophoresis (except

phosphorylase) were obtained from Boehringer Corp., Lewes, Sussex, U.K. Phosphorylase *b* was purified by the method of Fischer & Krebs (1958), and skeletal-muscle heat-stable protein kinase inhibitor by the method of Walsh *et al.* (1971), as far as the precipitation by 15% (w/v) trichloroacetic acid. All other biochemicals were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Sucrose-containing buffers were made from AnalaR sucrose (BDH Chemicals, Poole, Dorset, U.K.) and were treated with diethyl pyrocarbonate to inactivate ribonuclease before use (Palmiter, 1974).

Preparation of ribosomes and microsomal membranes

Bovine corpora lutea from animals in the oestrous cycle were collected from a local slaughterhouse and processed immediately on arrival at the laboratory. All succeeding operations were performed at 0–4°C. The corpora lutea were dissected free of connective tissue, cut into small pieces and rinsed with 0.25 M-sucrose in TKM buffer. TKM buffer is 50 mM-Tris/25 mM-KCl/4 mM-MgCl₂, adjusted to pH 7.5 with HCl at 20°C. The pieces of tissue were passed through a hand-operated tissue press and homogenized in 2.5 vol. of 0.25 M-sucrose in TKM buffer with a Potter–Elvehjem homogenizer, by using four strokes of a loose (clearance 0.66 mm) and four strokes of a tight (clearance 0.3 mm) pestle. The homogenate was centrifuged (4 min at 700g) to remove nuclei and cell debris. The supernatant was re-centrifuged (10 min at 10000g), and the resulting supernatant was aspirated and the floating fat layer discarded. The 10000g centrifugation was repeated, and the second supernatant was layered in 15 ml portions over 10 ml of 2 M-sucrose, 5 ml of 1.5 M-sucrose and 5 ml of 1.35 M-sucrose, all in TKM buffer. This discontinuous gradient was centrifuged for 16 h at 40000 rev./min in the MSE 43114-130 rotor (138000g at r_{av} , 7.72 cm). Three submicrosomal fractions were obtained: a pellet of free ribosomes, and two membranous fractions in the 1.5 M- and 1.35 M-sucrose layers. The lower, more-dense, fraction represents the rough endoplasmic reticulum. The upper, less-dense layer is referred to as the smooth-microsomal fraction. Both fractions were diluted with TKM buffer to about 1.1 M-sucrose and collected by centrifugation for 2 h at 180000g. All fractions were stored as pellets at –15°C. Free ribosomes were resuspended gently in TKM buffer; membranous fractions were resuspended in buffer containing 0.25 M-sucrose.

Preparation of rat luteal cytosol protein kinase

Corpora lutea were obtained from rats super-ovulated by the method of Parlow (1958) and were stored at –15°C. All subsequent operations were performed at 0–4°C. Corpora lutea from 15 rats were

passed through a hand-operated tissue press and homogenized in 5 vol. of 0.25 M-sucrose in TKM buffer with a Potter–Elvehjem homogenizer. The homogenate was centrifuged (10 min at 10000g) and the pellet resuspended in 5 vol. of homogenization buffer and re-centrifuged as above. The combined supernatants were then centrifuged (1 h at 166000g) and the supernatant was used as a source of enzyme. The fraction precipitated between 25 and 60% saturation of (NH₄)₂SO₄ was collected by centrifugation (20 min at 10000g), dissolved in 5 mM-potassium phosphate buffer/2 mM-EDTA/6 mM-mercaptoethanol, pH 7.4, and dialysed overnight against the same buffer. The preparation was assayed for protein kinase activity as described by Webb & Stansfield (1974). After correction for endogenous phosphorylation, the specific activity with mixed histone as substrate in the presence and absence of cyclic AMP was 10.2 and 1.1 pmol/min per mg respectively.

Assay of phosphorylation

The standard assay contained ribosomes or smooth-microsomal membranes (up to 250 µg), 50 mM-Tris/HCl buffer, pH 7.0, 2.5 mM-theophylline, 10 mM-NaF, 4 µM-cyclic AMP (when present), 1 mM-MgCl₂ and 20 µM-[γ-³²P]ATP (200–500 Ci/mol), in a final volume of 100 µl. Occasionally high concentrations of Mg²⁺ and ATP were used: these were 7.5 mM-MgCl₂ and 200 µM-[γ-³²P]ATP (20–50 Ci/mol). These and other variations to the assay are detailed in Figure legends. The reaction was started by the addition of MgCl₂ plus ATP to the other components, which had been preincubated for 5 min. After incubation at 30°C (usually 2 min unless stated otherwise) the reaction was stopped by the addition of 500 µl of cold 5% (w/v) trichloroacetic acid. The precipitated protein was then washed and counted for radioactivity to measure incorporation of ³²P into protein as described by Nimmo *et al.* (1976) for the assay of glycogen synthetase kinase-2. The assay was modified by performing two cycles of solution in NaOH and precipitation in trichloroacetic acid.

Identification of phosphorylated amino acids

Smooth microsomes were incubated in the presence or absence of cyclic AMP in the standard phosphorylation assay mixture scaled up to 250 µl. After 2 min at 30°C, a 50 µl portion was added to 500 µl of 5% trichloroacetic acid and subjected to the normal assay procedure. These results were used to estimate losses from the hydrolysed samples. The remaining 200 µl was added to 1 ml of cold 5% trichloroacetic acid, stood in ice for 10 min, and the precipitate recovered by centrifugation (2 min on a bench centrifuge). The precipitate was rapidly resus-

pended in cold 0.1M-NaOH and reprecipitated by adding 1 ml of cold 5% trichloroacetic acid. After collecting the precipitate by centrifugation as above, it was washed twice by resuspension and centrifugation with 1 ml portions of ethanol/diethyl ether (3:1, v/v). The precipitate was finally resuspended in 500 μ l of 2M-HCl and hydrolysed for 4 h at 110°C in sealed evacuated tubes. The contents were freeze-dried, dissolved in 50 μ l of water and spotted on Whatman 3MM paper, together with *O*-phosphothreonine, *O*-phosphoserine and $^{32}\text{PO}_4^{3-}$ markers. Electrophoresis in 2% formic acid/8% acetic acid (pH 1.9) was for 80 min at 50 V/cm. The paper was dried, cut transversely into 5 mm strips along the lanes of migration, and the strips were counted for radioactivity in 5 ml of scintillant (Bray, 1960). Unlabelled markers were located with ninhydrin.

Gel electrophoresis of phosphorylated proteins

Smooth microsomes were phosphorylated with and without cyclic AMP in the standard assay mixture. The reaction was stopped after 2 min at 30°C by adding 10 μ l of 10% (w/v) sodium dodecyl sulphate. Phosphorylated proteins were then reduced with dithiothreitol and analysed on 1% sodium dodecyl sulphate/5% polyacrylamide gels (Fairbanks *et al.*, 1971). The stained gels were scanned on a Joyce-Loebl Chromoscan 200 instrument using filter 5-040 and sliced manually into equal sections of about 1.5 mm. The gel slices were digested overnight in 200 μ l of 30% (w/v) H_2O_2 at 40°C and counted for radioactivity in 10 ml of scintillant (Bray, 1960).

Chemical analyses and marker enzymes

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, after precipitation with 5% trichloroacetic acid and redissolving in 0.5M-NaOH. RNA was determined as described by Wool & Cavicchi (1967). Phospholipid was determined by the method of Chen *et al.* (1956), using a factor of 25 to convert μ g of inorganic phosphorus into μ g of phospholipid. Marker enzymes assayed were NADPH-cytochrome *c* reductase (Masters *et al.*, 1967), NADH dehydrogenase (Wallach & Kamat, 1966), 5'-nucleotidase (Song & Bodansky, 1967) and glucose 6-phosphate dehydrogenase (Kelly *et al.*, 1955).

Results

Characterization of fractions

Free ribosomes had A_{260}/A_{280} ratios of 1.77 ± 0.07 (5) and A_{260}/A_{235} ratios of 1.44 ± 0.11 (5) (means $\pm$ S.D. with the number of preparations analysed in parentheses). The phospholipid content of free ribosomes was always less than 1%. RNA/protein ratios for the different fractions were: free ribosomes,

0.83 ± 0.2 (5); rough microsomes, 0.24 ± 0.09 (6); smooth microsomes, 0.084 ± 0.037 (6).

In two preparations of smooth microsomes, the following enrichments of specific activity over the post-nuclear supernatant were obtained: 5'-nucleotidase, 4.2- and 5.8-fold; NADPH-cytochrome *c* reductase, 3.9- and 4.2-fold; NADH dehydrogenase, 5.4-fold in both. Glucose 6-phosphate dehydrogenase was not detectable, although 98% of the homogenate activity was recovered in the pooled high-speed supernatants.

Endogenous phosphorylation of ribosomes and smooth membranes

The time-courses for the endogenous phosphorylation of ribosomes and smooth microsomes are shown in Fig. 1. In the presence of cyclic AMP both preparations self-phosphorylate very rapidly. Phosphorylation of ribosomes continues for a longer time and ultimately more phosphate is incorporated per

Fig. 1. Time-course of endogenous phosphorylation of (a) smooth microsomes and (b) free ribosomes

The standard assay was scaled up to 200 μ l, and 25 μ l samples were removed at various times and added to 500 μ l of 5% trichloroacetic acid. Results are expressed as pmol of phosphate incorporated/mg of smooth-microsomal or ribosomal protein. Additions were: ●, none; ○, 4 μ M-cyclic AMP; ▲, 25 μ g of rat luteal protein kinase + 4 μ M-cyclic AMP.

mg of protein in free ribosomes than in smooth microsomes. However, the rapid cessation of smooth-microsomal phosphorylation is the result of depletion of ATP by the abundant adenosine triphosphatases in this fraction. If a second addition of Mg^{2+} and ATP was made to microsomal incubations after the reaction had reached its plateau value, a second burst of phosphorylation of approximately equal magnitude was obtained, giving incorporations of over 400 pmol/mg.

In the absence of cyclic AMP, very little phosphorylation occurs in the smooth-microsomal fraction. Owing to the rapid rate of reaction, the activation by cyclic AMP is difficult to estimate, but in several experiments with different preparations it was between 5- and 10-fold. Under the particular conditions used, the self-phosphorylation of ribosomes was much less dependent on cyclic AMP, the activation being approx. 2-fold. In all cases, addition of rat luteal cytosol protein kinase produced only a slight increase in phosphorylation.

Characterization of the self-phosphorylation by smooth microsomes

Smooth microsomes were self-phosphorylated with various concentrations of ATP and 7.5 mM- $MgCl_2$. The results are illustrated in Fig. 2(a) and show that phosphorylation increases with ATP concentration and appears to be nearly saturated at 0.2 mM. However, at the higher concentrations the increase appears to be due to an increase in cyclic AMP-independent activity, and if this is subtracted (broken line in Fig.

2a) the requirement for ATP is saturated at around 50 μ M.

A similar result was obtained when Mg^{2+} concentration was varied in the presence of 20 μ M-ATP (Fig. 2b). Cyclic AMP-dependent phosphorylation appears to be saturated by 1 mM- Mg^{2+} , whereas the cyclic AMP-independent activity continues to increase as the Mg^{2+} concentration is raised to 15 mM. Since it is the cyclic AMP-dependent activity that is of interest from the point of view of hormonal regulation, conditions were chosen to maximize the effects of cyclic AMP. The concentrations adopted for further studies were 1 mM- Mg^{2+} and 20 μ M-ATP.

The effect of Ca^{2+} ions is shown in Fig. 3(a). In common with other protein kinases the endogenous phosphorylation is inhibited by this cation, inhibition in the presence or absence of cyclic AMP being almost complete at 5 mM- Ca^{2+} .

Cyclic AMP brings about maximum activation of the phosphorylation at about 5 μ M (Fig. 3b), with half-maximal activation at about 1 μ M. Cyclic GMP is also able to activate the reaction (Fig. 3b), but much higher concentrations are needed for full activation.

Effect of protein kinase inhibitor

The effect of the protein kinase inhibitor from skeletal muscle is shown in Fig. 4(a). At high inhibitor concentrations there is no significant activity in either the presence or the absence of cyclic AMP. If high concentrations of Mg^{2+} and ATP are used, however, the reaction is not completely inhibited

Fig. 2. *Effect of ATP and Mg^{2+} concentration on endogenous phosphorylation by smooth microsomes*

The reaction was assayed as described in the Experimental section except that the concentrations of ATP and Mg^{2+} were varied. (a) Effect of ATP concentration. For this experiment the Mg^{2+} concentration was 7.5 mM. ●, Control; ○, +4 μ M-cyclic AMP; ▲, values obtained with 4 μ M-cyclic AMP with control values subtracted. (b) Effect of Mg^{2+} concentration. ATP was at 20 μ M. Symbols used are as in Fig. 2(a).

Fig. 3. Effect of Ca^{2+} and cyclic nucleotide concentration on self-phosphorylation of smooth microsomes. The reaction was assayed as described in the Experimental section except for the variations in Ca^{2+} or cyclic nucleotide concentration. (a) Effect of Ca^{2+} ions: ●, control; ○, $+4 \mu\text{M}$ -cyclic AMP. (b) Effect of concentration of cyclic AMP (■) or cyclic GMP (□).

Fig. 4. Effect of the addition of the heat-stable protein kinase inhibitor from skeletal muscle on endogenous phosphorylation in the smooth microsomal fraction.

The reaction was assayed as described in the Experimental section except for the addition of the protein kinase inhibitor (0–50 μg of protein). The inhibitor (10 μl in 50 mM-Tris, 10 mM-theophylline, pH 7.0) was added just before the reaction was initiated by the addition of Mg^{2+} and ATP. (a) At low concentrations of Mg^{2+} (1 mM) and ATP (20 μM). (b) At high concentrations of Mg^{2+} (7.5 mM) and ATP (200 μM). ●, Control; ○, $+4 \mu\text{M}$ -AMP.

(Fig. 4b), and as the inhibitor concentration is increased the incorporation approaches a constant value which is independent of the presence of cyclic AMP.

Phosphorylation of endogenous substrates

The microsomal protein kinase will also phosphorylate added histone (Fig. 5). This reaction is also activated by cyclic AMP, but there is a substantial amount of phosphorylation in the absence of the nucleotide. It has been reported that histone can dissociate cyclic AMP-dependent protein kinase and hence release catalytic subunits which are fully active in the absence of cyclic AMP (Miyamoto *et al.*, 1973). The incubations shown in Fig. 5 were initiated by the addition of the membrane preparation. If the reaction was initiated by the addition of the Mg^{2+} and ATP, and the microsomes were preincubated with the histone, the dependence on cyclic AMP disappeared completely (results not shown). It may therefore be that the apparent lower dependence on cyclic AMP when histone is used as the substrate is due to its ability to dissociate the protein kinase rapidly.

Identification of phosphorylated amino acids

Smooth microsomes were allowed to self-phosphorylate in the presence or absence of cyclic AMP and at high and low concentrations of Mg^{2+} and ATP. Phosphorylated proteins were then extracted as described in the Experimental section and subjected to partial acid hydrolysis followed by paper electrophoresis at pH 1.9. Four peaks of radioactivity were obtained, one at the origin representing incompletely hydrolysed material, and three that had

migrated towards the anode and corresponded exactly with phosphothreonine, phosphoserine and phosphate markers. The ratio of incorporation of radioactivity into phosphoserine and phosphothreonine differed for the various treatments and the results are presented in Table 1. In the absence of

cyclic AMP the ratio is about 4:1 at both concentrations of Mg^{2+} and ATP. In the presence of cyclic AMP this ratio increases, and the increase in phosphoserine relative to phosphothreonine is especially marked when low concentrations of Mg^{2+} and ATP are used.

Electrophoresis of phosphorylated proteins

Smooth microsomes were phosphorylated in the presence and absence of cyclic AMP and the reaction was terminated by the addition of sodium dodecyl sulphate to a final concentration of 1%. The detergent solubilizes membrane proteins which, after reduction of disulphide bonds, can be analysed on 5% polyacrylamide gels in 1% dodecyl sulphate as described in the Experimental section. The gels were stained, scanned in a densitometer, and sliced to measure the distribution of radioactivity. The results are shown in Fig. 6. There are several peaks of radioactivity throughout the gel, all of which are phosphorylated much more extensively in the presence of cyclic AMP. There are several incompletely resolved phosphopeptides of high molecular weight (above 100000) and two distinct peaks with approximate molecular weights of 62000 and 45000. The pattern of Coomassie Blue stain is complex, and it is not possible to correlate with certainty any of the stained peaks with radioactive peaks.

Fig. 5. Time-course of phosphorylation of smooth microsomes with or without added histone

The standard assay was scaled up to 200 μ l, and 25 μ l samples were removed at various times and added to 500 μ l of 20% trichloroacetic acid to stop the reaction. The normal procedure was modified by using 20% trichloroacetic acid and 0.5M-NaOH, owing to the high acid solubility of histones. The reaction was started by the addition of smooth microsomes (in buffer) to the remaining components. Additions were: ●, control; ○, 4 μ M-cyclic AMP; ■, 400 μ g of mixed histone; □, 400 μ g of mixed histone+4 μ M-cyclic AMP.

Discussion

In this study we have demonstrated that a microsomal fraction from bovine corpus luteum contains both bound protein kinase(s) and endogenous proteins which can act as substrates for phosphorylation. When studied at low concentrations of Mg^{2+} (1 mM) and ATP (20 μ M), the reaction is similar in several respects to the phosphorylation catalysed by cyclic AMP-dependent protein kinases from cytosol (Walsh & Krebs, 1973). Thus cyclic AMP activates the reaction at low concentration

Table 1. Incorporation of ^{32}P from [γ - ^{32}P]ATP into threonine and serine residues of smooth-microsomal proteins incubated with and without 4 μ M-cyclic AMP

Two concentrations of Mg^{2+} and ATP were used as indicated in the Table. The specific radioactivities of [γ - ^{32}P]ATP used were: Expts. (a) and (b), 500 Ci/mol; Expts. (c) and (d), 50 Ci/mol. The incorporations have been corrected to allow for slight losses incurred by manipulation during the hydrolysis procedure. The actual radioactivity recovered in phosphothreonine and phosphoserine peaks has been multiplied by the expected total recovery and divided by the actual total recovery from the electrophoresis paper (minus background).

Expt.	[Mg^{2+}] (mM)	[ATP] (M)	Cyclic AMP	Phosphothreonine (c.p.m.)	Phosphoserine (c.p.m.)	Ratio phosphoserine/ phosphothreonine
(a)	1.0	20	—	98	348	3.6
(b)	1.0	20	+	230	3426	14.9
(c)	7.5	200	—	495	1947	3.9
(d)	7.5	200	+	420	2545	6.1

Fig. 6. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of phosphorylated smooth-microsomal proteins. The smooth-microsomal fraction was allowed to self-phosphorylate and then dissolved in sodium dodecyl sulphate as described in the Experimental section. (a) Gel was sliced and counted for radioactivity to detect ^{32}P incorporation. ●, Control; ○, $+4\mu\text{M}$ -cyclic AMP. (b) Scan of gel stained with Coomassie Blue. There was no detectable difference in staining pattern between control and cyclic AMP-treated incubations. The molecular-weight scale was constructed by using the following marker proteins in a parallel gel: rabbit skeletal-muscle phosphorylase *b* (100000); bovine serum albumin (67000); ox liver catalase (60000); hen's-egg albumin (45000); chymotrypsinogen A (25000); and cytochrome *c* (12500).

(below $1\mu\text{M}$); cyclic GMP also activates, but only at considerably higher concentrations; the reaction requires Mg^{2+} ions, but is inhibited by Ca^{2+} ions; phosphate is esterified almost entirely with serine residues; and the reaction is inhibited by the heat-stable inhibitor from skeletal muscle. The membrane-associated enzyme will also phosphorylate exogenous histone effectively. The lower degree of dependence on cyclic AMP for this reaction when compared with endogenous phosphorylation can be explained by the dissociation of catalytic and regulatory subunits by the histone (Miyamoto *et al.*, 1973). We have not attempted to solubilize the enzyme and do not know whether it differs from the cytosol enzyme in several other properties, as reported for the partially purified microsomal enzymes from several rat tissues (Uno *et al.*, 1976).

Two reports have appeared about the self-phos-

phorylation of rough and smooth microsomes from rat liver *in vitro* (Jergil & Ohlsson, 1974; Sharma *et al.*, 1976). These authors used ATP concentrations of $400\mu\text{M}$ and $200\mu\text{M}$ respectively, and in contrast with the present study found little or no stimulation of endogenous phosphorylation by cyclic AMP, although the phosphorylation of added histone was stimulated by the nucleotide. We have demonstrated that the cyclic AMP-dependent phosphorylation (i.e. the activity in the presence of cyclic AMP minus the activity in its absence) is saturated at lower concentrations of Mg^{2+} and ATP than is cyclic AMP-independent phosphorylation. A similar observation has been made with a plasma-membrane preparation from rat fat-cells (Chang *et al.*, 1974). At high concentrations of Mg^{2+} and ATP we may be observing an additional contribution from a distinct cyclic AMP-independent activity. This is supported by two observations: the cyclic AMP-independent activity is not inhibited by the heat-stable protein kinase inhibitor from skeletal muscle, and it incorporates phosphate into ester linkages with threonine as well as with serine residues. Purified cyclic AMP-dependent protein kinases, or the free catalytic subunit derived from them, appear to be almost completely specific for serine residues (Nimmo & Cohen, 1977). It is possible that cyclic AMP-dependence might be observed with rat liver preparations (Jergil & Ohlsson, 1974; Sharma *et al.*, 1976) if lower concentrations of Mg^{2+} and ATP were used. However, even at the highest concentrations used by us (15mM - Mg^{2+} and $200\mu\text{M}$ -ATP), we still observe about 2-fold activation by cyclic AMP (Fig. 2a). Hence there may be a genuine difference between rat liver and bovine corpus luteum with respect to microsomal protein phosphorylation. Our preparation of corpus luteum ribosomes also contains bound protein kinase(s) which will phosphorylate endogenous substrates at rates comparable with that of the self-phosphorylation of smooth microsomes, although ribosome phosphorylation appears to be much less dependent on cyclic AMP. Phosphorylation of bovine corpus luteum ribosomes *in vitro* has been reported previously (Azhar & Menon, 1975a), although these authors prepared their ribosomes by slightly different methods and the endogenous protein kinase activity was negligible. They therefore added partially purified cyclic AMP-dependent protein kinase from corpus luteum cytosol. With our preparation of ribosomes, the addition of luteal cytosol protein kinase did not significantly increase phosphorylation, presumably because the substrates were already saturated with enzyme, or because of differences in substrate specificity or in availability of substrate to bound and free enzymes. It has been suggested (Keely *et al.*, 1975) that the presence of protein kinase in particulate fractions can be an artifact of preparation, since the free catalytic

subunit of cyclic AMP-dependent protein kinase can bind at low ionic strength, apparently non-specifically, to particulate material or even to denatured protein. Although the protein kinases described in this paper might be eluted by solutions of higher ionic strength and may not represent true membrane proteins, we can rule out an artifact of the type described by Keely *et al.* (1975), since the cyclic AMP-independent activity we observe is not inhibited by the heat-stable inhibitor and thus cannot represent the free catalytic subunit derived from the cyclic AMP-dependent holoenzyme.

The intracellular location of the microsomal proteins here phosphorylated *in vitro* cannot be stated with certainty. At least some of them may be true membrane proteins, since they are not solubilized by washing in media of high or low ionic strength (D. G. Hardie & D. A. Stansfield, unpublished work). There was no measurable activity of the cytosol enzyme glucose 6-phosphate dehydrogenase, indicating that the membrane preparation has been adequately washed. We cannot exclude contamination with fragments of plasma membrane, and in fact the plasma-membrane marker, 5'-nucleotidase, was enriched in our smooth-microsomal fraction as much as two other presumed endoplasmic-reticulum markers, NADP-NADH cytochrome *c* reductase and dehydrogenase. There is cytochemical evidence that 5'-nucleotidase is located in endoplasmic reticulum as well as plasma membrane in rat liver (Widnell, 1972). In view of the highly abundant endoplasmic reticulum in cells of corpus luteum (Christensen & Gillim, 1969), any contamination with plasma membrane is likely to account for only a small proportion of the smooth-microsomal fraction. In addition, the distribution of phosphorylated polypeptides on sodium dodecyl sulphate/polyacrylamide gels (Fig. 6) is quite different from that observed in a similar study carried out with a plasma-membrane-enriched fraction from bovine corpus luteum (Azhar & Menon, 1975b).

At present we have no direct evidence to suggest that the phosphorylation of these microsomal proteins is involved in the control of steroidogenesis. However, the reaction is strongly activated by cyclic AMP, and the range of concentrations over which this occurs (0.1–10 nmol of cyclic AMP/ml) is of the same order of magnitude as the change in cyclic AMP concentration induced in corpus luteum slices by lutropin (0.4–8.5 nmol/g of tissue; Marsh *et al.*, 1966). In addition the reaction is very rapid, a large increase in phosphorylation being produced by cyclic AMP within seconds. To obtain more direct evidence it will be necessary to examine the phosphorylation of microsomal proteins *in vivo* or at least in intact cells which respond to lutropin. Phosphorylation of microsomal protein has been observed in rat liver *in vitro* (Zahlten *et al.*, 1972), and this phosphoryla-

tion was stimulated by glucagon, although not by dibutyryl cyclic AMP.

The rate-limiting step of steroidogenesis appears to be the conversion of cholesterol into pregnenolone in the mitochondrion (Hall & Koritz, 1964). From experiments with cycloheximide and chloramphenicol, Arthur & Boyd (1974) concluded that the continuing synthesis of a protein is necessary for steroidogenesis. This putative protein may be involved with the transport of cholesterol within the mitochondrion, but the inhibitor studies suggest that it is synthesized in the cytoplasm. Although phosphorylation of free ribosomes *in vitro* has not yet been shown to produce a change in function (Eil & Wool, 1973), cyclic AMP can modify protein synthesis in crude post-mitochondrial supernatants from rat liver (Klaipongpan *et al.*, 1975). This effect of cyclic AMP is lost if the membrane component is removed by detergent treatment. It is possible that cyclic AMP could selectively affect the synthesis of proteins on membrane-bound ribosomes via increased phosphorylation of endoplasmic-reticulum membrane proteins. Alternatively, phosphorylation of membrane proteins could affect the transport of metabolites or cofactors between cellular compartments.

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