ACTA CHEMICA SCANDINAVICA 12 (1958) 1997—2006

Cellular Localisation, Solubilization and Separation of Intestinal Glycosidases

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Invertase, maltase and trehalase activities of hog small intestinal mucosa are localized in the microsome fraction obtained from a homogenate on differential centrifugation.

Trypsin causes a solubilization of the glycosidases from the microsomes. During specified conditions the invertase and maltase activities are not affected by trypsin, and trehalase activity only to a minor extent. A method for preparation of soluble glycosidases based on this finding is described.

Trehalase activity of this soluble preparation in contrast to invertase and maltase activities, is destroyed by exposure to ethanol at +4°C. Intestinal trehalase, therefore, must be an enzyme separate from maltase and invertase.

Trehalase activity is also obtained as a separate peak by column chromatography on substituted cellulose (DEAE-cellulose), while invertase and maltase activities could not be completely separated. A change in the ratio of maltase to invertase activity during elution from 1.0 to 6.4, however, indicates the separate identity also of these two enzymes.

The disaccharide hydrolases of mammalian intestine are usually described as maltase, invertase and lactase. According to the specificity theory of Weidenhagen, intestinal invertase and maltase should only be two activities of one and the same enzyme, an α-D-glucopyranosidase, capable of hydrolyzing all α-D-glucopyranosides [1]. With the same nomenclature, lactase, which is well known to be a separate enzyme [2], should be a β-D-galactopyranosidase.

That intestinal extracts from certain mammals are also able to hydrolyze trehalose (1-*O*-α-D-glucopyranosyl-α-D-glucopyranoside) has been known for a long time [3], but it has never been shown if intestinal trehalase is a separate enzyme, or if it is identical with the α-D-glucopyranosidase supposed by Weidenhagen. In many lower organisms, trehalase has been found to be a special enzyme, separable from maltase [3].

Experimental results have been reported, which make the assumption of one single intestinal α-D-glucopyranosidase questionable. Intestinal extracts cannot hydrolyze methyl-α-D-glucopyranoside, which shows that not all α-D-

glucopyranosides can be hydrolyzed [4,5]. Recently it has been reported that activities against nigerose (3-*O*-α-D-glucopyranosyl-D-glucose) and *iso*maltose (6-*O*-α-D-glucopyranosyl-D-glucose) in intestinal extracts from swine have been partially separated from maltase activity [6–8]. All α-D-glucopyranosides which can be hydrolyzed by intestinal enzyme preparations therefore do not seem to be hydrolyzed by the same enzyme.

In the present paper are reported studies of glycosidases from swine small intestinal mucosa: invertase, maltase, trehalase and lactase. These glycosidase activities were found to be particle-bound in intestinal mucosa preparations, and their solubilization from the particles has been studied. Chromatography of the solubilized enzyme solution on substituted cellulose (DEAE-cellulose) according to Peterson and Sober [9] has indicated the presence therein of three separate α-D-glucopyranosidases, *i. e.* trehalase, invertase and maltase.

RESULTS AND DISCUSSION

Differential centrifugation of mucosa homogenate. Freshly prepared intestinal mucosa from swine was homogenized with 4 parts (volume/weight) of 0.25 M sucrose, previously chilled to prevent its enzymatic hydrolysis. In the first experiments a homogenizer of the Potter-Elvehjem type [10] with a plastic pestle was used. This was, however, found to give incomplete homogenization, with a great part of the enzymes remaining in intact cells. Later an "Ultra Turrax" homogenizer was used with a homogenization time of 2 min and a volume of about 100 ml. After homogenization, the sample was centrifuged at 600 *g* for 10 min to remove whole cells and nuclei. The supernatant from this centrifugation is henceforth named total homogenate.

In the experiments where the "Ultra Turrax" homogenizer was used, the total homogenate was more enzyme-rich, but both homogenization techniques gave the same results in the subsequent fractionation.

The total homogenate obtained contained 20—30 units of invertase per ml. The other glycosidases were determined in homogenates made with buffer instead of sucrose solution, or in homogenates from which the sucrose had been removed by dialysis against distilled water (this procedure does not affect the glycosidase activity). The ratios of the other glycosidase activities to invertase activity were relatively constant, being about 2.0 for maltase, 0.5 for trehalase, and 0.2 for lactase.

The total homogenate was fractionated in a "Spinco" preparative ultracentrifuge [11]. The mitochondria were first sedimented at 7 000 *g* for 20 min (rotor 40, 10 000 RPM). The sediment was resuspended in 0.25 M sucrose and centrifuged once more at 7 000 *g*. The combined supernatants were centrifuged at 100 000 *g* for 45 min (rotor 40, 40 000 RPM) to give the microsome fraction and the particle-free supernatant. The invertase activity was found to be mainly localized in the microsome fraction (Table 1).

The microsomes were washed with distilled water, and the remaining sucrose removed by dialysis before determination of maltase, trehalase, and lactase activities. The microsomes were found to have the same ratios of action upon maltose, trehalose, and lactose as the total homogenate. These activities

Table 1. Differential centrifugation of homogenate from intestinal mucosa. Homogenized with "Ultra Turrax" homogenizer in 4 parts (v/w) of 0.25 M sucrose. Volume of the homogenate 10 ml.

Fraction	Units of invertase	Invertase % of total	Milligrams of protein	Specific invertase activity
Total homogenate	330	(100)	315.0	1.05
Mitochondria (7 000 *g* for 20 min.)	21	6.4	28.5	0.74
Microsomes (100 000 *g* for 45 min.)	276	83.7	72.0	3.83
Particle-free supernatant	36	10.9	210.0	0.17

must therefore also be localized in the microsomes. (Of the small amounts of amylase present, less than 25 % was localized in the microsome fraction.)

Solubilization of the glycosidases from the microsome fraction. Different methods were tried to solubilize the glycosidases. Treatment with distilled water or repeated freezing and thawing, which has been found to release some particle-bound enzymes [12], were without effect in the present case. Larner and Gillespie, who had found intestinal maltase and oligo-1,6-glucosidase localized in the microsomes, used acetone powder extracts for fractionation [13]. They have not, however, reported any criterion that the enzymes really were brought into solution by the acetone treatment. We have found the glycosidase activities in acetone powder extracts from intestinal mucosa readily sedimentable in the ultracentrifuge at 100 000 *g* for 45 min (rotor 40, 40 000 RPM), which shows that no solubilization occurs with this method. Treatment with *n*-butanol according to Morton [12], resulted in a partial inactivation of the enzymes, without any solubilization. Nor did snake venom [14] give any solubilization (0.2 mg *Crotalus adamanteus* venom/ml in 0.1 M acetate buffer pH 6.5, incubated for 5 h at 37°C).

Sodium desoxycholate has been found to solubilize most of the proteins from isolated liver microsomes [15]. When intestinal microsomes were treated with 0.5 % sodium desoxycholate in 0.1 M phosphate buffer pH 7.5, the glycosidases remained in a perfectly clear supernatant upon centrifugation at 100 000 *g* for 45 min. Together with the glycosidases about 80 % of the microsome protein remained in the supernatant. In spite of this, it could however be demonstrated in different ways that the glycosidases still were particulate:

1) The desoxycholate extract was dialyzed against phosphate buffer pH 7.5 to remove the desoxycholate. The effect of the dialysis was controlled by isotope-carbon-labelled desoxycholate, added to give 13 620 c.p.m. per ml. After dialysis for 72 h less than 0.5 % of the radioactivity remained. When now centrifuged at 100 000 *g*, the glycosidases slowly sedimented into a transparent, yellow pellet. If sodium desoxycholate was added to the sample before centrifugation, the glycosidases again remained in the supernatant.

2) When a desoxycholate extract was brought to pH 5.0 by the addition of acetate buffer, it became turbid, and the glycosidases sedimented readily in

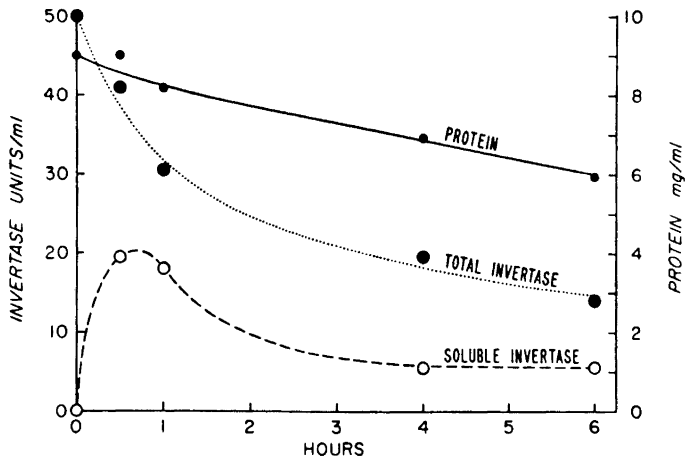

Fig. 1. Digestion of intestinal microsomes by trypsin in the absence of buffer. A suspension of microsomes in distilled water was added with 1 mg of trypsine per ml and incubated at 37°C. Soluble invertase was determined after ethyl alcohol precipitation (see the text) and (undigested) protein after precipitation with 5 % trichloroacetic acid.

an ordinary laboratory centrifuge (Wifug, 4 000 RPM for 10 min). This seems to be caused by agglutination of the particles at pH 5.0, a phenomenon which is well known [16].

3) When a desoxycholate extract was precipitated with ethyl alcohol in the cold, and the precipitate suspended in distilled water, all the invertase, maltase, and lactase activities were retained in the suspension (but the trehalase was partly inactivated by exposure to ethyl alcohol). When then centrifuged in Wifug at 4 000 RPM for 30 min, the glycosidases sedimented. The effect of ethyl alcohol precipitation seems to be the removal of desoxycholate, as the addition of desoxycholate after suspension of the precipitate retained the glycosidases in the supernatant.

When isolated microsomes were suspended in distilled water and incubated with trypsin, the glycosidases were inactivated parallel with the digestion of protein (Fig. 1). In the beginning of the reaction a solubilization of the glycosidases was, however, found to occur.

In the presence of phosphate buffer pH 7.5, the course of the reaction was found to be different (Fig. 2). In spite of the rapid digestion of protein, the invertase and maltase activities were unaffected even in a strong trypsin solution, and the enzymatic activity obtained in solution. The maltase activity indeed increased somewhat during the solubilization. The trehalase and lactase activities, on the other hand, decreased slightly during trypsin digestion. However, even in a solution containing 2 mg of trypsin per ml, incubated at 37°C for 4 h, less than 50 % of the lactase and trehalase activities were inactivated in the presence of phosphate.

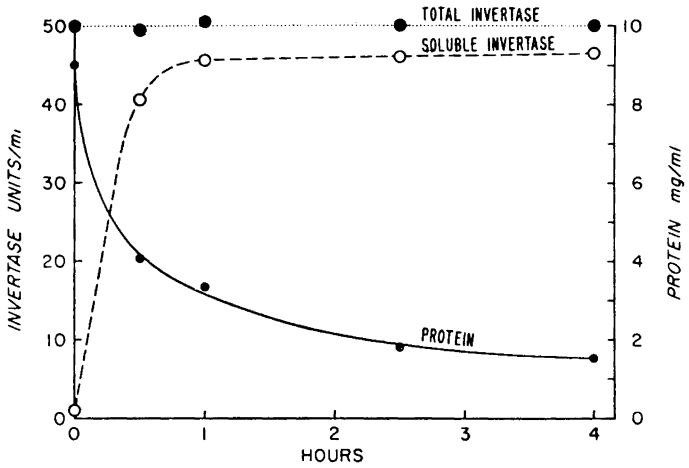

Fig. 2. Digestion of intestinal microsomes by trypsin in the presence of phosphate buffer. The same conditions as described in Fig. 1, but in the presence of 0.1 M phosphate buffer pH 7.5.

The influence of phosphate is not clearly understood. It was found that phosphate could be replaced by some other buffers as borate or tris-(hydroxymethyl) aminomethane but not by veronal buffer. Phosphate is not the only factor that is needed for protection of the glycosidases against trypsin digestion. When a solubilized glycosidase preparation, purified by ethyl alcohol precipitation, containing 5 mg of protein and 100 units of invertase per ml was incubated with 2 mg of trypsin per ml in 0.1 M phosphate buffer pH 7.5 at 37°C for 4 h, less than 10 % of the invertase and maltase activities remained. When, however, a boiled suspension of intestinal microsomes, with a protein content about equal to the purified glycosidase preparation, was added to the reaction mixture, 100 % of the maltase and 75 % of the invertase activities survived digestion under the same conditions.

The degree of solubilization of the glycosidases was measured after ethyl alcohol precipitation. A sample was precipitated in the cold with 5 parts of ethyl alcohol. After centrifugation, the sediment was suspended in distilled water to the original volume of the sample. The total glycosidases were determined on a sample of the suspension, and the rest was centrifuged in Wifug at 4 000 RPM for 20 min. The clear supernatant contained the soluble glycosidases, while the particle-bound glycosidases were found in the sediment.

Glycosidases from hog small intestinal contents. Intestinal contents from hog, obtained by gentle milking out by hand from an excised piece of intestine immediately after the slaughter, was found to contain rather much invertase (50—60 units per ml) and the same proportions of maltase, trehalase, and lactase as in the intestinal mucosa.

The glycosidases in intestinal contents seem to be in real solution. They are not sedimentable in the ultracentrifuge even after prolonged dialysis, they

Table 2. Comparison of the properties of invertase in the intestinal microsomes and the intestinal contents from swine.

	Intestinal microsomes	Intestinal contents
Optimum pH for sucrose hydrolysis	6.3–6.8	6.3–6.8
Optimum pH for melezitose hydrolysis	6.3–6.8	6.3–6.8
K_S for sucrose hydrolysis	2.5×10^{-2}	2.5×10^{-2}
K_S for melezitose hydrolysis	2.6×10^{-1}	2.8×10^{-1}
Action on melezitose *	0.002	0.002
Action on sucrose		
Action on raffinose	0	0

* Number of totally hydrolyzed melezitose molecules [22], divided with number of hydrolyzed sucrose molecules in 0.1460 M substrate concentration (5.0 % sucrose, 7.89 % melezitose . $2H_2O$) at pH 6.5 and temperature 37°C.

do not sediment in a centrifuge at pH 5.0, and they are readily soluble in distilled water after precipitation with ethyl alcohol.

To investigate if the glycosidases in the intestinal contents were identical with those obtained from the intestinal mucosa, some properties of the invertase from the two sources were compared (Table 2). The good agreement seems to show, that the enzyme preparations obtained from the two sources are identical.

When isolated microsomes were incubated with intestinal contents, a rapid solubilization of the glycosidases from the microsomes was found to occur.

Preparation of soluble intestinal glycosidases. The richest source for preparation of intestinal glycosidases was the material obtained by hard manual pressure or by pressing between two rolls pieces of the upper small intestine of hog. This material consists of the intestinal contents together with part of the intestinal mucosa and has an invertase activity of 100—150 units and a protein content of 50—60 mg/ml. If this material was frozen immediately, and thawed just before analysis, over 50 % of the invertase could be demonstrated to be in particular form, and in most cases it contained enough proteolytic enzymes to solubilize the glycosidases within 1—2 h at room temperature.

After storage for a few hours at room temperature, the material was chilled to 0°C and precipitated with 2 volumes of ethyl alcohol, previously chilled to —14°C. After centrifugation at 600 *g* for 10 min (International Refrigerated Centrifuge 2 000 RPM) the clear green supernatant was discarded, and the sediment suspended in its original volume of distilled water. By another centrifugation at 600 *g* a thick sediment, containing 2/3 of the protein, was obtained, while all glycosidase activity remained in the opalescent supernatant. The supernatant was then centrifuged in the Spinco ultracentrifuge at 35 000 *g* for 20 min (rotor 30, 20 000 RPM) to give a light yellow, clear supernatant. This supernatant was precipitated with 2 volumes of chilled ethyl alcohol and again centrifuged at 600 *g*. The sediment was yellow-stained, gelatinous, and

dissolved instantaneously in a small volume of distilled water to give a clear, yellow solution. The remaining ethyl alcohol was removed by dialysis against distilled water.

The solution thus obtained contained all the invertase, maltase, and lactase activities of the original material. The trehalase activity was found to be partly destroyed by exposure to ethyl alcohol at low temperature (Table 3), which clearly demonstrates that intestinal trehalase is an enzyme separate from maltase, invertase, and lactase. By keeping the solutions well chilled and avoiding unnecessarily long exposure to ethyl alcohol, about 50 % of the original trehalase activity could be obtained in the final solution.

The preparation obtained had a specific invertase activity of 55—60 units per mg of protein, which is about 50 times that in homogenates from intestinal mucosa.

Table 3. Action of ethyl alcohol on intestinal glycosidases. After ethanol treatment, the glycosidases are sedimented by centrifugation, and the sediment dissolved in water to the original volume. Invertase, maltase, and lactase activities are not affected by ethyl alcohol, but the trehalase activity is inactivated.

	Activity before treatment with ethyl alcohol units/ml	Activity after treatment with 60 vol. % ethyl days at +4°C units/ml	Per cent of activity remaining after ethyl alcohol treatment
Invertase	214	218	102
Maltase	325	324	99.5
Trehalase	78.5	5	6.5
Lactase	30	30	100

Separation of intestinal glycosidases on DEAE-cellulose. Columns of 0.5 g of DEAE-cellulose (dry weight) equilibrated with 0.01 M phosphate buffer pH 6.0 were used. A solution of solubilized glycosidases, containing 1 500 units of invertase, 3 000 units of maltase, 200 units of lactase, 400 units of trehalase, and 31 mg of protein, was dialyzed against 0.01 M phosphate buffer pH 6.0 and then applied to the column. The chromatogram was then developed with phosphate buffer pH 6.0 with a molarity continuously increasing from 0.01 to 0.16 M (Fig. 3). The rate of flow was 9 ml/h, and each fraction was collected over a 30 min interval.

1/4 of the total protein came out in the first three fractions, before any rise in molarity had occurred in the outcoming buffer. In this peak none of the glycosidases investigated could be detected. Already at 0.015 M phosphate the trehalase activity began to appear in the effluent. The lactase activity began to come out at 0.06 M phosphate and the invertase and maltase activities at 0.08 M phosphate. Thus trehalase, lactase, and invertase activities were clearly separated from each other. The maltase and invertase activities came out within the same interval, but the ratio of maltase activity to invertase activity increased from 1.0 at the beginning of the peak to 6.4 at its end (Table 4).

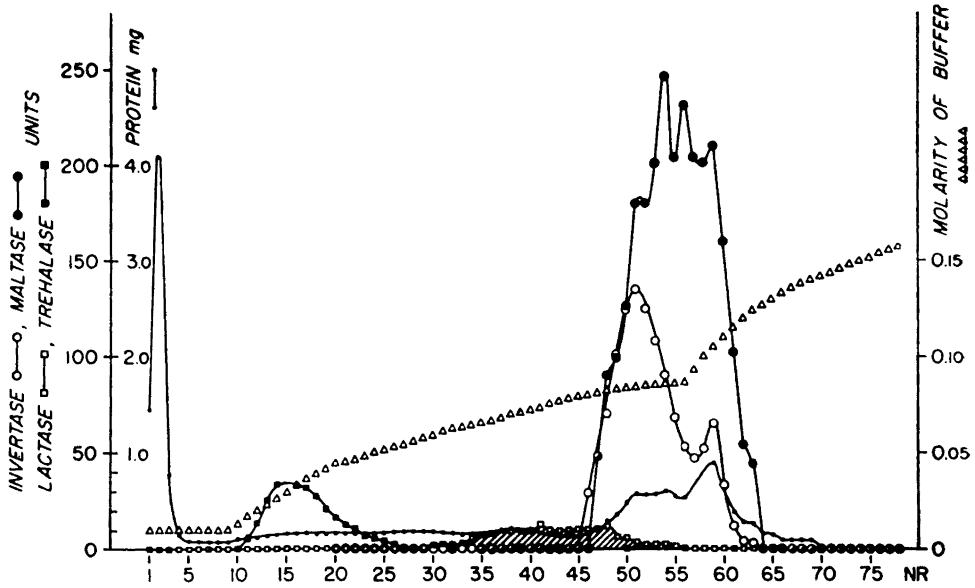

Fig. 3. Separation of intestinal glycosidases on DEAE-cellulose in phosphate buffer pH 6.0. The molarity of the effluent was determined by conductometry. The irregularity of the gradient around tube 52 is caused by a technical manipulation. For practical purposes all zero values for enzymatic activity are not shown in the figure.

It therefore seems probable that these activities are not caused by one single enzyme, as proposed by Weidenhagen [1] but that two different enzymes are involved in the hydrolysis of these sugars. It is, however, not possible at present, to say if there is one specific maltase and one specific invertase, or if

Table 4. Ratio maltase/invertase in fractions from the chromatography shown in Fig. 3.

Fraction No.	Invertase units/ml	Maltase units/ml	Ratio $\frac{\text{Maltase}}{\text{Invertase}}$
47	16.0	16.0	1.0
48	23.6	30.0	1.3
49	33.6	33.0	1.0
50	41.2	42.0	1.0
51	44.8	60.0	1.3
52	41.6	60.0	1.4
53	36.0	67.0	1.7
54	30.0	82.0	2.7
55	22.4	68.0	3.0
56	17.0	77.0	4.5
57	15.6	68.0	4.4
58	17.2	67.0	3.9
59	20.8	67.0	3.2
60	8.0	51.0	6.4

there are two enzymes with different relative activities against these two sugars. Chromatography of the enzymes on DEAE-cellulose at other pH values (from 5.0 to 8.5) did not give any better separation of the maltase and invertase activities. These enzymes were not absorbed to CM-cellulose.

In the most purified invertase fractions, the specific invertase activity was 250 units per mg of protein, which is 250 times that of intestinal mucosa, and 5 times that of the enzyme preparation applied on the column. The specific maltase activity reached 400 units per mg of protein, which is 200 times that of intestinal mucosa and 4 times that of the enzyme solution applied on the column.

MATERIALS AND METHODS

Intestinal mucosa. Pieces of hog intestine were chilled with crushed ice immediately after the slaughter. The pieces were cut from the upper part of the jejunum. The intestines were cut open, washed with tap water to remove the intestinal contents, and gently blotted with a piece of cloth. The mucosa was scraped off with a glass slide. The yield was 25—30 g of mucosa per meter of intestine.

Substrates. Sucrose and lactose were obtained from J. T. Baker Co., maltose from Merck A. G. (Germ.), *α,α*-trehalose and melezitose from Pfanstiehl Chemical Co. The trehalose was recrystallized from 80 % ethanol before use to remove traces of reducing substances. All the substrates used were chromatographically pure.

Assay of enzymatic activity. The reaction mixture was generally contained in 2.0 ml with 0.1388 M substrate concentration (4.75 % sucrose, 5.0 % maltose . H_2O, 5.0 % lactose . H_2O, and 5.25 % trehalose . $2H_2O$) in 0.05 M maleate buffer at optimum pH (6.5 for invertase and maltase, 6.0 for trehalase, and 5.6 for lactase). Toluene was used as preservative. The reaction mixture was incubated at 37°C for 60 min. With the non-reducing substrates (sucrose and trehalose) the degree of hydrolysis was determined with the 3,5-dinitrosalicylic acid method of Sumner [17] using a Beckman B spectrophotometer [18]. With the reducing substrates the method of Tauber and Kleiner [19] was used. Precipitation of proteins was made with the zinc-bariumreagent of Somogyi [20].

The hydrolysis followed a zero order reaction with a rate proportional to the amount of enzyme present up to over 10 % hydrolysis in 1 h. One unit of enzyme causes 1 % hydrolysis (= 1 mg of monohexoses produced) in 2.0 ml of reaction mixture at 37°C in 1 h.

Protein. Protein was determined with the method of Lowry *et al.*[21] A standard curve was made with human serum albumin (kindly supplied by A. B. Kabi).

Snake venom. Crotalus adamanteus venom was obtained from Ross Allen's Reptile Institute.

Sodium desoxycholate. Sodium desoxycholate (twice recrystallized) was kindly supplied by A. Norman.

Isotope-carbon labelled desoxycholic acid. 24-^{14}C-desoxycholic acid was a gift from S. Bergström. The activity was determined with a Tracerlab flow-counter.

Trypsin. Crystalline trypsin (Trypure Novo) was a gift from A.B. Ferrosan.

DEAE-cellulose. Diethylaminoethyl-cellulose was prepared from Whatman cellulose powder by the method of Peterson and Sober [9].

Conductivity measurements were with a Philips conductometer type PR 9500.

K_S *for the hydrolysis of sucrose and melezitose* were determined as described elsewhere [22].

Acknowledgement. This work has been supported by grants from *Statens Medicinska Forskningsråd* and the *Medical Faculty of the University of Lund.* Mrs. K. Hagebris is acknowledged for valuable technical assistance.

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Received August 27, 1958.