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Spectrophotometric Determination of Ribose-1-Phosphate

UMBERTO MURA, FRANCESCO SGARRELLA, AND PIER LUIGI IPATA

Laboratory of Biochemistry, Faculty of Science, University of Pisa, via A. Volta 4, 56100 Pisa, Italy

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In the course of studies on nucleoside monophosphate metabolism, the need was encountered for a method to determine ribose-1-phosphate. Published assays for ribose-1-phosphate depend either on chromatographic separation of the sugar-phosphate, or else on its acid lability which allows it to be determined as a phosphate. The present work describes a less laborious spectrophotometric assay which is both rapid and specific. The basis of the method is the absorbance change at 265 nm associated with the following two-stage enzymatic conversion: ribose-1-phosphate + adenine $\rightleftharpoons$ phosphate + adenosine (adenosine phosphorylase); adenosine + $H_2O \rightarrow$ inosine + NH_3 (adenosine deaminase). The change in absorbance was proportional to ribose-1-phosphate concentration at least up to 25 μg/ml. In tests of the assay, it was possible to detect ribose-1-phosphate formation from inosine and phosphate catalyzed by purine nucleoside phosphorylase. Further, the degradation of ribose-1-phosphate by various commercial phosphatases and several tissues or microbial extracts was observed.

In the course of our experiments on nucleoside monophosphate breakdown in *Bacillus cereus* (1), it became obvious that a better method of estimating ribose-1-phosphate formed by phosphorolytic cleavage of nucleosides would be valuable. To our knowledge, no simple standard method for ribose-1-phosphate determination has been reported so far, perhaps on account of the extreme acid lability of this sugar-phosphate. For example, in studies on phosphoribomutase from different sources, the formation of ribose-1-phosphate from ribose-5-phosphate has been measured by taking advantage of the acid lability of ribose-1-phosphate compared to ribose-5-phosphate. Thus, application of the normal Fiske–Subbarow phosphate method (2) measures the "ultra-labile" ribose-1-phosphate without hydrolyzing ribose-5-phosphate (3). A further complication in this instance was the necessity to determine also any true inorganic phosphate present in the system. We note that dephosphorylation of ribose-1-phosphate would go undetected in this method since the analysis does not distinguish phosphate and "ultra-labile" phosphate. In fact, nearly all commonly used methods for the determination of free phosphate or free ribose will cause complete hydrolysis of ribose-1-

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phosphate (2,4,5). Another procedure for ribose-1-phosphate determination (6) avoids the lack of specificity of the above method by using chromatographic separation of reactants. Both of these methods, however, are very laborious and time-consuming when large numbers of samples are to be analyzed.

We present here a method for the rapid determination of ribose-1-phosphate, which is based on its quantitative transformation to inosine via the successive action of adenosine phosphorylase and adenosine deaminase:

$$\text{ribose-1-phosphate} + \text{adenine} \rightleftharpoons \text{adenosine} + \text{phosphate};$$

$$\text{adenosine} + H_2O \rightarrow \text{inosine} + NH_3$$

The amount of ribose-1-phosphate is determined by the fall in absorbance at 265 nm, which accompanies the conversion of adenine to inosine.

METHODS

Adenosine deaminase (EC 3.5.4.4) was obtained from Boehringer and Soehne and was diluted with water to give a concentration of 20 μg of protein/ml before use. Purine nucleoside phosphorylase (1 mg/ml) (EC 2.4.2.1) and alkaline phosphatase (1 mg/ml) (EC 3.1.3.1) were obtained from Boehringer and Soehne. 5′-Nucleotidase from snake venom (EC 3.1.3.5) was obtained from Sigma Chemical Co. and was diluted with water to give a concentration of 0.4 mg/ml. Adenosine phosphorylase was prepared from *B. cereus* as described previously (7), and the Sephadex G-100 eluate was further purified by Sephadex G-200 gel filtration: The final preparation was free of any detectable adenosine deaminase activity, did not attack ribose-1-phosphate, and contained 0.94 mg of protein/ml, with a specific activity of 447 nmol of adenosine formed/min/mg of protein. Inosine phosphorylase activity of the final preparation was 0.64 nmol of hypoxanthine formed/min/mg of protein. Moreover, the preparation was free of any detectable phosphorylase activity on pyrimidine nucleosides.

The experiments were carried out at 37°C, in 1-cm pathlength cuvettes, in a final volume of 2 ml, and the change in optical density at 265 nm was determined with a recording Beckman ACTA C III spectrophotometer. The difference in molar extinction coefficients at 265 nm between adenine and inosine was taken as 6.390 for our experimental conditions (see Ref. 8).

Yeast autolysate, *B. cereus* extracts, and tissue homogenates were prepared as previously described and were dialyzed against 0.1 M Tris–Cl buffer, pH 7.4, before use (1,9,10). Determination of inosine and hypoxanthine were carried out as previously described (8). Total phosphate was determined by measuring the increase in inorganic phosphate after a 10-min hydrolysis in normal sulfuric acid at 100°C. Inorganic

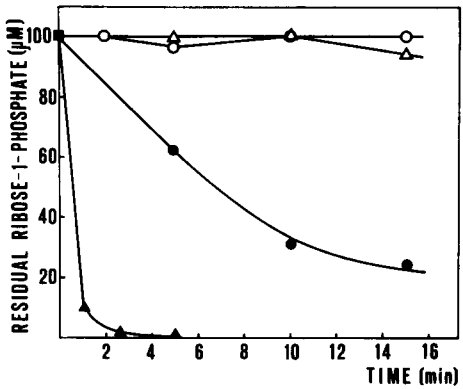

FIG. 1. Ribose-1-phosphate breakdown at different pHs. Samples of ribose-1-phosphate at a final concentration of 0.1 mM in Tris–Cl buffer 0.2 M, pH 7.4 (○), and 0.05 M citrate buffer, pH 5.5 (△), pH 4.5 (●), and pH 3.0 (▲), were held at 100°C. At the times indicated on the abscissa the samples were rapidly cooled, and residual ribose-1-phosphate was determined as described in Methods in 0.3-ml portions.

phosphate was determined according to Fiske and Subbarow (2). It must be emphasized that ribose-1-phosphate is completely hydrolyzed under the conditions of orthophosphate determination (3). However, it proved to be stable in Tris–Cl buffer 0.2 M, pH 7.4, even after 15 min of heating at 100°C (Fig. 1).

The detailed standard procedure for the determination of ribose-1-phosphate was as follows: 1 ml of Tris–Cl buffer, 0.35 M, pH 7.4, was pipetted into one cuvette, followed by different amounts of ribose-1-phosphate, in a maximal volume of 0.5 ml, 20 μl of 0.78 mM adenine, 100 μl of adenosine deaminase, and water to bring the volume to 2 ml. The absorbance at 265 nm (0.9 absorbance units) was first recorded for several minutes against a reference cuvette in which adenine was substituted by water; finally 50 μl of the adenosine phosphorylase preparation was added to both cuvettes, and the total change in optical density was recorded. Modifications of the standard conditions are described in the presentations of the experimental data.

RESULTS AND DISCUSSION

Figure 2 shows the results of an experiment in which three different amounts of ribose-1-phosphate were determined. In the absence of adenosine phosphorylase, the absorbance at 265 nm remained constant, showing the absence of adenosine phosphorylase activity in the adenosine deaminase. The changes in absorbance at 265 nm observed in Fig. 2A accounted for a recovery of about 94% as judged from the nominal concentration of the sugar-phosphate solution used. This small discrepancy most likely derives from difficulties in weighing accurately this

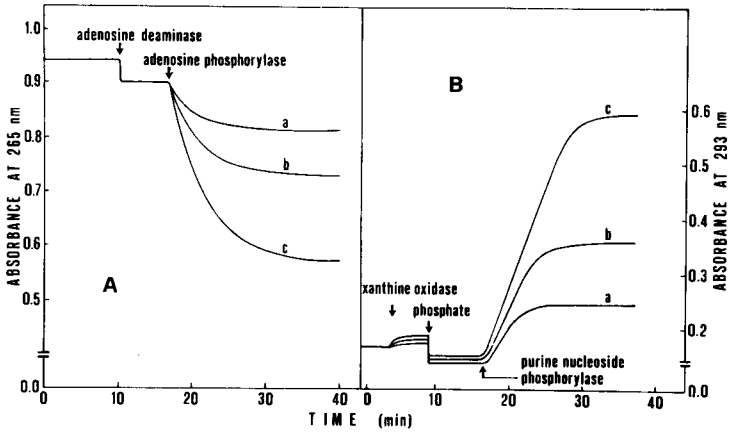

FIG. 2. Spectrophotometric determination of ribose-1-phosphate: (A) 23 (a), 47 (b), and 92 (c) nmol of ribose-1-phosphate were determined spectrophotometrically as described in the text. The changes in absorbance accounted for 22 (a), 43 (b) and 88 (c) nmol of ribose-1-phosphate. After 40 min, the reaction mixtures were heated 2 min at 100°C in sealed tubes and brought to room temperature. (B) Hypoxanthine and inosine were then determined as described previously (8): The wavelength was changed to 293 nm, and 20 μg of xanthine oxidase, 30 μmol of inorganic phosphate, and 5 μg of inosine phosphorylase were added in this order at the times indicated by arrows. The change in absorbance for inosine corresponded to 20 (a), 40 (b), and 86 (c) nmol, respectively.

hygroscopic and labile compound. As a further verification of the method, the amount of inosine formed at completion of the reaction was also measured (Fig. 2B). In fact, a close correspondence between inosine formed and the initial amount of ribose-1-phosphate was observed. In addition, a small amount of hypoxanthine was detected as evidenced by the small absorbance change following the addition of xanthine oxidase (see Fig. 2B), arising most probably from the slight inosine phosphorylase activity present in the adenosine phosphorylase (see Methods). This potential difficulty, however, lead only to a 2% overestimation of ribose-1-phosphate. A calibration curve for the method is shown in Fig. 3.

Although the stoichiometries reported above seemed satisfactory, some further checks were made. Incubation of ribose-1-phosphate with adenosine deaminase and adenosine phosphorylase prior to the addition of adenine, or alternatively, incubation of adenine with the same enzymes before the addition of ribose-1-phosphate, leads to no difference in the observed absorbance changes. Thus, the auxiliary enzymes contained neither adenase nor enzymes capable of acting on ribose-1-phosphate. The possible interference of other sugar and sugar-phosphates on the determination of ribose-1-phosphate was also investigated (Fig. 3). It is evident that the compounds tested did not interfere with the method, even at the lowest level of ribose-1-phosphate used.

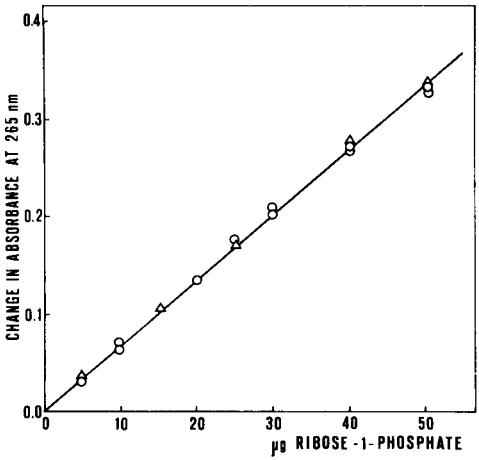

FIG. 3. Calibration curves of ribose-1-phosphate. Ribose-1-phosphate measured alone (○) or in the presence of a mixture of glucose, glucose-6-phosphate, glucose-1-phosphate, ribose, and ribose-5-phosphate (△), each at a final concentration of 60 μM.

As a final test, the method was applied to measure ribose-1-phosphate in various biochemical situations. First, we selected an enzymatic reaction, in which ribose-1-phosphate is produced; namely, that catalyzed by purine nucleoside phosphorylase: phosphate + inosine ⇌ ribose-1-phosphate + hypoxanthine. Xanthine oxidase was also added, in order to displace the equilibrium toward the formation of ribose-1-phosphate. It is evident from Fig. 4 that the amount of ribose-1-phosphate formed did not account for all of the inosine consumed. Clearly, ribose-1-phosphate must have undergone simultaneous transformation by some other process. Most likely this is due to dephosphorylation of ribose-1-phosphate, and in fact, as shown in Fig. 5, both of the commercial enzymes (purine nucleoside phosphorylase and xanthine oxidase) contained some contaminating activities which catalyze the transformation of ribose-1-phosphate. Correcting for this activity, the two curves in Fig. 4 virtually coincide. While this experiment provided proof of the capability of the method to detect the appearance of ribose-1-phosphate, evidently the system described above can be used as an assay for nucleoside phosphorylase only in the absence of interfering enzymatic activity. Since this was not the primary objective of this work, this problem was not considered further. Second, a series of commercial phosphatases were analyzed for their ability to act on ribose-1-phosphate (Fig. 5); only the alkaline phosphatase from calf intestine catalyzed the disappearance of ribose-1-phosphate. Finally, various crude extracts were analyzed for their ability to attack ribose-1-phosphate. The time course of ribose-1-phosphate disappearance catalyzed by a crude extract of *B. cereus* is

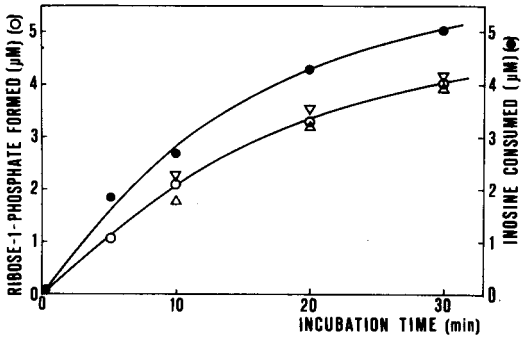

FIG. 4. Formation of ribose-1-phosphate catalyzed by commercial purine nucleoside phosphorylase. The reaction mixture contained, in a final volume of 1 ml, 0.1 μmol of inosine, 0.1 μmol of NaH_2PO_4, 0.194 M Tris–Cl buffer, pH 7.4, 2 μg of commercial inosine phosphorylase, and 20 μg of xanthine oxidase. The latter enzyme was added to displace the reaction toward ribose-1-phosphate formation. Ribose-1-phosphate formed (○) and inosine consumed (●) are shown as functions of time. The temperature was 37°C. At the times indicated, 0.3-ml aliquots were subjected to ribose-1-phosphate determination. To account for the contamination of the commercial enzymes with ribose-1-phosphate-consuming activity, the following correction was made: Using specific activities derived from Fig. 5 and the quantity of inosine consumed, the amount of ribose-1-phosphate degraded by contaminating enzymes was calculated assuming either a first-order (▽) or a zero-order (△) reaction. These quantities were subtracted from the respective values for inosine consumption.

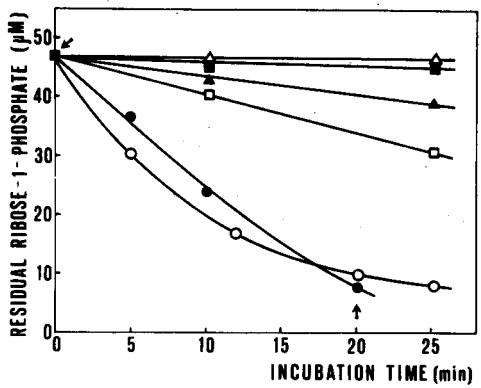

FIG. 5. Action of commercial enzymes and crude extracts of *B. cereus* on ribose-1-phosphate. The reaction mixture contained, in a final volume of 3 ml, 0.141 μmol of ribose-1-phosphate in 0.2 M Tris–Cl buffer, pH 7.4, and 0.5 μg of alkaline phosphatase (○), 9.0 μg of 5′-nucleotidase (△), 38.7 μg of 3′-nucleotidase (■), 6 μg of inosine phosphorylase (▲), 60 μg of xanthine oxidase (□), or 0.44 mg protein of a crude extract of *B. cereus* (●). From the reaction mixture 0.5-ml portions were withdrawn for the measurement of ribose-1-phosphate, and at the times indicated by arrows, total phosphate (10 min in 1 N H_2SO_4 at 100°C) and the sum of inorganic and "ultra-labile" phosphate were assayed.

shown in Fig. 5. The activities (μmol/min/mg of protein) of other extracts tested were: rat kidney (2.19×10^{-2}), rat liver (2.7×10^{-3}), rat muscle (1.06×10^{-3}), and Baker's yeast (8.97×10^{-4}). The extract from rat spleen, on the other hand, was inactive. In the case of the *B. cereus* extract, it was further shown that the reaction did not involve isomerization to ribose-5-phosphate, strongly suggesting a ribose-1-phosphate phosphatase activity (Fig. 5).

In our hands, then, the method described in this paper has proved to be both practicable and useful for the rapid determination of ribose-1-phosphate in various experimental situations. In fact, use of the tecnique has led, in preliminary studies (U. Mura, F. Sgarrella, P. L. Ipata, unpublished results), to the conclusion that ribose-1-phosphate phosphatase of *B. cereus* discussed earlier is specific for that sugar-phosphate.

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