PROPERTIES OF PARTICULATE HEXOKINASE OF THE KREBS-2 ASCITES TUMOR*

R. B. McCOMB AND W. D. YUSHOK
Biochemical Research Foundation, Newark, Del. (U.S.A.)

(Received October 28th, 1958)

SUMMARY

Hexokinase from the Krebs-2 ascites carcinoma has been partially purified and its properties have been investigated. It was obtained in good yield in particles which sediment at a centrifugal force of 4000 × *g* from isotonic sucrose homogenates of these cells. Treatment of the large particulate fraction with 0.6% deoxycholate in the presence of glucose led to a 22-fold purification with respect to original cell-free homogenate.

Particulate tumor hexokinase exhibited a plateau of maximal activity over the pH range 5.6 to 7.8 and was inhibited by its reaction products, glucose-6-phosphate and ADP.

The Michaelis constants of ascites tumor hexokinase for 11 sugars were determined as well as the maximum rate of phosphorylation of those compounds which served as substrates. The compound exhibiting the highest affinity for tumor hexokinase was glucosone, whose Michaelis constant was $8 \cdot 10^{-6}$. Competition between the various substrates and inhibitors when incubated with the purified enzyme preparation indicated that the observed phosphorylations were catalyzed by a single enzyme.

A comparison with the brain particulate enzyme reported by CRANE AND SOLS revealed no outstanding difference between it and particulate tumor hexokinase.

INTRODUCTION

Hexokinase of the Krebs-2 ascites carcinoma has been studied to determine whether it differs from that found in normal tissues and to ascertain whether the effect of certain sugar analogs on carbohydrate metabolism of the Krebs-2 ascites tumor is related to the affinity of the analog for tumor hexokinase. Previous investigations by YUSHOK[1,2,3] had shown that the inhibition of glucolysis and fructolysis in these cells by a number of sugar analogs paralleled their affinities, as reported by SOLS AND CRANE[4], for particulate brain hexokinase.

Particulate tumor hexokinase has been purified to a state free of interfering enzymes by a modification of the procedure used for brain hexokinase[5]. The properties

* A preliminary report of this work was presented before the 132nd meeting of the American Chemical Society at New York, 1957.

References p. 526.

of this purified enzyme have been investigated. No outstanding difference has been found between particulate hexokinase obtained from these cancer cells and that reported for brain[4], the normal tissue most studied. The small dissimilarities between certain properties of tumor and brain hexokinase were no greater than the reported differences between the hexokinase of brain and that of other normal tissue[5,6]. A correlation has been observed between the degree of inhibition of glycolysis in ascites tumor cells[2,3] by certain sugar analogs and the affinity of these compounds for particulate tumor hexokinase.

MATERIALS

Compounds used

Mannose and glucosamine were obtained from Pfanstiehl Laboratories, N-acetylglucosamine (NAGA) and lyxose from Mann Laboratories, 3-O-methylglucose from L. Light Company and glucose-6-phosphate, as the crystalline heptahydrate of the barium salt, from Sigma Chemical Company. Glucose was Baker's C.P. Analyzed. 2-Deoxyglucose (2DG) and glucosone were prepared in this laboratory, 2-deoxyglucose by Dr. F. B. CRAMER[7] and glucosone by Miss MARIE HUDSON[8]. Mannoheptulose was kindly supplied by Dr. N. K. RICHTMEYER. Adenosine triphosphate (ATP) and adenosine diphosphate (ADP) were obtained as the crystalline sodium salts from Pabst Laboratories.

Fructose (Pfanstiehl CP Special) was used either directly without purification or after recrystallization from methanol. The unrecrystallized Pfanstiehl product had less than 0.025 % glucose impurity as determined by a modification[9] of the specific glucose oxidase assay[10]. Galactose (Pfanstiehl CP) was freed of glycolyzable impurities by treatment with baker's yeast followed by recrystallization from 80 % ethanol.

All materials were of reagent grade or the equivalent. The sugars and sugar derivatives were of the D or natural configuration.

EXPERIMENTAL AND RESULTS

Preparation and homogenization of ascites tumor cells

Ascitic fluid was harvested from female white Swiss mice between the seventh and twelfth day after intraperitoneal inoculation of the animal with the tumor. The ascites cells were washed by centrifugation at 700 × *g* with 0.25 *M* sucrose containing 0.001 *M* potassium (ethylenedinitrilo) tetraacetate (EDTA), pH 7.5. They were washed two times for 1 min periods and a third for 3 min. The washed cells were then resuspended to four times the packed cell volume in the same medium. The washing operation and all following manipulations were performed in the cold.

Homogenates* were prepared from 80 ml of this cell suspension in four 20 ml batches by means of fifty passes with a 30 ml, Potter-type homogenizer equipped with a Teflon pestle. Strong hand pressure was exerted on the downward thrust; the whole operation took about 10 min for each batch. The combined broken cell

* An alternate method of homogenization was mechanical agitation with 0.2 mm glass beads obtained from the Minnesota Mining and Manufacturing Company, Minneapolis. A mixture of one part ascites cell suspension and one part by weight of beads was shaken for 15 sec at 3600 c/min through an amplitude of 0.8 cm in a shaker of electromagnetic vibrator type[12,13], or 3 parts beads and one part cell suspension were stirred from one to 5 min at maximum speed in a Virtis homogenizer as described by LAMANNA AND MALLETTE[14].

References p. 526.

suspension was centrifuged for 5 min at 700 × *g* and the sediment was then resuspended to 40 ml in sucrose-EDTA, subjected to 25 additional passes in the Potter homogenizer, and centrifuged as before. The two supernatant fluids were combined and recentrifuged for 5 min at 700 × *g* to yield a homogenate free of cells and nuclei.

Purification of hexokinase

Tumor particulate hexokinase was isolated by a modification of the procedure used by CRANE AND SOLS[5] for preparing brain hexokinase. The method may be adapted to any quantity of starting material.

Eighty ml of the cell-free homogenate were centrifuged for one h at 4000 × *g* and the supernatant fluid was removed by decantation and discarded. The sediment was resuspended to 40 ml volume with 0.001 *M* EDTA, pH 7.5, and redispersed by means of a Potter or Dounce[11] homogenizer.

To the resuspended particulate fraction was added 3.6 ml of 0.1 *M* glucose followed by 12 ml of 3.3% sodium deoxycholate, with stirring. The volume of the suspension was brought to 60 ml and the material centrifuged for 30 min at 12,000 × *g*. The sediment from the centrifugation was resuspended in 15 ml of 0.001 *M* EDTA, pH 7.5, and recentrifuged as before. The sediment was finally suspended in 10 ml of EDTA, pH 7.5. The preparation could then be stored at 4° for several weeks without appreciable loss of activity.

Preparation of phosphofructokinase

A mixture of crude phosphofructokinase–phosphoglucoisomerase (PFK–PGI) was prepared by the method of COLOWICK[15]. Contaminating hexokinase was inactivated by heating the crude extract 5 min at 55°.

Measurement of hexokinase activity

The hexokinase preparation was incubated at 30° with 0.01 *M* ATP, 0.007 *M* $MgCl_2$, 0.013 to 0.03 *M* tris(hydroxymethyl)aminomethane (Tris), pH 7.5, and substrate in a total volume of 1.0 or 1.5 ml. Potassium phosphate served equally well as buffer and was occasionally substituted for Tris. Hexokinase activity was determined by sugar disappearance; analysis of the hexose-6-phosphate reaction product; or by measurement of the increase of acidity accompanying the reaction. In the last case, a colorimetric indicator method was used[5]. Conditions for the colorimetric indicator method differ from those given above as follows: 0.003% bromthymol blue was included in the reaction mixture, buffer was omitted, the final ATP and $MgCl_2$ concentrations were 0.003 and 0.005 *M* respectively, and the final volume was 2.5 ml.

The unit of hexokinase activity, the same as that used for the brain enzyme[5], was defined as the amount of enzyme required to phosphorylate one micromole of glucose in 15 min at 30° under otherwise optimal conditions. Specific activity was defined as the units of activity per mg of protein. Protein was determined by the method of LOWRY *et al.*[16].

When hexokinase activity was determined by sugar disappearance, 0.2 or 0.5 ml portions were withdrawn from the reaction mixture at timed intervals and the reaction stopped by discharging the withdrawn material into 1.0 ml of 0.3 *M* $Ba(OH)_2$ or into an equal volume of acetone. Initial and final sugar concentrations were deter-

References p. 526.

mined by analysis of the Ba–Zn filtrate by the method of NELSON[17] or by analysis of the acetone inactivated reaction mixture by the specific glucose oxidase method[9]. Glucose and 2-deoxyglucose disappearance was measured by the latter procedure in the presence of phosphorylated products and other hexoses*.

When the analysis of the reaction products was used as a means of measuring hexokinase activity, the reaction was followed by either direct analysis of the sugar phosphate product[5] or by measurement of the change in absorbance of an acid–base indicator accompanying sugar phosphate formation[5]. The change in absorbance of the indicator was measured in microcolorimeter tubes with a Klett-Summerson photoelectric colorimeter equipped with a No. 56 filter.

Distribution of hexokinase in cell fractions

Much of the hexokinase activity originally present in cell-free homogenates of the Krebs ascites carcinoma was found to be associated with particulate material which sediments at 4000 × *g*, as shown in Table I. Isolation of the large particulate resulted in approximately a two-fold enhancement of specific activity over that found in the cell-free homogenate. Material sedimenting between 4000 and 20,000 × *g*, the small particulate fraction, had negligible activity. Most of the remaining activity was found in the supernatant fluid after centrifugation at 20,000 × *g*.

TABLE I

DISTRIBUTION OF HEXOKINASE IN CELL FRACTIONS FROM ASCITES TUMOR CELLS*

Cell fraction	*Relative activity*	*Specific activity*
Cell-free homogenate 700 × *g*, 5 min	100	2.47
Large particulate 4000 × *g*, 30 min	53 (22)**	5.42 (55)**
Small particulate 20,000 × *g*, 30 min	1	0.6
Supernatant fluid 20,000 × *g*, 30 min	39	1.33

* Homogenization with Potter homogenizer in 0.25 *M* sucrose. Activity measured by glucose disappearance in the presence of excess PFK–PGI.

** Deoxycholate-treated large particulate (final purified enzyme) sedimented at 12,000 × *g*, 30 min.

Data on the distribution of activity in material sedimented at 700 × *g* or less were not included in Table I, because of gross contamination of this nuclear fraction with unbroken and partially broken cells. The whole homogenate, which included the nuclear fraction, had a specific activity of 2.1, which is slightly lower than that of the cell-free homogenate.

The data in Table I are the averages of the results of three experiments among which variation was small. In preliminary experiments when the cell disruption was carried out in 0.1 *M* phosphate, the medium reported to give maximum enhancement of specific activity for brain particulate hexokinase[5], almost all the ascites tumor hexokinase activity originally present in cell-free extracts was found in the soluble

* Several commercial preparations of glucose oxidase were found to be contaminated with enzymes having sucrase activity. These preparations were avoided when analysis of homogenate prepared in sucrose was made. Glucose oxidase (Glucostat) obtained from Worthington Biochemical Corp. was free of sucrase and was found to be satisfactory for analysis of the fractions containing sucrose.

References p. 526.

fraction regardless of the mechanical means used to disrupt the cells. Because of the poor yield of particulate hexokinase in phosphate medium, all subsequent homogenizations were carried out in 0.25 *M* sucrose.

After the fairly drastic treatment of the large particulate fraction with deoxycholate, considerable hexokinase activity was found to be associated with particles that sedimented at 12,000 × *g*. The influence of deoxycholate on the activity, protein content and specific activity of the large particulate fraction of ascites tumor homogenates is shown in Fig. 1. Extraction of this fraction with 0.2 % deoxycholate in the presence of 0.006 *M* glucose only slightly reduced the sedimentable hexokinase activity although it removed nearly 90 % of the sedimentable protein. The combination of these two effects resulted in a sharp rise in the specific activity of the recovered hexokinase. The specific activity continued to increase after treatment with higher concentrations of deoxycholate until it reached a maximum at 0.6 %. This latter rise in specific activity was mainly due to an increase in recoverable hexokinase activity. At concentrations of deoxycholate higher than 0.6 % the yield and specific activity fell off sharply. All points on the curves represent deoxycholate treatment in the presence of glucose. As indicated by the isolated points in the same figure, when the particulate fraction was treated with 0.6 % deoxycholate in the absence of glucose, the hexokinase activity associated with the 12,000 × *g* sediment was only about one-half that recovered in the presence of glucose.

The deoxycholate-treated particulate fraction was free from other enzymes which might interfere in the determination of hexokinase activity. Adenosinetriphosphatase, phosphofructokinase, phosphoglucoisomerase, phosphomannoisomerase, myokinase and phosphomonoesterase activities could not be detected under the standard conditions used in hexokinase assay. The overall yield of the purified

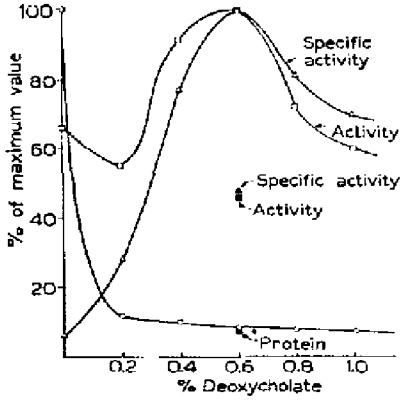

Fig. 1. Effect of deoxycholate on hexokinase activity and protein content of the large particulate fraction. Activity determined by glucose disappearance without correction for glucose-6-phosphate accumulation. Treatment with deoxycholate was performed in the presence of 0.006 *M* glucose except for the three points ▲, ■ and ●. The curves are based on the average of data of two experiments.

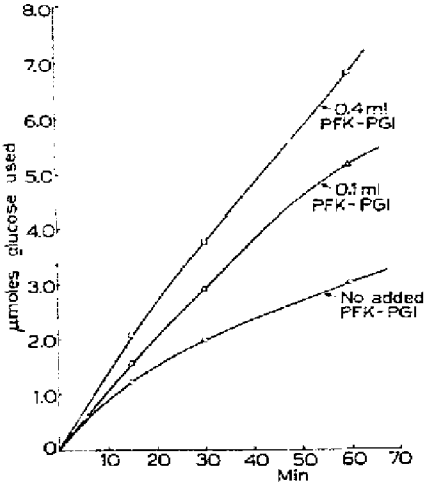

Fig. 2. Effect of phosphofructokinase–phosphoglucoisomerase (PFK–PGI) additions on glucose utilization by purified particulate hexokinase. The activity of the added PFK–PGI was such that 0.4 ml gave maximum stimulation of glucose utilization. No correction was made for inhibition due to accumulating ADP.

References p. 526.

enzyme was 22 % and the final specific activity was 55*. This represents an enhancement of specific activity 22 times that of the original cell-free homogenate.

Properties of the purified particulate hexokinase

Particulate ascites tumor hexokinase required both ATP and Mg^{++} as cofactors. The concentration of ATP necessary to give half maximal activity in the presence of 0.005 M $MgCl_2$ and 0.0003 M glucose was $1.7 \cdot 10^{-4}$ M. This value was determined by the method of LINEWEAVER AND BURK[18]. The concentration of $MgCl_2$ necessary to give half maximal activity when the enzyme was near saturation (0.01 M) with respect to ATP was found to be $1 \cdot 10^{-3}$ M.

The effect of pH on hexokinase activity is shown in Table II. The rate of phosphorylation of glucose was found to be maximal and constant from pH 5.6 to 7.8. Enzyme activity fell off rapidly under more alkaline or acid conditions.

TABLE II

EFFECT OF pH ON ASCITES TUMOR HEXOKINASE ACTIVITY

Hexokinase activity was measured by glucose disappearance (glucose oxidase method). Buffer: acetate–phosphate–Tris, 0.067, 0.033, 0.067 M respectively; adjusted to the indicated pH with N HCl or NaOH. Total glucose used was 0.4 μmoles per ml. A Beckman pH meter, model G, was used in determining the initial pH of the reaction mixture.

pH	*Relative activity*
4.5	22
4.8	53
5.1	78
5.6	100
6.6	95
7.0	100
7.85	96
8.4	68
8.8	9

The time-course of the hexokinase reaction with glucose, in the presence and absence of phosphofructokinase–phosphoglucoisomerase, is shown in Fig. 2. The inhibitory effect of accumulating glucose-6-phosphate on hexokinase activity is indicated by the decreasing rate with time of glucose utilization in the presence of the purified enzyme. This falling off in rate as the reaction proceeds was reversed by additions of phosphofructokinase–phosphoglucoisomerase, which removed glucose-6-phosphate as it was formed. Even in the presence of an excess of these glucose-6-phosphate-removing enzymes, there was a slight falling off of rate with time, presumably because of inhibition by ADP, the other reaction product. Independent experiments indicated that the enzyme was inhibited by ADP.

The K_i for the noncompetitive inhibition of both glucose and fructose phosphorylation by glucose-6-phosphate was determined in another set of experiments in which known quantities of glucose-6-phosphate were added. The inhibitor constants, calculated with the formula $K_i = I/(V_0/V_i - 1)$, were $4 \cdot 10^{-4}$ and $6 \cdot 10^{-4}$ respectively

* No further enhancement of specific activity of ascites tumor hexokinase was obtained by lipase treatment of the deoxycholate-extracted particulate fraction in the manner recommended for the purification of brain hexokinase[6].

References p. 526.

in the presence of the two substrates. Mannose, 2-deoxyglucose and fructose did not form inhibitory phosphate esters of tumor hexokinase as demonstrated by the linear rate of phosphorylation of these sugars.

Michaelis constants, determined for representative carbohydrate substrates and inhibitors by the method of LINEWEAVER AND BURK[18], have been listed in Table III. These constants were calculated from the results of experiments in which either the substrate or the inhibitor concentration was varied. Usually several methods of determining K_m were employed and the figures listed in Table III represent the averages of the values obtained. Variation in K_m determined by different methods was small.

TABLE III

MICHAELIS CONSTANTS OF KREBS ASCITES TUMOR HEXOKINASE

Compound	$K_m \cdot 10^4$		Method of K_m evaluation**	Analytical method
	Tumor	Brain*	Tumor	Tumor
Glucosone	0.078	0.1	Fructose competition[a] Glucose competition[b] Glucose competition[b] NAGA competition[a]	Colorimetric indicator Glucose disappearance Colorimetric indicator Colorimetric indicator
Mannose	0.15	0.05	Glucose competition[b]	Glucose disappearance
Glucose	0.28	0.08	NAGA competition[a] NAGA competition[a]	Colorimetric indicator Glucose disappearance
N-Acetylglucosamine	0.72	0.8	Fructose competition[a] Fructose competition[a] 2DG competition[a]	Colorimetric indicator Stable phosphate Colorimetric indicator
2-Deoxyglucose	1.24	0.27	Fructose competition[a] NAGA competition[a] Direct	2DG disappearance 2DG disappearance Colorimetric indicator
Mannoheptulose	1.31	0.5	Fructose competition[a] Glucose competition[b]	Colorimetric indicator Colorimetric indicator
Glucosamine***	1.7	0.8	2DG competition[b]	2DG disappearance
Fructose	25.2	16	Direct Direct	Colorimetric indicator Stable phosphate
Lyxose	41	13	Fructose competition[a]	Colorimetric indicator
Galactose	1750	1000	Direct	Colorimetric indicator
3-O-Methylglucose	§	§§	Fructose competition[a]	Colorimetric indicator Stable phosphate

* Data of SOLS AND CRANE[4].

** By LINEWEAVER-BURK treatment: *a*, competitor concentration varied, *b*, compound concentration varied. Direct signifies evaluation by means of the effect of substrate concentration on phosphorylation.

*** At pH 7.5, isoelectric point[4].

§ A reproduceable value could not be obtained because of limitations of the testing procedure. Values ranged from 340 to $1700 \cdot 10^{-4}$.

§§ Undetectable.

References p. 526.

The K_m values for fructose, 2-deoxyglucose, and galactose were calculated from the effect of substrate concentration on the rate of phosphorylation. A double reciprocal plot which illustrates the effect of fructose concentration on the rate of phosphorylation is shown in Fig. 3. The K_m for fructose* as calculated from these data was $2.6 \cdot 10^{-3}$.

Competitive inhibition by N-acetylglucosamine of fructose phosphorylation is illustrated also in Fig. 3. In this case the substrate, fructose, was varied at a single inhibitor concentration. The K_m calculated for this nonphosphorylated inhibitor was $7.4 \cdot 10^{-5}$.

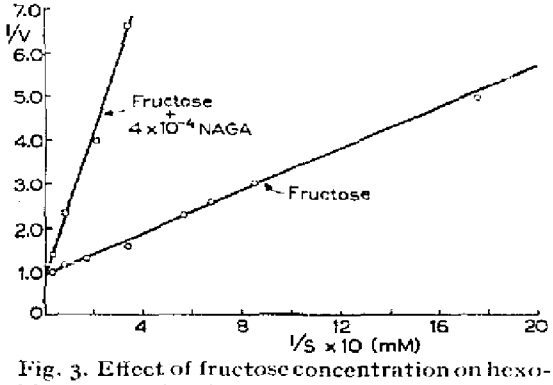

Fig. 3. Effect of fructose concentration on hexokinase activity in the presence and absence of NAGA. Activity determined by the colorimetric indicator method.

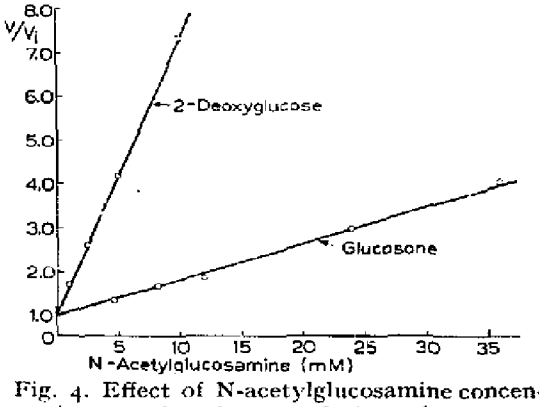

Fig. 4. Effect of N-acetylglucosamine concentration on the phosphorylation of 2-deoxyglucose and glucosone by tumor hexokinase. 2-Deoxyglucose phosphorylation determined by sugar disappearance and glucosone phosphorylation by the colorimetric indicator method.

Inhibition by N-acetylglucosamine of glucose, 2-deoxyglucose and glucosone phosphorylation was determined by varying the inhibitor concentration while holding the substrate concentration constant. An example of the competitive inhibition of 2-deoxyglucose and glucosone phosphorylation by this inhibitor is given in Fig. 4. From analysis of this graph and the previously determined K_m of N-acetylglucosamine, a K_m of $1.13 \cdot 10^{-4}$ was obtained for 2-deoxyglucose and $9.6 \cdot 10^{-6}$ for glucosone.

In several instances sugars capable of being phosphorylated were considered as competitive inhibitors and used in combination with other substrates to evaluate the K_m of either. This procedure was feasible whenever a great difference existed between the rates of phosphorylation of two substrates, *e.g.*, glucosone and fructose, or wherever one substrate could be determined without interference from the other, *e.g.*, 2-deoxyglucose in the presence of fructose. In these cases either of the methods illustrated in Figs. 3 and 4 was used to evaluate K_m.

The relative maximum rates of phosphorylation of several of these compounds have also been determined. These relative rates are given in Table IV. They were obtained at or near saturation of the enzyme with respect to the substrate tested or by extrapolation of values obtained at lower concentrations.

* The value was not changed by recrystallization of fructose from methanol. This indicated that the commercial product was essentially free of glucose and mannose. The value obtained for the K_m of fructose is reported to be markedly influenced by trace amounts of these sugars[4].

References p. 526.

TABLE IV

MAXIMUM RATE OF PHOSPHORYLATION BY ASCITES TUMOR HEXOKINASE

Compound	Maximum rate relative to glucose	
	Tumor**	Brain*
Glucosone	0.08	0.08
Mannose	0.61	0.4
Glucose	1.0	1.0
N-Acetylglucosamine	***	***
2-Deoxyglucose	1.2	1.0
Mannoheptulose	0.06	0.015
Fructose	2.4	1.5
Lyxose	***	***
Galactose	0.03	0.02
3-O-Methylglucose	***	***

* Data of SOLS AND CRANE[3].

** Determined at 0.02 *M* glucosone, 2-deoxyglucose and mannoheptulose, 0.005 *M* glucose and 0.0016 *M* mannose. Galactose and fructose values were obtained by extrapolation.

*** Undetectable.

DISCUSSION

For enzyme studies the ascites tumor offers an advantage over solid tumors in that it is composed of a nearly pure population of cancer cells with negligible contamination from enzymes of normal tissue. In addition, the free ascites cells can easily be separated from extracellular protein. These cells are very difficult to homogenize, however, and fairly drastic treatment is required in order to release the internal component of the cell. Since harsh methods of cell disruption may have a damaging effect on the released subcellular particles, the effect of the medium and the means of homogenization were investigated in relation to the recovery of hexokinase activity in tumor cell fractions. Although a relationship between the damage to particulate elements and the method of homogenization could not be demonstrated, the distribution of activity in various cell fractions was found to depend upon the medium in which the homogenization was carried out[19]. After homogenization of ascites cells in phosphate medium and centrifugation at 700 × *g*, the sediment, composed of intact cells, nuclei and partially broken cells, appeared to retain particulate hexokinase and only non-sedimentable hexokinase was released. Ascites tumor cells differ from brain in this respect. Homogenization of brain tissue in phosphate buffer afforded as good recoveries of hexokinase activity in the large particulate fraction as when isotonic sucrose was used[5].

In agreement with the results with solid tumors reported by BOYLAND[20], the total hexokinase activity of Krebs ascites tumor cells was found to be more than adequate to account for steady state anaerobic glucolysis in the intact cells. The hexokinase activity of whole homogenates of these tumor cells was approximated at 37.5° from the observed maximum activity at 30° and the temperature coefficient for brain hexokinase in this range[21]. The hexokinase activity in the homogenate calculated for these conditions was 15.3 μmoles of glucose used per hour per mg of protein compared with a value for intact Krebs ascites cells of 1.6 μmoles of glucose converted anaerobically to lactic acid per h per mg of cell protein[22]. This represents a potential capacity for glucose phosphorylation of greater than 9 times that actually

References p. 526.

observed during anaerobic glucolysis in the intact cell. It would appear from these calculations that hexokinase is not limiting in the overall process of glycolysis in intact ascites tumor cells.

The localization of considerable hexokinase activity of ascites tumor cells in the large particulate fraction of sucrose homogenates is similar to that in certain normal tissues, namely brain, kidney, heart, and intestinal mucosa[5]. This is in contrast to red blood cells[5] in which the enzyme is recovered in the soluble fraction. The localization of hexokinase in mitochondria of ascites tumor cells also has been reported by ACS *et al.*[23].

The continued association of hexokinase activity in material which sediments at relatively low centrifugal force, after rather drastic treatment with deoxycholate, is unusual in view of the removal of 92 % of the sedimentable protein and solubilization or destruction of the remaining interfering glycolytic enzymes and ATPase under these conditions. This firm association of much of the hexokinase activity with the large particulate suggests localization of the enzyme in mitochondria within the intact cell.

The localization of hexokinase in the mitochondria of ascites tumor cells may help explain the observation by CHANCE AND HESS[24] that added glucose produces an initial stimulation of respiration in these cells before the rest of the glycolytic system becomes active and steady state inhibition of respiration develops. The explanation of this transitory stimulation by glucose, given by CHANCE AND HESS, is that ADP formed by the hexokinase reaction diffuses to the respiratory chain of the mitochondria and stimulates respiration before appreciable glycolytic phosphorylation of ADP can occur. Since much of the ascites tumor hexokinase has been found to be associated with mitochondria, ADP formed during the hexokinase reaction should be more readily available for respiratory control.

The protection of ascites tumor hexokinase by glucose during the deoxycholate extraction procedure is apparently the well-known phenomenon of substrate stabilization of hexokinase[25,26]. In the present case, binding of a functional group is presumed to be responsible for the protection since only low concentrations of glucose are required. Glucose was also found to protect tumor hexokinase from heat denaturation[19].

Cofactor requirements for ascites tumor hexokinase are similar to those reported for the brain enzyme[21]. The response of enzyme activity to changing ATP and Mg^{++} concentration was similar for the enzyme from the two tissues. Also, as for the brain enzyme, ascites tumor hexokinase exhibits a plateau of maximum activity over a broad pH range.

Hexokinase from both brain and ascites tumor is inhibited by its reaction products, ADP and glucose-6-phosphate. The K_i for the non-competitive glucose-6-phosphate inhibition is in good agreement with the value reported for brain hexokinase[27]. Glucose-6-phosphate inhibition is typical of other mammalian hexokinases but not the yeast enzyme[5,28].

Glucosone-6-phosphate in its enol form meets the configurational requirements proposed by SOLS AND CRANE for product inhibitors of hexokinase[27]. Evidence was obtained which suggests that the phosphorylated product of glucosone, presumably glucosone-6-phosphate, inhibits tumor hexokinase. When phosphorylation of glucosone by tumor hexokinase was allowed to go to completion (no more acid

References p. 526.

production) and 0.002 M mannose then added to the reaction mixture, significant inhibition of the mannose phosphorylation was observed. However, because of the difficulty in obtaining satisfactory analyses of the components present at the completion of the glucosone–hexokinase reaction, the observed inhibition can not be attributed with certainty to glucosone-6-phosphate.

The reciprocal of the Michaelis constant is a good approximation of the true affinity of representative carbohydrate substrates and inhibitors for tumor hexokinase. In addition, the K_m and maximal rate of phosphorylation afford a good means of comparing tumor and brain hexokinase. With the exception of glucosone and N-acetylglucosamine, the absolute value of the K_m of the various sugars for tumor hexokinase was higher than the corresponding value for the brain enzyme. These K_m values for tumor hexokinase are, however, still well within the range of variation of K_m found among normal tissues. CRANE AND SOLS[6] report the K_m of skeletal muscle and liver hexokinase for glucose and mannose to be ten times higher than the corresponding value for brain. In general the relative order of affinities of the various compounds with tumor and brain were the same. Only glucosone and N-acetylglucosamine were noticeably out of sequence.

The low K_m of glucosone is of particular interest because of its exceedingly strong inhibition of glycolysis in intact ascites tumor cells[1]. Glucosone is also a strong inhibitor of substrate phosphorylation by yeast hexokinase. HUDSON AND WOODWARD[29] report a value of $6 \cdot 10^{-5}$ for the K_m of glucosone* with yeast hexokinase, which is considerably lower than the corresponding value for glucose. Hence with the yeast as well as the tumor enzyme, glucosone has the lowest K_m (highest affinity for the enzyme) of any sugar tested.

A comparison of the Michaelis constants of various carbohydrate substrates and inhibitors of tumor hexokinase with the affinities of the same compounds for the glycolytic system in the intact cell indicates a rough parallelism between the two systems. However, several potent inhibitors of tumor hexokinase, N-acetylglucosamine[2] and glucose-6-phosphate[1] when incubated with intact cells, exhibit no effect on ascites tumor glycolysis. Thus the location of hexokinase in the living cell and the ability of a specific compound to reach the active site of the enzyme, also must be considered in any comparison of the two systems. Finally, it must be realized that hexokinase in the intact cell is part of a balanced system and is totally dependent upon the uninterrupted operation of the whole glycolytic system for its supply of ATP and the removal of inhibitory end products. Even with these restrictions, however, there appears to be a relationship between the order of affinities of the two systems with most of the carbohydrates investigated. Glucosone, mannose and glucose demonstrate a strong affinity for tumor hexokinase as well as the glycolytic system in the intact cell. Mannoheptulose and glucosamine exhibit a moderate affinity for both systems and fructose and lyxose a weak affinity. Even though the K_m of glucosone is not low enough to explain completely its extremely potent inhibition of glycolysis in the intact cell, this compound has the highest affinity for tumor hexokinase of the 11 carbohydrates studied.

2-Deoxyglucose inhibits glycolysis of the ascites tumor cell to a greater extent than would be expected if affinity for hexokinase were the only factor determining

* The glucosone used by these authors[29] was the same material used in the present tumor hexokinase studies.

References p. 526.

this inhibition. In fact, 2-deoxyglucose at equimolar concentration of glucose, should exert little inhibition on glucolysis solely on the basis of its affinity for hexokinase, which is 22 % of that of glucose.

The inhibition of fructolysis in intact ascites tumor cells by galactose and 3-O-methylglucose is much greater than would be predicted from their affinities with hexokinase. A 4:1 ratio of galactose to fructose is reported to give approximately 50 % inhibition of fructolysis in the intact cell[1] whereas the ratio of Michaelis constants of tumor hexokinase for these two sugars is 70:1. Presumably the mode of inhibition of fructolysis by these two hexoses involves factors other than hexokinase.

The comparison of the properties of ascites tumor and brain hexokinases leads to the conclusion that the tumor enzyme is a typical mammalian hexokinase. As with the purified brain enzyme, competition experiments clearly demonstrate that a single enzyme in the purified tumor preparation is responsible for phosphorylation of all substrates tested. The similarity of the effect of certain carbohydrates on tumor hexokinase and the glycolytic system in the intact cell emphasize the important role hexokinase plays in the metabolism of neoplastic as well as normal tissues.

REFERENCES

1 W. D. YUSHOK, *Cancer Research* 18, pt. 2 (1958) 379.
2 W. D. YUSHOK AND W. G. BATT, *Am. Chem. Soc. Meeting, Atlantic City, Sept. 1956, Abstracts*, 22D.
3 W. D. YUSHOK AND W. G. BATT, *Am. Chem. Soc. Meeting, Miami, Apr. 1957, Abstracts*, 12D.
4 A. SOLS AND R. K. CRANE, *J. Biol. Chem.*, 210 (1954) 581.
5 R. K. CRANE AND A. SOLS, *J. Biol. Chem.*, 203 (1953) 273.
6 R. K. CRANE AND A. SOLS, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Academic Press, New York, Vol. I, 1955, p. 277.
7 F. B. CRAMER, *J. Franklin Inst.*, 253 (1952) 277.
8 M. T. HUDSON AND G. E. WOODWARD, *J. Franklin Inst.*, 264 (1957) 51.
9 R. B. MCCOMB AND W. D. YUSHOK, *J. Franklin Inst.*, 265 (1958) 417.
10 A. S. KESTON, *Am. Chem. Soc. Meeting, Dallas, Apr. 1956, Abstracts*, 31C.
11 A. L. DOUNCE, R. F. WITTER, K. J. MONTY, S. PATE AND M. A. COTTONE, *J. Biophys. Biochem. Cytol.*, 1 (1955) 139.
12 H. MICKLE, *J. Roy. Microscop. Soc.*, 68 (1948) 10.
13 P. M. NOSSAL, *Australian J. Expt. Biol. Med. Sci.*, 31 (1953) 583.
14 C. LAMANNA AND M. MALLETTE, *J. Bacteriol.*, 67 (1954) 503.
15 S. P. COLOWICK, in J. B. SUMNER AND K. MYRBACK, *The Enzymes*, Academic Press, New York, Vol. II, 1951, p. 138.
16 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
17 N. NELSON, *J. Biol. Chem.*, 153 (1944) 375.
18 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
19 R. B. MCCOMB AND W. D. YUSHOK, unpublished results.
20 E. BOYLAND, G. C. L. GOSS AND H. G. WILLIAMS-ASHMAN, *Biochem. J.*, 49 (1951) 321.
21 A. SOLS AND R. K. CRANE, *J. Biol. Chem.*, 206 (1954) 925.
22 W. D. YUSHOK, *Cancer Research*, 19 (1959) 104.
23 G. ACS, T. GARZO, G. GROSZ, J. MOLNAR, O. STEPHANECK AND F. B. STRAUB, *Acta Physiol. Acad. Sci. Hung.*, 8 (1955) 269.
24 B. CHANCE AND B. HESS, *Ann. N.Y. Acad. Sci.*, 63 (1956) 1008.
25 L. BERGER, M. SLEIN, S. COLOWICK AND C. CORI, *J. Gen. Physiol.*, 29 (1946) 379.
26 M. KUNITZ AND M. MCDONALD, *J. Gen. Physiol.*, 29 (1946) 393.
27 R. K. CRANE AND A. SOLS, *J. Biol. Chem.*, 210 (1954) 597.
28 H. WEIL-MALHERBE AND A. D. BONE, *Biochem. J.*, 49 (1951) 339.
29 M. T. HUDSON AND G. E. WOODWARD, *Biochim. Biophys. Acta*, 28 (1958) 127.