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AN INTERACTION BETWEEN OESTRADIOL AND PROGESTERONE IN AQUEOUS SOLUTIONS AND IN A MODEL MEMBRANE SYSTEM

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SUMMARY

The solubilities of oestradiol and of progesterone in Tris-buffered KCl and in egg phosphatidylcholine liposomes and the effects of these steroids on the K^+ permeability of the liposomes have been determined by radio-tracer methods. A suspension of the labelled steroids in aqueous medium was allowed to equilibrate with the same medium contained in dialysis sacs with and without liposomes also present in the sacs. The steroids had different solubilities in the two phases according to whether they were suspended alone in the external aqueous medium, or as an equimolecular mixture. When alone, progesterone was found to be approximately twice as soluble as oestradiol in the aqueous medium and three times as soluble in the lipid medium. When the external suspension contained an equimolecular mixture of the two steroids, they were taken up approximately equally into the dialysis sacs containing either aqueous medium or liposomes. The major effect was a marked increase in oestradiol lipid solubility where the two steroids were present together. The entry of progesterone into the lipid phase was retarded by the presence of oestradiol but ultimately reached the same concentration as when presented alone. Oestradiol alone decreased the K^+ permeability of liposomes, whereas progesterone increased the permeability. Progesterone *plus* oestradiol affected liposomal K^+ permeability similarly to progesterone alone. An hypothesis involving the possible formation of a chemical complex or small mixed micelle of the two steroids is offered to explain these observations.

INTRODUCTION

The interaction of oestradiol and progesterone in target tissues such as the uterus and mammary gland depends on the relative concentrations of administered steroids. At certain ratios they produce a more profound effect on cell metabolism than when either of them is acting alone, as observed, for instance, with the proliferation of the endometrium in the rabbit, the mucification of the vaginal epithelium in the guinea-pig, or the development of mammary tissue in several species[1].

Since the permeability of cellular or subcellular membranes may be involved in these actions, it is important to determine the effects of the two steroids on model

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membrane systems. BANGHAM and colleagues[2] have already examined the action of a series of steroids on the permeability of lipid membranes to ionic and non-ionic solutes using phospholipid liquid crystals (liposomes)[2]. Moreover, WEISSMANN and co-workers[3] have shown that the permeability changes caused by the steroids can be modified by pre-incorporation of other steroids such as cortisone in the liposomal membranes.

We have recently investigated the actions of oestradiol and progesterone on the potassium permeability of liposomes[4] and now report the effects of different combinations of these steroids in a similar system. The experimental design has allowed the simultaneous examination of several features of the oestradiol–progesterone interactions in this system, namely, the effect of the mixture compared with that of the individual steroids on aqueous and lipid solubilities, on the derived distribution coefficients, and on $^{42}K^+$ permeability of the liposomes.

MATERIALS AND METHODS

Materials

All reagents were A.R. grade and were used without further purification. The water was double glass-distilled over $KMnO_4$. The preparation and purification of ovolecithin has been described previously[5]. [6,7-$^3H_2$]Oestradiol 500 mC/mmole, and [4-^{14}C]progesterone, 36.1 mC/mmole, were obtained from the Radiochemical Centre, Amersham, Bucks., and their purity checked regularly by thin-layer chromatography. The specific activity of the steroids was adjusted in chloroform solution by the addition of unlabelled steroid (Sigma London Chemical Co., Ltd.) as appropriate.

Preparation of liposomes and steroid suspensions

The methods employed for the preparation of liposomes have been described previously[4]. Labelled oestradiol or labelled progesterone (15 μmoles, 6.03 μC/μmole) were mechanically shaken for 5 min with 10 ml buffered KCl (144 mM KCl–16 mM Tris–HCl (pH 7.4)); the suspension was diluted with 70 ml Tris-buffered KCl and sonicated for 30 min. Mixed suspensions of labelled oestradiol (15 μmoles, 0.03 μC/μmole) and unlabelled progesterone (15 μmoles) or labelled progesterone (15 μmoles, 0.03 μC/μmole) and unlabelled oestradiol (15 μmoles) were prepared in the same way.

Aqueous and lipid solubilities of oestradiol–progesterone mixtures

The techniques used have been described elsewhere[4]. In addition to the measurement of aqueous and lipid solubilities of oestradiol and progesterone individually, equimolar mixtures of the two steroids were sonicated to study the effect of steroid interactions on their solubilities.

$^{42}K^+$ release from liposomes

The rate of release of $^{42}K^+$ from liposomes was measured by the method of HEAP, SYMONS AND WATKINS[4]. The leakage was measured after a 20–24-h equilibration period under one set of conditions, and again during the next 24 h after transfer of some of the liposomes to fresh medium containing oestradiol and/or progesterone.

RESULTS

Solubilities and distribution coefficients

The aqueous and lipid solubilities of oestradiol and progesterone were individually determined by measuring the penetration of the labelled steroids into dialysis sacs containing buffered isotonic KCl only or suspensions of liposomes in buffered KCl. The solubilities were calculated from the radioactivity levels obtained in the sacs after 20 h at 37° by which time equilibrium had been established[4]. The aqueous and lipid solubilities found for progesterone and oestradiol when present separately in the dialysis system (Table I) were within the range of values reported in our previous communication[4]. Similarly, the distribution coefficients confirmed our earlier findings, the value for progesterone being somewhat higher than that found for oestradiol.

TABLE I

AQUEOUS AND LIPID SOLUBILITIES, AND DISTRIBUTION COEFFICIENTS OF OESTRADIOL AND PROGESTERONE EITHER ALONE OR IN COMBINATION

Liposome suspensions equilibrated for 20 h at 37°.

$$K = \frac{\text{solubility in lipid (nmoles/ml lipid)}}{\text{solubility in KCl (nmoles/ml Tris-buffered KCl)}}.$$

Steroids	*Aqueous solubility* (*μmoles/l*)	*Lipid solubility* (*nmoles/μmole*)	*Distribution coefficient* (*K*)
Progesterone alone	42.6	109.2	3268
in presence of oestradiol	34.9	28.9	1054
Oestradiol alone	23.5	30.1	1633
in presence of progesterone	36.5	50.3	1755

In contrast, when oestradiol and progesterone were sonicated together their aqueous solubilities became almost identical and the lipid solubility of oestradiol in the presence of progesterone was increased by about 67 % while that of progesterone was decreased by a similar proportion (Table I). Calculations of the lipid/buffer distribution coefficients (see Table I) after the first 20-h equilibration period showed that the value for oestradiol was not affected by the presence of progesterone in the dialysing medium, but the value for progesterone was reduced by 68 % when oestradiol was present. The lack of effect on the oestradiol values is due to the fact that the increase in aqueous solubility was paralleled by an increase in lipid solubility. In the case of progesterone, the decrease in lipid solubility was far greater than the decrease in buffer solubility, resulting in the large decrease in distribution coefficient.

In view of the apparent reduction in lipid solubility of progesterone in the presence of oestradiol, and the increased lipid solubility of oestradiol in the presence of progesterone, the experiment was repeated over a 42-h equilibration period (Table II). The penetration of progesterone alone into dialysis sacs containing liposomes showed little change after 42 h when compared with the value found after 20 h. However, when oestradiol was also present the rate of penetration of progesterone was much reduced and took up to 42 h to approach the figure found in 20 h with progesterone alone. In the case of oestradiol, there was a small increase in the amount

TABLE II

UPTAKE OF OESTRADIOL AND PROGESTERONE, ALONE AND IN COMBINATION, INTO DIALYSIS SACS CONTAINING LIPOSOMES SUSPENDED IN BUFFERED KCl AT 37°

Amount of lipid in each sac, Expt. A, 2.58 μmoles; Expt. B, marked by asterisk, 1.98 μmoles.

Treatment	*Uptake into dialysis sac (nmoles/μmole lipid)*	
	20 h	*42 h*
Progesterone from		
(a) progesterone	134	153*
(b) progesterone + oestradiol	49	130*
(c) liposomes equilibrated in oestradiol, transferred to saturated progesterone + oestradiol	—	114*, 102
(d) as in (c) but transferred to saturated progesterone	—	151*, 125
Oestradiol from		
(a) oestradiol	44	59*
(b) oestradiol + progesterone	72	137*
(c) liposomes equilibrated in progesterone, transferred to saturated progesterone + oestradiol	—	111*, 96
(d) as in (c) but transferred to saturated oestradiol	—	37*, 43

found in dialysis sacs after 42 h compared with 20 h, but there was much greater penetration at both times when progesterone was also present in the dialysing medium. At 42 h, the amounts of progesterone and oestradiol taken up by the liposomes on exposure to the equimolar mixture of the two steroids were closely similar.

The results suggest that the rate of uptake of progesterone was greatly reduced by the presence of oestradiol and only reached normal equilibrium values by 42 h. In contrast, the rate of uptake of oestradiol was increased by the presence of progesterone in the dialysing medium. This increase was still apparent even after a 42-h dialysis period.

To study whether pre-incorporation of oestradiol or progesterone into liposomes induced lipid conformational changes which affected the subsequent uptake of the other steroid, the liposomes were equilibrated with either progesterone or oestradiol for 20 h and then transferred to fresh buffered KCl containing saturated oestradiol + progesterone for up to 23 h. The subsequent uptake of progesterone by oestradiol-equilibrated liposomes, and of oestradiol by progesterone-equilibrated liposomes was 108 and 103 nmoles per μmole lipid, respectively (mean values from Table II). This indicates again that the penetration of both oestradiol and progesterone into dialysis sacs is similar when both steroids are present in the dialysing medium, and that the net effect gives lipid solubilities corresponding more closely to those of progesterone than oestradiol. The results also show that this effect is not significantly influenced by previously exposing the liposomes to one of the steroids.

That the effects observed with mixtures of oestradiol and progesterone in the external fluid are not apparent when steroids are presented sequentially, was shown in the following way. Oestradiol-equilibrated liposomes were transferred after 20 h to fresh buffer saturated with progesterone. The uptake of progesterone in the next 22 h was found to be the same as that of liposomes exposed only to progesterone (Table II).

TABLE III

EFFECT OF OESTRADIOL AND PROGESTERONE ON LEAKAGE OF $^{42}K^+$ FROM LIPOSOMES DURING INITIAL 20 h EQUILIBRATION PERIOD

Liposomes allowed to take up steroid from buffered KCl solution during 20 h at 37° and $^{42}K^+$ leakage over that period compared with that from control liposomes. Leakage calculated as % of original $^{42}K^+$ content of the liposomes which had diffused into the external medium at the end of the equilibration period. Column 2: % leak, steroid liposomes/% leak, control liposomes. Column 3: Figures of second column *minus* 1. This factor, multiplied by the control leakage, gives the increase in leakage of the steroid liposomes compared with controls[4]. Column 4: Figures of second column divided by the amount of steroid (in μmoles) incorporated in the liposomes per μmole lipid. For oestradiol + progesterone treatment, increment factor divided by sum of oestradiol and progesterone in liposomes per μmole lipid[4].

Treatment	*Steroid / Control*	*Increment factor*	*Molecular increment factor*
Oestradiol	1.24, 1.21	0.22	7.43
	1.25, 1.09	0.17	—
Progesterone	1.95, 1.75	0.85	7.82
	1.85, 1.32	0.59	—
Oestradiol + progesterone	2.24, 1.67	0.95	11.99
	2.39, 1.79	1.09	—

Similarly, the uptake of oestradiol by progesterone-equilibrated liposomes transferred to oestradiol was comparable to that of liposomes exposed only to oestradiol. Presumably the major part of the pre-incorporated steroid was lost to the medium and replaced by the second steroid in these two experiments.

Effect on lipid permeability to K^+ during 20-h equilibration period

Since the liposome suspensions were prepared[4] in isotonic buffered ^{42}KCl, it was possible to measure the effect of oestradiol and of progesterone individually, and of different combinations of both, on K^+ permeability of the liposome membranes. Table III shows that the $^{42}K^+$ leakage during the equilibration period was highest for liposomes being equilibrated in buffered KCl containing a mixture of oestradiol and progesterone. As the steroid content of the liposomes was monitored simultaneously with the $^{42}K^+$ leakage rate, the observed effects on K^+ permeability could be related to steroid content. On this basis the molecular effect of oestradiol and progesterone together during the period of equilibration was somewhat greater than that of either of the steroids alone (Table III).

Effect on lipid permeability to K^+ after equilibration

After 20 h, equilibrated $^{42}K^+$-labelled liposome suspensions were transferred to fresh saturated solutions of oestradiol, progesterone or oestradiol + progesterone as shown in Table IV. The rate of $^{42}K^+$ leak was estimated over the next 22 or 23 h in two experiments and the results expressed in terms of the ratio, % leak, steroid liposomes/% leak, control liposomes (see Table IV, footnote).

Oestradiol had a stabilising effect in oestradiol-equilibrated liposomes, reflected by the lower than unity steroid:control ratio (0.77) while the labilizing action of progesterone in progesterone-equilibrated liposomes was indicated by a significant

TABLE IV

EFFECT OF STEROIDS ON LEAKAGE OF $^{42}K^+$ FROM LIPOSOMES OF PHOSPHATIDYLCHOLINE

Liposomes were allowed to take up steroid from buffered KCl solution during 20 h at 37° (Treatment A) and $^{42}K^+$ leakage was measured at various times during the next 23 h after transfer to different medium (Treatment B). Control liposomes were transferred to fresh buffer. Leakages were calculated as % of the $^{42}K^+$ content of liposomes as estimated at the end of the equilibration period (20 h).

Steroid in dialysis fluid		*$^{42}K^+$ leak, steroid*/control*			
Treatment A (0–20 h)	*Treatment B (20–43 h)*	*Expt. 1*			*Expt. 2*
		22 h	*25 h*	*43 h*	*42 h*
A. Oestradiol	(i) Oestradiol	—	—	—	0.77
	(ii) Oestradiol + progesterone	1.23	1.59	1.72	1.94
	(iii) Progesterone	1.30	1.58	2.04	1.51
B. Progesterone	(i) Progesterone	—	—	—	2.32
	(ii) Oestradiol + progesterone	2.60	3.51	3.41	2.87
	(iii) Oestradiol	1.88	1.74	1.26	1.76
C. Oestradiol + progesterone	Oestradiol + progesterone	—	—	—	2.70
D. Oestradiol	Buffered KCl	—	—	—	0.65
Progesterone	Buffered KCl	—	—	—	1.42

* % leak, steroid liposomes/% leak, control liposomes.

increase in K^+ leak (steroid/control, 2.32). The greatest increases in K^+ leak, however, were observed with liposomes equilibrated with progesterone or oestradiol + progesterone and transferred to oestradiol + progesterone (Table IV, B, ii and C). The highest rate of K^+ leak observed was for progesterone-equilibrated liposomes during the first 5 h after transferring them to a progesterone + oestradiol solution.

In experiments where progesterone-equilibrated membranes were transferred to oestradiol alone (Table IV, B, iii), there was a gradual decrease in the $^{42}K^+$ leak, while in the reverse experiment (Table IV, A, iii) the $^{42}K^+$ leak increased. These results are in accord with the effects of progesterone and oestradiol on control (steroid-free) liposomes, and presumably reflect both the diffusion of the pre-incorporated steroid into the medium and uptake into the lipid of the second steroid in each case. When similar progesterone- or oestradiol-equilibrated liposomes were transferred to buffer, however, (Table IV, D) there appeared to be a negligible effect of both steroids during the next 22 h, with leakages within the range expected where a slow loss of the pre-incorporated steroid into the steroid-free medium was presumably taking place.

DISCUSSION

Our results can be summarized as follows:

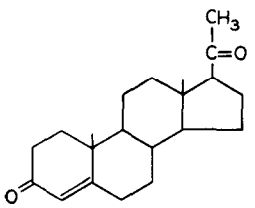
(1) When a sonicated suspension of equimolar amounts of solid oestradiol and progesterone is allowed to equilibrate with steroid-free KCl contained in a dialysis sac, approximately equal amounts of the two steroids enter the sac. This contrasts with the solubility of the two steroids measured separately in the same way, when twice as much dissolved progesterone as oestradiol is found to enter the sac.

(2) When the dialysis sac contains phosphatidylcholine liposomes, the uptake of the two steroids is approximately equal despite the fact that the solubility of progesterone in the phospholipid is 3-fold higher than that of oestradiol when the steroids are presented separately. Saturation of the lipid phase with the mixed steroid takes place more slowly than with the individual steroids but ultimately the uptake of progesterone from the mixture is the same as that occurring with progesterone alone while the uptake of oestradiol from the mixture is three times higher than is the case with oestradiol alone.

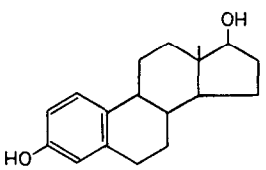
(3) The equal uptake of the steroids by the lipid always occurs when the dissolved equimolar mixture of the steroids is presented, even if the membranes have previously been completely equilibrated with one or the other steroid.

(4) The effect of the two steroids together on liposomal K^+ permeability is a marked increase, equal to or even higher than that normally produced by progesterone alone, and opposite to that of oestradiol alone which normally has a stabilizing (permeability-reducing) effect after complete equilibration.

The first three effects described above can be explained by the assumption that the molecular entities taken up by the buffered KCl inside the dialysis sac from an external suspension of the mixed solid steroids are not independent molecules of progesterone and oestradiol, but some type of molecular complex, or small mixed micelle containing approximately equal amounts of the two steroids, and, further, that this mixed entity enters the liposomal membranes as a unit. Our fourth observation would then suggest that such a progesterone–oestradiol complex is oriented in the membrane so as to produce a membrane conformational change similar to molecules of progesterone alone, the result being a membrane labilizing effect similar to that of progesterone rather than the stabilizing effect of oestradiol.



Progesterone



Oestradiol-17 β

The structures of oestradiol and progesterone present possibilities for intermolecular bonding of three different types: (i) hydrogen bonding between hydroxyl groups of oestradiol and carbonyl groups of progesterone, (ii) π-bonding between the aromatic ring of oestradiol and the $\alpha\beta$-unsaturated carbonyl system of progesterone and (iii) hydrophobic bonding between the hydrocarbon nucleus of each of the two steroids. Refined physical methods will be needed to test the possibility of discrete complex formation as against a less specifically assembled mixed micelle, and to define the type of intermolecular association not only between the two steroids, but also between the steroids and the phospholipid molecules.

Irrespective of the exact nature of the intermolecular interactions, the fact that the presence of progesterone greatly enhances the maximum uptake of oestradiol by phosphatidylcholine liposomes, while the presence of oestradiol greatly retards the rate of uptake of progesterone by the phospholipid, may well be important in the

biological interactions of these compounds. Thus, the permeability of cell or organelle membranes might be greatly influenced by the proportions not only of free molecules of oestradiol and of progesterone, but also of complexes between the two, if these exist in blood and other tissue fluids. The synergistic effects of oestradiol and progesterone on target tissues such as the uterus and vagina are most clearly observed when the ratio of administered steroids is greatly in favour of progesterone (*e.g.* 750:1; ref. 1). However, the results obtained from continuous infusion of labelled oestradiol or of labelled progesterone in rats[6] indicate that their target tissue concentrations may be of the same order (10^{-9} M). Our experiments using a model membrane system composed of only one type of phospholipid is clearly an oversimplification of the biological system and does not take into consideration the important binding role of tissue and transport proteins. Nevertheless our results do raise the interesting possibility that a direct molecular interaction between these two steroids may determine in part their mode of action.

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