et al. 1950). On the other hand, the first symptoms are noticed only a few months after birth, although there is little doubt that the absence of maltase is congenital. It therefore appears that the pathological manifestations are not due to the enzymic defect itself but rather to the progressive glycogen deposition, which causes the disruption of the muscular fibres. This is in agreement with the morphological aspect of the tissues, which contain large vacuoles of glycogen and a small amount of normal cytoplasm.

If the above interpretation is confirmed, it can be expected that other deposition diseases might be explained on the basis of the absence of other lysosomal enzymes.

SUMMARY

1. Human liver and heart and skeletal muscles contain an enzyme that hydrolyses maltose and glycogen into glucose and which catalyses transglucosylation from maltose to glycogen.

2. This α-(1→4)-glucosidase is absent from the tissues of children affected by Pompe's disease (cardiomegalic form of glycogen-storage disease).

3. The mechanism by which the absence of maltase can cause glycogen storage is discussed.

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Tissue Fractionation Studies

16. INTRACELLULAR DISTRIBUTION AND PROPERTIES OF α-GLUCOSIDASES IN RAT LIVER*

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The recent discovery that a α-(1→4)-glucosidase (or maltase) is lacking in the liver and heart and skeletal muscles of children affected by generalized glycogen-storage disease (Hers, 1961, 1963) has focused our attention on the study of this enzyme in animal tissues. The presence of maltase activity in liver tissue has been previously reported by several workers. The enzyme was detected by its ability either to liberate glucose from maltose or glycogen (Glock, 1936; Torres & Olavarria, 1961; Rosenfeld & Popova, 1962) or to catalyse transglucosylation (Whitby, 1954), or by both properties (Giri, Nagabhushanam, Nigam & Belavadi, 1955; Stetten, 1959). However, the published data

* Part 15: Sellinger, Beaufay, Jacques, Doyen & de Duve (1960).

are insufficient to characterize the enzyme or enzymes concerned and their possible relationship to glycogen disease. On the other hand, the recognition by Larner & Gillespie (1956) of the particulate nature of intestinal maltase has prompted us to determine the intracellular localization of the hepatic activity. In this paper it is shown that rat liver contains several α-glucosidases, which can be differentiated by their intracellular localization as well as by their pH–activity curves and by other properties. An acid maltase, similar to the enzyme present in human liver and lacking in generalized glycogenosis, has been localized in the lysosomes. A neutral maltase has been localized in both the soluble and the microsomal fractions.

EXPERIMENTAL

Tissue fractionation. The experiments were performed on the livers of adult albino rats. The particulate fractions used for the activation experiments as well as for the characterization of the liver maltases were obtained as follows:

Method 1. A 20% liver homogenate in 0·25M-sucrose or 0·25M-mannitol was prepared with a homogenizer of the Potter–Elvehjem type and fractionated by centrifuging in a Serval refrigerated centrifuge, model RC2, with the rotor SS-34. Three sediments and a soluble fraction were obtained by centrifuging the homogenate successively for 10 min. at 625*g* (sediment discarded), 10 min. at 22 500*g* (mitochondrial fraction) and 1 hr. at 36 000*g* (microsomal and soluble fractions). The last two sediments were washed twice with the suspending medium and finally resuspended in a volume (ml.) equal to twice the weight (g.) of the original liver.

Method 2. A more complete fractionation of the liver was performed according to de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). Density-gradient equilibration was achieved by the technique of Beaufay, Bendall, Baudhuin, Wattiaux & de Duve (1959), with the difference that 0·6 ml. of a concentrated light-mitochondrial fraction suspended in 0·25M-sucrose was layered on top of the gradient instead of being uniformly distributed within it at the beginning of the experiment.

Enzyme assays. Cytochrome oxidase was assayed according to Appelmans, Wattiaux & de Duve (1955), but at 25°; glucose 6-phosphatase and uricase by the techniques of de Duve *et al.* (1955), except that uricase was measured at 37° and in the presence of 0·1% Triton X-100; total acid phosphatase and acid deoxyribonuclease by the methods of Wattiaux & de Duve (1956), with the difference that for deoxyribonuclease the incubation medium contained 0·2M-KCl; free acid phosphatase as described by Gianetto & de Duve (1955).

Maltase was measured as described in the preceding paper (Hers, 1963). Commercial glucose oxidase contains a small amount of invertase which is responsible for a high blank in the presence of tissue extracts prepared in 0·25M-sucrose. As this invertase has a high temperature coefficient, the blank could be reduced by running the glucose-oxidase reaction for 2 hr. at 11° (temperature of tap water) instead of for 1 hr. at 37°. In some experiments, sucrose was replaced by mannitol (Leuthardt & Müller, 1948). As will be further described below, it was necessary to distinguish between total and free acid maltase activity. The former was assayed in the presence of 0·1% Triton X-100 and the latter with the precautions recommended by Gianetto & de Duve (1955), i.e. with an incubation time of 10 min. at pH 5, and with 0·25M-sucrose or 0·25M-mannitol in the medium. Proteins were determined according to Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Identification of several maltases in rat liver. When rat liver was fractionated by differential centrifuging (method 1), the property of hydrolysing maltose was found in the mitochondrial, the microsomal and the soluble fractions. As shown in Fig. 1, the pH influenced the activity of the three fractions in a different way: the enzyme in the mitochondrial fraction is an acid maltase whereas the two other fractions show optimum activity at neutral pH; the shape of the two curves is not, however, identical.

When the mitochondrial fraction had been frozen, acid maltase was no longer sedimentable. This property suggested that it could be bound to the lysosomes.

Lysosomal localization of acid maltase. The distribution pattern of acid and neutral maltases, as obtained by method 2, is shown in Fig. 2. Acid

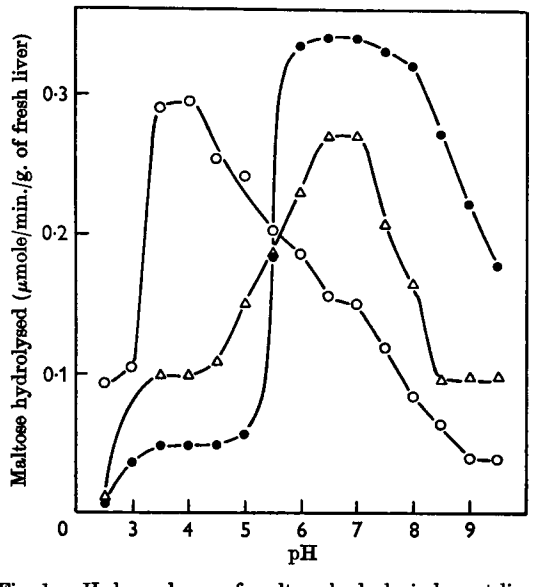

Fig. 1. pH dependence of maltose hydrolysis by rat-liver fractions. The activities are expressed as μmole of maltose hydrolysed/min. by an amount of each fraction corresponding to 1 g. of fresh liver. ○, Mitochondrial fraction; ●, microsomal fraction; △, soluble fraction.

maltase was measured at pH 3·5 and the neutral maltase at pH 8·5. These conditions were chosen to make the measurements as selective as possible. Acid phosphatase, cytochrome oxidase and glucose 6-phosphatase were used as reference enzymes for lysosomes, mitochondria and microsomes respectively. The results indicate that acid maltase behaved like a lysosomal enzyme and that the activity at pH 8·5 was mostly bound to microsomes.

Differential centrifuging does not, however, permit distinction between lysosomes and the group of particles which contain uricase, catalase and D-amino acid oxidase. This can be done by density equilibration in a density gradient (Beaufay *et al.* 1959; de Duve *et al.* 1960). The results of such a separation show that acid maltase behaved like acid phosphatase and acid deoxyribonuclease, and not like uricase (Fig. 3). The fact that the distributions of the various lysosomal enzymes are not superimposable has been previously observed and discussed by Beaufay *et al.* (1959).

Comparison between acid maltase and acid phosphatase. Under the conditions used for the measurement of 'free activity', all lysosomal enzymes are mostly latent; they are activated by various treatments which have in common damage to the structural integrity of the particles. These characteristic properties have been found for acid maltase.

Freshly prepared mitochondrial fractions incubated for 10 min. at pH 5 and 37° in the presence of 0·25M-mannitol hydrolysed maltose at a rate that was only 5% of what it was after freezing the preparation. This free activity increased with time when the preparation was preincubated in the same conditions (Fig. 4) and the activation paralleled that of acid phosphatase. Acid maltase was also activated, as was acid phosphatase, in a mitochondrial fraction exposed to media of low osmotic pressure or incubated in the presence of digitonin (Figs. 5 and 6).

Fig. 7 shows the excellent correlation existing between the free activities of acid maltase and acid

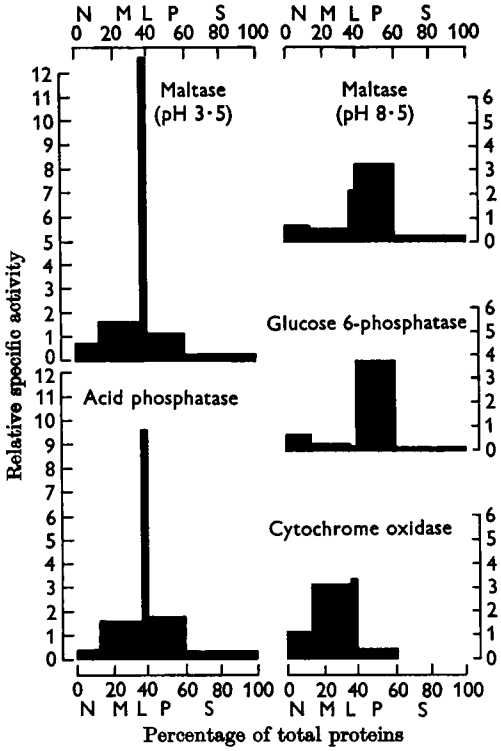

Fig. 2. Distribution pattern of enzymes. Fractions are plotted in the order of their isolation, i.e. (from left to right): N, nuclear fraction; M, heavy-mitochondrial fraction; L, light-mitochondrial fraction; P, microsomal fraction; S, final supernatant. Each fraction is represented separately on the ordinate by its own relative specific activity (percentage of total activity/percentage of total proteins). On the abscissa each fraction is represented (cumulatively from left to right) by its percentage of protein.

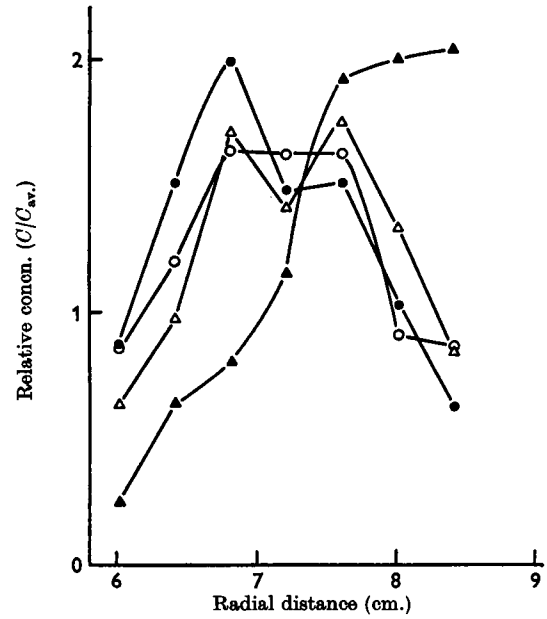

Fig. 3. Density equilibration in a linear gradient of 0·94–1·58M-sucrose in D_2O. The top and the bottom fractions are not shown in the Figure because of the irregularities of the gradient at these levels. Results are expressed in terms of the ratio of the concentration found (C) to the average concentration ($C_{av.}$), which was calculated by dividing the total amount recovered by the volume of the gradient. ●, Acid phosphatase; ○, acid maltase; △, acid deoxyribonuclease; ▲, uricase.

phosphatase of particles subjected to graded damage. It is apparent that in fresh preparations the free activity of acid maltase was lower than that of acid phosphatase.

Other properties of rat-liver α-glucosidases. Like human acid α-glucosidase, the three rat-liver fractions hydrolyse glycogen to glucose but at a rate that, in percentage of the activity on maltose (compared as number of glycosidic bonds hydrolysed), was approximately 40 for the lysosomal enzyme, 60 for the microsomes and 100 for the soluble fraction. All three fractions showed a low activity (from 1 to 10% of the activity on maltose) on isomaltose and none on cellobiose. As the relative activity on isomaltose varied from one preparation to another, it could be due to another hydrolytic enzyme. As is known to be the case for many maltases (see Gottschalk, 1951), glucose inhibits the activity in the three fractions. Galactose, fructose, xylose, arabinose, lactose, melezitose and melibiose were not inhibitory. Turanose, although not hydrolysed, caused 50 and 100% inhibition of the lysosomal maltase at 5 mM and 30 mM respectively. This inhibition was non-competitive (Fig. 8). The microsomal and the soluble enzymes were not inhibited by turanose.

With all three fractions, transglucosylation on glucose or on oligosaccharides was only barely detectable. The three preparations catalysed incorporation of ^{14}C from maltose into glycogen (see Hers, 1963) but this transglucosylation and the hydrolysis were not influenced by pH in a parallel manner. This seems to indicate that a variety of enzymes catalyse this reaction.

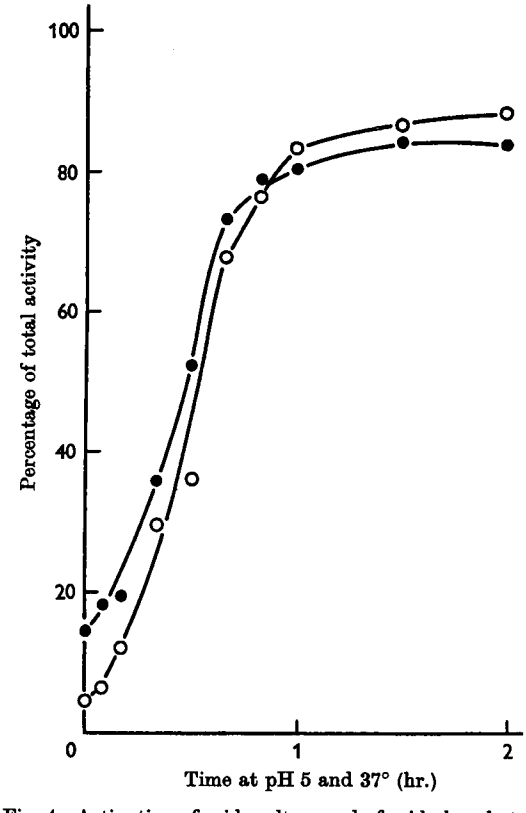

Fig. 4. Activation of acid maltase and of acid phosphatase by incubation at pH 5 and 37°. Free activities were measured after preincubation of the particles at 37°, and for the time indicated, in 0·25 M-mannitol containing 0·1 M-acetic acid–acetate buffer. Maltase was measured at pH 5. ●, Acid phosphatase; ○, acid maltase. The absolute value corresponding to 100% was 2·6 μmoles of β-glycerophosphate and 0·25 μmole of maltose hydrolysed/min. by the amount of particles representing 1 g. of fresh liver.

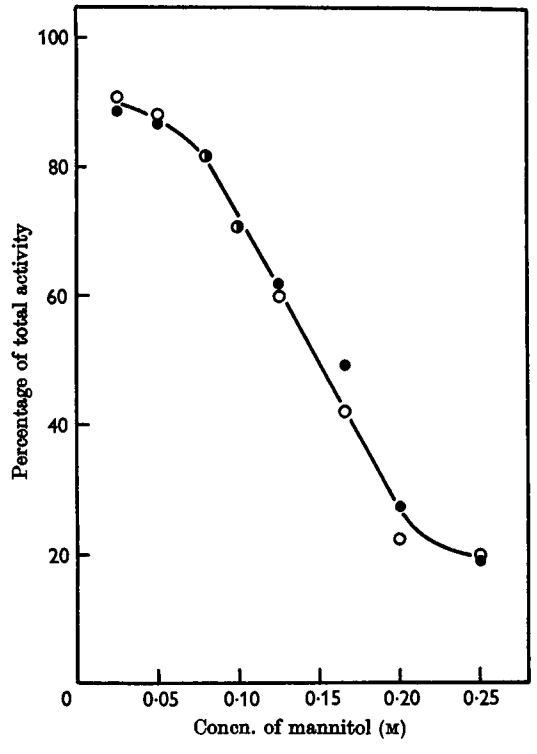

Fig. 5. Activation of acid maltase and acid phosphatase by osmotic disruption. Free activities were measured on particles kept for 15 min. at 0° in the concentration of mannitol indicated and then brought back into 0·25 M-mannitol by addition of suitable amounts of M-mannitol. Results are expressed as percentages of total activity. The absolute value corresponding to 100% was 1·7 μmoles of β-glycerophosphate and 0·26 μmole of maltose hydrolysed/min. by the amount of particles representing 1 g. of fresh liver. ●, Acid phosphatase; ○, acid maltase.

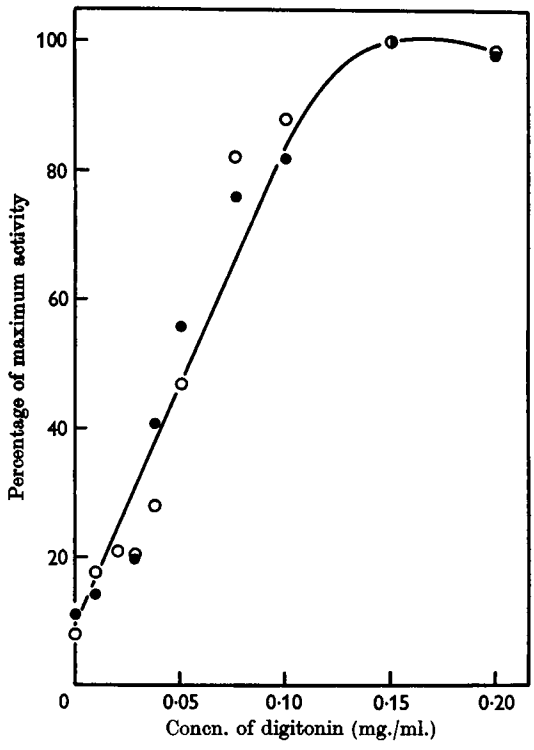

Fig. 6. Activation of acid maltase and acid phosphatase by increasing concentrations of digitonin. Free activities were measured in the presence of digitonin at the concentration indicated with an amount of particles corresponding to 2·5 mg. of original liver/0·2 ml. Results are expressed as percentages of highest observed activity. The absolute value corresponding to 100% was 2·2 μmoles of β-glycerophosphate and 0·35 μmole of maltose hydrolysed/min. by the amount of particles representing 1 g. of fresh liver. ●, Acid phosphatase; ○, acid maltase.

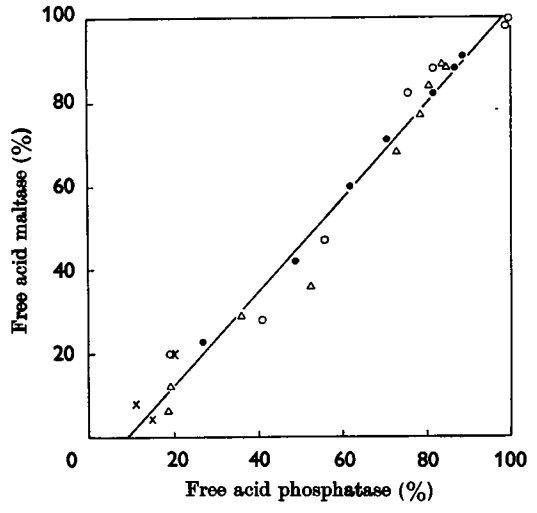

Fig. 7. Correlation between free acid maltase and free acid phosphatase of untreated particulate preparations (×) and of preparations incubated at pH 5 and 37° (△), exposed to media of lowered mannitol concentration (●) or treated with digitonin (○). Correlation coefficient: 0·99.

DISCUSSION

In the work described in this paper, differential centrifuging has been particularly useful to distinguish several isoenzymes that hydrolyse maltose and glycogen into glucose and which appear to belong to the group of the α-(1→4)-glucosidases.

A first enzyme, having its optimum activity at pH 4, can be identified with the acid α-glucosidase of human liver (Hers, 1963) by both its pH–activity curve and its property of being inhibited in a non-competitive manner by turanose. Its intracellular distribution, its structure-linked latency and its release under various conditions indicate that it is bound to lysosomes. Its possible role in glycogen degradation has been previously discussed in relation to its absence from the livers of children affected by the generalized cardiomegalic type of glycogenosis (Hers, 1963).

It is not possible at the present time to decide whether the maltase activities of the microsomal and of the soluble fractions are due to the same neutral α-glucosidase or to two distinct enzymes. The two fractions differ by some variations in their pH–activity curve and also by a greater relative activity on glycogen for the soluble enzyme. These slight differences in kinetic properties might, however, be due to a difference in physicochemical state of a single enzyme, or to the presence of interfering substances in the fractions; the possibility that either a soluble maltase has been partially adsorbed on microsomes or that a microsomal enzyme has been partially solubilized in the course of tissue fractionation cannot be precluded.

The exopolyglucosidase of rabbit liver that has been recently described by Rosenfeld & Popova (1962) shows an optimum activity of about pH 5 and is nearly as active at pH 6 as at pH 4. It could therefore be a mixture of the lysosomal and of the soluble enzymes.

SUMMARY

1. The intracellular distribution of rat-liver α-(1→4)-glucosidases (maltases) has been investigated by a fractionation scheme in which the mitochondrial fraction divided is into two sub-

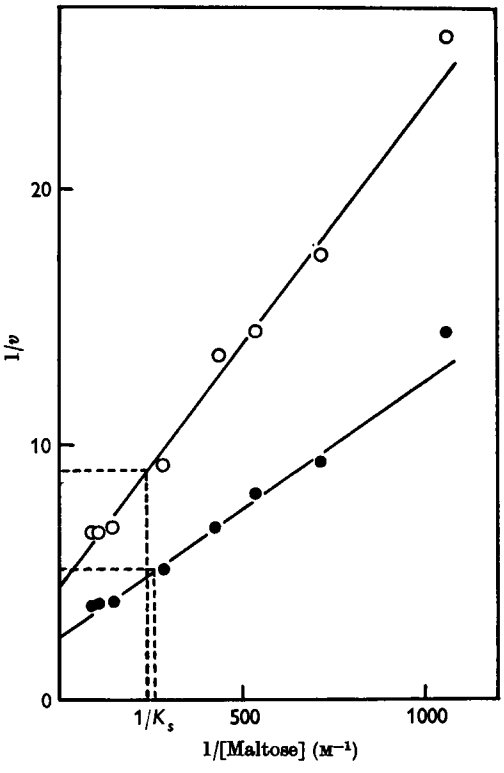

Fig. 8. Influence of turanose on the hydrolysis of maltose by acid maltase. The velocities (v) are expressed as μmoles of maltose hydrolysed/min. by an amount of particles corresponding to 1 g. of liver. Estimated K_s values are of the order of 4 mM both in the absence (●) and in the presence (○) of 5 mM-turanose.

fractions. An acid maltase was found to be concentrated in the lysosome-rich light-mitochondrial fraction, whereas neutral-maltase activity was found in both the microsomal and the soluble fractions.

2. The lysosomal localization of acid maltase has been confirmed by density equilibration in a density gradient, by its structure-linked latency and by its release under controlled damage.

3. Some kinetic properties of the rat-liver maltases have been investigated.

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