Radiochemical methods for the assay of phosphorylase in the direction of glycogenolysis

Greet GEVERS and Willy STALMANS

Afdeling Biochemie, Faculteit Geneeskunde, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

(Received 4 September 1980/Accepted 7 October 1980)

Two radiochemical procedures were explored for the determination of phosphorylase activity in the glycogenolytic direction. In the ' ^{32}P assay method' the formation of labelled glucose 1-phosphate from glycogen and $^{32}\text{P}_i$ is measured by the radioactivity that remains soluble after the precipitation of phosphomolybdate with triethylamine. In the ' ^{14}C assay method' the formation of labelled glucose 1-phosphate from peripherally ^{14}C -labelled glycogen and P_i is determined from the radioactivity that remains soluble after the precipitation of glycogen with ethanol. The ^{14}C assay method requires more preparative work but less circumspection than does the ^{32}P assay method. Both radiochemical methods can be applied where the classical spectrophotometric assay fails. They have the same accuracy and reproducibility, and allow more samples to be handled in parallel. They are not intended for use with crude tissue extracts.

The assay of glycogen phosphorylase (EC 2.4.1.1) is routinely performed in the non-physiological direction of glycogen synthesis. To evaluate the role of phosphorylase *b* in hepatic glycogenolysis we have recently attempted to define the relative activities of the two enzyme forms in simulated cellular conditions (Gevers & Stalmans, 1980). This approach involves the determination of glucose 1-phosphate produced by the phosphorolysis of glycogen. In the classical method phosphoglucomutase (EC 2.7.5.1) transforms the generated glucose 1-phosphate into glucose 6-phosphate, which is in turn oxidized by glucose 6-phosphate dehydrogenase (EC 1.1.1.49) with concomitant reduction of NADP^+ (Helmreich & Cori, 1964a; Maddaiah & Madsen, 1966). Several aspects, however, rendered the coupled spectrophotometric assay unsuitable for our current purposes. The milky appearance of α -particulate glycogen, which is the natural substrate for the liver enzyme, is incompatible with conventional spectrophotometry. Other drawbacks of the assay are intrinsically related to the use of auxiliary enzymes. The possible effect of glucose 6-phosphate, a known ligand of phosphorylase (Morgan & Parmeggiani, 1964), cannot be evaluated; the same applies to additions such as EDTA, NADP^+ and Mg^{2+} that are required for the secondary enzymes. Also, in the case of an apparent inhibition of phosphorylase by some experimentally added substance, it must be established that neither

of the auxiliary enzymes has in fact become rate-limiting. The present paper describes two radiochemical assay methods that do not suffer from those limitations, and yield satisfactory results with purified muscle phosphorylase.

Materials and methods

Chemicals, enzymes and analytical methods

Triethylamine from Baker Chemicals (Deventer, The Netherlands) was made 0.1M in water and adjusted to pH 5 with HCl. The mixture for precipitation of P_i consisted of 5 vol. of the former solution and 4.5 vol. of 67 mM-ammonium molybdate. $[\text{U-}^{14}\text{C}]\text{Glucose 1-phosphate}$ and $^{32}\text{P}_i$ were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Before use, samples (10 μCi) of the labelled phosphate were heated for 15 min at 100°C in 100 μl of 1M-HCl to hydrolyse polyphosphates, neutralized with a molar equivalent of NaOH, and diluted as required with unlabelled potassium phosphate, pH 7.4. Particulate glycogen was purified from dog liver by the phenol method (Laskov & Margoliash, 1963). Shellfish glycogen was purchased from E. Merck (Darmstadt, W. Germany), and Lumagel for liquid-scintillation counting from Lumac (Basel, Switzerland). All other chemicals were the purest grade available from various suppliers.

Amyloglucosidase (EC 3.2.1.3), phosphogluco-

mutase and glucose 6-phosphate dehydrogenase were obtained from Boehringer (Mannheim, W. Germany). Crystalline phosphorylase *b* was prepared from rabbit skeletal muscle by the method of Fischer & Krebs (1958), with slight modifications (De Lange *et al.*, 1968). One unit of phosphorylase *b* converts 1 μmol of substrate/min when assayed at 30°C as described for the liver enzyme (Stalmans & Hers, 1975), except for the omission of sulphate.

Glucose was determined by the glucose oxidase method (Dahlqvist, 1961). Glycogen was measured as glucose after hydrolysis by amyloglucosidase (Hue *et al.*, 1975). The enzymic assay of glucose 1-phosphate was performed as described by Bergmeyer & Michal (1974). Protein concentration was determined from absorbance at 280 nm.

Preparation of ^{14}C -labelled glycogen

A solution was prepared containing 4 g of particulate liver glycogen (22 mmol of glucosyl units), 100 μmol of AMP and 2 mmol of [^{14}C]glucose 1-phosphate (10^8 c.p.m.) at pH 6.1 in a volume of 100 ml. The mixture was warmed to 30°C, and after the addition of 400 units (10 mg) of muscle phosphorylase *b* it was incubated for a further 10 min. The reaction was arrested by the addition of 25 ml of 10% (w/v) trichloroacetic acid. The mixture was chilled and further diluted with 250 ml of cold water. Glycogen was precipitated by the addition of 750 ml of ethanol, recovered by centrifugation in the cold and dissolved in a minimum volume of water. The glycogen solution was dialysed against 10 litres of water for 8 h periods until the radioactivity of the diffusate became undetectable, and was then adjusted to a concentration of 100 mg/ml.

The incubation period with phosphorylase in the above procedure was established after a small-scale experiment where both the appearance of P_i and the incorporation of label into glycogen were followed. After 3 min the reaction had proceeded to about 10% from equilibrium, which was reached when 80% of the glucose 1-phosphate had been converted into P_i in the specified conditions. Further catalysis at equilibrium was allowed until 10 min, when 83% of the label had been incorporated into glycogen. A new constant specific radioactivity of glucose 1-phosphate was thereby reached, as evidenced by the fact that after 25 min of incubation the amount of label in the glycogen had not further changed. Since approx. 7% of the total glucosyl residues in particulate glycogen are non-reducing terminals (Laskov & Margoliash, 1963; Parodi *et al.*, 1967), the procedure as described results in an average elongation of the external branches with 1.0 glucosyl unit. Four stocks of labelled particulate glycogen have been prepared in this way.

The same labelling pattern was also observed in one preparation where shellfish glycogen was used.

Results and discussion

Phosphorolysis of glycogen with [^{32}P] P_i

Principle and standard assay procedure. Glycogen phosphorylase produces glucose 1- ^{32}P phosphate on incubation with [^{32}P] P_i and glycogen. P_i is then converted into phosphomolybdic acid, which is quantitatively precipitated with triethylamine (Sugino & Miyoshi, 1964). The initial velocity of the enzymic reaction is easily determined from the radioactivity that remains soluble, since the specific radioactivities of P_i and glucose 1-phosphate are necessarily identical.

Purified glycogen phosphorylase is incubated at 30°C in a final volume of 0.5 ml, containing 5 mg of hepatic particulate glycogen and 2.5 μmol ($125\,000$ c.p.m.) of potassium [^{32}P]phosphate at pH 7.4 (standard assay condition); other additions (e.g. salts or AMP, at pH 7.4) are specified when required. After 5 and 10 min of incubation a sample of 0.2 ml is transferred to tubes at 0°C containing 50 μl of 4 M-HClO₄ (and unlabelled P_i as indicated). Subsequently 0.95 ml is added of a cold precipitation mixture, containing 30 μmol of ammonium molybdate and 50 μmol of triethylamine (see the Materials and methods section). The tubes are kept at 0°C for a further minimum period of 10 min. Subsequent centrifugation in the cold for 10 min at 8000 g yields a yellow sediment and a clear colourless supernatant, of which 0.6 ml is withdrawn and mixed with 10 ml of Lumagel for liquid-scintillation counting.

Precipitation of P_i . In order to accommodate a useful range of phosphate concentrations to the assay of phosphorylase, we were forced to use some 5-fold more molybdate and triethylamine than previously recommended (Sugino & Miyoshi, 1964). The characteristics of the precipitation method were investigated on 0.2 ml samples of assay mixture without enzyme. As shown in Fig. 1, the optimal amount of phosphate per sample ranges from 2.5 to 12 μmol , of which only about 0.03% was not precipitated. A sharp rise in unprecipitated label occurred between 14 and 15 μmol of P_i , in agreement with the proposal (Sugino & Miyoshi, 1964) that the insoluble complex consists of phosphate, molybdate and triethylamine in the molar proportions 1:2:3. The blank values increased also progressively when the amount of P_i was below 2.5 μmol per sample (Fig. 1). We observed that the latter problem was adversely affected by glycogen (Fig. 2). At 1 μmol of P_i per sample, an increasing fraction of the label remained unprecipitated when the amount of particulate glycogen was raised

Fig. 1. Effect of phosphate concentration on the precipitation of labelled phosphate

Samples (0.2 ml) of assay mixture, containing 4 mg of particulate glycogen, 50000 c.p.m. of $[^{32}\text{P}]P_i$, and the indicated amounts of unlabelled P_i , were treated with HClO_4 and precipitation mixture. After centrifugation, the radioactivity was measured in the supernatant. Details were as described in the text. Each point shows the mean for five non-simultaneous determinations. Vertical bars, indicated where the scale permits, represent $\pm$ s.e.m.

Fig. 2. Effect of glycogen concentration on the precipitation of P_i

Samples (0.2 ml) of assay mixture, containing 50000 c.p.m. of $[^{32}\text{P}]P_i$, 1 μmol (○) or 5 μmol (●) of unlabelled P_i and the indicated amounts of particulate glycogen were treated as described in the legend to Fig. 1. Means $\pm$ s.e.m. for three non-simultaneous experiments are shown.

between 4 and 25 mg. The interference of glycogen was attenuated, but clearly not abolished, by a 5-fold increase in P_i concentration. Shellfish glycogen behaved similarly (results not shown). We have no explanation for these phenomena.

It appears thus that the assay procedure for phosphorylase as described above tolerates up to 60 mM- P_i and 30 mg of glycogen/ml. If higher substrate concentrations are required, the size of the sample for assay of labelled glucose 1-phosphate should be decreased, and the original sensitivity may be restored by a proportionate increase in the specific radioactivity of P_i . There is no lower limit for the concentration of phosphate; a rise in the blank value (Fig. 1) is effectively prevented by the supplementary addition of unlabelled phosphate at the end of the incubation (e.g. 2–5 μmol in the HClO_4 solution).

Normal blank values were observed when 'standard' assay mixtures were enriched with 0.15 M of various salts [KF , KCl , Na_2SO_4 , $(\text{NH}_4)_2\text{SO}_4$]. However, potassium acetate interfered, and from 0.45 M onwards inhibited entirely

the precipitation of phosphate (results not shown). The latter inhibition was pH-dependent; complete removal of phosphate was again obtained in the presence of even 1 M-acetate if more HClO_4 was added, so as to keep the pH of the final supernatant below 2.0. These observations have a bearing on work with liver phosphorylase *b*, which is commonly assayed in the presence of lyotropic salts at concentrations up to 0.7 M (Appleman *et al.*, 1966; Stalmans & Hers, 1975; Lederer & Stalmans, 1976).

Potential sources of error. Co-precipitation of glucose 1-phosphate with P_i does not occur; $[^{14}\text{C}]$ glucose 1-phosphate (2 or 20 mM) added to 'standard' reaction mixtures was completely recovered in the final supernatant. Another possible hazard, i.e. acid hydrolysis of the generated glucose 1- $[^{32}\text{P}]$ phosphate, would cause an underestimation of phosphorylase activity. To investigate the importance of the latter phenomenon, about 10% of substrate was converted into product by incubation with phosphorylase. After addition of HClO_4 and precipitation mixture, samples were

kept at 0°C for 10 min to 2.5 h before centrifugation. Since identical radioactivities were measured at any time, hydrolysis of the labelled product is not a practical problem in these conditions. Underestimation of phosphorylase activity might also occur on the use of crude enzyme preparations: conversion of labelled glucose 1-phosphate into other metabolites would not interfere, unless hydrolysis would liberate [^{32}P]P_i. This is bound to happen for instance with liver preparations that contain glucose 6-phosphatase. Data on the accuracy and the reproducibility of the ^{32}P assay method are shown below (see under 'Comparison of different methods').

Phosphorolysis of peripherally ^{14}C -labelled glycogen

Principle and standard assay procedure. Phosphorylase produces [^{14}C]glucose 1-phosphate from ^{14}C -labelled glycogen and unlabelled phosphate. Acid aqueous ethanol is used to separate soluble glucose 1-phosphate from insolubilized glycogen. The general composition of the standard assay mixture is as described above for the ^{32}P assay method, except for the use of the alternative labelled substrate: the standard mixture (0.5 ml) contains 5 mg (75 000 c.p.m.) of [^{14}C]glycogen, 2.5 μmol of unlabelled potassium phosphate, pH 7.4, and purified

enzyme. After 5 and 10 min of incubation a sample of 0.2 ml is transferred to tubes at 0°C containing 50 μl of 10% (w/v) trichloroacetic acid. Subsequently 0.5 ml of cold ethanol is added, and glycogen is packed by centrifugation for 5 min at 8000 g. The radioactivity in 0.6 ml of supernatant is measured as before. The initial reaction rate is calculated from the latter value and the specific radioactivity of the generated glucose 1-phosphate (see below).

Substrate properties of the labelled glycogen. The assay procedure is only valid inasmuch as glucose 1-phosphate is produced at a constant specific radioactivity. Since we use peripherally labelled glycogen, it is obvious that the latter requirement will only temporarily be met. We have used arsenolysis of glycogen by phosphorylase (Katz & Hassid, 1951; Helmreich & Cori, 1964b) to determine the practical limits of the assay. Since glucose 1-arsenate decomposes spontaneously to glucose and the substrate arsenate, the reaction is irreversible and the concentration of arsenate remains constant. Fig. 3 illustrates the analysis of one preparation of labelled glycogen (10 mg/ml). The time-dependent production of glucose proceeded linearly until a concentration of about 2 mM was reached (Fig. 3a). The subsequent decrease in the

Fig. 3. Analysis of peripherally ^{14}C -labelled glycogen by arsenolysis

In a final volume of 3 ml, 30 mg of labelled particulate glycogen (5.3×10^5 c.p.m.), 3 μmol of AMP and 150 μmol of sodium arsenate, pH 7.4, were incubated at 30°C with 4 units (0.1 mg) of muscle phosphorylase b. (a) At the indicated times, samples (0.2 ml) were withdrawn and each was mixed with 0.4 ml of cold ethanol containing 0.15 M-HCl. The supernatant after centrifugation was neutralized and assayed for glucose (●) and radioactivity (○). (b) The specific radioactivity of glucose is obtained from a replot of the data from (a).

rate of glycogenolysis results probably from a progressive inhibition of phosphorylase *b* by the increasing glucose concentration (Gevers & Stalmans, 1980). The liberation of radioactivity followed initially a time course similar to glucose production, but slowed more pronouncedly from 1 h onwards (Fig. 3a). As a result, the specific radioactivity of glucose remained constant up to a level of 90 000 c.p.m./ml, corresponding to 3.2 mM-glucose (Fig. 3b). This means that of the total label up to 50%, contained in less than 6% of the glycogen mass, was released by phosphorylase at constant specific radioactivity.

Four labelled preparations of α -particulate glycogen and one of shellfish glycogen have been analysed in this way, with similar results. The initial specific radioactivity of the liberated glucose ranged from 60 to 69% of that of the glucose 1-phosphate used for chain elongation.

Potential sources of error. Blank values obtained from 'standard assays' in the absence of enzyme amounted to 81 ± 4 c.p.m. (mean $\pm$ S.E.M.; $n = 18$). They were not affected by the addition of various salts. These blank values are small compared with the upper limits of the assay. From the analytical results described above it follows that in the presence of a sufficiently high phosphate concentration up to 12 000 c.p.m. can be actually measured within the linear zone of the assay. Although in 'standard' conditions the concentration of P_i rather than glycogen is limiting, we have observed that

even then up to 2000 c.p.m. can be measured without deviation from the initial reaction rate. It has also been checked that added glucose 1-phosphate was completely recovered in the alcoholic supernatant.

At first glance it would appear that the phosphorylase preparation does not even need to be very pure, since the enzymic conversion of glucose 1-phosphate into various other metabolites would generate products with a good solubility in acid 66% ethanol. However, the possible interference of 'glycogen hydrolases' should be evaluated by an assay without P_i , which can be replaced by, e.g., β -glycerophosphate. The latter approach revealed that the ^{14}C assay method cannot be applied to crude liver extracts (results not shown).

Comparison of different methods

Assay of muscle phosphorylase *b*. In order to check the accuracy of the radiochemical methods, purified muscle phosphorylase *b* was assayed in a parallel way by the enzymic determination of glucose 1-phosphate (Table 1). The activity of phosphorylase was varied over a wide range by the addition of positive effectors such as AMP (Cori *et al.*, 1938), IMP (Black & Wang, 1968) or a polycarboxylate (Sotiroudis *et al.*, 1979), or the inhibitors ATP (Morgan & Parmeggiani, 1964), caffeine (Kihlman & Overgaard-Hansen, 1955) or glucose (Cori *et al.*, 1943). For each determination a sample was analysed after two different time

Table 1. Comparison of radiochemical and spectrophotometric methods for the assay of phosphorylase

The substrate mixtures at pH 7.4 contained, in a final volume of 0.5 ml, 5 mg of shellfish glycogen, 5 mM- or 50 mM- P_i , 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer and other additions as indicated; the appropriate labelled compound was present in the radiochemical assays. According to the expected activity, 5 or 50 μ g of crystalline muscle phosphorylase *b* was added. After 5 and 10 min of incubation a 0.2 ml sample was withdrawn and either deproteinized for enzymic determination of glucose 1-phosphate (spectrophotometric assay) or processed as described in the standard radiochemical assays. When the ^{32}P assay method was performed at 5 mM- P_i the reaction was arrested with $HClO_4$ containing 2 μ mol of P_i . Results are means $\pm$ S.E.M. for four non-simultaneous determinations.

Substrate composition			Measured phosphorylase activity (nmol/min per mg)		
P_i (mM)	AMP (mM)	Other compound	^{14}C assay method	^{32}P assay method	Spectrophotometric method
5	—	—	6 ± 0.9	9 ± 2.7	10 ± 2.9
5	0.01	—	211 ± 6	185 ± 6	184 ± 5
5	0.1	—	3030 ± 79	3213 ± 81	3013 ± 148
5	1	—	5559 ± 136	5806 ± 265	5252 ± 223
5	0.01	70 mM-EDTA	1440 ± 76	1370 ± 67	1456 ± 68
50	0.01	—	1459 ± 78	1487 ± 50	1360 ± 30
50	—	1 mM-IMP	2695 ± 135	2317 ± 61	2333 ± 46
5	0.1	3 mM-ATP	441 ± 33	398 ± 13	385 ± 9
5	0.1	1 mM-Caffeine	85 ± 5	73 ± 3	100 ± 16
5	0.1	2 mM-Glucose	1325 ± 32	1340 ± 170	1472 ± 77
5	0.1	10 mM-Glucose	296 ± 34	261 ± 34	267 ± 39

intervals, which provided a check that initial velocities were indeed being measured. The same series of assays was then performed on different days. It is evident from the results in Table 1 that the three methods are strictly comparable with regard to accuracy and reproducibility.

Recommended applications. The radiochemical methods are not subject to the various limitations of the coupled spectrophotometric assay (see the introduction). They allow also more samples to be handled in parallel. A drawback is the necessity for rather purified enzymes; crude tissue preparations should not be used unless the amount of error (specified above) is shown to be negligible. The following considerations may help the reader who has to decide between the two proposed methods. The ^{14}C assay method requires the preliminary synthesis and analysis of labelled glycogen. However, all necessary biochemicals are commercially available, and the labelling procedure can be scaled up to meet the capacity of a preparative centrifuge. Major assets of this assay are the use of an isotope with a half-life beyond consideration, and the homogeneously low blank values in all experimental conditions. The application of the ^{32}P assay method requires a continued awareness of the influence of P_i , glycogen and buffers on the precipitation process. The ^{32}P assay method is undoubtedly the method of choice for determinations at very low P_i concentrations (e.g. 0.1 mM), where both the ^{14}C assay method and the spectrophotometric method become impracticable but the ^{32}P assay method retains full power.

References

- Appleman, M. M., Krebs, E. G. & Fischer, E. H. (1966) *Biochemistry* **5**, 2101–2107
- Bergmeyer, H. U. & Michal, G. (1974) in *Methods of Enzymatic Analysis*, 2nd edn. (Bergmeyer, H. U., ed.), pp. 1233–1237, Verlag Chemie, Weinheim, and Academic Press, New York
- Black, W. J. & Wang, J. H. (1968) *J. Biol. Chem.* **243**, 5892–5898
- Cori, C. F., Cori, G. T. & Green, A. A. (1943) *J. Biol. Chem.* **151**, 39–55
- Cori, G. T., Colowick, S. P. & Cori, C. F. (1938) *J. Biol. Chem.* **123**, 381–389
- Dahlqvist, A. (1961) *Biochem. J.* **80**, 547–551
- De Lange, R. J., Kemp, R. G., Riley, W. D., Cooper, R. A. & Krebs, E. G. (1968) *J. Biol. Chem.* **243**, 2200–2208
- Fischer, E. H. & Krebs, E. G. (1958) *J. Biol. Chem.* **231**, 65–71
- Gevers, G. & Stalmans, W. (1980) *Arch. Int. Physiol. Biochim.* **88**, B30–B32
- Helmreich, E. & Cori, C. F. (1964a) *Proc. Natl. Acad. Sci. U.S.A.* **51**, 131–138
- Helmreich, E. & Cori, C. F. (1964b) *Proc. Natl. Acad. Sci. U.S.A.* **52**, 647–654
- Hue, L., Bontemps, F. & Hers, H. G. (1975) *Biochem. J.* **152**, 105–114
- Katz, J. & Hassid, W. Z. (1951) *Arch. Biochem. Biophys.* **30**, 272–281
- Kihlman, B. & Overgaard-Hansen, K. (1955) *Exp. Cell Res.* **8**, 252–255
- Laskov, R. & Margoliash, E. (1963) *Bull. Res. Council. Isr. Sect. A* **4**, 351–362
- Lederer, B. & Stalmans, W. (1976) *Biochem. J.* **159**, 689–695
- Maddaiah, V. T. & Madsen, N. B. (1966) *J. Biol. Chem.* **241**, 3873–3881
- Morgan, H. E. & Parmeggiani, A. (1964) *J. Biol. Chem.* **239**, 2440–2445
- Parodi, A. J., Krisman, C. R., Leloir, L. F. & Mordoh, J. (1967) *Arch. Biochem. Biophys.* **121**, 769–778
- Sotiroidis, T. G., Oikonomakos, N. G. & Evangelopoulos, A. E. (1979) *Biochem. Biophys. Res. Commun.* **86**, 674–682
- Stalmans, W. & Hers, H. G. (1975) *Eur. J. Biochem.* **54**, 341–350
- Sugino, Y. & Miyoshi, Y. (1964) *J. Biol. Chem.* **239**, 2360–2364