mole of protein were estimated in the ether-extracted hydrolysate from the absorbance at 350 and 390 nm in 1 M HCl (*cf.* Table 11–1 of ref. 7).

X-ray patterns of crystals treated in the same way were made and compared with those of unmodified crystals. Two difference Fourier maps, the *Okl* and *hkO* projections, are shown in Fig. 1. Although the electron density peaks do not rise much above the background noise, six of them can be clearly seen. Peaks B, C, D overlap in the *hkO* projection. Peak A, elongated in the *hkO* projection, can most likely be ascribed to two partially overlapping peaks; this overlap also occurs in the other projection, as Peak A here has a rather high value. By correlation of the *y*-coordinates in the two projections, three-dimensional coordinates of the peaks can be calculated, thus determining which amino acid side chains have reacted.

Peak A can be ascribed to reaction with the α-NH_2 group and Lys-137, Peak B with Lys-54, Peak C with Tyr-176, Peak D with Tyr-161, and Peak E with Tyr-183. No reaction with Cys-25 or His-106 in the active site could be seen. Of course, sites of minor reactivity do not show up.

The present data exclude the participation of the α-NH_2 group in the action of papain, which apparently differs from chymotrypsin in this respect. Furthermore a number of other groups are not essential for catalysis either.

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Allosteric effects and phlorizin inhibition of intestinal trehalase

Intestinal sucrase (sucrose glucohydrolase)[1,2], and isomaltase (isomaltose glucohydrolase)[3], are activated by Na^+, and at least in the case of sucrase, the kinetic parameters of this Na^+ activation are essentially the same as those of Na^+ activation of sugar transport, although sucrase itself does not participate in sugar transport[4,5]. Sucrase shows an evident cooperative interaction both among substrate sites and among Na^+ sites[6].

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As a part of the same project, we now produce the data on intestinal trehalase (EC 3.2.1.28). At the same time, we report on phlorizin inhibition of this enzyme because it was suggested recently[7,8] that trehalase may be a part of the sugar transport system in kidney, and possibly in small intestine.

TABLE I

LACK OF EFFECT OF EITHER Na^+ OR K^+ ON RAT INTESTINAL TREHALASE

Buffer: 0.025 M lithium maleate. Data represent μmoles of substrate split per min per ml, at 37°.

	No Na^+, no K^+	*+50 mM Na^+*	*+12.5 mM K^+*
Unsolubilized			
pH 6.0	0.350	0.348	0.334
pH 5.4	0.298	0.293	0.297
Solubilized			
pH 6.0	0.465	0.500	0.480
pH 5.4	0.400	0.396	0.404

Lack of Na^+-activation. Table I shows that rat intestinal trehalase is not affected by Na^+ or by K^+, either at pH 6.0 (the pH optimum of this enzyme) or at pH 5.4 (the pH of maximum per cent activation of hamster intestinal sucrase by Na^+)[6]. Trehalase, therefore, is in this respect more akin to lactase (EC 3.2.1.23), which is also not affected by Na^+ (ref. 9), than the aforementioned α-glucosidases.

Cooperative interaction among substrate sites (Figs. 1 and 2). Like sucrase, trehalase shows a cooperative interaction between substrate sites. The slope of the Hill plots is 1.27 (Fig. 2; the maximum velocity was calculated from double reciprocal plots). This indicates that membrane-bound trehalase may be composed of at least two subunits[10,11]. Upon solubilization with trypsin, trehalase loses this cooperative interaction, the intrinsic apparent K_m being slightly changed, if at all. The cooperative interaction is lost during the solubilization step and not during the subsequent purification procedure. Therefore, this phenomenon is apparently due to the degradation of the membrane in the immediate neighborhood of trehalase (or to the separation of trehalase subunits by trypsin) rather than to the separation of a component occurring in crude membrane-bound trehalase.

Phlorizin inhibition. Renal trehalase is inhibited by this glucoside[12,13]. Fig. 3 clearly shows that phlorizin inhibits the small-intestinal enzyme also and that the inhibition is fully competitive in nature. The K_i is 2 mM at pH 5.4. Identical results were obtained with both solubilized and membrane-bound trehalases. This K_i value is some three orders of magnitude larger than the K_i of phlorizin inhibition of intestinal sugar transport (approx. 10^{-6} M at pH 7.3, refs. 14 and 15). It is unlikely that such a difference in K_i is due to the difference in pH. Rather it indicates that if intestinal trehalase plays any role at all in sugar transport in the small intestine[7,8], the carbohydrase is not the rate-limiting step. Phlorizin inhibits a large number of enzymes of carbohydrate metabolism (for a review, see ref. 16).

A number of other observations also indicate that trehalase does not play any role in intestinal sugar transport: (i) intestinal trehalase develops during the third

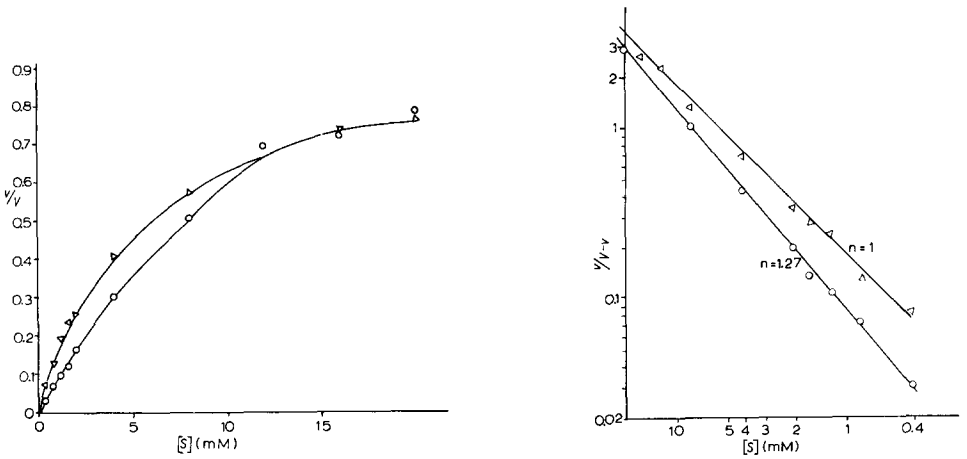

Fig. 1. Michaelis–Menten plots of membrane-bound (○—○) and of trypsin-solubilized (△—△) rat intestinal trehalase.

Fig. 2. Hill plots of membrane-bound (○—○) and of trypsin-solubilized (△—△) rat intestinal trehalase.

week of life in rats[17], *i.e.*, well after the development of intestinal sugar transport[18,19]; (ii) cats and other carnivores who do not have intestinal trehalase[20] absorb glucose; (iii) in human glucose–galactose malabsorption, intestinal trehalase is at the normal level (ref. 21 and personal communications from Drs. K. R. KERRY AND E. EGGERMONT, 1968); (iv) intestinal trehalase is external rather than internal to the sugar transport system[22], as is required by the aforementioned hypothesis[7,8]; (v) finally, the trehalase hypothesis implies phosphorylation of glucose to glucose 6-phosphate[7,8] which was conclusively shown not to occur during transport by CRANE[16].

Methods and enzyme preparations. As a crude rat intestinal trehalase, the

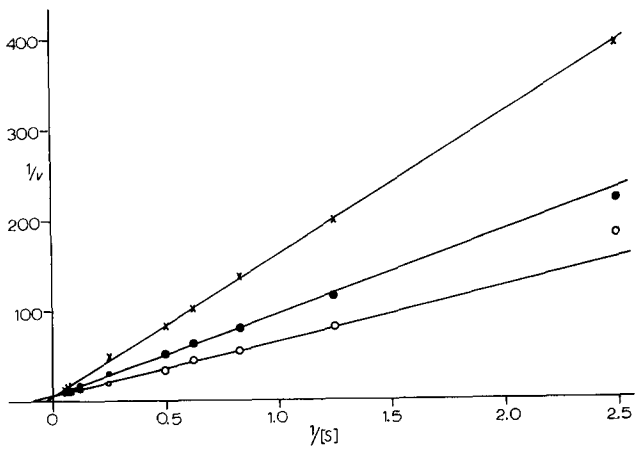

Fig. 3. Fully competitive inhibition of unsolubilized rat intestinal trehalase by phlorizin at pH 5.4, in sodium maleate buffer. ○—○, no phlorizin present; ●—●, 2 mM phlorizin; ×—×, 5 mM phlorizin. The K_m of trehalase is 10 mM. The K_i for phlorizin inhibition is 2 mM.

fraction sedimenting between 1500 × *g* and 105 000 × *g* was used. For some experiments, the enzyme was further purified by trypsin solubilization and DEAE-cellulose chromatography as described by DAHLQVIST[23] for the hog enzyme. The purified preparations used had a specific activity of approx. 0.18 units·mg^{-1} protein. (The international units used in the present paper are 21.6 times larger than the units used in ref. 23).

Unless stated otherwise, trehalase activity was determined in 0.05 M sodium or lithium maleate buffers, pH 6.0, in 0.14 M trehalose at 37°. Incubation was stopped by adding $Ba(OH)_2$ and subsequently $ZnSO_4$ (ref. 24). In the supernatant, free glucose was measured with a Tris–glucose oxidase–peroxidase reagent[25,26]. It was ascertained that zero-order kinetics were always followed.

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