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THE ACTION OF STEROIDS AND TRITON X-100 UPON PHOSPHOLIPID/CHOLESTEROL STRUCTURES*

GERALD WEISSMANN,† GRAZIA SESSA and SIGMUND WEISSMANN

Department of Medicine, New York University School of Medicine, New York, N.Y., and the Materials Research Laboratory, College of Engineering, Rutgers University, New Brunswick, N.J., U.S.A.

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Abstract—Purified lipids (ovolecithin, cholesterol, dicetylphosphate, in 70:10:20 molar ratios) swell spontaneously in salt solutions to form spherules which behave as salt-containing compartments limited by discrete membranes. These structures exhibit permeability characteristics for cations, anions, and water which are qualitatively similar to those occurring across natural membranes. After spherules were permitted to form in the presence of 0·145 M $H_2PO_4^-$, CrO_4^{2-}, glucose, or glycine, any excess marker ions or molecules which remained uncaptured in the aqueous phase between lamellae were removed by dialysis against 0·145 M NaCl/KCl. Subsequently, spherules were exposed to steroids (5 mM), Triton X-100, and streptolysin S, agents that affect the membranes of lysosomes, mitochondria, and erythrocytes. Progesterone, desoxycorticosterone (DOC), diethylstilbestrol, and 5 β-H steroids such as etiocholanolone, considerably accelerated the leakage of anions, glucose, and glycine into the surrounding medium, as did Triton and streptolysin. In contrast, cortisone, cortisol, and chloroquine, which protect natural membrane-bounded structures such as lysosomes, retarded leakage of the markers. It was possible to form spherules that contained cortisone *preincorporated* into the lipid lamellae. At an optimum molar preincorporation of 1 per cent, structures that contained cortisone (but not its inert metabolite tetrahydrocortisone) proved more resistant to leakage induced by other steroids. Negatively stained preparations in the electron microscope showed that the discrete spherules were formed of multiple concentric lamellae which were separated by compartments of approximately 50 Å distance. Triton X-100 completely disrupted these structures: only amorphous debris was formed. In contrast, DOC-treated samples showed the emergence of new forms: although small, discrete spherules remained, the predominant forms were a series of elongated, tubular, strand-like structures, loops and whirls of which maintained a lamellar substructure. These studies suggest that steroids interact with artificial lipid spherules to produce changes in their permeability which resemble steroid-induced changes in natural membranes, most likely by provoking a structural rearrangement of lipid layers.

PREVIOUS studies have documented the actions of steroids upon the membranes of cells and their organelles.[1–5] Progesterone, desoxycorticosterone, and etiocholanolone, for example, each appears to *labilize* the membranes of liver lysosomes,[1, 3, 5] leukocyte granules,[6, 7] and mitochondria.[2] In contrast, steroids containing oxygen groups at

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† Senior Investigator, the Arthritis Foundation.

C-11 or C-17 are relatively inert, while cortisone, cortisol, their acetates, and prednisolone appear to stabilize membranous structures.[1-5] These observations are in accord with the hypothesis of Willmer,[8] who suggested that steroids exert their biological effects by virtue of their capacity to enter into, and modify the permeability of, bimolecular lipid leaflets which bound cells and their organelles.

This hypothesis requires proof that steroids do, in fact, interact with membranes composed of purified lipids which might reasonably be expected to occur in natural membranes and, furthermore, that such an interaction produces changes in the permeability of purified lipid membranes that resemble those induced by steroids in natural membranes. Earlier studies of artificial systems have not conclusively proved that steroids can interact with purified lipids. Gershfeld and Heftmann[9] found that progesterone, etiocholanolone, and androsterone failed to effect either the surface pressure or potential of monolayers formed by cholesterol, or cholesterol/stearic acid films on water. In contrast, Taylor and Haydon[10] studied the uptake of progesterone into phospholipid/cholesterol monolayers and found that small but perceptible amounts of the steroid were incorporated at high pressure. Furthermore, Bangham and Horne[11] observed that smectic dispersions of lecithin/progesterone in water differed considerably from lecithin/cholesterol in water, when viewed by negative staining techniques in the electron microscope.

It has recently become possible to examine this problem more directly. Bangham *et al.*[12] have shown that purified phospholipids swell spontaneously in aqueous salt solutions to form liquid crystals which behave as salt-containing compartments limited by discrete bimolecular membranes. These structures exhibited permeability characteristics for simple univalent cations, anions, and water which were qualitatively similar to those occurring across biological membranes. Using such structures, upon which a net negative membrane charge was imposed by the preincorporation of dicetylphosphate, we have previously shown[13] that steroids which disrupt lysosomes and erythrocytes *increased* the leak of sequestered cation from the artificial spherules.* Other membrane-disruptive agents, such as streptolysin S and gramicidin, also augmented cation release. In contrast, cortisone, cortisol, cortisone acetate, and chloroquine, which protect lysosomes and erythrocytes, *diminished* the leak of sequestered cation.

Despite these observations, it remained possible that the release by membrane-active steroids of cations from lipid spherules represented an effect upon the binding or trapping of individual ions, rather than upon the lipid membranes themselves. Therefore the present study was undertaken in which it will be shown that membrane-active steroids release a variety of "marker" ions and molecules from artificial spherules of phospholipid and cholesterol. The markers included the complex monovalent anion $H_2PO_4^-$, the complex divalent anion CrO_4^{2-}, the *zwitterion* glycine, and glucose. Changes induced by steroids in the gross permeability of the structures were accompanied by characteristic alterations in the morphology of the lipid spherules, as observed by negative staining techniques in the electron microscope. The action of steroids will be contrasted with that of the nonionic detergent Triton X-100 (an alkylphenoxy, polyethoxy surfactant) which has previously been shown capable of

* Because of their predominant morphology, these structures will be referred to as spherules in order to distinguish them from the conventional myelin figures formed by lipids in water in the absence of added anion such as dicetylphosphate.

disrupting several biological membranes.[1, 3–7] Since these studies have shown that steroids influence the permeability of model lipid structures to cations, anions, glucose, and glycine, in a manner analogous to the action of steroids upon the permeability of natural membranes, they support, in general, the hypothesis of Willmer.[8]

MATERIALS AND METHODS

Ovolecithin was obtained from General Biochemicals, Chagrin Falls, Ohio, as eluted from silicic acid columns in chloroform; thin-layer chromatography showed it to be free of contamination by breakdown products. The chloroform solution of ovolecithin was stored at −20°C. Cholesterol was obtained from Nutritional Biochemicals, Cleveland, Ohio; dicetylphosphate (DCP) from K & K Biochemicals, Plainview, N.Y. Steroids, the properties of which have been previously described,[3, 4] were obtained from Steraloids Inc., Pawling, N.Y. Triton X-100 was the gift of Rohm & Haas, Inc., Philadelphia, Pa., and streptolysin S was generously supplied by Dr. A. W. Bernheimer of New York University. Glucose oxidase reagent was obtained as the commercially available Glucostat, from Worthington Biochemicals, Freehold, N.J. Chloroquine diphosphate, neutralized before use, was obtained from Winthrop Pharmaceuticals.

The phospholipid/cholesterol structures, with negative membranes imposed by the anion DCP, were prepared by a modification of procedures described in detail elsewhere.[12, 13] For each set of experiments, 10 μmoles ovolecithin, 2·86 μmoles DCP, and 1·43 μmoles cholesterol ($\times$ the number of aliquots, usually six) were added in chloroform to a round-bottomed 100-ml flask. The contents were evaporated in a rotary vacuum pump apparatus so that the dried lipid film was evenly distributed over the lower surface of the vessel. Finally, 1 ml of 0·145 M K_2CrO_4, KH_2PO_4, glucose, or glycine per aliquot was added to the flask, and the lipids were dispersed in the aqueous medium by anchoring the covered flask in contact with a vibrating mixer (Cyclo-mixer, Clay-Adams Co., New York, N.Y.) so that small drops of the dispersion were constantly thrown against the side of the flask during vibration (10 min, 23°). Thereafter, the lipid structures were permitted to complete their spontaneous swelling* for 18 hr at 4°. After a second period of vibratory mixing (5 min) the lipid spherules, which were suspended in aqueous solutions of the "markers," i.e. CrO_4^{2-}, $H_2PO_4^-$, glucose, or glycine, but which had by this time also sequestered these markers within the aqueous compartments of their lipid lamellae, were placed in Visking dialysis tubing. They were first dialyzed for 30 min at 23° against 900 ml of an equimolar mixture of NaCl/KCl (total molarity), and dialysis was continued against fresh salt solution until the last dialysate was free of detectable anion, glucose, or glycine. Usually four hourly changes of 900 ml each were sufficient. One-ml aliquots of the dialyzed spherules, now containing only those marker ions or molecules which had been sequestered, were placed in separate small dialysis sacs. Test steroids, dissolved in 0·05 ml ethanol, or 0·05 ml ethanol alone, were dispensed into the sacs and immediately mixed with the suspended spherules by inversion. To some suspensions, 0·2 ml Triton X-100 in NaCl/KCl was added to a final concentration of 0·1%, v/v. Streptolysin S was dissolved in 0·1 ml NaCl/KCl. The sacs were tied, placed in small, narrow-bore test tubes containing 5 ml NaCl/KCl solution, and

* This term has been discussed in detail elsewhere (Bangham *et al.*[13]).

shaken in a water bath maintained at 37°. At intervals noted below, the sacs were transferred to fresh test tubes also filled with NaCl/KCl, and the marker ions or molecules which had escaped through the dialysis tubing during these "leak" periods were determined. After each experiment, aliquots of each 900-ml dialysate, of the leak fluids, and of the suspensions remaining in the sacs, were analyzed for the marker ions or molecules by procedures detailed below.

In some experiments, the spherules, after incubation with test steroids or Triton X-100, were not placed in small dialysis tubing, but were immediately centrifuged at 100,000 *g* for 20 min in the SW 39 swinging-bucket head of a Spinco model L2 ultracentrifuge, and the release of chromate determined by analysis of the supernatant and of the pellet.

In experiments with glucose oxidase, the commercially available Glucostat reagent, containing the enzymes glucose oxidase, peroxidase, and chromogen, was made up in 0·145 M NaCl/KCl solution (90 ml salt solution being substituted for the distilled water required to solubilize each reagent vial) and 5 ml of this solution placed in the small test tubes during the "leak" periods.

When steroids were to be "preincorporated"* into the spherules, cortisone or tetrahydrocortisone was dissolved in chloroform to constitute 1 per cent (molar ratio) of the total lipid before the initial lipid mixture was taken to dryness.

Analysis. Chromate was determined by its absorbancy at 380 mμ, $H_2PO_4^-$ as *Pi* by the Fiske-SubbaRow method,[14] glucose by the glucose oxidase method,[15] and glycine by the ninhydrin method of Moore and Stein.[16]

For determination of the total marker ions or molecules remaining in the spherules after the leak periods, the sacs were opened, boiled for 20 min to destroy the spherules, centrifuged at 20,000 *g* in a Servall Superspeed centrifuge for 20 min to remove lipid debris, and the supernatants analyzed. This procedure was sufficient for chromate, glucose, and glycine. However, for phosphorus determinations, the tubes were read before addition of the reducing agent, and this blank turbidity value was subtracted from the final absorbance at 660 mμ.

Electron microscopy. Negative staining with potassium phosphotungstate was carried out according to the method of Bangham and Horne.[11] Suspensions of lipid spherules, swollen in 0·145 M glucose as described above, were exposed to ethanol alone (0·05 ml ethanol to 1·0 ml suspension), to Triton X-100 (0·1%, v/v), or to 5 mM DOC in ethanol, for 20 min at 23°. The suspensions were than placed for 3 hr in a vacuum flask at 4° (to permit transport of the material from New York to New Brunswick), mixed with an equal volume of 3% potassium phosphotungstate, and sprayed upon a cellulose-carbon-coated grid by means of an ordinary nebulizer. They were examined in an Hitachi HU 11A electron microscope.

RESULTS

Capture and release of CrO_4^{2-}

When the lipid spherules were permitted to swell in the presence of 0·145 M CrO_4^{2-}, an average of 9·15 per cent of the available anions was captured, presumably in

* This term is used for the addition of steroid to the lipid suspensions before swelling[12, 13] and should not be considered as an unequivocal assumption that all added steroid was actually inserted into the final spherules.

the aqueous compartments of the lamellar structures. Total recoveries of added CrO_4^{2-} in 20 experiments averaged 97 per cent (range 88–104 per cent); these figures represent the sum of anions recovered in the dialysates, the ions released during the leak period, and those that remained associated with the spherules after 1 hr.

TABLE 1. RELEASE OF CrO_4^{2-} FROM PHOSPHOLIPID/CHOLESTEROL SPHERULES

Agent added	No. of expts.	% Chromate released (as % released from control at 30 min) 30 min Mean ± S.E.M.	60 min Mean ± S.E.M.
Ethanol (control)	20	100	146 ± 2·93
Triton X-100	11	471 ± 16·6*	744·2 ± 23·2*
Diethylstilbestrol	8	245·3 ± 20·6*	366·0 ± 57·7*
DOC	4	209 ± 33·2*	319·2 ± 46·2*
Etiocholanolone	8	138·0 ± 12·1†	210·8 ± 20·5†
Progesterone	4	131·5 ± 7·9†	175·3 ± 9·6†
17-β-Estradiol	3	117·0 ± 7·4	171·0 ± 10·9
Androsterone	2	105·5 ± 3·9	156·0 ± 10·8
Cortisone	5	89·3 ± 2·6	126·0 ± 6·6
Cortisone acetate	4	81·3 ± 1·2†	108·0 ± 1·7†

Control samples released 0·58 μmoles ± 0·12 of CrO_4^{2-} at 30 min. Steroids added at 5 mM concentration in 0·05 ml ethanol; Triton X-100 present 0·1%, v/v.
* $P < 0·01$ vs. paired controls (Wilcoxon test).
† $P < 0·05$ vs. paired controls (Wilcoxon test).

In Table 1 are shown the effects of various agents upon the release of CrO_4^{2-} from the spherules. Since control samples released 0·58 μmoles of anion, and each ml of spherule suspension contained an average of 15·3 μmoles of anion before the leak period, it can be calculated that controls released 3·7% of captured CrO_4^{2-} at 30 min. These figures indicate that relatively little chromate was captured from the swelling medium, and that relatively little was lost (see below). Nevertheless, leakage was appreciably enhanced in the presence of Triton X-100 or various steroids. Those steroids which have previously been shown capable of inducing cation release from lipid spherules[13] (i.e. diethylstilbestrol, DOC, etiocholanolone, progesterone) were active in augmented CrO_4^{2-} leak. The 5 α-H isomer of etiocholanolone, androsterone, was as inert upon these model structures as upon lysosomes and erythrocytes.[3, 4] 17 β-Estradiol was similarly inactive. Cortisone and its acetate appeared to retard the release of CrO_4^{2-}, a finding in accord with the effect of these steroids upon natural membranes.[3–6]

Figure 1 shows the results of an experiment in which steroids (5 mM) were compared with the effects of chloroquine (2·5 mM). It has previously been found that chloroquine is optimally "protective" for membranes at half the molar concentration of steroids when the latter are dissolved in ethanol.[5] Diethylstilbestrol and DOC accelerated the release of anion, whereas cortisone, cortisone acetate, and chloroquine retarded release from the lipid spherules.

It remained possible that Triton or the steroids might release anions, not by virtue of a direct effect upon formed membranous structures, but by an effect upon the rates of dialysis of CrO_4^{2-} or its binding by unstructured lipid. Therefore, experiments

were performed in which it was shown that none of the added agents significantly altered the rates of anion leakage through dialysis sacs in the absence of formed spherules or when the swollen spherules were permitted to stand for 6 days in the cold. The latter procedure resulted in loss of the lamellar structure.

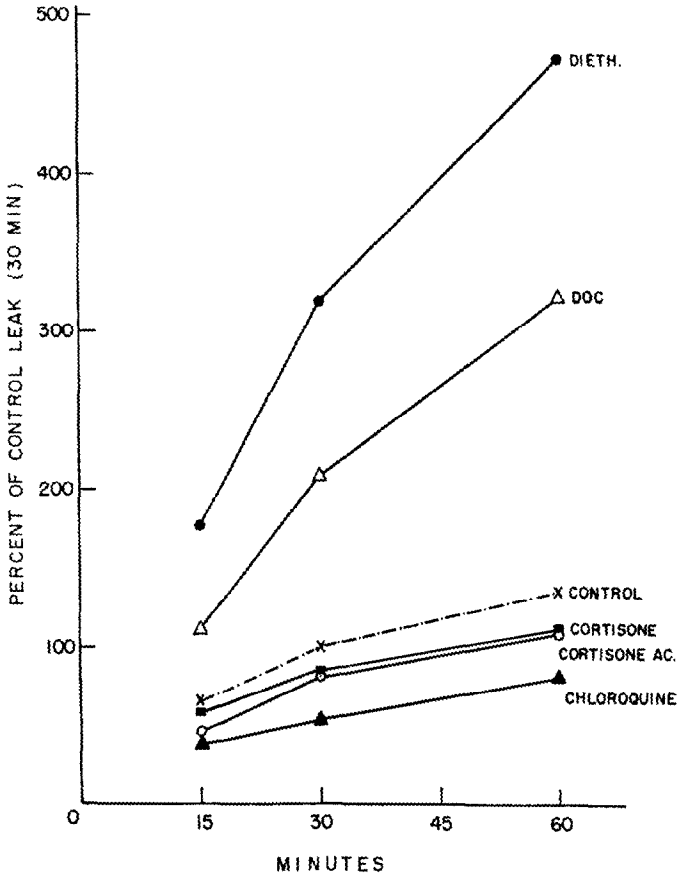

FIG. 1. Release of CrO_4^{2-} from artificial spherules composed of phospholipid/cholesterol. Steroids added to a final concentration of 5 mM in 0·05 ml ethanol, chloroquine at 2·5 mM. Controls had 0·05 ml ethanol added. Dialysis sacs containing 1 ml spherule suspension (see text for preparation) were permitted to leak chromate into tubes containing 5 ml of 0·145 M NaCl/KCl, and anions which had escaped from the spherulites through the dialysis tubing were determined. Results expressed as the cumulative percentage of anions released from control suspensions at 30 min. Dieth. = diethylstilbestrol; DOC = desoxycorticosterone; cortisone ac. = cortisone acetate.

In other experiments, 1 ml aliquots of spherules swollen in 0·145 M $CrO_4^{=}$ were exposed to ethanol, Triton X-100, and diethylstilbestrol (5 mM), but were not transferred to smaller dialysis sacs. Instead, they were centrifuged for 20 min at 100,000 *g* in a swinging-bucket rotor of the ultracentrifuge. Each milliliter of suspension had sequestered 18·5 μmoles of CrO_4^{2-} before treatment. Control spherule suspensions released 4·8 μmoles of anion (26 per cent of the total captured anion vs. 3·7 per cent in dialyzed samples), Triton-treated suspensions released 10·6 μmoles, and diethylstilbestrol-treated suspensions released 6·1 μmoles of anion into the clear

supernatants. These experiments indicated not only that dialysis was unnecessary to demonstrate steroid action on the spherules but also that centrifugation imposed an unacceptable degree of fragility upon the lipid structures.

Capture and release of $H_2PO_4^-$

Spherules permitted to swell in 0·145 M $H_2PO_4^-$ captured 20·2 per cent of available anion. The recoveries of added anion averaged 95·0 per cent (range 89–99 per cent) in six experiments. From Table 2 it may be seen that controls released 2·7 μmoles of anion at 30 min. Since each ml of spherule suspension contained an average of 32 μmoles $H_2PO_4^-$, it may be calculated that an average of 8·4 per cent of anions was released from controls. Therefore the spherules captured and released approximately twice as much monovalent anion as divalent anion (CrO_4^{2-}) of similar size. This observation would confirm the premise that exchange with the surrounding salt solution was on an equivalency basis.

TABLE 2. RELEASE OF $H_2PO_4^-$ FROM PHOSPHOLIPID/CHOLESTEROL SPHERULES

Agent added	No. of expts.	% $H_2PO_4^-$ Released (as % released from controls at 30 min) 30 min Mean ± S.E.M.	60 min Mean ± S.E.M.
Ethanol (control)	8	100	159 ± 10·2
Triton X-100	5	376 ± 147·0	715 ± 258·0
DOC	3	230 ± 72·0	476 ± 212·0
Etiocholanolone	3	150 ± 20·9	289 ± 70·7
Diethylstilbestrol	3	148 ± 30·6	170 ± 81·7
Progesterone	3	106 ± 5·8	169 ± 9·9
Cortisone acetate	2	94·7	142
Cortisol	2	93·4	131
Cortisone	2	82·5	127
Streptolysin S	2	165·0	222

Controls released 2·70 ± 1·83 μmoles $H_2PO_4^-$ at 30 min. Steroids added at 5 mM concentration in 0·05 ml ethanol. Triton present, 0·1%, v/v; streptolysin S, 2000 hemolytic units/ml.

Table 2 also shows the effect of steroids upon the release of anion from the lipid spherules. Although diethylstilbestrol did not release significant quantities of $H_2PO_4^-$, the other steroids that induced chromate leak were able to release $H_2PO_4^-$. Cortisone and cortisol (but not cortisone acetate) did not appreciably affect the leak of anion from the structures. Differences among these steroids in "protection" were not regarded as significant. In these experiments, steroids were compared not only with Triton but also with streptolysin S, a protein active upon biological membranes. Both of these released $H_2PO_4^-$ from the spherules; Triton appeared to increase leakage to a considerably greater extent than the lysin.

Capture and release of glucose

Spherules permitted to swell in glucose (0·145 M) captured 43·8 per cent of available glucose. In six experiments, recoveries averaged 103 per cent (range 98–107 per cent). From Table 3 it may be seen that controls released 10·9 per cent μmoles glucose at 30 min. Since each ml of spherule suspension contained 77·8 μmoles

glucose, it is apparent that control suspensions released a mean of 14·0 per cent of the total glucose that had been captured. These figures indicate that the spherules both captured and released more glucose than the two anions.

Table 3 shows the effects of steroids on release of glucose. DOC, etiocholanolone, and progesterone induced leakage at rates greater than controls; diethylstilbestrol was, surprisingly, relatively inert. Cortisol acetate, but not cortisone, was effective in retarding leakage of glucose from the spherules. These findings were compared to those of Triton X-100, which induced severe leakage, and to streptolysin S, the effects of which were comparable to those of the steroids.

TABLE 3. RELEASE OF GLUCOSE EROM PHOSPHOLIPID/CHOLESTEROL SPHERULES

Agent added	No. of expts.	% Glucose released (as % released from controls at 30 min) 30 min Mean ± S.E.M.	60 min Mean ± S.E.M.
Ethanol (control)	6	100	166 ± 5·5
Triton X-100	6	154 ± 7·8*	286 ± 27·9†
DOC	4	148 ± 6·9	234 ± 10·9*
Etiocholanolone	6	117 ± 6·2	224 ± 35·2*
Progesterone	4	122 ± 2·8	193 ± 14·1*
Diethylstilbestrol	3	106 ± 4·4	174·0 ± 2·9
Cortisone	3	99·0 ± 3·3	165 ± 2·5
Cortisol acetate	3	83·3 ± 1·5	154 ± 5·7
Streptolysin S	2	145·0	201

Controls released 10·9 ± 3·8 μmoles glucose at 30 min. Steroids added at 5 mM concentration in 0·05 ml ethanol. Triton X-100 present at 0·1 %, v/v; streptolysin S at 2000 hemolytic units/ml.
* $P < 0·05$ vs. paired controls (Wilcoxon test).
† $P < 0·01$ vs. paired controls (Wilcoxon test).

Capture and release of glycine

Spherules swollen in the presence of 0·145 M glycine captured a mean of 25 per cent of available amino acid. Recoveries were comparable to the other markers. From Table 4 it may be seen that controls released a mean of 8·3 μmoles at 30 min. Since each milliliter of spherule suspensions contained an average of 38 μmoles glycine, control samples released 25·8 per cent of the total captured amino acid by 30 min. Thus the spherules captured less, but released more, of the *zwitterion* glycine than of glucose.

Table 4 shows the effects of steroids upon the release of glycine. Etiocholanolone, DOC, and progesterone all appear to augment glycine leakage but, in these experiments, diethylstilbestrol was the most active of the added steroids, as in release of chromate. Both cortisone and cortisone acetate retarded the release of the amino acid from lipid spherules; Triton showed its usual drastic effect.

The effect of preincorporation of 1 % cortisone upon capture and release of anions and glucose

We have previously shown that phospholipid/cholesterol structures containing 1 per cent cortisone preincorporated were more resistant to leakage of cation induced by other steroids.[13] Table 5 shows the results of experiments in which 1 per cent cortisone was preincorporated into lipid suspensions before these were permitted to

swell in the presence of 0·145 M CrO_4^{2-}, $H_2PO_4^-$, or glucose. The results have been expressed as the percentage of the total marker anion or glucose which was capable of being released at 30 min. by Triton, in order to permit expression of the data in comparable forms and to relate these studies to those previously reported. Spherules prepared with 1% cortisone preincorporated did not capture more or less of these

TABLE 4. RELEASE OF GLYCINE FROM PHOSPHOLIPID/CHOLESTEROL SPHERULES

Agent	No. of expts.	Glycine released* 30 mm % of control at 30 min	Glycine released* 60 min % of control at 30 min
Control	5	100·0	142·0
Triton X-100	4	317·8	452·0
Diethylstilbestrol	2	187·5	283·5
Etiocholanolone	2	130·0	190·0
DOC	2	128·0	204·0
Progesterone	2	113·0	155·0
Cortisone	4	91·9	136·0
Cortisone acetate	4	86·1	129·0

* Results expressed as means. Steroids added at 5 mM concentration in 0·05 ml ethanol; Triton X-100 at 0·01%, v/v. Controls released 8·26 μmoles at 30 min.

TABLE 5. RELEASE OF "MARKER" MOLECULES FROM PHOSPHOLIPID/CHOLESTEROL SPHERULES WITH AND WITHOUT 1 PER CENT CORTISONE PREINCORPORATED

	Chromate*			$H_2PO_4^-$*		Glucose*	
	Control	1% Cortis	1% THE†	Control	1% Cortis	Control	1% Cortis
Ethanol (control)	29·8	20·6	31·2	51·5	19·7	68·5	64·6
Triton X-100*	100	100	100	100	100	100	100
Diethylstilbestrol	49·0	37·0	48·5	57·8	32·7	79·5	59·2
Etiocholanolone	31·6	22·8	29·8	60·0	19·5	85·5	61·2
Desoxycorticosterone	36·0	23·6	34·2	72·5	38·2	101·0	71·0
μmoles released (TX)‡	2·64	2·44	2·53	8·42	9·37	17·1	18·9

* Results are expressed as percentage of release induced by Triton X-100 at 30 min and represent the means of two experiments.
† THE = tetrahydrocortisone.
‡ Micromoles released by Triton X-100 at 30 min.

markers than did control suspensions. The effects of Triton were not modified by the presence of cortisone in the membranes. In direct contrast, it may be seen that leakage of markers induced by diethylstilbestrol, etiocholanolone, or DOC was significantly diminished if the membranes contained cortisone. If, however, the inert metabolite of cortisone, tetrahydrocortisone, was added to the lamellae, leakage induced by the subsequent addition of disruptive steroids did not differ from control membranes.

Effect of steroids and Triton X-100 *upon glucose oxidase activity*

A model system was devised to test the possibility that steroids or detergent would directly influence the rate at which an enzyme encountered its substrate. Sacs of

glucose-laden spherules were therefore permitted to leak glucose into tubes containing glucose oxidase, peroxidase, and chromogen in NaCl/KCl. In such experiments, the swelling mixture had been constituted by adding enough NaCl/KCl to 29 μmoles glucose /ml to bring the total molarity to 0·145 M. Some of the spherule suspensions were prepared to contain 1% cortisone preincorporated. Figure 2 shows that Triton

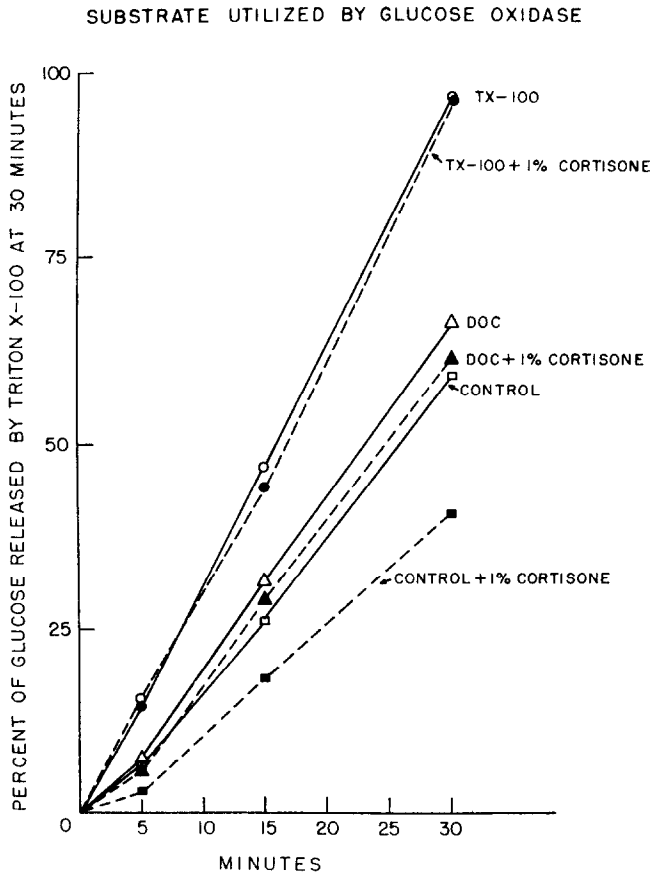

FIG. 2. Release of glucose and its oxidation by glucose oxidase. Spherules of phospholipid/cholesterol were prepared to contain glucose, and dialysis sacs containing 1 ml spherule suspension, with either 0·05 ml ethanol (control), or 0·05 ml ethanol + desoxycorticosterone (4 mM), or Triton X-100, 0·1% v/v, were permitted to leak glucose into a medium composed of 0·145 M NaCl/KCl, glucose oxidase, peroxidase, and chromogen. Activation of the linked assay system was immediately observed as tubes became coloured by chromogen in Triton-treated suspensions. Spherule suspensions which had been formed to contain 1% cortisone preincorporated into the lipid lamellae are indicated by dotted lines. Results are expressed as percentage of glucose released by Triton X-100 to exclude any effect of cortisone preincorporation upon dialysis rates.

and DOC accelerated the formation of reaction product in the linked enzyme assay. During the course of the experiment, Triton-containing tubes appeared almost immediately to become colored by the chromogen. Preincorporation of cortisone did not affect the release of substrate by Triton X-100. However, in DOC-treated suspensions, and especially in control samples, formation of reaction product was

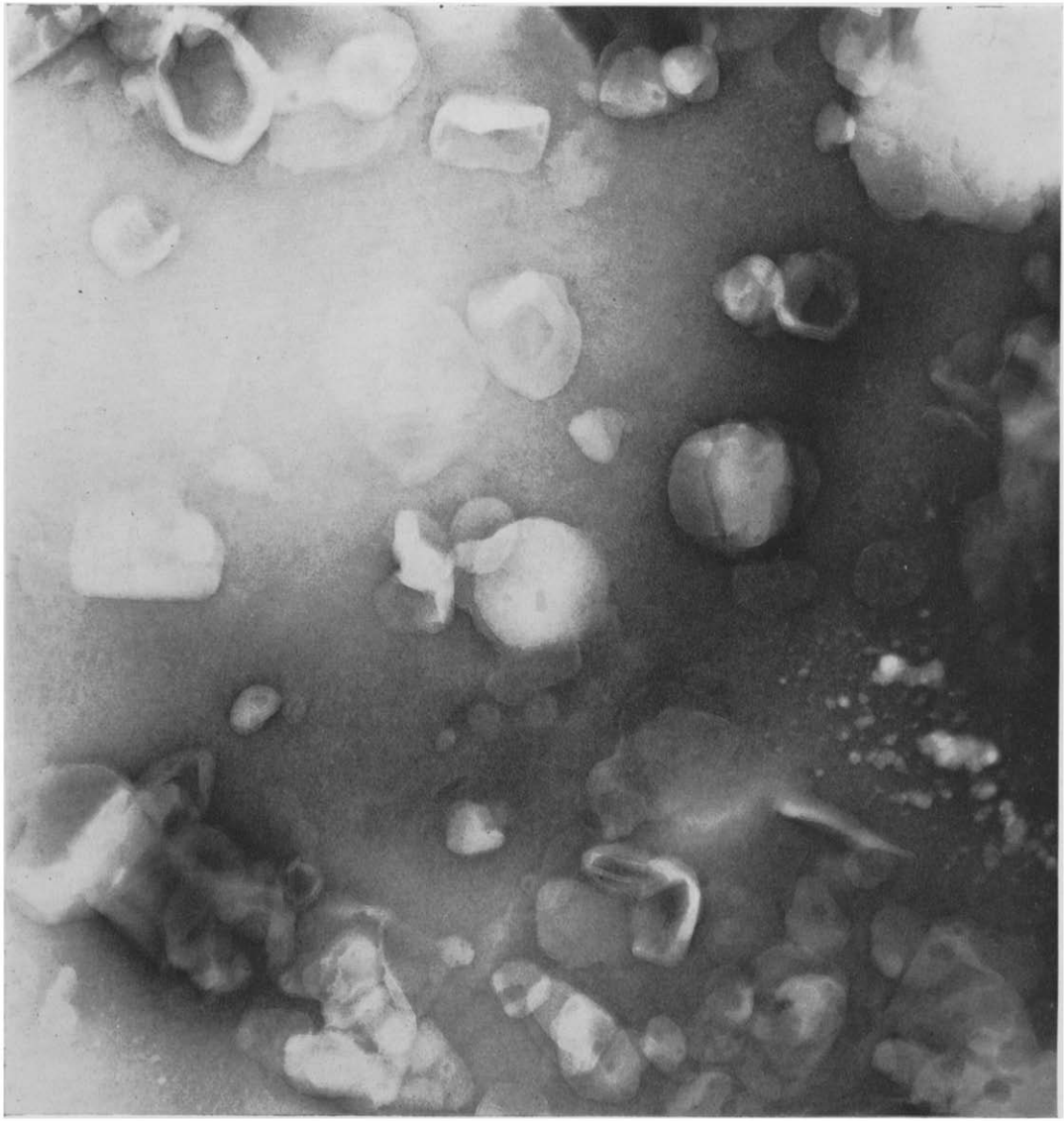

FIG. 3. Phospholipid/cholesterol spherules swollen in 0·145 M glucose and negatively stained with 3 % potassium phosphotungstate. Arrows indicate lamellar substructure; magnification × 100,000. Preparation treated for 20 min at 23° with 0·05 ml ethanol/ml suspension.

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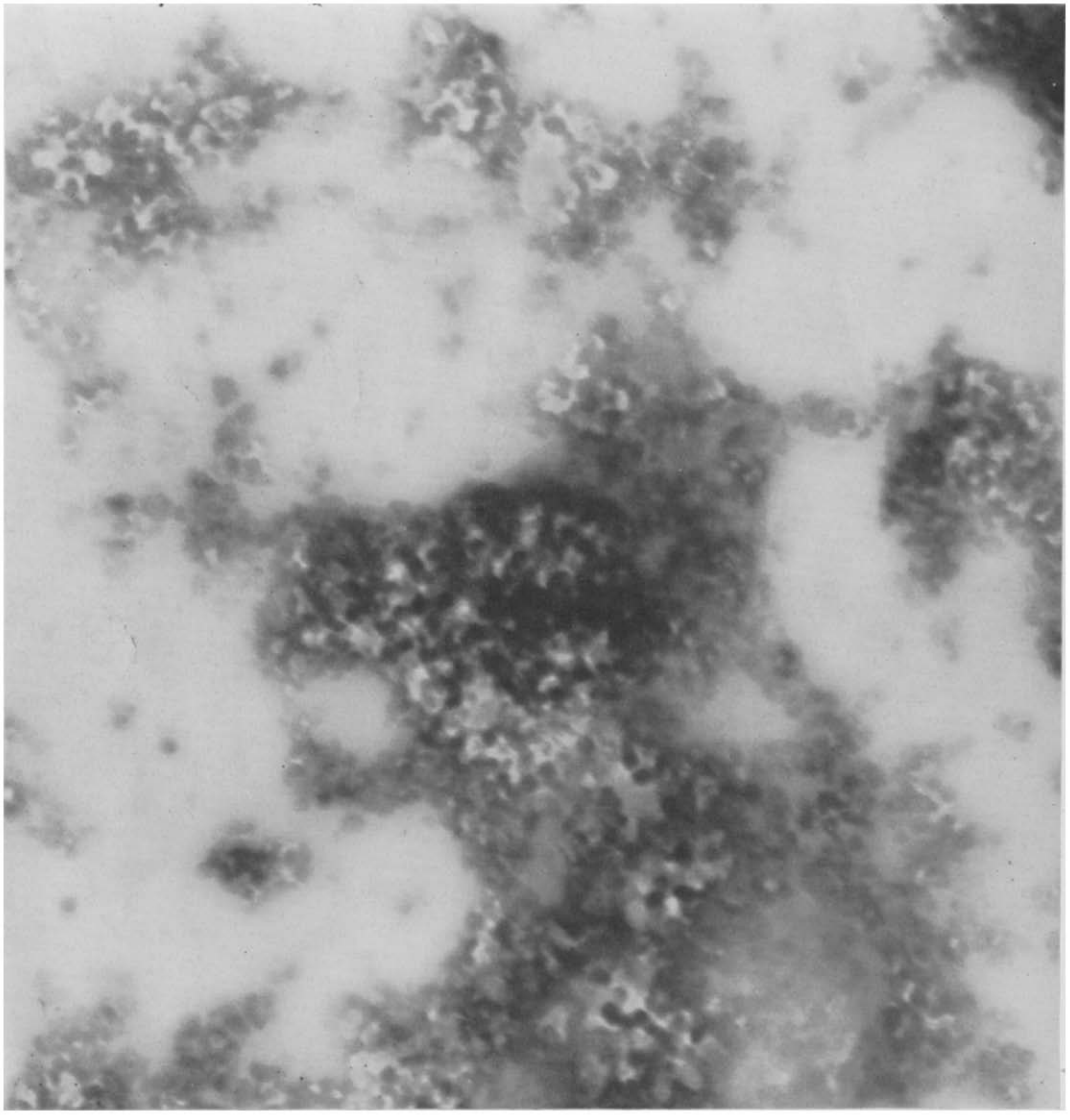

FIG. 4. Preparation identical with Fig. 3 but treated for 20 min at 23° with Triton X-100; magnification × 100,000.

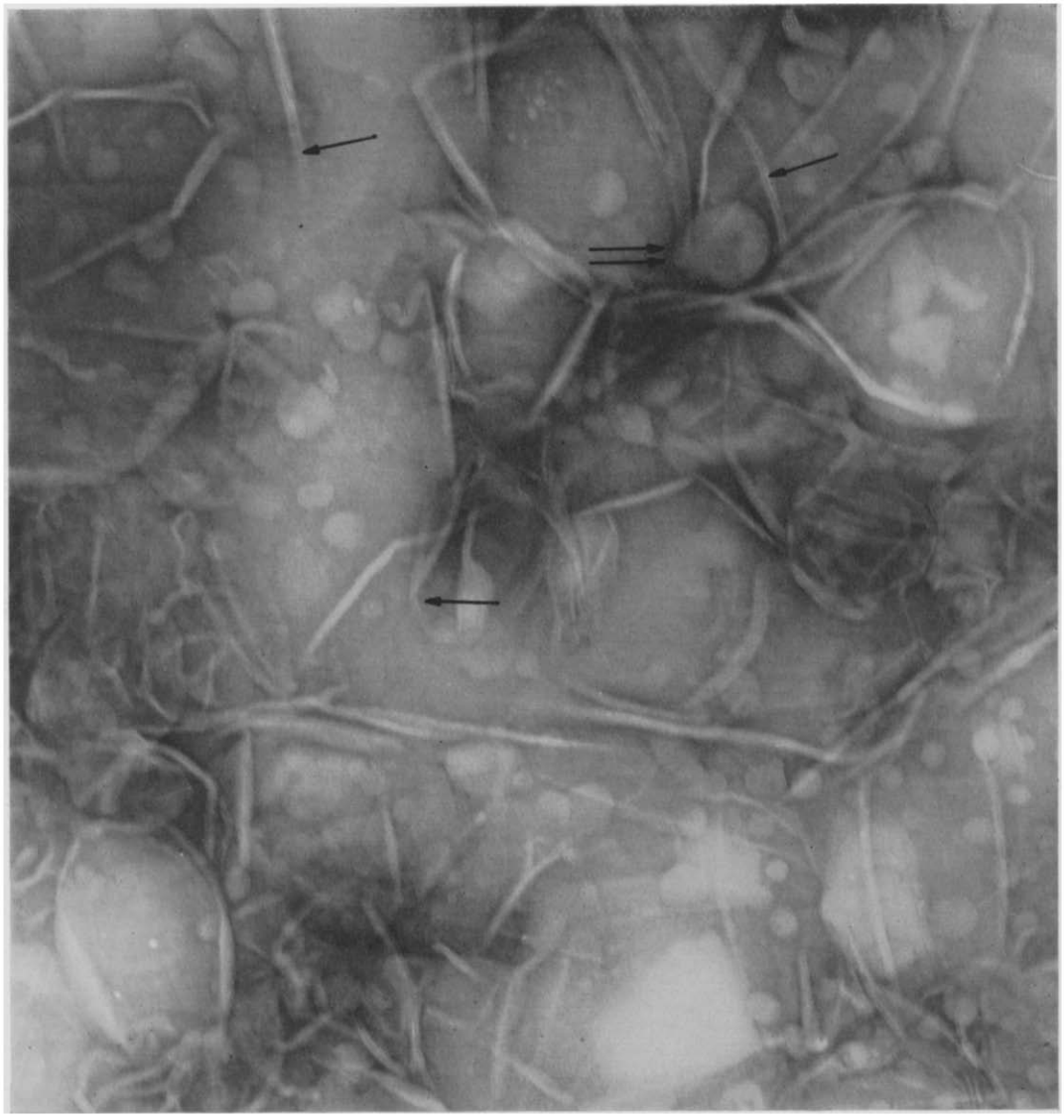

FIG. 5. Preparation as in Fig. 3 but treated with 5 mM desoxycorticosterone in 0·05 ml ethanol for 20 min at 23°. Arrows point to tubular arrays, double arrows to merger of these strands with lamellae of the spherules. Magnification × 100,000.

considerably retarded in tubes that contained sacs filled by spherules formed with 1 per cent cortisone. In similar experiments, chloroquine, cortisone, and cortisone acetate, but not tetrahydrocortisone (all at 5 mM), retarded the formation of reaction product.

Appearance of phospholipid/cholesterol spherules in the electron microscope and their modification by steroids and Triton -X100

When viewed by negative staining techniques, the lipid structures in glucose appeared as discrete spherules, sometimes superimposed upon each other. Most of these measured between 0·2 and 0·8 μ, although larger, composite forms were observed. In appropriate specimens, the spherules appeared to be composed of concentric lamellae, usually 45–55 Å apart (Fig. 3). Therefore these structures resembled those previously described by Bangham *et al.*, with the important difference that spherules prepared with 0·145 M glucose appeared to be much more discrete. Only very rarely were any elongated rod-like structures observed. In specimens which had been incubated with Triton X-100, the distinct spherular organization appeared entirely lost, and no lamellar structure could be identified. The grids appeared to be filled with amorphous material in which no evidence of substructure could be discerned (Fig. 4). In direct contrast to these and to control structures, samples which had been treated with DOC showed the emergence of entirely new forms (Fig. 5). Although smaller, discrete spherules could still be identified, the predominant forms were a series of elongated strand-like structures, loops and whirls of which maintained a lamellar substructure. These strands were frequently observed as a series of double- or triple-layered systems which could conceivably be interpreted as tubular, and which resembled forms infrequently observed by Bangham and Horne.[11] However, the strands were often seen to arise as layered forms which merged with the outermost layers of the native spherules. The distance between such lamellar strands was maintained at approximately 50 Å, but in some multilayered strands this distance was quite variable. Longer exposure to steroids produced a series of frayed strands and flat sheets.

DISCUSSION

The results of these studies provide strong support for the hypothesis of Willmer[8] and, together with previous work,[12, 13] demonstrate that steroids interact with artificial structures composed of biologically relevant lipids. The model system has proved quite useful in that the phospholipid/cholesterol spherulites have now been shown capable of sequestering not only cations[12, 13] but anions, glucose, and glycine. Further studies will show whether these model counterparts of membranes which bound cells and their organelles can be persuaded to swell in the presence of hemoglobin, hydrolases, respiratory enzymes, or nucleic acids. At present, however, the spherules appear to resemble natural structures in their release of dialyzable ions and molecules after exposure to lysolecithin, gramicidin, streptolysin S, Triton X-100, and the steroids. The integrity of the spherules can be promoted by the addition of cortisol and cortisone and their acetates, and of chloroquine. Preincorporation of cortisone, but not its inert isomer tetrahydrocortisone, also stabilized the spherules. Therefore the models responded in the same fashion as do naturally found structures (lysosomes, mitochondria, erythrocytes) to lytic and protective agents.[1–6] Each of these structures can also be stabilized by cortisol and its analogues and by chloroquine. At least one

form of lysosome, the myelin figure, even bears a reasonable morphologic resemblance to the artificial spherules, possessing a concentric lamellar substructure.[17]

A discussion of the permeability of the artificial spherules to various ions and to water, and of the theoretical considerations upon which their formation is based, have been presented in detail elsewhere.[12] Indeed, the term "permeability" is used to indicate the gross passage of marker ions or molecules from the entrapped state within the spherules to the surrounding medium. Certain problems, however, which were not immediately apparent after studies of monovalent cation leakage from the spherules,[12, 13] have been raised by the present work. In general, the steroids that caused cation release also induced leakage of anions, glucose, and glycine. But although there was no appreciable difference between the amounts of Na^+ or K^+ released by any individual steroid, there appeared to be considerable variation in the capacity of a given steroid to effect leakage of individual anions, glucose, or glycine. Thus diethylstilbestrol was among the most active agents in inducing the release of cations, chromate, and glycine. This agent, however, was relatively less active in provoking leakage of glucose, and induced no appreciable release of $H_2PO_4^-$. While such a discrepancy could be due in part to the relatively flexible structure of the diethylstilbestrol molecule, or to its interaction with $H_2PO_4^-$ or glucose, no data are available to support these possibilities. Similarly, DOC has been shown to be considerably more, and etiocholanolone to be considerably less, effective in inducing release of anions, glucose, and glycine than in promoting leak of cations.[13]

Such variation among the several "active" steroids may be explained by postulating that individual steroids induce quite distinctive changes in the permeability of lipid structures to specific ions or molecules. No such interpretation can as yet be made, since the observation could be equally well explained by the possibility that the specific ionic environment may modify the rearrangement of lipid which is brought about by various steroids. For example, potassium and calcium phosphotungstate differ in their effects upon the stability of phospholipid membranes, certainly to the extent that lipid mixtures stained by the salts assume slightly different dry configurations.[18, 19] Notwithstanding these unexplained findings, the experiments presented above would appear to exclude the possibility that steroids simply influence the release of marker ions by a counterion displacement effect, a possibility that remained after previous studies.[13] First, anions as well as cations can be released from the structures, and both monovalent and divalent anions of approximately similar ionic dimensions (i.e. $H_2PO_4^-$, $CrO_4^=$), as well as cations are released. Second, neutral molecules such as glucose and *zwitterions* such as glycine are also released from the spherules by steroids. Finally, the electron microscopic studies demonstrate not only that the membrane-disruptive detergent Triton X-100 definitely destroyed the lamellar organization of the spherules but that DOC directly interacted with the lipid structures. These observations, together with the demonstration that dialysis *per se* is unnecessary for steroid-induced effects to be manifest, would exclude any sort of nonspecific action of the steroids upon the dialysis, binding, or trapping of the marker ions or molecules.

In order to facilitate the comparison of these studies with those published previously,[12, 13] it was decided to add the marker ions and molecules at constant molarity (0·145 M). This imposed upon the final bulk solution considerable variation in pH, osmolarity, and ionic strength. For example, bulk solutions containing spherules

filled with $H_2PO_4^-$ have a pH of 4·50, with CrO_4^{2-} of 8·15, glucose of 5·80, and glycine of 5·81 after dialysis against 0·145 M NaCl/KCl. However, the pH at the lipid lamellae should be relatively independent of these variations, since the membranes have imposed upon them (by incorporation of the anion dicetylphosphate) a negative charge that would tend to cancel changes in the local ionic environment imposed by changes in the bulk pH.[12] Despite their variations in pH, $Cr_2O_4^{2-}$ and $H_2PO_4^-$ appeared to exchange with the dialysate on an equivalency basis (see above), suggesting that exchange diffusion was not remarkably altered by these variations. Similarly, although more glucose than glycine was trapped, a finding suggesting that the water compartment available to less polar molecules is greater, spherules containing either glucose or glycine responded in similar fashion to added steroids. Therefore, while it is possible that variations in pH etc. accounted for the differences observed in leakage of individual ions by any given steroid, there was no *consistent* relationship of leakage to variations in pH, ionic strength, or osmolarity of the marker molecules or ions.

The simplest explanation for the effect of steroids upon the lipid structures is that these amphipathic molecules were inserted as "rogue" molecules into the lamellae of ovolecithin and cholesterol, and that this insertion disrupted the cohesive forces holding the lipids together in such a way as to permit markers present in the aqueous compartment to escape. This interpretation would be consistent with the findings of Taylor and Haydon,[10] who observed the penetration of lipid films by progesterone, but who were unable to determine whether the steroids were inserted vertically (one polar group at the interface) or horizontally (both polar groups at the interface) into monolayers. The partial unraveling of lamellar structures, with the formation of strands and tubules, as seen in the electron micrographs of DOC-treated suspensions, would also be compatible with such a mechanism. However, since negatively stained preparations of lipid membranes do not necessarily reflect the precise arrangement assumed by lipids in the wet state,[11, 18, 19] some caution must be exercised in assuming that unraveling of lamellar structures takes place in aqueous dispersion. Indeed, although the liquid crystalline nature of the phospholipid/cholesterol spherules seems clear from their physical properties,[12] the lamellar structure is by no means the only possible arrangement of phospholipids in the smectic mesophase. Luzzatti and Husson,[20] and Stoeckenius,[21] in a combined crystallographic and electron microscopic study, have shown that brain phospholipids may exist as two liquid crystalline phases: a lamellar phase, built up by the ordered sequence of lipid and water planar sheets, and a hexagonal phase, which was a hexagonal array of circular cylinders, each cylinder being a thin water channel covered by the hydrophilic groups of the lipid molecules, the hydrocarbon chains filling the gaps between cylinders. Furthermore, Lucy and Glauert[22] have suggested, on the basis of negative staining techniques, that lamellar or hexagonal phases (especially as seen in preparations of lipids treated with saponin) can best be explained by postulating that small globular micelles constitute the basic building blocks of both phases. Luzzatti and Husson[20] found that brain phospholipids in the presence of water underwent transition from the liquid crystalline phase to the coagel phase at temperature close to 37°, and suggested that the permeability of biological membranes may be regulated by phase transformations. Although such a phase transition would be unlikely to occur in the bulk of lipids at the concentrations employed in our experiments, it is possible that local changes to a coagel

could be induced by steroids. It is perhaps more likely that steroids modified the liquid crystalline structure itself, perhaps through a local transformation of the lamellar phase to the hexagonal or tubular phase. Such a possibility is supported by the electron microscopic finding of newly unraveled strand-like structures in spherules treated with DOC. Haydon and Taylor,[23] on the other hand, have suggested that when cholesterol (or, by analogy, progesterone and DOC) becomes incorporated into bimolecular (lamellar) leaflets of lecithin, these may break up into more or less spherical aggregates of molecules. Were steroids to induce the formation of such spherical aggregates at successively exposed surfaces of the spherules, strand-like or tubular structures, such as those which we have observed, might be formed.

Whatever the exact mechanism proves to be whereby steroids induce structural changes in phospholipid/cholesterol spherules, the studies presented above clearly show that steroids disrupt the organization and function of lipid membranes in salt solutions. It is also clear that certain structural requirements for membrane action must be met. For example, the angulated 5 β-H steroid etiocholanolone is capable of releasing ions, glucose, and glycine from the spherules, whereas its planar 5 α-H isomer androsterone is incapable of these effects. Oxygen groups at the 11 or 17 position abolished the membrane activity of steroids; although corticosterone was unable to induce leakage of cations,[13] DOC has proved effective in promoting the release of cations, anions, glucose, and glycine. These structural requirements correlate well with those necessary for the disruptive effects of steroids upon liver lysosomes,[3, 5] leukocyte granules,[7] mitochondria,[2] and erythrocytes.[4] They are also identical with those found by Selye[24] to determine the anesthetic potency of steroids. Bangham (personal communication) has recently found that a series of local anesthetics affects the exchange diffusion of ions from phospholipid/cholesterol spherules. Since anesthesia has been considered to result from an interaction of the anesthetic agent with lipid membranes in conductive tissue,[25] it becomes clear that the membranes that bound model spherules respond to the specific configuration of disruptive agents in the same manner as biological membranes. Indeed, the protection of model structures by cortisol, cortisone, and chloroquine would support earlier suggestions[26] that these agents exert their pharmacologic action, at least in part, through their stabilization of biological membranes. Finally, the demonstration that steroids *in vitro* can regulate the availability to enzyme of substrate molecules (e.g. glucose to glucose oxidase), suggests that steroids have the potentiality of exerting similar actions *in vivo*.

The concentrations of steroids used in these studies are far above physiologic levels obtainable *in vivo*. However, the lipid content of these artificial spherules (expressed as μmole P/ml suspension) is approximately 100-fold the lipid content of mitochondrial or lysosomal suspensions previously studied (Weissmann, unpublished). In suspensions of natural organelles, steroids exert lytic or protective functions at concentrations of 10^{-4}M when the steroid is dissolved in ethanol, or at 10^{-6}M when dissolved in dimethylsulfoxide.[5] These observations suggest that the relative insolubility of the steroid in the suspending medium would explain the high concentrations required for action *in vitro*. The observation that "preincorporation" of a *maximum* of 1 per cent cortisone into lipid spherules rendered these less permeable to sequestered ions suggest that only modest amounts of steroids actually enter the lamellae. Despite these observations, it remains possible that the effects of steroids at these

high concentrations represent instances of pharmacologic, rather than physiologic, action.

Finally, in the absence of biophysical studies of the spherules as they exist in suspension, it is not certain that the postulated multilamellar structures seen by electron microscopy reflect the actual organization of the spherules in suspension. Thus, the release of anions, glucose, and glycine may represent re-compartmentalization of various "pockets" of markers, rather than actual diffusion across a bilayer. The present studies do not permit one to judge finally between these possibilities; however other studies of the *kinetics* of marker release[12, 13] suggest that leakage of markers represents exchange diffusion across bilayers.

Since steroids and other membrane-active agents can be shown to exert lytic or protective functions upon structures composed entirely of purified lipids, the suggestion that the permeability of natural membranes depends upon the integrity of their lipid skeletons becomes more plausible. Although the presence of a functional protein has been held to be an integral structural feature of the membranes which bound cells or their organelles,[27] the experiments we have presented indicate that some membrane phenomena, at least, can be explained without assignment of a crucial role either to this protein, to associated polysaccharide, or to active cell metabolism.

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