BBA 65542

A RADIOCHEMICAL ENZYMATIC ACTIVITY ASSAY FOR GLYCEROL KINASE AND HEXOKINASE

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(Received July 11th, 1966)
(Revised manuscript received October 11th, 1966)

SUMMARY

1. A simple radiochemical sampling assay for hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) and glycerol kinase (ATP:glycerol phosphotransferase, EC 2.7.1.30) has been developed. The paper describes tests carried out in order to show that this assay is valid for the accurate measurement of enzyme activity. Some of the problems associated with coupled assays of enzyme activity are not encountered with this radiochemical assay.

2. Radioactive substrate was incubated with enzyme for varying periods of time, and the reaction was stopped by addition of alcohol; other methods of stopping the reaction are discussed. Samples of the reaction mixture were pipetted on to discs of DEAE-cellulose paper and the substrate was removed by washing the paper discs with water. The anionic product remained adsorbed on the paper. The radioactivity on the paper, which is proportional to the enzyme activity, was measured in a liquid scintillation counter.

3. The measured rate of product formation by glycerol kinase or hexokinase was the same whether the product was determined radiochemically or enzymatically.

4. When [^{14}C]glucose with a specific activity of approx. 120 mC/mmole was used as substrate for hexokinase, the sensitivity of the radiochemical assay was higher than that of an assay depending on the fluorescence of pyridine nucleotides.

INTRODUCTION

The development of techniques for determining the activity of individual enzymes *in vitro* has played a large part in the recent rapid advance in knowledge of regulation and interrelationships of intermediary metabolism. Most of these methods are based on the enzymatic oxidation or reduction of the pyridine nucleotides (NAD^+ or $NADP^+$), and involve either spectrophotometric or fluorimetric measurements.

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These methods, however, are not applicable to all enzymes and, even if applicable, they may have serious limitations. Many enzymes do not react with pyridine nucleotides and cannot be coupled to reactions involving these nucleotides. In some cases, although the enzyme can be coupled to a reaction involving a pyridine nucleotide, the activity cannot be assayed by a direct spectrophotometric method at a physiological pH, because the reduction of the pyridine nucleotide is thermodynamically unfavourable *e.g.* glycerol kinase (ATP:glycerol phosphotransferase, EC 2.7.1.30). Some problems associated with enzyme activity measurements which involve coupled assay systems have been discussed by BERGMEYER[1]. It is probable that such problems could complicate the kinetic interpretation of enzyme activity measurements based on assays involving coupling enzymes, particularly if the activity of the coupling enzyme is low, or if the assay system contains three or more component enzymes.

A radiochemical assay for measuring the activity of kinase enzymes was described by SHERMAN[2]. However, this assay, as described by SHERMAN, was found to be completely inadequate for accurate measurement of the activities of hexokinase or glycerol kinase. From the paucity of experimental detail provided by SHERMAN it was not possible to understand why the assay did not function, and, therefore, a systematic investigation into this radiochemical assay was carried out. The details given in RESULTS describe the tests that were carried out in order to provide evidence that the radiochemical assay, which is described in this paper, is satisfactory for accurate measurement of enzyme activity. This is particularly important for glycerol kinase, for which no satisfactory alternative assay is available for investigating the kinetics of the enzyme at a physiological pH.

The radiochemical assay which was finally adopted depends on the principle which was originally described by SHERMAN[2]. After enzymic conversion, radioactive glycerol and α-glycerophosphate (or glucose and glucose 6-phosphate) were separated using DEAE-cellulose paper. The uncharged substrate was washed off the paper with water, while the anionic product remained adsorbed on to the paper. The radioactivity adhering to the paper was measured in a liquid scintillation counter giving an estimate of the enzyme activity. The advantages of this method and its possible significance as a general method of enzyme activity assay are discussed.

EXPERIMENTAL

Radiochemicals

[1-^{14}C]Glucose, [1-^{14}C]glycerol and [1-^{14}C]glucose 6-phosphate were obtained from Radiochemical Centre, Amersham.

α-[^{14}C]Glycerophosphate was prepared by the method (unpublished) of Dr. N. KUHN of this Department. 20 μC of [1-^{14}C]glycerol (500 μmoles) and 600 μmoles of ATP were converted to α-[^{14}C]glycerophosphate by the method of WIELAND[3]. α-[^{14}C]-Glycerophosphate was separated from the reaction mixture by differential barium precipitation (see UMBREIT, BURRIS AND STAUFFER[4]). The barium cation was replaced by sodium using an ion-exchange column.

Paper discs

DEAE-cellulose paper discs, 23 mm diameter, were obtained from Reeve Angel and Co. Ltd., London.

Filter holder

Pyrex hydrosol microanalysis filter holder of Millipore Filter Corp. Bedford (Mass.) catalogue No. XX 10 025 00, was obtained through V. A. Howe and Co. Ltd.

Scintillator

Toluene (sulphur free) was used as solvent. 2,5-Diphenyloxazole (3 g/l) and 1,4-bis-(5-phenyloxazol-2-yl) benzene (0.1 g/l) were used as the primary and secondary scintillators, respectively.

Seroblock

Seroblock (model 13, Precision Scientific Co., Chicago, U.S.A.) was obtained from Scientific Techniques Ltd., London.

Chemicals and enzymes

Glycerol kinase, (ATP:glycerol phosphotransferase, EC 2.7.1.30); hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1); glucose 6-phosphate dehydrogenase (D-glucose-6-phosphate:$NADP^+$ oxidoreductase, EC 1.1.1.49); glycerophosphate dehydrogenase (L-glycerol-3-phosphate:NAD^+ oxidoreductase, EC 1.1.1.8); phosphoenolpyruvate (tricyclohexylammonium salt), NADH, $NADP^+$, ATP and Tris were obtained from C. F. Boehringer and Soehne G.m.b.H., Mannheim, Germany. Bovine plasma albumin (Fraction V) was obtained from the Armour Pharmaceutical Co. Ltd., Eastbourne.

Incubation buffers

The incubation buffer for glycerol kinase usually consisted of 83 mM Tris, 1.6 mM EDTA, 22 mM NaF, 33 mM mercaptoethanol, 5 mM ATP, 5 mM $MgSO_4$ and 4 mM [1-^{14}C]glycerol (specific activity approx. 10 μC/ml) at pH 7.5. The incubation buffer for hexokinase usually contained 50 mM Tris, 1 mM EDTA, 20 mM mercaptoethanol, 10 mM ATP, 10 mM $MgSO_4$ and 5 mM [1-^{14}C]glucose (specific activity approx. 10 μC/ml), at pH 7.5. Incubations were carried out at 25° or 28° in a seroblock.

Incubation procedure

Samples (100 μl) of incubation medium were pipetted into small polystyrene tubes in a seroblock, and enzyme solution (20 μl) was transferred to the fine end of a small disposable Pasteur pipette and blown into the incubation medium at zero time. After incubation 100 μl of 98% ethanol were injected into the tubes which were removed from the seroblock. Samples (20 μl) of the solution from these tubes were carefully pipetted on to the centres of discs of DEAE-cellulose paper which were held at one edge between two glass plates. It was important that the 20 μl of solution remained as discrete circles in the centre of the paper discs, and did not approach the edges. The papers were allowed to dry at room temperature (20–30 min) and were then washed on a Pyrex hydrosol microanalysis filter holder with a suitable volume of water (100 or 200 ml). In most cases one or two discs of unused DEAE-cellulose paper were placed underneath the radioactive discs on the filter holder prior to washing. This improved the retention of the [^{14}C]glucose 6-phosphate or α-glycerophosphate (see Table I). After washing, the papers were oven-dried (90°) for approx. 30 min, dropped into vials containing scintillator fluid and counted in an IDL Tritomat

TABLE I

EFFECT OF WASHING ON RETENTION OF [^{14}C]GLUCOSE, [^{14}C]GLYCEROL, α-[^{14}C]-GLYCEROPHOSPHATE AND [^{14}C]-GLUCOSE 6-PHOSPHATE ON DEAE-CELLULOSE PAPER DISCS

Samples (20 μl) of [^{14}C]glycerol (0.040 μmole, specific activity approx. 5.0 μC/ml), [^{14}C]glucose (0.050 μmole, specific activity approx. 1.6 μC/ml), α-[^{14}C]glycerophosphate (0.043 μmole, specific activity approx. 0.07 μC/ml) and [^{14}C]glucose 6-phosphate (0.050 μmole, specific activity approx. 1.2 μC/ml), which were contained in a mixture of assay buffer *plus* an equal volume of 98% ethanol, were pipetted on to one disc of DEAE-cellulose paper and allowed to dry at room temperature. This disc was then placed on the filter holder, either alone or on top of one or two unused discs of DEAE-cellulose paper. The discs were then washed, dried and counted as described in EXPERIMENTAL. When two or three papers were used they were always counted together in one scintillation vial. The [^{14}C]glucose 6-phosphate and α-[^{14}C]glycerophosphate always contained small quantities of [^{14}C]glucose and [^{14}C]glycerol, respectively. Therefore the values for the zero washing volume are calculated by adding together the counts/min retained by three papers after washing and the counts/min retained from the filtrate by three fresh DEAE-cellulose paper discs.

Number of unused paper discs included in washing	*Volume of water for washing*	*Counts/min*				*Per cent retention*			
		Glycerol	*Glucose*	*α-Glycero-phosphate*	*Glucose 6-phosphate*	*Glycerol*	*Glucose*	*α-Glycero-phosphate*	*Glucose 6-phos-phate*
0	0	112 604	37 836	1940	33 209	—	—	—	—
	50	1 120	313	1694	30 022	0.99	0.83	87.3	90.4
	100	885	223	1656	29 197	0.79	0.59	85.4	87.9
1	0	111 118	38 348	1940	33 209	—	—	—	—
	50	1 328	305	1797	30 435	1.19	0.79	92.6	91.6
	100	948	220	1854	31 111	0.85	0.57	95.6	93.7
	200	536	143	1807	31 479	0.48	0.37	93.1	94.8
2	0	111 369	39 658	1940	33 209	—	—	—	—
	50	1 214	326	1867	32 297	1.09	0.82	96.2	97.3
	100	669	218	1869	32 876	0.60	0.55	96.9	99.0
	200	309	161	1878	32 429	0.28	0.41	96.8	97.7

scintillation counter model 6020 to a minimum of 10 000 counts. If three papers were used in the washing process they were counted together in one scintillation vial. The position of the papers relative to one another in the vial was important, as was the position of the papers in relation to the phototubes of the scintillation counter. The maximum count rate was obtained if the uppermost radioactive paper was placed in the centre of two other papers in the counting vial and the faces of the paper discs were perpendicular to the plane of the phototubes of the scintillation counter.

A zero-time count rate was obtained by injecting 100 μl of alcohol into a tube prior to addition of the enzyme and counting in a similar manner to the other solutions. This count rate is presumably due to ^{14}C-labelled substrate retention on the DEAE-cellulose paper. This rate is subtracted from the count rates obtained after incubation with the enzyme, so that the progress curve passes through the origin.

Glucose 6-phosphate was measured by the method of NEWSHOLME AND RANDLE[5] and α-glycerophosphate was measured by the method of HOHORST[6].

METHODS AND RESULTS

The amount of [^{14}C]glucose 6-phosphate or α-[^{14}C]glycerophosphate retained on the discs of DEAE-cellulose paper was directly proportional to the concentration of the phosphorylated compound pipetted on to the disc of DEAE-cellulose paper (up

to 100 mμmoles). It was found that up to 40 μl of radioactivity could be pipetted on to a single disc of DEAE-cellulose paper without overloading the paper provided two unused papers were included in the washing procedure.

As the ability of the DEAE-cellulose paper to retain the radioactive organic phosphates depends largely on ionic binding, the presence of other anions could compete with the organic phosphates for the binding sites on the DEAE-cellulose paper. In all experiments reported in this paper the concentration of anions was not sufficient to reduce the retention of the radioactive organic phosphates. However, it was observed that ATP concentrations of 20 mM and 40 mM decreased the retention of radioactivity (α-[^{14}C]glycerophosphate) by 15 and 40% respectively, whereas concentrations up to 10 mM had no effect. Similarly, varying the pH from 5 to 9 had no effect, but at pH 9.9 and pH 10.4 the retention of radioactivity was decreased by 11 and 22% respectively. Thus in any experiment in which reasonably high concentrations of anions are to be used, their effect on the binding of the radioactive anion must be investigated. However, the percentage loss of radioactivity appears to be constant and could therefore be taken into account in an estimation of enzyme activity.

Retention of radioactivity on DEAE-cellulose paper discs

Samples of radioactivity were pipetted onto one paper, which was washed with varying volumes of water, either alone or with one or two unused DEAE-cellulose paper discs placed underneath the radioactive disc on the filter-holder. As shown in Table I the number of paper discs makes little difference to the amount of [^{14}C]glucose or [^{14}C]glycerol adsorbed after washing with 200 ml of water: approx. 99.7% is removed by the washing procedure. However, increasing the number of paper discs increases the retention of the [^{14}C]glucose 6-phosphate and α-[^{14}C]glycerophosphate; approx. 97% of the radioactivity is retained on the paper discs when two unused DEAE-cellulose paper discs are included in the washing procedure as described in the EXPERIMENTAL section. The rate of washing with water was approx. 30 ml/min; it was observed that increasing the rate (*e.g.* 60 ml/min) decreased the efficiency of removal of [^{14}C]glycerol from the DEAE-cellulose paper. Thus, it can be seen that a very high degree of differential separation of [^{14}C]glucose and [^{14}C]glucose 6-phosphate, or [^{14}C]glycerol and α-[^{14}C]glycerophosphate, is obtained by this simple, rapid procedure. This degree of separation is of fundamental importance to this radiochemical assay.

Inhibition of enzyme reaction

The usual means of rapid inhibition of enzyme activity (*e.g.* the addition of protein precipitants) were found unsuitable for the radiochemical assays. The use of $Ba(OH)_2$ and $ZnSO_4$, $HClO_4$ or trichloroacetic acid, all resulted in poor recoveries of α-[^{14}C]glycerophosphate or [^{14}C]glucose 6-phosphate. This could be due to either competition for binding sites in the DEAE-cellulose paper by the anions added with the precipitant, or, in the case of $Ba(OH)_2$ and $ZnSO_4$, adsorption of the organic phosphate on to the precipitate. Therefore, ethanol was used; an equal volume of 98% ethanol completely inhibited both hexokinase and glycerol kinase activities, and resulted in a linear progress curve (Fig. 1). Other organic solvents were tried and both acetone and *n*-butanol were inhibitory, while diethylether only partially inhibited the enzymes. In the case of butanol, which is immiscible with water, the sample for counting had to be pipetted from the bottom aqueous layer in the tube. This had the

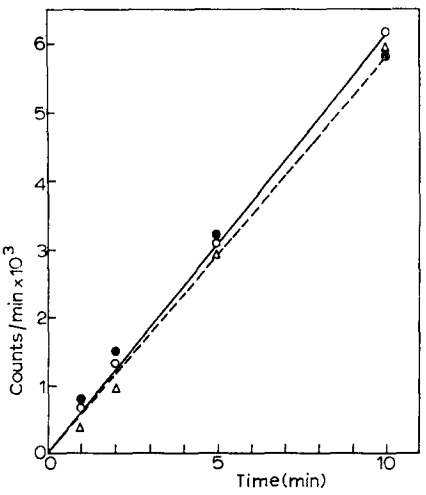

Fig. 1. Inhibition of glycerol kinase and hexokinase. Samples (20 μl) of enzyme solutions (see Fig. 2) were incubated with 100 μl of incubation buffer at 25°. ○—○, glycerol kinase inhibited by addition of an equal volume of ethanol to tubes incubated for various periods of time; △— —△, hexokinase inhibited by addition of an equal volume of ethanol to tubes incubated for various periods of time. In both cases 20-μl samples were pipetted onto DEAE-cellulose paper discs and treated as described in the EXPERIMENTAL section. ●—●, glycerol kinase inhibited by removal of samples from one incubation tube at various time intervals, and immediately pipetted onto DEAE-cellulose paper discs, which were maintained at −70° by placing the paper discs on solid CO_2. The cold papers were placed on the filter holder and washed rapidly with distilled water. Subsequent procedures as described in EXPERIMENTAL.

advantage of doubling the count rate obtained in comparison with the use of acetone or alcohol.

It was shown that samples of the incubation mixture could not be pipetted directly on to the DEAE-cellulose paper, because the enzyme was not inactivated. However, this was possible if DEAE-cellulose paper discs were kept at −70° on solid CO_2 and the sample pipetted onto this chilled paper. Under these conditions the sample was rapidly frozen on the DEAE-cellulose paper. Subsequent rapid washing of the paper on the filter holder, followed by the usual drying and counting procedures, produced a linear progress curve; and this could not be distinguished from that produced by inhibition of the enzyme with alcohol (see Fig. 1). Presumably the substrate was removed by the washing process too rapidly to permit enzymatic reaction. In all experiments reported in this paper the enzyme activity was stopped by addition of alcohol. This process proved simpler than the freezing procedure, and more accurate timing of inhibition was obtained by the rapid injection of alcohol. However, in certain cases, *e.g.* the use of a very expensive isotope, the freezing method may offer some advantage.

Radiochemical assay and enzyme concentration

The effects of increasing the concentrations of commercial preparations of hexokinase and glycerol kinase on the enzyme activity assay, as measured by radioactivity adsorbed on the DEAE-cellulose paper, are shown in Fig. 2. For both enzymes the

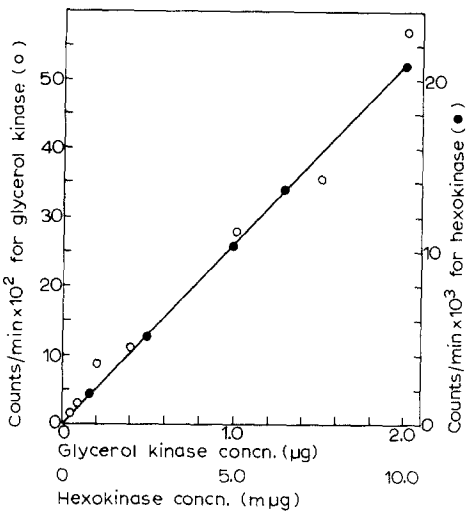

Fig. 2. Effect of concentration of glycerol kinase and hexokinase on rate of radioactivity retention on DEAE-cellulose paper discs. The commercial $(NH_4)_2SO_4$ suspensions of glycerol kinase and hexokinase were diluted just prior to use with albumin solution (2 mg/ml). Samples (20 μl) of each concentration were incubated with 100 μl of incubation medium at 25°, and the reaction inhibited by addition of alcohol. At each concentration of enzyme a progress curve was plotted and the activity calculated as radioactivity retained per min. The activity is plotted as countr/min per ml. ○--○, glycerol kinase; ●—●, hexokinase.

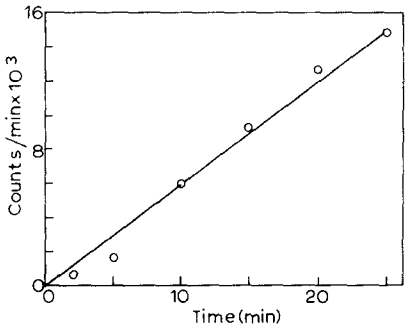

Fig. 3. Progress curve for low concentrations of hexokinase. The commercial $(NH_4)SO_4$ suspension of hexokinase was diluted just prior to use with albumin solution (2 mg/ml). Samples (20 μl) of this solution containing 50 $\mu\mu$g of enzyme were incubated with 100 μl of incubation medium at 25°, and the reaction inhibited with alcohol. Subsequent procedures as described in EXPERIMENTAL.

plots obtained are linear. The concentration range for glycerol kinase was 0.2–2 μg of enzyme per assay tube, that for hexokinase was 0.5–10 mμg per assay tube. The difference in sensitivity reflects the use of [^{14}C]glucose with a much higher specific activity than was possible with glycerol. Hexokinase activity was measured without any difficulty at a concentration of 50 $\mu\mu$g of enzyme per assay tube (Fig. 3).

TABLE II

MEASUREMENT OF PRODUCT FORMATION BY RADIOCHEMICAL AND ENZYMATIC METHODS

Samples (20 μl) of the enzyme were incubated with 100 μl of incubation buffer and at various time intervals the reactions were stopped by addition of 100 μl of 98% ethanol. Samples (20 μl) were pipetted on to DEAE-cellulose paper and washed dried and counted as described in EXPERIMENTAL. Samples (50 μl) were taken for enzymatic assay. See EXPERIMENTAL for details.

Time of incubation (min)	*mμmoles α-glycerophosphate produced*		*mμmoles glucose 6-phosphate produced*	
	Radiochemical assay	*Enzymatic assay*	*Radiochemical assay*	*Enzymatic assay*
1	42	42	124	123
2	80	66	198	192
5	196	193	385	376
10	348	358	388	381

Comparison of activity measured radiochemically and enzymatically

Glycerol kinase and hexokinase were incubated for various periods of time with [^{14}C]glycerol and [^{14}C]glucose, respectively. The radioactive products of the reactions were measured both radiochemically and enzymatically, and the results are shown in Table II. These two procedures produced results that are in extremely good agreement, which indicates that the radiochemical method can measure accurately the concentration of the product of the enzyme reaction.

DISCUSSION

The main drawback of a sampling assay, whether radiochemical or not, is that there is a considerable expenditure of time in measurement of the enzyme activity. An advantage of the radiochemical assay described in this paper is the relative simplicity of the sampling and estimation procedures, which minimizes the amount of work involved in any given assay. Moreover, apart from a liquid scintillation counter, no major equipment is required, and the assays are easily performed by technical assistants.

Another advantage that this radiochemical enzymatic assay may offer is a high degree of sensitivity; this is obviously dependent upon the specific activity of the substrate. At a specific activity of glucose of 120 mC/mmole it was possible, without any difficulty, to measure the activity of 50 $\mu\mu$g of hexokinase which had a specific activity of 45 μmoles/mg per min. This sensitivity is approximately two orders of magnitude greater than is possible with fluorimetric techniques measuring the change in fluorescence due to oxidation or reduction of pyridine nucleotides. Using the fluorescent technique described by DALZIEL[7], the recorder could give a full scale (10 inch) response to 0.1 μM NADH with a noise level corresponding to 0.005 μM NADH (K. DALZIEL, personal communication).

The combined properties of sensitivity and simplicity suggest that this radiochemical type of assay might be of value in measuring enzyme activities that are not easily assayed by other means. A radiochemical assay would be theoretically possible for any enzyme provided that the radioactive substrate could be separated from the product on DEAE-cellulose paper, preferably by a simple washing technique on a filter holder as described above. Moreover, the availability of discs of CM-cellulose paper and cellulose phosphate paper increases the range of enzymes whose activities could be assayed in such a manner.

ACKNOWLEDGEMENTS

We thank Professor Sir HANS KREBS, F.R.S., for his interest and encouragement. J.R. is a recipient of a Medical Research Council Training Scholarship. This work was aided by a grant from the U.S. Public Health Service AM 08715.

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