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NON-GENOMIC EFFECTS OF STEROIDS

INTERACTIONS OF STEROID MOLECULES WITH MEMBRANE STRUCTURES AND FUNCTIONS

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Contents

I. Introduction

Since the development of tracers of high specific activity permitting the demonstration of steroid binding sites in different target tissues, most research in this field has been devoted to furthering the understanding of the nature and role of these receptors. The resulting hypothesis of a universal mechanism of steroid-hormone action has obscured previously proposed theories of steroid action, and in particular the model of membrane-steroid interaction. However, as previously stated by Thompson and Lippman [1], the now classical receptor-mediated pathway of steroid hormone action is not sufficient to account for all the known effects of steroids. Baulieu and co-workers [2] have, for example, recently demonstrated that progesterone and other steroid molecules are able to promote the maturation of *Xenopus laevis* oocytes, although these cells do not contain steroid receptors. They therefore suggested that progesterone may trigger its action by interacting with the cell membrane. It thus appears that, in addition to the receptor-mediated mechanism, steroids may also operate through other mechanisms and, in particular, through an effect on plasma membrane.

In the present paper, we wish to reexamine some of the previously published results in view of this possible duality.

Let us first recall the major properties of steroid-receptor interaction. Classically, the mechanism of action of steroid hormones includes the following steps [3–5]: it is generally accepted that the steroid enters the target cell by simple diffusion and combines with a high affinity cytoplasmic receptor. The steroid-protein complex then undergoes activation and is transferred to the nucleus where it binds to selective sites in the chromatin. The interaction between the hormone-receptor complex and the genome leads to a modulation of RNA and protein synthesis. This synthesis of specifically induced proteins is finally responsible for the physiological response to the hormone (Fig. 1).

One of the main arguments supporting the assumption that steroids act via macromolecular

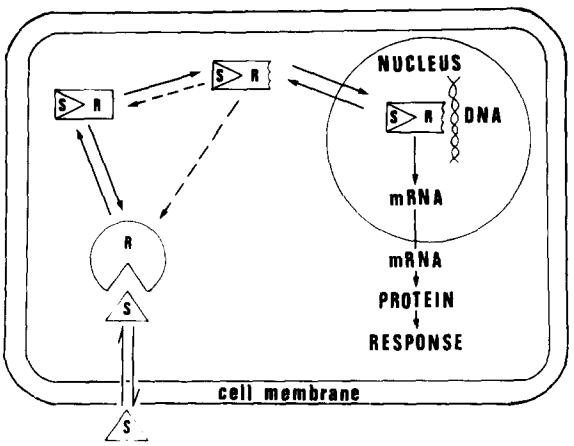

Fig. 1. Classical, receptor-mediated, mechanism of steroid-hormone action. From Baxter and Harris [5].

synthesis is the fact that inhibitors of RNA and protein synthesis will also block steroid-induced responses. The demonstration of a lag period ranging from at least 20–30 min to several hours and even days between the entry of the molecule into the cell and the manifestation of the hormonal response [6] together with the fact that the induced macromolecular synthesis may continue for several hours or days after steroid removal [7] are also in favour of this scheme. One outstanding property of all steroid receptors is their ability to recognize subtle differences in steroid structure. In a given tissue, receptors exhibit a high affinity for molecules that are biologically active in that tissue, a lower affinity for related steroids with low biological activity and no binding of inactive steroids.

On the basis of their biological activities and structures six major classes of hormonal steroids which bind to different receptors have been characterized: glucocorticoids, mineralocorticoids, estrogens, androgens, progestins and vitamin D compounds. In general, despite possible overlap between receptor binding profiles, each member of a given category binds essentially to its specific receptor. Another feature of the steroid receptors is their limited capacity. Usually, the saturation of the binding sites is achieved at concentrations close to the circulating hormonal levels so that the dose-response curves of a receptor-mediated action of steroids reach a plateau at relatively low hormone concentrations.

In view of the above, it is now possible to define the characteristics of any steroid action which likely represents a non genomic effect: instantaneous onset or a very short latency as well as rapid recovery following steroid removal; insensitivity to inhibitors of RNA and protein synthesis.

The demonstration that steroids belonging to different hormonal categories may exert similar effects has sometimes been used as an argument in favour of a direct steroid action, not mediated through receptor occupancy [2]. This however, does not represent an obligatory characteristic of an extragenomic action since, several of the direct membrane effects described below are restricted to a single class of steroid molecules.

Using these criteria, we would now like to review some of the previously reported actions of steroids on membrane properties in order to determine whether or not these are receptor-mediated. Rather than an extensive review of all the extragenomic effect of steroids, including those exerted at the post transcriptional level, we have concentrated on some areas which illustrate the possible mechanisms of such direct effects at the membrane level. As will be discussed later, this distinction between receptor-mediated and extragenomic actions of steroids is perhaps not so clear, since a given molecule may exert both types of actions either sequentially in the same target cell or in parallel in different organs.

II. Receptor-mediated effects of steroids on membrane composition and properties

The plasma membrane plays a key role in the maintenance of cell functions and survival. It first represents a highly selective permeability barrier between the cytoplasm and the extracellular space. Therefore, many transport systems will ensure the transfer of selected molecules across the lipid layers. They afford nutriment and maintain the constancy of pH and ionic concentrations within the cell, one consequence of this ion transport being the persistance of membrane potential. In addition, the plasma membrane conveys signals from the cell's environment, which are recognized usually by specific receptors located at the outer surface of the cell and which, by various mecha-

nisms, trigger modulations in cell metabolism. Finally, the membrane participates in the contact and cooperation between cells.

Given the wide spectrum of membrane functions and the multiplicity of the recognized effects of steroid on cell biology, it is not surprising that these molecules are able to modify several of the membrane functions. It appears however, that most of the steroid effects on membrane properties do not represent direct effects of the drugs, but merely receptor-mediated actions. We will now briefly review these receptor-mediated effects which are listed in Table I.

IIA. Membrane composition

The effects of steroids on membrane composition are well documented in the case of glucocorticoids and aldosterone. They include changes in all the classes of molecules involved in the composition of the plasma membrane (cholesterol, phospholipids, polysaccharides and proteins) and have been demonstrated in numerous cell types such as HeLa cells, liver cells, hepatoma tissue, fibroblasts, lymphoid cells, bladder and lung. Similar effects of estrogens and androgens in accessory sex organs have been reviewed by Spaziani [8], whereas Nemere and Norman [9] have recently reviewed the action of vitamin D on intestinal cell membranes.

TABLE I

RECEPTOR-MEDIATED EFFECTS OF STEROIDS ON MEMBRANE COMPOSITION AND FUNCTIONS

Membrane composition	Cholesterol synthesis, lipid synthesis, polysaccharides, proteins and receptors
Membrane functions	Permeability to water, monovalent and divalent cations, glucose and macromolecular precursors, proteins 'Permissive effects' on the action of agents acting through cAMP Effect on membrane enzymes
Shape and organization of membranes	Plasma membranes Lysosomal membranes Mitochondrial membranes

IIA-1. Inhibition of cholesterol synthesis

Glucocorticoids have been shown to induce a marked decrease in cholesterol synthesis in various target tissues [10–13]. This action, which may well be involved in the control of cell proliferation by steroids, appears to be typical of a receptor-mediated effect [14]. Indeed, Ramachandran et al. [15] showed that this action of dexamethasone was primarily linked to an inhibition of hydroxymethylglutaryl coenzyme A synthase synthesis.

IIA-2. Modulation of lipid synthesis

The effects of steroids on lipid synthesis and turnover appear highly variable according to the target cell studied. Glucocorticoids have been shown to inhibit fatty acid synthesis in HeLa cells and lymphocytes [16,17], whereas in lung they are known to enhance the synthesis of lecithins involved in the constitution of the pulmonary surfactant [18–20]. Aldosterone has been demonstrated to stimulate both phospholipid synthesis and fatty acid turnover in toad bladder cells [21,22].

These effects of steroids on cholesterol and phospholipid biosynthesis could indirectly affect many of the membrane properties. Indeed, the respective proportion of cholesterol and phospholipids is a major factor controlling membrane fluidity [23]. In addition, many of the membrane-associated enzymes appear highly dependent upon their lipid environment [24].

IIA-3. Effect on membrane polysaccharides

There is some evidence that steroids, particularly glucocorticoids, can also alter membrane sugar synthesis. Carubelli and co-workers [25] described an increase in sialic acid production after treatment with hydrocortisone, whereas Wong [26] showed a similar effect of cortisol on hyaluronic acid synthesis. In cultures of fetal lung cells, Heifetz and Snyder [27] described a marked stimulation of sulfated glycoconjugates synthesis after 6 days of hydrocortisone treatment. Ramachandran et al. [28] described a stimulation of fucose and mannose incorporation into membrane glycoproteins following treatment of HeLa cells with dexamethasone.

IIA-4. Action on membrane proteins

IIA-4a. Protein synthesis. Little is known about

the regulation of membrane protein biosynthesis by steroids. Using two-dimensional gel electrophoresis O'Farrell and Ivarie [29] showed that dexamethasone is able to stimulate amino acid incorporation into two different plasma membrane proteins of the HTC cell. Geheb and co-workers [30] showed that aldosterone enhances the synthesis of four to six membrane proteins in toad bladder cells, this induction being inhibited in the presence of actinomycin D.

IIA-4b Modulation of membrane hormonal receptors. Steroid hormones, which are known to control the level of their own receptors, are also able to modify the synthesis and/or turnover of membrane receptors for non-steroid hormones. These effects have been well documented for insulin and catecholamine receptors, but also for other peptide hormones [31]. The number of insulin receptors appears to be modulated by glucocorticoids, but also probably by the sex steroids. It has been shown, for example, that cortisol treatment in vivo induced a marked rise in monocyte insulin receptors, whereas these binding sites were decreased in rat liver cells by dexamethasone treatment [32,33]. In addition, Beck-Nielsen et al. [34] showed that the number of monocyte insulin receptors decreased in pregnant women and suggested that this diminution could be related to sex steroid action.

α- as well as β-adrenergic receptors are modulated by steroids in a complex manner. Roberts et al. [35] showed that estradiol increases the number of α-receptors in myometrium, whereas Williams and Lefkowitz [36] described a decrease in uterine cell α-receptors following progesterone treatment. Adrenalectomy also produces marked changes in catecholamine receptors. Guellaen et al. [37] described an increase in both α- and β-receptors in rat liver membranes, whereas there was a decrease in β-receptors in lung preparations following adrenalectomy [38].

Manipulations of steroid levels have also been shown to induce a modulation of numerous peptide hormone receptors including prolactin, thyrotropin releasing hormone, gonadotrophins and others (see the review by Tell and co-workers, [31]).

IIA-4c. Effect on immunological receptors. In addition to their effects on hormonal receptors, steroids and, particularly glucocorticoids, have been shown to modify the expression of immunological markers in various cell types. Dexamethasone was shown to induce the appearance of receptors for immunoglobulins and complement in some clones of myeloid cells in culture, whereas it decreased the number of Fc receptors in monocytes [39,40].

In HTC cells, glucocorticoids treatment led to an increased expression of surface antigens [41], whereas Pietras and Szego [42] presented evidence for an elevation of lectin binding sites following incubation of isolated endometrial cells in the presence of estradiol.

IIB. Membrane functions

IIB-1. Steroids and membrane permeability

IIB-1a. Water permeability. A single physiological dose of estrogen, administered to the immature or adult ovariectomized rat, promotes dramatic changes in the composition and metabolism of the uterus including water and sodium uptake, hexose uptake, increased precursor incorporation into RNA and stimulation of RNA polymerase activity and protein synthesis [8,43,45,46].

The mechanism of the early, estrogen-induced, increase in water permeability remains a matter of debate, since arguments exist both for a direct effect of estrogen and for a classical receptor-mediated action. The hypothesis of a non-genomic effect is based on the following observations: water uptake occurs before any detectable increase in protein and RNA synthesis [8], is not blocked by inhibitors of RNA synthesis [47], but can be counteracted by simultaneous injection of compounds with low affinity for uterine estrogen receptors such as cortisol [43], deoxycorticosterone and testosterone [44] and dexamethasone [46]. Tchernitchin [45] postulated that this non-genomic action of 17β-estradiol is dependent upon the very rapid accumulation of eosinophils in uterus, observed within minutes following steroid injection. On the other hand, Markaverich et al. [50] described that dexamethasone antagonism of uterine growth was mediated through occupancy of a new type of nuclear estrogen receptors (Type II) which appears to be involved in the uterotrophic action of estrogens [49].

It also appears that some of the in vivo effects of estrogens on uterine cells are indirect conse-

quences of an action of estradiol on the release of vasoactive mediators such as histamine, catecholamines and prostaglandins [8,45,47,48].

In non-mammalian species and particularly in fish, cortisol plays a role in osmoregulation. During sea water adaptation of the eel, there is a marked stimulation of intestinal water transport which is abolished following hypophysectomy. Ando [51] reported that cortisol injection induced in the fresh-water eel a significant increase in water flux and osmotic permeability across the intestinal epithelial cells. A similar effect was obtained in vitro after 7 h exposure of isolated intestinal fragments to 10^{-6} M cortisol.

Cortisol also exerts in mammals an effect on water permeability as reviewed by Forman and Mulrow [52].

IIB-1b. Ion transport. (i) *Aldosterone and sodium transport*: aldosterone has long been recognized as one of the factors controlling sodium transport in vertebrates. Its mode of action in rat kidney and toad bladder has been extensively reviewed in the past [53,54] and clearly involves a specific interaction of the hormone with cytoplasmic receptors and the induction of protein synthesis. These aldosterone induced proteins have been suggested to play three distinct but not necessarily independent roles:

Facilitation of passive Na^+ entry across the apical plasma membrane. Using amiloride, a selective blocker of apical Na^+ conductance, to identify Na^+ channels, it was shown that aldosterone elicited within 6 h a 2.4-fold increase in sodium conductance and a corresponding increase in the number of apical channels [55].

Increased $(Na^+ + K^+)$-pump activity as a result of activation of preexisting pump or of an increase in the number of pumps. Recently, Horster and co-workers [56] showed that aldosterone was able to restore $(Na^+ + K^+)$-ATPase activity in isolated nephrons dissected from adrenalectomized rabbits. Given the time course of this effect, which became apparent after 60 min of treatment, they suggested that the action of aldosterone was associated with changes in the enzyme lipid environment, in good agreement with the results of Goodman and co-workers [21].

Augmentation of the energy supply, presumably in relation with enhanced mitochondrial oxidative phosphorylation, since aldosterone was shown to stimulate the activity of the enzyme citrate synthetase [57].

(ii) *Effect of other steroids on monovalent cations transport*: glucocorticoids: recent experiments have suggested that independently of their possible interaction with mineralocorticoid receptors, glucocorticoids may control the movements of monovalent cations. Johnson and co-workers [58] have shown that administration of dexamethasone in adrenalectomized rats induced a significant increase in urinary potassium excretion without altering sodium excretion. They also showed that comparable amounts of aldosterone had no influence on renal potassium excretion. Freiberg et al. [59] demonstrated that dexamethasone, but not aldosterone, was able to increase the Na^+/H^+ exchange and to decrease the Na-dependent phosphate uptake in renal brush border membrane vesicles.

Estradiol: in addition to its effect on uterine water permeability, estradiol was shown to increase sodium uptake [43]. Johnson et al. [60] described a marked sodium retaining effect of estrogen in dogs, visible within 2–4 h of treatment.

Progesterone: whereas synthetic progestogens have been shown to induce sodium retention, progesterone exerts a significant natriuretic action in man, probably via competition at the level of the aldosterone receptors [61].

It has also been suggested that ecdysone, the steroid hormone in insects, may in part control gene expression by modulating potassium permeability of nuclear membranes [62].

(iii) *Steroids and divalent cations*: glucocorticoids have been known for a long time to interfere with calcium metabolism. Cortisol has been shown to decrease calcium uptake by intestinal cells within 1 h of treatment [63], whereas the bone resorption associated with long-term treatment by glucocorticoids is well known. This effect of glucocorticoids appears to be essentially associated with an alteration of mitochondrial properties (see below).

1,25 Dihydroxycholecalciferol (vitamin D-3), which controls in part the overall metabolism of calcium in vertebrates, is now considered as a member of a new class of steroid-like compounds acting through an interaction with cytosolic receptors [9,64].

Although this has been poorly investigated, it is likely that the transport of other cations might be modulated by steroid hormones: zinc uptake and retention appear for example to be in part controlled by steroids. Cox [65] demonstrated in HeLa cells a stimulatory effect of glucocorticoids on ^{65}Zn retention, which was dependent upon RNA and protein synthesis.

IIB-1c. Effect of glucocorticoids on glucose transport. The ability of corticosteroids to modulate glucose uptake, which provides the basis for the term 'glucocorticoids', has been extensively documented in various tissues such as adipocytes, fibroblasts, macrophages and lymphoid cells [66]. This action is one of the earliest measurable effects of the glucocorticoids and is observed as early as 15–20 min following steroid addition in rodent thymus cells [67]. It was however clearly demonstrated by Munck and co-workers [68] that this effect is mediated through receptor occupancy and requires protein synthesis.

IIB-1d. Amino acids and nucleosides uptake. Glucocorticoids have been demonstrated to exert on amino acids and nucleosides uptake an effect comparable to that observed for glucose.

The uptake of the non-metabolisable α-aminoisobutyric acid is stimulated by glucocorticoids in liver cells, whereas it is inhibited in muscle cells, fibroblasts and lymphoid cells [66].

Similarly, the inhibitory action of glucocorticoids on uridine uptake and incorporation into macromolecules has long been used to assess the potency of a given glucocorticoid molecule on lymphocytes [69]. In mouse thymocytes, which are particularly sensitive to glucocorticoid action, this inhibition is visible after 60–90 min incubation and unquestionably corresponds to a classical steroid effect.

Estrogens have also been shown to stimulate by a receptor-dependent process the uptake of nucleic acid precursors in uterine cells within several hours of in vivo hormone injection, as well as during in vitro incubation [70].

IIB-1e. Effect on protein transport. In addition to low molecular weight compounds, steroids may also modulate the transport of proteins across cell membranes. Finlay and co-workers [71] showed that estradiol markedly enhanced the uptake of α_1-protease inhibitor and other plasma proteins into mouse uterine cells.

IIB-2. Long term 'permissive effects' of steroids

It is now accepted that cyclic nucleotides are not intracellular intermediates necessary for steroid effects [72–75]. It appears, however, that under certain conditions steroids are required for the full expression of other hormonal activities, particularly those mediated through a stimulation of cyclic AMP (cAMP). Most of these so-called permissive effects of steroids correspond to classical receptor-mediated actions of steroid hormones. Indeed, they always require a lag period before emergence of an effect and are blocked by inhibitors of RNA and protein synthesis. Exton et al. [76] showed, for example, that the stimulation of glucose production by glucagon and epinephrine was impaired in liver from adrenalectomized rats and that normal stimulation could be restored within 60 min following glucocorticoid infusion.

Several explanations have been proposed to account for these permissive effects of steroids, but the situation remains unclear. The regulation of β-adrenergic receptors in response to modulation of the circulating levels of steroid hormones may in part play a role in this phenomenon [37,38]. It was also suggested that corticosteroids could modulate intracellular cAMP action, by decreasing its hydrolysis by the phosphodiesterase enzymes. Indeed, adrenalectomy was shown to increase phosphodiesterase activity in various target tissues [77], whereas Ross et al. [78] reported a decreased enzyme activity in HTC cells grown for 24 h in the presence of dexamethasone.

On the other hand, the experimental results of Exton et al. [76] showed that the level of cAMP elicited by glucagon stimulation in the liver of adrenalectomized rats was similar and even higher than that in intact animals. It was thus pointed out that steroids are not required for normal cAMP accumulation, but may be necessary for maintenance of normal cell responsiveness to the hormonal stimuli.

Liu and Greengard [79] showed that cortisol treatment specifically decreased ^{32}P-incorporation in a rat liver cytosolic protein, whose phosphorylation is also regulated by a cAMP-dependent protein kinase. They therefore suggested that this protein may represent a common intermediate in steroid and cyclic nucleotide action.

Finally, Rasmussen and Tenenhouse [80] sug-

gested that steroids could modulate cyclic nucleotide action by controlling intracellular calcium concentration (Fig. 2).

IIB-3. Effects of steroids on membrane enzymes

Many enzyme activities are affected by steroid treatment. Na-K-dependent ATPase activity, which represents the major system of transport for sodium and potassium ions, is decreased by estrogens in rat uterus and liver, but increased by aldosterone in toad bladder and kidney cells [81–83]. Calcium-dependent ATPase activity was shown to be reduced in rat liver membranes following castration [84].

The regulation of alkaline phosphatase by steroids was first studied by Costlow and Melnykovych [85]. These authors showed an increased activity of this enzyme in HeLa cells after prednisolone treatment. More recently, Bachelet et al. [86] demonstrated in rat intestine an early stimulation of alkaline phosphatase activity 30 min after treatment by $1,25\text{-}(OH)_2D_3$.

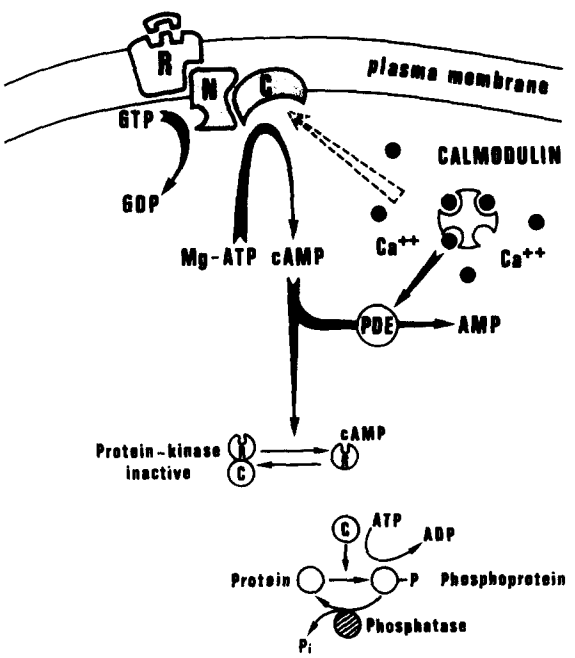

Fig. 2. Receptor-mediated activation of adenylate cyclase and messenger function of cyclic AMP. R, membrane receptor; N, regulatory protein; C, adenylate cyclase; PDE, phosphodiesterases; R, regulatory subunit; C, catalytic subunit of the cAMP-dependent protein kinase.

In HTC cells, several membrane enzymes have been reported to be controlled by glucocorticoids. Rousseau et al. [87,88] showed that 48 h treatment with 10^{-7} M dexamethasone simultaneously induces an increase in alkaline phosphodiesterase type I activity and a decrease in nucleoside ADP diphosphatase. There are also some indications that steroids may alter adenylate cyclase activity. Pejoan et al. [89] described an increase in enzyme activity following adrenalectomy as well as a decrease in basal activity following hydrocortisone injection in adrenalectomized animals.

Recent experiments have suggested that glucocorticoids and probably other steroids [90,91] may control in part the synthesis of prostaglandins and other derivatives of arachidonic acid via an action at the level of phospholipases. The non-steroidal antiinflammatory drugs, such as aspirin or indomethacin, act mainly by blocking directly the enzyme cyclooxygenase. This enzyme catalyzes the transformation of the fatty acid precursor arachidonate into endoperoxydes, which in turn give rise to prostaglandins under the action of various prostaglandin synthetases. In contrast, the glucocorticoids are not active in cell free preparations and do not inhibit cyclooxygenase activity [92]. The demonstration that glucocorticoid-induced inhibition of prostaglandin synthesis could be overcome by the addition of exogenous arachidonate led to the suggestion that steroids operate mainly by reducing the amount of precursor available for prostaglandin production. Given the fact that in most of the cell types investigated, there are only negligible amounts of unesterified fatty acids and that arachidonate is generally esterified in the 2 position of membrane phospholipids [93], attention has been drawn to the mechanism of deacylation as a possible target for steroid action. Gryglewski et al. [94] first suggested that glucocorticoids could inhibit phospholipase A_2 activity. Then, Hong et al. [95] demonstrated that steroids inhibited the release of arachidonic acid from membrane phospholipid stores, whereas Blackwell et al. [96] reported a decreased hydrolysis of labeled phospholipids by perfused guinea-pig lungs following steroid infusion.

For several years, it was believed that this steroid effect was a consequence of the stabilization of

membranes by steroids [97]. However, the concentrations of steroids necessary to produce membrane stabilization are, in general, several orders of magnitude higher than those required to block prostaglandin synthesis, and several authors demonstrated that steroid action is only detectable after a lag period of 30–60 min and is blocked by inhibitors of macromolecule synthesis [98,99]. Finally, it was shown in rat renomedullary cells in tissue culture that glucocorticoids bind to specific receptors with a good correlation between the affinity for these receptors and the inhibitory action on prostaglandin synthesis [100]. These results clearly demonstrate that the action of antiinflammatory glucocorticoids on prostaglandin production is a receptor-mediated event. Recently, Hirata and co-workers [101] as well as Backwell et al. [102] isolated steroid-induced proteins (lipomodulin, macrocortin) able to inhibit arachidonic acid release by blocking phospholipase activity (Fig. 3).

Similarly, the effects of sex steroids on prostaglandin secretion, demonstrated in various target tissues, probably represent long-term hormonal actions of steroids [103–105].

IIC. Effects of steroids on shape and organization of membranes

IIC-1. Plasma membranes

Steroids are not only able to alter the biochemical composition of the membrane, they also induce marked changes in its structure and organization.

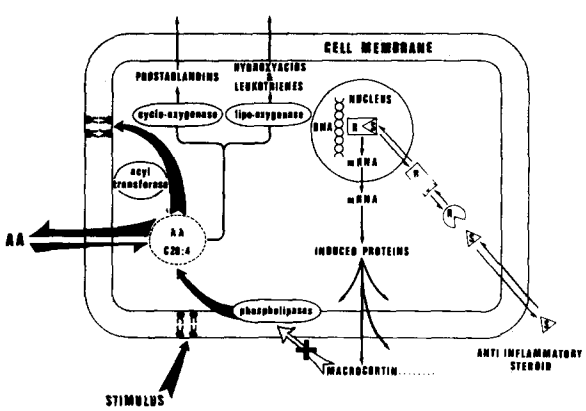

Fig. 3. Mechanism of action of anti-inflammatory steroids on the metabolism of arachidonic acid (AA).

Dexamethasone has been shown to induce changes in cell adhesiveness, modifications of membrane microvilli, rounding and altered sensitivity to detergent-induced lysis in HeLa cells as well as in HTC cells [41,88]. These changes were associated with alterations of the surface charge and electrophoretic mobility of the cell [106,107].

The increased adhesiveness of HTC cells to glass walls was seen within 6–8 h of treatment by dexamethasone and was attributed to a steroid action on the cytoskeleton or to a decreased production of the serine protease plasminogen activator [108]. More likely, it results from a steroid-induced modulation of the synthesis of membrane glycoproteins.

Dexamethasone was also shown to induce the differentiation of myeloid leukemia cells in culture. This process, which was only observed after long-term treatment (up to 7 days), was associated with changes in the membrane phenotypes and the appearance of membrane markers [39].

Similarly, Jung Testas and Baulieu [109] showed that estrogens and androgens (40 nM) enhanced adhesiveness of mouse L_{929} fibroblasts grown in petri dishes, whereas Yates and co-workers [110] described an effect of testosterone on the morphology and cytoskeleton arrangement of the S_{115} mouse mammary tumor cell line. These effects of steroids on the structure of plasma membrane always required RNA and protein synthesis. These types of membrane alteration may well explain the changes in the recirculation of leucocytes and polymorphonuclear cells observed in man as well as in rodents after in vivo glucocorticoid treatment [111,112].

IIC-2. Lysosomal membranes

Lysosomes are involved in immune and inflammatory processes as well as in a variety of diseases and tissue injuries. Since it has been recognized for a long time that corticosteroids are powerful anti-inflammatory agents, many investigations have been carried out to study the effects of these drugs on lysosomes.

Although it is widely accepted that steroids are able to decrease the release of lysosomal enzymes, the mechanism of this inhibition still remains undefined. It was postulated first that steroids act

by stabilizing the lysosomal membrane, but this assumption is now questioned [113,114]. Indeed, many of the experiments showing lysosomal membrane stabilization were performed in vitro at suprapharmacological concentrations (i.e. 10^{-4}, 10^{-3} M) which are never achieved in vivo. Moreover, the appropriate controls, i.e. measuring the effect on lysosomal enzyme release of the solvents used to dissolve steroids (ethanol, DMSO, poly(ethylene glycol)...), were often omitted.

There are some indications suggesting that steroids may exert long-term actions on lysosomal properties probably via a receptor-mediated mechanism. Cortisone administration was shown for example, to decrease acid hydrolase concentration in rat brain [115], whereas Wiener et al. [116] demonstrated an increase in the number of lysosomes per surface unit following cortisone injection. Bird et al. [117] showed that liver lysosomes isolated from adrenalectomized rats are more fragile in vitro than those isolated from intact animals. Conversely, the release of lysosomal enzymes (cathepsin, β-glucuronidase, acid phosphatase...) provoked by retinoids, traumatic or endotoxin shock can be diminished by injection of anti-inflammatory steroids [118]. Using isolated polymorphonuclear cells, Smith [119] and Ignarro et al. [120] demonstrated that glucocorticoids were able to inhibit both phagocytosis and lysosomal enzyme release elicited by latex or zymosan particles within 20–40 min of incubation. This action, which was still measureable at 10^{-7} M methylprednisolone, could not be obtained in the presence of aldosterone or deoxycorticosterone.

IIC-3. Mitochondrial membranes

More than 20 years ago, it was suggested that uncoupling of oxidative phosphorylation might represent one mode of action of steroid hormones [121].

Indeed, numerous effects of steroids on both mitochondrial structure and functions have already been reported. It is necessary to distinguish between the effects observed after in vivo steroid treatment or after in vitro incubation of intact cells with physiological concentrations of steroids, which are likely to correspond to receptor-mediated actions, and those observed after in vitro incubation of isolated mitochondria which represent direct steroid action and will be discussed elsewhere.

Several authors showed first that in vivo treatment with glucocorticoids induced a profound alteration of the mitochondrial structure. There was in general an increase in the size of individual mitochondria associated with a diminution of their number. This phenomenon was considered to represent a fusion between these organelles [122–124]. In some cells however such as thymus cells, or in some parts of the cells, the steroid treatment led to the degeneration of the mitochondria [125].

It was also demonstrated that glucocorticoid treatment was associated with changes in oxygen consumption [126,127] and usually, with an uncoupling of oxidative phosphorylations [128].

In addition, glucocorticoids were shown to modify the mitochondrial calcium transport. Kimberg and Goldstein [129] as well as Kimura and Rasmussen [130] described a decreased ability of rat liver mitochondria isolated from steroid-treated animals to accumulate calcium ions and suggested that this effect was due to an altered ability of the mitochondria to regulate intramitochondrial ATP content. On the other hand, Eilam et al. [131] reported a marked increase in the amount of exchangeable calcium within the mitochondria following a 3 h treatment of cultured bone cells with triamcinolone acetonide. Most of these effects were observed after several days of treatment by glucocorticoids, but morphological alterations of mitochondria could be observed within 1–2 h of steroid injection [132].

In addition, Roosevelt and co-workers [133] showed that the effect of in vivo injection of cortisol on the properties of rat brain mitochondria can be abolished by the simultaneous injection of either actinomycin D or cycloheximide.

IID. Conclusion

In order to survive, every living organism must accomodate to changes constantly occurring in the environment. Hormones that are released in response to these environmental changes are the chemical messengers responsible for cell adaptation.

In this section, we have summarized the wide variety of effects that steroids exert on membrane

properties and showed that, in many cases, they represent 'classical' steroid actions corresponding to a progressive adaptation of the target cells to changes in the external environment.

Adaptation of the organisms could also be achieved through the proliferation and/or differentiation of new cell types. This process, which is in part under the control of hormonal factors, could promote the emergence of cells with different membrane phenotypes.

III. Direct effects of steroids

IIIA. Anaesthetic steroids

The anaesthetic properties of steroid molecules have been recognized for a long time, ever since Selye in 1941 [134] described the effect of steroid administration on rat behaviour. In contrast with the steroid effects described above, which are usually restricted in a given target cell to a single class of steroid molecules, no such specificity could be found for anaesthetic steroids. Atkinson and coworkers [135] have tested a broad range of steroids and showed that 67 out of 168 possess hypnotic properties. These compounds either belonged to the class of steroids with hormonal activity or were devoid of any hormonal properties (Table II). Holzbauer [136] attempted to establish the structure-activity relationship of steroids with anaesthetic activity, but failed to clearly define the structural requirement for this action. Nevertheless, it appears that oxygen in position 3, 17 and 20 is important for anaesthetic potency, whereas the presence of a hydrogen atom in position 5 is often associated with enhanced activity.

TABLE II

MAIN ANAESTHETIC STEROIDS

According to Selye [134], Atkinson et al. [135] and Holzbauer [136].

Deoxycorticosterone
Deoxycorticosterone acetate
Progesterone
Pregnandione
Pregnanolone
20-OH-pregnane-3,20-dione (viadril)
3α-OH-5α-pregnane-11,20-dione (alfaxolone)
Androsterone
Testosterone
Methyltestosterone
Etiocholanolone
Diethylstilbestrol

The concentrations necessary for the expression of the anaesthetic effects are usually within the pharmacological range. The dose-response curves greatly vary according to the compounds tested, but the values given by Atkinson et al. [135] were within the range 28–420 mg/kg, which corresponds to the concentrations given by Selye (5–50 mg/100 mg body weight) in rats [134]. Similarly, Richard and Hesketh [137] showed that alfaxolone, the active principle of althesin, was able to depress electrical activity of cat olfactory neurones at a concentration of $5 \cdot 10^{-5}$ M.

Another property common to these anaesthetic molecules is the rapidity of their action. The lag period between steroid administration and the onset of hypnotic activity never exceeds 15 min and can even be shorter than 1–3 min [134,135].

Since it is generally accepted that anaesthetic compounds act at the level of the cell membrane [138] and given the characteristics of their action, one may consider that anaesthetic steroids exert direct effects on membranes.

IIIB. Effects of steroids on cell excitability

Various steroids particularly estrogens, have been shown to affect the electrophysiological activity as well as the excitation-contraction coupling in several types of cell.

IIIB-1. Effects of steroids on membrane potential

The effects of steroids on the central nervous system have been widely documented and, in most cases, appear to be mediated by interaction of the hormones with specific receptors located in well-defined brain regions [139–141].

However, changes in the electrical activity of neurones could be observed within minutes or even seconds after changes in circulating steroids and may well indicate a direct action of these drugs on brain cell membranes.

As stated above, Richard and Hesketh [137] showed that alfaxolone was able to decrease the

potential response elicited by electrical stimulation in cat olfactory cortex. McEwen reported, after local application of glucocorticoids by iontophoresis or after intraperitoneal injection, a marked decrease in the firing rate of single or multiple units in the hypothalamus and hippocampus. This effect was observed as early as 30 s following intraperitoneal injection [139].

Using implanted microelectrodes Dufy et al. [142,143] have studied the changes in electrical activity of hypothalamic neurons after intravenous injection of estradiol benzoate. They showed an almost instantaneous diminution of the firing rate in the great majority of the cells tested. In addition, this effect was not blocked by inhibitors of macromolecule synthesis injected at the same time or before the steroid. Similar effects were also observed by Kelly et al. [144,145] after microelectrophoretical application of 17β-estradiol on preoptic septal neurons, and by Pfaff et al. [146] after intraperitoneal injection of corticosterone in rats. Teyler et al. [148] demonstrated a stimulation of electrical activity in transverse hippocampal slices from male rats incubated in the presence of 10^{-10} M 17β-estradiol.

The secretion of ACTH in the rat is under the control of two distinct phases of corticosteroid negative feedback inhibition. Jones and Hillhouse [149] showed that the release of corticotrophin-releasing factor from rat hypothalamus incubated in vitro with acetylcholine was inhibited by corticosterone within less than 5 min, thus suggesting that the fast feedback action of corticosteroids could be mediated through an extragenomic mechanism of physiological importance.

There are also some indications suggesting that steroids may alter the membrane potentials in non-neuronal cell types. These rapid effects of steroids on membrane potential were confirmed by experiments on cell cultures. Dufy and co-workers have studied a tumoral cell line $GH_3/B6$ which releases prolactin in response to estrogen stimulation [147]. In these cells, the addition of 17β-estradiol in the vicinity of the cell membrane increases the percentage of cells showing spontaneous electrical activity and induces action potentials in about one-third of the cells. This effect was obtained at a final concentration of steroid in the range of 10^{-10}–10^{-8} M and was observed within 1–2 min following steroid addition. Moreover, this effect was blocked by D_{600} a selective inhibitor of calcium transport but also by the inactive compound 17α-estradiol [147]. Harder and Coulson [150] have studied the resting potential of dog coronary artery cells and showed that this potential raises progressively from −50 to −75 mV in the presence of increasing concentrations of the estrogenic compound diethylstilbestrol. This action measured after 20 min incubation was still detectable at 10^{-7} M and was attributed to a progressive increase in potassium conductance leading to membrane hyperpolarisation. Using a fluorescent probe to monitor the membrane potential of rat thymocytes, Morgan et al. [151] showed that estradiol (10 μM) was able within 30 s to inhibit the changes of membrane potential induced by the addition of extracellular calcium ion.

IIIB-2. Steroid and contractile activity

Uterine smooth muscle has been known for almost a century to be profoundly influenced by ovarian steroids. The mechanism responsible for these steroid effects remain however largely unknown. In particular there are marked discrepancies between the in vitro and in vivo effects of estrogens on uterine cells. As recently emphasized by Batra [152], this may be due to the fact that the changes in uterine contractility recorded after in vivo estrogen treatment correspond in fact to a combination of multiple steroid actions.

Some of these effects affect the uterine cells only secondarily. It is obvious that the changes in uterine blood flow observed after injection of estrogens may be primarily due to an action on uterine blood vessels. It is also likely that estrogens could alter uterine cell contractility via an action on the release of prostaglandins from ovaries [105].

Among the diverse effects of estrogens on uterine smooth muscle, the great majority correspond to receptor-mediated events in particular those leading to hypertrophy and hyperplasia as well as to change in several metabolic processes. It appears, however, that some of the effects of estrogens may represent direct non-genomic effects. It was suggested that estrogens induce alterations in membrane ionic permeability and alter muscle contractility by modulating the reactivity of the cells to other agents. Under in vitro condi-

tions, estrogens generally exert an inhibitory action on spontaneous myometrial activity.

Batra and Bengtson [153] showed that diethylstilbestrol, but also 17β-estradiol and progesterone, in the range 2.10^{-6}–2.10^{-5} M are able to decrease both the frequency and the magnitude of uterine cells contractions. This effect was observed after less than 10 min incubation in vitro and was associated with a decrease in cell calcium uptake. Similar effects of various progestogens on uterine cell contractions were also observed by Kubli-Garfias et al. [154].

The action of steroids on cell contractility does not appear to be restricted to uterine cells, but can also be observed in other smooth muscle cells. McCalden [155] showed that estradiol was able to alter spontaneous contraction of rat venous smooth muscle cells in vitro. In the presence of 0.1–10 μg/ml 17β-estradiol, there was an increase in the rate of contraction associated with a decreased amplitude which may indicate a progressive hyperpolarisation of the membrane. However, this effect was not observed earlier than 2 h after the beginning of the incubation and the precise mechanism of this steroid action may thus be questioned.

Kahn [156] demonstrated, in contrast, that treatment of frog skeletal muscle preparations by diethylstilbestrol (2–10 μM) did not modify the frequency or the amplitude of cell contraction, but induced a marked lengthening of the relaxation phase without any alteration of the resting potential. This effect was almost instantaneous and was attributed by the author to an effect on calcium sequestration within the reticulum.

A negative inotropic action of cortisone was demonstrated by Soustre [157] at concentrations below 10^{-4} M in frog auricular fibers, which appears to be related to a decrease in the slow calcium conductance.

As stated above, many steroid molecules are able also to modulate muscle contractions elicited by other agents. Seaman and co-workers [158] showed that the contractions of guinea-pig ileum induced by histamine, nicotine, acetylcholine, prostaglandins, angiotensin II and electrical stimulation could be partly blocked (approx. 50% inhibition) in the presence of steroids. This inhibitory effect is produced as early as 2 min following drug addition and was observed not only in the presence of glucocorticoids (10^{-4} M), but also with progesterone, testosterone, and oestriol ($2 \cdot 10^{-5}$ M). In contrast, Brink et al [159] showed that in vitro treatment of tracheal spirals with hydrocortisone rapidly (< 30 min) enhanced the contractile activity of histamine.

Several experiments performed in vitro support the hypothesis that glucocorticoids exert direct presynaptic effects at the neuromuscular junction. Leeuwin and co-workers [160] showed in isolated phrenic nerve-diaphragm preparation, that the muscle contraction caused by short tetanic stimulations (10–40 Hz) are blocked in the presence of d-turbocurarine, but that this effect was markedly antagonised by prednisolone (10^{-5} M). In addition, dexamethasone (2.10^{-7} M) also antagonized the inhibition by d-turbocurarine of both choline uptake and incorporation into acetylcholine by isolated rat diaphragm. Using the same phrenic nerve-diaphragm preparation Dengler et al. [161] showed that the repetitive firing in the motor nerve as well as the augmentation of muscle contraction induced by neostigmine, an inhibitor of cholinesterase, was greatly reduced within minutes of treatment by prednisolone (3.10^{-6} M). Moreover, this inhibitory effect of glucocorticoids on neostigmine-induced stimulation of nerve activity and associated muscle contraction disappeared within 5 min of washing with steroid-free medium. Van Wilgenburg et al. [162] demonstrated that incubation of rat diaphragm for 30 min in a medium containing prednisolone resulted in an increase in the amplitude of the miniature endplate potentials associated with an enlargement of the synaptic vesicles.

IIIC. Short term interactions of steroids with cyclic nucleotides

As stated above, the major part of the so-called 'permissive effects' of steroids represent long term action mediated through modulation of protein synthesis. There are however some in vitro experiments suggesting that steroids could also rapidly alter the cell responsiveness to agents acting via a modulation of cyclic nucleotides.

IIIC-1. Effect of steroids on cAMP content

Because of the parallel effects of steroids and

cyclic AMP on several cell functions, it has been suggested repeatedly that steroids may act by increasing cAMP levels [163].

Early reports have indeed shown that acute administration of 17β-estradiol or diethylstilbestrol in ovariectomized rats markedly increased the cAMP content of uterine cells as early as 15 s following intravenous injection [164]. Similar results were obtained by Weismann and co-workers [165] in rat hypothalamus incubated in the presence of 20 μM diethylstilbestrol. This hypothesis was however seriously questioned since, in most of the tissues investigated, steroids were shown to have no direct effect on cyclic AMP concentration [166].

Recent experiments have shown that the progesterone-induced meiotic division of *Xenopus* oocytes is associated with a very rapid (within minutes) decrease in intracellular cAMP levels [167]. Sadler and Maller [168] have demonstrated, in addition, that progesterone did not act by an activation of phosphodiesterase activity, but rather by an inhibition of adenylate cyclase through an action on the guanine nucleotide regulatory protein. This mechanism, however, appears to be restricted to this particular system.

In an attempt to explain these controversial results, it was suggested that the increase of cAMP level measured in uterine cells after injection of estradiol or diethylstilbestrol, may represent an indirect rather than a direct effect of steroids. Indeed, the diethylstilbestrol-elicited stimulation of uterine cAMP could be blocked by pretreatment with β-adrenergic blocking agents such as propranolol [169]. Similarly, the increase of cAMP induced by diethylstilbestrol in rat hypothalamus is abolished in the presence of both α- and β-blockers [170].

It was thus postulated that steroid treatment may lead to a release of biogenic amines such as epinephrine and histamine in the uterus [48], and dopamine in the hypothalamus, which in turn trigger adenylate cyclase activation. The mechanism responsible for this steroid-induced release of amines remains unknown, although it was recently shown that [^{3}H]estradiol specifically accumulates in brain catecholaminergic neurons [171].

On the other hand, several reports have recently presented evidence for an activation of guanylate cyclase activity by glucocorticoids and estrogens [172–174]. Vesely [174] showed that cortisol, at concentrations as low as 10^{-10} M, was able to markedly stimulate cGMP accumulation in various rat tissues after 3–10 min incubation at 37°C. In this study, it was shown that the order of maximal responsiveness was cortisol > prednisone prednisolone > triamcinolone, an order which does not correspond to the known affinity of these compounds for glucocorticoid receptors. The mechanism and the physiological significance of this phenomenon would thus require further investigation.

IIIC-2. Rapid 'permissive actions'

Several authors have reported a rapid permissive action of glucocorticoids on intracellular cAMP content. Tolone and co-workers [175] showed for example that hydrocortisone (10^{-6} M), which does not affect the basal level of cAMP, markedly potentiates the elevation of cAMP induced by 10^{-5} M epinephrine and 10^{-5} M prostaglandin E_1 in isolated rat mast cells. Similar effects were also observed by Lee and Reed, Mendelsohn and co-workers and Marone et al. [176–178] in human circulating lymphocytes. We have recently investigated the mechanism of this permissive action of glucocorticoids in isolated mouse thymocytes [179]. In these cells, the addition of dexamethasone or corticosterone, which did not modify the basal cyclic AMP content, markedly potentiated the cAMP content when stimulated with L-isoproterenol. This potentiating action of glucocorticoids was almost instantaneous (within 60 s of steroid addition) and was not inhibited in the presence of an inhibitor of protein synthesis. It appears, however, that the effect of dexamethasone greatly varied according to the concentration of isoproterenol tested. At submaximal concentrations (i.e. 10^{-7} and 10^{-6} M isoproterenol), the action of dexamethasone became significant at 10^{-7} M. In contrast, at 10^{-5} M isoproterenol, the action of dexamethasone was no longer visible whatever the concentration of steroid tested.

The mechanism of this glucocorticoid effect which presents the characteristics of a non-genomic action remains unknown. We showed however, in good agreement with Tolone and co-workers, that glucocorticoids do not affect either

the affinity or the number of β-receptors on lymphocytic membranes. But the addition of the chelating agent EGTA blocks this potentiation induced by corticosteroids [175,179].

Among the various hypotheses proposed to explain the permissive action of glucocorticoids, a potential inhibitory effect of these drugs on phosphodiesterase activity has received widespread consideration. However, in vitro determinations of phosphodiesterase activity showed that inhibition of enzyme activity in the presence of glucocorticoids was only observed at concentrations higher than 10^{-4} M [77,180,181]. Under these conditions, inhibition did not exceed 10–25%, whereas potentiation of isoproterenol action in intact cells can be observed at 10^{-7}–10^{-6} M dexamethasone. Nevertheless, these results do not exclude the possibility that steroids may modulate in vivo phosphodiesterase activity, probably through an indirect mechanism. Another possible explanation of this permissive action would be the suggestion that steroids may in some ways (alteration of membrane fluidity, phospholipid methylation and/or changes in membrane calcium permeability...) affect the coupling, either between the membrane receptors and the regulatory protein, or between this regulatory protein and adenylate cyclase. This suggestion is reinforced by the demonstration that colchicine treatment is also able to potentiate the stimulation of cAMP production elicited by prostaglandin E_1 [182].

Several other examples of the permissive actions of steroids in various tissues have been described. Weissman [165] described a potentiation of dopamine-induced cAMP accumulation in rat hypothalamus incubated in vitro with 20 μM diethylstilbestrol, whereas Cloix and co-workers [183] demonstrated that several metabolites of vitamin D were able to rapidly (5–15 min) modulate the action of PTH on adenylate cyclase activity in isolated kidney plasma membranes.

IIIC-3. Effect of steroids on extraneuronal uptake

The experiments of Iversen [184] suggest that at least part of the stimulatory effect of steroids on catecholamine action could be accounted for by an inhibition of extraneuronal uptake 2. The mechanism of this steroid action has been poorly investigated, but it was demonstrated that not only corticosterone, but also estradiol, deoxycorticosterone and, to a lesser extent progesterone and diethylstilbestrol, were also able to block extraneuronal uptake of exogenous amines at concentrations within the range of 10^{-6}–10^{-4} M [185]. Recently Bryan and O'Donnel suggested that corticosterone may act as a competitive inhibitor of the adrenalin uptake process [186].

Whatever is the exact mechanism leading to a steroid-induced potentiation of catecholamine action, this effect might play an important role in some of the therapeutic uses of steroids [187,188]. Geddes et al. [189] showed, for example, that glucocorticoids at concentrations close to 10^{-5} M are able to rapidly (2–5 min) enhance the relaxant effect of isoprenaline on guinea-pig airway smooth muscles.

IIID. Effects of steroids on transport

IIID-1. Effect on ion transport

Among the various systems involved in the transport of ions across cell membranes, the ($Na^+ + K^+$)-dependent ATPase is probably the best known. Several authors have postulated that this enzyme may be a digitalis receptor, which mediates the inotropic effects of ouabain and other digitalis-like compounds [190]. In addition to ouabain many other molecules with a four ring steroid skeleton are able to interfere with ($Na^+ + K^+$)-ATPase activity.

Labella and co-workers [191] showed that chlormadinone acetate, medroxyprogesterone acetate, megestrol acetate, cyproterone acetate and even progesterone and 17α-hydroxyprogesterone are able to compete with ouabain for its binding sites and to block ($Na^+ + K^+$)-ATPase activity at concentrations close to 10^{-5} M. Similarly, Yamamoto et al. [192] showed in atrial muscle fragments that prednisolone-3,20-bisguanylhydrazone (10^{-7}–10^{-4} M) enhances within 3–10 min of treatment the contractile activity of the fiber and induces a marked decrease in ($Na^+ + K^+$)-ATPase activity, as measured by following ^{86}Rb influx. In addition Finotti and Palatini [193] demonstrated that the antialdosterone compound canrenone was also a good competitor for the ouabain binding sites on partially purified guinea-pig brain ($Na^+ + K^+$)-ATPase.

Aldosterone has also been suggested to interact directly with the Na-pump. Hamlyn and Duffy [194] showed that incubation of rat erythrocytes with low concentrations of aldosterone ($3 \cdot 10^{-10}$ M) led to a marked stimulation of ATP hydrolysis, whereas this effect was not observed in the presence of either cortisol or dehydroepiandrosterone. More recently, Moura and Worcel have suggested that aldosterone may exert a rapid action on passive sodium permeability in the rat tail artery [195].

It appears, in addition, that accumulation of steroid molecules in the vicinity of the cell membrane could indirectly alter ($Na^+ + K^+$)ATPase activity. Alivisatos et al. [196] demonstrated that cholesterol and testosterone (10^{-6}–10^{-5} M) may increase, whereas progesterone decreases ($Na^+ + K^+$)-ATPase in synaptosomal membranes. Massa and co-workers [197] showed that cortisol (10^{-6} M) induces changes in ($Na^+ + K^+$)-ATPase fluoride sensitivity in rat erythrocytes.

The finding that steroidal compounds are able to interfere with monovalent ion transport is of considerable pathophysiological significance, since several authors have suggested the existence of a circulating endogenous digitalis-like compound, which may play a major role in the genesis of hypertensive states [198,199].

Although the other systems of ion transport have been less carefully investigated, there are some indications that steroids may also interfere with calcium permeability.

Several years ago, Baulieu and co-workers [2] have suggested that progesterone and several other steroid molecules are able to promote the maturation of *Xenopus laevis* oocytes by increasing the intracellular calcium concentration. This assumption was based on indirect evidence such as the induction of maturation by the calcium ionophore A23187 and its inhibition by the chelating agent EGTA. Since this proposal, a direct demonstration of the progesterone-induced increase in free calcium concentration in these oocytes has been provided by several authors [200,201].

There is also evidence, in several other tissues, for an action of steroid molecules on calcium transport. Batra and Bengtson [153] described a decrease in calcium uptake by myometrial cells in the presence of $2 \cdot 10^{-5}$ M diethylstilbestrol, whereas, in contrast, Batra and Müntzing [202] showed that estramutine phosphate (estradiol-17β-3-chloroethylcarbamate-dihydrogenphosphate) stimulated calcium uptake in male accessory glands.

Pietras and Szego [203] have reported a very rapid stimulation (< 5 min) of ^{45}Ca uptake by endometrial cells in the presence of 10^{-9} M 17β-estradiol, whereas we showed that various glucocorticoids (10^{-7}–10^{-6} M) are able to promote an early uptake of calcium in isolated mouse thymocytes [204].

On the other hand, Ignarro and Cech [205] demonstrated that dexamethasone (10^{-5} M) was able after 10 min of preincubation to block the calcium influx promoted by phagocytosis or ionophore treatment in polymorphonuclear leucocytes.

The mechanism of these effects of steroids on calcium permeability remains almost entirely unknown. However, Shlatz and Marinetti [206] described a marked stimulation of the binding of calcium to isolated rat liver plasma membranes after a 10 min incubation in the presence of 10^{-8} M hydrocortisone.

Experiments performed to study the mechanism of vitamin D-induced stimulation of calcium transport across chick duodenal mucosa suggest that an increase in calcium permeability may be associated with alterations in membrane phospholipids composition.

The mechanism of action of vitamin D-3 (1,25-dihydroxycholecalciferol) on intestinal calcium transport was thought to be comparable to that of other steroid hormones and mediated through the induction of a specific calcium binding protein [9,57]. Bikle and co-workers [207] showed, however, that inhibition by cycloheximide of the vitamin D-induced increase in calcium binding protein did not block the increase in calcium permeability across chick intestine. Matsumoto et al. [208] demonstrated that 1,25-$(OH)_2D_3$ enhances the synthesis of phosphatidylcholine and the incorporation of unsaturated fatty acids into this phospholipid in the enterocytes of vitamin D-deficient chick. This effect was independent of protein synthesis and occurred with a time course very similar to that of vitamin D-induced increase in calcium permeability (i.e. within 1–2 h).

These authors suggested that alterations in

specific lipid domains may play a regulatory role in the calcium transport process.

IIID-2. Effect of steroids on glucose transport

By studying the action of steroids on glucose transport in adipocytes, Livingston and Lockwood [209] have described two different models of inhibition. A classical inhibitory effect was observed within 1–2 h of incubation in the presence of 0.1 μM dexamethasone and was specific for glucocorticoid hormones. On the other hand, inhibition of glucose transport can also be observed within 1–2 min of treatment with 0.1 mM steroid. This effect, however, was obtained not only in the presence of dexamethasone, but also with 17β-estradiol and progesterone [209].

A similar inhibition of glucose transport was observed by Lacko and co-workers [210] in erythrocytes. In these experiments, the effect was measurable as early as 5 s after treatment with 0.1–0.5 mM steroid. The order of steroid potency was: deoxycorticosterone > testosterone > androstene 3,17-dione > corticosterone > cortisol and aldosterone.

In addition, Plageman and Renner [211] demonstrated in Novikoff hepatoma cells that prednisolone acts as a competitive inhibitor of glucose transport with an inhibition constant $K_i = 2$ mM.

IIID-3. Effect of steroids on nucleosides transport

We have recently studied the action of various steroid compounds on the uptake of tritiated uridine in isolated mouse thymocytes. As already demonstrated by others, dexamethasone induced after a 2–3 h incubation period a progressive inhibition of uridine uptake and incorporation, which was visible at 10^{-9} M and reached a maximum at 10^{-6} M. In contrast, testosterone, progesterone and the non-steroidal estrogenic compound diethylstilbestrol exerted a very rapid action on uridine uptake. This effect was visible almost immediately, reaching a maximum after 15–45 min incubation and then decreasing with time. Moreover, this inhibitory effect was usually not detectable at concentrations below 10^{-6} M and abruptly increased at concentrations above 10^{-5} M [212]. Similar results were also obtained by Waddel and co-workers in human lymphoblastoid cells incubated with millimolar concentration of methylprednisolone [213].

IIIE. Steroids and lysosomes

As discussed above, the effects of steroids on lysosomes have usually been studied under experimental conditions that make it difficult to distinguish between a receptor-mediated and a direct action of steroids on lysosomal membranes. There is however evidence suggesting that steroids may directly alter lysosomal membrane properties.

Almost 20 years ago, Weissman [214] studied the effect of several steroids in vitro on β-glucuronidase release from isolated rat liver lysosomes. He showed that enzyme liberation could be enhanced by progesterone, etiocholanolone, pregnandione, pregnanolone, testosterone, androstenedione and deoxycortisol, but was inhibited by cortisol. Concentrations higher than 10^{-5} M were required to be maximally effective. More recently, the stabilising effect of glucocorticoids has been investigated in cell types directly involved in inflammatory processes such as polymorphonuclear leucocytes. Even if we consider only those observations made during incubation of isolated lysosomes with steroid molecules, marked discrepancies exist between the results obtained by different groups. Several explanations may account for these variations. First, Persellin and Ku [215] pointed out that steroid action could greatly vary from one tissue to another in the same animal and also between different species. Similarly, Ignarro [216] outlined the necessity of using very pure cell preparations because lysosomes from different origins may respond in a different way to drugs. In addition, it appears that determination of steroid effects on lysosomal enzyme release is critically dependent upon the experimental procedure used, and, particularly upon temperature and drug concentrations. Lewis and co-workers [217] showed for example that prednisolone at 37°C decreases enzyme release from isolated rabbit lysosomes at all concentrations between 10^{-2} and 10^{-7} M, whereas at 20°C or 45°C, 10^{-7} M prednisolone slightly enhances enzyme liberation. Many steroids may also exert a biphasic action on lysosomal membranes: while stabilizing them at low concentrations, they usually lead to membrane disruption at higher concentrations [218]. A similar biphasic pattern of steroid-induced stabilization-lysis has already been demonstrated for

erythrocytes and liposomes [138,219]. It was suggested that this direct stabilizing effect of steroids involves steroid-lipid interaction, whereas the lytic effect could be due to protein denaturation [138]. Nevertheless, these in vitro experiments performed with isolated lysosomes demonstrate that steroids are able to affect lysosomal membrane properties directly and suggest that similar phenomena may occur in vivo under pharmacological situations.

Several reports by Szego and co-workers [220,221] have suggested that effects on lysosomes are not restricted to antiinflammatory steroids. Indeed, these authors have shown that within 2–15 min following in vivo administration of estrogen, there was a rapid intranuclear accumulation of lysosomal enzymes in steroid target cells such as uterine endometrial cells or cells of the preputial gland.

It was thus suggested that estrogen interact specifically with lysosomal membranes and induce a liberation of acid hydrolase in the nucleoplasm, thus inducing genic derepression. Whatever is the biological importance of this mechanism in steroid-induced uterine growth, it does not appear to correspond to a classical, receptor-mediated, steroid action. Indeed, these rapid effects of estrogens on lysosomal enzyme liberation could be counteracted both by glucocorticoids and by the β-blocking agent, propranolol, suggesting that estradiol either directly fragilizes lysosomal membranes or acts indirectly perhaps through a modulation of histamine release (see above).

IIIF. Steroids and mitochondrial electron transport

Experiments carried out with isolated mitochondria exposed in vitro to steroids have shown that these compounds are potent inhibitors of the electron transport chain.

Wade and Jones [222] showed that the addition of several steroids such as progesterone, estradiol, testosterone, pregnandiol and 17-hydroxyprogesterone ($6.4 \cdot 10^{-4}$ M) to isolated mitochondria markedly decreased oxygen uptake, whereas progesterone only produced an uncoupling of oxidative phosphorylation as determined by measuring the P : O ratio.

Similarly Yielding and Tomkins [223,224] showed that many hormonal steroids are able to inhibit oxygen consumption in isolated mitochondria. The most efficient compounds were progesterone and the non steroidal estrogenic compound diethylstilbestrol which induced a 50% inhibition at concentration close to 10^{-7}–10^{-6} M, whereas glucocorticoid compounds, corticosterone and cortisone, were only active at 10^{-4}–10^{-3} M concentration. In contrast, Gomez-Puyou [225] showed that triamcinolone acetonide ($4 \cdot 10^{-4}$ M) was able to stimulate oxygen consumption in the presence of various substrates.

In addition to this action on oxygen uptake, steroids have been demonstrated to markedly stimulate ATPase activity in isolated mitochondria [226]. These effects were observed in the presence of a large variety of steroid molecules, the most efficient being progesterone, 17α-ethyltestosterone and deoxycorticosterone [227]. On the other hand, Batra has described an inhibitory action of diethylstilbestrol ($4 \cdot 10^{-6}$ M), estradiol and ethynylestradiol on ^{45}Ca uptake by isolated myometrial mitochondria [228,229].

Most of these effects on isolated mitochondria did not appear to be specific of a given class of hormonal steroids and were observed only at pharmacological concentrations (in the range 10^{-5}–10^{-3} M), which are far higher than circulating steroid levels. These alterations of the respiratory chain produced by steroids were, in general, instantaneous and did not require any lag period. Several authors, however, have pointed out that high concentrations of steroids are also able to produce structural alterations in isolated mitochondria. The swelling of mitochondria is usually visible in less than 60 s after steroid treatment [227]. It was therefore suggested that alterations of the oxidative respiratory chain observed in the presence of steroids could be secondary to changes in mitochondria structures and mitochondrial membrane permeability. It thus appears, as suggested by Symons and co-workers [230], that the uncoupling of oxidative phosphorylation is not likely to represent a significant mechanism of action of hormone steroids.

IIIG. Steroids as regulators of enzyme activities

One of the mechanisms by which steroids are able to exert extragenomic actions would be a

direct interaction of the steroid molecules with enzymes.

Tomkins and Maxwell [121] long ago have reviewed the interactions of steroids with a wide variety of enzymes. In some cases, steroids may behave as co-factors and participate in the reaction under control, as in the oxidation catalyzed by phenolases and peroxidases, which are stimulated by estrogens. In other cases, the regulation of enzyme activity is due to a reversible alteration of the protein structure in the presence of steroids.

Many of these experiments have been performed with purified enzyme preparations and therefore represent direct action of the steroid molecules on the protein structure. Although these alterations of enzyme activities are usually observed at relatively high steroid concentrations (close to 10^{-6} M), it is possible that these in vitro mechanisms possess an in vivo counterpart.

Studies performed on the control of cholesterol biosynthesis in cell cultures suggest, for example, that molecules with the four-ring sterol backbone may possibly act as allosteric regulators. Kandutsch et al. [231,232] showed that the activity of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme-A reductase, one of the key steps in the cholesterol biosynthetic pathway, can be almost abolished by the addition of oxygenated sterols (25-OH-cholesterol,7-ketocholesterol...) to the culture medium.

The mechanism of this rapid inhibition (60–120 min) still remains a matter of debate, but does not appear to require RNA synthesis and gene activation, in contrast to glucocorticoid-induced inhibition of cholesterol formation [233,234]. Noland and co-workers [235] have recently purified a sterol carrier protein, which is able to bind 25-OH-cholesterol. A reasonable explanation of the inhibitory effects of these oxygenated sterols would thus imply that this sterol carrier protein, when bound to 25-OH-cholesterol, may act as an inhibitor of HMG CoA-reductase activity [236].

Another potentially important action of steroids is their interaction with the enzymes of catecholamine metabolism. In addition to the well known effects of glucocorticoids on the biosynthesis of several enzymes in the catecholamine pathway and particularly on phenylethanolamine-*N*-methyltransferase and dopamine-β-hydroxylase [237], several authors have shown that estrogens and probably also glucocorticoids may directly interfere with enzyme function: Lloyd and Weisz [238] demonstrated that catechol estrogens, one of the major group of estrogen metabolites, which are present in relatively high amounts in pituitary and hypothalamus, are competitive inhibitors of tyrosine hydroxylase.

Breuer and Köster [239] as well as Ackerman and co-workers [240] demonstrated that catechol estrogens are competitive inhibitors of the enzyme catechol-*O*-methyltransferase. Thus by inhibiting one of the degradative pathways of catecholamines, estrogens may potentiate their action. Similarly, it was suggested long ago by Kalsner that the potentiation of the response of rabbit aortic strips to catecholamines, observed in the presence of hydrocortisone, was due to an action of the steroid on catechol-*O*-methyltransferase activity [187].

IIIH. Miscellaneous

In addition to the various systems described above, there is scattered data in favour of a direct membrane action of steroids in different target cells.

IIIH-1. Sex steroids and platelets

Platelets in mammals are not considered as target cells for steroid hormones, since they do not contain a nucleus or transcriptional mechanisms. Furthermore, there are some indications that they do not contain specific glucocorticoid receptors (Russo-Marie, unpublished data).

Recent experiments, however, have shown that sex steroids are able to interfere with platelet aggregation. Johnson and co-workers [241] have demonstrated that incubation of platelet-rich plasma with testosterone led to an enhancement of their aggregability in the presence of ADP. This effect was obtained within 10–30 min of incubation with 10^{-8} M testosterone at 22°C and was abolished in the presence of a 10-fold excess of the antiandrogen flutamide or in the presence of estradiol. These authors postulated that the effect of androgens on platelet aggregation was related to a modification of platelet membrane permeability. Florence and Rahman [242] showed that estrogens and progestogens at concentrations close

to 10^{-5} M are able to modify the electrophoretic mobility of bovine platelets. In addition, ethynylestradiol increases the sensitivity of the platelets to the action of ADP. These authors thus suggested that the thrombogenic effect of oral contraceptives might be related to a direct action on platelet adhesiveness and aggregation.

In addition, it was recently suggested by Rabbani and co-workers [243] that the acute (in less than 5 min) protective action of glucocorticoids injection on arachidonate-induced toxicity in mice was due to a direct effect of these drugs on platelet aggregation.

IIIH-2. Effect of steroids on membrane proteins distribution

The presence of sheep erythrocytes or antiimmunoglobulin antibodies induces a redistribution of the lymphocyte surface immunoglobulins toward one pole of the cell. This capping phenomenon requires energy production and appears in part controlled by the cytoskeleton. Recently, Ashman and Young-Karlan [244] showed that 20 min incubation with 10^{-5}–10^{-4} M hydrocortisone hemisuccinate not only delayed the time of maximal capping, but also decreased its amplitude. This effect was readily reversible after washing and could also be obtained in the presence of progesterone or in the presence of $2 \cdot 10^{-4}$ M propranolol. Similarly, Maki and co-workers [245] demonstrated that 10 min treatment with hydrocortisone (10^{-4} M) decreased the interaction of human lymphocytes with HeLa cells infected with measles virus, thus suggesting that steroids may rapidly alter the distribution of membrane binding sites.

IIIH-3. Melanosome aggregation by corticosteroids

Edwards and co-workers [245] have recently extended earlier studies and shown that the MSH-induced melanosome dispersion in frog skin melanophore could be reversed by cortisol. They showed in addition that this effect of cortisol did not correspond to a classical receptor-mediated action. Indeed, cortisol action occurred with no apparent lag period, was dose-dependent between 10^{-8} and 10^{-5} M steroid and was rapidly reversible. Moreover, this effect did not require macromolecular synthesis, whereas the activity of a given steroid molecule did not correlate with its lipid solubility or with its affinity for known steroid receptors. The authors postulated that cortisol may interfere with some step in the activation of adenylate cyclase leading to a partial alteration of microtubule assembly.

IIIH-4. Interaction of steroids with non-steroidal membrane receptors

Several authors have suggested that direct interactions of steroids upon membrane receptors might play a role in the neurogenic actions of these hormones.

Inaba and Kamata [247] showed that 17β-estradiol (10^{-6} M), and estrone decrease the binding of [^{3}H]dopamine and [^{3}H]noradrenaline to synaptic membranes of rat brain, whereas it was also shown that 2-hydroxyestradiol interacts with dopamine receptors in rat anterior pituitary cell membranes [248]. Sokolovsky and co-workers [249] presented evidence for an interaction of β-estradiol and progesterone with the muscarinic system in rat hypothalamus and adenohypophysis. Although sex steroids did not appear to bind to muscarinic receptors, they induced a decrease of the affinity of these receptors for the muscarinic antagonist used as tracer. On the other hand, it was also suggested that cortisol may inhibit the binding of $ACTH_{1-24}$ to adrenal cortex membranes [250].

IV. Mechanisms of membrane-steroid interactions

In the preceding sections, we have reviewed several non-receptor-mediated actions of steroid molecules. Many of these direct effects of steroids relate to changes in membrane function.

Two questions thus arise from this statement: first, what could be the mechanisms of steroid-membrane interaction? The second question dealing with the possible physiological significance of these interactions will be discussed in detail in the last section of this review.

IVA. Steroids as surface active agents

Among the mechanisms proposed to explain steroid action, the hypothesis of membrane stabilization has received widespread consideration. This theory is indeed supported by numerous ex-

perimental results as well as by theoretical considerations.

Long ago, several authors reported the existence of a good correlation between the biological effects of steroids and the partition coefficient of these compounds between lipids and water [219,251]. Lacko et al. [210] showed, for example, that the rapid inhibition of glucose transport by steroids was correlated with their lipid solubility. In addition, when considering several membrane effects of steroids such as anaesthetic properties, lysosomal membrane stabilization and inhibition of transports, it generally appears that these various effects are produced by the same molecules, therefore suggesting the existence of a common mechanism for all these steroid actions at the membrane level.

Seeman [138] showed for example, a fairly good correlation between the effect of a given steroid on erythrocyte hemolysis and its anaesthetic action in tadpole, whereas Bangham et al. [252] reported a similar relationship between the effect of steroids on the release of acid phosphatase from lysosomes and the increase in cation leakage from liposomes. Furthermore, the stabilizing effect of steroids on the membrane was immediately reversible after washing of the molecule, thus showing that the interaction with the membrane was relatively weak and readily dissociable [138].

In addition, a possibility of membrane-steroid interactions was deduced from the knowledge of the major role played by cholesterol molecules in the architecture of the membrane and the control of its fluidity. This role has been extensively reviewed in the past [23,24] and will not be presented here in detail. Briefly, at a temperature below the transition of the membrane bilayer from a crystalline state to a liquid-crystalline state, cholesterol exerts a liquefying action towards an intermediate gel state of the membrane bilayer, whereas above this temperature cholesterol acts to condense the membrane structure.

Given the analogy between the structure of the cholesterol molecule and that of steroids, it was suggested that these steroids could be inserted vertically in the phospholipid bilayer instead of a cholesterol moiety [253]. This hypothesis is very similar to that of membrane expansion which was proposed by several authors to account for the action of anaesthetic molecules: the association and the entry of a foreign molecule within the membrane structure would lead to a dissociation of the lipid-lipid interactions as well as to an alteration of the three-dimensional structure of membrane proteins; this disorganization inducing in turn a progressive blockade of membrane ionic channels [254,255] (Fig. 4). In this scheme, known as the Willmer's theory [253], the differences between the properties of the various steroid molecules would be due to the nature of the terminal groups emerging from the membrane and namely those carried by the C_3 carbon and the chain on the C_{17}. This assumption was supported by the finding that several aminosteroids are able to decrease the temperature of transition from a gel to a crystalline state of phospholipid bilayers within seconds of drug addition [256].

However, when studying the distribution of various steroids at natural or artificial lipid-water interfaces, several authors have shown that steroid molecules are not really incorporated in the membrane structure, but lie flat or on their edge on the surface of the phospholipid polar heads [257–260]. It was also shown that some steroids with known

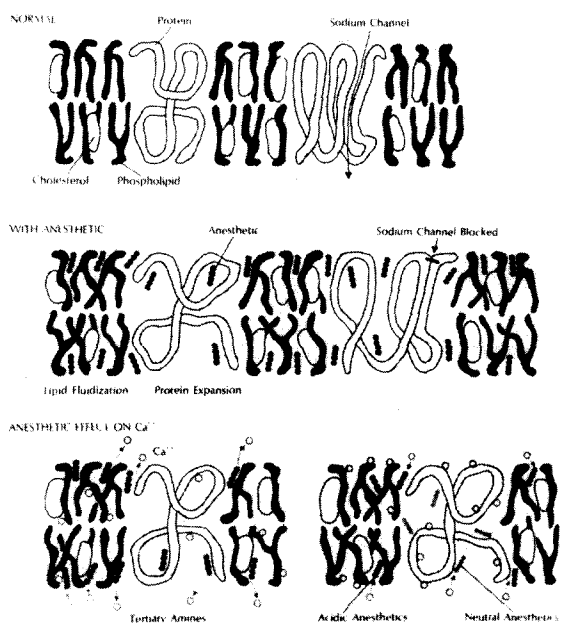

Fig. 4. Mechanism of action of anaesthetic drugs. From Seeman [251].

anaesthetic properties such as pregnenolone, alfaxolone, pregnane-3,20-dione or the glucocorticoid hydrocortisone do not change the transition temperature of membranes [256].

The accumulation of hydrophobic molecules around the membrane, as well as their interaction with membrane components, is likely to produce alterations in membrane properties, particularly at concentrations above 10^{-5} M, at which the process of stabilization or lysis of the cell membrane are usually observed [261,262]. It should also be reminded that molecules with the steroid backbone such as deoxycholate and digitonin are widely used as detergents to solubilize membrane proteins. Goldstein and co-workers [163] have described the various actions of local anaesthetics on polymorphonuclear functions. These include a decrease in osmotic fragility, a decrease in cell to cell and cell to support adhesiveness, partial inhibition of exocytosis, changes in cell shape, alteration of spreading, partial inhibition of locomotion and particle ingestion. These events, which were in part attributed to the ability of the anaesthetic compounds to displace calcium ions from the membrane sites, are strangely similar to those observed in the presence of high concentrations of steroids, suggesting again, that many of the membrane steroid actions may represent broad nonspecific interactions of these molecules with membrane components.

It should be borne in mind, however, that steroids are hydrophobic molecules, which are almost insoluble in aqueous buffers at concentrations above 10^{-5}–10^{-4} M. Many of the experiments devised to study the action of steroids on membrane properties have thus been carried out in the presence of organic solvents such as ethanol or DMSO which may considerably alter the experimental results [264]. Ethanol concentrations above 1% have been shown for example to alter several membrane functions such as aminoacid uptake or adenylate cyclase activity [265,266].

The extrapolation of in vitro results concerning steroid action to the in vivo pharmacological situation should be performed with caution, unless the experiments carried out with high steroid concentrations have been carefully controlled.

IVB. Selective interactions of steroid molecules with membranes

Several indications suggest the existence in the membrane of sites able to discriminate between closely related steroid molecules.

In the system described by Baulieu and coworkers [267], a wide variety of steroid compounds have been tested, not only for their ability to induce germinal vesicle breakdown, but also for their interaction with progesterone-induced oocyte maturation. It appears that several steroids, ineffective by themselves, are able to counteract by varying degrees the action of progesterone. Given their demonstration, using a polymer-bound progesterone that the steroid action was on the outside of the cell membrane, these authors suggested that these antagonist steroids may compete with progesterone for selective binding sites on the cell membrane.

Richard and Hesketh [137] have recorded the membrane potential of isolated guinea-pig olfactory cortex under electrical stimulation and described an inhibition of this evoked potential in the presence of the anaesthetic steroid alfaxolone. They showed also that the isomer Δ_{16} alfaxolone, which exerts very little inhibition of electrical activity, is able to block the action of alfaxolone when added to the cell preparation before it. In addition, preincubation with Δ_{16}-alfaxolone, followed by extensive washing, failed to counteract the inhibitory action of the anaesthetic compound. They therefore suggested that Δ_{16}-alfaxolone may act as an antagonist competing with alfaxolone for the same binding sites on the cell membrane. Dufy et al. [142,143] demonstrated that addition of 17β-estradiol increases electrical activity of G_3H cells in culture within seconds of steroid addition, whereas the stereoisomer 17α-estradiol, although inactive, blocks the action of 17β-estradiol. Similar results were obtained by Kelly et al. [144,145] with estradiol epimers in their study on the firing of hypothalamic neurones.

The nature of these putative 'selective' sites for steroids on the cell membrane remains unknown, but does not appear to correspond to classical receptor proteins. Indeed, the characteristics of these interactions between steroids and the membrane are different from those of usual receptor

protein-steroid associations. In the case of *Xenopus* oocytes, not only progesterone but also cortisol, testosterone and many other steroids without hormonal activity are able to trigger maturation. In addition, germinal vesicle breakdown can also be provoked by other membrane-active agents, such as local anaesthetics and propranolol, acting either at the same site as the steroids or elsewhere. Finally the affinity of these compounds for the membrane site appears relatively weak as most of these effectors are only active at concentrations of 10^{-6} M or above [267].

It has been suggested that the steroid recognition structures on membranes may be formed by phospholipids or phospholipid clusters. This hypothesis is again based on the similarities observed between the effects of steroids on membranes and those of anaesthetic compounds. Lee [254] has proposed a model of anaesthetic action involving an interaction of the drugs with phospholipid clusters. This model postulated that some of the membrane proteins are almost permanently embedded in a core of lipids, which remains in a gel state. This rigid environment maintains the tertiary structure of the protein in a particular conformation. It was recently demonstrated in good agreement with this model, that several membrane enzymes or receptors, when purified, are unable to function properly unless they receive an appropriate phospholipid surrounding [268–270]. In this model, the addition of an anaesthetic molecule or of a steroid, will lead to a transition of this lipid core towards a liquid state and thus to an alteration in protein function. In this respect, it is worth noting that the calcium ion, which is believed to play an important role in the control of lipid transition and anaesthetic action, appears also to be involved in steroid membrane interactions [271]. Indeed, steroids have been shown to modulate calcium influx in *Xenopus* oocytes, uterine cells and mouse thymocytes [201–204]. Moreover, Dufy and co-workers [147] demonstrated that the action of estradiol on the electrical activity of C_3H cells could be blocked by D_{600} a well known calcium blocker. Morgan et al. [151] showed in mouse thymocytes that the fluorescence of the dye 3,3′-dipentyloxocarbocyanine iodide, which reflects the polarization state of the membrane is decreased in the presence of calcium, but can be restored by estradiol.

In conclusion, although the phospholipid nature of these putative membrane sites for steroids remains to be proved, the possibility of a precise interaction of steroid molecules with phospholipid aggregates, as well as the importance of calcium ions in steroid-membrane action, may well represent promising lines for future research.

IVC. Steroid uptake: passive diffusion or carrier-mediated uptake?

The different approaches used to investigate this problem have been recently extensively reviewed by Szego and Pietras [272], by Giorgi and Stein [273,274] as well as by Ballard [275], and will be only briefly summarized in this section.

IVC-1. Kinetic experiments

Assuming that the association of the steroid with its cytoplasmic receptor is extremely rapid at 37°C and does not represent a rate-limiting step [276], any discrepancy between this rate of association and that of steroid uptake by whole cells or tissue fragments would thus reflect an interaction between the tracer steroid and some component of the cell membranes. Therefore, the comparison between the kinetics of steroid-receptor interaction and that of cell uptake has been used by numerous authors to discriminate between a passive diffusion and any carrier mediated entry of steroids into their target cells.

However, despite extensive investigations, the results remain unclear. Whereas Milgrom et al. [277] in uterine cells, Harrison et al. [278] in AtT_{20} D/1 pituitary cells and Rao et al. [279] in isolated hepatocytes presented evidences in favour of a carrier-mediated uptake of steroids, Plageman and Erbe [280] and Giorgi and Stein [273] in hepatoma cells as well as Müeller and Wotiz [281] in isolated uterine cells claimed that there was no argument to support such a system for the movement of steroids across the membranes. Giorgi and Stein [273], in particular, clearly established the following points: the rate of entry of steroids into the cells appears independent of the substrates concentrations, there is no competition for transport between steroids, and the permeability of the steroids strongly correlates with their partition coefficient in *n*-octanol.

Thus, in the absence of any conclusive evidence for a carrier-mediated mechanism, it is reasonable to assume that steroid transport occurs by simple diffusion through the lipid bilayer of the plasma membrane [275]. This, however, does not exclude the possibility that steroid transport could in some particular cells be achieved by a more complex mechanism [282–284]. Harrison and co-workers [278] have, for example, demonstrated in the pituitary tumor cell line AtT_{20} D/1 a very marked decrease in the uptake of [^{3}H]triamcinolone acetonide at 4°C as compared to that observed at 25°C, whereas the level of cytosolic binding sites was similar in both cases. Using the same experimental procedure, we failed however to show any significant difference in the level of [^{3}H]dexamethasone uptake between 4°C and 37°C in isolated mouse thymocytes [285].

IVC-2. Effect of membrane alterations on steroid uptake

Several authors have compared the action of thiol group blockers on steroid uptake as well as on steroid receptor interaction. Harrison et al. [286], Rao et al. [287] and Milgrom and co-workers [277] suggested that the inhibition of steroid uptake in the presence of these SH-blocking agents was an additional argument in favour of a carrier-mediated entry of steroids. In contrast, Levinson et al. [288] showed that only those reagents, which cross the membrane barrier, inhibit both the uptake and the association of the steroid to its receptor, whereas the non-permeant compounds are without effect on whole cell binding.

IVD. Are there steroid receptors on cell membranes?

The major argument in favour of the cytoplasmic location of the steroid receptors is the fact that most of the steroid binding sites (with the properties of hormonal receptors, i.e. high affinity, limited capacity and stereoselective specificity) are recovered in a $105\,000 \times g$ supernatant (i.e. cytosol) after cell homogeneization. It is however possible that the soluble nature of the receptors is artefactual, arising during cell homogeneization and fractionation. Many authors have thus, in the past, investigated whether or not steroid receptors are, in part or in totality, associated with plasma or organelle mebranes.

IVD-1. Steroid binding to isolated plasma membranes

This direct method has been used by numerous workers but has led to unreliable and conflicting results. This is illustrated by the recent reports of Pietras and Szego [289] and of Müeller et al. [290]. Whereas the former group reported the existence of estradiol receptors associated with a purified fraction of rat uterine plasma membrane, Müeller and co-workers [290] claimed that the small amount of estradiol binding sites recovered in rat uterine membrane fraction was due to a contamination by cytosolic receptors. Indeed, one of the major problems encountered in this type of experiment is the development of a separation method permitting the isolation of a well defined plasma membrane fraction, free of contamination by other membrane fractions. This necessity was clearly demonstrated in the work of Pietras and Szego [289]. These authors have studied in parallel seven procedures commonly used for plasma membrane isolation. They have determined in these experiments the factor of purification of the membrane fractions (using marker enzymes), the extent of the contamination by other cell fractions and the amount of [^{3}H]estradiol specifically associated with each preparation. They showed that, according to the composition of the incubating buffer, its ionic strength and the method of cell homogeneization, the proportion of the 5′-nucleotidase activity (a membrane enzyme) recovered in the cytosolic fraction ranged from 9 to 60%, whereas 20.4 to 78% of the bound tracer was found in cytosol. Using the experimental protocol that led to a maximal enrichment of 5′-nucleotidase activity in the membrane fraction and a minimal contamination by either mitochondrial or microsomal membranes, they demonstrated that about 25% of the receptors present in the cell homogenate may constitute membrane associated receptors. It thus appears that many of the reports devoted to membrane receptor determinations are not fully conclusive, because they often lack the appropriate controls of membrane integrity and purity.

In most of the experiments carried out to study glucocorticoid membrane receptors there is a common feature which supports their existence, distinct from the classical cytosolic receptors. In almost all the species and the organs tested, these

membrane receptors exhibit more affinity for natural glucocorticoids (such as corticosterone and cortisol) than for synthetic compounds (such as dexamethasone or triamcinolone acetonide), whereas the opposite is true for cytoplasmic receptors [291–294]. The former pattern of specificity is similar to that of the type III receptors found in rat kidney cytosol or to that of CBG-like proteins described by several authors in various tissues [295–296]. This could suggest that these steroid binders, found in cytosolic preparations, could be more or less associated with membrane in intact cells.

IVD-2. Cytochemical evidences

Nenci and co-workers [297,298] using a cytochemical approach with fluorescent estrogen analogs bound to serum albumin, have presented evidence in favour of steroid binding sites in the plasma membranes of human breast cancer cells. These binding sites present several characteristics of estrogen receptors: the binding process was saturable and abolished by pretreatment with natural estrogen; binding sites were only visible on estrogen target cells and not on other cell types; in addition, these binding sites were characterized by a precise stereospecificity. Interestingly, these membrane sites easily recognized natural estrogens, but did not exhibit appreciable affinity for synthetic estrogens or antiestrogens. These authors demonstrated also a time and temperature-dependent redistribution of these binding sites (capping) at the surface of the cell. Similar results were also reported by others in mammary tumor cells [299] and *Xenopus* oocytes, using photoaffinity labeling [300]. The significance of this cytochemical approach has been recently questioned by Daxenbichler and Weiss [301].

IVD-3. Effect of membrane alterations on steroid-receptor interactions

Several authors have reported a decrease in the association of steroids to their receptors (using whole cell uptake) following alteration of the cell membrane by various agents and, suggested, that this could represent an argument in favour of membrane associated receptors.

Harrison et al. [302,303] as well as Picard and co-workers [304] demonstrated that treatment of intact glucocorticoid target cells by phospholipase A_2 or neuraminidase, but not by proteolytic enzymes leads to a decrease in steroid binding, when tested in a whole cell assay. In contrast, cytosolic receptors remain unaffected in the presence of phospholipase A_2 and neuraminidase, but are destroyed by proteases. These authors concluded that, at least, a fraction of the steroid receptors may be located within the cell membrane and is inactivated following membrane alterations.

These results however could well be explained by the existence of other mechanisms. Indeed, these different enzymatic treatments have been shown to enhance membrane permeability [305] and could thus lead to the leak and/or entry of factors, which may interfere with steroid-receptor interactions such as proteins, ATP, calcium ions... [306–308]. In addition there have been several reports associating a decrease in steroid receptors with a possible change in membrane permeability. We have shown that treatment of isolated mouse thymocytes with the calcium ionophore A23187 induced a decrease in whole cell binding of dexamethasone (Homo et al. unpublished data), and Cidlowski and Munck [309] showed that treatment of rat thymocytes by the mitogen concanavalin A led to a decrease in glucocorticoid receptors. This effect was observed at concentrations of mitogen far higher than those required to induce blast transformation and which have been described to enhance calcium permeability and even to decrease cell viability [310].

V. Discussion

It appears that there is in fact a graded variety of interactions of steroid molecules, over a wide range of concentrations, not only with classical steroid receptors, but with several types of binding sites with different affinities and specificities. The question now arises as to the physiological significance of these interactions and particularly of those at the membrane level. Given the fact that direct actions of steroids on the membrane are usually demonstrated at high concentrations, which are several orders of magnitude higher than the usual circulating steroid hormone concentrations, it is generally accepted that these effects represent nonspecific pharmacological actions. In this sec-

tion we will briefly discuss this statement and suggest that the direct interaction of steroids with the plasma membrane as well as extragenomic steroid effects could be relevant to some in vivo situations.

VA. What is the significance of 'physiological concentrations'?

Steroid hormones in the bloodstream are usually found in very low concentrations (i.e. 10^{-10}–10^{-7} M). Furthermore, a large fraction of these compounds is tightly bound to plasma transport proteins and does not have free access to target cells. It thus appears that the physiological roles of these steroids occur within a limited range of concentrations and that capture of these compounds will only be performed by high affinity receptors. Several reserves should, however, be placed on the above statement.

First, several authors showed that tissue concentrations may well be higher than plasma concentrations. Henkins et al. [311] demonstrated for example that cortisol concentration in the cat brain was almost 30 times higher than that in plasma. Therefore circulating hormone concentrations do not accurately reflect those in target tissues.

As outlined by Spain [312], corticosteroids appear to affect the inflammatory process by localizing in the inflamed or injured areas at higher concentrations than in other tissues or organs of the body. This increased concentration appears, however, to be a result of the increased blood flow and increased permeability associated with the early inflammatory reaction, rather than the consequence of a specific trapping mechanism inflamed tissue. Amaral and Werthamer [313] have recently shown that incubation of RPMI 1788 lymphocytes, resistant to cortisol, in the presence of transcortin induces an accumulation of transcortin within the cell associated with a subsequent cortisol-induced inhibition of DNA synthesis. The existence of transcortin-like binders within cells or the possible uptake of plasma transcortin by some cells may contribute to steroid accumulation.

Second, in several organs where steroid synthesis or metabolism takes place such as ovary, testes, adrenal glands, placenta and even liver, adipocytes or brain, the steroid concentration could well reach values allowing interaction with low affinity sites [314–316].

Third, steroids are widely used in the treatment of many different diseases and thus very high circulating concentrations of synthetic or natural steroids could be achieved in the course of these treatments [317].

In addition, high levels of steroids could also occur under pathological conditions such as Cushing's disease, endocrine tumors or even during stress and pregnancy. For example, Basset et al. have shown that, in rats exposed to stress situations, the circulating corticosterone level reached values of 35–90 μg/100 ml of plasma (i.e. $2–3 \cdot 10^{-6}$ M) [318]. MacCalden [155] demonstrated that several of the effects of estradiol on uterine smooth muscle contractility observed in vitro were also demonstrated in rat uterus during late pregnancy. Conversely, the concentration of free steroids in blood may also vary as a function of the circulating levels of plasma steroid-binding proteins. Indeed, the concentration of transcortin has been shown to fluctuate under various conditions [319,320].

VB. Heterogeneity of target tissues

In a given target tissue, the number of steroid receptors is usually measured in cytosolic preparations and expressed on an average basis (i.e. per mg protein, per mg DNA or per cell) assuming that the binding sites are equally distributed throughout cells. Autoradiographic studies have, however, ruled out this hypothesis. In brain, but also in heart and kidney, the radioactive ligand appears to be concentrated in discrete areas [321]. Similar phenomenon may also occur at the level of individual cells, ensuring steroid concentration in particular cell compartments (such as lysosomes...). Receptor determinations performed after separation of cell subpopulations in various target tissues have shown a marked heterogeneity of receptor levels in the different cell types [322,323]. It thus appears that some cells in a target tissue are enriched in cytosolic receptors and are likely to concentrate the steroid above plasma levels.

VC. Permissive effects of steroids

The demonstration that steroids are able to exert rapid permissive actions on the effect of many hormonal and non hormonal stimuli, such as catecholamines, parathormone, prostaglandins..., suggest that steroids, at concentrations where they are inactive by themselves, may indirectly control and modulate several functions particularly at the membrane level.

VD. Is 'nonspecific' binding really nonspecific?

The number of hormonal receptors is usually taken as the difference between total tracer binding and nonspecific binding, i.e. residual binding in the presence of 100–1000-fold excess of unlabeled ligand. In contrast to the so-called specific binding sites, which are characterized by limited capacity and high affinity, this nonspecific binding corresponds to an almost unsaturable population of sites with a lower affinity.

In vivo experiments have shown that this 'nonspecific' binding is readily washed out within a few hours following tracer injection [324,325]. These binding sites are usually believed to represent adsorption and/or weak association of the steroid ligand with lipophilic components of the membranes. In cytosolic extracts this nonspecific binding does not exceed 10–20% of the total binding, whereas in whole cell assays, it could reach up to 50–60% of the total binding. In some cell types such as macrophages, it is even necessary to carefully wash out this nonspecific binding in order to accurately measure the real receptors which are present in low concentrations [326].

The significance of these low affinity steroid binding sites has usually been neglected, although some authors have suggested that they could play a role in steroid action. Parchman et al [327] demonstrated, for example, that this population of low affinity sites markedly rose with aging in rat liver cells. These low affinity 'nonspecific' sites whose specificity has not been carefully investigated, may be present in sufficient amounts to account for some of the direct effects of steroids on membrane properties, through for example changes in membrane fluidity or structure [219,328].

VI. Conclusions

The understanding that rapid extragenomic actions of steroids not only exist, but also may take place in vivo considerably extends the potential field of steroid action. Although the nature of the membrane sites involved in these direct actions is not yet established, it remains essentially to determine their exact functions, since there are up to now very few indications regarding these roles. Nenci and co-workers [329] have recently listed several possibilities and suggested that the estrogen binding sites located on the plasma membrane may either represent classical receptors associated with the membrane, adsorbed or integrated sex binding globulin or may exert several functions such as vectorial transport of estrogen, ion channeling, interaction with the cyclic nucleotide system or subcortical cytoskeleton [330,331], participation in surface architecture and charges, or even may play no biological role whatsoever. It is also tempting to speculate that these membrane binding sites may play an important role in the rapid behavioural effects of sex steroids as well as in the fast adrenal steroid feedback on neuroendocrine tissues [149,332], and may explain some non-receptor-mediated actions of steroids, which have been recently described [333–335].

Pietras and Szego [336,337] have established a relationship between the presence of estradiol membrane receptors on liver cells and cell responsiveness to estradiol. Rat liver cells dissociated by collagenase treatment were passed through a column containing estradiol molecules covalently bound to nylon fibers [336]. A small percentage of these cells (6%) was retained on the column and was only eluted in the presence of 17β-estradiol, but not in the presence of glucocorticoids or androgens. It was therefore concluded that a minor fraction of rat liver cells possesses specific membrane receptors for 17β-estradiol. In addition, the level of cytosolic estradiol receptors was almost three times higher in those cells containing membrane binding sites, whereas their sensitivity to estradiol treatment was also enhanced as determined following [^{3}H]thymidine incorporation and cell respiration.

Towle and Sze [338] have recently reported the existence, in well characterized synaptic plasma

membranes isolated from rat brain, of specific binding sites for glucocorticoids and sex steroid. Several characteristics of these binding sites indicate that they are probably different from the brain cytosolic receptors present in the same tissues. Indeed, the affinity of these membrane sites for their ligand was about one order of magnitude lower than that of cytosolic receptors. In addition, these membrane binding sites were heat stable, whereas cytosolic receptors were easily inactivated following 30 min incubation at 30°C.

These authors showed also that incubation of the membrane fractions in the presence of corticosterone induced a marked stimulation of [^{14}C]tryptophan uptake with a good correlation existing between the dose-dependence of the membrane binding and the increase of tryptophan uptake [338,339].

We believe that additional experiments performed on purified membrane fractions to relate steroid binding with some membrane functions will shed light on the role of membrane steroid binding sites. Another question to be investigated is whether or not a given steroid molecule may exert both types of effect in the same target cell and what could be the relationship, if any, between rapid and genomic steroid actions. Kanazir and co-workers [340] have proposed some years ago a general mechanism of steroid hormone action which integrates both genomic and extragenomic effects of steroids. In this model, the native steroid receptor is a multimeric macromolecule comprising several regulatory subunits. The binding of the steroid molecule to one subunit initiates the disaggregation of the complex. The free subunits then act as rapid modulators of several metabolic pathways including membrane enzymes, transport..., whereas the steroid binding unit is transferred to the nucleus and triggers RNA and protein synthesis. Attempts to establish the relevance of this model to steroid action as well as carefull investigations of the extragenomic effects of steroids will in the future provide a further insight into our knowledge of the mode of action of steroid hormones.

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