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THE BINDING OF HORMONES AND RELATED COMPOUNDS BY NORMAL AND CHOLESTEROL-DEPLETED PLASMA MEMBRANES OF RAT LIVER*

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Abstract—The effects of removing part of the unesterified cholesterol from rat liver plasma membranes upon their ability to bind a number of different hormones and related compounds has been studied.

Amongst steroids, the more polar compounds containing an 11 βOH group (dexamethasone, cortisol, corticosterone) are bound to a greater extent by cholesterol-depleted membranes than by control membranes whilst the less polar compounds (deoxycorticosterone, progesterone, testosterone, diethylstilbœstrol) are bound to a lesser extent.

The increase in binding of cortisol is related to the degree of cholesterol depletion.

In comparison experiments using rat liver mitochondrial preparations, which normally possess a very low level of cholesterol, it is found that when this level is raised, the binding of corticosterone decreases and that of testosterone increases.

Binding of adrenaline and thyroxine by cholesterol-depleted plasma membranes is greater than that of controls whereas the binding of peptide hormones (insulin, glucagon, oxytocin, vasopressin) is much lower.

The results suggest that membrane sterols play an important part in regulating the uptake of biologically active compounds.

THERE has been much speculation about the ways in which hormones, particularly steroids, interact with cellular membranes. The lipid components of the latter have been particularly emphasized as sites for interaction[1] and a number of investigations have been made in which monolayers or aqueous dispersions of lipids have been used as membrane models.[2–7] It is possible to deplete erythrocytes and liver plasma membranes of a large part of their unesterified cholesterol without altering the level of the other major constituents.[8, 9] The process is reversible[9] and has been used in this study to investigate the relationship between the binding of a number of hormones and related compounds and the cholesterol content of the liver plasma membrane. Various hormones have been shown, directly or indirectly, to exert a physiological action upon this membrane,[10–13] but unlike most other tissues, the problem of hormone uptake in the liver is not confined to those exerting a physiological action. The liver is the major site of hormone detoxication[14–17] and at times such as the end of pregnancy and in some pathological conditions, large quantities of different hormones are carried to the liver cells. Perfusion experiments[15] have confirmed the ability of these cells to take up very large amounts of steroids. The mechanism by which

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such large molecules enter the cell is unknown and this makes their binding by the plasma membrane, as a likely first step in the process, of some interest.

MATERIALS AND METHODS

Crystalline beef insulin, lysozyme, L-thyroxine, L-adrenaline, testosterone, progesterone and diethylstilboestrol were obtained from British Drug Houses Ltd. (Poole, Dorset); cortisol, corticosterone and purified human plasma albumin from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.); dexamethasone (extracted from clinical tablets) from Merck, Sharpe and Dohme Ltd. (Hoddesdon, Herts.); deoxycorticosterone from Mann Research Laboratories (New York, U.S.A.); glucagon from Eli Lilly and Co. Ltd. (Basingstoke, Hants.); synthetic oxytocin and vasopressin from Ferring AB (Malmo, Sweden); and [^{125}I]insulin, [1,2-^{3}H] cortisol and [4-^{14}C]aldosterone from The Radiochemical Centre (Amersham, Bucks). The radioactive steroids were purified by thin layer chromatography[18] until only a single spot was obtained.

Plasma membranes. These were prepared by a modification[19] of the method developed by Kamat and Wallach for ascites cells.[20] The preparation obtained by this method consists of closed vesicles rather than open sheets of membrane.

Membranes were depleted of cholesterol by incubation overnight at 37° with cholesterol-depleted plasma lipoproteins.[8, 9] No change in membrane protein or phospholipid was detected during this operation but the unesterified cholesterol was lowered to 46–53 per cent of the original level.

Mitochondrial preparations. The mitochondrial pellet[21] from one rat liver was resuspended in 20 ml of 0·44 M sucrose and 5 ml of the suspension incubated for 16 hr with the normal, untreated lipoproteins from 25 ml of human plasma. The mitochondria were washed three times with 0·44 M sucrose. After incubation, the level of unesterified cholesterol had roughly doubled. Incubations with steroids were carried out as for plasma membranes except that 0·44 M sucrose was used as the medium.

Analytical methods. Membrane proteins and peptides were estimated using the Folin–Ciocalteu reagent and unesterified cholesterol by the method of Leffler and McDougald.[22] Steroids were estimated from their ultraviolet absorption spectra[23, 24] as were adrenaline (λmax 296; ϵ $5{\cdot}4 \times 10^3$) and thyroxine (λmax 323; ϵ $5{\cdot}05 \times 10^4$), the last two measurements being made in alkaline solution.

[^{3}H] cortisol and [^{14}C] aldosterone were determined in a Packard Tricarb Scintillation Spectrometer and (^{125}I) insulin in a solid crystal (NaI) scintillation counter. Materials were counted to a statistical error of < 2 per cent.

Incubation conditions. The membranes and compounds under investigation were dispersed at the required concentration in 5 ml of 0·15 M NaCl in polypropylene centrifuge tubes. Control and test experiments were carried out together and contained equal quantities of normal and cholesterol-depleted mitochondria. Where necessary, the steroids were first dissolved in the minimum volume of ethanol. This gave a final ethanol concentration of $<0{\cdot}4\%$ (v/v). Incubations were carried out at 37° for 75 min after which the membranes were sedimented at 80,000 *g* for 30 min. Controls containing no membranes were carried out to ensure that there was no sedimentation of the dispersed test compound or adsorption to the vessel walls. In no case was either detected. The very close agreement between the results of individual

experiments in which different concentrations of membranes and test compound were used, also rules out the possibility of errors arising from such phenomena.

Measurement of binding. The uptake of non-radioactive compounds was obtained by estimation of the amount of the test compound remaining in the supernatant after incubation. Binding of radioactive compounds was determined by direct measurement of the isotope in the membrane pellet after washing it twice by resuspending in saline and resedimenting. Membranes labeled with [^{14}C]- or [^{3}H] steroids were spread on glass fibre discs[25] and those containing [^{125}I] insulin were dissolved in NaOH (2N) for assay. Uptakes by membranes represented 5–80 per cent of the test compound present except for that of oxytocin by untreated membranes which represented 90% of the total in the system.

Expression of results. Binding is expressed in terms of the original level (i.e. before depletion) of membrane unesterified cholesterol so that untreated and sterol-depleted membranes can be directly compared. This basis was chosen because isolated liver plasma membranes can lose proteins on incubation[26] and it was of interest to relate steroid binding by untreated membranes to their natural sterol content. Control experiments indicated, however, that there was very little loss of protein by the membranes but corrections had still to be made to the binding levels obtained with peptides. These corrections amounted to only 6·6–20 per cent of the measured uptakes. It follows from this that where no uptake was detected, a small amount, masked by simultaneous release of membrane protein, could have occurred.

RESULTS

Steroids. The uptakes of various steroids by liver plasma membranes are given in Table 1. It can be seen that when untreated membranes are used there is relatively

TABLE 1. BINDING OF STEROIDS BY NORMAL AND CHOLESTEROL-DEPLETED LIVER PLASMA MEMBRANES

Steroid	Concn (μM)	Steroid uptake (μmoles/μmole original membrane cholesterol)	
		Untreated membranes Mean ± S.E.M.	Depleted membranes Mean ± S.E.M.
Dexamethasone	38–49	0·28 ± 0·01	0·53 ± 0·03
Cortisol	36–45	0·30 ± 0·02	0·32 ± 0·01
Corticosterone	46–59	<0·02	0·22 ± 0·02
Deoxycorticosterone	16–27	0·12 ± 0·01	0·09 ± 0·01
Progesterone	29–35	0·42 ± 0·01	0·25 ± 0·01
Testosterone	28–60	0·35 ± 0·02	0·24 ± 0·01
Diethylstilbœstrol	33	0·49 ± 0·02	0·34 ± 0·01

In control experiments, untreated membranes were added to give a concentration of 59–91 μg of membrane cholesterol per 5 ml of medium. The same quantity of membranes were added in the test experiments but their cholesterol level had been lowered to between 46 and 53 per cent of that of the corresponding control. Each result is the mean of 4 determinations (± S.E.M.) using 2 different membrane preparations.

little variation in these uptakes except in the case of corticosterone which is bound to a very low extent. There is no obvious reason for this exception but it may be noted that corticosterone is also bound by erythrocytes less well than other steroids.[27]

The steroid uptakes can be shown from the results of Ashworth and Green[28] to represent less than 1 per cent of the dry weight of the membranes. Following removal of cholesterol, there is a marked change in the steroid binding properties of the membranes. The binding of dexamethasone and corticosterone increases greatly while that of cortisol shows only a slight change. On the other hand, the binding of deoxycorticosterone, progesterone, testosterone and diethylstilbœstrol shows a marked decrease.

The above results were obtained using relatively high concentrations of steroids and it was of interest to determine whether similar results would be obtained at concentrations approaching those found *in vivo* and when the steroids were dispersed in blood plasma (Table 2). The radioactive steroids available were cortisol and aldosterone. Although aldosterone was not used in the experiments recorded in Table 1,

TABLE 2. BINDING OF HORMONES FROM SALINE AND PLASMA BY NORMAL AND CHOLESTEROL-DEPLETED LIVER PLASMA MEMBRANES

Hormone	Concn (M)	Medium	Hormone uptake	
			Untreated membranes (μmoles/mmole original membrane cholesterol)	Depleted membranes
[^{14}C]Aldosterone	$8{\cdot}3 \times 10^{-8}$	Saline	$10{\cdot}0 \pm 1{\cdot}2$	$19{\cdot}5 \pm 1{\cdot}15$
		Plasma	4·2	19·2
[^{3}H]Cortisol	$2{\cdot}6 \times 10^{-6}$	Saline	$19{\cdot}8 \pm 4{\cdot}4$	$33{\cdot}3 \pm 6{\cdot}3$
		Plasma	44·1	52·7

The untreated membranes in the control experiments provided 58–95 μg of cholesterol per 5 ml. of incubation medium. Those in the test experiments provided 51–53 per cent of the corresponding control cholesterol level. Saline results are means of 4 determinations (± S.E.M.) using 2 different membrane preparations. Plasma results are means of duplicate experiments on the same membrane preparation.

it behaves similarly to the other polar steroids in being bound to a greater extent by cholesterol-depleted membranes than by untreated ones. Cortisol behaves in the same way and at this lower concentration shows a more marked increase in binding after sterol depletion than in the earlier experiments. When the saline is replaced by plasma, there are some quantitative differences but the general pattern of binding is the same.

Non-steroids. The most notable finding from the studies made with non-steroids (Table 3) was that of all compounds studied, the only two for which no binding by untreated membranes could be detected were the two which show no hormonal activity, albumin and lysozyme. However, both of these compounds were used at high concentration and an uptake of up to 0·5 μg of albumin per μg of membrane cholesterol could not be detected. The experiment with albumin was therefore repeated using 76 μg of albumin and 400 μg of membrane sterol per ml. Again no uptake by untreated or cholesterol-depleted membranes could be detected indicating that the level of uptake of this protein was less than 0·1 μg per μg of membrane sterol. Albumin and lysozyme differ in several ways from the other peptides (e.g. in being larger) and without more controls, too much emphasis cannot be attached to these differences in binding properties. The binding of nonsteroid hormones is affected by removal of membrane sterol as much as that of the steroids. Both adrenaline and thyroxine appear to

show increased binding after sterol depletion whereas all peptide hormones show a much lower binding, the maximum level being only 39 per cent of the uptake obtained with untreated membranes.

TABLE 3. BINDING OF ADRENALINE, THYROXINE AND PEPTIDES BY NORMAL AND CHOLESTEROL-DEPLETED LIVER PLASMA MEMBRANES

Compound used	Concn (μg/ml)	Uptake (μg/μg original membrane cholesterol)	
		Untreated membranes Mean $\pm$ S.E.M.	Depleted membranes Mean $\pm$ S.E.M.
Adrenaline	38	0·24 $\pm$ 0·01	0·34 $\pm$ 0
Thyroxine	11–28	0·56 $\pm$ 0·02	0·77 $\pm$ 0·08
Insulin	75–180	3·88 $\pm$ 0·03	<0·3
Glucagon	21–48	2·34 $\pm$ 0·11	0·72 $\pm$ 0·06
Oxytocin	87	1·63 $\pm$ 0·07	0·63 $\pm$ 0·01
Vasopressin	47–50	1·47 $\pm$ 0·03	<0·2
Lysozyme	502	<0·2	<0·2
Albumin	146	<0·5	<0·5

In control experiments, untreated membranes were added to give 45–86 μg of membrane cholesterol per 5 ml of incubation medium. The same quantity of membranes was added in test experiments but their cholesterol level had been lowered to 45–54 per cent of that of the corresponding control. Each result is the mean of 4 determinations ($\pm$ S.E.M.) using 2 different membrane preparations.

[^{125}I] insulin was used in one experiment to check whether the binding pattern was the same at near-physiological concentrations and also the effects of plasma upon the process. Using 1·7 μg of insulin and 17 μg of membrane cholesterolper ml of saline in the control experiments, a binding of 16 μg/mg membrane cholesterol was obtained. With depleted membranes (8·0 μg membrane cholesterol per ml) 8 μg/mg were bound. When the saline was replaced by plasma, uptakes of 8 and 4 μg/mg original membrane cholesterol were obtained in control and test experiments respectively. Thus at the lower levels, the differences between untreated and cholesterol-depleted membranes are still shown although they are not so marked as in the previous experiments. In the presence of plasma, there is an all-round reduction in insulin binding but the differences between the untreated and depleted membranes remain.

Washing of membranes. The effects of washing the membranes with 0·15 M NaCl after binding of dexamethasone, cortisol and thyroxine were investigated. The membranes (untreated) were allowed to bind the hormones under the conditions described in Table 1 and were then washed three times by resuspending in saline and recentrifuging. The washings were found to contain 5 per cent of the bound cortisol and no detectable dexamethasone or thyroxine. Thus even these polar compounds are not easily removed. These figures show that trapping of material within the membrane pellet does not affect the results and this is expected since the volume of the pellet was only a fraction of a percentage of the total volume of the suspension.

Graded depletion of membrane cholesterol. If it is the removal of cholesterol molecules which alters the binding properties of liver membranes, then it should be possible to correlate the change in binding with the proportion of cholesterol removed. An experiment was therefore carried out in which the binding of [^{3}H]cortisol was measured at several stages during the depletion of the membrane sterol. It can be

seen (Fig. 1) that the binding does vary with the cholesterol content of the membrane. The figures for the control and final membrane preparations obtained in this experiment fall within the range of those obtained in the earlier experiments shown in Table 2.

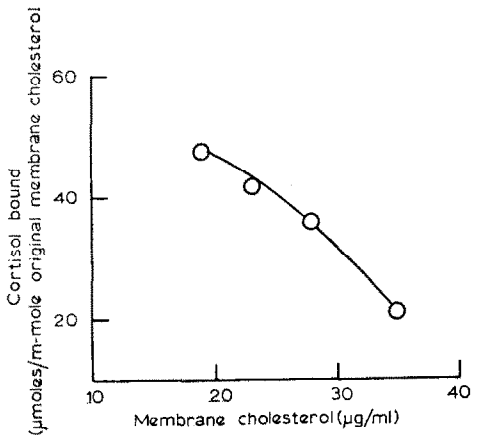

FIG. 1. Binding of cortisol by cholesterol-depleted liver plasma membranes. Liver plasma membranes were incubated with cholesterol-depleted plasma at an initial concentration of 35 μg of membrane sterol per ml. Samples were removed at 1, 2 and 24 hr when the membrane sterol levels were 28, 23 and 19 μg/ml. The membranes were washed and resuspended at the same concentration in 0·15 M NaCl containing [^{3}H]cortisol ($3·3 \times 10^{-6}$ M).

Use of cholesterol-enriched mitochondria. As a further test of the relationship between steroid binding and the cholesterol content of cellular membranes, the effect of enriching membranes with cholesterol was examined. Liver mitochondrial preparations normally have a much lower sterol content (3–6 per cent of the lipid) than plasma membranes (15–20 per cent of the lipid), but this can be greatly increased by incubation with untreated plasma lipoproteins. The binding of the extra sterol does not appear to differ from that normally present as judged by its ability to exchange with plasma cholesterol.[21] It has now been established (Graham and Green, unpublished observations) that the oxidation of many substrates, including fatty acids, and the process of respiratory control are not damaged by the incorporation of the sterol. Rat liver mitochondria take up corticosterone *in vitro* and this is bound to the membranes rather than to the intramitochondrial components.[29–30] A liver mitochondrial fraction was therefore chosen as a suitable test preparation and corticosterone and testosterone were taken as examples of steroids which respond in their binding in different ways to cholesterol depletion. The results given in Table 4 show that the untreated mitochondria take up about the same amount of testosterone per μmole of sterol as the plasma membranes but they take up much more corticosterone. After the mitochondrial sterol level had been doubled, uptake of corticosterone decreased while that of testosterone increased. These are the results one would predict from the earlier findings with the plasma membranes (Table 1). Any binding by contaminants derived from the endoplasmic reticulum has been neglected since rat liver mitochondria show a greater affinity for corticosterone than the components of the microsomal fraction.[31]

TABLE 4. BINDING OF STEROIDS BY NORMAL AND CHOLESTEROL-ENRICHED RAT LIVER MITOCHONDRIA

Steroid	Concn (μM)	Uptake (μmoles/μmole original membrane cholesterol)	
		Untreated mitochondria Mean ± S.E.M.	Enriched mitochondria Mean ± S.E.M.
Corticosterone	30	1·11 ± 0·03	0·78 ± 0·01
Testosterone	23	0·31 ± 0·02	0·53 ± 0·03

The two control mitochondrial preparations contained 32 and 21 μg of cholesterol per ml and the cholesterol-enriched preparations 68 and 43 μg/ml respectively.

DISCUSSION

The uptake of hormones by the liver cell plasma membrane is of specific interest both from the point of view of their physiological action and their detoxication. Of more general importance is the pharmacological action of steroids on membrane structures such as mitochondria,[30, 32] erythrocytes,[33–36] lysosomes[37–38] and liposomes.[3, 4] All three phenomena probably involve different levels of the agents concerned and it is difficult to know how far they are interrelated. Most of the present experiments have been conducted with concentrations of hormones much greater than those which occur *in vivo* and Bush[39] has warned against the dangers of trying to correlate physiological effects with results obtained *in vitro* at such concentrations. However, a given level of binding cannot be said to be unphysiological until the rate at which the hormone crosses the membrane *in vivo* is known. If this process is rate-limiting then even although the plasma level of a given compound is low, the number of molecules bound to the membrane could be very high. The similarities in behaviour of cortisol and insulin at different concentrations and the finding that steroid binding by mitochondrial and plasma membrane preparations can be regulated in the same way, indicate that the results obtained in this study could be of relevance to all three phenomena discussed above.

The marked effect of removal of membrane cholesterol on the uptake of hormones and related compounds (Tables 1–3) is consistent with the view that membrane lipids are directly exposed at the sites concerned. Evidence from a variety of physical techniques, suggests that it is unlikely that removal of part of the cholesterol would result in any significant change in the conformation of the bulk of the protein of cellular membranes[40–42] although a change in a small portion of the membrane protein following removal of cholesterol cannot be excluded. However, it seems most probable that at the levels of uptake under discussion, direct binding to membrane lipids is of major importance. Earlier studies on the exchange of membrane cholesterol[21, 43] strongly supported the concept of membranes as mosaics of lipid and protein.

Steroids. Bojesen and Jensen[44] have proposed that in studies made *in vitro*, binding of steroids often merely reflects the lipid/water partition coefficients and is not a specific phenomenon. In fact, in view of the wide variations in membrane lipid composition and in the hydrophilic/hydrophobic balance of steroids, this could provide for very specific uptakes as Siekevitz[45] has pointed out. However, the steroid uptakes shown in Table 1 do not correlate with the published oil/water partition coefficients.

Snart and Wilson[46] measured the uptake of steroids by liposomes composed of phospholipid and varying amounts of cholesterol but their results do not agree with the present ones. In their system, the presence of cholesterol lowered the uptake of progesterone and deoxycorticosterone whereas with liver membranes, uptake is greater at higher cholesterol levels. The differences could arise from the effect of the protein in the membranes on the lipid structure or from the differences in phospholipid composition of the two systems but they suggest that caution must be exercised in using dispersions of pure lipids as membrane models. Nuclear magnetic resonance studies[47] have also shown that the cholesterol/phospholipid interaction is not the same in erythrocyte membranes as in simple aqueous dispersions of the two lipids.

A popular theory of steroid action maintains that they are inserted into the lipid bilayers of cellular membranes or of liposomes as 'rogue' molecules either replacing or in addition to the natural cholesterol.[1, 6, 37, 38] These 'rogue' molecules could then alter the permeability properties of the membrane. If steroids acted on cellular membranes in this way then one would expect the binding of all to respond to lowering of membrane cholesterol in the same way, i.e. all should increase or all decrease. This is not the case. Willmer[1] proposed that certain steroids would be aligned with the polar group at C-3 towards the aqueous phase whilst others would have the groups at C-17 to C-21 towards the aqueous phase. The two types of steroid binding pattern seen in this study do not correspond to the two proposed modes of orientation since if they did, deoxycorticosterone would behave like cortisol and corticosterone and not, as it does, like progesterone.

Evidence from monolayer studies[2, 5, 6] indicates that except at the lowest surface pressures, only progesterone of all steroids used in the present study can penetrate phospholipid films (with or without cholesterol). Taylor and Haydon[5] found that at high pressures even progesterone contributed only 1–4 per cent of the molecules in the monolayer. From the phospholipid/cholesterol ratios of the plasma membranes[28] it can be calculated that the progesterone taken up in the experiments detailed in Table 1 represents 8–9 per cent of the membrane lipid molecules. It thus seems more probable that in the present experiments, it is adsorption to the surface of the lipids which is important. This is in keeping with the findings of Munck[48] and of Gershfeld and Heftmann[2] who investigated the interaction of steroids at heptane/water interfaces and phospholipid/water interfaces respectively. The steroids whose binding is increased after removal of membrane cholesterol (Tables 1 and 2) are the more polar molecules which possess an 11 βOH group. Munck[48] showed that at water/heptane interfaces, cortisol lies on edge and testosterone, deoxycorticosterone, diethylstilboestrol and progesterone all lie flat. It is easy to imagine that removal of membrane cholesterol reduces the hydrophobic areas to which the less polar steroids attach. The resulting closer packing of the phospholipid molecules could then provide more sites for the binding of the 11β hydroxysteroids which attach via the polar groups along one edge.[39] It is interesting in this connection to note that Pak and Gershfeld[49] have suggested that bound steroids exert effects by altering the aqueous environment next to the receptor site. It is known that the binding of cholesterol molecules in membranes depends strongly on the ordering of water molecules around them[43] so uptake of steroids at such sites could have the proposed effect.

Peptides. The finding that uptake of peptide hormones by liver cell membranes is affected as much as that of steroids by cholesterol removal, raises the possibility that

they too, interact directly with exposed lipids. Eley and Hedge[50] and Sanyal and Snart[7] showed that insulin, oxytocin and vasotocin were adsorbed by lipid monolayers and there is evidence suggesting that the physiological effects of peptide hormones depend on their interaction with membrane lipids. Thus low concentrations of phospholipases can produce effects on membranes which alter or mimic those of vasopressin and insulin.[51, 52] Evidence from monolayer studies also shows the importance of cholesterol in lipid-peptide interactions. Several different proteins and peptides have been shown to interact more strongly with monolayers of cholesterol than with those of lecithin and other lipids.[7, 50, 53, 54] In this case, the importance of cholesterol hydroxyl groups has been emphasized. The very marked effect of removing membrane cholesterol upon hormone binding is consistent with the view[7, 54] that some regular arrangement of cholesterol hydroxyl groups is required for their uptake.

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