A pathway for the interconversion of hexose and pentose in the parasitic amoeba *Entamoeba histolytica*

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Isotope studies indicate that hexose-to-pentose interconversion by axenic *Entamoeba histolytica* conserves the C-1 and C-6 hexose carbon atoms. Transketolase was readily identified in amoebal extracts, but transaldolase could not be demonstrated. However, sedoheptulose 7-phosphate is a substrate for the PP_i-dependent amoebal phosphofructokinase, and sedoheptulose 1,7-bisphosphate is cleaved by amoebal aldolase to dihydroxyacetone phosphate and erythrose phosphate. Since these three enzymes catalyse physiologically reversible reactions, a non-oxidative pathway for hexose–pentose interconversion exists in amoebae in the absence of transaldolase. By using known amoebal enzymes, the conversion of ribose into fructose was confirmed *in vitro*. Some kinetic parameters of amoebal phosphofructokinase, transketolase and aldolase were determined.

Nearly one-half of the ribose moieties in amoebal AMP are synthesized from glucose (Susskind *et al.*, 1980). The predominant pathway of glucose metabolism in *Entamoeba histolytica* is the Embden–Meyerhof pathway (Bragg & Reeves, 1962). Since the parasite lacks the first two enzymes of the oxidative pentose phosphate pathway (Hilker & White, 1959; Bragg & Reeves, 1962; Warren *et al.*, 1964), it appears unlikely that a hexose monophosphate shunt pathway exists. The present study shows that C-1 and C-6 on glucose are conserved during hexose–pentose interconversion, and evidence will be presented for a pathway by which three amoebal enzymes reversibly catalyse this interconversion.

Some of the results described here are taken from a dissertation (Susskind, 1979) submitted to the Graduate Faculty of the Louisiana State University Medical Center in partial fulfilment of the requirements of the Doctor of Philosophy degree.

Materials and methods

Organism

Axenic *Entamoeba histolytica*, strain 200:NIH, was maintained in TP-S-1 medium (Diamond,

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1968). Penicillin G, 900 units/ml, was added. Calceferol was omitted from the vitamin mixture.

Harvesting amoebae

Amoebae were harvested after 72–90 h cultivation. Cells were resuspended in the spent growth medium, transferred to a graduated tube and packed by centrifugation for 5 min at 850 g. The volume of packed amoebae was noted. Cells were then thrice washed by centrifugation in buffer A [10 mM-K₂HPO₄/20 mM-KCl/0.5 mM-MgCl₂/140 mM-NaCl/0.1 mM-Ca(NO₃)₂, adjusted to pH 7.0 with HCl].

Growth of amoebae with [¹⁴C]glucose

E. histolytica trophozoites were grown in 125-ml flasks containing a TP-S-1 medium prepared with one-half the prescribed amount of glucose. A 3–5 µCi portion of [¹⁴C]glucose was added to each flask. The specific radioactivity of the glucose was based on radioactivity counts and measurements of total carbohydrate in the medium. The flasks were inoculated with 1.2 × 10⁶ amoebae per flask, and incubated at 36°C in a sealed chamber.

Cell extracts

Buffer A-washed cells were suspended in an equal volume of ice-cold 20 mM-imidazole/HCl buffer, pH 7.0, and ruptured with 15 strokes of a conical ground-glass tissue grinder at 900 rev./min. The homogenates were diluted with 2 or 3 vol. of the same buffer and centrifuged for 30 min at 35 000 g and

at 4°C. The supernatant solutions were dialysed for 20 h against 500 vol. of buffer at 4°C, and then stored at -20°C until used.

Extracting and purifying RNA

Buffer A-washed cells were suspended in 5 vol. of a buffer containing 50 mM-Tris/HCl, pH 8, 150 mM-NaCl and 0.3% sodium dodecyl sulphate. Bentonite was added (4 mg/ml of suspension). The cells were chilled in ice and broken in a tissue grinder as described above. The homogenate was diluted with an equal volume of the suspending buffer. Protein was precipitated from the homogenate by the hot-phenol method described by Brawerman (1973), and the RNA was precipitated with ethanol and centrifuged. The RNA pellet was dissolved in 50 mM-sodium acetate buffer, pH 5.1, and the solution was clarified by centrifugation for 20 min at 39000 g at 4°C. The supernatant solution was precipitated with cetyltrimethylammonium bromide to remove traces of remaining polysaccharide (Ralph & Bellamy, 1964). RNA was quantified by its absorbance at 260 nm. Yeast RNA (Sigma, type XI) served as the standard.

Substrate assays

Ribose and ribose 5-phosphate were assayed with an orcinol-containing reagent as described by Schneider (1957). Total carbohydrate was determined by the phenol/H₂SO₄ method of Montgomery (1957), with glucose as the standard. Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard. Triose phosphate was assayed in a cuvette containing, per ml: 50 µmol of imidazole buffer, pH 7.0, 0.16 µmol of NADH, 2 units of glycerol-3-phosphate dehydrogenase and 20 units of triosephosphate isomerase. Decrease in A_{340} was measured. In some instances 'triose phosphate equivalents' were measured. This was accomplished by adding 0.25 unit of aldolase to the preceding assay mixture. This value represents triose phosphate (mol) plus twice the fructose biphosphate (mol) present.

Fructose 6-phosphate was assayed in a cuvette containing, per ml: 50 µmol of imidazole buffer, pH 7.0, 2.5 µmol of MgCl₂, 0.32 µmol of NADP, 1 unit of glucosephosphate isomerase, and 5 units of glucose-6-phosphate dehydrogenase. Increase in A_{340} was measured.

Sedoheptulose 7-phosphate was assayed in a cuvette containing, per ml: 50 µmol of imidazole buffer, pH 7.0, 40 µmol of ATP, 25 µmol of phosphoenolpyruvate, 2.5 µmol of MgCl₂, 0.16 µmol of NADH, 4 units of rabbit muscle 6-phosphofructokinase, 2.5 units of pyruvate kinase, and 2.5 units of lactate dehydrogenase. Decrease in A_{340} was measured.

Standard enzyme assays

The standard assay for transketolase activity (EC 2.2.1.1) contained, per ml: 50 µmol of imidazole/HCl, pH 7.0, 2.5 µmol of MgCl₂, 0.2 µmol of thiamin pyrophosphate, 2.0 µmol of ribose 5-phosphate, 0.3 µmol of xylulose 5-phosphate, 0.16 µmol of NADH, 2 units of glycerol-3-phosphate dehydrogenase and 20 units of triosephosphate isomerase. After equilibration at 30°C, reaction was started by the addition of the amoebal enzyme. The rate of decrease in A_{340} was monitored at 30°C. A cuvette lacking ribose 5-phosphate served as a control.

The standard assay for aldolase activity (EC 4.1.2.13) contained, per ml: 50 µmol of Tris/HCl, pH 8.6, 2.0 µmol of fructose 1,6-bisphosphate, 0.16 µmol of NADH, 2 units of glycerol-3-phosphate dehydrogenase, and 20 units of triosephosphate isomerase. Reaction was started by the addition of the amoebal enzyme after equilibration at 30°C. The rate of decrease in A_{340} was measured.

Transaldolase was assayed by the methods of Cooper *et al.* (1958) and Tsolas & Horecker (1970).

The assay for the pyrophosphate-requiring 6-phosphofructokinase (EC 2.7.1.90) was described by Reeves *et al.* (1974).

The molar absorption coefficient for NADH and NADPH was taken as $6.22 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Assay for ribose 5-phosphate utilization

Ribose 5-phosphate utilization in cell extracts was measured at 30°C in tubes containing, per ml: 50 µmol of imidazole/HCl buffer, pH 7.0, 2.5 µmol of MgCl₂, 0.2 µmol of thiamin pyrophosphate and cell-extract protein (2–4 mg/ml). Reaction was started by the addition of 8 µmol of ribose 5-phosphate. A control lacked the added substrate. After 1 h, tubes were diluted 5-fold with buffer, capped, heated for 1 min at 100°C, centrifuged to remove denatured protein, and the remaining substrate was assayed with the orcinol reagent.

Chemicals

[U-¹⁴C]glucose, [1-¹⁴C]glucose and [6-¹⁴C]glucose were from Amersham Radiochemical Centre, Amersham, Bucks., U.K. Linking enzymes used in the assays were from Boehringer and Sons, Mannheim, Germany. Enzyme suspensions in (NH₄)₂SO₄ solution were centrifuged, the pellet was dissolved in 1 mM-sodium EDTA, pH 7.0, and dialysed at 4°C overnight against 500 vol. of the EDTA solution.

Results

Incorporation of labelled glucose into amoebal RNA

Entamoeba histolytica were grown with [U-¹⁴C]-glucose, [1-¹⁴C]glucose or [6-¹⁴C]glucose. RNA was extracted from the cells after 84 h of cultivation. Glucose utilized was the total carbohydrate

in TP-S-1 medium at the time of inoculation less the amount in the amoeba-free culture fluid at the time of harvesting. Data from these experiments are listed in Table 1.

Amoebal transketolase

Cell extracts utilized 8.1 ± 1.7 nmol of ribose 5-phosphate/min per mg of protein in four preparations. Triose phosphate equivalents and sedoheptulose 7-phosphate formed, per mol of ribose 5-phosphate, were 0.46 ± 0.04 and 0.40 ± 0.04 respectively. No fructose 6-phosphate was formed in these experiments. The formation of these products by cell extracts indicated that amoebae possess transketolase. These products accounted for 86% of the pentose utilized. Failure to produce fructose 6-phosphate suggested that the amoebae lack transaldolase. We have repeatedly been unable to detect transaldolase activity in amoeba.

A freshly prepared cell extract was applied to a Sephacryl 200-S column, bed volume 100 ml, equilibrated with 20 mM-imidazole/HCl buffer, pH 7.0. Fractions rich in both phosphofructokinase and transketolase were eluted with the buffer, pooled, and applied to a column of DEAE-cellulose, bed volume 30 ml, which had been equilibrated with the buffer. The column was washed with the buffer until the A_{280} of the effluent approached zero. The enzymes were then eluted with buffer containing 0.1 M-NaCl. Transketolase and phosphofructokinase were separated by the second column fractionation. The partially purified enzymes were stored at -10°C after the addition of 30% (v/v) glycerol. Thiamin pyrophosphate, 0.2 mM, and 1 mM-MgCl₂ were also added to the transketolase preparation.

The identity of the amoebal transketolase was established according to the following three criteria (Racker, 1971).

(1) It has a cofactor requirement for thiamin pyrophosphate and Mg²⁺ (Fig. 1). The order of the addition of the cofactor had an effect on transketolase. When Mg²⁺ was followed by the addition of thiamin pyrophosphate, there was a lag period before enzyme activity reached the maximum rate (Fig. 1). Optimum transketolase activity occurred at pH 7.0.

(2) It catalyses the formation of glyceraldehyde 3-phosphate from xylulose 5-phosphate in the presence of either erythrose 4-phosphate or ribose 5-phosphate.

(3) It catalyses the utilization of glyceraldehyde 3-phosphate in the presence of fructose 6-phosphate. The kinetic values for amoebal transketolase are summarized in Table 2.

Amoebal phosphofructokinase

Sedoheptulose 7-phosphate was assayed by substituting it for fructose 6-phosphate in the standard

Fig. 1. Dependence for activity by the apoenzyme of amoebal transketolase on added thiamin pyrophosphate and Mg²⁺

The conditions were those of the standard assay (see the Materials and methods section), but lacking thiamin pyrophosphate and MgCl₂. Three such cuvettes were equilibrated with enzyme. After 5 min (arrow) the following were added: both cofactors (A); thiamin pyrophosphate only (B); and MgCl₂ only (C). Then, after 10 min (arrow), for B and C, the respective missing cofactor was added.

Table 1. Incorporation of D-[¹⁴C]glucose into amoebal RNA

Position of label	10 ⁻⁶ × Glucose radioactivity (d.p.m./mmol)	Glucose utilized (mmol)	10 ⁻³ × Radioactivity in recovered RNA (d.p.m.)	Percentage of radioactivity utilized recovered in RNA
U	4.8	4.1	4.3	0.022
U	8.8	8.2	19.3	0.027
1	4.4	6.0	6.4	0.024
1	2.0	8.1	3.8	0.024
6	5.5	4.6	3.5	0.021
6	2.5	5.0	2.6	0.021

Table 2. K_m of amoebal transketolase for various substrates

Varied substrate	Co-substrate (mM)	Apparent K_m (mM) of varied substrate
Ribose 5-phosphate	Xylulose 5-phosphate (0.3)	0.027*
Xylulose 5-phosphate	Ribose 5-phosphate (2.0)	0.23*
Glyceraldehyde 3-phosphate	Fructose 6-phosphate (12.0)	1.60†
Erythrose 4-phosphate	Xylulose 5-phosphate (0.1)	0.005‡

* Determined by changing the substrate in the standard transketolase assay.

† Determined by measuring the disappearance of glyceraldehyde 3-phosphate after 10 min incubation at 30°C in a tube containing $MgCl_2$, thiamin pyrophosphate, the co-substrate and enzyme.

‡ Determined spectrophotometrically by the rate of formation of glyceraldehyde 3-phosphate.

assay for the latter. That the product of the reaction with sedoheptulose 7-phosphate was the corresponding 1,7-bisphosphate was indicated by paper chromatography of the product of a reaction conducted in the absence of added aldolase. The paper-chromatographic technique employed the ammonium acetate/ethanol solvent and the periodate/benzidine staining technique of Wawszkiewicz (1961). The K_m of sedoheptulose 7-phosphate for amoebal phosphofructokinase is $64 \mu M$, which compares with a reported K_m value of $38 \mu M$ for fructose 6-phosphate (Reeves *et al.*, 1974). The K_m for PP_i was about $10 \mu M$ with either co-substrate.

Amoebal aldolase

Aldolase was purified from a cell extract fractionated on a Sephacryl 200-S column, 300 ml bed volume, equilibrated with 20 mM-imidazole/HCl, pH 7.0. Aldolase was eluted from this column after phosphofructokinase, and our preparation was slightly contaminated by this enzyme. The kinetic constants of amoebal aldolase for sedoheptulose 1,7-bisphosphate and fructose 1,6-bisphosphate were determined in cuvettes having a 5 cm light-path. The K_m for the former substrate was $13 \mu M$; for the latter, $3 \mu M$. The relative V_{max} for the former is one-half that for the latter. Triosephosphate isomerase was not required in the assay system when sedoheptulose bisphosphate was the substrate, since the products of its cleavage by aldolase are dihydroxyacetone phosphate and erythrose 4-phosphate. The addition of triosephosphate isomerase to the cuvette did not alter the rate of this reaction.

Demonstration of pentose-to-hexose interconversion

A reaction mixture devised for the interconversion of pentose and hexose *in vitro* contained enzymes that had been partially purified from amoebal cell extracts. The formation of fructose 6-phosphate from xylulose 5-phosphate and ribose 5-phosphate was measured. The hexose phosphate was formed only when the three amoebal enzymes transketolase, aldolase and phosphofructokinase were present (Fig. 2). Its formation was dependent on added PP_i .

Fig. 2. Demonstration *in vitro* of a proposed amoebal pentose phosphate pathway

The complete reaction system contained, in a volume of 1 ml: 50 μM of imidazole/HCl, pH 7.0, 0.10 μM of thiamin pyrophosphate, 0.05 μM of $MgCl_2$, 0.10 μM of PP_i , 0.62 μM of xylulose 5-phosphate, 2.0 μM of ribose 5-phosphate, 0.32 μM of $NADP^+$, 1 unit of yeast phosphoglucose isomerase, 5 units of yeast glucose 6-phosphate dehydrogenase, 0.07 unit of amoebal aldolase (except B) and 0.15 unit of amoebal phosphofructokinase. After equilibration at 30°C, the reaction was started by the addition of 0.03 unit of amoebal transketolase (arrow at left). A_{340} was monitored spectrophotometrically. A, complete system; B, contained 70 munits of yeast aldolase in place of the amoebal aldolase, and phosphofructokinase was not added until 35 min (arrow on right); C, minus amoebal aldolase; D, minus PP_i ; E, minus transketolase; F, minus glucose 6-phosphate dehydrogenase.

Discussion

Ribose phosphate is necessary for synthesis of nucleotides *de novo* or their formation by the salvage

of free bases. Glucose carbon has been shown to be one source of nucleotide ribose, and the glucose incorporated into amoebal ribonucleotides is recovered only in the ribose moiety (Susskind *et al.*, 1980). The results reported here show that labelled ribonucleotides were recovered when the amoebae were grown with $[1-^{14}\text{C}]$ glucose or $[6-^{14}\text{C}]$ glucose. Thus neither the hexose monophosphate nor the glucuronic acid pathway appear to be operating in *E. histolytica*. The percentage of labelled glucose carbon recovered in RNA was the same when *E. histolytica* was cultivated with $[\text{U}-^{14}\text{C}]$ glucose, $[1-^{14}\text{C}]$ glucose, or $[6-^{14}\text{C}]$ glucose, confirming that the amoebal pentose–hexose interconversion pathway is characterized by the conservation of glucose carbon.

The usual mechanism for the non-oxidative interconversion of hexose and pentose is the transketolase–transaldolase pathway (Horecker *et al.*, 1954; Racker, 1955; Marks & Feigelson, 1957; Horecker, 1965; Rognstad & Katz, 1974). However, in the Calvin cycle a series of reactions catalysed by transketolase, aldolase and sedoheptulose biphosphatase are involved in the regeneration of pentose from hexose (Racker & Schroeder, 1958; Bassham, 1971). The latter two enzymes replace transaldolase in the formation of sedoheptulose 7-phosphate, a key intermediate. Aldolase catalyses a condensation, yielding sedoheptulose 1,7-bisphosphate (Horecker & Smyrnitotis, 1952), and its dephosphorylation at C-1 leaves sedoheptulose 7-phosphate. In many organisms, sedoheptulose biphosphatase activity is a property of fructose biphosphatase (EC 3.1.3.11) (Racker & Schroeder, 1958; Pontremoli & Horecker, 1971).

Since *E. histolytica* lacks transaldolase, an alternative pentose–hexose interconversion scheme similar to that involved in the Calvin cycle was con-

sidered and is shown in Scheme 1. We have demonstrated that *E. histolytica* possesses transketolase, that amoebal aldolase utilizes sedoheptulose bisphosphate as a substrate, and that the reversible amoebal phosphofructokinase catalyses conversion of sedoheptulose bisphosphate into sedoheptulose 7-phosphate. We have reconstituted this system *in vitro* by using partially purified amoebal enzymes and demonstrated that it functions in the pentose-to-hexose direction. Since each reaction involved is readily reversible, it is reasonable to suppose that the system can function in the opposite direction. That it does so *in vivo* is likely in view of our inability to demonstrate transaldolase in amoebal extracts.

The two predominant pathways in Nature for the synthesis of pentose from hexose are the hexose monophosphate shunt and the transketolase–transaldolase pathway. Despite the widespread distribution of aldolase (Horecker *et al.*, 1972) and sedoheptulose biphosphatase activity (Pontremoli & Horecker, 1971) in plants, animals and microorganisms, there is little indication that the alternate pathway plays an important role in net pentose synthesis. Gibbs & Horecker (1954) found a small amount of label from $[1-^{14}\text{C}]$ ribose incorporated into C-4 and C-6 of hexose by pea (*Pisum sativum*) leaf preparations. These results, and the labelling pattern in pentose from $[1-^{14}\text{C}]$ glucose obtained by Plaut & Broberg (1956) with *Ashbya gossypii* would be consistent with the operation of the alternate transketolase pathway. However, Plaut & Broberg (1956) also obtained data using $[6-^{14}\text{C}]$ glucose that indicated the presence of a hexose monophosphate shunt, and the data of Gibbs & Horecker (1954) indicated that the major pathway in pea leaves and roots was the transketolase–transaldolase pathway.

Both the hexose monophosphate shunt and the transketolase–transaldolase pathways are energy-

Scheme 1. A schematic pathway for hexose–pentose interconversion in *E. histolytica*

Amoebal enzymes comprising the enzyme system are: (a) transketolase; (b) aldolase; (c) phosphofructokinase (PP_i).

conserving in terms of retaining phosphate ester bonds. The proposed pathway of pentose formation in *E. histolytica* is also energy-conserving. In most organisms, sedoheptulose biphosphatase activity results in the hydrolysis of a phosphate ester (Pontremoli & Horecker, 1971) to orthophosphate. However, with the amoebal phosphofructokinase this reaction is accompanied by the esterification of orthophosphate to form PP_i, which is a source of energy for this organism (Reeves, 1976). Our results indicate that, in *E. histolytica*, pentose phosphate is formed by a series of C₂-plus-C₃ condensations catalysed by transketolase, aldolase, phosphofructokinase (PP_i) and transketolase again. Glucose carbon and all phosphate bonds are conserved. This pathway of hexose-pentose interconversion differs significantly from that of the human host, and also from the interconversion occurring in the Calvin cycle.

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