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Research Article

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Variations in cholesterol and phospholipid contents of liver and PMN leukocyte lysosome membranes could possibly account for the different reactivity to corticosteroids observed. We believe that the anti-inflammatory activity of adrenal corticosteroids can best be explained by their inhibitory effects on cellular metabolism rather than by their direct interaction with lysosomal membranes.

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ABSTRACT Lysosomal membrane stabilization has been proposed as a mechanism for the anti-inflammatory action of corticosteroid hormones. This hypothesis was based on studies with liver organelles. We studied the action of steroids on intact lysosomes isolated from human peripheral blood polymorphonuclear (PMN) leukocytes. Both androstenedione and progesterone, 10^{-8} – 10^{-6} M, caused leakage of acid hydrolase markers from these organelles, thus resembling their effects on liver lysosomes. But none of the anti-inflammatory steroids tested protected organelle membranes from either detergent lysis (Triton X-100) or heat incubation (37°C , 90 min). Hydrocortisone (HC), HC sodium succinate, HC acetate, HC hemisuccinate, prednisone, and dexamethasone were without detectable stabilizing activity at concentrations of 10^{-8} – 5×10^{-8} M. Release of the lysosomal marker, β -glucuronidase, was not retarded by any of the compounds studied. In addition, PMN leukocyte lysosomes isolated from human volunteers receiving prednisolone were not more stable than control organelles, nor did serum from steroid-treated humans protect intact lysosomes from detergent lysis.

Variations in cholesterol and phospholipid contents of liver and PMN leukocyte lysosome membranes could possibly account for the different reactivity to corticosteroids observed. We believe that the anti-inflammatory activity of adrenal corticosteroids can best be explained by their inhibitory effects on cellular metabolism rather than by their direct interaction with lysosomal membranes.

INTRODUCTION

The anti-inflammatory action of adrenal corticosteroid hormones is believed to be mediated in part by their

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effects on lysosomal membranes. Early studies by de Duve, Wattiaux, and Wibo (1) and by Weissmann and Thomas (2) showed that hydrocortisone retarded the release of acid hydrolases from isolated lysosomes. Since the contents of these subcellular organelles can induce inflammation, inhibition of their release would modify and diminish the inflammatory process (3). These observations, however, were based on studies with liver lysosomes, subcellular organelles not usually involved with extrahepatic inflammation. Recent studies using phagocytic cells and bactericidal systems have failed to document stabilization by hydrocortisone of the lysosomes in these cells (4–6). Although Wright and Malawista (4) found less extracellular release of granular enzymes in 5×10^{-4} M cortisol, neither altered degranulation nor impaired intracellular digestion by lysosomal hydrolases was detected by Mandell, Rubin, and Hook (5) or by Wiener, Marmary, and Curelaru (6). And no effects were observed by these authors using hydrocortisone in the usual physiologic and pharmacologic concentrations. Since the polymorphonuclear (PMN)¹ leukocyte is the dominant cell in acute inflammatory reactions—reactions that can be suppressed by corticosteroids—we have studied the effect of adrenocortical hormones on lysosomes isolated from these cells.

METHODS

Intact leukocyte lysosomes were isolated from normal human venous blood PMN leukocytes by the method of Chodirker, Bock, and Vaughan (7). Briefly, after hypotonic lysis of red cells in 0.2% saline and washing in 0.34 M sucrose, leukocytes (75–90% PMN) were disrupted by rapid pipetting in 0.2 M sucrose containing aqueous heparin, 50 U/ml. Intact large granules composed mainly of lysosomes were isolated by differential centrifugation (the organelles sedimenting between 800 *g* for 15 min and 25,000 *g* for 10 min), washed, and suspended in 0.3 M sucrose containing heparin so that the absorbance 520 nm of the suspension was 0.6–0.8. Approximately 30 ml of a uni-

¹ Abbreviations used in this paper: DMSO, dimethyl sulfoxide; PMN, polymorphonuclear.

TABLE I
*Effect of Incubation at 37°C for 90 min on Release of Enzymes from Human PMN Leukocyte Lysosomes**

Lysosomal fraction assayed	β -Glucuronidase†		Acid phosphatase‡	
	A _{550 nm}	Percent of total	A _{420 nm}	Percent of total
A. Activity in supernate before incubation	0.076	15±1.1	0.223	28±1.3
B. Activity in supernate after 37°C incubation	0.172	35±2.7	0.379	47±3.1
C. Activity released by incubation (B - A)	0.096	20±2.9	0.156	19±3.6
D. Activity recovered in pellet after incubation	0.321	65±4.3	0.427	53±6.7
E. Total enzyme content (B + D)	0.493	100±6.3	0.806	100±8.9

* Intact granulocyte lysosomes were stressed by 37°C incubation and enzyme activities assayed in clear supernates. See text for details.

† Presented are means±1 SEM of 16 experiments, each with three to six replicates. All values have been corrected for substrate blanks.

‡ Presented are means±1 SEM of four experiments, each with four to six replicates. All values have been corrected for substrate blanks.

|| After 37°C-90 min incubation and centrifugation at 25,000 *g*-10 min, the pelleted intact organelles were resuspended to original volume, disrupted by freezing and thawing seven times, and the liberated enzyme activities assayed in the clear supernates after centrifugation to remove disrupted membranes.

form lysosome suspension could be obtained from 50 ml of venous blood. The lysosomes, isolated and maintained at 2°C until use, were stable without release of their enzyme contents for periods in excess of 48 h. All experiments presented were performed on organelles within 16 h of isolation.

Lysosome stress procedure. The intact lysosome suspension, 0.6 ml, was mixed with 6 μ l of either the steroid to be tested solubilized in dimethyl sulfoxide (DMSO) or with DMSO alone. The final concentration of DMSO was 1% in all tubes. After mixing, the lysosome suspensions were allowed to stand for 20 min at room temp and then were incubated for 90 min at 37°C with gentle mixing. After incubation, intact lysosomes were removed by centrifugation at 25,000 *g* for 10 min and the clear supernates were assayed for enzyme activities. In some experiments the centrifuged intact lysosomes were resuspended to original volume in 0.3 M sucrose, frozen and thawed seven times to disrupt remaining intact organelles and to release their soluble contents, and centrifuged 25,000 *g* for 10 min. The resulting supernates were assayed for enzyme activities. In some studies, lysosomes were suspended in 0.15 M phosphate-buffered saline, pH 7.4, and in other studies in 0.3 M sucrose. Identical results were obtained with each medium.

A second lysosome stress procedure was employed in some experiments. PMN leukocyte lysosomes suspended in 0.3 M sucrose containing 30 U aqueous heparin/ml were mixed with solubilized steroid or with solvent alone at room temp. The absorbance 520 nm of the suspension of intact lysosomes was stable until the addition of Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.). This nonionic detergent disrupts organelle membranes, releasing their soluble contents, resulting in a rapid decrease in absorbance of the lysosomal suspension (8). Volumes used were 0.8 ml of lysosome suspension ($A_{520 \text{ nm}} = 0.6$) and 0.1 ml steroid followed by 0.1 ml detergent, final concentration 0.01%

(vol/vol). Absorbance was measured before the addition of detergent and at intervals of 60 s after its addition with rapid mixing.

To study the effect of in vivo adrenocorticosteroids, normal adult males received prednisolone by mouth, 60 mg/day in divided doses. After 3 days, venous blood PMN leukocyte lysosomes isolated from three steroid-treated individuals were pooled and stressed with heat incubation, and the liberated enzyme activity was quantitated as a percent of total enzyme content of the suspension. The results were contrasted with a lysosome suspension obtained from untreated controls. Six control subjects were needed to provide sufficient numbers of PMN leukocytes for these stress experiments. The absorbance of the organelle suspensions from both treated and control subjects was made identical before testing. In addition, sera obtained daily for 3 days before and during steroid administration were studied for their effects on detergent-stressed normal lysosomes. 0.1 ml of serum was mixed with 0.8 ml of the intact lysosome suspension before the addition of 0.1 ml of 0.1% Triton X-100 and the absorbance change measured. This method for the detection of serum membrane reactants that modify organelle permeability has been previously described (9-10). A lysosomal stabilizer in the serum will retard the rate of absorbance change followed addition of the membranolytic detergent.

Activity of the lysosomal enzyme, β -glucuronidase, was measured by the method of Fishman, Springer, and Brunetti (11) with phenolphthalein glucuronide as substrate. The time of 37°C incubation of 0.2-ml samples at pH 4.5 was 6 h. Absorbance 550 nm was determined after addition of glycine buffer, pH 10.4. Activity as determined by using a beef liver β -glucuronidase standard (Nutritional Biochemicals Corporation, Cleveland, Ohio) showed one Fishman U = 0.0025 OD_{550 nm}/h at 37°C. Acid phosphatase was determined by the method of Andersch and Szczepinski using *p*-nitrophenylphosphate as substrate (12). The time

of incubation at 37°C, pH 4.8, of 0.2-ml samples was 5 h. Absorbance 420 nm was measured after addition of 0.1 N NaOH. 1 U of enzyme activity as standardized against wheat germ acid phosphatase (Sigma Chemical Co., St. Louis, Mo.) was 0.340 OD_{420 nm}/30 min at 37°C.

Steroid hormones studied were 4-pregnene-11 β ,17 α ,21-triol-3,20-dione (hydrocortisone) and 1,4-pregnadiene-17 α ,21-diol-3,11,20-trione (prednisone) from Sigma Chemical Co., hydrocortisone sodium succinate from Upjohn Co., Kalamazoo, Mich., 9 α -fluoro-16 α -methylprednisolone (dexamethasone) from Merck & Co., Inc., Rahway, N. J., and hydrocortisone acetate, hydrocortisone hemisuccinate, 4-androstene-3,17-dione (androstenedione), and Δ 4-pregnene-3,20-dione (progesterone) from Mann Research Labs Inc., New York. After solubilization in DMSO, all steroids were diluted in lysosomal buffers for use in lysosome stress tests. All compounds were studied at multiple dilutions for possible inhibitory effects on lysosome enzyme activities.

RESULTS

Incubation at 37°C exerts a significant and reproducible stress on intact isolated human leukocyte lysosomes (Table I). In the absence of steroid hormones, incubation for 90 min induced a release of 20% of the total β -glucuronidase and 19% of the total acid phosphatase contents of the lysosome suspension. Total content was determined by summing the activities present in the 0.3 M sucrose suspension after incubation and the enzyme content released by subsequent freezing and thawing of the remaining intact lysosomes. Cellular acid phosphatase from other than lysosomal sites was believed to account for the differences observed between the two enzymes assayed (13). Because β -glucuronidase is more localized to the large granule (lysosomal) fraction of PMN leukocytes (14), this enzyme was considered the

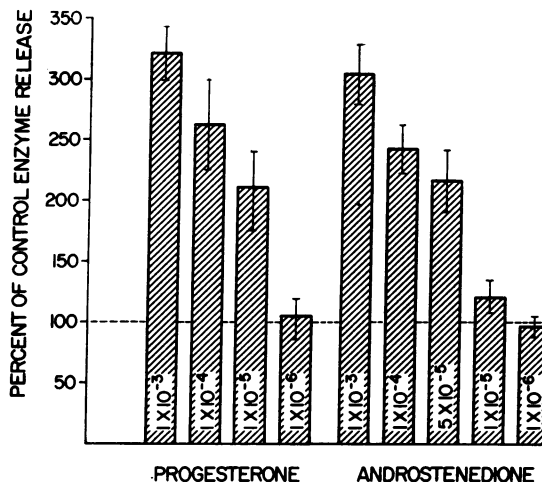


FIGURE 1 Effect of steroids on β -glucuronidase release from human PMN leukocyte lysosomes. Intact lysosomes were incubated at 37°C for 90 min in varying molar concentrations of steroid solubilized in DMSO. Enzyme activities liberated from lysosomes in DMSO alone served as controls, were corrected for β -glucuronidase activity in supernates of lysosomes kept at 2°C, and set as 100% release. Shown are means \pm 1 SD of four experiments, each with two to six replicates.

most important marker in subsequent studies on lysosome membrane permeability. Some experiments were performed with 0.15 M phosphate-saline buffer, pH 7.4, rather than sucrose as the suspending medium. Results obtained were within the same ranges as those seen in sucrose and data were combined.

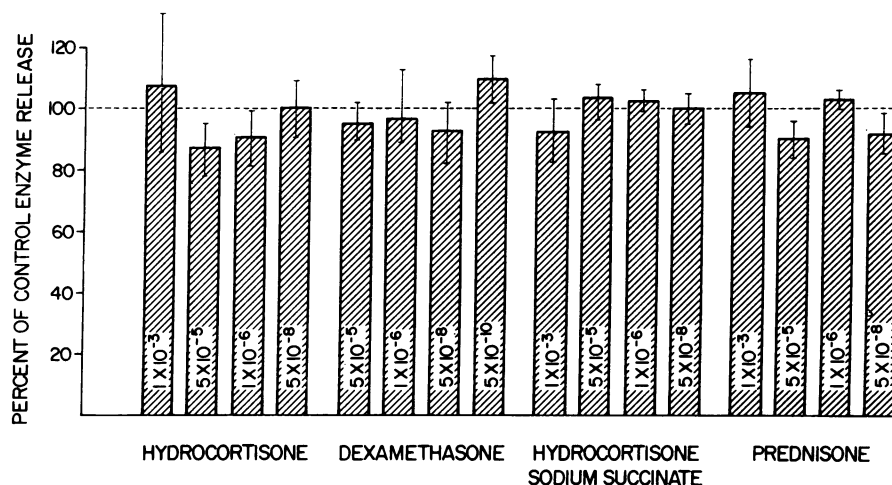


FIGURE 2 Effect of anti-inflammatory steroids on β -glucuronidase release from human PMN leukocyte lysosomes. Intact lysosomes were incubated at 37°C for 90 min in varying molar concentrations of steroid solubilized in DMSO. Enzyme activity liberated from lysosomes in DMSO alone served as a control and represented 100% release. Shown are means \pm 1 SD of four experiments, each with four replicates for all steroids except prednisone, which was studied three times, each with four replicates.

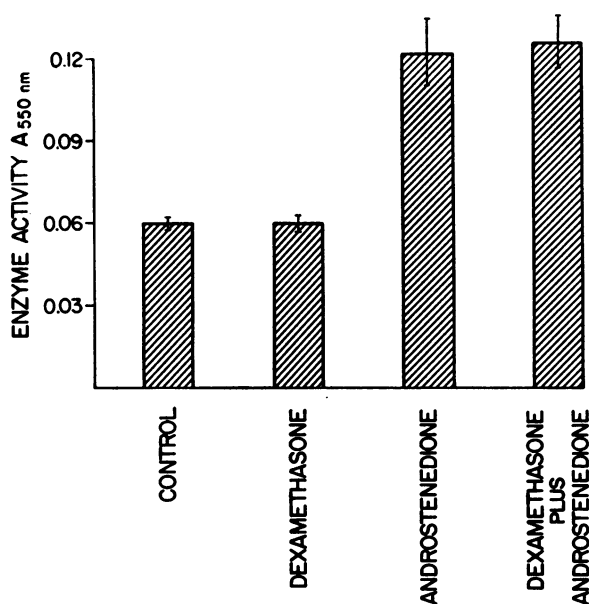


FIGURE 3 Effect of preincubation with 5×10^{-6} M dexamethasone on labilizing activity of 5×10^{-6} M androstenedione. β -Glucuronidase released from lysosomes incubated at 37°C for 90 min was corrected for enzyme activity measured in supernates of organelle suspensions maintained at 2°C . After centrifugation, 0.2-ml samples were incubated with phenolphthalein glucuronide and change in absorbance 550 nm was determined. See text for details. Shown are means ± 1 SD of two experiments, each with three replicates.

The effects of progesterone and of androstenedione, 10^{-3} – 10^{-6} M, are shown in Fig. 1. These steroids, neither oxygenated nor hydroxylated at the C-11 position, have previously been shown to induce membrane rupture and allow leakage of enzymes from liver lysosomes (15). It can be seen that these steroids also labilize lysosomes from human PMN leukocytes. Almost the total lysosome content of β -glucuronidase was released after incubation with 10^{-3} M progesterone. The membranolytic effect was dose related both for progesterone and androstenedione. Neither steroid inhibited the enzyme measured.

The anti-inflammatory steroids previously shown to stabilize liver organelles, however, were not capable of retarding release of enzyme from PMN lysosomes (Fig. 2). Hydrocortisone, dexamethasone, prednisone, and hydrocortisone sodium succinate did not prevent the release of β -glucuronidase from lysosomes stressed by heat incubation. These steroids were studied at various concentrations including the usual physiologic and pharmacologic ranges. Less of the lysosomal enzyme assayed was found in the supernate after incubation of the organelles with either hydrocortisone hemisuccinate or with hydrocortisone acetate, in concentrations ranging from 10^{-3} to 10^{-6} M. The activities of both of the soluble granulocyte lysosomal enzymes studied were inhibited by

the addition of these two compounds, this effect being dose related. Thus, the finding of less enzyme in the supernate was due to enzyme inhibition by these corticosteroid preparations rather than to lysosomal membrane stabilization. In no case could any of the anti-inflammatory steroids studied be found to stabilize PMN leukocyte lysosomes to a statistically significant degree.

Lysosomes preincubated for 20 min at 23°C with dexamethasone, 5×10^{-6} M, were then challenged with the labilizer, androstenedione, 5×10^{-6} M (Fig. 3). After 37°C incubation for 90 min, lysosomes released as much enzyme as when incubated in androstenedione alone. β -Glucuronidase release in dexamethasone alone did not differ from the control.

PMN leukocyte lysosomal membrane stabilization by an anti-inflammatory steroid was also investigated by a different stress technique (Fig. 4). Hydrocortisone hemisuccinate at either 5×10^{-3} or 5×10^{-4} M could not protect isolated organelles from the lytic effects of a nonionic detergent. A stabilizing agent would be expected to retard the change in absorbance of the lysosomal suspension after the addition of the membranolytic Triton X-100. In no case was the $A_{520 \text{ nm}}$ change of the steroid-treated lysosomes statistically different from controls.

Studies with an anti-inflammatory steroid (dexamethasone) and with a membrane labilizer (progesterone)

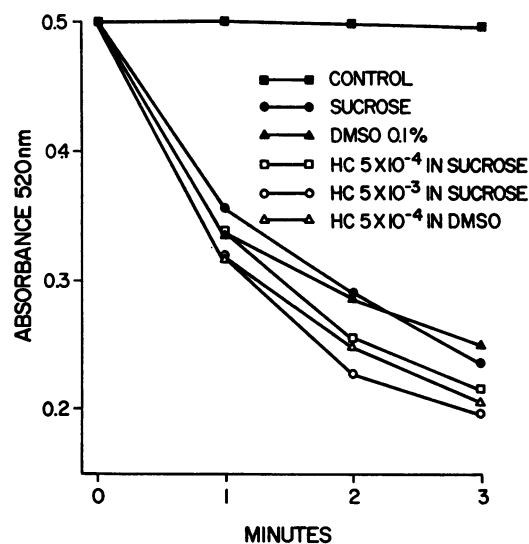


FIGURE 4 Effect of hydrocortisone hemisuccinate (HC) on lysis of PMN leukocyte lysosomes by Triton X-100 (0.01%). Steroid was solubilized either in ethanol and diluted in 0.3 M sucrose or in 1% DMSO, and preincubated with intact organelles, and change in absorbance 520 nm was measured after addition of nonionic detergent. Solvents without HC were also studied. Control shows $A_{520 \text{ nm}}$ change without Triton X-100. Presented are the means of 8–12 replicates for each point.

were performed with normal rat liver as the lysosome source (Fig. 5). Liver organelles contained in the subcellular fraction sedimenting between 1,000 *g* for 10 min and 25,000 *g* for 10 min were stressed with 37°C incubation for 45 min (16). This incubation period caused the liver organelles to leak $16.5 \pm 1.5\%$ of their total enzyme content in the absence of steroid. Enzyme release was augmented by the PMN leukocyte lysosome labilizer, progesterone, at several concentrations. Unlike its action on PMN leukocyte lysosomes, however, dexamethasone prevented the release of β -glucuronidase from heat-stressed liver organelles. Significantly less enzyme was detected at both 5×10^{-5} and 1×10^{-4} M concentrations, and dexamethasone did not inhibit the liver lysosomal enzyme studied.

Lysosomes isolated from normal human volunteers receiving prednisolone were stressed by heat incubation and the β -glucuronidase activity released was assayed. A simultaneously studied organelle suspension obtained from control subjects released $38.8 \pm 1.9\%$ (SEM) of the total granule content of β -glucuronidase after incubation at 37°C for 90 min. The lysosomes from steroid-treated volunteers released $37.9 \pm 2.9\%$. These values represent data obtained from five control and six steroid studies, each with three replicates. The plasma cortisol concentrations studied at the time PMN leukocytes were obtained averaged $14.7 \mu\text{g}/100 \text{ ml}$ in control subjects and $0.8 \mu\text{g}/100 \text{ ml}$ in volunteers receiving prednisolone. Thus in vivo anti-inflammatory steroids did not render

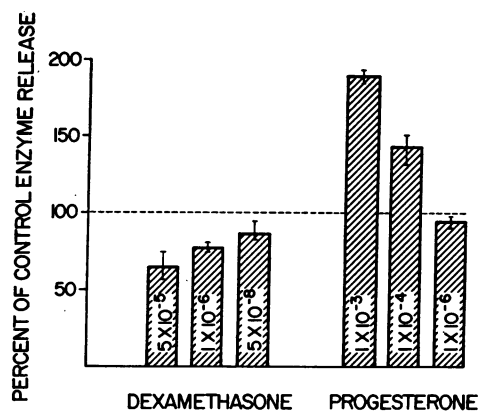


FIGURE 5 Steroid effects on β -glucuronidase release from rat liver lysosomes. Liver organelles in 0.25 M sucrose-0.02 M Tris, pH 7.1, ($A_{520 \text{ nm}}$ of 1:10 dilution = 1.000) were preincubated for 20 min at 2°C with steroid hormone or its solvent and then heat-stressed at 37°C for 45 min. Enzyme liberated in solvent alone was set as 100% release. Shown are means \pm 1 SD of six determinations. *P* values (Student's *t* test) versus the control for 5×10^{-5} and 1×10^{-4} dexamethasone and for 1×10^{-3} M progesterone are < 0.02 , and for 1×10^{-5} M progesterone < 0.01 . Differences observed at the other steroid concentrations were not significant.

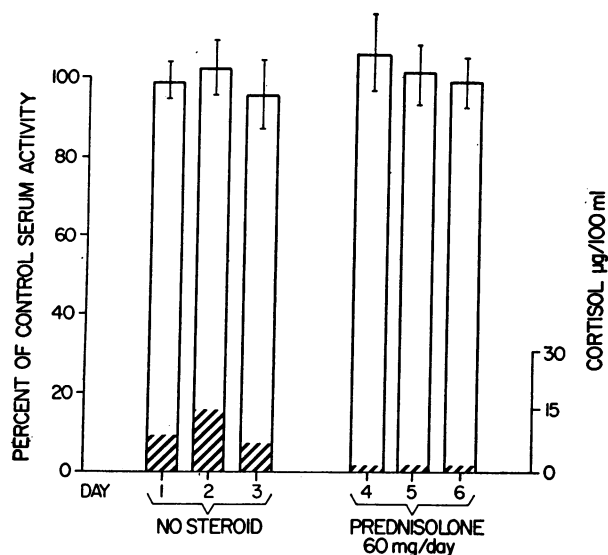


FIGURE 6 Effect of in vivo prednisolone on isolated lysosomes. Sera obtained daily before and during prednisolone administration were mixed (final concentration 1:10) with intact organelles before the addition of Triton X-100. Absorbance 520 nm change in control sera was set at 100% activity. Shown are means and ranges of six experiments, each with 4-8 replicates. Shaded areas depict 8 A.M. plasma cortisol concentrations.

PMN leukocyte lysosomes more resistant to heat stress. Furthermore, serum obtained during prednisolone treatment did not protect isolated organelles from lysis by Triton X-100 (Fig. 6). Sera obtained from subjects receiving adrenocorticosteroid had the same effect as control sera. The membranolytic action of the detergent was not impeded. As anticipated, plasma cortisol concentrations were depressed by the exogenous prednisolone, indicating that the volunteers had ingested the steroid.

DISCUSSION

The methods used in these experiments are the techniques commonly employed to study the effects of steroid hormones on lysosomal membranes (1, 2, 8, 15, 16). Two standard stress procedures were studied: heat incubation and detergent lysis, both known to induce a predictable leakage of hydrolases from intact lysosomes. Using these methods with liver organelles, we could reproduce the observations previously published on the labilizing and stabilizing effects of certain hormones. However, substituting lysosomes derived from the cell most concerned with acute inflammatory reactions, the PMN leukocyte, we could not demonstrate membrane stabilization by anti-inflammatory corticosteroids. Direct membrane interaction was clearly evident in the case of progesterone and androstenedione, two compounds known to

induce leakage of liver organelle membranes (15). But the anti-inflammatory steroid hormones we tested exerted no stabilizing activity on PMN leukocyte lysosomes. And the agents were studied at various concentrations including both physiologic and pharmacologic ranges. With only two steroids, hydrocortisone hemisuccinate and hydrocortisone acetate, could we find less enzyme activity in the supernates after stressing lysosomes by heat incubation. But in both cases this was due to enzyme inhibition by these hormone preparations rather than to lysosomal membrane stabilization. de Duve et al. (1) observed less free acid phosphatase activity released from liver organelles in cortisone acetate and cautioned about the possible misinterpretation of lysosome membrane stabilization by what in fact were enzyme inhibitors. And finally, the administration of prednisolone to human volunteers not only failed to modify the integrity of their PMN leukocyte lysosomes, but also did not impart a stabilizing activity to their sera when tested with normal leukocyte granules.

Numerous publications have dealt with the effects of corticosteroids on liver lysosomal membrane integrity. Not all studies (17, 18) have confirmed the stabilization hypothesis. Either no effect or mild stabilization has been noted with anti-inflammatory steroids, depending upon experimental conditions. These diverse observations have also been found using PMN leukocyte lysosomes. Thus, Weissmann, Becher, and Thomas (8) showed that 10^{-8} M hydrocortisone would not protect rabbit peritoneal PMN leukocyte lysosomes from streptolysin O lysis. And Willis, Davison, Ramwell, Brocklehurst, and Smith (19) could not alter the release of β -glucuronidase from isolated lysosomes by hydrocortisone even at concentrations up to 1 mg/ml. Both hydrocortisone and paramethasone were found to be without effect at 10^{-8} – 10^{-6} M on rat blood leukocyte lysosomes but capable of stabilizing rabbit and guinea pig peritoneal exudate PMN organelles using hypotonic incubation media (20, 21). The variables found by these authors to alter lysosomal membrane permeability have included species and organ sources of organelles, tonicity and pH of media, and the types of stress procedures employed. In addition, lysosomes from both liver (22) and PMN leukocytes (23, 24) are heterogeneous in composition and form. These variables could account for some of the published discrepancies on how drugs affect membrane stability. We used experimental conditions more physiologic for our studies with lysosomes. Whereas both liver and PMN leukocyte lysosomes can be labilized by progesterone and androstenedione, only liver organelles were stabilized by the anti-inflammatory steroids, and then to only a mild degree.

The reasons for the different reactivity of liver and PMN leukocyte lysosomes to anti-inflammatory corti-

costeroids are unclear; our observations could be due to variations in membrane structure. Although the studies performed thus far (25, 26) are in agreement concerning protein content (both liver and leukocyte organelle membranes containing approximately 50% protein/wet wt) differences have been found in the lipids of these membranes. Rat liver lysosomal membranes contained cholesterol and phospholipid at a ratio of 1:3.4 (25), and a cell membrane fraction from rabbit PMN leukocyte granules had a ratio of 1:1.4 (26). If steroids alter permeability by their action at the lipid-water interface of membranes, these differences in the composition of the membrane lipids could explain our results.

Using viable cell preparations, numerous authors, including Mandell, Rubin, and Hook (5) and Wiener et al. (6), have shown that hydrocortisone prevents the release of lysosomal hydrolases during phagocytosis. This association with anti-inflammatory corticosteroids has also been observed with in vivo models of inflammation (27, 28) and lysosome rupture (29). But direct membrane interaction by these compounds and resulting stabilization need not be invoked to explain their anti-phlogistic actions. Corticosteroids have a wide range of biological activities that could account for their suppressive effects on inflammation (30–34). Interference with glucose transport (33) and inhibition of ATP generation (34), or suppression of NADH oxidase activity (5) are several actions that could lead to depressed cell function. After phagocytosis, these effects of hydrocortisone would prevent the intracellular metabolic activity necessary for merger of organelle membranes with the endocytic vacuole, the subsequent degranulation, and the extrusion of lysosome contents from the cell. In light of our experimental observations presented above, these are more plausible explanations for the mechanism of anti-inflammatory steroid action than the hypothesis of direct membrane stabilization.

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