ADVANCES IN

STEROID BIOCHEMISTRY AND PHARMACOLOGY

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CELLULAR BIOCHEMICAL ASSESSMENT OF STEROID ACTIVITY

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1. INTRODUCTION: THE POSSIBLE CELLULAR BIOCHEMICAL EFFECTS OF STEROIDS

Steroids can influence cellular biochemistry in a number of different ways. They may act by influencing gene activity; this aspect has been reviewed by Grant (1969) and will not be discussed further here. They may inhibit particular enzymes by direct action on the enzyme molecule. Or they may alter enzyme activity by interference at the co-enzyme or substrate-level, as appears to be the case in some types of steroid transhydrogenation mechanisms (see Grant, 1969) or by competitive inhibition reactions of the normal type which are not peculiar to steroids. On the other hand, steroids have a particular propensity for altering the chemistry of cells by what amounts predominantly to their physical effects, on cellular and on sub-cellular membranes. These possibilities will be considered

briefly before discussing the special methods of cellular biochemistry which have been developed to allow the fuller assessment of these effects to be made.

A. DIRECT EFFECT ON ENZYMES

1. Non-Competitive Inhibition

It now seems quite clear that steroids can have a direct and inhibitory influence on the activity of certain purified enzymes. Marks and Banks (1960) showed that 4.10^{-5} M pregnenolone, dehydroisoandrosterone (dehydroepiandrosterone), epiandrosterone, androstane-3,17-dione and related steroids caused between 75–85% inhibition (depending on the exact steroid used) of the activity of purified human red blood cell glucose -6-phosphate dehydrogenase, and of 73–90% of the activity of a crude glucose 6-phosphate dehydrogenase preparation from rat adrenal. Inhibition (20–30%) was found when the concentrations of these steroids was diminished to 4.10^{-7} M. Other steroids caused some inhibition of this activity, particularly when used at the higher concentrations. The effect of pregnenolone and of dehydroisoandrosterone (dehydroepiandrosterone) on glucose 6-phosphate dehydrogenase from rat and human tissues varied slightly with the tissue; these steroids had no effect on this dehydrogenase isolated from yeast or from spinach. Equally they did not inhibit 6-phosphogluconate dehydrogenase derived from any of these sources or isocitrate dehydrogenase from rat and human tissues. Thus the inhibition appeared to be remarkably selective, and was not due to competitive inhibition with respect either to the co-enzyme or the substrate. They pointed out that all the inhibitory steroids had a ketone group at C_{17} or C_{20}; a hydroxyl group at this position produced little inhibition. Equally, no inhibitory effect was produced if the steroid contained a ketone group or an α or β hydroxyl group at C_3; saturation or unsaturation at C_{4-5} or C_{5-6} had little effect. The significance of these findings has been extended by the application of newer cellular biochemical investigations, as will be discussed below (Section III A 3).

At first sight it seems difficult to understand how steroids can influence the activity of proteins, such as glucose 6-phosphate dehydrogenase. With this enzyme at least, the inhibitory effect of the steroids does not appear to be through competition at the active site of the enzyme; consequently the suggestion is that the steroids produce some effect on the protein molecules akin to the allosteric effects discussed by Monod *et al.* (1963), and developed further by Monod *et al.* (1965) more specifically for conformational changes in the protein molecules of enzymes. Levy *et al.* (1966) confirmed the results of Marks and Banks (1960), namely that dehydroepiandrosterone inhibits mammalian glucose 6-phosphate dehydrogenase in a non-competitive manner. They suggested that this type of inhibition is due to conformational and polymeric change. It is well known that most enzymes occur as macromolecules which are composed

of sub-units. Klotz *et al.* (1970) listed about 100 enzymes which appear to fit this rule. Glucose 6-phosphate dehydrogenase in particular has been much studied in this respect. Yoshida (1966) found the molecular weight of the crystalline dehydrogenase from human erythrocytes to be 240,000; it was inactivated if the bound NADP was removed from it. He suggested that the active enzyme was composed of 6 sub-units with 6 molecules of NADP bound to the whole assembly. It readily dissociates into two partially active trimeric assemblies. With glucose 6-phosphate dehydrogenase derived from brewers' yeast, Yue *et al.* (1969) found that the apoenzyme (i.e. lacking bound NADP) had a molecular weight of 101,600 and was made up of two polypeptide sub-units each of molecular weight of 51,000. The addition of NADP caused the sub-units to aggregate into the dimeric active macromolecule (also see Frieden, 1971). Levy *et al.* (1966) suggested that, for glucose 6-phosphate dehydrogenase from the mammary gland, the enzyme exists in two monomeric forms, the X and Y form, which can be in rapid and mobile equilibrium one with the other. The X form can be reversibly dissociated into inactive sub-units and is converted into the active Y form by the presence of NADP or NADPH. They postulated that inhibitory steroids, like dehydroepiandrosterone, can be bound only to the active Y monomer, so that the binding of such steroids can occur only in the presence of NADP. The change from the X to the Y form may be solely a change in the conformation of the protein molecule as Levy *et al.* (1966) indicate (following the views of Monod *et al.*, 1965), or it may involve a greater change in the flexible shape of the enzyme, as favoured by Koshland and Neet (1968).

These results, and the concepts of conformational change and of steroids affecting the quaternary structure of enzymes, have been worked out with purified enzymes. They become of even greater moment when the enzymes are attached to solid surfaces, as are most of the intra-cellular enzymes (and as discussed below). However even these effects may be difficult to analyse completely with isolated enzymes. As Monod *et al.* (1965) pointed out: 'one should note, however, that the capacity to mediate physiologically significant interactions might be more frequent and widespread among proteins than has been realized so far. As we have seen, these properties are frequently very labile and may easily be lost during extraction and purification of an enzyme'.

2. Competitive Inhibition

Steroids can also influence enzymic activity in cells either by fulfilling a co-enzyme function, as in certain transhydrogenation mechanisms (i.e. so stimulating enzymic activity) or by competitive inhibition. An example of steroids functioning as if they were co-enzymes is the well-known case of oestradiol, which appears to be an essential factor in the transhydrogenation reaction $NADPH + NAD^+ \rightleftharpoons NADH + NADP^+$ in placental preparations and for soluble fractions of rat liver (Talalay, 1961; also see Tomkins and Maxwell,

1963; Karavolas and Engel, 1966; Grant, 1969). The reaction appears to take the following form:

$$\text{NADPH} + \text{H}^+ + \text{ketosteroid} \rightleftharpoons \text{hydroxysteroid} + \text{NADP}^+$$
$$\text{NAD}^+ + \text{hydroxysteroid} \rightleftharpoons \text{ketosteroid} + \text{NADH} + \text{H}^+$$
$$\text{NADPH} + \text{NAD}^+ \rightleftharpoons \text{NADH} + \text{NADP}^+$$

Oestradiol activates this transhydrogenation at very low (catalytic) concentrations, as if it were acting as a hydrogen carrier or a co-enzyme. It is not inconceivable that other steroids might act competitively in this specific situation.

A different type of competitive inhibition is that described for tryptophan oxygenase in the supernatant fraction of rat liver homogenates (Braidman and Rose, 1970). This enzyme, which has a haem co-factor, was inhibited by deoxycorticosterone and progesterone (10^{-4} M). Other Δ^4-3-oxo steroids, such as cortisol, did not inhibit this enzyme; a double bond in the A ring seems to be essential for inhibitory action. The effect of increasing concentrations of haematin in the assay system, and the Lineweaver-Burk plots indicated that the inhibitory steroids competitively inhibited by involvement with the haem co-factor of the enzyme, as had been postulated by Oelkers and Dulce (1964).

Other aspects of competitive inhibition could involve competition for specific binding sites in the cells, either in the cell membrane and cytoplasm or in chromatin.

B. INDIRECT EFFECTS ON ENZYME ACTIVITIES

1. Physical Effects

Cellular and sub-cellular membranes are constructed of lipids (phospholipids and glycolipids), proteins and steroid. A typical analysis of the lipids of a cell membrane, taken from the erythrocyte, would be: 50-60% phosphatides, 20-30% free cholesterol (Cook, 1968). According to whichever model structure is preferred, for example the Davson and Danielli (1952) bimolecular leaflet or the Lucy (1968) globular micelle structure or any other, steroids are conceded to play a major role at least in the stabilization of the lipid components. Willmer (1961) has reviewed the earlier evidence concerning the significance of steroids in cell surfaces and in lipid monolayers. He drew attention to the fact that the closeness of the packing of the phospholipid components depends on the relative proportion of cholesterol to phospholipid, that is it is strikingly altered by changes in the steroid component. More recent investigations, using artificial thin films or droplets (of the type discussed by Bangham, 1968; Bangham and Haydon, 1968) have largely confirmed the role of steroids in such membranes. Moreover, electron spin resonance studies, with steroid spin labels in biological

membranes (Hubbell and McConnell, 1969) and with spin-labelled lipids (Waggoner *et al.*, 1969) have shown decisively that steroids increase the viscosity of lipid membranes. In terms of cellular biochemistry, the physical changes induced in cellular and sub-cellular membranes by different types of steroids could have the following effects:

(1) Alteration in the permeability of the cell membrane. Such a change can alter the water-balance inside and outside the cell, and the flow of ions and of nutrients; it may affect the pinocytotic activity of the cell surface (Holter, 1965; see Chayen and Bitensky, 1973) and may influence cell-to-cell surface interactions.

(2) Alteration in the permeability of sub-cellular organelles and structures. This is an important effect because it controls the flow of substrates into and out of such sub-cellular structures and, in this way, can play a critical part in the control of cellular metabolism.

(3) By changing the physico-chemical characteristics of the solid structures to which most intra-cellular enzymes are attached, steroids can markedly change the activity of the enzymes. This aspect of the control of intra-cellular activity arises from concepts which have become current in biochemistry only relatively recently. Previously biochemistry dealt predominantly with enzyme activities measured on isolated enzymes acting in solution. But it is now apparent that most of the enzymes inside cells occur in or on solid structures. These include not only those enzymes located on, or in, the plasma membrane but also many of the mitochondrial enzymes, distributed on the membranes of the outer surface or of the christae mitochondriales (see Lehninger, 1965); those inside lysosomes and peroxisomes, in which the nature of the binding of the enzymes to solid surfaces is still not clear; and the enzymes which are bound to the endoplasmic reticulum.

In fact, based on the appearances demonstrated by electron microscopy, Lehninger (1966) has calculated that between 40 and 90% of the mass of cells is composed of membranes, on which—or as part of which—are active enzymes. With the availability of solid matrices, on to which enzymes can be bound (e.g. Hornby *et al.*, 1966; McLaren and Packer, 1970), it has been shown that the physico-chemical properties of matrix-bound enzymes, and their activities generally, may be very different from the physico-chemical properties of the same, purified enzymes when present in solution. From such work in particular has come a great deal of work on the differences between the activity of enzymes in homogeneous phase reactions (i.e. in aqueous solution) as against heterogeneous phase reactions, namely when the enzyme is in a solid matrix, be it an artificial matrix or a natural matrix such as a mitochondrion. These studies, on enzyme reactions in heterogeneous systems, have recently been reviewed by

McLaren and Packer (1970). In particular, changes in enzyme activity can be induced by changing the nature of the charge, and the charge-density, on the matrix around the enzyme molecules (e.g. Hornby *et al.*, 1968; Filipusson and Hornby, 1970); by changes in the lipid component of the matrix or membrane in the vicinity of the enzyme (Mazanowska *et al.*, 1966); and by configurational change in the enzyme at an interface (Quarles and Dawson, 1969). Conformational changes in succinate dehydrogenase, associated with substrate (Kearney, 1957) and hypobaric conditions (Aithal and Ramasarma, 1969) have also been reported. All of these can be influenced by steroids if these substances alter the properties of the natural matrix in which the enzymes are embeded.

C. THE ROLE OF CELLULAR (MULTIPHASE) BIOCHEMISTRY

The direct effect of steroids (Section I A), for example their inhibitory effect on enzymes or their interaction with nucleohistone, can be studied by conventional biochemical procedures. These methods generally involve the disruption of the tissue by homogenization and the separation by differential centrifugation of the sub-cellular components into a foreign medium; they frequently require the purification of the enzyme, or the active compound, from other cellular systems. These procedures are liable to alter, sometimes very considerably, the permeability of the membranes of sub-cellular organelles and thus the rate of entry of substrates and other reactants into the organelle; in this way the apparent rate of activity of intra-organelle enzymes will be enhanced. Disruptive biochemical analysis measures the activity of the isolated enzyme, which may be very different from its activity when attached to its natural matrix or when it is able to interact with other cellular systems. Moreover, it is unlikely to be able to record the effect of the more subtle configurational changes which occur in an enzyme, or other active group, when acting as part of a functional, changing membrane system. These are theoretical possibilities. It is now pertinent to see how these arguments are borne out in practice.

1. Effect of Isolating Mitochondria

Lehninger (1951) showed that mitochondria became permeable to NADH after damage such as is produced during homogenization. Bendall and de Duve (1960) subjected mitochondria to increased time of homogenization and found increased activities of glutamate, malate and β-hydroxybutyrate dehydrogenases due to increased permeability of the mitochondrial membranes to the substrates for these enzymes. With prolonged homogenization, these activities approximated to that which they obtained by treating the mitochondria with a surface active agent. Chayen *et al.* (1966) showed that even relatively gentle physical disturbance, which was insufficient to cause the break-down of tissue, produced

enhanced mitochondrial glutamate dehydrogenase activity, apparently due to increased permeability of the mitochondrial membranes.

One control of mitochondrial respiratory activity is the permeability of the mitochondrial membrane. If the preparatory methods severely disturb the nature of the membrane, so that it is rendered unduly permeable, it may become impossible to observe whether steroids can influence its natural permeability and so influence mitochondrial respiration. Clearly for this type of work it is necessary to study the mitochondria without isolating them, with the concomitant mechanical trauma, into a foreign medium; the ionic balance, tonicity, pH and lack of colloids, will all tend to alter irrevocably the nature of their membranes. The newer techniques of cellular biochemistry, to be discussed below, have shown that certain steroids can stabilize mitochondrial membranes and make them resistant to the damaging influence of inflammatory amines (Chayen *et al.*, 1970b).

2. Effect of Isolating Lysosomes

The whole concept of enzyme latency, and of the controlling function of organelle membranes, has been established largely by work done on lysosomes. These are sub-cellular organelles, usually about 0.5 μ in diameter, which contain many, if not most, of the hydrolase enzymes of the cell in a fully active state. They cannot express their activity inside the cell because they are sequestered behind a semi-permeable organelle membrane. The subject of lysosomes in biology generally, and in pathology, has been fully reviewed in the three-volume work, edited by Dingle and Fell (1969) and Dingle (1973). Conventional biochemistry would be sufficient to establish the amount and the activity of such lysosomal hydrolases in a sample of tissue. But it is becoming increasingly clear that the function of these organelles, particularly in conditions involving cell injury (Bitensky, 1963a) and inflammation (Weissmann, 1966, 1968, 1969; Chayen and Bitensky, 1971), is related to the condition of the lysosomal membrane. Thus apart from their normal involvement in endocytosis, with the formation of secondary lysosomes and phagosomes, they may also 'leak' inflammatory substances and even degradative enzymes which can act on the extra-cellular matrix. This subject has been extensively investigated by Dingle (e.g. 1968, 1969) who suggested that the 'leakage' of such lysosomal material may be by fusion of the abnormal lysosomal membranes with the cell surface and the ejection of 'packages' of lysosomal contents into the extra-cellular environment. However this may be, the primary consideration is not how much lysosomal activity is present in the cells (as can be determined readily by conventional biochemistry) but how stable is the lysosomal membrane which normally sequesters this activity from the rest of the cell and from the extra-cellular matrix. This question of the state of the lysosomal membrane has also become of some interest in view of the suggestions by Weissmann (1966) and