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A radioassay for the hexokinase reaction

Un radioensaye para la reacción hexoquinasa

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A method for the measurement of the hexokinase reaction using labelled glucose as a substrate is described. The radioactive product, glucose 6-phosphate, is adsorbed on Dowex-1 formate resin and subsequently eluted and counted. As little as 0.01 international milliunits can be easily detected under the conditions described.

RADIOASSAY HEXOKINASE

Most of the methods currently used for the assay of the hexokinase reaction (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1)

Glucose + ATP → Glucose 6-phosphate + ADP

are based on the spectrophotometric measurement of nicotinamide nucleotide reduction or oxidation brought about by auxiliary enzymes acting upon the products of the reaction (1, 2). The major advantages of these methods are that they are rapid, simple and reproducible. The main drawbacks, however, are the danger of high blank values due to contamination of the hexokinase sample and/or the auxiliary enzymes with NAD(P)-dependent hexose dehydrogenases (3), and a sensitivity not high enough for some purposes.

During a comparative study of the hepatic hexokinases of small vertebrates it became necessary for us to assay very low levels of the enzymes. A seemingly convenient procedure in this regard, that avoid most of the disadvantages mentioned above, was proposed several years ago by Sherman (4) and later modified by Newsholme *et al.* (5). The assay is based on the use of radioactive glucose as a substrate and subsequent separation on DEAE-cellulose filters of the labelled glucose 6-P[1] formed. This is possible since the substrate has no electrical charge, whereas the product is ionized even at low pH ($pK_1 = 0.94$). In our hands, however, the procedure was found unreliable because of the rather poor retention of glucose 6-P on the filters, and because of high blank counts due to charged impurities in the commercial radioactive glucose available. Both effects resulted in somewhat erratic values and in a decreased sensitivity of the assay.

The general idea behind the original procedure of Sherman (4) is, however, appealing, especially by the fact that the sensitivity of the assay is limited only by the specific radioactivity of the labelled substrate. We circumvented the above mentioned disadvantages by substituting the DEAE-cellulose filters by small Dowex 1 columns. The method has been in routine use in this laboratory during the last three years, and the results obtained in a comparative study of the hexokinase isoenzymes will be reported elsewhere (6). The present report describes the assay and illustrates its general applicability.

MATERIAL AND METHODS

Dowex 1 X-8, 200-400 mesh, was purchased from BioRad laboratories. [^{14}C]-D-glucose and [^{14}C]-D-glucose 6-P (both uniformly labelled) were from New England Nuclear. Crystalline hexokinase type C-301, NADP and ATP were purchased from Sigma. Glucose 6-P dehydrogenase was obtained from Boehringer. Bovine serum albumin was from Pentex. Microcrystalline DEAE-cellulose (DE-52) was a Whatman product. All other chemicals were of the purest grade commercially available. A male, adult, fed albino rat from the colony of this Department was used as the source of liver hexokinase isoenzymes.

[1]The abbreviations used are: glucose 6-P: glucose 6-phosphate; NADP: nicotinamide adenine dinucleotide phosphate; DEAE-cellulose: diethylaminoethyl-cellulose; EDTA: ethylenediaminotetracetic acid.

Enzyme assay

Glucose phosphorylating activity was measured in a medium containing (final concentrations): 80 mM Tris-HCl buffer pH 7.5, 1.6 mM EDTA, 12.6 mM $MgCl_2$, 10 mM ATP, 0.5 mM [^{14}C]-glucose (specific radioactivity: 1 μC/μmole) and enzyme in a final total volume of 0.5 ml. A similar system in which ATP was omitted served as a blank. After incubation at 30° the reaction was stopped by the addition of 1 ml of 100 mM unlabelled glucose in 0.1 N formic acid. The whole mixture was then applied to a small column (0.8 × 1 cm) of Dowex 1 formate resin (around 200 mg). Unreacted [^{14}C]-glucose was then washed out with three five-ml portions of 0.1 N formic acid. [^{14}C]-glucose 6-P was eluted with 2.5 ml of 0.4 M ammonium formate, pH 5.3. Usually 0.5 ml of the ammonium formate washing were plated, evaporated and counted in a Nuclear Chicago low-background gas flow counter at 20 per cent efficiency. Liquid scintillation counting was performed mixing 0.5 ml of the ammonium formate washing with 5 ml of a mixture of standard toluene-PPO-POPOP scintillation liquid and Triton X-100 (2:1 v/v). An Unilux spectrometer (Nuclear Chicago) was used at an efficiency of 60 per cent.

In experiments designed to study the working conditions and the reproducibility of the method crystalline yeast hexokinase was used. The activity was checked using the spectrophotometric method of Slein *et al.* (1). The incubation mixture was similar to that described above, except for the inclusion of 0.2 units of glucose 6-P dehydrogenase and 0.5 mM NADP. The increase in absorbance at 340 nm was followed in a Gilford 2400 recording spectrophotometer thermostatted at 30°. There was excellent agreement between the activities measured with the spectrophotometric and the radioassay.

Handling of the dowex columns

The Dowex columns were regenerated by succesive washings with 2 ml of a mixture of 3 N formic acid-0.5 M sodium formate, 2 ml of concentrated formic acid, and an excess of glass distilled water. The regeneration step can be conveniently performed in a manifold apparatus cheaply made with a disposable 7103 filter from Falcon Plastics, whose lid was perforated with a stopper puncher to support 10 columns. The filter apparatus was connected to a water pump to obtain a slight negative pressure (Fig. 1).

Separation of isoenzymes

The procedure for the preparation of the rat liver extracts, the centrifugation step and the treatment with CM-cellulose were exactly those described previously (7). An aliquot of 0.5 ml from the CM-cellulose-treated fraction was applied to a DEAE-cellulose column made with a B-D 2 ml syringe. Resolution of the isoenzymes was accomplished with a linear gradient from 0 to 0.5 M KCl in 0.01 M Tris-0.001 M EDTA buffer, pH 7. The total gradient volume was 34 ml. Fractions of 0.3 ml were collected in a Gilson fraction collector at 4°. The total content of every other tube was used for the enzyme assay.

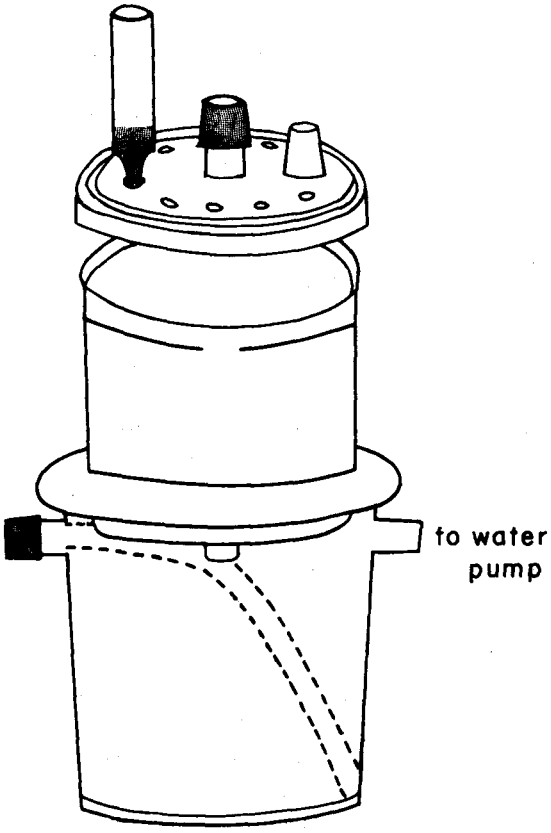

Fig. 1. An inexpensive manifold for the regeneration and washing of the Dowex columns. For simplicity only one column is shown in position.

RESULTS AND DISCUSSION

The sharp separation of [^{14}C]-glucose from [^{14}C]-glucose 6-P in the Dowex columns using the elution schedule described under *Enzyme assay* is illustrated in Table 1. After 15 ml of 0.1 N formic acid have passed through the columns, all the radioactivity from [^{14}C]-glucose was eluted and all the [^{14}C]-glucose 6-P remained absorbed. Ammonium formate on the other hand, released completely the phosphoric ester. Water can be used to elute the unreacted glucose but a charged radioactive impurity (about 0.3 per cent) is then retained on the resin, thus obscuring the glucose 6-P radioactivity, especially when low enzyme activities are assayed. This unidentified impurity can be washed off with 0.1 N formic acid.

A time curve using 10 ng (0.2 milliunits) of crystalline yeast hexokinase is shown in

TABLE I

SEPARATION OF [^{14}C]-GLUCOSE FROM [^{14}C]-GLUCOSE 6-P IN DOWEX 1 COLUMNS

Approximately 100,000 cpm of [^{14}C]-glucose (1 μCi/μmole), 7,000 cpm of [^{14}C]-glucose 6-P (1 mCi/μmole), or a mixture of both, were applied to separate columns of Dowex 1 formate resin. The columns were succesively washed five times with 3-ml portions of 0.1 N formic acid, and thrice with 1-ml portions of 0.4 N ammonium formate, pH 5.3. Aliquots of 0.5 ml from the washings were plated, evaporated and counted in a gas flow counter. Figures are total cpm.

Eluting agent	*Volume ml*	*Column 1* [^{14}C]-glucose *cpm*	*Column 2* [^{14}C]-*glucose 6-P cpm*	*Column 3 Mixture cpm*
Formic acid	3	93,924	21	98,454
Formic acid	3	2,322	20	2,730
Formic acid	3	504	18	462
Formic acid	3	120	14	90
Formic acid	3	38	14	18
Ammonium formate	1	14	5,776	5,704
Ammonium formate	1	11	1,152	1,364
Ammonium formate	1	7	82	148

Fig. 2. The progress of the reaction was linear at least up to 20 min. This is also true for as much as a tenfold increase in enzyme concentration.

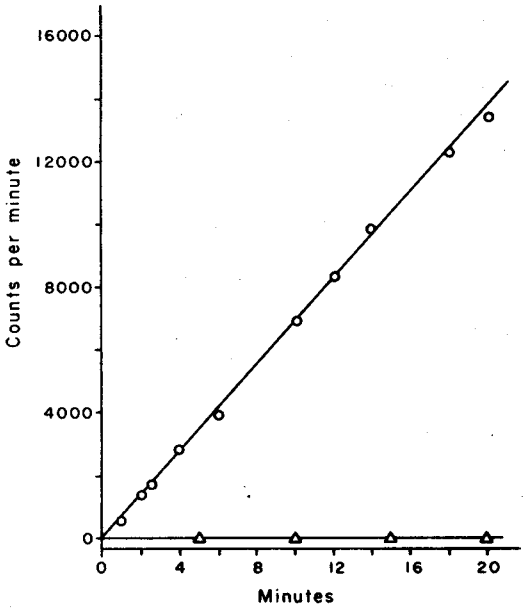

Fig. 2. A progress curve of the hexokinase reaction. The commercial ammonium sulfate suspension of enzyme was diluted immediately prior to use with an albumin solution (1 mg/ml) and incubated as described under *Enzyme assay.* After terminating the reaction at the indicated times the incubation mixtures were filtered through the Dowex columns. The washing step was performed with 15 ml of 0.1 N formic acid. Ammonium formate (2.5 ml) was then added to the columns and the effluent collected in test tubes. Aliquots (0.5 ml) were then plated, evaporated and counted. o, complete system; Δ, ATP omitted.

We have thus used 10 min as a convenient reaction time in the studies reported here.

An enzyme concentration curve using the same crystalline preparation is depicted in Fig. 3. As little as 0.5 ng of enzyme could be easily detected under the conditions employed. Even lower amounts than this may be detected if the specific radioactivity of the substrate is increased. This sensitivity is of the same order as that reported by Newsholme *et al.* (5) and represents an improvement of about threee orders of magnitude over the spectrophotometric techniques. Not more than 5 international milliunits of hexokinase should be used under the conditions described; higher amounts produce more glucose 6-P than our small Dowex columns can retain (about 0.15 μmoles), and in this case the assay must be performed during shorter times.

With the radioassay described it was easy to distinguish three low-K_m hexokinases in rat liver using as starting material as little as 200 mg of tissue (Fig. 4). The pattern obtained is indistinguishable from that seen in previous work using larger amounts of material (7).

As described, the method only measures the low-K_m hexokinase isoenzymes (8) but we have also used it to measure the high-K_m glucokinase, although the necessity of using very high concentrations of glucose (50 to 100 mM) makes the assay too expensive. The method

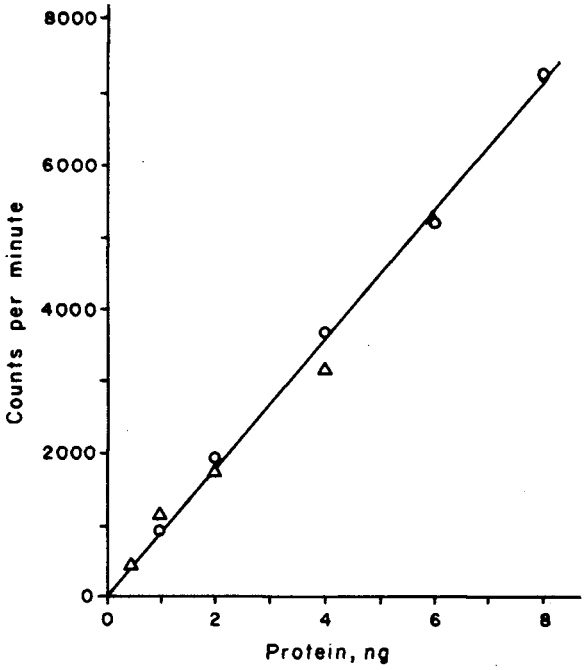

Fig. 3. The effect of hexokinase concentration. Crystalline hexokinase diluted in albumin solution was incubated for 10 min at the concentrations indicated and the tubes processed as described in the legend to Fig. 2. o and △ indicate replicate experiments performed in different days.

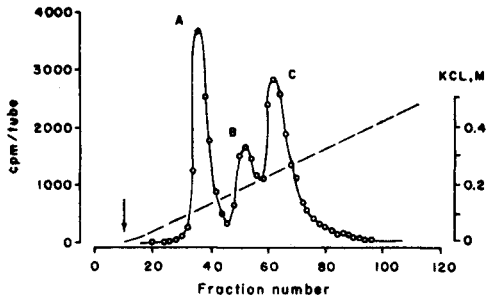

Fig. 4. DEAE-cellulose column chromatography of a crude extract from rat liver. To every other fraction 0.2 ml of a mixture containig the assay reagents (see under *Ensyme assay)* was added at zero time after 2 min preincubation at 30°. The tubes were then incubated during 10 min and processed as described in the legend to Fig. 2 except that liquid scintillation counting of the ammonium formate eluant was preferred. Enzyme activity is expressed as cpm/tube per minute of incubation. Control tubes in which either ATP or enzyme were omitted gave baseline values of about 60 cpm. The broken line represents the gradient concentration of KCl which started at the point indicated by the arrow. A, B and C correspond to the denomination of the three rat liver nexokinases (7).

has also been used to measure the phosphorylation of hexoses which cannot be converted enzymically to glucose 6-P as required in some spectrophotometric techniques. It should also prove convenient for the measurement of initial velocity without the drawbacks implicit in the use of auxiliary enzymes. It is clear however that precautions have to be taken to insure the absence of glucose 6-phosphatase activity which may hydrolyze the product.

While writing up this report we learned that Ashcroft and Randle (9) had used a similar procedure for the assessment of glucose phosphorylation in mouse islet extracts. However, the scarcity of experimental data provided in their paper precludes comparison with the present work.

RESUMEN

Se describe un método para la medición de la reacción de la hexoquinasa que utiliza como sustrato gluçosa marcada. El producto radioactivo, glucosa-6-fosfato, se absorbe en resina Dowex 1 formiato, y luego es eluido y contado. En las condiciones descritas pueden detectarse fácilmente cantidades de enzimas del orden de 0,01 miliunidades internacionales.

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