Accuracy was assessed by adding known quantities of each oestrogen (50–2500 pg) separately to 2.5 ml samples taken from a pool of male plasma and determining the masses of each oestrogen present. The equation of the regression line for the recovery of oestradiol-17 β was $y = 1.03x + 24$ (endogenous concentration, determined separately, 33 pg/2.5 ml) and for the recovery of oestrone was $y = 0.99x + 79$ (endogenous concentration 74 pg/2.5 ml). No interference by one oestrogen in the determination of the other was detected.

A high degree of specificity was achieved by a combination of the insensitivity of the cytosol binding 'protein' to non-oestrogenic steroids (Korenman, 1969; Corker & Exley, 1970; and our observations) and selective chromatography.

Application of the method to the determination of oestrone and oestradiol-17 β throughout the menstrual cycle will be presented.

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The Intracellular Distribution of Adrenal 3 β -Hydroxy Steroid Dehydrogenase and 3-Oxo Steroid Δ^4 – Δ^5 -Isomerase Studied with the Zonal Rotor

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The conversion of pregnenolone into progesterone during steroid biosynthesis in the adrenal cortex requires two enzymes, an NAD⁺-dependent 3 β -hydroxy steroid dehydrogenase and a 3-oxo steroid Δ^4 – Δ^5 -isomerase. It is well established that these enzymes are located in the microsomal fraction obtained by differential centrifugation after tissue homogenization (Beyer & Samuels, 1956). The observed association of dehydrogenase and isomerase activities with the mitochondrial fraction has been attributed to contamination by microsomal enzymes (for discussion see Hayano *et al.* 1956). This view has recently been questioned and the possibility of a bimodal distribution of these enzymes in the adrenal cell has been suggested (McCune *et al.*, 1970). Experiments involving rate-zonal centrifugation of a crude mitochondrial preparation on sucrose gradients in the HS zonal rotor (Measuring and

Scientific Equipment Ltd.) have now indicated that the dehydrogenase and isomerase are firmly bound to the mitochondria, as well as to the endoplasmic reticulum, of the bovine adrenal cortex.

The crude mitochondrial fraction prepared from 5g of bovine adrenal cortex by conventional differential centrifugation was loaded on to a continuous sucrose density gradient (10–55%, w/v) linear with rotor radius. Separation was allowed to proceed for 45 min at 7500g, after which 15 ml fractions were collected and centrifuged at 24000g for 15 min. The pellets were assayed for marker enzymes as follows: for mitochondria, cytochrome oxidase, succinate dehydrogenase, steroid 11 β -hydroxylase and cholesterol side-chain-cleavage enzyme system; for microsomal fraction, 5'-nucleotidase and steroid-21-hydroxylase; for lysosomes, acid phosphatase. In addition, the protein and cholesterol contents of each fraction were determined. Certain fractions were also examined by electron microscopy.

Biochemical and electron-microscopic examination of the fractions show the microsomal particles to be concentrated at the light end of the gradient, whereas the mitochondria sediment as a broad band through the gradient. The width of this band reflects considerable variation of mitochondrial size.

A comparison of the distribution of dehydrogenase and isomerase enzymes with that of the marker enzymes revealed that the enzymes responsible for the conversion of pregnenolone into progesterone are firmly associated with mitochondria and microsomal fraction. The significance of this observation in relation to pathways of steroid biosynthesis in the adrenal cortex will be discussed, together with the advantages of the zonal rotor for preparing mitochondria with minimal microsomal contamination.

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Phosphorylation of the Erythrocyte Membrane: Stimulation by Adenosine 3':5'-Cyclic Monophosphate

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Cyclic AMP (adenosine 3':5'-cyclic monophosphate) is thought to be implicated in the control of cation permeability of membranes. It is possible

that the control may be effected via phosphorylation of a membrane protein. We have therefore investigated the phosphorylation of the erythrocyte membrane and its stimulation by cyclic AMP on isolated membranes, erythrocyte 'ghosts' and intact erythrocytes.

Membrane phosphorylation in the presence or in the absence of cyclic AMP was measured by the transfer of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to membrane components. Erythrocyte membranes were prepared by the method of Hoozeven *et al.* (1970). Cyclic AMP and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ were incorporated into erythrocyte 'ghosts' by the reversed-haemolysis method of Whittam (1962). Intact erythrocytes were allowed to synthesize endogenous $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ by incubating fresh citrated blood with $[\text{P}^{32}]\text{P}_i$ for 45 min. Dibutyl cyclic AMP (6-*N*,2'-*O*-dibutyladenosine 3':5'-cyclic monophosphate) was used in experiments with intact erythrocytes.

In all experiments phosphorylation of the membrane was stimulated by cyclic AMP in intact erythrocytes, 'ghosts' or isolated membranes. Noradrenaline and prostaglandin, which had been shown to enhance the synthesis of cyclic AMP in rat erythrocytes (Sheppard & Burghardt, 1970), also stimulated the incorporation of ^{32}P into components of the membrane. Inhibition of phosphodiesterase enhanced the cyclic-AMP-stimulated phosphorylation.

Separation of the membrane lipid and protein (Boyer & Bieber, 1967) showed that 80% of the bound ^{32}P was in the latter fraction and that the stimulatory effect of cyclic AMP on ^{32}P incorporation was confined to the protein. Hydrolysis of the membrane protein, followed by high-voltage paper electrophoresis and radioautography, yielded phosphoserine and phosphothreonine as the only amino acids containing ^{32}P .

On fractionation of the membrane proteins by electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulphate, six or seven discrete bands were obtained. The three bands representing the highest-molecular-weight proteins contained most of the ^{32}P , and the additional label resulting from stimulation by cyclic AMP was mainly in band 3 (mol.wt. approx. 100000).

Our findings indicate that human erythrocytes are capable of responding to cyclic AMP by increased incorporation of ^{32}P in membrane protein. Whether this observation is of physiological significance and whether it is a property of mature erythrocytes or of reticulocytes remains to be established.

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Mode of Action of Adenosine 3':5'-Cyclic Monophosphate in Insulin Secretion

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It has been shown that the intracellular concentration of cyclic AMP (adenosine 3':5'-cyclic monophosphate) may play a role in determining the rate of insulin release from islets of Langerhans (Montague & Cook, 1971). Since the effects of cyclic AMP in a variety of tissues appear to be related to its binding to a specific receptor protein, which may lead directly to the activation of a protein phosphokinase, we have investigated the occurrence of cyclic-AMP-binding protein in islets of Langerhans and its relationship to cyclic-AMP-dependent protein phosphokinase activity in the same tissue.

Islets of Langerhans prepared by collagenase digestion of rat or guinea-pig pancreas were homogenized at 4°C in 10mM-sodium phosphate buffer, pH 6.5, containing 0.3M-sucrose, and the homogenate was separated by centrifugation into nuclear, mitochondrial, granule, microsomal and supernatant fractions (Howell *et al.*, 1969). Cyclic-AMP-binding and protein phosphokinase activities in the fractions were determined by the methods of Walton & Garren (1970) and Miyamoto *et al.* (1969). Over 90% of the total of both activities present in the homogenate was recovered in the supernatant fraction. Purification of this fraction by ammonium sulphate precipitation followed by polyacrylamide-gel electrophoresis resulted in a single component possessing both cyclic-AMP-binding and cyclic-AMP-dependent protein phosphokinase activities. The cyclic-AMP-dependent protein phosphokinase was capable of phosphorylating not only exogenous histone but also soluble endogenous islet proteins.

Colchicine is known to bind specifically to microtubular protein, to interfere with microtubular function and in islets of Langerhans to inhibit the secretion of insulin. These observations have led to the suggestion that contraction of a microtubular system may provide the mechanism of translocation of insulin-storage granules through the cell during their release (Lacy *et al.*, 1968). It was of considerable interest therefore to find that the cyclic-AMP-stimulated phosphorylation of endogenous islet proteins was significantly inhibited in the presence of colchicine (0.5mM), although phosphorylation of exogenous histone was unaffected under these conditions. It seems possible that the mode of action