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Characterization of Hog Intestinal Trehalase

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Hog intestinal trehalase is a specific enzyme, separable from the intestinal invertase, maltase, and *iso*maltase activities. In the present paper the partial purification and the characteristics of hog intestinal trehalase will be described.

In contrast to the hog intestinal invertase and maltase activities, hog intestinal trehalase had *no* transglycosylase activity. Nor could any synthesis of trehalose be detected.

Hog intestinal trehalase has very high specificity for *aa*-trehalose. The preparation did not hydrolyze sucrose, maltose, *iso*maltose, phenyl α-D-glucopyranoside, or *ββ*-trehalose. It hydrolyzed *αβ*-trehalose slowly, but heat inactivation revealed that this sugar was *not* hydrolyzed by the same enzyme as *aa*-trehalose.

The high specificity and the lack of transglycosylase activity makes it probable that hog intestinal trehalase acts upon its substrate in a more symmetrical way than do the other glycosidases.

That intestinal mucosa from certain mammals (rabbit, horse, cow) has trehalase activity *, has been known since the end of the 19th century [1–3]. Until recently it has not been known, however, if the intestinal trehalase activity is caused by a specific enzyme, or if it is only one activity of an intestinal α-D-glucopyranosidase which acts on many different substrates, as proposed by Weidenhagen [3,4].

In the course of a study of hog intentinal α-D-glucopyranosidase activities it was recently observed, however, that the hog intestinal trehalase activity was separable from the hog intestinal invertase and maltase activities. The trehalase activity was inactivated by exposure to ethanol, and it appeared as a separate peak in chromatography upon DEAE-cellulose [5]. The hog intestinal trehalase activity could also be separated from the invertase and maltase activities by heat inactivation [6]. Hog intestinal trehalase, therefore, is a specific enzyme, as has earlier been reported in the case of trehalase from fungi (*Aspergillus niger* [7], yeast [3]) and insect larvae (*Galleria mellonella* [8]).

In the present paper the partial purification of trehalase from hog intestinal mucosa and some characteristics of hog intestinal trehalase will be described.

* *Trehalase* activity (without prefix) means hydrolytic activity against the *naturally* occurring trehalose, *aa*-trehalose (1(α-D-glucopyranosyl) α-D-glucopyranoside).

MATERIALS AND METHODS

Hog intestinal mucosa was obtained from the slaughterhouse immediately after slaughter, and was chilled with crushed ice during the transport to the laboratory.

Substrates. *αα-Trehalose* (1-(α-D-glucopyranosyl) α-D-glucopyranoside) (dihydrate) of analytical grade purity, with $[\alpha]_D^{20}$ +178.3°, was obtained from Pfanstiehl Chemical Co. *Sucrose*, cryst., was obtained from J. T. Baker Co. and *maltose* (monohydrate), cryst., from Merck A. G. (Germany).

αβ-Trehalose (1-(α-D-glucopyranosyl) β-D-glucopyranoside, *neo*trehalose) and *ββ-trehalose* (1-(β-D-glucopyranosyl) β-D-glucopyranoside, *iso*trehalose) were obtained as their *octaacetates*, a generous gift from *Professor H. Bredereck* (Technische Hochschule, Stuttgart) [9]. Deacetylation was performed by shaking with excess of NaOH (200 mg of trehalose octaacetate and 40 ml of 0.1 N NaOH) at room temp. for 16 h. The Na-acetate formed and the excess of NaOH were removed by filtration through a column with 2 cm diam., containing 4 g of Dowex 2 (OH-form), 4 g of Dowex 50 (H-form), and 4 g of Dowex 2 (OH-form) as separate layers. The flow rate was 0.5 ml/min. The column was washed with 150 ml of water. The neutral filtrate was evaporated at reduced pressure to a volume of about 10 ml, lyophilized and dried *in vacuo* at 50°C over P_2O_5. About 90 mg of each sugar was obtained.

The sugars obtained did not reduce Somogyi's copper reagent. They had the same mobility as αα-trehalose on the paper chromatogram. $[\alpha]_D^{20}$ was +79.0° for αβ-trehalose (3 % in water) and −41.4° for ββ-trehalose (4.2 % in water).

Phenyl-α-D-glucopyranoside was obtained as its *tetraacetate* by the method of *Helferich* [10] as modified by *Montgomery et al.*[11] $[\alpha]_D^{20}$ of the tetraacetate was +168.0° (7.2 % in $CHCl_3$), m. p. 113−114°C (reported [10] $[\alpha]_D^{20}$ +168°, m. p. 115−116°C). Deacetylation was performed as described above in the case of trehalose octaacetates. The phenyl-α-D-glucopyranoside was crystallized from water and dried *in vacuo* at 50°C over P_2O_5. $[\alpha]_D^{20}$ was +186.0° (anhydrous, 3 % in water).

Isomaltose (6(α-D-glucopyranosyl) D-glucose) was obtained by enzymatic hydrolysis of dextran (kindly supplied by *A. B. Pharmacia*) as described elsewhere [12].

Determination of trehalase activity was performed at 0.1388 M substrate concentration at 37°C in the presence of 0.05 M maleate buffer pH 6.0. 1 ml of enzyme solution (containing 0.5−3 units of trehalase) was mixed with 1 ml of substrate solution (10.5 % * αα-trehalose (dihydrate) in 0.1 M maleate buffer pH 6.0 with a few ml of toluene per litre as a preservative). After 60 min. at 37°C the reaction was interrupted by the addition of 2 ml of Sumner's 3,5-dinitrosalicylic acid reagent [13]. A blank was prepared with the same composition, but the 3,5-dinitrosalicylic acid reagent was added *before* the substrate solution. The tubes were boiled in a water-bath for 10 min, chilled with tap water, and diluted with 20 ml of distilled water. The red colour was measured in a Beckman B spectrophotometer at 530 mμ. The colour was stable for at least 20 h. The amount of glucose produced was calculated from a standard curve with known amounts of glucose. One unit of trehalase is the amount which produces 1 mg of glucose (= 1 % hydrolysis in 2 ml of reaction mixture) during the conditions used.

Determination of K_s for trehalase activity was made by measuring the rate of hydrolysis in 0.0500−0.0007 M trehalose solutions. The degree of hydrolysis was never allowed to exceed 10 %. The amount of glucose produced in these dilute substrate solutions was determined with the method of Somogyi and Nelson [14,15] after deproteinization with Somogyi's Zn-Ba reagents [16]. Calculation of K_s was performed as described earlier [17].

The hydrolysis of sucrose, maltose, isomaltose, and phenyl-α-D-glucopyranoside was measured at 0.1388 M substrate concentration at 37°C in the presence of 0.05 M maleate buffer pH 6.0. The amount of glucose produced from these substrates was determined with the methods described elsewhere [12,18].

The hydrolysis of αβ-trehalose and ββ-trehalose was measured at 0.0139 M substrate concentration at 37°C in the presence of 0.05 M maleate buffer pH 6.0. The amount of glucose produced was determined with the reagents of Somogyi and Nelson [14,15] after deproteinization with the Zn-Ba-reagents [16].

* Per cent means gram of solute in 100 ml of solution.

Determination of protein was made with the method of Lowry et al. [19] by use of human serum albumin (kindly supplied by *A. B. Kabi*) as a standard.

Determination of inorganic phosphate was made with the method of King [20]. The blue colour produced was measured with a Beckman B spectrophotometer at 700 mμ.

Paper chromatography of sugars was performed as described earlier [21].

^{14}C-Labelled glucose was obtained from the *Radiochemical Centre* (England). The distribution of the radioactivity on paper chromatograms was measured as described earlier [21].

Crystalline trypsin (Trypure Novo) was kindly supplied by *A. B. Ferrosan.*

RESULTS AND DISCUSSION

As the source of trehalase hog intestinal mucosa was used. While the mucosa of the duodenum and the proximal jejunum had a strong trehalase activity (trehalase/invertase activity quotient 0.50—0.75), the trehalase activity of the distal part of the small intestine was weak (trehalase/invertase activity quotient about 0.10). For the preparation of trehalase the proximal 100 cm of the small intestine was therefore used.

Solubilization and purification of trehalase. The mucosa and the intestinal contents of the proximal part of the small intestine were squeezed out manually within a few minutes after the slaughter. From 10 pigs (about 10 metres of small intestine) 200 ml of viscous liquid was thus obtained, which yielded 55 mg/ml of protein, 96 units/ml of trehalase, 140 units/ml of invertase, and 210 units/ml of maltase. For solubilization of the glycosidases 30 mg of crystalline trypsin, freshly dissolved in 20 ml of water, was added during stirring [5]. After 4 h at room temperature the solution was centrifuged at 600 *g* for 10 min and the supernatant was filtered through a bed of Solkafloc cellulose powder SW 40 A. The filtrate was dialyzed against 0.01 M phosphate buffer pH 6.0.

The solution was then applied to a 6 × 20 cm column, containing 80 g (dry weight) of DEAE-cellulose [5], equilibrated with 0.01 M phosphate buffer pH 6.0. The column operations were performed at +4°C. The column was first eluted with 4 l of 0.01 M phosphate buffer pH 6.0, which eluted 40 % of the protein applied to the column, but only 8 % of the trehalase activity. This fraction was discarded. The main part (80 %) of the trehalase activity, together with 5 % of the protein, was eluted with 4 l of 0.05 M phosphate buffer pH 6.0. This buffer did not elute the invertase and maltase activities from the column.

For concentration of the enzyme, this fraction was diluted with 4 vol. of distilled water, and filtered through a column containing 10 g (dry weight) of DEAE-cellulose, equilibrated with 0.01 M phosphate buffer pH 6.0. All the trehalase activity was adsorbed to the column. The enzyme was eluted with 200 ml of 0.20 M phosphate buffer pH 6.0, dialyzed against distilled water and lyophilized. Since the powder obtained was found to contain considerable amounts of inorganic phosphate, it was dissolved in 25 ml of distilled water and again dialyzed against distilled water. On lyophilization of the dialyzed solution 160 mg of a white powder, with a trehalase, activity of 34 units/mg, was obtained. This corresponds to 25 % of the trehalase activity of the original solution. The powder had the same absorption coefficient according to Lowry's method for protein determination as pure human serum

Table 1. Hydrolysis of *aa*-trehalose by hog intestinal trehalase. Course of the reaction. The reaction mixture contained 0.1388 M *aa*-trehalose, 0.05 M maleate buffer pH 6.0, 1 mg/ml of purified trehalase containing 1 mg of protein and 34 units of trehalase per mg. The temperature was 37°C. — Up to between 15 and 20 % hydrolysis the reaction followed zero order kinetics.

t Time, h	x per cent of hydrolysis	k_{zero} x/t
0.083	5.3	63.7
0.166	10.6	63.7
0.250	15.9	63.7
0.333	20.5	61.4
0.500	30.5	61.0
0.667	39.3	58.9
0.833	48.3	58.0
1.000	52.9	52.9
2.000	80.3	40.1
3.000	91.7	30.6
4.000	97.3	24.4

albumin. The specific trehalase activity of the powder (trehalase activity/mg of protein) was 20 times that of the original solution.

Stability of hog intestinal trehalase activity. In crude hog intestinal glycosidase preparations the trehalase activity, in contrast to the invertase and maltase activities, was not stable when stored at low temperature. After storage at —16°C for a month, the trehalase activity of a crude preparation had decreased by 25 %. At higher temperatures the decrease was more rapid. After storage at —16°C for 6 months, no more than about 15 % of the trehalase activity remained, while the invertase and maltase activities were unaffected.

The trehalase activity of the purified, lyophilized powder, however, was very stable. After storage at —16°C for 6 months, *no* decrease of the trehalase activity of this preparation could be detected.

Kinetics of hog intestinal trehalase. Hog intestinal trehalase had its optimum pH around 6.0 (Fig. 1). The hydrolysis of *aa*-trehalose by hog intestinal trehalase proceeded to completion with glucose as the only product. Up to 20 % hydrolysis the reaction followed zero order kinetics (Table 1). Within wide limits the initial velocity of the reaction was proportional to the amount of enzyme present (Fig. 2).

The K_s value was determined at 3.0×10^{-3} M *aa*-trehalose (at 37°C in the presence of 0.05 M maleate buffer pH 6.0). The V_{max} was 1.02 times the initial velocity at 0.1388 M substrate concentration.

Influence of inorganic phosphate on hog intestinal trehalase activity. The trehalase activity by extracts from some *insects* is stimulated by the presence of inorganic phosphate, which has led to the conclusion that a phosphorolysis may be involved in the trehalase action by these preparations [23]. Later researchers, however, found that the hydrolysis of trehalose by insect trehalase proceeds to completion with glucose as the sole product. Since their preparations had no activity on glucose-1-phosphate, the cleavage of trehalose by their preparations must be a purely hydrolytic reaction [8].

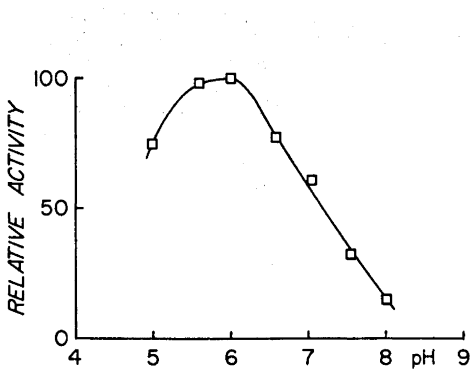

Fig. 1. Influence of pH on the hydrolysis of *aa*-trehalose by hog intestinal trehalase. Buffers: 0.05 M acetate pH 5.0, 0.05 M maleate pH 5.5—6.5, 0.025 M veronal pH 7.0—8.0. Temperature 37°C. Substrate concentration 0.1388 M.

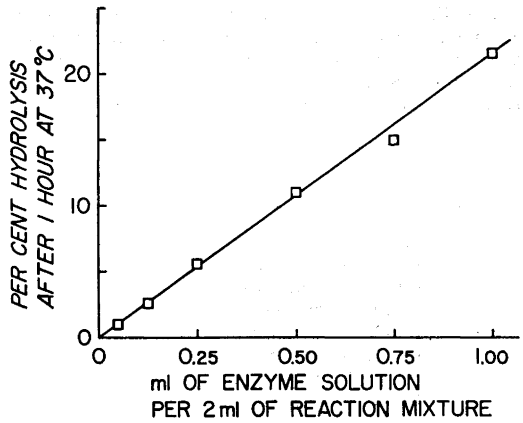

Fig. 2. Proportionality between the degree of hydrolysis after 1 h and the amount of enzyme added. Experimental conditions: 0.1388 M *aa*-trehalose solution in the presence of 0.05 M maleate buffer pH 6.0. The enzyme solution was a purified preparation of hog intestinal trehalase, containing 0.65 mg of protein and 22 units of trehalase per ml. Up to 20 % hydrolysis, the degree of hydrolysis after 1 h was proportional to the amount of enzyme added.

The influence of inorganic phosphate on the hog intestinal trehalase activity was studied with the results seen in Table 2. The reaction mixture contained 25 μg/ml of the lyophilized trehalase preparation, 0.1388 M *aa*-trehalose, and varying amounts of phosphate or maleate buffer pH 6.0. Without addition of phosphate buffer, the reaction mixture was found to contain less than 0.2 μg/ml of inorganic phosphorous. The trehalase activity was *not* stimulated by the addition of phosphate buffer. The presence of concentrated phosphate buffer rather decreased the rate of hydrolysis to some extent.

Transglycosylation properties of hog intestinal trehalase. Most glycosidases are known to have transglycosylase activity, *i.e.* they are able to transfer the glycosyl residue of their substrate to some receptor other than water [24]. In a concentrated substrate solution the substrate may itself act as receptor, which results in the formation of oligosaccharides. Also other compounds, containing a free alcohol group, may act as receptors. If the substrate is a disaccharide, the transfer of the glycosylic residue to a free hexose may lead to the regeneration of the substrate or the production of other disaccharides [21,24].

A reaction mixture, containing 30 % (= g/100 ml) of *aa*-trehalose (dihydrate) and 41 units/ml of hog intestinal trehalase in 0.05 M maleate buffer pH 6.0 with toluene as preservative, was incubated at 37°C for 23 h. When 5 μl spots of the reaction mixture were chromatographed, only glucose and trehalose were found (Fig. 3). Thus no oligosaccharide formation could be

Table 2. Influence of inorganic phosphate on the activity of hog intestinal trehalase. Experimental conditions: 0.1388 M substrate concentration, pH 6.0, temperature 37°C, and time of reaction 60 min. The reaction mixture contained 25 μg/ml of purified hog intestinal trehalase. The degree of hydrolysis was determined with the 3,5-dinitrosalicylic acid reagent. — The presence of phosphate did *not* stimulate the trehalase activity.

Buffer	Degree of hydrolysis, %
No buffer	1.79
0.010 M maleate	1.80
0.025 M maleate	1.85
0.050 M maleate	1.83
0.005 M phosphate	1.86
0.025 M phosphate	1.85
0.050 M phosphate	1.74
0.100 M phosphate	1.59

demonstrated. During the corresponding conditions, hog intestinal invertase and hog intestinal maltases produce a series of oligosaccharides in addition to their hydrolysis products [12,21].

In another experiment 1 mg/ml of ^{14}C-labelled glucose was added to a reaction mixture with the same composition as above. After 5 and 23 h at 37°C 5 μl samples of the reaction mixture were applied to a paper chromatogram. After run for 2 days, the distribution of the radioactivity on the chromatogram was measured as described earlier [21]. Each 5 μl spot contained 6 250 c.p.m. of ^{14}C-labelled sugar as measured with the counter. All the radioactivity was localized in the *glucose* spot. Glucose is therefore *not* incorporated into trehalose or other oligosaccharides by intestinal trehalase. During the corresponding conditions the intestinal maltases form ^{14}C-labelled maltose and other oligosaccharides [12], and intestinal invertase, too, forms a ^{14}C-labelled disaccharide [21].

That hog intestinal trehalase has no transglycosylase activity was also indicated by the fact that the rate of glucose formation (as measured with the 3,5-dinitrosalicylic acid reagent) did not decrease when the substrate concentration was increased to 0.794 M (30 % *aa*-trehalose dihydrate). The

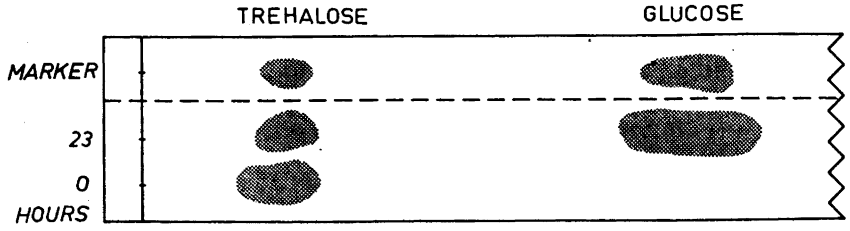

Fig. 3. Hydrolysis of a 30 % solution of *aa*-trehalose (dihydrate) by hog intestinal trehalase. Experimental conditions, see text. The chromatogram was developed for 2 days. In contrast to the other intestinal glycosidases, trehalase did *not* form any oligosaccharides by transglycosylation.

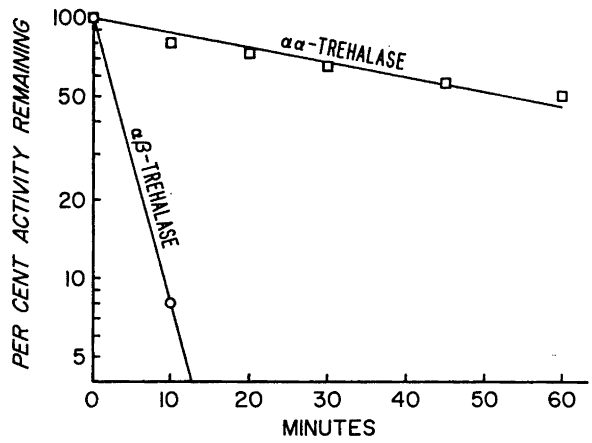

Fig. 4. Separation of $\alpha\beta$-trehalase and $\alpha\alpha$-trehalase activity by heat inactivation. A solution of purified hog intestinal trehalase, containing 5 mg/ml of protein in 0.01 M phosphate buffer pH 6.0, with 165 units/ml of $\alpha\alpha$-trehalase and 1 unit/ml of $\alpha\beta$-trehalase, was heated at 65°C. While the $\alpha\beta$-trehalase activity was rapidly inactivated, the $\alpha\alpha$-trehalase activity only decreased slowly.

hog intestinal invertase and maltase activities decrease in concentrated substrate solutions, probably owing to their transglycosylase activity [12,17,21].

In some cases, the equilibrium for reactions catalyzed by glycosidases has been found to be such that the synthesis of the substrates from the hydrolysis products could be demonstrated [22]. A solution containing 30 % glucose and 41 units/ml of trehalase in 0.05 M maleate buffer pH 6.0 and with toluene as a preservative was incubated at 37°C. After 23h only glucose was found on a paper chromatogram. The equilibrium for the reaction catalyzed, therefore, must be far to the side of hydrolysis.

Specificity of hog intestinal trehalase. The trehalase preparation had no hydrolytic activity on *sucrose*, *maltose*, *isomaltose*, *phenyl* α-D-*glucopyranoside*, or *ββ-trehalose*. The experimental conditions were such that a hydrolysis with a rate only 1/100 of that for the hydrolysis of $\alpha\alpha$-trehalose would have been detected with sucrose, maltose and *iso*maltose as substrates, and only 1/1 000 with phenyl α-D-glucopyranoside and $\beta\beta$-trehalose.

The preparation hydrolyzed *αβ-trehalose* with a rate which was 0.006 times the rate for the hydrolysis of $\alpha\alpha$-trehalose. The hydrolysis of $\alpha\beta$-trehalose proceeded *to completion*. The K_s for the hydrolysis of $\alpha\beta$-trehalose was determined at 9.1×10^{-3} and the V_{max} at 1.65 times the rate of hydrolysis at 0.0139 M substrate concentration. — Heat inactivation at 65°C (performed as described earlier [6]), however, revealed that $\alpha\alpha$-trehalose and $\alpha\beta$-trehalose were hydrolyzed by different enzymes (Fig. 4). The hydrolytic activity on $\alpha\beta$-trehalose was completely destroyed after 20 min while 73 % of the original $\alpha\alpha$-trehalase activity persisted after that time. Hog intestinal trehalase, therefore, has *no* activity on $\alpha\beta$-trehalose. The specificity of the contaminating enzyme, which hydrolyzed $\alpha\beta$-trehalose, is not known.

Mode of action upon the substrate. Glycosidases generally act upon their substrates asymmetrically, *i.e.* they are more firmly bound to the *glycon* component (the glycosyl residue) of their substrates than to the *aglycon*. This appears from their greater specificity for the glycon [4,17,22] and by the phenomenon of transglycosylation [24]. This fact is particularly apparent in the case of invertases. In sucrose either the glucosylic or the fructosylic part may be regarded as glycon and aglycon, respectively. Consequently, there are two kinds of invertases, namely, glucosido- and fructosidoinvertases, with activity on different α-D-glucopyranosides or β-D-fructofuranosides, and with transglucosylase or transfructosylase activity, respectively [4,17,21,24].

Hog intestinal trehalase, on the other hand, has no transglycosylase activity. Nor does it seem to be able to hydrolyze any other α-D-glucopyranoside than αα-trehalose. These facts make it probable that hog intestinal trehalase has a more symmetrical way of attacking its substrate than have the other glycosidases. This property is possibly common to many different trehalases, since trehalases from many different sources seem to have a marked specificity for their substrate [3,8,22].

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REFERENCES

1. Bourquelot, E. and Gley, E. *Compt. rend. soc. biol.* **47** (1895) 555.
2. Fischer, E. and Niebel, W. *Sitzber. Preuss. Akad. Wiss.* **75** (1895) 73.
3. Myrbäck, K. *Ergeb. Enzymforsch.* **10** (1949) 168.
4. Weidenhagen, R. in Nord F. F. and Weidenhagen, R. (Ed) *Handbuch der Enzymologie*, Vol. 1 Leipzig 1940.
5. Borgström, B. and Dahlqvist, A. *Acta Chem. Scand.* **12** (1958) 1997.
6. Dahlqvist, A. *Acta Chem. Scand.* **13** (1959) 945.
7. Bourquelot, E. *Compt. rend.* **116** (1893) 826.
8. Kalf, G. H. and Rieder, S. W. *J. Biol. Chem.* **230** (1958) 691.
9. Bredereck H., Höschele, G. and Ruck, K. *Chem. Ber.* **86** (1953) 1277.
10. Helferich, B. and Schmitz-Hillebrecht, E. *Chem. Ber.* **66** (1933) 378.
11. Montgomery, E. M., Richtmyer, N. K. and Hudson, C. S. *J. Am. Chem. Soc.* **64** (1942) 690.
12. Dahlqvist, A. *To be published.*
13. Sumner, J. B. *J. Biol. Chem.* **62** (1924) 287.
14. Somogyi, M. *J. Biol. Chem.* **195** (1952) 19.
15. Nelson, N. *J. Biol. Chem.* **153** (1944) 375.
16. Somogyi, M. *J. Biol. Chem.* **160** (1945) 69.
17. Dahlqvist, A. *Acta Chem. Scand.* **12** (1958) 2012.
18. Dahlqvist, A. *Acta Chem. Scand.* **13** (1959) 1817.
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* **193** (1951) 265.
20. King, E. J. *Biochem. J.* **26** (1932) 292.
21. Dahlqvist, A. and Borgström, B. *Acta Chem. Scand.* **13** (1959) 1659.
22. Gottschalk, A. in Sumner J. B. and Myrbäck, K. The Enzymes, Vol. I, Part 1, New York 1950.
23. Frèrejacque, M. *Compt. rend.* **213** (1941) 88.
24. Edelman, J. *Advances in Enzymol.* **17** (1956) 189.

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