Further Observations on the Activation of Lysosomal Acid α -Glucosidase by Cortisone Derivatives

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1. Cortisone acetate activates the acid α -glucosidase in rat liver slices and in isolated liver lysosomes. 2. The reaction is steroid specific and moreover does not occur with lysosomal acid phosphatase or β -galactosidase. 3. After pretreatment of the lysosomes with cortisone, substrate (maltose) binding to the soluble lysosomal acid α -glucosidase is not affected, but the steroid does increase the $V_{\max}$ value. Membrane-bound enzyme is not activated by cortisone. 4. 4-[14 C]Cortisone is preferentially bound to the lysosomal membrane and the possible involvement of this structure in the activation phenomenon is discussed.

Bourne *et al.* (1971) established that cortisone derivatives activated acid α -glucosidase in lysosomes both *in vivo* and *in vitro*. The present paper describes further studies on the interaction of cortisone and other steroids with the lysosomal enzyme, which were carried out in an attempt to locate the site of hormone activation.

Materials and Methods

Materials

A.R.-grade chemicals were used whenever possible; other chemicals were of the best available purity.

Cholesterol was purchased from British Drug Houses Ltd., Poole, Dorset, U.K., and cortisone acetate and adrenosterone were from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. 4-Pregnen-21-ol-3,11,20-trione (21-hydroxypregn-4-ene-3,11,20-trione) and 4-pregnene-17 α ,21-diol-3,20-dione (17 α ,21-dihydroxypregn-4-ene-3,20-dione) were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., and [4- 14 C]cortisone acetate (15.8 mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

Animals and liver slices. The treatment of animals and the preparation of liver slices were described by Bourne *et al.* (1971).

Acid α -glucosidase and acid phosphatase assays. These determinations were carried out as described by Bourne *et al.* (1971).

β -Galactosidase assay. The enzyme solution (0.4 ml)

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was added to a solution of McIlvaine buffer (pH 5.5, 0.3 ml) and 30 mM *p*-nitrophenyl β -D-galactoside (0.3 ml), preheated to 30°C (Dey & Pridham, 1969). This temperature was maintained for 15 min and the reaction stopped by addition of 0.1 M Na_2CO_3 (5 ml). The release of *p*-nitrophenol was measured by reading the E_{405} . The activities are expressed as μmol of *p*-nitrophenyl β -D-galactoside hydrolysed/min per mg of protein at 30°C.

Protein. Protein was measured by a modification of the Folin method (Lowry *et al.*, 1951) with crystalline human serum albumin [Sigma (London) Chemical Co. Ltd.] as standard.

Preparation of soluble and membrane fractions of lysosomes. Lysosomes were isolated from rat liver tissue as described by Bourne *et al.* (1971). The organelles were frozen and thawed three times in 1 mM-EDTA–25 mM-NaCl, pH 6.7 (1 ml), and the membranes centrifuged down at 100 000g (1 h). The supernatant solution (soluble fraction) was decanted off and stored at –60°C. The membranes were washed four times in 1 mM-EDTA–25 mM-NaCl, pH 6.7 (1 ml), with vigorous stirring. After each washing the suspension was frozen and thawed three times and the membranes were isolated by centrifugation at 100 000g (1 h). The washings were stored at –60°C. Before being assayed for α -glucosidase, the membranes were stirred vigorously in 1 mM-EDTA–25 mM-NaCl, pH 6.7 (1 ml), to produce a fine suspension. Assay method 1 (Bourne *et al.*, 1971) was employed for the determination of activity of the soluble and membrane fractions and the membrane washings.

Reaction of [4- 14 C]cortisone acetate with rat liver lysosomes. A rat liver lysosome preparation equivalent to 1 mg of protein was incubated with Krebs–Ringer bicarbonate solution (Krebs & Henseleit, 1932) (1 ml) containing 0.25 M-sucrose and 0.02 M-[4- 14 C]cortisone acetate (0.1 ml, 0.25 μCi) in a shaking incubator at 37°C for 1 h. The lysosomes were then

Table 1. *Effect of cortisone acetate and structurally related steroids on the acid α -glucosidase activity of rat liver slices and lysosomes*

Liver slices (each approx. 300mg) were placed in a stoppered tube in a shaking incubator with steroid (4mg) in Krebs–Ringer bicarbonate solution (10ml) for 2h at 37°C. The slices were individually homogenized in 1 mM-EDTA–25 mM-NaCl, pH 6.7 (2ml), with a glass–Teflon Potter–Elvehjem homogenizer, and then frozen and thawed three times and centrifuged for 1 h at 100000g. Acid α -glucosidase in the supernatant solution was determined by assay method 1 (Bourne *et al.*, 1971). The experimental values represent the arithmetical mean of ten determinations with the s.e.m. shown in parentheses. The lysosomes (equivalent to 1 mg of protein) were incubated at 37°C for 1 h with steroid (40 μ g) in Krebs–Ringer bicarbonate–0.25 M-sucrose solution (1 ml). The organelles were isolated by centrifugation and a soluble fraction was prepared as described in the Materials and Methods section. The acid α -glucosidase activity of this fraction was determined by assay method 1. Experimental values represent the arithmetical means of three determinations, with the s.e.m. shown in parentheses.

Steroid	$10^{-2} \times$ Acid α -glucosidase activity (μ mol of maltose hydrolysed/min per mg of protein at 37°C)	
	Liver slices	Lysosomes
Control (no steroid)	0.95 (± 0.09)	2.98 (± 0.50)
Cortisone acetate	4.70 (± 0.40)	15.00 (± 0.40)
Cholesterol	0.81 (± 0.06)	1.73 (± 0.01)
4-Pregnen-21-ol-3,11,20-trione	0.79 (± 0.02)	1.83 (± 0.02)
4-Pregnene-17 α ,21-diol-3,20-dione	0.86 (± 0.25)	2.38 (± 0.01)
Adrenosterone	1.20 (± 0.02)	—

sedimented at 22500g (20min) and washed with 5×1 ml of Krebs–Ringer bicarbonate–0.25 M-sucrose solution. The final washing possessed negligible radioactivity. Membrane and soluble fractions were then prepared as described above; negligible radioactivity was present in the last membrane washing. The soluble fraction and membrane washings were pooled and evaporated to dryness (under reduced pressure at room temperature), and the residue was extracted with benzene–ethanol (9:1, v/v) (0.2ml). The extract was applied to 4cm $\times$ 4cm pieces of Whatman no. 3 paper, which were then dried under an i.r. lamp. The membrane fractions were suspended in the benzene–ethanol and also applied to paper strips. Radioactivities were determined, to 1% error, with a Beckman LS100 liquid-sintillation counter by immersing the strips in a toluene scintillant [toluene (1 litre) containing 2,5-diphenyloxazole (5g) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.3g)].

Results and Discussion

When liver slices were treated, separately, with a number of structurally related steroids only cortisone acetate produced a significant activation of acid α -glucosidase (Table 1). In comparison with the control, all the other steroids, with the exception of adrenosterone, partially inhibited the activity. Cortisone did not affect the optimum pH (4.4) of the

Fig. 1. *Lineweaver–Burk plots of soluble acid α -glucosidase activities of cortisone acetate-treated and untreated purified rat liver lysosomes*

Lysosomes (equivalent to 1 mg of protein) were incubated for 1 h at 37°C with ($\circ$) and without ($\bullet$) cortisone acetate (40 μ g) in Krebs–Ringer bicarbonate–0.25 M-sucrose solution (1 ml). Acid α -glucosidase activities of the soluble fractions (prepared as described in the Materials and Methods section) were measured by assay method 1 (Bourne *et al.*, 1971). Reaction rates (v) are expressed as μ mol of maltose hydrolysed/min per mg of lysosomal protein.

acid α -glucosidase in these experiments. A similar, specific, activation of the enzyme was observed (Table 1) when cortisone acetate was incubated with isolated rat liver lysosomes; the other steroids were, again, slightly inhibitory.

A kinetic analysis of the rates of hydrolysis of maltose by lysosomal acid α -glucosidase obtained from organelles that had been preincubated with cortisone acetate (40 μ g/mg of lysosomal protein) showed that the steroid had little effect on enzyme-substrate binding. K_m values derived from a Lineweaver-Burk plot (Fig. 1) are identical (11.1 mM) for both control and cortisone acetate-treated organelles. The V_{max} values, however, were 312.8 and 594.6 μ mol

of maltose hydrolysed/min per mg of protein respectively. Cortisone acetate therefore significantly increases the catalytic efficiency of the enzyme. A similar result has been obtained by Cox *et al.* (1971) in a study of the cortisone induction of alkaline phosphatase of HeLa 65 cells.

The possible role of the lysosomal membrane in the activation of acid α -glucosidase by steroid was first examined by incubating rat liver lysosomes with [$4\text{-}^{14}\text{C}$]cortisone acetate (Table 2); approx. 78 % of the radioactivity taken up was retained by the lysosomal membrane fraction.

In Fig. 2 the results are shown of an experiment to determine whether the hormone activated the lysosomal membrane-bound and/or soluble enzymes. In this study the total enzyme activity increased by 100 % after a 1 h incubation with the higher concentration (40 μ g/mg of soluble lysosomal protein) of cortisone acetate: the lower steroid concentration had little effect. Three successive washings, each followed by freezing and thawing, were necessary to remove all of the soluble acid α -glucosidase from the membrane fraction. When cortisone acetate (40 μ g/mg of protein) was added to a membrane fraction prepared in the absence of this steroid no latent acid α -glucosidase activity was liberated. The greatest increase in enzyme activity appeared in this soluble fraction (lysosomal contents plus washings), which was about three times that of the control. The total activity of the membrane-bound enzyme remained approximately constant.

The possibility that cortisone acetate produces a generalized activation of various lysosomal enzymes was checked by incubating various concentrations of the steroid with lysosomes and then assaying the soluble fraction from the organelles for acid phosphatase and β -galactosidase activities. The results

Fig. 2. Effect of cortisone acetate on soluble and membrane-bound acid α -glucosidase of purified rat liver lysosomes

Lysosomes (equivalent to 1 mg of protein) were incubated with and without cortisone acetate for 1 h at 37°C. Soluble and membrane fractions were prepared as described in the Materials and Methods section. The membranes were washed four times (washes w₁–w₄) with 1 mM-EDTA–25 mM-NaCl, pH 6.7 (1 ml); each washing was followed by freezing and thawing three times and centrifugation at 100000g (1 h). The membranes were then suspended in 1 mM-EDTA–25 mM-NaCl with vigorous stirring (final volume 1 ml). The soluble and membrane fractions and membrane washings w₁–w₄ were then assayed for acid α -glucosidase activity by assay method 1 (Bourne *et al.*, 1971). The results shown are each the arithmetical mean of five determinations. (a) Control (no steroid added); (b) with 27 μ g of cortisone acetate/mg of lysosomal protein; (c) with 40 μ g of cortisone acetate/mg of lysosomal protein.

Table 2. Incorporation of [$4\text{-}^{14}\text{C}$]cortisone acetate into rat liver lysosomes

Purified rat liver lysosomes (equivalent to 1 mg of protein) were incubated with [$4\text{-}^{14}\text{C}$]cortisone acetate (0.25 μ Ci) for 1 h at 37°C. The organelles were then washed and membrane and soluble fractions prepared; radioactivities were determined with a liquid-scintillation counter. Experimental details are given in the Materials and Methods section.

	[$4\text{-}^{14}\text{C}$]Cortisone acetate (c.p.m.)
Total incubated	230685
Total recovered	218666
Lysosome washings plus incubation volume	212223
Lysosomal contents	1411
Lysosomal membranes	5032

Fig. 3. Effect of cortisone acetate on lysosomal (a) acid phosphatase and (b) β -galactosidase activities

Purified rat liver lysosomes (equivalent to 1 mg of protein) were incubated at 37°C in Krebs-Ringer bicarbonate-0.25 M-sucrose solution (1 ml) containing different concentrations of hormone for 1 h. The organelles were isolated from the suspensions by centrifugation at 22500g (20 min). Soluble fractions of the organelles (prepared as described in the Materials and Methods section) were assayed for enzyme activities. Acid phosphatase activity is expressed as $10^{-2} \times \mu\text{mol}$ of phosphate liberated/min per mg of lysosomal protein and β -galactosidase as μmol of *p*-nitrophenyl β -D-galactoside hydrolysed/min per mg of lysosomal protein; both were measured at 30°C.

are illustrated in Fig. 3, which shows that cortisone acetate does not activate either of the enzymes but tends to inhibit, particularly for β -galactosidase. Weissmann (1965) and Lewis *et al.* (1970) have reported that corticosteroids do not affect the activity of acid phosphatase in lysosomes.

The exact mechanism of cortisone activation of lysosomal acid α -glucosidase is still not clear from the

results described above. *In vitro* a particular steroid structure appears to be necessary for activation to occur, and the reaction is at least to some extent enzyme-specific, as the catalytic efficiencies of lysosomal acid phosphatase and β -galactosidase are not increased by cortisone. It should be noted that Kyaw & Mellors (1972) report that cortisone acetate elevates the soluble lysosomal acid phosphatase and some other hydrolases in rat liver, but these workers were administering the hormone *in vivo*.

The lysosomal membrane appears to be important, as acid α -glucosidase cannot be activated once it is released from the organelle (Bourne *et al.*, 1971), and also cortisone binds preferentially to the membrane. The latter observation does not, of course, exclude a role for the cortisone that penetrates to the interior of the lysosome. In fact, it is possible that the steroid passes through the membrane by a specific permeation process and then activates the soluble enzyme directly by producing a change in conformation or by displacing an endogenous inhibitor. The membrane may also function to hold the enzyme in a favourable conformation for interaction with the cortisone. A second possibility is that cortisone acts indirectly and releases material, perhaps protein or lipid, which then interacts with the acid α -glucosidase.

Previous work in this general field has indicated that sex hormones react with the lysosomal membrane. For example, Szego *et al.* (1971) have shown that intravenous administration of low doses of oestrogens and testosterone to ovariectomized rats enhances the lytic susceptibility of the organelles, resulting in a diminished structural latency of some acid hydrolases.

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