Glucokinase from *Entamoeba histolytica* and Related Organisms*

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ABSTRACT: *Entamoeba histolytica* grown in a glucose-containing medium is relatively rich in soluble glucokinase. A method for the visualization of glucokinase activity following electrophoresis on cellulose acetate strips revealed that each of nine strains of typical *E. histolytica* yielded the same two electrophoretically distinct isoenzymes. Two strains of atypical *E. histolytica* displayed only a fast-migrating glucokinase which was also characteristic of *Entamoeba moshkovskii*. Two strains of *Entamoeba invadens* each gave a single glucokinase band migrating more slowly than the isoenzymes from *E. histolytica*. The electrophoretic behavior of glucokinase provides a means of characterizing typical *E. histolytica* and distinguishing it from closely related organisms which may not be human pathogens. Comparative kinetic studies were made on the two glucokinase isoenzymes from a typical *E. histolytica*. The kinetic properties were, in most respects, indistinguishable. Comparative kinetic studies were made on crude glucokinase from seven typical strains of *E. histolytica*. All had identical kinetic properties. These properties, determined at pH 7.0 in phosphate buffer, were: apparent K_m for glucose, 4×10^{-5} M; for adenosine triphosphate (ATP), 2×10^{-4} M. Apparent K_i's competitive with glucose: *N*-acetyl-D-glucosamine, 5×10^{-5} M; D-glucosamine, 2×10^{-4} M; and D-xylose, 2.4×10^{-3} M. Apparent K_i competitive with ATP: adenosine monophosphate (AMP), 7×10^{-6} M. Substrates were D-glucose, D-mannose, 2-deoxy-D-glucose, *N*-acetyl-D-glucosamine, and D-glucosamine, the last four being phosphorylated at 27–42% the rate of glucose in millimolar concentration. D-Fructose was not a substrate. ATP was the preferred phosphate donor; guanosine triphosphate (GTP), inosine triphosphate (ITP), and uridine triphosphate (UTP) were relatively ineffective. Adenosine diphosphate (ADP), guanosine monophosphate (GMP), and inosine monophosphate (IMP) were competitive inhibitors for ATP, but with K_i's at least fivefold greater than that of AMP. The strong inhibition of amebal glucokinase by AMP was confirmed with an enzyme preparation essentially free from adenylate kinase.

When grown in a glucose-containing culture medium the human parasite, *Entamoeba histolytica*, is particularly rich in glucokinase activity (ATP:[1] D-glucose 6-phosphotransferase, EC 2.7.1.2). Aqueous extracts of the cells frequently yield sufficient soluble enzyme to phosphorylate per minute glucose amounting to 4% of the dry weight of the cells. This high enzyme activity approaches that of the hexokinase content of dry yeast cells (Sols *et al.*, 1958). Bragg and Reeves (1962) had found a glucose-phosphorylating enzyme in the cells of an atypical ameba, but were undecided as to whether it was a glucokinase or a hexokinase since fructose was also phosphorylated by the same preparation. Apparently, glucokinase has not been reported previously from a typical strain of *E. histolytica*.

Experimental Procedures

The Organisms. Stock tube cultures of *E. histolytica* and related organisms are maintained at the Louisiana State University laboratory in a medium containing Trypticase, glucose, horse serum, thiomalate, penicillin, and the cells of a penicillin-sensitive anaerobic bacteria provisionally designated *Bacteroides symbiosus* (Reeves *et al.*, 1957). The strains of typical *E. histolytica* employed and the date and geographical location of the isolation of each are as follows: DKB strain, England, 1924; NRS strain, England (from a *Macacus sp.* monkey), 1929; 200 strain, Washington, D. C., 1947; K9, Korea, 1951; the F22, BH, JH, JS, and JI strains, New Orleans, 1947, 1955, 1956, 1959, and 1966, respectively. All except NRS were isolated from naturally infected, diseased humans. The two strains of atypical *E. histoly-*

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[1] Abbreviations used: TPN^+, oxidized triphosphopyridine nucleotide; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; NAD^+ and NADH, oxidized and reduced nicotinamide–adenine dinucleotides; $NAPD^+$ and NADPH, oxidized and reduced nicotinamide–adenine dinucleotide phosphates; GTP, ITP, and UTP, guanosine, inosine, and uridine triphosphates, respectively; IMP and GMP, inosine and guanosine monophosphates.

tica were those designated Laredo and Huff. They were isolated from human sources, but were not positively associated with disease. Their atypical character is reflected in their ability, unlike the typical strains, to multiply at room temperature. The two strains of *Entamoeba invadens* were those designated IP and PZ. This is the species parasitic in snakes. Two cultures of *Entamoeba moshkovskii*, the sewerage ameba, were also employed in this investigation.

Inoculae taken from tube cultures were used to seed petri dish cultures. These were grown in an anaerobic incubator, harvested, and lyophilized under the conditions described by Reeves and Ward (1965). The typical *E. histolytica* and the Laredo organisms were grown at 36°, the others at 28–32°. The trophozoites were free from cysts. The lyophilized powders were sealed in glass under vacuum and stored at −20° until used.

Materials. The auxillary enzymes employed in the spectrophotometric test systems were Boehringer products. The cellulose acetate strips and sheets were obtained from the Gelman Co. under the designation Sepraphore III. The nucleotides were obtained from commercial sources and their solutions were neutralized and assayed enzymatically or spectrophotometrically by standard procedures prior to use. The collodion bags were products of Membranfiltergesellschaft, Gottingen, and were obtained from Schleicher & Schuell Co. Fructose 6-phosphate containing only 1% glucose 6-phosphate was obtained from Sigma. The EDTA solution employed had been adjusted to pH 7.0 with sodium hydroxide.

Amebal Extracts. Lyophilized powders, representing known volumes of packed cells, were weighed, suspended in four cell volumes of cold water or 1 mM EDTA, and gently agitated for 30 min. All manipulations were conducted at 0–4°, unless otherwise noted. The broken-cell suspensions were centrifuged for 10 min at 30,000*g* and the supernatant fluids were withdrawn and reserved. The residues were washed by centrifugation with an additional cell volume of fresh fluid and the supernatants were combined to yield the amebal extracts. These were used for the preparation of crude enzyme and, after dialysis, for electrophoretic studies. Equivalent yields of glucokinase activity were obtained following mild sonic disintegration of fresh trophozoites, but a greater proportion of the amebal protein was solubilized by this procedure.

Crude Enzyme. Streptomycin sulfate was added to amebal extract in the proportion of 0.03 ml of a 20% solution/ml of extract. After standing for 15 min the precipitate was removed by centrifugation and discarded. To the supernatant solution was added an equal volume of cold, saturated ammonium sulfate solution which had been adjusted to pH 7.4. The precipitate which separated upon standing for 30 min was removed by centrifugation. Solid ammonium sulfate (214 mg/ml) was added with stirring to the supernatant solution. After standing for 1 hr the precipitate was collected by centrifugation, redissolved in 1 mM EDTA, and dialyzed twice against 100 volumes of the EDTA solution. Further purification steps are described in the Results section.

Electrophoretic Methods. The buffer for electrophoreses contained 4.88 g of sodium barbital and 3.24 g of sodium acetate/l. It was adjusted to pH 8.0 with hydrochloric acid. The cellulose acetate strips were presoaked in the buffer, placed in the electrophoresis chamber, and the enzyme solution was streaked across the strips. The full width required approximately 0.01 ml of solution. The electrophoreses were regularly run for 1 hr at room temperature with a voltage drop of about 12 v/cm along the strip.

After electrophoresis the strips were placed in the developing solution to locate the glucokinase activity. This solution contained 50 mM Tris-HCl (pH 8), 0.3 mM TPN^+, 5 mM $MgSO_4$, 5 mM ATP, 6 mM glucose, 0.4 mg/ml of *p*-nitroblue tetrazolium, 25 μg/ml of glucose 6-phosphate dehydrogenase, and 20 μg/ml of phenazine methosulfate. This solution is similar to one used by Katzen and Schimke (1965) in their studies on liver enzymes. When the applied sample contained 0.4 unit/ml or more of glucokinase a visible indication of enzyme activity usually appeared within 5–15 min of incubation at room temperature. No visable bands appeared when glucose or ATP was omitted from the developing solution.

Assay Methods. Glucokinase activity was assayed spectrophotometrically by measuring either the rate of formation of, or disappearance of, reduced pyridine nucleotide. (1) The standard glucose 6-phosphate formation assay employed 50 mM buffer, 0.6 EU/ml of glucose 6-phosphate dehydrogenase, 0.3 mM $NADP^+$, 4 mM $MgSO_4$, 2 mM ATP, and 4 mM D-glucose. The rate of increase in optical density at 340 mμ was taken as proportional to the rate of glucose 6-phosphate formation. No corrections were applied to these values. Buffers used included Tris-HCl, potassium phosphate, and imidazole-HCl at the pH stated in the text. (2) The standard ADP formation assay employed 50 mM potassium phosphate buffer (pH 7.0), 4 mM $MgSO_4$, 2 mM ATP, 4 mM D-glucose, 0.1–0.2 mM NADH, 1 mM phosphoenolpyruvate, 0.75 EU/ml of pyruvate kinase, and 1 EU/ml of lactate dehydrogenase. The rate of decrease in optical density at 340 mμ was taken as proportional to the rate of ADP formation. Small no-substrate corrections to account for ATPase and NADH oxidase activities were applied to the ADP formation assays. In both test systems glucokinase was employed at levels in which the system response was linear with respect to enzyme concentration. The glucokinase concentrations were usually in the range 0.004–0.01-unit/ml final volume. Much of the kinetic data was obtained using a Beckman DU spectrophotometer equipped with the Gilford 200 attachment for absorbancy recording. Unless otherwise noted the determinations were made at 25°. The molar absorbancy of NADPH at 340 mμ was taken to be 6.27×10^3, that of NADH, 6.22×10^3.

The glucokinase unit is defined as the amount of enzyme causing the formation of 1 μmole/min of glucose 6-phosphate at 25° in the Tris-HCl buffer (pH 8.0) in the standard glucose 6-phosphate formation assay described

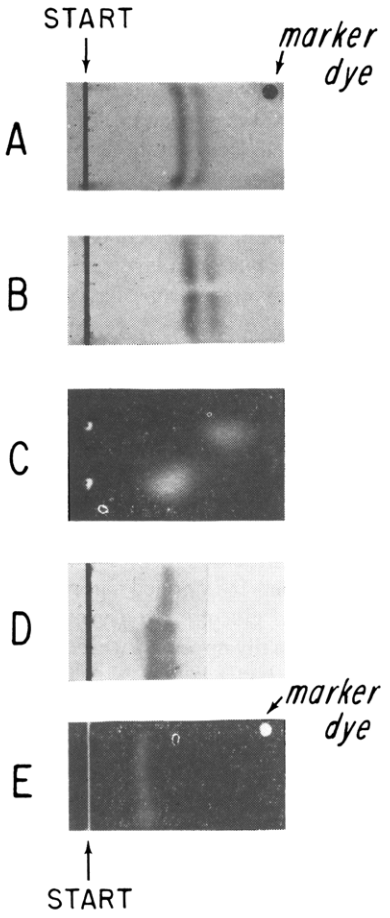

FIGURE 1: Comparative behavior of ambebal glucokinases following cellulose acetate strip electrophoresis. The conditions were those described under Experimental Procedures. (A) The two isoenzymes from the DKB strain, with a spot indicating the position of a marker dye. (B) Comparative electrophoresis of glucokinase from the DKB strain (upper) and the 200 strain (lower). (C) The resolved fast- and slow-migrating isoenzymes from the DKB strain. (D) The glucokinase from the Laredo strain (upper) compared with that from the DKB strain (lower). (E) The glucokinase from the PZ strain of *E. invadens*, with a spot indicating the position of the marker dye allowing comparison with the DKB strain in A. Prints A, B, and D were made from black and white negatives while prints C and E from color film positives.

above. Specific activity is expressed as units of glucokinase per milligram of protein.

The glucokinase preparations were assayed for other enzymes which might influence the interpretation of the results. ATPase was assayed by the standard ADP formation system minus glucose; adenylate kinase, the same system plus 0.5–2.0 mM AMP, correcting for ATPase activity; NADH oxidase and NADPH-oxidase were assayed in the phosphate buffer (pH 7.0) using 0.1 mM concentrations of the reduced nucleotides; glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were assayed in the phosphate buffer with 0.3 mM $NADP^+$ or NAD^+ in the presence of 1 mM substrate. The assay for glucose phosphate isomerase employed the phosphate buffer, 0.3 mM $NADP^+$, 0.6 EU/ml of glucose 6-phosphate dehydrogenase, and 2.5 mM fructose 6-phosphate (amebal enzyme added last). Phosphoglucomutase was assayed by the same system with 1 mM glucose 1-phosphate replacing the fructose 6-phosphate and with 0.05 mM glucose 1,6-diphosphate.

Except where otherwise noted apparent K_m's were calculated from Lineweaver–Burk plots at four appropriate substrate concentrations. Apparent inhibition constants (K_i's) are reported only in instances where the inhibition was shown to be competitive against the named substrate. Protein assays were made by the method of Lowry *et al.* (1951) using a crystalline bovine serum albumin standard.

Results

Electrophoretic Studies. All nine investigated strains of typical *E. histolytica* showed two glucokinase bands migrating toward the anode. These were observed when amebal extracts were prepared as rapidly as possible from either fresh or lyophilized cells. We saw no evidence of the formation of one isoenzyme at the expense of the other. A typical electrophoretic finding is illustrated in Figure 1A. In most instances the slower band was visibly darker. To compare the relative electrophoretic mobilities of glucokinases from various strains, without strip-to-strip variations, one half of a strip was streaked with glucokinase from the DKB strain as a reference and the other one-half was streaked with enzyme from a second strain. Using this technique it was found that the mobilities of the two enzymes from each of the typical strains were, respectively, equal to those of the reference DKB strain (Figure 1B). Two bands were also observed for these enzymes following starch gel electrophoresis, but the cellulose acetate strip technique was preferred because of its smaller sample size, speed, and convenience.

Electrophoresis of extracts from the two atypical strains, Laredo and Huff, and from *E. moshkovskii* each gave a single glucokinase band migrating close to or exactly with the faster of the two bands of the DKB reference. A comparison between the Laredo and DKB glucokinases is shown in Figure 1D. The Laredo band retained the same relative position when mixed with the isolated slow-migrating isoenzyme from the DKB strain. Figure 1E illustrates the slow-migrating band which was obtained from both cultures of *E. invadens*. When the DKB and *E. invadens* extracts were mixed and applied to the starting line three glucokinase bands were obtained.

Properties of E. histolytica Glucokinase. The extracts from the lyophilized powders of typical *E. histolytica* regularly contained 5–7 units of glucokinase activity and 7–9 mg of protein/ml. The glucokinase activity was not sedimented by centrifugation of an amebal extract for 2 hr at 105,000*g*.

The crude enzyme solutions contained 60–80% of the original glucokinase at specific activities ranging from 1 to 2. They were relatively stable at pH 7, losing only 10–20% of their activity/week when stored at 4° in 1 mM EDTA. A similar loss was sustained upon freezing and thawing. AT 52° the loss was pronounced after 10 min, but in the presence of 4 mM glucose the glucokinase was stable for 10 min at 56°. MgATP accelerated loss of activity at elevated temperatures. Both isoenzymes appeared to have similar stabilities as indicated by strip electrophoreses following aging or heating of the enzyme solutions. Both withstood dialysis against water or 1 mM EDTA for 1–2 days.

Phosphoryl Donors. Using amebal extract from the DKB strain potential phosphoryl donors were tested in phosphate buffer (pH 7.0) in the presence of 1 mM $MgCl_2$. At 1 mM concentration GTP, ITP, and UTP were, respectively, 15, 5, and 4% as effective as was ATP. The last two activities might well be due to adenine nucleotide impurities.

Divalent Cation Requirement. The cation requirements were tested with the standard glucose 6-phosphate formation assay in phosphate buffer (pH 7.0) modified by the substitution of other divalent metal salts for the magnesium sulfate. The enzyme solutions were amebal extracts from the DKB and Laredo strains which had been dialyzed overnight against two changes of 1 mM EDTA (pH 7). No activity was observed with either enzyme in the presence of calcium chloride, zinc sulfate, cupric sulfate, or nickelous nitrate. With the DKB enzyme 4 mM manganous chloride and cobaltous chloride gave 34 and 37%, respectively, the activity of magnesium sulfate. With the Laredo enzyme the corresponding values were 40 and 44%.

pH Optimum. In phosphate buffer the crude enzyme from the DKB strain had a broad optimum from pH 7.0 to 8.5. In imidazole, Tris, or glycylglycine buffer the activity decreased sharply below pH 8. The activity was irreversibly lost upon storage of the enzyme below pH 5.

Purification of E. histolytica Glucokinase. The crude enzyme solutions still contained three potentially interfering enzyme activities in significant quantity. These were adenylate kinase, glucose phosphate isomerase, and phosphoglucomutase. ATPase activity amounted to only 1–2%, and NADH oxidase, 0.5–1.0% of the glucokinase activity. Glucose dehydrogenase, glucose 6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase were not found under conditions which should have detected their activity if equivalent to 0.3% of that of the glucokinase. These activities were tested for both NAD^+ and $NADP^+$.

Neither phosphoglucomutase nor glucosephosphate isomerase activities interfered with the kinetic studies; however, the latter could be separated from the glucokinase by passage of crude enzyme through a DEAE-Sephadex column. The former activity was not significant in the absence of added glucose diphosphate. Glucokinase essentially free from adenylate kinase and ATPase was prepared in the following manner. An undialyzed crude enzyme (1 ml) from the DKB strain containing 12 units of activity (sp act. 0.7) was placed on a Sephadex G-200 column (3 cm^2 × 14 cm) previously equilibrated with 0.02 M potassium phosphate (pH 6.4). The glucokinase was eluted with the 0.02 M phosphate buffer. The activity followed a leading protein peak and was quantitively recovered. It was eluted as a single peak with no evidence of resolution of the isoenzymes. The activities of the peak fractions were in the range 2–3 units/mg of protein. A pooled fraction from the Sephadex column containing 5.6 units of glucokinase and an equivalent amount of adenylate kinase activity was allowed to concentrate to dryness by vacuum dialysis in a collodion bag membrane filter immersed in the phosphate buffer. Redissolving the contents of the filter in 0.6 ml of the buffer yielded the glucokinase activity contaminated by less than 0.2% of the adenylate kinase activity.[2] The ATPase activity of this solution was 0.1% of the glucokinase activity. After centrifuging from insoluble protein the specific activity of this glucokinase was 4.2. There was no evident resolution of the isoenzymes. This preparation was employed in AMP and ADP inhibition studies.

The highest specific activity for the amebal glucokinase was achieved by adsorption on calcium phosphate gel followed by elution. A diluted amebal extract (10 ml) (DKB strain) containing 25 units of glucokinase and 30 mg of protein was treated with 1 ml of a calcium phosphate gel suspension containing 14.5 mg of solids/ml. After standing for 10 min the suspension was centrifuged at 10,000*g*. The supernatant solution was withdrawn, assayed for protein and enzyme, and the treatment was repeated. Most of the enzyme was contained on the third and fourth adsorbates. These were washed with water and extracted with 0.8 M ammonium sulfate solution (pH 7.4). The extracts were combined, dialyzed, and concentrated by vacuum dialysis. This preparation contained 11.6 units of glucokinase of sp act. 9. Upon repetition of the adsorption cycle, with 0.5-ml portions of gel suspension, most of the enzyme was contained on the first adsorbate. Elution and dialysis gave 6.4 units of enzyme of sp act. 10. It contained both fast- and slow-migrating isoenzymes and was less stable on storage, or freezing and thawing, than was the crude enzyme.

Resolution of the Two Isoenzymes from the DKB Strain. Crude glucokinase (0.25 ml containing 4.4 units of enzyme, sp act. 2) was heavily streaked along 10.5 in. of an 11 × 6.5 in. sheet of the Sepraphore III paper. Electrophoresis was conducted at room temperature for 1.5 hr at a current of 12 ma. Guide strips were cut and developed to locate the enzyme bands. Two bands were evident, but they were not widely separated. Lateral strips were cut as follows: (1) beyond and bisecting the fast band, (2) bisecting the fast band and the area between the bands, (3) bisecting the area between the bands and the slow band, and (4) bisecting the slow band and extending toward the starting line. Each strip

[2] The success of this step was apparently due to the inactivation of adenylate kinase at the membrane surface. It was essential to employ glucokinase of specific activity of 2 or greater in order to secure the essentially complete inactivation of the contaminating adenylate kinase.

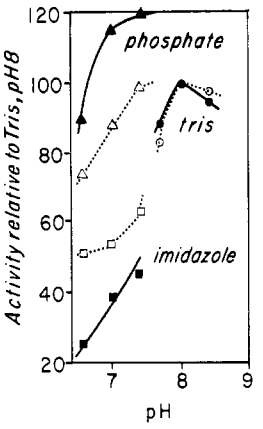

FIGURE 2: The influence of pH and buffer upon the activity of electrophoretically separated isoenzymes. The enzyme preparations are described in the text under Results. The broken-line curves represent the fast-migrating fraction 2, the solid lines, the slow-migrating fraction 4. The standard glucose 6-phosphate formation assay was employed and results are related to Tris-HCl (pH 8) which is taken as equal to 100 for each isoenzyme.

was extracted with 0.8 M ammonium sulfate (pH 7.4) to yield fractions 1–4, respectively. The total recovery of enzyme activity was 64%: fraction 1, 0.11 unit, sp act. 0.5; fraction 2, 0.5 unit, sp act. 1.8; fraction 3, 1.7 units, sp act. 4.7; and fraction 4, 0.5 unit, sp act. 0.7.

Portions of each fraction were concentrated by vacuum dialysis and again subjected to electrophoresis. Fraction 1 showed only the fast-migrating enzyme band. Fraction 2 was principally the fast band, but was contaminated by a barely detectable trace of the slower component. Fraction 3 was a mixture of both isoenzymes, and fraction 4 showed only the slow-moving component. The resolved slow- and fast-migrating enzymes retained their original mobilities with respect to each other, as is illustrated in Figure 1C. This was the case when they were subjected to electrophoresis separately or when they were mixed prior to electrophoresis.

It was noted that the activity of the crude enzyme diminished sharply below pH 8 in buffers other than phosphate. Studies on the effects of pH and buffer on the resolved slow- and fast-migrating enzymes, illustrated in Figure 2, show pronounced stimulation by phosphate in the region pH 6.5–7.4, and that this effect was considerably greater for the slow-migrating isoenzyme.

Kinetic Studies on the Resolved Isoenzymes. The remaining portions of fractions 1, 2, and 4 were dialyzed overnight against two changes of mM EDTA and employed for the determination of kinetic properties. These studies were made in phosphate buffer at pH 7.0. Some of the results obtained with these fractions are listed in Table I. Inspection of the table reveals that most of the kinetic properties of the fast- and slow-migrating isoenzymes are identical, within the limitations of the experimental methods. Each enzyme had the same five

TABLE I: Kinetic Properties of Glucokinase Isoenzymes from the DKB Strain of *E. histolytica* (phosphate buffer, pH 7.0).

Substrates	Fast-Migrating Isoenzyme (fraction 2) K_m(mM)	V_{max}	Slow-Migrating Isoenzyme (fraction 4) K_m (mM)	V_{max}
D-Glucose[a]	0.02–0.03[c]	(100)[b]	0.04	(100)[b]
D-Glucosamine[b]	0.10	45	0.08	46
N-Acetyl-D-glucosamine[b]	0.06	40	0.04	40
D-Mannose[b]	0.12	44	0.45	70
2-Deoxy-D-glucose[b]	0.10	59	0.33	70
ATP[a]	0.20	—	0.20	—

Inhibitors[a]	K_i *vs.* Glucose (mM)	
N-Acetyl-D-glucosamine	0.03	0.03
D-Glucosamine	0.1	0.1
2-Deoxy-D-glucose	0.9	1.0
D-Mannose	0.7	0.8
D-Mannosamine	1.0	1.8
D-Xylor	1.0	1.4
D-Mannoheptulose	1.8	4.0
	K_i *vs.* ATP (mM)	
5′-AMP	0.008	0.005

[a] Determined by the standard glucose 6-phosphate formation assay (see Experimental Procedure) modified relative to substrate concentrations, and for the K_i's by the inclusion of inhibitors. [b] Determined by the standard ADP formation assay (see Experimental Procedure) modified relative to substrate and its concentration. These values were derived from data at 1 and 0.1 mM substrate concentration. [c] The higher value was obtained using the fraction 1 fast-migrating enzyme.

effective substrates; however, glucose was definitely the best substrate for both isoenzymes. The four alternate substrates were competitive inhibitors of glucose phosphorylation, as were the nonsubstrates D-mannosamine, D-xylose, and D-mannoheptulose.

Other Kinetic Studies. The gel-purified enzyme was employed in kinetic studies at pH 8.2. Its K_m for glucose was determined by three different methods: in Tris-HCl buffer by the glucose 6-phosphate formation method (illustrated by Figure 3), in the same buffer by the ADP formation method, and in an unbuffered solution using the Sargent pH-Stat. All three methods gave the same apparent K_m value, 0.04 mM. The competitive inhibition of glucose phosphorylation by *N*-acetyl-D-glucosamine is also illustrated in Figure 3.

Other results obtained with the gel-purified enzyme

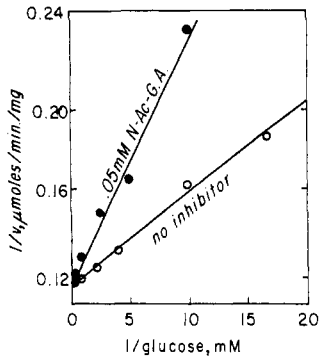

FIGURE 3: A reciprocal plot of velocity *vs.* glucose concentration in the presence and absence of *N*-acetyl-D-glucosamine (N-Ac-G.A.). Data were determined by the standard glucose 6-phosphate formation assay modified as regards substrate concentration, inhibitor, and buffer (30 mM Tris-HCl, pH 8.2). Each cuvet contained 1.35 μg of the gel-purified enzyme protein in a final volume of 1 ml. The reaction was initiated by the addition of glucose.

in Tris-HCl buffer (pH 8.2) were as follows: K_m for ATP, 0.2 mM; for D-mannose, 0.9; for *N*-acetyl-D-glucosamine, 0.04 mM; K_i for acetylglucosamine *vs.* glucose, 0.04 mM; for AMP *vs.* ATP, 0.002 mM; and for ADP *vs.* ATP, 0.02 mM.

The adenylate kinase free enzyme was used for studies on the inhibition of ATP by ADP and AMP. The findings with ADP are illustrated in Figure 4, with AMP in Figure 5. From the data of these figures the apparent K_m for ATP was calculated to be 0.29 mM; the apparent

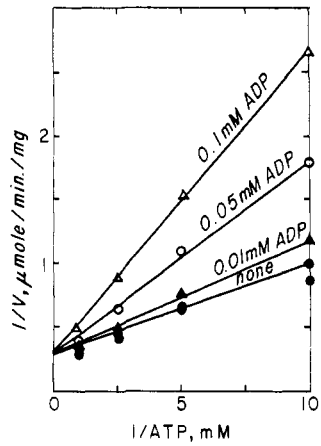

FIGURE 4: A reciprocal plot of velocity *vs.* ATP concentration in the presence and absence of ADP. The standard glucose 6-phosphate formation assay with phosphate buffer (pH 7) was modified as regards ATP and inhibitor (ADP) concentration. Reaction was initiated by the addition of 0.9 μg of the adenylate kinase free enzyme protein; final volume, 0.4 ml.

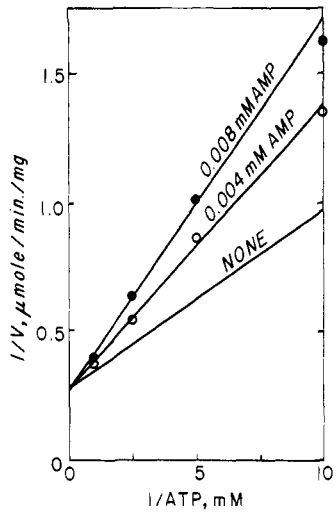

FIGURE 5: A reciprocal plot of velocity *vs.* ATP concentration in the presence and absence of AMP. The standard glucose 6-phosphate formation assay with phosphate buffer (pH 7) was modified as regards ATP and inhibitor (AMP) concentration. Reaction was initiated by the addition of 0.9 μg of the adenylate kinase free enzyme protein; final volume, 0.4 ml. The data for the lower curve are indicated in Figure 4.

K_i for AMP *vs.* ATP, 0.008 mM; and the apparent K_i for ADP *vs.* ATP, 0.045 mM. These K_i's were somewhat greater when tested in 1 mM $MgSO_4$ instead of the 4 mM salt employed in the reported experiments.

Using similar experimental conditions, but with a DKB amebal extract, IMP and GMP were found to be inhibitors competitive against ATP. The K_i's were 0.6–0.2 mM for IMP and 0.09–0.05 mM for GMP. Since the amebal adenylate kinase is selective for the adenine nucleotides (R. E. Reeves, 1966, unpublished data) these results should not be unduly influenced by the adenylate kinase contaminant of this enzyme.

Comparative Kinetic Properties of the Crude Enzymes from Typical and Atypical E. histolytica. For comparative studies extracts were made from lyophilized cells representing from 0.12 to 1.0 ml of packed cell volumes of the 200, DKB, K9, F22, JH, JS, and NRS strains of typical *E. histolytica* and the atypical Laredo strain. The crude enzymes were prepared as described under Experimental Procedure. The amount of enzyme obtained in the amebal extracts ranged between 9 and 27 units/ml of packed cells. Units of enzyme per gram of dry cells ranged from 68 to 230. Each crude enzyme preparation from the typical strains had a specific activity between 1 and 1.6; that for the Laredo strain was 0.27.

Each of the crude glucokinases was tested against ten possible substrates, and apparent K_m's for glucose and ATP were determined. Each was tested against four competitive inhibitors, three *vs.* glucose and one *vs.* ATP. Since no significant differences were detected among the typical strains the results are summarized in Table II as averages, the range of values being noted

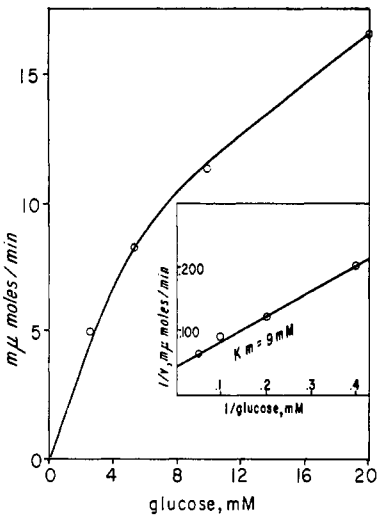

FIGURE 6: The influence of glucose concentration on the reaction rate of the bacterial glucokinase. Each cuvet contained in a final volume of 1 ml: 50 mM Tris-HCl (pH 8), 0.6 EU of glucose 6-phosphate dehydrogenase, 0.3 mM $NADP^+$, 4 mM $MgCl_2$, 2 mM ATP, 0.05 mM NADPH, and the indicated concentration of glucose. Reaction was initiated by the addition of 31 μg of bacterial enzyme protein. The cuvets were read in a double-beam spectrophotometer *vs.* a no-substrate control (see text for additional conditions). The insert shows a reciprocal plot of the same data.

parenthetically. The results for the Laredo strain are listed separately in the table. They differ only with respect to the greater K_i for D-glucosamine.

In addition to the three inhibitors competitive with glucose listed in Table II other substances were tested against glucose (glucose 6-phosphate formation assay) employing the enzyme from the DKB strain. D-Mannose, D-mannosamine, D-mannoheptulose, and 2-deoxy-D-glucose were competitive inhibitors. D-Arabinose, L-arabinose, L-sorbose 1-phosphate, D-galactose, and D-ribose did not show appreciable inhibition (K_i > 100 mM). Glucose 6-phosphate was a weak inhibitor of glucose phosphorylation and was not competitive against glucose. Other experiments with the crude DKB enzyme were carried out in Tris-HCl buffer at pH 8.0 and the results were in agreement with those reported for the gel-purified enzyme in the same buffer at pH 8.2.

The Bacterial Glucokinase. The bacteria upon which the amebae feed in culture have about 7 units of soluble glucokinase/g of moist cells. Frozen bacterial cells (0.86 g) were suspended in 8 ml of a solution containing 0.03 M NaF and 1 mM EDTA. The cells were broken for 1.5 min with the aid of a Branson sonifier. After centrifugation the supernatant solution was streaked across one-half the width of a cellulose acetate strip and a DKB amebal extract was streaked across the other half. Following the usual electrophoresis and development the bacterial glucokinase appeared to be a single band migrating slower than the *E. histolytica* isoenzymes.

TABLE II: Average Properties of Crude Glucokinases from Seven Strains of Typical *E. histolytica* and the Laredo Strain.

Property	Seven Typical *E. histolytica* Average	(range)	Laredo Strain
K_m for glucose (mM)	0.04	(0.034–0.05)	0.04
K_m for ATP (mM)	0.20	(0.18–0.25)	0.2
Substrate Activity[a] (mM) (*vs.* glucose = 100)			
2-Deoxy-D-glucose	42	(32–52)	63
D-Mannose	32	(25–43)	48
D-Glucosamine	32	(25–47)	35
N-Acetyl-D-glucosamine	27	(22–34)	27
Apparent Inhibition Constants (mM) (*vs.* glucose)			
N-Acetyl-D-glucosamine	0.05	(0.04–0.07)	0.04
D-Glucosamine	0.2	(0.09–0.27)	1.3
D-Xylose	2.4	(1.3–2.9)	4.3
	(*vs.* ATP)		
5′-AMP	0.007	(0.003–0.01)	0.002

[a] Substrate tests with D-xylose, D-mannoheptulose, D-fructose, and D-mannosamine were all less than 1% that of glucose at millimolar concentration, save for one instance with D-fructose (NRS strain, 3%). Tests with D-galactose ranged from 1 to 3.5% and were probably attributable to glucose impurity. Other substances tested only on the DKB strain and having less than 1% the substrate activity of glucose were sorbitol, D-xylose, D-arabinose, L-arabinose, D-ribose, and D-mannitol.

Kinetic studies on the bacterial enzyme were made on a similar preparation, except in this extraction the NaF was replaced by 0.1 M KCl. Owing to the high activity of NADPH oxidase in the bacterial extract the standard glucose 6-phosphate formation assay was modified by the addition of 0.05 mM NADPH to the no-substrate control and to the substrate-containing samples. The latter were read *vs.* the control in a double-beam spectrophotometer (Beckman DB) during the interval in which the decay of absorbance in the control cuvet had previously been determined to be linear with respect to time. The initial rates of reaction at various glucose concentrations are illustrated in Figure 6, the insert being a reciprocal plot of the same data. From this data the apparent K_m for glucose was calculated to be 9 mM for the bacterial enzyme. In a similar experiment its apparent K_m for ATP was found to be 0.75 mM.

Discussion

All of the investigated strains of typical *E. histolytica* appear to contain the same two electrophoretically dis-

tinguishable glucokinase isoenzymes. The possibility that one enzyme is of bacterial origin seems remote, not only because the amebal cells were thoroughly washed from accompanying bacteria prior to enzyme extraction, but also because of the widely different properties found for the soluble bacterial glucokinase. The extraction of enzyme may be made so rapidly and simply, owing to the fragility of the amebae, that we are inclined to believe that a second form does not appear as a result of degradation, and the resolved enzymes do not spontaneously interconvert.

The kinetic properties of the two typical *E. histolytica* isoenzymes are so similar that the crude enzyme may be adequately characterized although it contains a mixture of the two isoenzymes. It can be distinguished from other well-characterized glucokinases. Its glucose K_m is two orders of magnitude smaller than that reported by Walker (1963) for liver glucokinase. Unlike the glucokinase of *Aerobacter aerogenes* reported by Kamel *et al.* (1966) the amebal enzyme does not require the glucose configuration at carbon 2 and its inhibition by ADP is an order of magnitude greater than that of this bacterial enzyme. Unlike the glucokinase from *Brevibacterium fuscum* reported by Saito (1965) the amebal enzyme phosphorylates D-mannose. Its K_m's for glucose and ATP are considerably lower and its inhibition by ADP is two orders of magnitude greater than was found for the *B. fuscum* enzyme. Unlike the glucokinase from *E. coli* reported by Asensio (1960) the amebal enzyme phosphorylates *N*-acetyl-D-glucosamine and its glucose phosphorylation is strongly inhibited by this substance.

The spectrum of substrates of amebal glucokinase resembles that of liver glucokinase, as reported by Walker (1966). Its failure to phosphorylate fructose distinguishes the amebal enzyme from the hexokinases of yeast, protozoa, and metazoa. The presumption that the five substrates for the amebal enzyme are all acted upon by the glucokinase is reinforced by the similar behavior observed for the crude and partially purified enzymes, and by the behavior of the two resolved isoenzymes. It is also significant that the four alternate substrates appear as competitive inhibitors of the phosphorylation of glucose, with inhibition constants not greatly different from their substrate K_m's.

Of particular interest are the low apparent inhibition constants of AMP and ADP found for the amebal glucokinase. Product inhibition by ADP had been noted for glucokinases or hexokinases by Asensio (1960), Kamel *et al.* (1966), Saito (1965), Salas *et al.* (1965), Grossbard and Schimke (1966), and Seed and Baquero (1965), but only the first three investigations reported strictly competitive kinetics *vs.* ATP. In the case of the amebal enzyme the inhibition by ADP was competitive and greater than that reported for the other enzymes. The inhibition by AMP was almost an order of magnitude greater than that of ADP, and the K_i of AMP was much smaller than the K_m of ATP as substrate. The inhibitory effect of AMP seems not to have been investigated for other glucokinases free from adenylate kinase. Zewe *et al.* (1964) studied AMP inhibition of yeast hexokinase and Hanson and Fromm (1967), of rat skeletal muscle hexokinase II. AMP was much less inhibitory to the hexokinases than was found for the amebal glucokinase.

There was a striking uniformity in the electrophoretic and kinetic properties of glucokinase from typical strains of *E. histolytica* of diverse geographical origin, from England to Korea, and having dates of isolation ranging from 1924 to 1966. All of these strains, save NRS, were originally from naturally infected, diseased human hosts. The two atypical strains, although isolated from human hosts, have not been conclusively linked to pathogenicity in man (Richards *et al.*, 1966). These two organisms, plus *E. moshkovskii*, appear to contain only the faster migrating of the two isoenzymes characteristic of the typical strains. The glucokinase from *E. invadens* was qualitatively different from the other amebal enzymes studied.

Since no component of parasitic amebae had previously been well characterized by both qualitative and quantitative parameters there have been no firm biochemical markers which might serve to characterize the potentially pathogenic amebae and to distinguish them from nonpathogenic strains and closely related species. It appears that the detailed characterization of amebal enzymes, such as glucokinase, may offer the possibility of making such distinctions.

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Further Studies on 20α-Hydroxysteroid Dehydrogenase of Rat Testes*

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ABSTRACT: A 20α-hydroxysteroid dehydrogenase has been partially purified from rat testicular homogenates. This enzyme catalyzes the reduction of 17α-hydroxyprogesterone to 17α,20α-dihydroxypregn-4-en-3-one. The equilibrium of this reaction strongly favors the dihydroxy compound. Sulfhydryl compounds inhibit the enzyme while sodium ethylenediaminetetraacetate (EDTA-Na_2) stimulates it. The optimum temperature for the reduction reaction is between 30 and 42° with no sharp maximum, while the optimum pH for this reaction is about 7. The Michaelis constants were 6.3–6.7 $\times$ 10^{-5} M for 17α-hydroxyprogesterone and 2.0–2.5 $\times$ 10^{-5} M for reduced nicotinamide–adenine dinucleotide phosphate.

Soluble fractions of testicular tissue from several species of mammals contain a 20α-hydroxysteroid dehydrogenase which specifically catalyzes the reduction of the 20-carbonyl group of 17α-hydroxyprogesterone[1] (Shikita and Tamaoki, 1965). The substrate specificity of this testicular enzyme differs from that of the 20α-hydroxysteroid dehydrogenases present in hepatic (Recknagel, 1957), ovarian (Wiest, 1959), placental (Purdy *et al.*, 1964), and adrenal (Matthijssen *et al.*, 1964) tissues, and it is this unique specificity which prompted the present study.

Experimental Section

Tissue Preparation. Rats of the Wistar strain (age 2–3 months) were killed by a blow on the head. The testes were removed shortly thereafter, decapsulated, and homogenized in twice their weight of ice-cold 0.25 M sucrose. The homogenates were centrifuged by a conventional differential centrifugation method described elsewhere (Shikita and Tamaoki, 1965). The 105,000*g* supernatant solution was frozen and stored at −20° until sufficient material had been accumulated for the enzyme purification.

Assay of the Enzyme. 17α-Hydroxy[4−^{14}C]progesterone (10 μg, 30–50 $\times$ 10^3 cpm) in 1 ml of methylene dichloride was added to a 50-ml round-bottom centrifuge tube. Propylene glycol (2 drops) was added to this solution, and the methylene dichloride was removed first by a nitrogen jet at 40° and then under vacuum for 10 min at room temperature. Unless otherwise specified, 4 ml of 0.1 M Tris-Cl buffer (pH 7.4) containing 0.5 μmole of NADPH was added to each tube. Incubations were initiated by the addition of 1 ml of enzyme solution, and were preformed under aerobic conditions at 37° for 30 min with continuous shaking. The enzymatic reaction was stopped by the addition of 20 ml of methylene dichloride. After the organic phase was removed, the incubation mixture was extracted with two 20-ml portions of methylene dichloride. The 17α,20α-dihydroxy compound obtained by evaporating the combined extracts was purified by thin layer chromatography (silica gel G and GF) in a benzene–acetone (4:1, v/v) solvent system and detected by ultraviolet light. It was then extracted from silica gel with a mixture of ethyl alcohol and chloroform (1:1, v/v) and determined quantitatively by meas-

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[1] The following are the systematic names for the steroids mentioned in the text: androstenedione, androst-4-ene-3,17-dione; 17α-hydroxyprogesterone, 17α-hydroxypregn-4-ene-3,20-dione; progesterone, pregn-4-ene-3,20-dione; $NADP^+$ and NADPH oxidized and reduced nicotinamide–adenine dinucleotide phosphates; NAD^+ and NADH, oxidized and reduced nicotinamide–adenine dinucleotides.