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Studies on Type II Glycogenosis

EFFECTS OF CORTISONE DERIVATIVES ON ACID α-GLUCOSIDASE

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Cortisone causes a marked increase in the activity of liver acid α-glucosidase 2h after injection into male Wistar rats. Studies on rat liver tissue slices, isolated lysosomes and cultured skin fibroblasts have demonstrated similar elevations of acid α-glucosidase activity after incubation with cortisone. Cortisone-treated human liver tissue, obtained by needle biopsy, also shows an increase in acid α-glucosidase activity. Neutral α-glucosidase activity was not stimulated by cortisone *in vivo* or in liver slices.

All but one of the various types of glycogen storage diseases are characterized by specific enzyme lesions (Brown & Brown, 1968). In type II glycogenosis lysosomal acid α-glucosidase is not detectable and the absence of this activity is believed to result in tissue glycogen accumulation (Hers, 1963; Brown, Brown & Jeffrey, 1970). In the present study the effect of cortisone and cortisol (hydrocortisone) on acid α-glucosidase activity in rats has been examined in relation to possible treatment of cases of type II disease.

MATERIALS AND METHODS

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

Glucose oxidase (15424 EGAC) and horseradish peroxidase (15302 EPAB) were obtained from the Boehringer Corp. (London) Ltd., London W.5, U.K.; *o*-dianisidine dihydrochloride and cortisone acetate were purchased from British Drug Houses Ltd., Poole, Dorset, U.K.; cortisol (hydrocortisone) sodium succinate was obtained from Glaxo Ltd., Greenford, Middx., U.K.

Difco tissue culture media (Waymouth 752/1 MB, Eagle's minimal medium) and antibiotics, and bovine serum (T. C. desiccated), were obtained from Baird and Tatlock Ltd., London W.1, U.K.; quarter-strength Ringer solution tablets were obtained from Oxoid Ltd., London E.C.4, U.K.

[U-^{14}C]Maltose was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Acid α-glucosidase assay method I. Sodium acetate (24μmol, pH3.6), maltose (5.8μmol) and enzyme fraction (0.1ml, 100000*g* supernatant) in a total volume of 0.4ml were incubated together at 37°C for 15min and the reaction was stopped by addition of 0.18M-$ZnSO_4$ (0.1ml) and 0.36M-NaOH (0.1ml) solutions. The protein precipitate was removed by centrifugation and a portion of the supernatant solution (0.4ml) assayed by the glucose oxidase method (Fleming & Pegler, 1963; Catley, 1967).

Specific activity is defined as μmol of maltose hydrolysed/min per mg of protein at 37°C.

Acid α-glucosidase assay method II (Dancis, Hutzler, Lynfield & Cox, 1969). Sodium acetate (0.024mM, pH3.6), 0.4μM-[U-^{14}C]maltose (0.2ml, 0.025μCi), 0.2μM-D-glucose and fibroblast suspension (0.1ml) in a total volume of 0.5ml (pH3.6) were incubated at 37°C and the reaction was stopped by the addition of propan-2-ol (1.0ml). The protein precipitate was removed by centrifugation and the supernatant solution fractionated by descending paper chromatography on Whatman no. 3 paper with ethyl acetate–acetic acid–water–formic acid (18:3:1:4, by vol.) solvent. [^{14}C]Glucose liberated by enzymic hydrolysis was determined by counting the radioactivity on appropriate 1cm paper strips from the chromatograms with a liquid-scintillation counter. A glucose marker was used to locate the radioactive areas of the chromatogram. Enzyme activity is defined as percentage of maltose hydrolysed in 40min at 37°C.

Acid phosphatase assay. The reaction mixture contained 0.25M-sodium β-glycerophosphate (0.1ml), 0.125M-sodium acetate buffer, pH5.0 (0.2ml) and enzyme fraction (0.1ml). After incubation for 30min at 30°C, an equal volume of 10% (w/v) trichloroacetic acid was added, and a sample of the deproteinized solution was assayed for P_i by the method of Fiske & Subbarow (1925). The activity was calculated as μmol of phosphate formed/min at 30°C per mg of protein.

Glutamate dehydrogenase assay. Glutamate dehydrogenase assay was carried out with a Boehringer diagnostic kit (15995 TGAD).

Animals. Male Wistar rats, weighing between 150 and 200g, were used both in the studies *in vivo* and to provide material for the liver enzyme and tissue-culture studies. The animals were fed on a commercial stock diet *ad lib.* and were killed as needed by cervical fracture.

Studies in vivo. Cortisone acetate (in saline) was injected intraperitoneally (Hanoune & Feigelson, 1969) and the liver excised immediately after death and homogenized in a 1:1 (w/v) 1 mM-EDTA–25 mM-NaCl (pH 6.7) solution in a Waring Blendor. The homogenate was frozen and thawed six times and centrifuged for 1 h at 100000*g*. α-Glucosidase activity was determined by assay method I.

Treatment of liver slices. The method of Deutsch (1936) was used to prepare rat liver slices (wet wt. 200–300 mg each) which were placed in a shaking incubator at 37°C in stoppered tubes containing Krebs–Ringer bicarbonate solution (Krebs & Henseleit, 1932) (10 ml) with and without added cortisone acetate. The slices were individually homogenized in 1 mM-EDTA–25 mM-NaCl, pH 6.7 (2 ml), in a glass–Teflon Potter–Elvehjem homogenizer, frozen and thawed three times and finally centrifuged at 100000*g* for 1 h. α-Glucosidase activity was measured by assay method I.

Treatment of lysosomes. The livers of rats that had been starved for 24 h were excised and a light-mitochondrial fraction was prepared by the method of Schneider & Hogeboom (1950, 1955*a*,*b*). Further fractionation was effected with a 650 ml linear-with-volume gradient of 17–55% sucrose and centrifuging for 4 min at 11000 rev./min (excluding acceleration and deceleration times) in a B-XIV titanium zonal rotor (MSE Ltd., London S.W.1, U.K.). Acid phosphatase and glutamate dehydrogenase activities served as markers for the lysosomal and mitochondrial fractions, respectively; the former fraction was eluted from the rotor between 150 and 300 ml. This was diluted with 0.25 M-sucrose to a final molarity of 0.4 with respect to sucrose, and the organelles were isolated by centrifugation at 54000*g* (30 min) (Aronson & de Duve, 1968). Lysosomal incubations were carried out at 37°C in Krebs–Ringer bicarbonate solution (Krebs & Henseleit, 1932) (1 ml) containing 0.25 M-sucrose (85.6 mg of solid sucrose added/ml of Krebs–Ringer bicarbonate solution) (Rosenberg & Janoff, 1968) with and without cortisone acetate and the treated lysosomes were removed by centrifugation at 22500*g* (20 min) and then ruptured by freezing and thawing three times in 1 mM-EDTA–25 mM-NaCl, pH 6.7 (1 ml). Organelle membranes were removed by centrifugation at 100000*g* for 1 h and the supernatant solution was assayed for acid α-glucosidase activity by using method I. All preparations were carried out at 0–4°C.

Tissue culture. A small section (1 mm × 2 mm) of skin was removed from the shaved abdomen of a rat immediately after death and was sterilized in Ringer solution containing penicillin (1000 units/ml) and streptomycin (0.5 mg/ml). The explants were cultured in Eagle's minimal medium, supplemented with 10% (w/v) bovine serum, for 7 days at 37°C, treated with 0.5% trypsin, and sub-cultured in 12 oz medicine bottles containing Waymouth's medium, supplemented with 6% (w/v) bovine serum. All media were sterilized by filtration through millipore filters. The cells were allowed to grow to confluence. All media contained penicillin (50 units/ml) and streptomycin (50 μg/ml). Cells were harvested by trypsinization followed by centrifugation or by use of a rubber 'policeman' and the acid α-glucosidase activity of suspensions of fibroblasts in 24 μM-sodium acetate buffer, pH 3.6 (0.1 ml), was measured, by assay method II, after incubation at 37°C with and without cortisol sodium succinate.

Treatment of human liver tissues. A needle biopsy specimen (5 mg) was divided into five approximately equal sections which were suspended in Krebs–Ringer bicarbonate solution (1 ml) containing various concentrations of cortisone acetate. (Individual digests contained approximately equal concentrations of protein.) Incubations were carried out at 37°C for 1 h and assay method II was used for acid α-glucosidase after tissue maceration.

Protein. Protein was measured by a modification of the Folin method (Lowry, Rosebrough, Farr & Randall, 1951) with crystalline human serum albumin as standard.

RESULTS AND DISCUSSION

Figs. 1(*a*) and 1(*b*) show clearly that injection of cortisone acetate into rats increases the concentration of acid α-glucosidase in the liver. The stimulatory effect reaches a maximum with a cortisone concentration of 24 mg/kg body wt. and with fixed amounts of the hormone enzyme activity is highest 2 h after injection. To see if repeated hormone injection would further increase the enzyme activity, acid α-glucosidase activity was measured 2 h after a 12 mg/kg body wt. injection and again

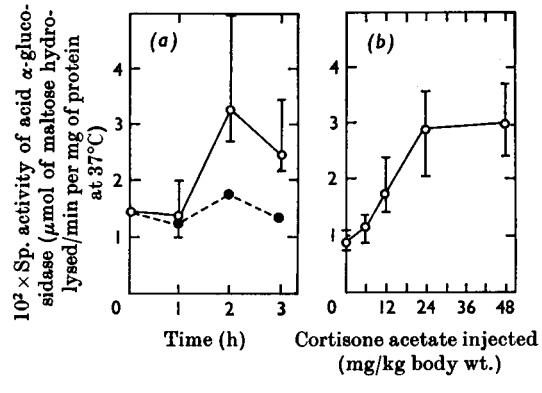

Fig. 1. (*a*) Effect of intraperitoneal injection of cortisone acetate (12 mg/kg body wt.) into rats on liver acid α-glucosidase activity after various times. ●, Animals injected with saline (1 ml); ○, cortisone acetate (in saline, 1 ml)-treated animals. (*b*) Effect of different concentrations of cortisone acetate on liver acid α-glucosidase activity measured 2 h after injection. The livers were excised immediately after death and individually homogenized in 1 vol. (v/w) of 1 mM-EDTA–25 mM-NaCl, pH 6.7, in a Waring Blendor. The homogenate was frozen and thawed six times, centrifuged at 100000*g* for 1 h and the supernatant was assayed by assay method I for acid α-glucosidase activity. Six rats were used for each experimental point in (*a*) and (*b*). The enzyme activity is expressed in μmol of maltose hydrolysed/min per mg of protein at 37°C and the arithmetical mean and range of the results are shown.

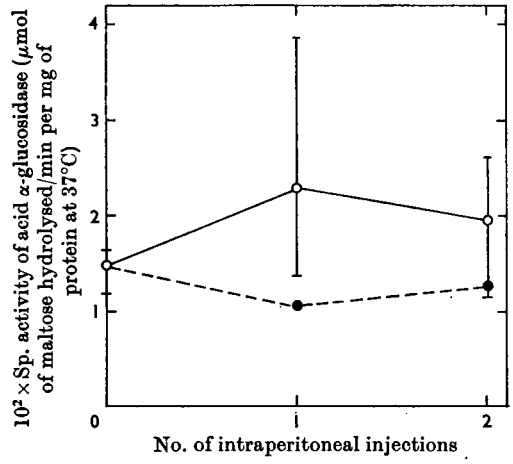

Fig. 2. Effect of two equal intraperitoneal injections (12mg/kg body wt.) of cortisone acetate on rat liver acid α-glucosidase activity. The second injection was given 2h after the first and the assays were carried out 2h after the injection. ○, Cortisone acetate (in 1ml of saline)-treated animals; ●, animals injected with saline only (1ml). Six rats were used per experimental point and the results were obtained and expressed in the same way as those shown in Fig. 1.

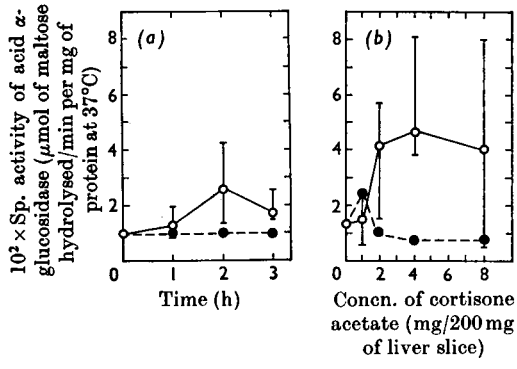

Fig. 3. (*a*) Acid α-glucosidase activity in rat liver slices (about 300mg each) after incubation with cortisone acetate (2mg) for different times. ○, Cortisone-treated liver slices; ●, controls. (*b*) Effect of different concentrations of cortisone acetate (2h incubations) on acid α-glucosidase in rat liver slices (about 200mg each). ○, Cortisone-treated liver slices; ●, controls. Each slice was placed in a stoppered tube (containing 10ml of Krebs–Ringer bicarbonate solution) on a shaking incubator with or without added cortisone acetate. The slices were individually homogenized in 1mM-EDTA–25mM-NaCl, pH6.7 (2ml), in a glass–Teflon Potter–Elvehjem homogenizer, frozen and thawed three times and centrifuged for 1h at 100000*g*. Acid α-glucosidase activity expressed as μmol of maltose hydrolysed/min per mg of protein was determined by assay method I. The experimental points represent the arithmetical and range mean of ten determinations.

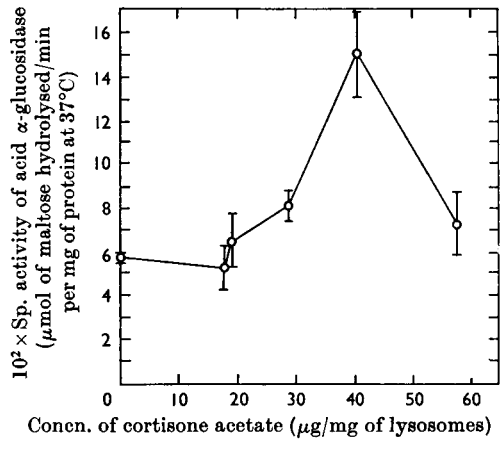

Fig. 4. Effect of different cortisone acetate concentrations on the acid α-glucosidase activity in whole rat liver lysosomes (for extraction procedure see the text). The lysosomes were incubated at 37°C in different concentrations of hormone for 1h before assay. The acid α-glucosidase activity is expressed as μmol of maltose hydrolysed/min per mg of lysosome protein and was determined on a 100000*g* supernatant, after freezing and thawing three times in 1mM-EDTA–25mM-NaCl, pH6.7 (1ml). Experimental points are the mean of three determinations.

Table 1. *Effect of cortisol on the acid α-glucosidase activity in rat skin fibroblasts*

Rat skin fibroblasts grown to confluence in Waymouth's medium at 37°C were harvested and then incubated with different concentrations of cortisol sodium succinate for 40min at 37°C. Acid α-glucosidase activity was measured by assay method II (described in the Materials and Methods section).

Concn. of cortisol (μg/ml) ...	0	10	100	1000
Maltose hydrolysed (%) ...	3.9	4.3	15.3	10.1

2h after a second such injection (Fig. 2). No further increase in activity was observed after the second injection. Cortisone acetate also increased the acid α-glucosidase activities of rat liver slices (Figs. 3*a* and 3*b*), isolated lysosomes (Fig. 4) and cultured rat skin fibroblasts (Table 1). In all cases inhibition of the enzyme occurred at high concentrations of cortisone.

Treatment of a dialysed 30–60% saturated ammonium sulphate fraction of acid α-glucosidase from rat liver with cortisone acetate produced no activation.

Neutral α-glucosidase activity did not appear to be stimulated by cortisone in experiments either *in vivo* or with tissue slices.

A needle-biopsy specimen of normal human liver

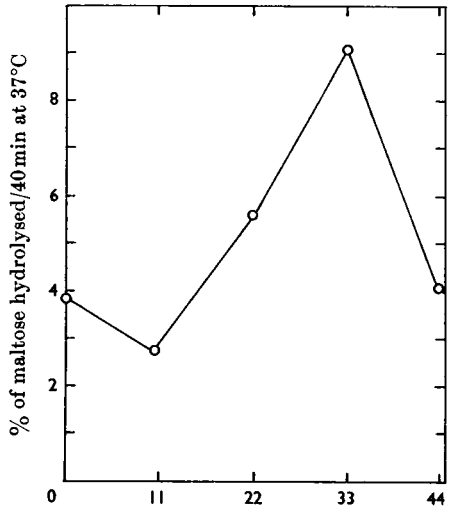

Fig. 5. Effect of incubation for 1 h at 37°C with different concentrations of cortisone acetate on acid α-glucosidase activity of human liver tissue. A needle-biopsy specimen was divided into five approximately equal parts and, after freezing and thawing in acetate buffer, pH 3.6 (0.1 ml), enzyme determinations were carried out with assay method II.

behaved similarly to rat liver in that cortisone acetate activated acid α-glucosidase and at higher concentrations produced an inhibition (Fig. 5).

The molecular weight of rat liver lysosomal acid α-glucosidase is approx. 114000 (Jeffrey, Brown & Brown, 1970). Our own (unpublished) gel-filtration experiments confirm that the enzyme has a high molecular weight and a probable subunit structure. Cortisone only appears to be an effective activator when the enzyme is in an 'organized state' (i.e. intra-cellular or intra-organelle). In this connexion it is not known whether type II glycogenosis results from a complete absence of the enzyme protein or possibly from an incomplete assembly of a number of subunits. The latter could result from a failure of the cell to synthesize one or more subunits or from the lack of some other factor that is responsible for higher-order structures of the protein.

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