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The stabilization-lysis action of anti-inflammatory steroids on lysosomes

D. A. LEWIS, A. M. SYMONS AND R. J. ANCILL

Pharmacology Group, School of Pharmacy, Bath University of Technology, Bath BA2 7AY, U.K.

The effect of anti-inflammatory steroids on lysosomal enzyme release has been investigated. Most of the steroids stabilized lysosomes at pharmacological concentrations (10^{-4}–10^{-6}M) but lysed them at higher concentrations. Etiocholanolone, a steroid pyrogenic in man, had no stabilizing effect. The concentration of steroid would therefore seem critical in determining its subcellular action. Experiments with albumin suggest that anti-inflammatory steroids (at 5×10^{-4}M) have little effect in aiding its thermal denaturation whereas other steroids greatly increase denaturation. Increasing concentrations of cortisol and prednisolone however caused greater denaturation of albumin. Although the correlation between albumin solutions and lysosomal membrane proteins is tenuous it is suggested that the lytic effect of anti-inflammatory steroids could be due to protein denaturation. Their stabilizing effect, however, probably involves steroid-lipid interactions.

Corticosteroids stabilize lysosomes and this property has been proposed as the basis of their anti-inflammatory activity (Weissmann & Dingle, 1961). Many of the *in vitro* experiments made on isolated lysosomes have been at a fixed steroid concentration. However, there is some evidence that at high concentrations the stabilizing action of these steroids on lysosomes is lost (Seeman, 1968). This investigation was designed to determine the action of steroids on lysosomes over a wide range of concentrations. In addition, the effect of steroids on the stability of acid phosphatase (EC 3.1.3.2) and of albumin has been examined because steroid-protein interactions may be involved in the action of steroids on lysosomes.

EXPERIMENTAL

The action of steroids on lysosomes

The action of dexamethasone, 9α-fluoroprednisolone, β-methasone alcohol, methyl prednisolone acetate, prednisolone, prednisolone stearoylglycolate and triamcinolone acetonide on lysosomes was examined. These steroids have a stabilizing action on lysosomes when at a final concentration of 5×10^{-4}M in our lysosomal preparation (Symons, Lewis & Ancill, 1969). Etiocholanolone, a steroid with a lytic action on lysosomes, was also examined.

Lysosome enriched suspensions in 0·05M tris-acetate buffered 0·25M sucrose (pH 7·4) were prepared from rabbit liver by methods previously described (Symons & others, 1969). The protein concentration of each suspension was determined by the method of Lowry, Rosebrough & others (1951). Steroids were deposited as thin films in 50 ml conical flasks by evaporating to dryness portions of a 1,4-dioxan solution. Portions (5 ml) of the lysosome suspensions were added to the flasks, which were then incubated for 90 min in a shaking reaction incubator at 37° (100 oscillations/min). In other experiments the flasks were incubated at 20° and 45°. Intact

lysosomes and debris were then removed by centrifuging at 20 000 *g* for 20 min at 4° in a Beckman Model L2 Ultracentrifuge. The supernatants were examined for acid phosphatase activity and β-glucuronidase activity (EC 3.2.1.31) by methods previously described (Symons & others, 1969). In one experiment *N*-acetyl-β-glucosaminidase (EC 3.2.1.30) activity was assayed. A 0·1 ml portion of the supernatant was added to 0·5 ml of 1·5 mM p-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Koch–Light) and 0·5 ml of 0·2M acetate buffer pH 4·5. After incubation for 30 min at 37° in a shaking reaction incubator 5 ml of 0·1N NaOH was added and the amount of *p*-nitrophenol released was determined at 410 nm.

The effect of steroids on acid phosphatase

A lysosome suspension was prepared from rat liver by methods previously described (Symons & others, 1969). The suspension was frozen and thawed six times and the debris removed by centrifuging at 20 000 *g* for 20 min at 4°. The acid phosphatase activity of the supernatant was determined and 2 ml portions were transferred to stoppered test-tubes. The steroids were added to the tubes dissolved in 0·1 ml of ethanol, except for prednisolone stearoylglycolate which was dissolved in 0·1 ml of 1,4-dioxan. The solvent alone was added to the control tubes. The tubes were heated for 90 min in a water bath at 37°. Portions of the solutions were then examined for acid phosphatase activity. The experiment was repeated at 45°.

The effect of steroids on the stability of albumin solutions

The steroid was dissolved in 0·2 ml of either ethanol, 1,4-dioxan or propane-1,2-diol and added to stoppered test-tubes containing 5 ml of 1% w/v egg albumin dissolved in 0·9% w/v sodium chloride solution buffered at pH 5·2 with 0·1M phosphate. The solvent alone was added to the control tubes. The tubes were gently shaken to disperse the steroid and the extinction of the solution determined at 420 nm in a spectrophotometer (Unicam S.P. 500). The tubes were placed in a water bath, at a temperature, and for a period determined by preliminary experiments. After heating, the absorbances of the solutions were again determined. To avoid delay in the determination of the absorbances of the solutions only two steroids were compared with one control in each experiment.

RESULTS

The action of steroids on lysosomes

The effect of etiocholanolone, dexamethasone, methyl prednisolone acetate on the release of acid phosphatase and β-glucuronidase from lysosomes is shown in Fig. 1. The effect of prenisolone stearoylglycolate and β-methasone alcohol on the release of these two enzymes plus that of triamcinolone acetonide on acid phosphatase is shown in Fig. 2. Fig. 3 shows the effect of prednisolone on the two enzymes and the effect of 9α-fluoroprednisolone on the release of β-glucuronidase. The control values have been fixed at 100% and values in excess of 100% represent a lytic action and values below 100% represent a stabilizing action by the steroids on the lysosomes. The anti-inflammatory steroids, with the exception of prednisolone stearoylglycolate, gave stabilization-lysis curves where the steroids stabilized the lysosomes at the lower steroid concentrations but lysed them at higher concentrations. The optimum stabilization concentration range for all the anti-inflammatory steroids examined was 10^{-4}–10^{-6}M.

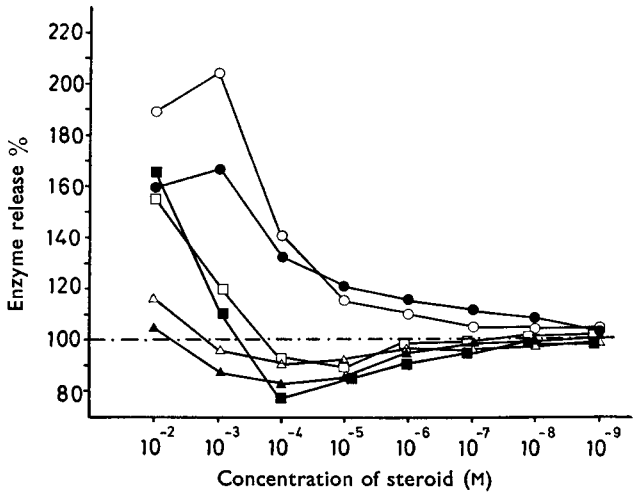

FIG. 1. The effect of etiocholanolone (circles), dexamethasone (triangles), and methyl prednisolone acetate (squares), on the release of lysosomal enzymes. Closed symbols represent acid phosphatase and open symbols β-glucuronidase. The protein concentration of the three lysosomal preparations were 4·1, 4·0 and 6·6 mg/ml respectively.

Prednisolone stearoylglycolate stabilized the lysosomes at 37° over the whole concentration range examined although this action declined at higher concentrations, but the curve was similar to that for the other steroids. The stabilizing properties of the steroid were much less at 20° than at 37°. At 20° the steroid had a lytic action on the lysosomes at the higher concentrations. At 45° the steroid had a lytic action over the entire concentration range (Fig. 4).

Etiocholanolone had a lytic-action on the lysosomes over the range of steroid concentrations examined which increased with concentration except for the highest concentration where there was a slight decline.

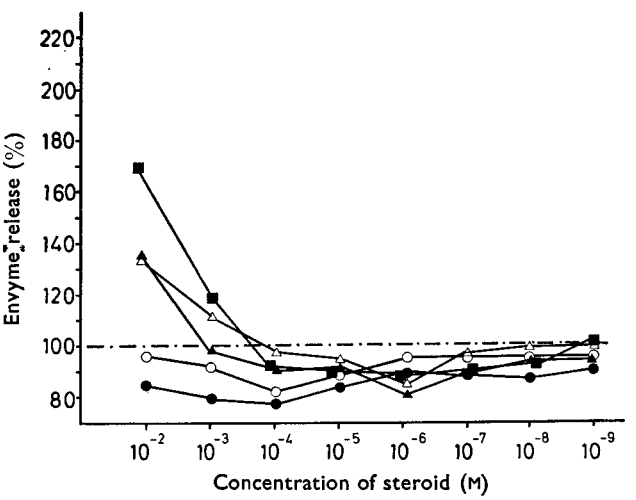

FIG. 2. The effect of prednisolone stearoylglycolate (circles), β-methasone alcohol (triangles), and triamcinolone acetonide (squares), on the release of lysosomal enzymes. Closed symbols represent acid phosphatase and open symbols β-glucuronidase. The protein concentration of the three lysosomal preparations were 6·4, 4·7 and 5·2 mg/ml respectively.

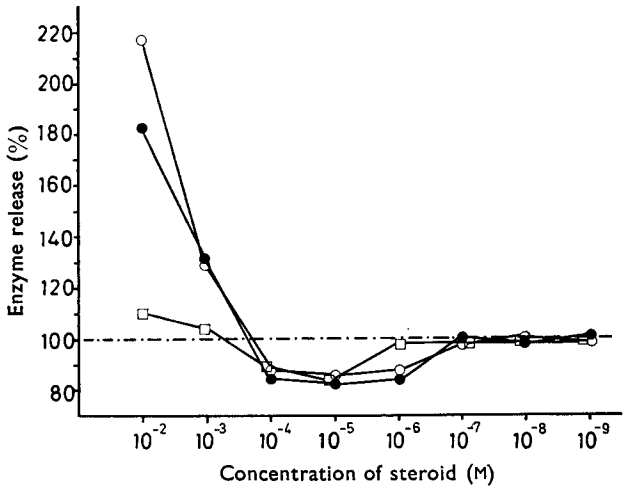

FIG. 3. The effect of prednisolone (circles), and 9α-fluoroprednisolone (squares), on the release of lysosomal enzymes. Closed symbols represent acid phosphatase and open symbols β-glucuronidase. The protein concentration of the two lysosomal preparations were 3·7 and 6·7 mg/ml respectively.

The effect of steroids on acid phosphatase

The results in Table 1 are in general agreement with those of Weissmann (1965) who has reported that steroids do not affect acid phosphatase activity at 37°. From Table 1 it would appear that low acid phosphatase levels in the lysosome experiments were due to membrane stability rather than inactivation of the enzyme. In other experiments where higher concentrations of steroids were used the results were similar. Presumably steroid solubility was a limiting factor in these experiments.

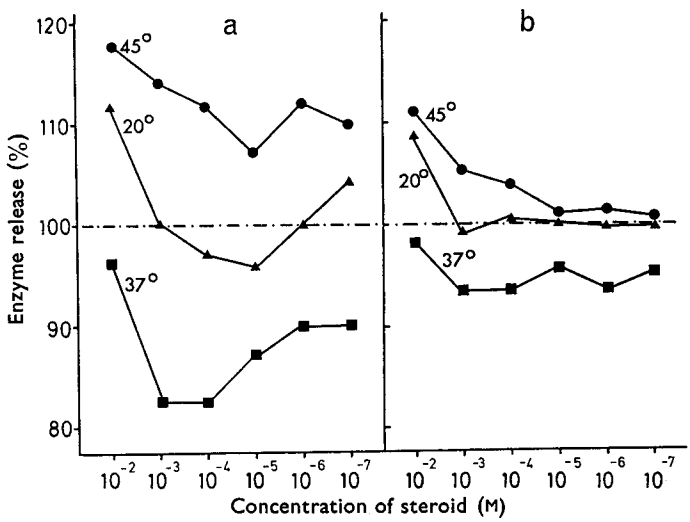

FIG. 4. a. The effect of prednisolone stearoylglycolate on the release of acid phosphatase from lysosomes at 20°, 37° and 45°.

b. The effect of prednisolone stearoylglycolate on the release of *N*-acetyl-β-glucosaminidase from lysosomes at 20°, 37° and 45°. Protein concentration of lysosomal preparation was 6·9 mg/ml.

Table 1. *Effect of steroids on acid phosphatase.* Each value is the mean of four experiments ± standard deviation.

Steroid added to final concentration of 5×10^{-4}M	Activity (%) of acid phosphatase after 90 min incubation at 37°	45°
None (control)	100	100
Cortisol	104 ± 5	112 ± 3
Cortiscosterone	100 ± 1	109 ± 5
Dexamethasone	113 ± 3	103 ± 1
Etiocholanolone	106 ± 2	114 ± 3
Epiandrosterone	100 ± 4	99 ± 3
9α-Fluorocortisol	103 ± 3	106 ± 2
β-Methasone alcohol	107 ± 2	106 ± 2
Methyl testosterone	96 ± 7	99 ± 1
Methyl prednisolone acetate	110 ± 2	115 ± 5
Prednisolone stearoylglycolate	108 ± 2	121 ± 5
Triamcinolone acetonide	110 ± 2	117 ± 3

The effect of steroids on the thermal stability of albumin solutions

The effect of steroids on the thermal denaturation of albumin solutions is shown in Table 2. The difference between the final and initial absorbance values of the solutions to which steroids were added have been calculated as a percentage of the increase of the absorbance values of the controls. Values in excess of 100% indicate that the steroid increased the amount of denaturation and values below 100% indicate that the steroid decreased the amount of denaturation. Steroids with a lytic action on lysosomes, namely etiocholanolone, dehydroepiandrosterone, testosterone (Weissmann, 1965) and methyl testosterone (personal observation) greatly increased the rate of thermal denaturation of the albumin solution. Some of the anti-inflammatory steroids also increased the rate of denaturation of the protein but less so than the androgens. Prednisolone stearoylglycolate effected a decrease indicating a protective action on the protein at the concentration examined. The denaturing action of cortisol, prednisolone and etiocholanolone increased with concentration (Table 3).

DISCUSSION

The results show clearly that steroid concentration is a critical factor in the action of steroids on lysosomes. In addition they show that many anti-inflammatory steroids have a biphasic action on lysosomes, in that they stabilize them at relatively low concentrations but lyse them at higher concentrations. The investigations of Bangham, Standish & Weissmann (1965) on the interaction of steroids with artificial lipid membranes suggest that the action of steroids on biological membranes may result directly from their interaction with lipid, independent of other membrane components. The chemical composition of rat liver lysosome membranes has been reported to be similar to that of plasma membranes. Typical analysis figures reported are phospholipid 0·43 mg/mg protein and cholesterol 0·13 mg/mg protein (Thinès-Sempoux, 1967). The lysosomal membrane is therefore rich in both lipid and protein. It is probably an over simplification to regard the lysosomal membrane as consisting of inert structural protein and lipid. At low concentrations the actions of steroids on lysosomes parallel closely the action of steroids on the permeability of artificial lipid membranes. Therefore it is unlikely that the mild protein denaturing actions of anti-inflammatory steroids we observed with albumin are of significance in their actions on lysosomes at low concentrations. However, it is possible that

Table 2. *Effect of steroids on the thermal denaturation of albumin.* Each value is the mean of three experiments ± standard deviation.

Steroid added to give a final concentration of 5×10^{-4}M	Action on lysosomes at pharmaceutical concentrations (10^{-4}–10^{-6}M)	Heating time min	Temp. °C	Solvent	Increase in denaturation of albumin (%) compared to control value of 100
Cholic acid	Lytic	15	60	Ethanol	266 ± 12
Dehydroepiandrosterone	"	10	60	Propane-1,2-diol	197 ± 12
Etiocholanolone	"	10	59	Ethanol	243 ± 40
Etiocholanolone	"	10	60	Propane-1,2-diol	225 ± 2
Methyl testosterone	"	10	60	Propane-1,2-diol	237 ± 10
Methyl testosterone	"	15	55	Ethanol	267 ± 21
Testosterone	"	10	60	Propane-1,2-diol	219 ± 3
Corticosterone	Stabilization	10	59	Ethanol	111 ± 2
Corticosterone	"	5	62	Propane-1,2-diol	127 ± 2
Corticosterone	"	10	60	Propane-1,2-diol	121 ± 4
Cortisol	"	5	62	Propane-1,2-diol	142 ± 2
Cortisol	"	10	59	Ethanol	116 ± 5
Cortisone	"	10	59	Ethanol	129 ± 7
9α-Fluorocortisol	"	5	62	Propane-1,2-diol	98 ± 4
9α-Fluorocortisol	"	10	59	Ethanol	96 ± 8
9α-Fluoroprednisolone	"	10	59	Ethanol	108 ± 2
Fluoxymesterone	"	10	59	Ethanol	98 ± 4
Fluoxymesterone	"	5	62	Propane-1,2-diol	98 ± 4
Methyl prednisolone acetate	"	15	55	Ethanol	128 ± 5
Prednisolone	"	10	60	Propane-1,2-diol	112 ± 1
Prednisolone	"	10	59	Ethanol	122 ± 3
Prednisone	"	5	62	Propane-1,2-diol	121 ± 4
Prednisone	"	15	55	Ethanol	124 ± 8
Prednisolone stearoyl-glycolate	"	15	55	1,4-Dioxan	76 ± 9

Table 3. *The effect of increasing steroid concentrations on the thermal denaturation of albumin.* Each value is the mean of three experiments ± standard deviation. Albumin solutions were heated at 50° for 15 min.

Steroid added	Concentration of steroid M	Increase in denaturation of albumin (%) compared to control value of 100
Cortisol	0	100
	10^{-4}	106 ± 1
	2×10^{-4}	112 ± 2
	3×10^{-4}	118 ± 5
	4×10^{-4}	120 ± 3
	5×10^{-4}	123 ± 0
Etiocholanolone	0	100
	10^{-4}	105 ± 1
	2×10^{-4}	135 ± 3
	3×10^{-4}	146 ± 3
	4×10^{-4}	152 ± 3
	5×10^{-4}	157 ± 1
Prednisolone	0	100
	10^{-4}	103 ± 1
	5×10^{-4}	108 ± 3

the stronger denaturing actions of androgens may have aided their lytic actions on lysosomes. In the presence of excess steroid (e.g. at high concentrations) the possibility that steroid-protein interactions occur increases. It has been shown in this investigation that the denaturing action of three steroids on albumin increased with concentration. Therefore it is possible that many steroid-protein interactions may affect the stability of the lysosomal membrane. Denaturation leads to a disorganization of protein structure and this will lead to a loss in stability of the membrane.

Although most of the steroids examined denatured albumin, prednisolone stearoylglycolate stabilized the protein and also stabilized the lysosomes over the entire concentration range examined (10^{-2}–10^{-9}M) at 37° while at 45° it had a lytic action on the lysosomes. The reason for this is not clear. It is possible that high acid phosphatase levels result from the stabilizing action of the steroid on the enzyme at 45° (Table 1). A recent report (Brown & Schwartz, 1969) stated that dexamethasone has a lytic action on lysosomes at 10^{-4}–10^{-5}M after incubation for 90 min at 45°. Although prednisolone stearoylglycolate stabilized the lysosomes at 10^{-4}–10^{-5}M at 20° the stabilization was much less than that at physiological temperature.

Whether the lytic action of steroids at high concentrations on lysosomes is of pharmacological significance is not clear. The concentrations of steroids needed to produce a lytic action on lysosomes in the *in vitro* experiments were well in excess of concentrations likely to occur *in vivo* as a result of short term steroid therapy. However, it is possible that long term steroid therapy may result in an accumulation of steroids in cells or membranes. It is of interest that the oral administration of steroids occasionally gives rise to gastrointestinal ulceration. Ulceration would be accelerated by lyosomal damage since acid hydrolases (e.g. cathepsins) would be released intracellularly. Many other drugs show a biphasic pattern of stabilization-lysis on various membranes. This subject has recently been reviewed by Seeman (1966).

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