Kemp, R. G., and Krebs, E. G. (1967), *Biochemistry 6*, 423.
Klotz, I. M. (1953), *Proteins I*, 771.
Marcus, F., and Hubert, E. (1968), *J. Biol. Chem. 243*, 4923.
Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.
Newsholme, E. A. (1963), *Biochem. J. 89*, 38P.
Opie, L. H., and Newsholme, E. A. (1967), *Biochem. J. 104*, 353.
Pogell, B. M. (1962), *Biochem. Biophys. Res. Commun. 7*, 225.
Pogell, B. M., Tanaka, A., and Siddons, R. C. (1968), *J. Biol. Chem. 243*, 1356.
Pontremoli, S. (1966), *Methods Enzymol. 9*, 625.
Pontremoli, S., Grazi, E., and Accorsi, A. (1968a), *Biochemistry 7*, 1655.
Pontremoli, S., Grazi, E., and Accorsi, A. (1968b), *Biochemistry 7*, 3628.
Pontremoli, S., Luppis, B., Wood, W. A., Traniello, S., and Horecker, B. L. (1965), *J. Biol. Chem. 240*, 3464.
Rosen, O. M. and Rosen, S. M. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 1156.
Sarngadharan, M. G., Watanabe, A., and Pogell, B. M. (1968), *Federation Proc. 27*, 791.
Scala, J., Patrick, C., and Macbeth, G. (1968), *Arch. Biochem. Biophys. 127*, 576.
Taketa, K., and Pogell, B. M. (1963), *Biochem. Biophys. Res. Commun. 12*, 229.
Taketa, K., and Pogell, B. M. (1965), *J. Biol. Chem. 240*, 651.
Walaas, E. (1958), *Acta Chem. Scand. 12*, 528.
Watanabe, A., Sarngadharan, M. G., and Pogell, B. M. (1968), *Biochem. Biophys. Res. Commun. 30*, 697.
Weitzman, P. D. J., and Wilson, I. B. (1966), *J. Biol. Chem. 241*, 5481.

Purification and Properties of Rabbit Muscle Amylo-1,6-glucosidase–Oligo-1,4→1,4-transferase*

T. E. Nelson,† E. Kolb,‡ and J. Larner

ABSTRACT: The glycogen phosphorylase limit dextrin-debranching system of muscle amylo-1,6-glucosidase–oligo-1,4→1,4-transferase was purified twofold over previous preparations by a new procedure and its properties reinvestigated. The preparation was found to be homogeneous by both physical and biological criteria and apparently contains two distinct enzymatic activities.

The pH optima in cationic buffers was found to be at 6.6 whereas that in anionic buffers was shifted to 7.2. Protonated hydroxylalkyl-substituted amines were found to inhibit the glucosidase–transferase in a simple linear noncompetitive manner. A mechanism for this type of inhibition is suggested.

The classical mammalian debranching system of glycogen phosphorylase limit dextrin (Cori and Larner, 1951; Larner and Schliselfeld, 1956) consists of two enzymatic activities. Oligo-1,4-transferase (EC 2.4.1.24 α-1,4-glucan:α-1,4-glucan-4-oligoglucan transferase) disproportionates the symmetric phosphorylase limit dextrin of glucogen to form an asymmetric structure having a single glucosyl residue branch and amylo-1,6-glucosidase (EC 3.2.1.33 dextrin:6-glucohydrolase) then liberates the glucosyl residue. This generates a linear chain which is further susceptible to the action of phosphorylase (EC 2.4.1.1 α-1,4-glucan:orthophosphate glucosyltransferase) (Walker and Whelan, 1960; Abdullah and Whelan, 1963; Brown *et al.*, 1963; Illingworth and Brown, 1962; Abdullah *et al.*, 1964; Brown and Illingworth, 1964; Brown and Brown, 1966; Hers *et al.*, 1964).

There have however been discrepancies in the reported properties of the glucosidase–transferase system. The pH optimum for hydrolysis has been shown to be 7.2–7.4 in one case (Larner and Schliselfeld, 1956) and 5.8–6.5 in others (Brown and Illingworth, 1964; Brown and Brown, 1966; Hers *et al.*, 1964, 1967; Taylor and Whelan, 1966). In addition, Tris and glycylglycine have been found to be inhibitory in some cases and not in others (Brown and Brown, 1966; Hers, 1964; Brown, 1964). The pH optimum for incorporation of glucose-^{14}C into glycogen has likewise been reported as not coinciding with that of hydrolysis (Hers *et al.*, 1964, 1967).

* From the Department of Biochemistry, College of Medical Sciences, University of Minnesota, Minneapolis, Minnesota 55455. *Received November 19, 1968.* This work was supported in part by a grant from the U. S. Public Health Service, National Institutes of Health (AM 09071). A portion of this has been presented as a preliminary note (Nelson *et al.*, 1968).

† Recipient (1966–1968) of a U. S. Public Health Service postdoctoral fellowship (5-F2-GM-23, 725-02). Present address: Department of Biochemistry, Baylor University College of Medicine, Texas Medical Center, Houston, Texas 77025.

‡ Present address: Department of Biochemistry, Mayo Graduate School of Medicine, Rochester, Minn.

In the most highly purified preparation of the enzyme reported the glucosidase activity has been inseparable from the transferase activity (Brown and Illingworth, 1964; Brown and Brown, 1966). The ratio of glucosidase activity, as measured by hydrolysis of a glucosidase-specific substrate (6^3-α-glucosylmaltotetraose, "fast B_5"), to transferase activity (as measured by hydrolysis of glycogen phosphorylase limit dextrin) was the same throughout purification. The purified preparation was homogenous in the ultracentrifuge and has been suggested to constitute a two-headed enzyme (Cori, 1964). The preparation however contains some phosphorylase *b* activity (Brown and Brown, 1966) and there have been reports of multiple activities upon column chromatography (Taylor and Whelan, 1968) in partially purified preparations.

In an attempt to resolve these questions, the pH optimum, inhibitory properties of buffers, and other properties were reinvestigated with a more highly purified preparation of the glucosidase–transferase prepared by a new procedure.

Experimental Section

Methods

Determination of Enzymatic Activity. GLUCOSIDASE–TRANSFERASE STATIC ASSAY. The method of Brown and Brown (1966) was used modified so that determinations were made with a single sample aliquot after 10 min. The enzyme (0.020 ml) at suitable dilution is incubated in a total volume of 0.20 ml containing 0.50% w/v glycogen phosphorylase limit dextrin, 0.05 M sodium maleate, 0.05% gelatin, 0.0005 M EDTA, and 0.01 M 2-mercaptoethanol (pH 6.6) (at 25°). The enzyme diluent consists of the above ingredients minus the limit dextrin and with 0.005 M EDTA. The reaction is started by adding the enzyme to the incubation mixture (13 × 100 mm Pyrex tubes) at 30°. After 10 min the tube is immersed in a boiling-water bath for 80 sec followed by immersion in a cold water bath. Sequencing of determinations was done with the aid of a stop watch. Zero time controls were made by using 2-min heat-denatured enzyme at the same dilution. The production of glucose was measured by adding a hexokinase glucose 6-phosphate dehydrogenase NADP reagent and observing the production of NADPH spectrophotometrically at 340 mμ (Larner and Schliselfeld, 1956; Brown and Brown, 1966; Slein, 1965). The reagent (0.80 ml) was added to the assay tubes and mixed by inversion. The difference between sample and zero time was recorded after the increase in adsorption stopped (*ca.* 10 min). Glucose standards were run concomitantly as a check on accuracy. The determinations were made in a Zeiss spectrophotometer equipped for 1.0-ml cells having a 1-cm light path. The glucose reagent was the same as that described below for the dynamic assay minus the limit dextrin.

GLUCOSIDASE–TRANSFERASE DYNAMIC ASSAY. The method of Larner and Schliselfeld (1956) was used modified so as to extend the capacity. In this assay the production of glucose from limit dextrin by the glucosidase–transferase is coupled with the glucose reagent. The assay was done in a Beckman Model DK-2 recording spectrophotometer equipped with a time-scan attachment and 1-cm light-path DK microcells having a minimum capacity of 50 μl. The enzyme incubation solution consisted of 5 μl of enzyme at suitable dilution, 10 μl of other additions or water, and 135 μl of 0.01 M glycylglycine, 1.5×10^{-2} M magnesium sulfate, 0.05% gelatin, 7.5×10^{-3} M dithiothreitol, 1×10^{-3} M ATP, 5×10^{-4} M NADP, 1.0 unit/ml of hexokinase (EC 2.7.1.1 ATP:D-hexose 6-phosphotransferase), 0.35 unit/ml of glucose 6-phosphate dehydrogenase (EC 1.1.1.49 D-glucose 6-phosphate:NADP oxidoreductase), and 0.5% glycogen phosphorylase limit dextrin. After the short lag period (Larner and Schieselfeld, 1956) the reaction was followed for 10 min. The temperature of the cuvet chamber was *ca.* 30°.

GLUCOSIDASE ASSAY. The hydrolytic action of the glucosidase alone was determined using 0.5% w/v glucosyl Schardinger dextrin as a substrate according to the method of Taylor and Whelan (1966) except that 0.05 M maleate, 0.0005 M EDTA, and 0.01 M 2-mercaptoethanol (pH 6.6) were substituted as the buffer.

MISCELLANEOUS ENZYME ASSAYS. Maltase (EC 3.2.1.20 α-D-glucoside glucohydrolase) activity was determined, using a 0.5% maltose as substrate in place of limit dextrin by the static assay procedure described above. In addition, a duplicate incubation was conducted for 24 hr at 30° and a 10-μl portion analyzed by paper chromatography using the descending solvent system 1-propanol–ethyl acetate–H_2O (7:1:2, v/v) (Albon and Gros, 1952). The spots were detected using the Trevelyan modification of the Tollen silver nitrate method (Trevelyan *et al.*, 1950); α-amylase (EC 3.2.1.1 α-1,4-glucan-4-glucanohydrolase) was determined according to Whelan (1964a). Phosphorylase was determined according to Hedrick and Fischer (1965) in the presence of AMP. Apophosphorylase *b* was determined according to the method of Shaltiel *et al.* (1965) after incubation of the diluted enzyme in 1×10^{-5} M pyridoxal phosphate for 20 min at 30° in 0.05 M maleate (pH 6.5) containing 0.005 M EDTA, 0.05% gelatin, and 0.04 M 2-mercaptoethanol. UDPG glucose:α-1,4-glucan α-4-glucosyltransferase (EC 2.4.1.11) was determined by the method of Villar-Palasi *et al.* (1966).

Other Analytical Methods. The sedimentation velocity of the purified preparation was measured in a Spinco Model E analytical ultracentrifuge using an AN-D rotor equipped with a 4° sector, 12-mm light-path standard cell. The phase-plate angle of the photographs illustrated was 40°. The average temperature during the run (180 min) was 7°. The sedimentation velocity was computed and corrected to 20° for the second through eighth frames by standard methods (Svedberg and Peterson, 1940) and the values were averaged. The protein (3.2 mg/ml) was sedimented at 52,640 rpm in 0.05 M Tris–0.005 M EDTA–0.01 M 2-mercaptoethanol (pH 7.2) at 25°.

Polyacrylamide gel electrophoresis was performed using the slab method in an EC 470 standard vertical gel cell having a 6-mm slab (EC Apparatus Corp.,

Philadelphia, Pa.). A 6% gel with 5% cross-linking (Raymond and Nakamichi, 1964) was prepared by dissolving 12 g of acrylamide and 0.6 g of *N,N'*-methylenebisacrylamide in 210 ml of 0.01 M Tris–0.01 M EDTA (pH 7.8) at 25°. After the acrylamide dissolved 0.12 ml of *N,N,N',N'*-tetramethylenediamine was added and the pH was readjusted to 7.8. Ammonium persulfate was then added (150 mg) and the slab was poured as soon as the persulfate dissolved. The gelled slab was preelectrophoresed for 1 hr at 200 V (*ca.* 100 mA). The running buffer was the same as that used to form the gel but containing 0.01 M 2-mercaptoethanol. Enzyme samples were dialyzed against the running buffer before application. The entire apparatus was kept at 0–4° during the run using a refrigerated thermostated water bath and a circulating pump. The chamber buffers were recirculated during the run *via* a cooling coil immersed in the water bath. Enzyme samples were layered into the slots (25% sucrose w/v solution) and electrophoresed into the gel at 50 V (*ca.* 25 mA) using a trace of brom phenol blue as a marker. After the protein migrated into the gel it was electrophoresed at 200 V (*ca.* 100 mA) for 24 hr. The slab was stained for protein by immersion for 18 hr in 0.25% Amido-Black 10-B in acetic acid–methanol–water (1:1:5, v/v) (Raymond and Nakamichi, 1964). The gel was electrolytically destained using a device similar to that described by Ferris *et al.* (1963) and the gel stored in 15% acetic acid. The same procedure was used for other pH values and buffers.

Protein was determined by an adaptation of the Miller modification of the Folin–Lowry method (Miller, 1959) modified as follows to measure insoluble protein. Protein was precipitated by addition of an equal volume of 10% trichloroacetic acid. The precipitate was washed once with 5% trichloroacetic acid (50–100 volumes excess) to remove 2-mercaptoethanol and the precipitate was dissolved in an appropriate volume of 1.0 N NaOH. An aliquot of this solution, usually 0.5 ml, was used for assay. To 0.50 ml of 1.0 N NaOH (containing the protein sample aliquot in 1.0 N NaOH) was added 1.0 ml of H_2O and then 0.50 ml of 25% Na_2CO_3, 2% sodium potassium tartrate (Rochelle's salt), and 1% $CuSO_4$ (mixed in that order). The usual method was followed from this point. This procedure was employed rather than the usual method for soluble proteins since 2-mercaptoethanol was found to interfere with the determination.

Carbohydrate concentration was determined by the phenol–sulfuric acid method of Dubois *et al.* (1956).

pH determinations were made with a Radiometer Model 26 pH meter standardized at the temperature and range of usage. All pH values are at the concentration and temperature stated. Temperatures in parentheses following a buffer pH value indicate the temperature at which that value was obtained, not the temperature at which the buffer was used.

All buffers and other salts were in either the sodium or the chloride counterion form unless otherwise specified. The pK_a values cited in Table III are either accepted handbook values or those supplied by the manufacturers.

Ion-exchange resins were recycled before use according to the manufacturer's suggestions.

Enzyme Purification. The enzyme was purified from an extract of rabbit muscle. The following is a typical purification procedure. One adult white rabbit weighing 7–8 lb was permanently anesthetized by administering 1 mg/2-lb body weight of 7% seconal in 0.9% saline. The animal was bled and the back and leg muscles were excised and placed on ice. The muscle (540 g) was cut into small pieces and homogenized in a large Waring Blendor using 2.5 volumes of 0.1% $KHCO_3$ containing 0.004 M EDTA, *ca.* pH 7.5 (at 25°). The muscle was ground three times for 30 sec at high speed with 1-min intervals between grinding to prevent heating. This and all subsequent operations were conducted at 0–4°. The muscle homogenate was centrifuged at 16,000*g* in a refrigerated centrifuge for 30 min and the supernatant was filtered through glass wool. Oyster glycogen (10% solution) was added to the extract to give a concentration of 1 mg/ml. After 10 min the pH was lowered with stirring to 5.3 at 0° with 1 N acetic acid and allowed to remain for 10 min. The extract was then centrifuged at 16,000*g* for 20 min and the supernatant was adjusted to pH 7.8 at 0° by addition of 2 M Tris base, after the addition of 0.20 M EDTA, pH 7, to 0.01 M. The solution was then brought to 45% ammonium sulfate saturation by the addition of 25.6 g of solid/100 ml, 0° formulation (Di Jeso, 1968). Solid ammonium sulfate was added over a period of 30 min with constant stirring. The solution was stirred an additional 30 min and then allowed to stand at least an additional 6 hr or overnight. The solution was centrifuged at 16,000*g* for 30 min and the residue was dissolved in (about 5 ml/200-ml capacity centrifuge bottle) 0.05 M Tris–0.005 M EDTA–0.01 M 2-mercaptoethanol (pH 7.8), at 0°. The dissolved residue was dialyzed against (20 volumes excess) 0.005 M Tris–0.0005 M EDTA–0.01 M 2-mercaptoethanol (pH 7.2), at 25° for 18 hr with one change of buffer after 6 hr.

The dialyzed solution was applied to a DEAE-cellulose column (diameter to height 1:20) having about 1-ml bed volume/4 mg of protein (*ca.* 1 ml of resin/g of muscle excised) equilibrated with the buffer used for dialysis. The column was washed after application of the enzyme with 1 l. of the dialysis buffer at a flow rate of 30 drops/min. A minor inert protein peak was eluted midway through this wash. The enzyme was eluted by a 2-l. linear salt gradient consisting of equal volumes of the dialysis buffer and 0.05 M Tris–0.005 M EDTA–0.01 M 2-mercaptoethanol containing 0.25 M NaCl (pH 7.2), at 25°. The eluate at this point was collected by an LKB automatic fraction collector in 20-ml fractions. The eluate from the column was monitored by means of an LKB ultraviolet monitor and recorder. A typical trace is shown in Figure 4. A minor inert protein peak was also eluted at the start of the gradient. The glucosidase–transferase activity was located by means of the dynamic assay described and the peak fractions were pooled. Phosphorylase *b* was resolved by adding to the pooled fractions at 0° a quantity of 2 M cysteine hydrochloride, 2 M imidazole, and 0.5 M citrate at 40° (mixed in that order and adjusted to pH

4.4 with 1 N HCl at 60°) to give 0.4 M cysteine, 0.4 M imidazole, and 0.1 M citrate. The pH was adjusted to pH 4.8 at 0° with 1 N acetic acid and kept at that pH for 2 hr. The pH was then raised to 7.8 at 0° with 2 M Tris base after the addition of EDTA solution to 0.01 M. Any precipitate which forms at pH 4.8 (after about 20 min) redissolves upon raising the pH to 7.8 and should be disregarded. The solution was then brought to 75% saturation with ammonium sulfate by the addition of 46.8 g/100 ml and was allowed to remain at least 24 hr and then centrifuged at 30,000*g* for 30 min. The residue was dissolved in 0.05 M Tris–0.005 M EDTA–0.01 M 2-mercaptoethanol (pH 7.8), at 0° (*ca.* 5 ml/200-ml centrifuge bottle) and dialyzed against 0.05 M Tris, 0.005 M EDTA, and 0.01 M 2-mercaptoethanol (pH 7.2), at 25° (20 volumes excess), for 18 hr with one change after 6 hr. The solution was then brought to 30% saturation with ammonium sulfate (16.4 g/100 ml) after addition of EDTA solution to 0.01 M. The solution was allowed to remain for 3 hr and then centrifuged at 16,000*g* for 30 min. The residue was dissolved in (*ca.* 10 ml/200-ml centrifuge bottle) 0.05 M Tris, 0.005 M EDTA, 0.01 M 2-mercaptoethanol (pH 7.8), at 0°, and dialyzed against a 20-volume excess of 0.005 M Tris, 0.0005 M EDTA, 0.01 M 2-mercaptoethanol (pH 7.2), at 25°, for 18 hr with one change after 6 hr (protein concentration *ca.* 10 mg/ml). The dialysate was then diluted tenfold with 0.0025 M phosphate, 0.0025 M citrate, 0.005 M EDTA, 0.01 M 2-mercaptoethanol (pH 6.5), at 25°. After a precipitate formed (20–60 min) the solution was centrifuged at 16,000*g* for 30 min and the supernatant was applied to a phosphocellulose column equilibrated with 0.005 M phosphate, 0.0005 M EDTA, 0.01 M 2-mercaptoethanol (pH 6.5), at 25°. The column was then washed with 1 l. of the equilibration buffer at 30 drops/min. The column (dimensions as before) had a bed volume of 1 ml of resin/mg of protein (*ca.* one-third the bed volume of the DEAE-cellulose column). A minor peak was eluted during the wash. The glucosidase–transferase was eluted with a 1-l. linear salt gradient at 15 drops/min consisting of equal volumes of the equilibration buffer and 0.05 M phosphate, 0.005 M EDTA, and 0.01 M 2-mercaptoethanol containing 0.25 M NaCl (pH 6.2), at 25°. The gradient was followed by a 500-ml wash with the pH 6.2 buffer. The enzyme was eluted midway through the gradient as a protracted peak continuing halfway through the additional wash. The enzyme was located as before using the dynamic assay, and the peak fractions were pooled and adjusted to pH 7.8 (at 0°) with 2 M Tris base after addition of EDTA solution to 0.01 M. The pooled fractions were then precipitated by addition of ammonium sulfate to 75% saturation and the solution was allowed to remain at least 24 hr. The 75% solution was centrifuged at 20,000*g* for 45 min and the residue was dissolved in the above 0.05 M Tris buffer (pH 7.8), at 0° (about 2 ml/200-ml centrifuge bottle). The dissolved residue was then dialyzed against a 50-volume excess of 0.05 M Tris, 0.005 M EDTA, and 0.001 M dithiothreitol (pH 7.2), at 25°, for 18 hr with one change after 6 hr, the dialysate was centrifuged at 30,000*g* for 30 min, and any residue was discarded. The preparation is stable without loss of activity, for several months.

Materials

Glycogen phosphorylase limit dextrin was available in this laboratory, prepared as described previously (Larner *et al.*, 1952). Glucosyl-Schardinger dextrin was a gift of Professor W. J. Whelan, University of Miami School of Medicine, Miami, Fla. Waxy maize starch (98% amylopectin) was a gift of Dr. B. A. Lewis, University of Minnesota, St. Paul, Minn. Maltose (three-times recrystallized) was available in this laboratory, as was panose. Rabbit liver glycogen was obtained from Nutrition Biochemicals, Cleveland, Ohio, and deionized by passage of a 1% solution over a mixed-bed ion-exchange resin (Ambelite MB-3) before use.

Hexokinase was purchased from the Sigma Chemical Co., St. Louis, Mo., as the purified mixed isozyme grade from yeast and had a specific activity of 400 units/mg. ATP, disodium salt, Tris base, and pyridoxal phosphate were purchased from Boehringer Mannheim. Dithiothreitol, 2-(*N*-morpholino)ethanesulfonic acid, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, and *N*-tris(hyroxymethyl)methyl-2-aminoethanesulfonic acid were purchased from Calbiochem, Los Angeles, Calif. Diethanolamine, ethanolamine, imidazole, acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N'N'*-tetramethylethylenediamine, and brom phenol blue (3',3'',5',5''-tetrabromophenosulfonphthalein) were obtained from Distillation Products Industries, Rochester, N. Y., as reagent grade. Amido-Black 10-B and sodium cacodylate (sodium dimethylarsenate) were purchased from K & K laboratories, Inc., Plainview, N. Y.

DEAE-cellulose was obtained from Brown Co., North Stratford, N. H., as Selectacel course grade, type 20. Phosphocellulose was obtained from Sigma Chemical Co., as the course grade.

Results

Determination of Enzymatic Activity. Amylo-1,6-glucosidase–oligo-1,4→1,4-transferase activity was routinely measured by the release of glucose from glycogen phosphorylase limit dextrin. Glucose was measured spectrophotometrically by the production of NADPH using the coupled enzyme system of hexokinase glucose 6-phosphate dehydrogenase initially employed by Larner and Schliselfeld (1956). The static method of Brown and Brown (1966) was used, modified so that determinations could be made with a single sample aliquot.

The degradation of the limit dextrin is linear with respect to time and proportional to enzyme concentration over a measurable range (Figure 1).

The dynamic assay of Larner and Schliselfeld (1956) modified for use on a microscale was also employed. In this procedure the glucosidase–transferase system is coupled directly to the NADPH generating system. The correlation between the two methods in terms of the same units was found to be within experimental error (less than 10%).

TABLE I: Purification Procedure.

Stage	Vol (ml)	Glucosidase–Transferase: Total Protein (mg)	Total Units	Sp. Act. (units/mg)	Purification, -fold	Recov. (%)	Phosphorylase: Total Units of Phosphorylase *b*	Total Units of Apophosphorylase *b*
Muscle homogenate	1080	22,250	1,825	0.082		100		
pH 5.3 supernatant	1150	17,700	1,485	0.084		81		
Ammonium sulfate residue (0–45% saturation)	70	1,890	847	0.45	6	51		
DEAE-cellulose column Pooled fractions	670	482	556	1.15	14	32	18,200	1,500
Phosphorylase *b* resolution Ammonium sulfate residue	72	475	407	0.86	11	24	0	6,670
Ammonium sulfate residue (0–30% saturation)	42	268	320	1.19	15	19	0	1,260
Isoelectric precipitation Supernatant	420	134	252	1.87	23	15	0	575
Phosphocellulose column Pooled fractions	13.8	13.3	110	8.26	101	6	0	0

Enzyme units are those in current usage (Brown and Brown, 1966). One unit of enzyme activity is that amount which produces 1 μmole of glucose/min at 30° under the conditions described.

Purification. The results of the over-all procedure are shown in Table I. The initial acidic pH precipitation step utilizes the fact that glycogen-bound phosphorylase *a* and UDPG glucose:α-1,4-glucan α-4-glycosyltransferase can be precipitated at acid pH. The major protein contaminant in the DEAE-cellulose column fractions containing glucosidase–transferase activity is phosphorylase *b* (Figure 2). Attempts to increase the separation of the two activities by using another pH were unsuccessful. Phosphorylase *b* was therefore removed by first converting it into apophosphorylase *b*. The resolving conditions described by Shaltiel *et al.* (1965) were modified to give total conversion. The bulk of the apophosphorylase *b* was then separated by isoelectric precipitation. This step is required for subsequent procedures, since the preparation at this stage spontaneously precipitates under conditions of even moderate ionic strength (0.05 M) at pH values below 7. The final purification was achieved by phosphocellulose column chromatography, where the glucosidase–transferase separates from apophosphorylase *b*. Phosphorylase *b* does not separate well from the glucosidase–transferase under the same conditions.

The purified preparation is homogeneous in the ultracentrifuge (Figure 3). The average sedimentation constant was determined to be 7.68. This value is in reasonable experimental agreement with the previously reported value of 8.68 (Brown and Illingworth, 1964). Upon acrylamide gel electrophoresis (Figure 4), the purified glucosidase–transferase migrates as a single component at pH values between 6 and 8 with the exception of pH 7.8 (at 25°) in 0.01 M Tris where there is a doublet (Figure 4). Using these conditions the preparation was preparatively chromatographed on the

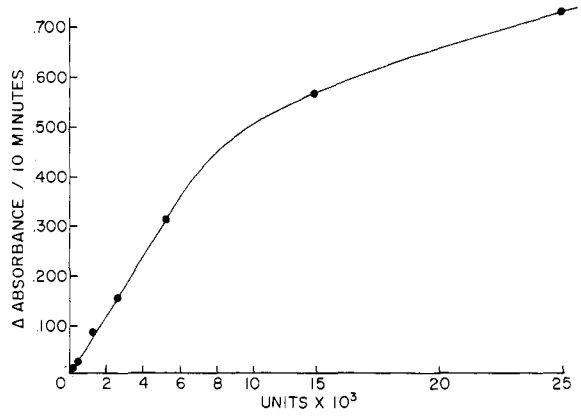


FIGURE 1: Action of glucosidase–transferase on phosphorylase glycogen limit dextrin as a function of enzyme concentration. The standard static assay was conducted as described in the text using 0.20 ml of the incubation mixture. The enzyme employed had a specific activity of 7.7 units/mg.

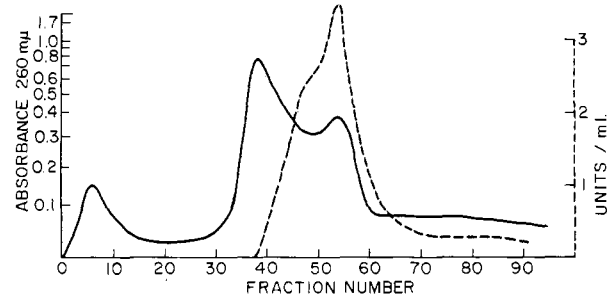


FIGURE 2: DEAE-cellulose column chromatography. The ultraviolet monitor trace is diagrammatically represented. Absorbance at 260 mμ is shown on one absicca and units per milliliter on the other. The procedure is described in the text. The first major peak contains phosphorylase *b* and the second the glucosidase–transferase. The initial minor protein peak at the start of the gradient contains inert material. An absorbance of 0.100 corresponds to *ca.* 0.55 mg as determined colorimetrically. Fractions 40–74 were pooled. Fraction 40 is about the midpoint of the gradient.

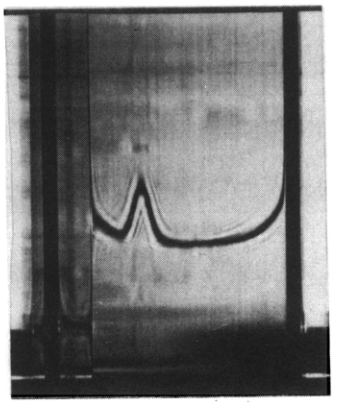


FIGURE 3: Sedimentation velocity pattern. The sedimentation velocity photographs were obtained in an analytical ultracentrifuge as described in the text. The exposure shown was made 60 min after the start of acceleration.

gel and marker strips were stained for protein. The bands of the doublet were sectioned out and the enzyme was eluted by mechanical disruption of the gel. Each band was found to contain equal quantities of the glucosidase and transferase. The doublet at this pH (there is none at pH 7.6 or 8.0) is apparently a physical characteristic of the preparation under these conditions and not the separation of enzymatic activities or an inert component.

The purified preparation was found to contain no detectable α-amylase or maltase (Table II). UDPG glucose:α-1,4-glucan α-4-glucosyltransferase, phosphorylases *a* and *b*, and apophosphorylase *b* were also absent. The preparation is thus, by the criteria applied, both physically and biologically homogeneous. It is of interest to note that although the final specific activity of the glucosidase–transferase shown in Table I is 8.3 units/mg, the phosphocellulose peak fraction with the highest activity had a specific activity of 9.5 units/mg.

The quantity of the enzyme present in muscle can be computed on the basis of the specific activity of the purified preparation (assuming a final specific activity of 9.5) and the activity measured to the tissue extract. The glucosidase–transferase accounts for 0.03% or 0.3 mg/g of total protein. This contrasts to 0.3% for phosphorylase (Fischer and Krebs, 1962). Since the turnover number of the present glucosidase–transferase is about 1000 μmoles of glucose/10^5 g of protein per min (based on a specific activity of about 10) and that of phosphorylase in the degradative direction is about 2000 μmoles of glucose 1-phosphate/10^5 g of protein per min, the relative action of the glucosidase–transferase is at least an order of magnitude lower than that of phosphorylase. This may indicate that the debranching system is rate limiting in the degradation of glycogen.

pH Optimum and Inhibitors. In order to determine the pH optimum of the purified preparation in the range 4–9 a number of different buffers were selected. It soon became apparent that the activity of the enzyme was not only a function of the pH but also of the buffer employed. Since buffers frequently affect the pH optimum

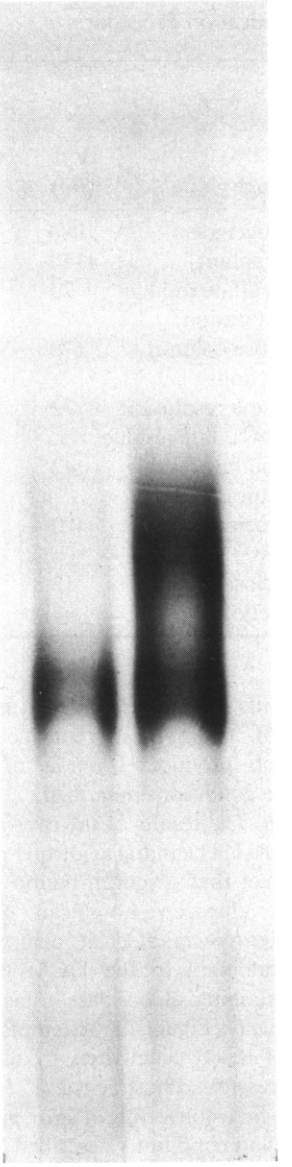


FIGURE 4: Polyacrylamide gel electrophoresis. Gel electrophoresis was performed as described in the text. Lane A (left) is the preparation after DEAE-cellulose chromatography (*ca.* 3 mg). Lane B (right) is the preparation (*ca.* 1.5 mg) after removal of phosphorylase (*ca.* 1.5 mg) by isoelectric precipitation (*cf.* Table I). The direction of migration is toward the bottom (anode) as shown.

(Dixon and Webb, 1964) the activity of the enzyme was determined in a representative series of cationic and anionic buffers. The results are shown in Figure 5. All of the anionic buffer optima cluster at 6.6 with the exception of citrate where the optimum is at 6.0. The optima of the cationic buffers, on the other hand, are at 7.2 with the exception of imidazole which has an optimum at 6.6.

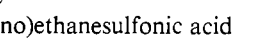
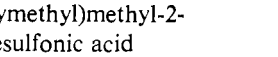
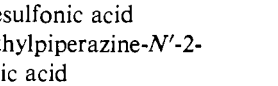
All of the cationic buffers inhibited enzymatic activity to varying degrees with the apparent exception of glycylglycine (no inhibition was noted at either 0.01 or 0.05 M). Since all of the cationic buffers were substituted amines it was of interest to investigate the

TABLE II: Relative Rates on Possible Substrates.[a]

Substrate	Concn (w/v %)	Enzyme Concn (unit/ml)	Rate
Glycogen phosphorylase limit dextrin	0.50	0.021	100
Rabbit liver glycogen	0.50	0.52	2.0
Amylopectin	0.50	0.52	<0.1
Panose	0.50	0.52	<0.1
Maltose	0.50	0.52	<0.1
Glucosyl Schardinger dextrin	0.50	0.021	122

[a] The amount of enzyme used for phosphorylase limit dextrin was chosen to give the maximum measurable rate. This rate was arbitrarily set at 100. Relative rates of greater than 0.1 were within the range of reliability of the assay.

TABLE III: Inhibition by Various Amines.[a]

Name	Structure	pK_a at 25°	% Act.
Without amine			100
2-(*N*-Morpholino)ethanesulfonic acid		6.2	86
N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid		7.5	91
N-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid		7.6	91
Tris	$(HOCH_2)_3CNH_2$	8.1	68
Diethanolamine	$(HOCH_2CH_2)_2NH$	9.0	63
Ethanolamine	$HOCH_2CH_2NH_2$	9.4	74
Ethylamine	$CH_3CH_2NH_2$	10.8	87
Triethylamine	$(CH_3CH_2)_3N$	10.6	98
Choline	$HOCH_2CH_2\overset{+}{N}(CH_3)_3$	8.9	99
Taurine	$H_2NCH_2CH_2SO^-{}_3$	9.1	93
Hydroxylamine	$HONH_2$	6.0	88
Ammonium chloride	NH_4Cl	9.2	94

[a] 0.01 M in 0.01 M citrate, pH 6.0. The enzyme concentration used in this experiment was 0.021 unit/ml.

extent that various substituents had on inhibition. The results are shown in Table III. The taurine structure in itself does not cause appreciable inhibition. The same is true of triethylamine, a tertiary amine. Ammonium ion is likewise a poor inhibitor.

The inhibition of the amines appeared to be related to the presence of hydroxyalkyl substituents. The series diethanolamine, ethanolamine, and ethylamine illustrate this. It is also evident that the amino group needs to be relatively unhindered since choline is a very poor inhibitor whereas ethanolamine is not.

The correlation between hydroxyalkyl groups and the degree of inhibition was investigated further because of the analogy to carbohydrate structure. The inhibition constants were determined for diethanolamine, ethanolamine, ethylamine, and Tris at pH 6.6, the anionic buffer pH optimum. The K_I for Tris was also determined at pH 7.2, the cationic buffer pH optimum. In all cases a noncompetitive inhibition of the simple linear type was observed (Cleland, 1963). This is illustrated in the case of Tris as shown in Figure 6A,B. The inhibition constants are given in Table IV. The K_I for Tris determined by two independent methods was the same. In addition the K_I was identical in either the cationic or anionic system.

Michaelis Constants. The Michaelis constants of the purified preparation for glycogen phosphorylase limit dextrin were determined at the anionic buffer pH optimum of 6.6 (maleate) and at the cationic optimum (glycylglycine) of 7.2, and found to be identical. K_m as a function of pH is constant in glycylglycine from 6.1 to 7.5 (Larner and Schlieselfeld, 1956). The K_m in both systems was 0.030% or 6.3×10^{-5} M expressed as outer tier branch point glucolase (*cf.* Larner and Schliselfeld, 1956).

Independent Measurement of Amylo-1,6-glucosidase Activity. Determination of the glucosidase activity in the preparation independently of the transferase

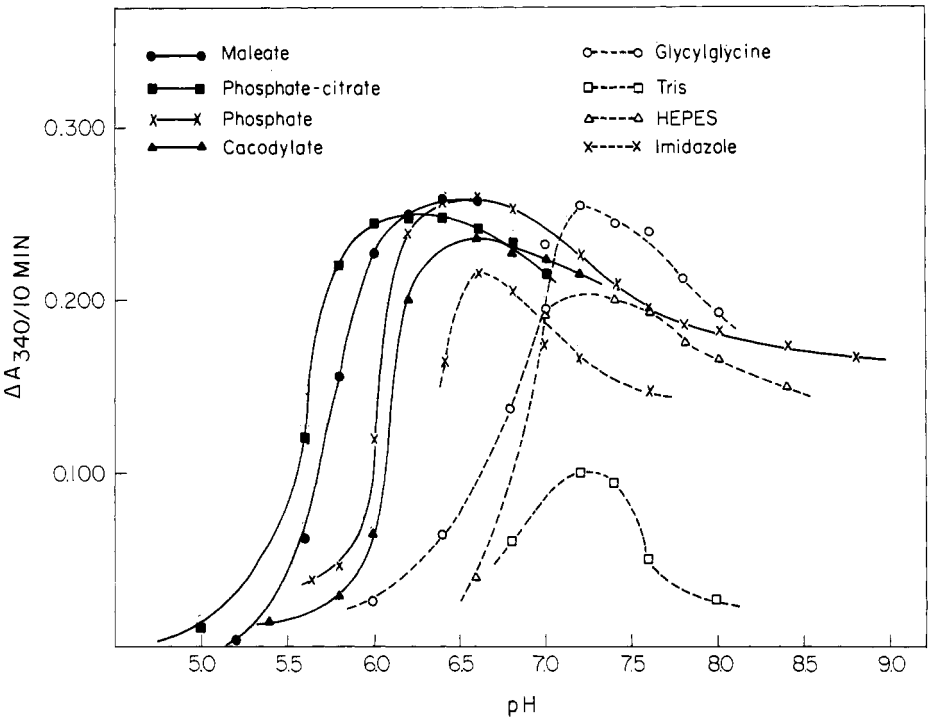


FIGURE 5: The hydrolysis of glycogen phosphorylase limit dextrin by the glucosidase–transferase in various buffers. Incubations were conducted using the standard static assay conditions described in the text. The incubation mixture (0.20 ml) contained 4.2×10^{-3} unit of enzyme with a specific activity of 7.7 units/mg. All the buffers were at 0.01 M concentration. In addition to those shown 2-(*N*-morpholino)ethanesulfonic acid, *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, citrate, and α-glycerol phosphate were also tested.

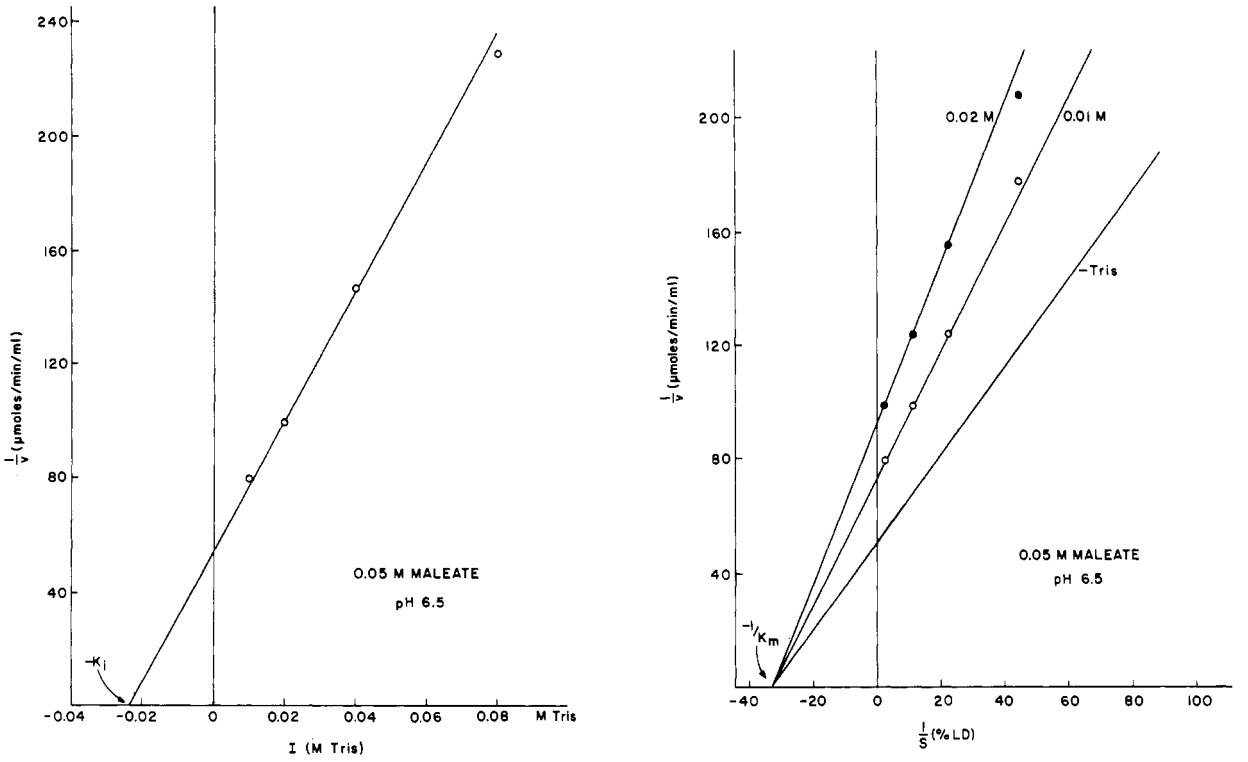


FIGURE 6: Action of the glucosidase–transferase on glycogen phosphorylase limit dextrin in the presence of Tris. (A, right) Double-reciprocal plot in 0.05 M maleate buffer (pH 6.5) containing 0.018 unit of enzyme/ml of incubation mixture. (B, left) Reciprocal of velocity *vs.* inhibitor concentration in 0.05 M maleate buffer (pH 6.5) containing 0.017 unit of enzyme/ml of incubation mixture. The standard static procedure was used as described in the text. Incubation mixtures were prepared for each concentration and 0.20-ml aliquots were removed at 2, 4, and 6 min. The velocities shown represent extrapolated initial velocities.

TABLE IV: Inhibition Constants.

Inhibitor	System	Method of Calculation	K_i (M)
Tris	0.05 M maleate, pH 6.5	Slope, $1/v$ *vs.* $1/S$ plot	0.022
Tris	0.05 M maleate, pH 6.5	Abscissa, $1/v$ *vs.* I plot	0.024
Tris	0.05 M maleate, pH 6.5	Absicca, slopes *vs.* I plot	0.024
Tris	0.05 M glycylglycine, pH 7.2	Slope, $1/v$ *vs.* $1/S$ plot	0.024
Diethanolamine	0.05 M maleate, pH 6.5	Slope, $1/v$ *vs.* $1/S$ plot	0.016
Ethanolamine	0.05 M maleate, pH 6.5	Slope, $1/v$ *vs.* $1/S$ plot	0.056
Ethylamine	0.05 M maleate, pH 6.5	Slope, $1/v$ *vs.* $1/S$ plot	0.424

activity was done by using glucosyl Schardinger dextrin (Taylor and Whelan, 1966) as a substrate on an equal weight basis to limit dextrin. The method of determination with the glucosyl Schardinger dextrin was essentially that described by Taylor and Whelan (1966) with the exception that 0.05 M maleate (pH 6.6) was employed as the buffer. The results are given in Table II. The increased rate of the glucosidase–transferase preparation on glucosyl Schardinger dextrin *vs.* that on phosphorylase limit dextrin may indicate that the transferase is rate limiting in the degradation of the limit dextrin (Table II).

Discussion and Conclusions

The highest level of purity attained with the present glucosidase–transferase preparation would appear to be about as high or perhaps as much as twice that of the best preparation previously described (Brown and Brown, 1966). The preparation is homogeneous in the ultracentrifuge and upon polyacrylamide gel electrophoresis. The preparation contains no detectable α-amylase and maltase activities (compare Table II with Brown *et al.*, 1963). Phosphorylase and UDPG glucose:α-1,4-glucan α-4-glucosyltransferase have been removed. The preparation is homogeneous with respect to the physical and biological criteria employed.

The fact that the preparation contains both glucosidase and transferase activities, which are independently demonstrable, is of interest. Brown and Brown (1966) have shown that the two activities are constant throughout purification, and it is possible that this is a "double-headed" enzyme as Cori (1964) has suggested. The present results do not contradict these conclusions although they do not preclude the possibility of two discrete enzymes very closely related in physical properties, or of an aggregate or complex of two proteins which upon separation lose activity.

The low activity observed on glycogen and on amylopectin (Table II) is less than what one would have expected from previous studies (Brown *et al.*, 1966). This suggests a high degree of specificity of the transferase for the 4 unit A chain of the phosphorylase limit dextrin structure.

The Michaelis constant for phosphorylase limit dextrin (0.03%) was found to be twice as high as previously reported: 0.014% (Larner and Schliselfeld, 1956) and 0.017% (Brown and Brown, 1966). In addition, it was the same in maleate buffer at pH 6.6 and glycylglycine buffer at 7.2. There is no clear explanation for this discrepancy. It is possible that the less purified preparations used previously contained enzymatic contaminants which also produced glucose from limit dextrin. Such activities have been found in muscle homogenates (Whelan, 1964b), and although they apparently do not significantly compete with the glucosidase–transferase under saturation substrate conditions, if their K_m's were lower they would do so at lower levels of limit dextrin. This would have the effect of lowering the apparent K_m of the glucosidase–transferase for limit dextrin.

The shift in the pH optimum of the enzyme preparation in the presence of anionic and cationic buffers explains many of the conflicting findings that have been reported. The optimum of 7.2–7.4 in glycylglycine reported by Larner and Schliselfeld (1956) does not conflict with that of 6.5 in phosphate–citrate reported by Taylor and Whelan (1966), nor that of 6.0 in citrate reported by Brown and Brown (1966). The slightly more acidic optimum reported by Hers *et al.* (1967) can not be interpreted on the basis of the present results since mixed buffer systems were used. The inhibition by Tris that has been noted (Brown and Brown, 1966; Hers, 1964; Brown, 1964) is substantiated by the present results. The reported inhibition by glycylglycine (Hers, 1964) is not apparent in these experiments.

It is of interest that maleate, cacodylate, and phosphate, although unrelated to structure, give the same optimum (through the range of pH 5–8 in the case of phosphate) at pH 6.6. The shift of the optimum in other buffers is probably due to an interaction of the enzyme with the particular buffer ion involved. Most of the cationic buffers tested (all of them substituted amines) probably interact with the enzyme as judged by the inhibition they cause.

The inhibition produced by the amine buffers was in all cases greatest in the range where the amine was charged (see Figure 5 and Table III). Therefore, the inhibition is related to the protonated form of the amine. In the case of Tris, for example, the inhibition did not diminish at pH 7.2 from that of 6.5. This is contrary to the case of intestinal maltases where the inhibition due to hydroxylalkylamines was found to be competitive and postulated as being due to the unprotonated species (Larner and Gillespie, 1956).

The present results indicate that the degree of in-

hibition produced by a particular amine is related to the degree of hydroxyalkyl substitution. The fact that this inhibition was of the simple linear noncompetitive type (Cleland, 1963) is of mechanistic interest.

Simple linear noncompetitive inhibition indicates that the inhibitor is acting as a dead-end type (Cleland, 1963), *i.e.*, it is, by combining with the enzyme, stabilizing or inducing a form which cannot bind to substrate. A possible mechanism for this type of inhibition is that the amine is binding at (or near) the active site by means of its hydroxyalkyl groups. This might allow the positively charged amino group to electrostatically interact, by virtue of its proximity, with a negatively charged group, and in so doing, to induce or stabilize an inactive conformation. It is of interest to note in this regard that glycerol phosphate does not inhibit or shift the optimum. Although it has hydroxyalkyl groups it is negatively charged. In addition choline, an hydroxyalkyl quaternary amine where the amino group is sterically hindered, is also not an effective inhibitor.

The shift in pH optimum caused by the cationic buffers cannot be explained by the inhibition caused by the hydroxyalkylamines. The shift appears to be independent of the inhibition since glycylglycine causes a shift but is not inhibitory, whereas imidazole is inhibitory yet causes no shift (see Figure 5).

References

Abdullah, M., Taylor, P. M., and Whelan, W. J. (1964), *in* Control of Glycogen Metabolism, Whelan, W. J., and Cameron, M. P., Ed., London, Churchill Ltd., p 123.

Abdullah, M., and Whelan, W. J. (1963), *Nature 197*, 979.

Albon, N., and Gros, D. (1952), *Analyst 77*, 410.

Brown, D. H. (1964), *in* Control of Glycogen Metabolism, Whelan, W. J., and Cameron, M. P., Ed., London, Churchill Ltd., p 170.

Brown, D. H., and Brown, B. I. (1966), *Methods Enzymol. 8*, 515.

Brown, D. H., Brown, B. I., and Cori, C. F. (1966), *Arch. Biochem. Biophys. 116*, 479.

Brown, D. H., and Illingworth, B. (1964), *in* Control of Glycogen Metabolism, Whelan, W. J., and Cameron, M. P., Ed., London, Churchill Ltd., p 139.

Brown, D. H., Illingworth, B., and Cori, C. F. (1963), *Nature 197*, 980.

Cleland, W. W. (1963), *Biochim. Biophys. Acta 67*, 104, 173, 188.

Cori, C. F. (1964), *in* Control of Glycogen Metabolism, Whelan, W. J., and Cameron, M. P., Ed., London, Churchill Ltd., p 169.

Cori, G. T., and Larner, J. (1951), *J. Biol. Chem. 188*, 17.

Di Jeso, F. (1968), *J. Biol. Chem. 243*, 2022.

Dixon, M., and Webb, E. C. (1964), Enzymes, 2nd ed, New York, N. Y., Academic, p 443.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem. 28*, 350.

Ferris, T. G., Easterling, R. E., and Budd, R. E. (1963), *Am. J. Clin. Pathol. 39*, 193.

Fischer, E. H., and Krebs, E. G. (1962), *Methods Enzymol. 5*, 369.

Hedrick, J. L., and Fischer, E. H. (1965), *Biochemistry 4*, 1337.

Hers, H. G. (1959), *Rev. Intern. Heptol. 9*, 35.

Hers, H. G. (1964), *in* Control of Glycogen Metabolism, Whelan, W. J., and Cameron, M. P., Ed., London, Churchill Ltd., p 170.

Hers, H. G., Verhue, W., and Mathiew, M. (1964), *in* Control of Glycogen Metabolism, Whelan, W. J., and Cameron, M. P., Ed., London, Churchill Ltd., p 151.

Hers, H. G., Verhue, W., and Van Hoof, P. (1967), *European J. Biochem. 2*, 257.

Illingworth, B., and Brown, D. H. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 1679.

Larner, J., and Gillespie, R. E. (1956), *J. Biol. Chem. 223*, 709.

Larner, J., Illingworth, B., Cori, G. T., and Cori, C. F. (1952), *J. Biol. Chem. 199*, 641.

Larner, J., and Schliselfeld, L. H. (1956), *Biochim. Biophys. Acta 20*, 53.

Miller, G. L. (1959), *Anal. Chem. 31*, 964.

Nelson, T. E., Kolb, E., and Larner, J. (1968), *Biochim. Biophys. Acta 151*, 212.

Raymond, S., and Nakamichi, M. (1964), *Anal. Biochem. 1*, 225.

Shaltiel, S., Hedrick, J. L., and Fischer, E. H. (1965), *Biochemistry 5*, 2108.

Slein, M. W. (1965), *in* Methods of Enzymatic Analysis, revised ed., Bergmeyer, H. U., Ed., New York, N. Y., Academic, p 117.

Svedberg, T., and Pederson, K. O. (1940), The Ultracentrifuge, London, Oxford University.

Taylor, P. M., and Whelan, W. J. (1966), *Arch. Biochem. Biophys. 113*, 500.

Taylor, P. M., and Whelan, W. J. (1968), *Proc. 4th Fed. European Biochem. Soc., Oslo, 5.*

Trevelyan, W. E., Proctor, D. P., and Harrison, J. S. (1950), *Nature 166*, 444.

Villar-Palasi, C., Rosell-Perez, M., Hizukuri, S., Huijing, F., and Larner, J. (1966), *Methods Enzymol. 8*, 374.

Walker, G. J., and Whelan, W. J. (1960), *Biochem. J. 76*, 264.

Whelan, W. J. (1964a), *Methods Carbohydrate Chem. 4*, 252.

Whelan, W. J. (1964b), *in* Control of Glycogen Metabolism, Whelan, W. J., and Cameron, M. P., Ed., London, Churchill Ltd., p 175.