STUDIES ON THE METABOLISM OF *ECHINOCOCCUS GRANULOSUS*

III. GLYCOLYSIS, WITH SPECIAL REFERENCE TO HEXOKINASES AND RELATED GLYCOLYTIC ENZYMES

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(Received September 5th, 1958)

SUMMARY

1. Phosphorylative glycolysis has been demonstrated in cell-free extracts of hydatid cyst scolices. An appropriate phosphate pool appears to be necessary for optimal *in vitro* activity.

2. Hydatid cyst scolices contain four hexokinases catalyzing specifically the phosphorylation of glucose, fructose, mannose and glucosamine. 2-deoxyglucose is not phosphorylated by crude or purified preparations. The four hexoses are phosphorylated in position 6. Gluco-, fructo-, and mannokinase activities are inhibited by glucose-6-phosphate, while mannose-6-phosphate inhibits only gluco- and mannokinases. ADP inhibits competitively fructose phosphorylation by fructokinase. Glucokinase is sensitive to PCMB, the inhibition being partially reversed by cysteine.

3. Cell-free preparations of hydatid cyst scolices contain in addition to various phosphatases, myokinase, phosphoglucomutase, phosphoglucose, and phosphomannose isomerases, phosphofructokinase, aldolase, glycerophosphate dehydrogenase, and lactic dehydrogenase. The extracts could also catalyze mutation of fructose-1-phosphate to fructose-6-phosphate and isomerization of glucosamine-6-phosphate to glucose-6-phosphate.

INTRODUCTION

The view that glycolytic reactions form part of the mechanism of lactic acid formation by some parasitic helminths has gained support through the demonstration of various enzymes which take part in the phosphorylative oxidation of glucose[1,2]. However, it should be kept in mind that the presence in cell-free extracts of enzymes involved in the series of reactions usually designated as the EMBDEN-MEYERHOF process does

The abbreviations used throughout are: ADP, adenosinediphosphate; ATP, adenosinetriphosphate; AMP, adenosine-5-phosphate; DPN, oxidized diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; TPN, oxidized triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; G-6-P, glucose-6-phosphate; G-1-P, glucose-1-phosphate; F-6-P, fructose-6-phosphate; F-1-P, fructose-1-phosphate; M-6-P, mannose-6-phosphate; GNH_2-6-P, glucosamine-6-phosphate; HDP, fructose-1,6-diphosphate; Gly-P, α-glycerophosphate; DAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3-phosphate; PCMB, *p*-chloromercuric benzoate; TCA, trichloroacetic acid.

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not necessarily indicate that they are actually taking part in the carbohydrate metabolism of the cell *in vivo*. Thus, *Trichinella spiralis* larvae, whose lactic acid production under aerobic or anaerobic conditions is negligible[3], have enzymes for glycolysis, including an active lactic dehydrogenase[4]. A similar situation has been observed in the cells of *Corynebacterium creatinovorans*[5].

Lactic acid is, from a quantitative standpoint, the most important end-product of the anaerobic and aerobic glycogen fermentation by whole hydatid cyst scolices[6]. Although earlier work in this laboratory suggested the possibility that lactate might be produced in this organism through a metabolic sequence resembling the EMBDEN-MEYERHOF cycle[7], no critical data exist concerning the mechanism of anaerobic carbohydrate degradation in *E. granulosus*. The present experiments yield evidence for phosphorylative glycolysis as a metabolic pathway to lactic acid in cell-free preparations of hydatid cyst scolices. Furthermore, the presence of four specific hexokinases, catalyzing respectively the phosphorylation of glucose, fructose, mannose, and glucosamine in cell-free extracts, is reported, and partial purification and certain properties of the enzymes are described.

METHODS

Parasitological material

Liver hydatid cyst scolices were obtained from newly slaughtered sheep as previously described[7].

Enzyme preparations

The scolices were washed 3 or 4 times with 30–40 vol. of either distilled water or 0.001 *M* KH_2PO_4 buffer, pH 7.0. The supernatant fluid was removed by suction. The scolices were then suspended in an appropriate amount of one of the above media, as described in tables and figures, and disrupted at 2–4° in a French pressure cell[8] at a pressure of 5,000 lb./square inch. The preparation thus obtained was centrifuged at 20,000 × *g* for 60 min at 2° in an International refrigerated centrifuge (Model PR-2, head No. 290) and the supernatant fluid used as a source of enzyme. Since preliminary evidence suggested the possibility of the presence of more than one hexokinase in the scolices, the supernatant fraction of 0.001 *M* KH_2PO_4 (pH 7.0) extracts, obtained as indicated above, was fractionated with the aid of hydroxylapatite[9] as follows. Approximately 0.8 to 1.0 g (dry weight) of hydroxylapatite suspended in 0.001 *M* KH_2PO_4 buffer of pH 7.0 was centrifuged in a lusteroid tube at 10,000 × *g* for 10 min and the supernatant fluid discarded. To the hydroxylapatite pellet 8.0–10.0 ml of the supernatant fraction containing 70.0–90.0 mg total protein were added and thoroughly mixed with the gel with the aid of a stirring rod. The mixture was then centrifuged at 10,000 × *g* for 10 min at 2° and the supernatant fluid discarded. The gel was then eluted stepwise with 5.0–6.0 ml portions of KH_2PO_4 buffer, pH 7.0, of increasing ionic strength, from 0.005 *M* to 0.22 *M*. Each eluate was assayed for hexokinase activity as described later. Active eluates could be stored for 3–4 days with only slight loss in activity. However, when the eluates were frozen, 80–100 % of the activity was lost over a 24–48 h period. All the above operations were carried out at 2–4°.

Acetone powder extracts were prepared as follows. Distilled-water-washed

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scolices suspended in 10.0–12.0 ml of distilled water were added dropwise into 40 vol. of cold acetone (— 10°) under constant magnetic stirring and centrifuged at 3,500 × *g* for 10 min at — 10°. The supernatant fluid was discarded and the sediment washed with 30 vol. of cold acetone (— 10°) and centrifuged. The sediment was washed finally with 20 vol. of cold, anhydrous ether (— 10°) and the powdered material was spread in thin layers on filter paper and dried at room temperature. The material was stored at — 20° in a vacuum desiccator over $CaCl_2$ until used. For assay of enzymic activity, 250 mg of acetone powder were suspended in 2.5 ml of distilled H_2O and homogenized in an all-glass Potter-Elvehjem homogenizer immersed in crushed ice, and extracted during 30 min at room temperature. The slightly opalescent supernatant fluid remaining after centrifugation at 20,000 × *g* for 60 min at 2° was used for the assay.

Analytical procedures

Overall glycolysis was estimated in the Warburg apparatus, at pH 7.6, and at 38° in an atmosphere of nitrogen containing 5 % carbon dioxide. The gas mixture was passed over heated copper to remove traces of oxygen. A glycolytic reaction mixture similar to that of LE PAGE[10], with ATP replaced by ADP, was used. The enzyme preparation was mixed with the medium in the main compartment of the chilled Warburg flasks before these were placed in the bath. After 7 min of gassing and 3 min of equilibrium, inorganic phosphate, contained in the side arm of the vessel, was tipped into the main compartment, at which time the zero-time flasks were removed. The criteria of activity were the appearance of lactic acid, as determined in aliquots of the reaction mixture after deproteinization with cold TCA[11] and the net uptake of glucose[12] after deproteinization with $Ba(OH)_2$–$ZnSO_4$[13]. Inorganic phosphate and pyruvic acid were determined according to LE PAGE[14] and FRIEDEMAN AND HAUGEN[15], respectively.

Hexokinase activities were measured by three procedures. In the first method disappearance of hexose was determined as follows. Protein and phosphate esters were precipitated with $Ba(OH)_2$–$ZnSO_4$[13] and hexose utilization determined from the difference between the free hexose concentration of the control and final samples, ATP being added to the zero-time tubes immediately after deproteinization. Glucose, mannose, and glucosamine were estimated according to NELSON'S[16] or HAGEDORN AND JENSEN'S[12] procedures; fructose, by the method of ROE[17]; 2-deoxyglucose, according to SOLS AND CRANE[18]. In the second method, decrease in labile phosphate was estimated on aliquots of the reaction mixture which were heated in 1.0 *N* H_2SO_4 at 100° for 11 min[19] and after removal of any turbidity, inorganic phosphorus was determined according to LE PAGE[14]. Finally, hexokinase activities were followed spectrophotometrically by determining the formation of G-6-P in the presence of TPN, Zwischenferment, and an excess of either phosphoglucose isomerase or both phosphoglucose isomerase and phosphomannose isomerase, as required. The rate of reduction of TPN determined at 340 mμ constitutes a measure of the hexokinase reaction[20]. In some cases, identification of the end-product of the hexokinase reaction was achieved by paper chromatography of the sugar phosphates contained in the barium-soluble, alcohol-insoluble fraction[21], with methanol, formic acid, and water as solvents[22].

Phosphoglucomutase activity was determined spectrophotometrically. The G-6-P

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formed from G-1-P was oxidized by TPN in the presence of Zwischenferment and Mg^{++} and the increase in optical density at 340 mμ due to the reduction of TPN followed.

Phosphohexokinase was also measured spectrophotometrically by the test suggested by WARBURG AND CHRISTIAN[23]. The formation of HDP from F-6-P by the action of phosphohexokinase was estimated by following the decrease in optical density at 340 mμ due to the reoxidation of DPNH in the presence of excess crystalline muscle aldolase and Gly-P dehydrogenase. Phosphatase activities were determined essentially according to WEINBACH[24]. Phosphoglucose and phosphomannose isomerases were followed spectrophotometrically according to SLEIN[25,26]. Myokinase (adenylate kinase) was detected by coupling the enzyme to the hexokinase reaction. In the presence of excess yeast hexokinase, glucose, and Mg^{++}, glucose was phosphorylated by the ATP formed in the myokinase reaction and the rate of formation of G-6-P was followed spectrophotometrically at 340 mμ in the presence of Zwischenferment and TPN, the increase in optical density due to the reduction of TPN now being a measure of myokinase.

Protein was determined according to the method of WARBURG AND CHRISTIAN[27] or by the turbidimetric procedure of KUNITZ[28]. Volatile fatty acids were estimated quantitatively by steam-distillation and titration[29], acetyl phosphate, by the hydroxamate method of LIPMANN AND TUTTLE[30].

All spectrophotometric determinations were done in a Beckman model DU spectrophotometer. $6.22 \cdot 10^6$ was used as the molecular extinction coefficient of DPNH and TPNH.

Reagents

Phosphoglucose and phosphomannose isomerases were prepared from rabbit muscle according to SLEIN [25,26]. Crystalline muscle aldolase and Gly-P dehydrogenase were prepared by the method of TAYLOR *et al.*[31] and BEISENHERZ *et al.*[32], respectively. Zwischenferment was obtained by extracting 50 g (dry weight) of commercial brewer's yeast with 0.1 *M* $NaHCO_3$ saturated with 95 % N_2–5 % CO_2 for 16 h at 37°. Thereafter, KORNBERG's procedure was essentially followed[33]. Sodium pyruvate was prepared according to PRICE AND LEVINTOW[34], F-1-P from HDP under the action of crystalline muscle aldolase[35], and assayed by its 7 min acid-labile contents. Fructose (Nutritional Biochemicals Corporation) was recrystallized from methanol; glucose (Analar) and mannose (Merck) were recrystallized from ethanol; 2-deoxyglucose and M-6-P (Nutritional Biochemicals Corporation) as well as glucosamine (Pfanstiel Chem. Co.) were used without further purification. TPN (Sigma) was 92 % pure when assayed with Zwischenferment and G-6-P[33]; DPN (Sigma) was 90 % pure when assayed according to COLOWICK, KAPLAN AND CIOTTI[36]; DPNH was a gift from Dr. E. C. WEINBACH, National Institutes of Health, U.S. Public Health Service; HDP and F-6-P (Schwarz), the barium salts, were standardized according to ROE[17]; G-6-P (Sigma), the barium salt, was standardized spectrophotometrically at 340 mμ from the amount of TPNH formed in the presence of an excess Zwischenferment and Mg^{++}[33]; G-1-P, ATP, and ADP (Sigma) were standardized by their 7 min acid-labile contents. All compounds obtained as barium salts were freed from barium before use.

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RESULTS

Acetone powder extracts of hydatid cyst scolices glycolyzed glucose to the extent of approximately 2 moles of lactate per mole of glucose utilized (Table I). Concomitantly, 2 moles of added pyruvate disappeared from the medium and 2 moles of inorganic phosphate were esterified in the complete system of Table I. When glucose was omitted from the medium, lactic acid production was only 0.59 μmoles per mg protein, as compared with 1.49 μmoles of the complete system. It was concluded that the lactic acid produced in the absence of added glucose was derived from HDP. In the absence of HDP, glucose phosphorylation was nil and lactic acid production dropped to 0.1 μmole per mg protein. The small lactic acid production in the absence of added HDP may be attributed mainly to the activity of lactic dehydrogenase, which is present in these extracts, reoxidation of DPNH at pH 7.0 being readily carried out by acetone powder extracts in the presence of pyruvate. Contrary to what has been observed in other tissues, such as tumor homogenates[10], upon withdrawal of fluoride, glycolysis was reduced to some extent, as shown in Table I.

TABLE I

GLYCOLYSIS IN ACETONE POWDER EXTRACTS OF HYDATID CYST SCOLICES

Complete system, 25.0 μmoles $KHCO_3$; 1.0 μmoles DPN; 15.0 μmoles nicotinamide; 2.0 μmoles ADP; 2.0 μmoles HDP; 10.0 μmoles KH_2PO_4 buffer, pH 7.6 (side arm); 5.0 μmoles Na-pyruvate; 45.0 μmoles KF; 15.0 μmoles $MgCl_2$; 5.0 μmoles glucose; 0.25 ml acetone powder extract. Final volume, 1.0 ml; gas phase, 95 % N_2–5 % CO_2. Incubated for 30 min at 38°. Reaction stopped either with TCA or $Ba(OH)_2$–$ZnSO_4$. The figures represent the averages of at least three determinations.

System	Δ Glucose μmoles/mg protein	Δ Lactate μmoles mg protein
Complete	0.74	1.49
Minus Glucose		0.59
Minus HDP	0.0	0.10
Minus F^-	0.60	1.08
Minus pyruvate-F^-	0.24	0.10
Minus ADP	0.0	0.16
Complete plus hexokinase	1.14	1.98

The need of fluoride and added HDP for optimal activity suggests that dephosphorylative reactions compete with glucose phosphorylation, the former reactions being predominant in the absence of a suitable inhibitor such as fluoride, or an appropriate phosphate reservoir, as HDP. As a result, glucose phosphorylation decreases or is completely abolished.

Added ADP was necessary for demonstrating glycolysis in acetone powder extracts of hydatid cyst scolices (Table I). When ADP was replaced by ATP or ATP plus AMP, the level of lactate production was of the same order of magnitude as that observed with ADP. This observation suggested the presence of an active myokinase in the acetone powder extracts. On addition of ADP to the extracts in the presence of Mg^{++}, glucose, yeast hexokinase, TPN, and Zwischenferment, TPN was reduced (Fig. 1), a demonstration that ADP was being converted to ATP and AMP, the former then transferring its terminal phosphate group to glucose by the action of hexokinase; the G-6-P thus formed being in turn oxidized by TPN in the presence of Zwischenferment.

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In the absence of pyruvate, which was added to act as a substrate for the reoxidation of DPNH formed furing glycolysis, glucose phosphorylation markedly dropped (Table I). A similar observation has been made in *Ascaris lumbricoides*[37] and *Trichinella spiralis*[1] cell-free extracts.

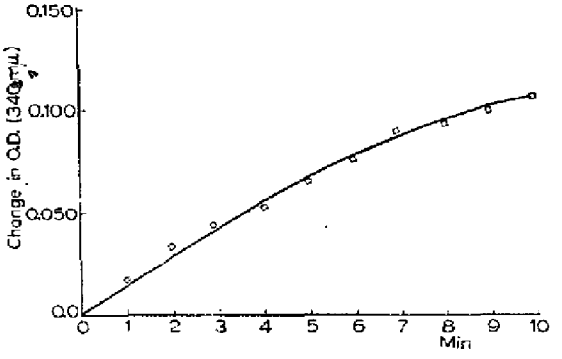

Fig. 1. Myokinase activity of hydatid cyst scolices acetone powder extracts. The reaction mixture contained 5.0 μmole $MgCl_2$; 20.0 μmoles glycylglycine buffer of pH 7.6; 2.0 mg yeast hexokinase; 0.28 μmoles TPN; 0.4 U. Zwischenferment; 510.0 μg protein acetone powder extract; final volume, 3.0 ml. The blank cuvette contained the same components except ADP. Reaction started by adding 2.0 μmoles ADP.

In the anaerobic breakdown of glucose to pyruvate, of the four ATP synthesized, one is utilized for the phosphorylation of glucose to yield G-6-P, this reaction being catalyzed by hexokinase; thus, when glucose is the substrate utilized for glycolysis, hexokinase becomes the rate-limiting enzyme, which appears to be the case in our system; glycolysis was appreciably increased upon the addition of hexokinase to the complete system, thereby indicating that acetone powder extracts are not a good source of hexokinase (Table I).

The effect of fluoride upon glycolysis indicated the presence in acetone powder extracts of phosphatases which could hydrolyze not only ATP but also some of the intermediate phosphorylated sugars. The phosphatase activity of the acetone powder extracts was tested by incubation for 30 min at 38° with various phosphorylated sugar derivatives and the nucleotides, ATP, ADP, and AMP. ATPase, fructose diphosphatase, and glucose-6-phosphatase were the most active of all the phosphatases studied. The hydrolysis of ADP was slower. G-1-P was not hydrolyzed at all and F-6-P, M-6-P, and AMP, only to a small extent (Table II). The fructose diphosphatase was corrected for the amount of fructose-6-phosphatase of the extracts.

TABLE II

PHOSPHATASE ACTIVITIES OF ACETONE POWDER EXTRACTS OF HYDATID CYST SCOLICES

Substrates, 4.0 μmoles; $MgCl_2$, 5.0 μmoles; buffer glycylglycine, pH 7.6, 20.0 μmoles; 0.1 ml acetone powder extract. Final volume, 0.40 ml. Incubated for 30 min at 38°. Reaction stopped with TCA.

Substrate	*Inorganic P μmoles/mg protein*
ATP	0.74
HDP	0.67
G-6-P	0.51
ADP	0.44
M-6-P	0.27
AMP	0.17
F-6-P	0.14
G-1-P	0.0

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Whole hydatid cyst scolices excrete aerobically and anaerobically small amounts of acetic acid[6]. Acetone powder extracts contained very small amounts of volatile fatty acids, but no increase of them was observed after incubation, nor was formation of acetyl phosphate detected in the experiments reported in Table I.

Aqueous homogenates and their supernatant fractions, as well as acetone powder extracts of hydatid cyst scolices, catalyzed the phosphorylation of glucose, fructose, mannose, and glucosamine in the presence of ATP and Mg^{++}. 2-deoxyglucose was not phosphorylated. It is known that G-6-P inhibits brain, muscle, and liver hexokinases[38] therefore, its effect, as well as that of M-6-P, was tested on the hexokinase activity of the scolex preparations. G-6-P inhibited the phosphorylation of glucose, fructose, and mannose, as it does with brain hexokinase[38]. However, M-6-P only inhibited glucose and mannose phosphorylation, fructose phosphorylation not being affected (Table III). This observation suggested that, contrary to what has been observed for yeast[20] and brain[18] hexokinases, the phosphorylation of glucose, fructose, mannose, and glucosamine, could be catalyzed by individual, specific enzymes, as has been reported for *Schistosoma mansoni*[2]. This hypothesis was confirmed when supernatant fractions of hydatid cyst scolex homogenates were fractionated with the aid of hydroxylapatite as described under METHODS. The eluates obtained with KH_2PO_4 buffer of pH 7.0 of molarities varying from 0.01 to 0.05 *M* catalyzed only the phos-

TABLE III

EFFECT OF G-6-P AND M-6-P ON THE HEXOKINASE ACTIVITIES OF SUPERNATANT FRACTIONS OF HYDATID CYST SCOLEX AQUEOUS EXTRACTS

Substrates, $7.1 \cdot 10^{-3}$ *M*; ATP, 6.0 μmoles; $MgCl_2$, 15 μmoles; glycylglycine buffer, pH 7.0 10.0 μmoles; NaF, 10.0 μmoles; 0.20 ml supernatant fraction; final volume, 0.50 ml; incubated for 30 min at 38°. Reaction stopped with $Ba(OH)_2$–$ZnSO_4$.

Additions	*Molarity*	*Relative rate of phosphorylation of*		
		Glucose	*Fructose*	*Mannose*
G-6-P	—	100	100	100
	$5.7 \cdot 10^{-3}$	46	61	52
	$2.8 \cdot 10^{-3}$	77	82	66
M-6-P	—	100	100	100
	$5.7 \cdot 10^{-3}$	69	100	25
	$2.8 \cdot 10^{-3}$	95	100	73

TABLE IV

PURIFICATION OF HYDATID CYST SCOLEX HEXOKINASES

System as in Table III, with the exception that neither glycylglycine buffer nor fluoride were added for the assay of eluates.

Fraction	*Volume ml*	*Total protein mg*	*μmoles of hexose phosphorylated per mg protein*			
			Glucose	*Fructose*	*Mannose*	*Glucosamine*
Supernatant	8.5	74.5	1.2	0.77	0.83	0.28
0.01–0.05 *M* eluate	6.0	1.8	0.0	30.0	0.0	0.0
0.05–0.07 *M* eluate	6.0	1.8	0.0	0.0	9.7	0.0
0.17–0.18 *M* eluate	6.0	5.4	7.4	0.0	0.0	0.0
0.19–0.20 *M* eluate	6.0	2.4	0.0	0.0	0.0	2.1

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phorylation of fructose; 0.05 to 0.07 *M* phosphate buffer eluates catalyzed the phosphorylation of only mannose; an eluate catalyzing glucose phosphorylation was obtained between 0.17 to 0.18 *M* phosphate buffer; finally, the fraction eluted with 0.19 to 0.20 *M* phosphate buffer was specific for glucosamine phosphorylation (Table IV). The ATPase activity of the purified kinases was negligible. The K_m values for ATP, Mg^{++}, and substrates for purified gluco-, fructo-, and mannokinases are shown in Table V. K_m for fructose was lower for hydatid cyst fructokinase than for the *Schistosoma* enzyme[2] or brain[18] hexokinase, while K_m for glucose was of the same order of magnitude for hydatid cyst scolices and *Schistosoma* glucokinases[2], much higher than for brain hexokinase[18]. On the other hand, K_m for ATP was much lower for hydatid cyst glucokinase than for the corresponding *Schistosoma* enzyme[2] and higher than brain hexokinase[18]. Hydatid cyst scolex hexokinases resembled the *Schistosoma* enzymes[2] as far K_m for Mg^{++} is concerned.

BERGER *et al.*[30] found no evidence for essential -SH groups in yeast hexokinase. However, brain hexokinase is inhibited by sulfhydryl inhibitors[18]. Hydatid cyst scolex hexokinases are sensitive to sulfhydryl inhibitors, such as PCMB. For demonstrating this effect, reaction mixtures containing glucokinase; PCMB; 10.0 μmoles $MgCl_2$; and 5.0 μmoles glucose in a final volume of 0.50 ml were incubated during 10 min at room temperature, the tubes then were placed in a bath at 38°, the reaction started by adding 8.0 μmoles ATP and incubated for an additional 30 min. The

TABLE V

K_m VALUES FOR PURIFIED HYDATID CYST SCOLEX HEXOKINASES

System as in Table IV.

	K_m (molarity)		
	Glucokinase	*Fructokinase*	*Mannokinase*
Glucose	$1.07 \cdot 10^{-1}$		
Fructose		$6.2 \cdot 10^{-1}$	
Mannose			$1.5 \cdot 10^{-1}$
ATP	$2.85 \cdot 10^{-1}$	$1 \cdot 10^{-3}$	$3.5 \cdot 10^{-3}$
Mg^{++}	$1.33 \cdot 10^{-3}$	$1 \cdot 10^{-3}$	$2.2 \cdot 10^{-3}$

TABLE VI

INHIBITION OF PURIFIED GLUCOKINASE BY PCMB AND PROTECTION BY L-CYSTEINE

System as indicated in the text.

Additions	*Molarity*	*μmoles of glucose phosphorylated per mg protein*	*Inhibition percentage*
None	—	4.0	—
PCMB	$1.4 \cdot 10^{-3}$	2.45	38
	$2.5 \cdot 10^{-3}$	0.78	81
PCMB plus L-Cysteine	$1.4 \cdot 10^{-3}$ / $1.4 \cdot 10^{-2}$	3.5	12
PCMB plus L-Cysteine	$2.5 \cdot 10^{-3}$ / $1.4 \cdot 10^{-2}$	1.85	54

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activity was inhibited by PCMB and this effect could be partially reversed when L-cysteine was included during the 10-min incubation period (Table VI).

ADP has been reported as a non-competitive inhibitor for *Schistosoma* glucokinase[2] and as a competitive inhibitor for brain hexokinase[18]. ADP also inhibits non-competitively liver fructokinase[40]. As for the hydatid cyst scolex enzymes concerned, ADP inhibits competitively fructokinase, its inhibitory effect being reversed by increasing the concentration of ATP (Table VII).

TABLE VII

COMPETITIVE INHIBITION OF PURIFIED FRUCTOKINASE BY ADP

15.0 μmoles $MgCl_2$; 5.0 μmoles fructose; 0.2 ml enzyme; other additions as indicated. Incubated for 30 min at 38°. Reaction stopped with $Ba(OH)_2$–$ZnSO_4$.

	Fructose utilization per mg protein relative rate	
ADP	$8.5 \cdot 10^{-3}$ *M ATP*	$1.7 \cdot 10^{-2}$ *M ATP*
—	100	100
$2.85 \cdot 10^{-4}$ *M*	82	100
$5.7 \cdot 10^{-4}$ *M*	49	86

Yeast[41] and *Schistosoma*[2] hexokinases catalyze the phosphorylation of glucose, fructose, mannose, and glucosamine in position 6. On the other hand, rabbit testis glucokinase[42] and liver fructokinase[43] catalyze the phosphorylation of glucose and fructose respectively in position 1. Supernatant fractions of hydatid cyst scolex homogenates, as well as acetone powder extracts, were not suitable for studying the site of phosphorylation, since they contained phosphoglucomutase and were moreover capable of catalyzing the conversion of F-1-P to F-6-P, which in turn was isomerized to G-6-P (Fig. 2). These preparations contained also phosphoglucose and phosphomannose isomerases (Fig. 3). However, purified glucokinase contained little phosphoglucomutase activity. This minimized the possibility that G-1-P would be converted to G-6-P, since it is the end-product of the reaction. When the activity of glucokinase was followed by the formation of G-6-P according to method 3 (see under METHODS), the rate of formation of G-6-P from glucose and ATP was much greater than from G-1-P (Fig. 4), thus indicating that glucose phosphorylation occurred at position 6.

Although homogenate supernatant fractions could catalyze the conversion of F-1-P to F-6-P, purified fructokinase preparations were free from this activity and the rate of formation of G-6-P from F-1-P in the presence of muscle phosphoglucose isomerase was nil, while appreciable amounts were formed from fructose and ATP (Fig. 5). Conclusive evidence that phosphorylation of fructose occurred in position 6 was obtained by two additional procedures. In the first one, reaction mixtures containing 10.0 μmoles fructose, 8.0 μmoles ATP, 15 μmoles $MgCl_2$, and enzyme in a final volume of 0.50 ml were incubated for 2 h at 38°, deproteinized with TCA, initial and final samples fractionated according to LE PAGE[11] and the sugar phosphates of the barium-soluble, alcohol-insoluble fractions chromatographed on paper[22]. A spot with an R_F value of 0.43 was obtained in the final samples, as compared with an R_F value of 0.42 for an authentic sample of F-6-P and of 0.30 for F-1-P. In the second procedure, when the activity of fructokinase was determined simultaneously by

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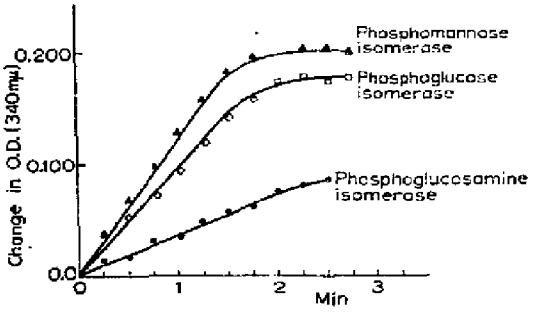
methods 1 and 2 (see METHODS), a good correlation between the sugar utilized and the decrease in acid-labile phosphate was obtained[19].

Mannose-6-P appeared also as the end-product of purified mannokinase activity of hydatid cyst scolices. No formation of G-6-P from mannose and ATP was observed when muscle phosphomannose isomerase was omitted, while TPN was reduced when phosphomannose isomerase was present (Fig. 6).

Fig. 2. Phosphoglucomutase and phosphofructomutase activities of aqueous homogenate supernatants of hydatid cyst scolices. The experimental cuvette contained 0.28 μmole TPN; 10.0 μmoles $MgCl_2$; 10.0 μmoles glycylglycine buffer, pH 7.6; 0.2 U Zwischenferment. The blank cuvette contained all the components but the substrate. Reaction started by the addition of 4.0 μmoles of substrate.



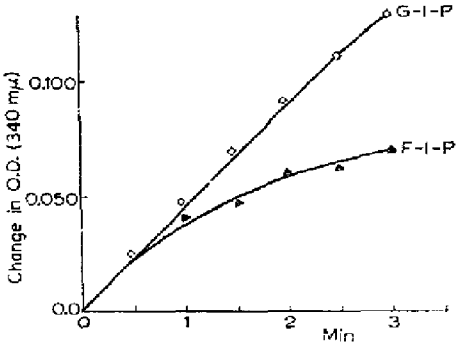
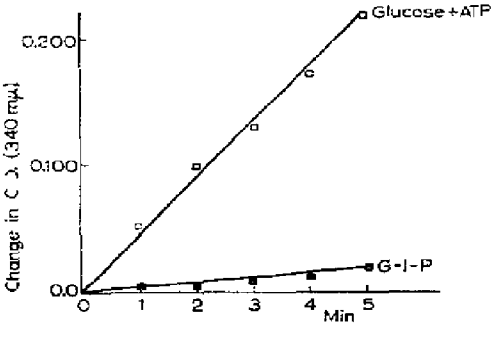


Fig. 3. Phosphoglucose, phosphomannose, and phosphoglucosamine isomerase activities of supernatant fractions of aqueous homogenates of hydatid cyst scolices. System for phosphoglucose and phosphomannose isomerases was, 10.0 μmoles $MgCl_2$; 10.0 μmoles glycylglycine buffer, pH 7.6; 0.28 μmole TPN; 0.4 U. Zwischenferment; 202 μg protein supernatant; final volume, 3.0 ml. Reaction started by the addition of 2.0 μmoles substrate. The blank cuvette contained the same components but for the substrate. System for phosphoglucosamine isomerase was, 8.0 μmoles ATP; 0.28 μmole TPN; 0.4 U Zwischenferment; 5.0 μmoles $MgCl_2$; 10.0 μmoles glycylglycine buffer, pH 7.6. Reaction started by addition of 5.0 μmoles glucosamine. The blank cuvette contained the same components minus glucosamine. Final volume, 3.0 ml.

Fig. 4. Formation of G-6-P from glucose plus ATP and from G-1-P by purified hydatid cyst scolices glucokinase. The experimental cuvette contained 4.0 μmoles ATP; 10.0 μmoles $MgCl_2$; 0.2 U. Zwischenferment; 0.14 μmoles TPN; 10.0 μmoles glycylglycine buffer of pH 7.6; 180.0 μg protein glucokinase. The blank cuvette contained the same components but substrates. Reaction started by the addition of either 4.0 μmoles glucose or G-1-P. Final volume, 3.0 ml.



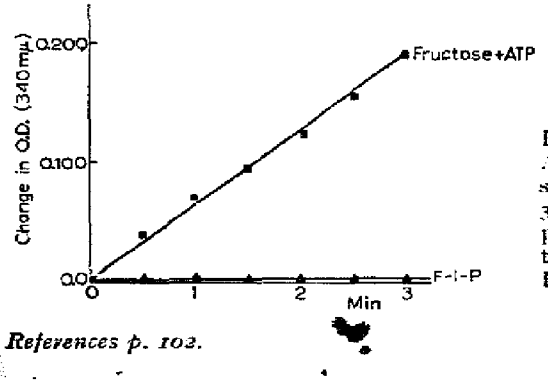


Fig. 5. Formation of F-6-P from fructose plus ATP and from F-1-P by purified hydatid cyst scolices fructokinase. System as in Fig. 4, with 30.0 μg protein fructokinase and 600.0 U. muscle phosphoglucose isomerase. Reaction started by the addition of either 4.0 μmoles fructose or F-1-P. The blank cuvette contained the same components minus substrate.

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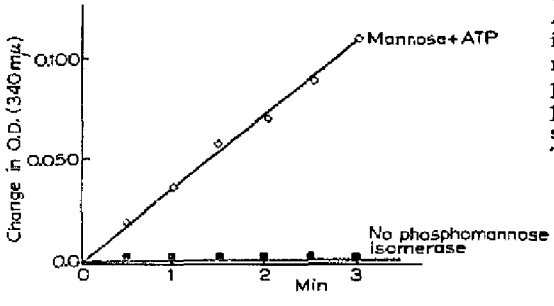

Fig. 6. Formation of M-6-P from mannose and ATP with or without muscle phosphomannose isomerase by purified hydatid cyst scolices mannokinase. System as in Fig. 5 with 160.0 μg protein mannokinase and 200 U. muscle phosphomannose isomerase as indicated. Reaction started by the addition of 4.0 μmoles mannose. The blank cuvette contained all the components but the substrate.

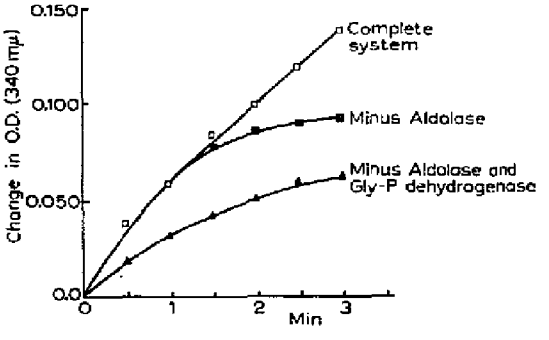

Fig. 7. Phosphofructokinase, aldolase, and Gly-P of hydatid cyst scolices cell-free extracts. Complete system: Tris(hydroxymethyl)aminomethane buffer ("Tris" buffer), pH 7.5, 100.0 μmoles; ATP, 4.0 μmoles; DPNH, 0.2 μmole; $MgCl_2$, 5.0 μmoles; muscle aldolase, 200.0 μg protein; muscle Gly-P dehydrogenase, 60.0 μg protein; supernatant of hydatid cyst scolex homogenate, 1.21 mg protein. Final volume, 3.0 ml. Reaction started with 4.0 μmoles of F-6-P. The blank cuvette contained H_2O. A control cuvette read against H_2O containing the same components but F-6-P was used as a correction for the endogenous reoxidation of DPNH.

Although glucosaminekinase was not studied as extensively as the other kinases, evidence obtained indicates that glucosamine is also phosphorylated in position 6, since a decrease in acid-labile phosphate corresponding to the amount of sugar utilized was observed when the activity was assayed according to methods 1 and 2.

Aqueous homogenate supernatant fractions could also catalyze the deamination of glucosamine-6-phosphate to G-6-P. This could be demonstrated by incubating the supernatant fractions with glucosamine, yeast hexokinase, Mg^{++}, and ATP; GNH_2-6-P formed by the action of hexokinase on glucosamine in the presence of ATP was converted to G-6-P, which in turn oxidized TPN in the presence of Zwischenferment (Fig. 3). No reduction of TPN was observed when glucosamine or ATP were omitted.

The following reactions were also catalyzed by the above supernatant fractions:

$$\text{F-6-P} + \text{ATP} \longrightarrow \text{HDP} + \text{ADP} + \text{H}^+ \quad (1)$$

$$\text{HDP} \rightarrow \text{DAP} + \text{GAP} \quad (2)$$

$$\text{DAP} + \text{DPNH} + \text{H}^+ \longrightarrow \text{Gly-P} + \text{DPN} \quad (3)$$

$$\text{sum: F-6-P} + \text{ATP} + \text{DPNH} + \text{H}^+ \longrightarrow \text{Gly-P} + \text{DPN} + \text{ADP} + \text{H}^+ + \text{GAP}$$

When supernatant fractions were incubated in the presence of ATP, DPNH, Mg^{++}, crystalline muscle aldolase and Gly-P dehydrogenase, DPNH was reoxidized upon addition of F-6-P (Fig. 7). The supernatant fractions contained sufficient amounts of aldolase and Gly-P dehydrogenase to catalyze reactions (2) and (3) in the absence of added muscle enzymes, although at a lower rate. It should be realized that ATP was resynthesized in this system owing to the presence of myokinase in the supernatant fractions (Fig. 1).

The fact that these preparations could catalyze the conversion of F-1-P to F-6-P suggested the possibility that hydatid cyst scolices might contain, in addition to

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HDP aldolase, an F-1-P aldolase. However, no splitting activity was found when aldolase was assayed according to SIBLEY AND LEHNINGER[44], with F-1-P as substrate.

DISCUSSION

The experiments reported here demonstrate that under appropriate experimental conditions, cell-free extracts of hydatid cyst scolices are capable of degrading glucose as well as HDP to lactate by the reactions of the Embden–Meyerhof scheme. Although not every enzyme of this cycle has been individually assayed, the entire reaction sequence appears to be covered by the present experiments. As Table I shows, HDP was required for glucose phosphorylation, none being observed when HDP was omitted from the reaction mixture. HDP appears to serve, as in insect material[45], as a phosphate reservoir. The importance of an adequate phosphate pool is stressed by the effect of fluoride. When dephosphorylating reactions were not controlled by the addition of fluoride, the rate of glycolysis was markedly reduced. Dephosphorylation of various glycolytic phosphorylated intermediates, as well as of ADP, ATP, and AMP, was catalyzed by acetone powder extracts of hydatid cyst scolices (Table II). Whether dephosphorylation of ATP and ADP was catalyzed by specific enzymes or by nonspecific apyrases remains to be established. The possibility that ADP was not really hydrolyzed but transformed into ATP and AMP by the action of myokinase, ATP being in turn hydrolyzed by an ATPase, should be contemplated. AMP was also dephosphorylated to a small extent, indicating the presence of a 5'-nucleotidase. Glucose-6-phosphatase, which has been reported only for a few tissues[46], was also present.

Present evidence indicates that hexokinases may be classified mainly into two groups: (a) those kinases which non-specifically catalyze the phosphorylation of various hexoses, such as yeast[39] and brain[18] hexokinases; (b) hexokinases which specifically catalyze the phosphorylation of individual hexoses, such as liver fructokinase[13] and the *Schistosoma* enzymes[2]. The hexokinases of hydatid cyst scolices belong to this second group, glucose, fructose, mannose, and glucosamine being phosphorylated by individual kinases, each one separable from the others by protein fractionation procedures (Table IV). The *E. granulosus* enzymes differ from hexokinases of the first type, as well as from *Schistosoma* kinases in their inability to phosphorylate 2-deoxyglucose and in some kinetic characteristics. Another difference with the *Schistosoma* enzymes is the competitive inhibition of fructokinase by ADP, while ADP inhibits non-competitively *Schistosoma* glucokinase[2]. G-6-P does not inhibit the *Schistosoma* fructokinase[2], while inhibition of the corresponding *Echinococcus* enzyme by this ester is evident. However, as in the case of the *Schistosoma* enzymes, each hexose was phosphorylated in position 6 by the *Echinococcus* kinases.

Cell-free preparations of hydatid cyst scolices have phosphofructokinase activity, as well as aldolase and Gly-P dehydrogenase (Fig. 7). The role of the reaction by which F-1-P is converted to F-6-P is not clear, F-1-P being unable to serve as a substrate for an aldolase reaction as described for jack bean seeds[47].

An interesting finding seems to be the conversion of GNH_2-6-P to G-6-P (Fig. 3). Although the reversibility of this reaction was not studied, it could represent a pathway for the synthesis of GNH_2-6-P. Whether this reaction represents a simpel diamination of GNH_2-6-P or whether some intermediate such as F-6-P is formed, remains to be established.

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ACKNOWLEDGEMENTS

This work was supported by grant E-1867 of the Division of Research Grants and Fellowships of the National Institutes of Health, U.S. Public Health Service.

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