J. Pharm. Pharmac., 1972, **24**, 942–949 *Received September* 23, 1972

The interaction of some oral contraceptive steroids with lipid monolayers and with erythrocytes

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Some steroids used in oral contraceptive combinations have been found to interact with cholesterol and lecithin spread as monolayers on water and to protect erythrocytes against haemolysis. No simple relation between steroid structure and these interactions was found, although in general the more water-soluble members of a group of structurally related compounds interacted to a lesser extent. No distinction could be observed in the behaviour of progestogens and oestrogens in the systems studied. It is suggested in the light of the evidence presented here and from data in the literature that these steroids may adsorb at lipid surfaces and may penetrate lipid membranes. This gives substance to the hypothesis that some steroids adsorb at platelet surfaces and alter the surface properties or the permeability of the platelet membrane sufficiently to cause increased platelet aggregation.

Steroids are known to adsorb at oil-water interfaces (Munck, 1957; Baret & Roux, 1968) and at the glass-solution surface (Doisy, Huffman & others, 1941) and to interact with lipids spread as monolayers on water (Taylor & Haydon, 1965; Willmer, 1961). The present work is part of an investigation to determine whether it is possible that the steroids used in oral contraceptive combinations interact with the surfaces of platelets and thereby alter the tendency of the platelets to aggregate. The role that platelets play in haemostasis has been established (Brinkhous, 1967; Haanan & Jurgens, 1968; Maupin, 1969). Any interference with their aggregation is therefore likely to be of importance in seeking the causes of the thrombotic episodes alleged to occur in patients taking oral contraceptive steroids over long periods of time.

At least two possibilities present themselves to explain a membrane-mediated effect. One is that the steroid molecules, by interacting with the platelet membranes, decrease the surface potential of the platelets and accentuate the tendency to aggregation—which may be the precursor of clot formation. The second possibility is that by interaction with the platelet surface membrane, permeability is increased or decreased, whereby the flux of platelet components such as adenosine diphosphate, which are implicated in the aggregation process, is altered (O'Brien, 1962). Several membrane-stabilizing drugs such as chlorpromazine and imipramine actually decrease platelet aggregation by inhibiting the release of ADP from the platelets (Mills & Roberts, 1967).

This part of the investigation has determined the extent of interactions of ten steroids and one synthetic oestrogen, stilboestrol, with cholesterol and lecithin monolayers. Cholesterol and lecithin occur in roughly equimolar amounts in the platelet membrane. The hypothesis that the demonstrated interactions can alter the

forces of interaction between platelet and platelet is currently being tested (Florence & Rahman, unpublished work). Intake of oestrogens can affect human platelet electrophoretic mobility and the response of the platelets to adenosine diphosphate (Elkeles, Hampton & Mitchell, 1968).

MATERIALS AND METHODS

Materials

The progestogens, chlormadinone acetate, megestrol acetate, progesterone, norethinodrel, norethinodrone, lynestrenol and medroxyprogesterone acetate were used without further treatment after receipt from suppliers. Chlormadinone acetate was a gift from the Lilly Research Centre Ltd., through the courtesy of Dr. C. B. Macfarlane. The oestrogens, ethynyl oestradiol, mestranol and oestradiol were also used as received. Stilboestrol (BDH) was Biochemical grade, used as received. The steroids were all examined by gas-liquid chromatography to preclude contamination.

L-α-Lecithin (Sigma Type III E ex egg yolk) was obtained as a 10% w/v hexane solution and diluted to the required concentration with Analar benzene for spreading. Cholesterol was BDH Biochemical grade, dissolved in Analar benzene for spreading on water. Water was freshly distilled from an all-glass apparatus, and stored in a glass container.

Methods

Surface pressures were measured using a Langmuir trough of glass construction (Polymer Consultants Ltd.). A glass Wilhelmy plate (4·8 cm perimeter) was used in conjunction with a 0–0·5 g torsion balance (White Electrical Instrument Co.). Experiments were made at room temperature (22 $\pm$ 1°).

Spreading solvents were tested for purity by dropping 0·2 ml onto a swept water surface, allowing solvent evaporation to occur, and compressing the surface. No changes in surface tension were observed when the surface was compressed. A limiting area/molecule for cholesterol of 39Å^2 compared well with the value of 40Å^2 quoted in the literature (Willmer, 1961; Taylor & Haydon, 1965).

Interaction between the steroids and lecithin or cholesterol was investigated either by spreading the lipids on water and then on saturated aqueous solutions of the steroid, or by spreading equimolar solutions of steroid and lipid and comparing the resulting surface pressure-area curves with those obtained by spreading the lipids alone. As the two methods of studying the interaction involved the penetration of the film by the steroid or the diffusion of the steroid from the monolayer, the process was observed over 2 h in all cases. At least three determinations of the surface pressure-area isotherm were made in each experiment. The first was recorded 5 min after spreading of the film; after completion of the sweep, the barrier was returned to its zero position. After 1 h a second experiment was performed and a third after 2 h. In some cases, e.g. with cholesterol–chlormadinone acetate, the apparent area per molecule of 53–54Å^2 did not change after the first reading. The values recorded in Table 1 are considered to be equilibrium areas.

Steroid solubilities were determined at 25° by equilibrating an excess quantity of steroid with water for 48 h, removing the supernatant and recording the ultraviolet spectrum in 1 cm or 4 cm cells of a dilution in ethanol. E (1%, 1 cm) values were obtained from the literature or determined from calibration graphs obtained in ethanol–water mixtures. Lynestrenol does not absorb sufficiently for this technique

to be used. Norethinodrel has no native ultraviolet absorption spectrum, but it is readily quantitatively converted into norethinodrone, which absorbs in the ultraviolet region, by the action of hydrochloric acid (Bastow, 1967).

Haemolysis. Erythrocyte suspensions for haemolysis experiments were prepared by centrifuging citrated blood at 1500 *g* for 10 min. The plasma was carefully removed and 1 ml of the erythrocyte layer was mixed with 154 mM NaCl in 10 mM phosphate buffer at pH 7, to give a total volume of 12·5 ml. This served as stock suspension. Haemolysis experiments were made as follows:

0·1 ml of the stock erythrocyte suspension was added to 1 ml of 130 mM NaCl in 20 mM sodium phosphate buffer, pH 7, with 7 ml of steroid solution in water. For the control water (7 ml) was used instead of the steroid solution. The mixture was kept at room temperature for 5 min and then centrifuged for 45 s at 1500 *g*. The haemoglobin content of the clear supernatant was measured by recording the absorbance at 543 nm using a Unicam SP600. All experiments were made at least in triplicate.

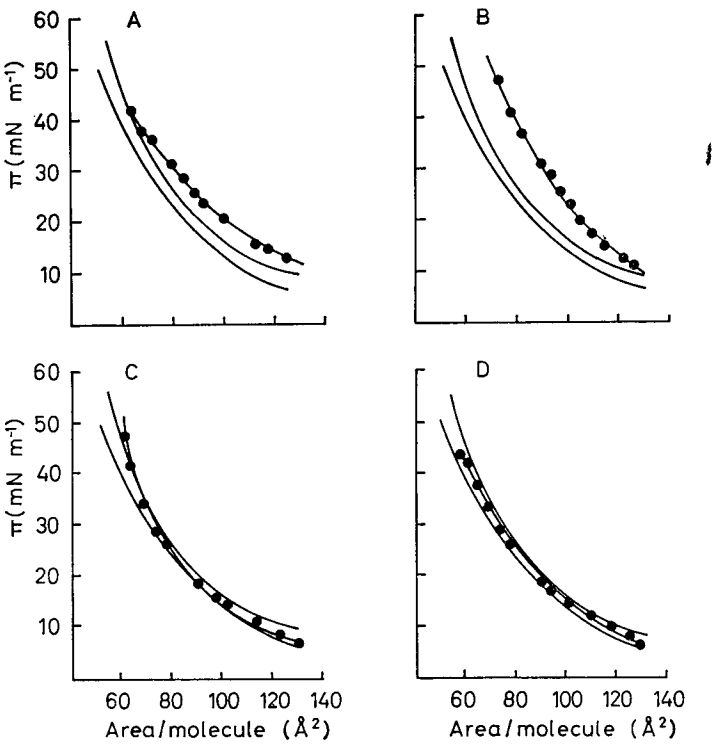

FIG. 1. Surface pressure (π)—area (A) for lecithin and some lecithin-steroid combinations. Lines with no points represent limits of results for lecithin from 10 experiments. A, in presence of ethinyloestradiol; B, in presence of lynestrenol; C, in presence of progesterone; D, in presence of oestradiol.

RESULTS

Representative surface pressure (π)—area (A)—plots are shown in Figs 1 and 2. The error in determining the π-A curve for the lipids themselves is shown from the spread of results from all the experiments conducted. Interaction, if it occurs, should be manifested by an increase in surface pressure at any given area. If the steroid

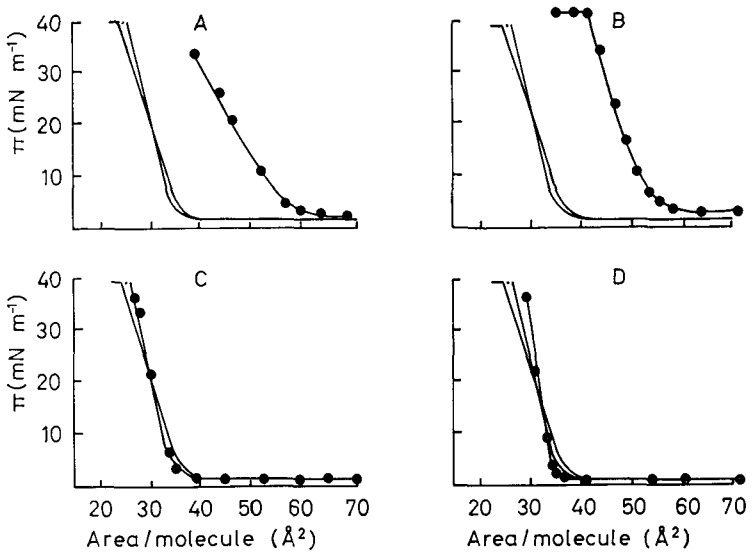

FIG. 2. Surface pressure (π)—area (A) curves for cholesterol and some representative cholesterol-steroid combinations. Lines with no points represent limits of results obtained in 10 experiments with cholesterol. A, results in presence of progesterone and B, chlormadinone, showing interaction, and C, medroxyprogesterone acetate and D = oestradiol showing lack of obvious interactions.

Table 1. *Apparent area per molecule of lecithin and cholesterol in mixed steroid-lipid monolayers.*

Steroid	Lipid	Limiting area/molecule Å²	Area at π = 20 mN m⁻¹	Interaction
—	Lecithin	98	84–91	—
A. *Oestrogens*				
Ethynyl oestradiol ..	"	133	103	Yes
Stilboestrol	"	125	—	Yes
Mestranol	"	98 (110*)	96 (105*)	?
Oestradiol	"	98	87	No
B. *Progestogens*				
Chlormadinone acetate ..	"	110 (107*)	103 (98*)	Yes
Lynestrenol	"	114	97	Yes
Megestrol acetate ..	"	110 (102*)	96	Yes
Norethinodrel	"	104	88	No
Norethindrone	"	101 (91*)	86 (82*)	No
Progesterone	"	98	88 (86*)	No
Medroxyprogesterone acetate	"	96 (100*)	90 (100*)	No
—	Cholesterol	39	37·5	—
A. *Oestrogens*				
Mestranol	"	44 (53*)	40 (42*)	Yes
Stilboestrol	"	46	41	Yes
Ethynyl oestradiol ..	"	39	—	No
Oestradiol	"	38 (43*)	39·5	No
B. *Progestogens*				
Progesterone	"	56	46	Yes
Chlormadinone acetate ..	"	49 (53*)	47·5	Yes
Lynestrenol	"	44	41	Yes
Megestrol acetate ..	"	38	—	No
Norethinodrel	"	39	—	No
Medroxyprogesterone acetate	"	37	—	No
Norethindrone	"	39	—	No

* Determined from mixed solutions of steroid and lipid, spread on water.

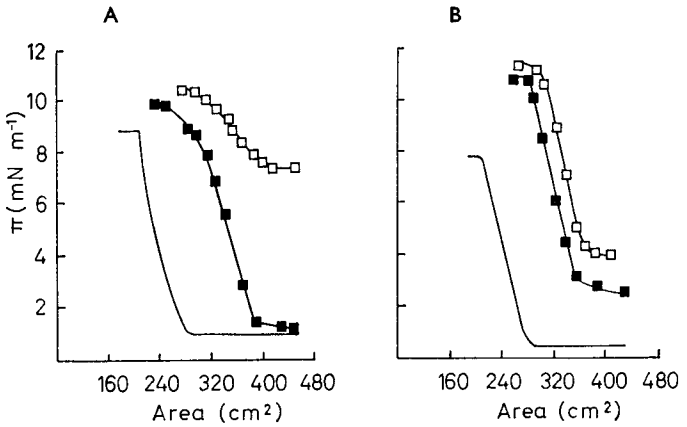

FIG. 3. Surface pressure (π)—area (A) curves obtained A, from lipids extracted from blood and spread on water (—), B, from plasma lipids, in the presence of chlormadinone acetate (■) and medroxyprogesterone acetate (□). The abscissa is the area occupied by a dilution of the lipid extract containing an unknown quantity of lipid(s).

Table 2. *Aqueous solubilities of steroids studied.*

Steroid	Temperature °C	Solubility μmol litre^{-1}	Solubility μg ml^{-1}	Source
Oestradiol	20	16·5	4·5	Gale & Saunders (1971)
	25	18·4	5	This work and Kabasakalian & others (1966)
	37	15·0	4·1	Heap & others (1970)
Ethynyl oestradiol	20	34·4	—	Gale & Saunders (1971)
	25	33·7	10	Kabasakalian & others (1966)
Stilboestrol	25	29·1	7·8	This work
Mestranol	37	4·8	1·5	Sundaram & Kincl (1969)
Norethinodrel	25	12·7	3·8	This work
Norethindrone	25	13·4	4	This work
	37	30·2	9	Sundaram & Kincl (1969)
Progesterone	25	27·7	8·7	This work
	—	28·6	9·0	Kabasakalian & others (1966)
	37	138·0	27·0	Sundaram & Kincl (1969)
	—	48·0	15·1	Heap & others (1970)
Chlormadinone acetate	25	4·9	2·0	This work
Medroxyprogesterone acetate	25	12·4	4·8	This work
Megestrol acetate	37	5·2	2·0	Sundaram & Kincl (1969)
Lynestrenol	25	*	*	

* No ultraviolet absorption.

spread from a mixture is not retained in the monolayer the pressure-area curve obtained should be identical to that of the lipid alone. Where penetration occurs, or where the steroid is retained in the monolayer, a marked increase in π should be noted. Examples are given in Figs 1 and 2 of the behaviour of steroids which interact and of those steroids which are thought not to interact with the lipids studied. From the π-A plots the data given in Table 1 was assembled. Here the limiting areas for lecithin and cholesterol molecules are listed in the absence of, and presence of, the oestrogens and progestogens studied. The areas per molecule calculated in the presence of steroids are apparent areas as the calculation is based on the content of lipid originally spread on the surface. The apparent areas per molecule at a surface pressure of 20 mN m^{-1} are also listed in Table 1.

Lipids extracted from the platelets and spread on water showed interactions with the selected steroids tested (Fig. 3).

Table 3. *Protection against haemolysis by steroids at saturation concentration.*

Steroid	Absorbance* at 543 nm	Number of experiments	% protection†
Control (water)	1·09	(6)	—
Progesterone	0·97	(6)	11
Oestradiol	0·89	(6)	18·3
Stilboestrol	0·68	(6)	37·6
Control	1·07	(5)	—
Chlormadinone acetate	0·85	(5)	20·6
Ethynyl oestradiol	0·98	(5)	8·4
Norethinodrel	1·02	(5)	4·7
Control	1·05	(4)	—
Mestranol	0·87	(4)	17·1
Lynestrenol	0·98	(4)	6·7
Medroxyprogesterone acetate ..	0·92	(4)	12·4
Control	1·06	(3)	—
Northindrone	1·03	(3)	2·8
Megestrol acetate	0·90	(3)	15·1

* See text.

† Calculated as $\frac{A_{control} - A_{steroid}}{A_{control}} \times 100$.

The group of steroids discussed in this paper all have low aqueous solubilities (Table 2) as expected from this type of compound, but all had sufficient solubility in the subphase to fail to form monomolecular films when spread on water alone.

Results of the haemolysis experiments are shown in Table 3 as the percentage protection afforded by the steroids at their maximum solution concentrations.

DISCUSSION

There is no obvious explanation for the fact that some of the steroids studied interact with the pure monolayers and that some do not. There may be two reasons for a lack of steroid interaction with the lipids. A high aqueous solubility would mean that the drug had a low lipid partition coefficient. A bulky molecular shape would preclude close packing of steroid and lipid and prevent strong interactions between the two species in spite of favourable lipophilicity. One can therefore only make comparisons between steroids having closely related structures.

Chlormadinone acetate interacts with cholesterol monolayers but megestrol acetate and medroxyprogesterone acetate do not. Of the three compounds, medroxyprogesterone acetate is most soluble in water (4·8 μg/ml). Megestrol acetate has a solubility less than 2 μg/ml at 25° (2 μg/ml at 37°). Chlormadrinone acetate has a solubility of 2·0 μg/ml. The difference in degree of interaction is undoubtedly marginal, the acetoxy group of each molecule preventing any significant interactions from occurring.

Lynestrenol, norethinodrel and norethindrone have closely related structures, but only lynestrenol interacts with cholesterol. Lynestrenol differs from norethindrone only in the absence of a keto-group at the 3-position and thus is likely to be less water-soluble than norethindrone or norethinodrel, so the trend is expected.

The conversion of a ring A-en-one, C_{10} methyl system to ring A phenol results, according to the analysis by Heap, Symons & Watkins (1970), in a decrease in aqueous solubility but little change in lipid affinity. Hence as norethindrone is non-interacting, 17-ethynyloestradiol would be expected to be also, and this found to be so. The

3-methyl ether of ethynyloestradiol, mestranol, is less soluble in water than is norethisterone and thus likely to be more lipid-soluble. This could explain the difference exhibited by these compounds (Table 1). Oestradiol itself does not interact with cholesterol and this may be related to the possession of two hydroxy groups at opposite ends of the molecule: this would tend to encourage a horizontal orientation at the interface, although stilboestrol which also has a dipolar structure interacts. Taylor & Haydon (1965) have suggested that molecules which prefer a horizontal orientation and interact by attaching themselves to the underside of the monolayer would not cause changes in lipid π-A curves. It may be that interactions other than those detected by this monolayer technique occur with the lipid films.

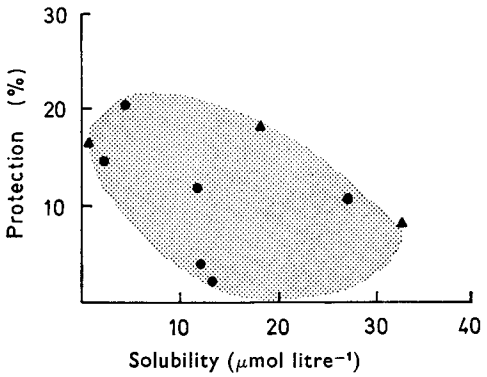

FIG. 4. The relation between the protection against osmotic stress haemolysis in the presence of steroids at saturation concentration, and the solubility of the steroids studied. ▲ oestrogens. ● progestagens.

In Fig. 4 it is evident that only an imprecise relation between aqueous solubility and the degree of protection against haemolysis can be drawn. While imprecise, the trend is as expected: in general as drug solubility increases the interaction with a lipid membrane will decrease, and thus the most soluble materials will be unable to protect the erythrocyte membrane against lysis.

Equilibrium solubility values depend not only on the hydrophobic nature of the steroid molecules, but also on the energy required to disrupt the crystal: the latter depends on, *inter alia*, the closeness of packing of the steroid molecules and hence is related to molecular shape. The average solubility of the steroids which interact with both lecithin and with cholesterol is about 12 μM, and that of the non-interacters is about 16 μM in the series studied. The behaviour of stilboestrol does not fit into the trends shown in Fig. 4.

While difficult to collate, the data presented in this paper show that a number of commonly used oral contraceptive steroids have some affinity for cholesterol and lecithin monolayers and for erythrocyte membranes. Interaction of the steroids with the mixed lipids extracted from platelets has also been demonstrated. It may be that the extent of the interaction of the steroid with erythrocytes and platelets is such that little change occurs in their physical properties but it has been observed that changes which *might* have significance *in vivo* do occur. Changes in the electrophoretic mobility of bovine platelets and in the extent of aggregation of the platelets by adenosine diphosphate have been noted in the presence of some of the steroids studied here (Florence & Rahman, 1972).

Acknowledgements

We thank Dr. B. A. Hems of Glaxo Research Ltd. and Professor P. H. Elworthy (of the University of Manchester) for making funds available which allowed the project to proceed.

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