Simultaneous Absence of α-1,4-Glucosidase and α-1,6-Glucosidase Activities (pH 4) in Tissues of Children with Type II Glycogen Storage Disease*

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ABSTRACT: α-1,4-Glucosidase and α-1,6-glucosidase activities in whole homogenates of human tissues have been measured at pH 4 using maltose and glycogen as substrates for the first activity, and isomaltose as the substrate for the second. The tissues studied were liver, skeletal muscle, heart, leucocytes, and fibroblasts grown in tissue culture. Both types of glucosidase activity are missing in all tissues of children who have type II glycogen storage disease which is known to be a heritable defect with familial incidence. This finding provides indirect evidence in support of the conclusion reached in the accompanying two papers that a single lysosomal protein has both α-1,4-glucosidase and α-1,6-glucosidase activities. Although such an α-glucosidase has been isolated from rat liver lysosomes, the lysosomal origin of the corresponding human enzyme has not been shown conclusively. A study was made of the inhibition by trehalose and by α-methyl glucoside of α-glucosidase action at pH 4 in homogenates of human liver and heart. The action of α-glucosidase on glycogen is more sensitive to inhibition by these substances than is its action on maltose or isomaltose.

The two preceding papers (Jeffrey *et al.*, 1970a,b) describe the purification and some physical and kinetic properties of an α-glucosidase isolated from rat liver lysosomes. The enzyme was shown to have α-1,6-glucosidase as well as α-1,4-glucosidase activity, and to catalyze the total conversion of glycogen into glucose. Debranching of glycogen was found to be the rate-limiting step in its total hydrolysis. Hers (1963) found that an α-1,4-glucosidase active at pH 4 is present in normal human tissues, and that this presumably lysosomal enzyme is absent from the liver, heart, and skeletal muscles of children who have a fatal form of glycogen storage disease known as type II glycogenosis ("Pompe's disease"). Electron micrographs of liver, heart, kidney, and skeletal muscle from such patients show that a large amount of glycogen is sequestered within intracellular bodies which may be lysosomes (Baudhuin *et al.*, 1964; Zellweger *et al.*, 1965; Cardiff, 1966; Hernandez *et al.*, 1966; Hug *et al.*, 1966; Witzleben, 1969). Auricchio and Bruni (1967) and Auricchio *et al.* (1968) have purified an α-1,4-glucosidase, which is active at acid pH toward maltose and glycogen, form normal human kidney. This enzyme was found to be similar to the rat liver lysosomal glucosidase with respect to the effect of some inhibitors upon it. However, the possibility that the enzyme from human kidney also may have α-1,6-glucosidase activity was not studied. In this paper, we present data on the level of α-1,4-glucosidase and α-1,6-glucosidase activities which can be measured at pH 4 in whole homogenates of human liver, skeletal muscle, heart muscle, leucocytes, and cultured fibroblasts. In addition, it is shown that children with type II glycogen storage disease lack both of these glucosidase activities in all of their tissues which have been analyzed. Thus, the biochemical findings in this congenital human disease provide additional evidence in favor of the conclusion reached in the two preceding papers that the α-glucosidase of rat liver lysosomes, as well as the analogous enzyme which is present in human tissues, has dual specificity with respect to the type of bond upon which it is able to act. The relevance of this finding to the biochemical explanation for glycogen storage in the tissues of individuals with type II glycogenosis is also discussed.

Materials and Methods

Materials. With the generous cooperation of numerous physicians throughout the United States human tissue samples were obtained from patients by biopsy or at autopsy performed within 6 hr after death. Care was taken to ensure that the tissues were frozen immediately, either by immersion in liquid nitrogen or by pressing them between blocks of Dry Ice. They were then placed in closed containers to prevent dehydration, and these were packed in Dry Ice and shipped to Saint Louis. Upon arrival, the tissue specimens were transferred to small, plastic bags which were kept frozen in air-tight vials at −90° until used for analysis. The results of repeated assays done at various times have shown that the activities of many enzymes of glycogen metabolism are well maintained for periods of 6 months or longer when human tissues are taken and stored as described above. However, an effort was always made to analyze each tissue sample as promptly as possible after its receipt in the laboratory.

The substrates, maltose, isomaltose, and glycogen were obtained and purified as described by Jeffrey *et al.* (1970a). D-(+)-Trehalose and methyl α-D-glucopyranoside were products of Sigma Chemical Co.

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Preparation of Tissue Homogenates. Samples of frozen tissue (100 to 200 mg) were weighed quickly on a direct-reading torsion balance and then homogenized in nine volumes of ice-cold 0.15 M KCl. A Tenbroeck glass homogenizer (Kontes Glass Co.) was used by hand, and care was taken that thorough disintegration of the tissue was achieved. The homogenate was kept at 0° until aliquot portions of it were assayed as described below. Leucocytes were isolated in this laboratory from heparinized blood, essentially by the method of Huijing (1964). The well-washed film of cells then was suspended in either ice-cold 0.05 M NaCl or 0.05 M NaF (to inhibit glycogen phosphorylase *a* phosphatase) and frozen. Sometimes leucocyte preparations were received from other medical centers. These cells had often been isolated by dextran sedimentation and then frozen in 0.05 M NaCl or 0.05 M NaF. Frozen leucocyte suspensions were either homogenized in a tightly fitting Tenbroeck homogenizer or were disrupted using a Branson Sonifier (Model W 140 D) equipped with a microtip. Cultured fibroblasts were harvested by trypsinization and centrifugation. The cells were washed and suspended in 0.15 M NaCl prior to shipment in the frozen state. Some cell lines were established and grown in this laboratory, and washed suspensions of these cells were assayed for various enzyme activities without the cells having been frozen first. In either case, it was found that disruption of fibroblasts was achieved most satisfactorily by use of the Branson Sonifier as described above for leucocytes.

Enzyme Assays. Reaction mixtures for the assay of α-1,4-glucosidase activity contained 0.02 M maltose–0.05 M potassium acetate buffer (pH 4.0), and homogenate equivalent to 5 to 10 mg of tissue in a final total volume of 0.2 ml. Incubation was at 37° for a period of time within which the glucosidase reaction proceded at a linear rate. Homogenates of liver were incubated for either 30 min or 1 hr and muscle homogenates for 1 hr. The incubation mixtures for leucocytes and fibrobalsts contained 50–100 μg of protein and were incubated from 1 to 3 hr. All incubations were terminated by heating in boiling water for 1 min. After cooling, the contents of each tube was diluted to 0.5 ml with water and then centrifuged to remove denatured protein. Glucose was determined enzymatically as described by Jeffrey *et al.* (1970a), and glucosidase activity is expressed as micromoles of maltose hydrolyzed per minute per gram of tissue. In the case of leucocytes and fibroblasts the activity is expressed as millimicromoles of maltose hydrolyzed per minute per milligram of protein. Protein was determined by the method of Lowry *et al.* (1951) adapted to a microscale. Crystallized bovine plasma albumin (Armour) was used as the protein standard.

Reaction mixtures for the assay of α-1,4-glucosidase with glycogen as substrate contained 1% polysaccharide (isolated from rabbit liver). Otherwise, they were similar in composition to those prepared with maltose as the substrate, and they were incubated and analyzed in the same way. Glucosidase activity is expressed as micromoles of glucose formed (glucosidic bond hydrolyzed) per minute per gram of tissue, or as millimicromoles of glucose formed per minute per milligram of protein (leucocytes, fibroblasts).

Reaction mixtures for the assay of α-1,6-glucosidase contained 0.08 M isomaltose as substrate. Otherwise, they were similar in composition to those prepared with maltose or glycogen, and they were incubated and analyzed as before. The α-1,6-glucosidase activity is expressed as micromoles of isomaltose hydrolyzed per minute per gram of tissue, or as millimicromoles of isomaltose hydrolyzed per minute per milligram of protein (leucocytes, fibroblasts).

In all glucosidase assays suitable tissue blanks and reagent blanks were incubated under the same conditions and simultaneously with the experimental samples, and the quantity of glucose found in each of these control tubes was subtracted from that found in the corresponding complete system. The amount of glucose found initially in tissue blanks depended chiefly upon the amount of blood which was present in tissues obtained by surgical procedures. Because of the presence in all tissue homogenates of endogenous glycogen, there was often an appreciable formation of glucose during incubation in the absence of added substrate. Although this may have been due largely to the action of the glucosidase which has an acid pH optimum, the magnitude of its contribution to the apparent total activity of this enzyme has been neglected in calculating the results which are presented in this paper. Thus, the glucosidase activity is calculated from the increment in glucose formation due to incubation with added maltose, glycogen, or isomaltose.

In most cases assays were also done for several other enzymatic activities in each tissue homogenate. These included the enzymes implicated in the etiology of other types of glycogen storage disease: glucose 6-phosphatase (here assayed for only in liver), glycogen phosphorylase, phosphofructokinase, oligo-α-1,4→1,4-glucan transferase–amylo-1,6-glucosidase (the glycogen "debranching enzyme" system), and α-1,4-glucan–α-1,4-glucan 6-glycosyltransferase (the glycogen "branching enzyme"). A recent discussion of procedures used to measure the activities of these enzymes in human tissues and a review of the significance of the biochemical findings in relation to understanding the enzymatic basis of the various types of glycogen storage disease has been published (Brown and Brown, 1968). In the present work the results of such assays were used to identify the enzyme lesion, if any, which was present in each tissue used. Tissues which are designated here as having been taken from children with type II glycogenosis are those which had normal levels of activity of all enzymes for which assay was made except for α-glucosidase (pH 4) which was found to be absent (see below).

Results

Table I contains the results of determinations of the α-glucosidase activity measured at pH 4 in whole homogenates of liver, skeletal muscle, heart, leucocytes, and cultured fibroblasts from control subjects, and from children with type II glycogen storage disease, as well as from children with a variety of other types of glycogen storage disease. Since the levels of activity measured in tissues from the last group of children were always within the range of values obtained for tissues from control subjects, the data from these two groups have been combined and are shown in Table I as the control level of α-glucosidase as assayed with each of the three substrates. By examination of the range of values given it can be seen that the enzymatic activity measured in the tissue from one individual may vary as much as two- to threefold from that of another. It has been shown that this variation is not attributable to uncertainty in the enzyme assay nor to differences in the method of collecting and preserving the tissues, but, rather, that it is a

TABLE I: α-Glucosidase Activity in Human Tissues.[a]

Tissue[b]		α-1,4-Glucosidase		α-1,6-Glucosidase
		Maltose	Glycogen	Isomaltose
		μmoles of Bonds Hydrolyzed/min per g of Tissue		
Liver	Control	0.60–1.6 (34)[c]	0.30–1.0 (23)	0.10–0.25 (20)
	Type II	0–0.03 (8)	0–0.03 (5)	0–0.02 (8)
Skeletal muscle	Control	0.05–0.15 (17)	0.04–0.20 (5)	0.013–0.035 (4)
	Type II	0.00 (6)	0.00 (2)	0.00 (4)
Heart	Control	0.40–0.45 (5)	0.09–0.27 (5)	0.04–0.13 (5)
	Type II	0.00 (3)	0.00 (3)	0.00 (3)
		mμmoles of Bonds Hydrolyzed/min per mg of Protein		
Leucocytes	Control	1.2–4.4 (10)		0.30–0.70 (6)
	Type II	0.00 (4)		0.00 (2)
Fibroblasts[d]	Control	3.5–7.0 (17)	2.4–6.2 (7)	0.36–0.84 (7)
	Type II	0–0.03 (4)	0.00 (2)	0.00 (3)

[a] Homogenates were prepared, incubated at pH 4, and analyzed as described in the text. The α-glucosidase activity has been expressed in terms of the number of micromoles of glucosidic bonds hydrolyzed in order to permit comparison of the three substrates used. [b] Tissues removed by biopsy or at autopsy from children with type II glycogen storage disease were received from Dr. Lester Baker (Philadelphia), Dr. David Goldring (Saint Louis), Dr. Kurt Hirschhorn (New York), Dr. Ronald Lauer (Iowa City), Dr. Joshua Lynfield (New York), Dr. Wayne Marsh (Omaha), and Dr. James Monteleone (Saint Louis). Tissues from children with other types of glycogen storage disease were received from Dr. George Donnell (Los Angeles), Dr. Dick Ellis (El Paso), Dr. J. A. Haworth (Winnipeg, Canada), Dr. Rodney Howell (Baltimore), Dr. David Hsia (Chicago), Dr. Charles Lobeck (Mdiason), Dr. Marvin Rallison (Salt Lake City), Dr. Howard Sloan (Bethesda), Dr. Thomas Starzl (Denver), Dr. Hulda Wohltmann (Charleston), and Dr. Hans Zellweger (Iowa City). [c] The numbers in parentheses refer to the number of tissues analyzed. [d] Fibroblasts grown in tissue culture have been received from Dr. Kurt Hirschhorn (New York), Dr. Rodney Howell (Baltimore), Dr. Patricia Monteleone (Saint Louis), Dr. Elizabeth Neufeld (Bethesda), and Dr. William Sly (Saint Louis).

true difference in enzyme content at the time of taking the tissue sample (see also, Brown and Brown, 1968). The control level of α-1,4-glucosidase activity in leucocyte homogenates shown in Table I is somewhat higher than that reported previously (Brown and Zellweger, 1966). This difference probably is due to the use of 0.05 M potassium acetate buffer, pH 4, in the present series instead of the 0.02 M acetate buffer, pH 4.5, used in the earlier work. It is clear from the data of Table I that children with type II glycogen storage disease are lacking both α-1,4-glucosidase and α-1,6-glucosidase activities measured at pH 4, and that this simultaneous lack of these two activities is demonstrable in at least five tissues. A preliminary report published in abstract form by Den Tandt and Van Hoof (1969) also states that liver and muscle samples from patients with type II glycogen storage disease show either a strongly reduced or a complete absence of isomaltase activity at pH 4. The very low level of α-1,4-glucosidase and α-1,6-glucosidase activities occasionally measured in homogenates of liver tissue from some children with this disease (see Table I) should not be regarded as significant, as these apparent activities are within the limits of precision of the assays. Steinitz and Rutenberg (1967) reported that kidney was the only organ in which there was a substantial α-glucosidase activity, assayed at pH 4 with maltose as substrate, in each of three children with type II glycogenosis whom they studied. These authors were unable to decide whether the glucosidase deficiency discovered by Hers (1963) sometimes does not exist in the kidney, or whether the strikingly high activity of presumably nonlysosomal α-glucosidase (maltase) found at all pH values between 4 and 8 in homogenates of kidney from control subjects can obscure the detection of a true enzyme defect within the kidney lysosome of type II children. We have recently had the opportunity to study this question and have found that the use of glycogen rather than of maltose as the substrate for α-glucosidase at pH 4, permits a much clearer demonstration that in kidney, as well as in all other tissues examined, a deficiency of α-1,4-glucosidase activity exists in children with type II glycogenosis (B. I. Brown and D. H. Brown, 1969, unpublished data). Because of the small number of kidney samples available to us, the data from these experiments are not included in Table I.

Since the enzyme lesion in type II glycogenosis, a congenital disease of familial incidence, results in the simultaneous disappearance of two kinds of α-glucosidase activity, these two activities are most probably attributable in normal human tissues to a single protein. It has already been shown in the two preceding papers that in rat liver a single lysosomal protein possesses both α-1,4-glucosidase and α-1,6-glucosidase activity. Thus, the present findings in human tissues are in accord with the demonstrated properties of the corresponding enzyme from rat liver, except that the intracellular localization of the human enzyme within lysosomes has not been directly demonstrated.

The complex kinetic behavior of purified rat liver lysosomal α-glucosidase has been described in the preceding paper (Jeffrey *et al.*, 1970b). Many of the kinds of experiments which were done there would give ambiguous results if they were to

TABLE II: Action of Inhibitors on α-Glucosidase in Homogenates of Human Liver.[a]

Tissue Source[b]	Activity in Absence of Inhibitor (μmole of Bonds Hydrolyzed/min per g of Tissue)			Inhibition (%)			
	α-1,4-Glucosidase		α-1,6-Glucosidase	Trehalose		α-Methyl Glucoside	
	Maltose 10 mM	Glycogen 1%	Isomaltose 45 mM	20 mM	50 mM	10 mM	50 mM
M.S. (I)[c]	1.15				13	2	34
		0.37		50	73	24	100
L.F. (III)[d]	0.73			0	18	0	10
		0.27		63	88	75	100
			0.063		15		0
S.R. (III)[e]	0.60			0	15	0	33
		0.20		66	77	64	100
			0.059		0		0
L.V. (IV)[c]	1.39			0	12	5	19
		0.56		52	73	45	82
			0.089		0		
W.G. (VI)[f]	0.84			0	8	16	33
		0.45		48	79	76	93
			0.086		0	0	0

[a] Incubation of whole tissue homogenates was at pH 4 and 37° for 1 hr when maltose or glycogen were used as substrates or for 100 min when isomaltose was the substrate. For details of the analytical procedure, see the text. [b] The initials of the patient from whom liver biopsy or autopsy samples were taken are given for purposes of identification. The roman numeral following the initials denotes the type of glycogen storage disease which the patient had. For a discussion of this generally used classification, see Brown and Brown (1968). Type I signifies a deficiency of glucose 6-phosphatase. Type III signifies a deficiency of the glycogen "debranching enzyme," oligo-α-1,4→1,4-glucantransferase—amylo-1,6-glucosidase. Type IV signifies a deficiency of the glycogen "branching enzyme," α-1,4-glucan: α-1,4-glucan 6-glycosyltransferase. Type VI signifies a type of glycogen storage disease without any demonstrated enzyme lesion. [c] Tissue received from Dr. Thomas Starzl, Denver. [d] Tissue received from Dr. Martin Jimenez, Havana, Cuba. [e] Tissue received from Dr. Vincent Kelley, Seattle. [f] Tissue received from Dr. James Monteleone, Saint Louis.

be used in a study of the corresponding unpurified enzyme present in human tissues. In the latter cases, the presence of endogenous glycogen, and possibly also of a mixture of oligosaccharides derived from it by the action of α-amylase, would make uncertain the interpretation of mutual inhibition studies of α-glucosidase action involving the use of added maltose, glycogen, or isomaltose. The occurrence in human liver and skeletal muscle of an oligo-α-1,4-glucan glucohydrolase which has maximal activity at pH 7 on maltosidically linked oligosaccharides has been described (Brown and Brown, 1965). This enzyme, as well as any other glucosidase with a similar pH optimum, has some activity at pH 4, and the action of these enzymes would complicate the interpretation of kinetic studies involving various substrates. Several inhibitors of α-glucosidase action at acid pH which are not substrates have been described. D-(+)-Turanose was found by Lejeune *et al.* (1963) to be apparently specific as an inhibitor of the lysosomal enzyme of mammalian tissues. The previous paper (Jeffrey *et al.*, 1970b) contains a discussion of the findings of other investigators with regard to turanose inhibition, as well as reporting our own results from using it in kinetic studies on the purified rat liver lysosomal enzyme. Rosenfeld and Belenki (1968) described the inhibitory effects of D-(+)-trehalose and of methyl α-D-glucopyranoside on an enzyme which they partially purified from rabbit liver and which they have called "γ-amylase." This enzyme has many of the properties of an α-glucosidase with an acid pH optimum. These authors made the striking observation that trehalose and α-methyl glucoside had differential effects as inhibitors of glucose formation by "γ-amylase" when it acted on glycogen or maltose. Their results indicated that both of these inhibitors specifically affect only the hydrolysis of glycogen by the enzyme and have no effect on glucose formation from maltose. In view of these findings, it was of interest to study what effect these substances might have on the rate of glucose formation from maltose, glycogen, and isomaltose when these substrates are incubated separately at pH 4 with human tissue homogenates. Table II shows results of studies done using liver tissue from five different children. Trehalose and α-methyl glucoside are strongly inhibitory when glycogen is the substrate for the α-glucosidase active at pH 4. α-1,4-Glucosidase action on maltose is also inhibited but to a significantly lesser extent. It is of interest that very little or no inhibition of α-1,6-glucosidase by trehalose and α-methyl glucoside was found. Table III shows that similar results were obtained using homogenates of heart tissue from five different human subjects except that in this tissue

α-methyl glucoside seems to have a significant inhibitory effect on isomaltose hydrolysis. The principal difference between the results obtained in this work and those of Rosenfeld and Belenki (1968) in their study of rabbit liver "γ-amylase" is that trehalose inhibition does not appear to be specific for glycogen hydrolysis when whole homogenates of human tissue are used. However, taken as a whole, our data do indicate that α-1,4-glucosidase action on glycogen is more sensitive to inhibition than is the action of α-glucosidase on maltose and isomaltose. Whether conditions can be found under which trehalose would have a regular inhibitory effect on α-1,6-glucosidase activity is uncertain in view of the fact that adequate kinetic studies have not yet been done with a purified glucosidase prepared from human tissue. The data on inhibition shown in Tables II and III are qualitatively consistent with the hypothesis discussed in the preceding paper (Jeffrey *et al.*, 1970b) that a single, catalytically active binding site on the lysosomal glucosidase has affinity for both maltose and isomaltose, while glycogen binding and hydrolysis occurs at a second site which is separate from the first but capable of interacting with it. Purification of the human enzyme is necessary before more definitive studies of the kinetics of its action can be done.

TABLE III: Action of Inhibitors on α-Glucosidase in Homogenates of Human Heart.[a]

Tissue Source[b]	μmole of Bonds Hydrolyzed/min per g of Tissue: α-1,4-Glucosidase (− Inhibitor) Glycogen 1.3%	μmole of Bonds Hydrolyzed/min per g of Tissue: α-1,6-Glucosidase (− Inhibitor) Isomaltose 75 mM	Inhibition (%): Trehalose 40 mM	Inhibition (%): α-Methyl Glucoside 50 mM
H.[c]	0.088		66	61
		0.041	0	29
S. N.[d]	0.17		82	68
		0.073	0	21
D.[d]	0.20		32	54
		0.088	0	16
W. H.[d]	0.15		78	72
		0.062	0	
J. P. (III)[e]	0.27		80	76
		0.13	15	36

[a] Incubation of whole tissue homogenates was at pH 4 and 37° for 1 hr. For details of the analytical procedure, see the text. [b] The initials of the patient from whom heart biopsy or autopsy samples were taken are given for purposes of identification. When no roman numeral follows the initial, the patient did not have glycogen storage disease. Type III signifies that the patient had glycogen storage disease due to a lack of the glycogen "debranching enzyme," oligo-α-1,4→1,4-glucan transferase–amylo-1,6-glucosidase (for a discussion of this classification, see Brown and Brown, 1968). [c] Tissue received from Dr. Carl Smith, Saint Louis. [d] Tissue received from Dr. Ben D. McCallister, Rochester, Minnesota. [e] Tissue received from Dr. Kurt Hirschhorn, New York.

Discussion

The results of enzyme analysis of a variety of human tissues from ten different patients establish the fact that both α-1,6-glucosidase and α-1,4-glucosidase activities at pH 4 are missing in children who have type II glycogen storage disease. It is of interset that the data of Table I include the results of enzyme assays on fibroblasts cultured from the skin of two brothers, only one of whom had type II glycogenosis. The fibroblasts from the child with the heritable disease lacked both kinds of glucosidase activity while those from his sibling had normal levels of α-1,6-glucosidase as well as α-1,4-glucosidase. The fact that these two activities appear to be inherited together indirectly strengthens and also extends the conclusion reached in the preceding two papers that a single, lysosomal protein has both types of α-glucosidase activity. However, in the present work the lysosomal origin of the human enzyme has not been shown, since, unlike the rat liver enzyme, it has not yet been purified from any of the tissues studied here. Nevertheless, for reasons which have been discussed earlier, it is likely that the human α-glucosidase which is active at pH 4 also is localized within an intracellular organelle whose structure and properties are such that it can become filled with glycogen under circumstances when the α-glucosidase contained within it is inactive or missing, as it has been shown to be in type II glycogen storage disease. That this enzyme has the capacity to catalyze the total hydrolysis of glycogen to glucose has also been shown (Jeffrey *et al.*, 1970b). Accordingly, the accumulation of glycogen which can occur in its absence seems now to be better explained. However, no estimate can yet be given of the fraction of total glycogen catabolism in mammalian tissues which is due normally to the action *in vivo* of this hydrolytic enzyme.

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References

Auricchio, F., and Bruni, C. B. (1967), *Biochem. J. 105*, 35.
Auricchio, F., Bruni, C. B., and Sica, V. (1968), *Biochem. J. 108*, 161.
Baudhuin, P., Hers, H. G., and Loeb, H. (1964), *Lab. Invest. 13*, 1139.
Brown, B. I., and Brown, D. H. (1965), *Biochim. Biophys. Acta 110*, 124.
Brown, B. I., and Brown, D. H. (1968), *in* Carbohydrate Metabolism and Its Disorders, Vol. 2, Dickens, F., Randle, P. J., and Whelan, W. J., Ed., London, Academic, p 123.
Brown, B. I., and Zellweger, H. (1966), *Biochem. J. 101*, 16C.
Brown, D. H., Jeffrey, P. L., and Brown, B. I. (1969), *Abstr. 6th Meeting FEBS, Madrid*, 283.
Cardiff, R. D. (1966), *Pediatrics 37*, 249.
Den Tandt, W., and Van Hoof, F. (1969), *Abstr. 6th Meeting FEBS, Madrid*, 283.
Hernandez, A., Marchesi, V., Goldring, D., Kissane, J., and Hartmann, A. F., Jr. (1966), *J. Pediatrics, 68*, 400.

Hers, H. G. (1963), *Biochem. J. 86*, 11.
Hug, G., Garancis, J. C., Schubert, W. K., and Kaplan, S. (1966), *Amer. J. Dis. Child. 111*, 457.
Huijing, F. (1964), *Clin. Chim. Acta 9*, 269.
Jeffrey, P. L., Brown, D. H., and Brown, B. I. (1970a), *Biochemistry 9*, 1403.
Jeffrey, P. L., Brown, D. H., and Brown, B. I. (1970b), *Biochemistry 9*, 1416.
Lejeune, N., Thinès-Sempoux, D., and Hers, H. G. (1963), *Biochem. J. 86*, 16.
Lowry, O., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Rosenfeld, E. L., and Belenki, D. M. (1968), *Bull. Soc. Chim. Biol. 50*, 1305.
Steinitz, K., and Rutenberg, A. (1967), *Israel J. Med. Sci. 3*, 411.
Witzleben, C. L. (1969), *Lab. Invest. 20*, 424.
Zellweger, H., Brown, B. I., McCormick, W. F., and Tu, J.-B. (1965), *Ann. Paediat. 205*, 413.

Determination of Trypsin–Inhibitor Complex Dissociation by Use of the Active Site Titrant, *p*-Nitrophenyl *p'*-Guanidinobenzoate*

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ABSTRACT: The active site titration of trypsin with *p*-nitrophenyl *p'*-guanidinobenzoate was used to determine the extent of reaction of three naturally occurring inhibitors with trypsin. The equilibrium between free and inhibitor-bound trypsin was determined by measuring the initial burst of *p*-nitrophenol liberated with and without inhibitor. Dissociation of the trypsin–inhibitor complex was followed by measuring the subsequent increase of *p*-nitrophenol liberated with time. Hence, both kinetic and equilibrium data were obtained. All protein inhibitors tested reduced the initial burst. The presence of excess ovoinhibitor eliminated the initial burst. Rates of dissociation of the trypsin–inhibitor complexes increased in the order: soybean trypsin inhibitor $\simeq$ chicken ovomucoid $<$ chicken ovoinhibitor $\ll$ benzamidine. Competitive inhibition by ovoinhibitor could be shown directly at $\leq 10^{-5}$ M *p*-nitrophenyl *p'*-guanidinobenzoate. Dissociation of the trypsin$_1$–ovoinhibitor complex followed apparent first-order kinetics up to at least 65% completion. Inhibition curves obtained by active site titration agree with those obtained by rate assay. These data suggest that this active site titration can be used to determine trypsin in the presence of an excess of dissociable endogenous inhibitors.

Rate assays of remaining trypsin activity are commonly used to assay naturally occurring trypsin inhibitors (Laskowski and Laskowski, 1954; Vogel *et al.*, 1968). In using rate assays to study the action of ovoinhibitors from chicken egg white (Davis *et al.*, 1969), we observed progressive increase in trypsin esterase activity at high inhibition (unpublished data). The effect was usually small, and it resembled the displacement observed by Green (1953) but with less substrate. The curvature could not be attributed entirely to substrate activation (Trowbridge *et al.*, 1963) because use of benzoyl-L-arginine ethyl ester as substrate instead of tosyl-L-arginine methyl ester did not completely eliminate the effect. Hence, use of a direct assay method based on stoichiometric titration of the free-trypsin active sites (all-or-none assay) available at the time of addition of substrate (titrant) seemed desirable. The advantages of such all-or-none assays of enzyme concentration over rate assays of enzyme activity have been pointed out by Koshland *et al.* (1962) and by Bender *et al.* (1966).

Our objective was to assay inhibitors by all-or-none assay of the active trypsin remaining, as a suitable titrant, *p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride (NPGB)[1] is available (Chase and Shaw, 1967). Since soybean trypsin inhibitor prevents the reaction of trypsin with another active site titrant (Elmore and Smyth, 1968), and the competitive inhibitor, benzamidine, slows the reaction between trypsin and NPGB (Chase and Shaw, 1967, 1969), we might be able to determine the stoichiometry, the equilibrium, and the kinetics (dissociation of inhibitor–trypsin complex) of the reaction between ovoinhibitor and trypsin. In addition, trypsin has high affinity for NPGB and *p*-guanidinobenzoyl-trypsin deacylates very slowly (Chase and Shaw, 1967, 1969). (NPGB can be viewed here as either a substrate with a very low rate of deacylation or as a nearly irreversible inhibitor.)

The results reported in this paper show that NPGB titration can be used to assay protein inhibitors of trypsin. Ovoinhibitor is differentiated directly from chicken ovomucoid and soybean trypsin inhibitor in this assay by the more rapid postburst hydrolysis of NPGB by trypsin in the presence of ovoinhibitor.

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[1] Abbreviations used are: NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate·HCl; OI, chicken egg white ovoinhibitor; OM, chicken egg white ovomucoid.