Chapter 15

Modified Procedure for Phosphate Estimation which Stabilizes Color and Improves Sensitivity

Introduction

The first quantitative method for colorimetric estimation of phosphate was described by Fiske and Subba Row (1). A mixture of sodium sulfite, sodium bisulfite and 1, 2, 4 aminonaphthol sulfonic acid was used as reducing agent to produce color in the form of molybdenum blue species. The method is rapid but suffers from the disadvantage that the color is unstable and optical density readings have to be taken within a short time span of 8-12 min. Subsequently, modifications using different reducing agents such as ascorbic acid, hydroquinone, 2,4-aminophenol, thiosulfate, stannous chloride, hydrazine sulfate etc. have been described (2, 3). However, the problem of instability of the color persists [2, 3]. Hurst[3] used a mixture of hydrazine sulfate and stannous chloride as reducing agent, under these conditions the color developed maximally in 3 min and was stable up to 30 min. The problem of color stability was solved by Bartlett (4) by increasing the sulfuric acid (H_2SO_4) concentration from 0.3 N to 1.2 N and by introducing a boiling step to accelerate the process of color development. By this procedure the color was stabilized and also the sensitive improved (4). However, this method has no applicability in the enzyme assays, especially when a substrate such as ATP which contains labile phosphate groups is used. Hence it is desirable to develop a method which produces stable color, and has higher sensitivity and applicability in enzyme assay.

Earlier reports suggested that higher H_2SO_4 concentration improved stability and optical density measurements at around 820 rather than at 660 nm improved sensitivity (1, 3, 4). In the light of these observations attempt was made to check if increasing

concentration of H_2SO_4 in the conventional method (1) helps in improving both the parameters. The results of these studies are described here.

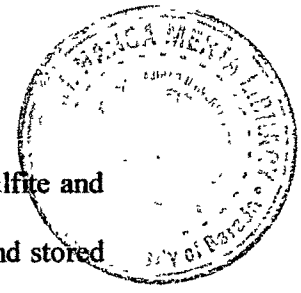
Materials and Methods

Chemicals

Glucose-6-phosphate (G 6 P) was purchased from Sigma Chemical Co., U. S. A. Bovine serum albumin fraction V (BSA) and Sodium salt of adenosine 5' triphosphate (ATP) were obtained from SRL, India. 1, 2, 4 aminonaphthol sulfonic acid was purchased from Glaxo Laboratories (India) Ltd. India. Sodium dodecyl sulfate (SDS) was obtained from Koch-Light, England. All other chemicals were of analytical-reagent grade and were purchased locally.

Procedure for Phosphate Estimation

Aliquots of solution containing 1-8 μg Pi were taken and the volume was made up to 3.2 ml with distilled water. To this 0.5 ml of H_2SO_4 was added to achieve final concentrations of 0.5, 0.625, 0.8 and 1.0 N respectively. This was followed by the addition of 0.2 ml of 5 % ammonium molybdate (freshly prepared in distilled water). Finally the color was developed by the addition of 0.1 ml of reducing agent solution (40 mg triturate/ml of distilled water) with vigorous shaking. Optical density measurements and recording of absorption spectra were carried out in a Shimadzu UV model 160 A or JASCO V 530 UV/VIS spectrophotometer.



The triturate was prepared by grinding 1.2 g sodium sulfite, 1.2 g sodium bisulfite and 0.4 g 1, 2, 4 aminonaphthol sulfonic acid using a porcelain mortar and pestle and stored in an amber color bottle (5).

Acid Hydrolysis of ATP and G 6 P

For determining the extent of acid hydrolysis of G 6 P and ATP, the two phosphate esters were incubated under phosphate estimation conditions in the presence of 0.5, 0.625 or1.0 N H_2SO_4. Concentrations of G 6 P were 1.0 and 5.0 mM and that of ATP were 2.0 and 5.0 mM. Release of Pi was monitored over a 24 h period. In case of 0.5 N H2SO4, where instability of the color posed a problem, G 6 P and ATP at two concentrations cited above were incubated with 0.5 N H_2SO_4 and aliquots were taken at specified time intervals to monitor the amount of Pi released by conventional Fiske and Subba Row procedure (1). The extent of substrate hydrolysis was determined from the amount of Pi released for which the corresponding values of slope were used (e. g. see Fig.6).

Effect of Sugars and Salts

To examine if the sugars and salts interfere in color development, assays were performed (using 4 µg Pi) in the presence of 1- 10 mM glucose and sucrose, 5- 100 mM NaCl or KCl and 1-10 mM $MgCl_2$ employing different concentration of H_2SO_4 as detailed above. Similarly, interference by proteins was determined by including 10- 500 µg BSA/ tube in the presence of SDS (final concentration 0.25% w/v) under all the conditions as detailed above. Color development was monitored for up to 24 h.

Results

Effect of H_2SO_4 concentration

0.5 N H_2SO_4

In the initial experiments where 0.5 N H_2SO_4 was used, the concentration-dependent increase in the optical density was linear only up to 15 min, and lost linearity at the end of 30 min. At the end of 1 h, besides the standard samples, the blank itself developed intense blue color because of which recording of the optical density of the sample tubes became difficult. In view of this, further experiments were carried out only with the three higher concentrations of H_2SO_4 and the time course of color development was followed for up to 24 h.

0.625 N, 0.8 N and 1.0 N H_2SO_4

In the presence 0.625 N, 0.8 N as well as 1.0 N H_2SO_4 the color in blank as well the standards was found to be stable even up to 24 h. The time course of color development determined by measurements at 660 and 820 nm is shown in Fig.1. It can be noted that the intensity of the color increased with time under all the conditions and 1.0 N H_2SO_4 proved to be most efficient in the process. Also the magnitude of increase in the color density was higher when monitored at 820 nm under these conditions. This suggested that with progress of time there was a shift towards formation of molybdenum blue species which absorbs at 820 nm. To verify this point we recorded the absorption spectra of standard Pi solution at different time intervals. From the absorption spectra shown in Fig. 2 it is evident that indeed there is a shift in absorption maximum with time towards higher wavelength. The spectra also depict that 1.0 N H_2SO_4 is most efficient in the

Figure 1

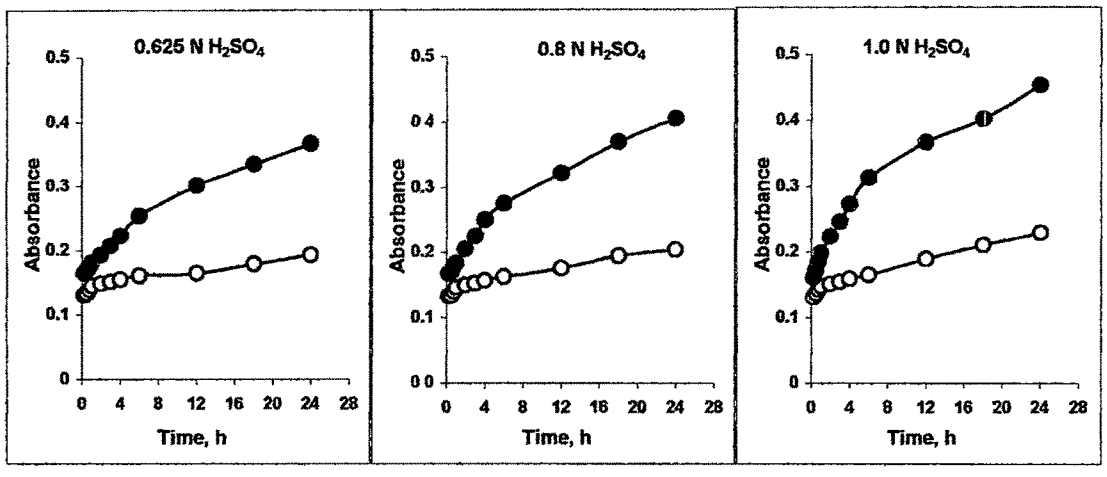


Figure 2

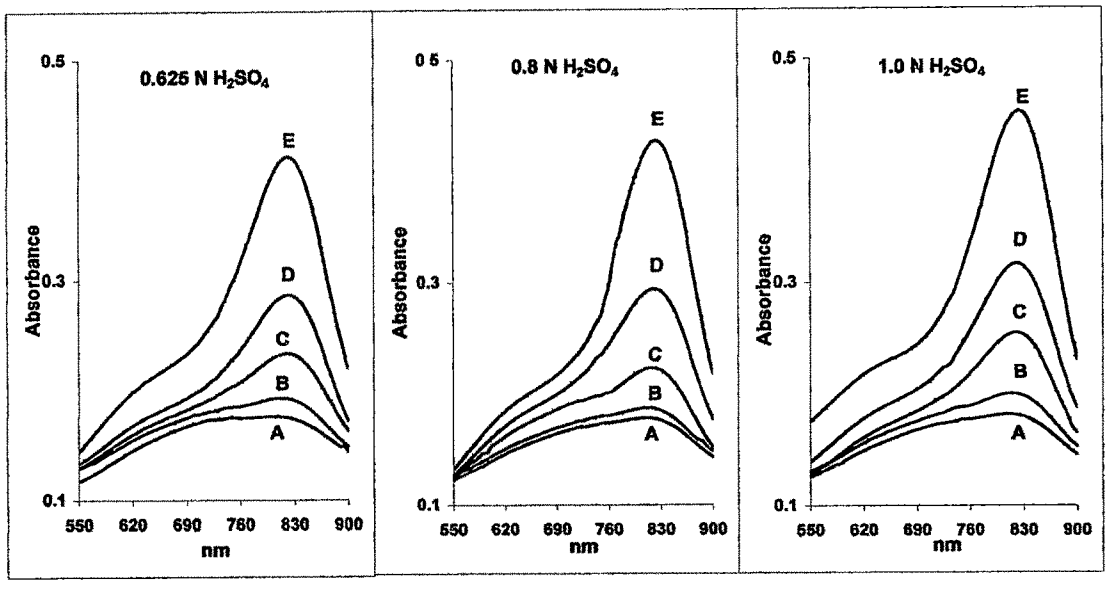


Figure 1. Time course of color development in Pi assay. Color was developed using 4 μg Pi. Concentrations of H_2SO_4 were 0.625 N, 0.8 N and 1.0 N. Optical density measurements at 660 nm and at 820 nm were made at specified time periods as indicated. Each data point represents average of 3 independent observations. Variation ranged from 2-3 %.

Optical density measurements at 660 nm, —○— and at 820 nm, —●—.

Figure 2. Typical Spectra depicting course of color development for different time in Pi assay. Color was developed using 4 μg Pi. Concentrations of H_2SO_4 were 0.625, 0.8 N and 1.0 N. A, 15 min.; B, 1 h; C, 6 h; D, 12 h and E, 24 h.

process. The progressive shift in the absorption maximum with time under the assay conditions employing 0.625 N and 1.0 N concentrations of H_2SO_4 is shown in Fig. 3; with 0.8 N H_2SO_4 picture was intermediate (data not shown). We then decided to monitor the relative improvement in sensitivity of Pi estimation in terms of a standard curve at different time intervals. For this purpose the optical density readings were recorded at 660 and 820 nm. These standard curves are shown respectively in Fig. 4, 5 and 6. It may also be noted here that all the plots were linear up to the Pi concentration of 8 µg. The changes in slope with time, monitored at 660 and 820 nm are shown in Fig. 7 which once again emphasize the efficacy of 1.0 N H_2SO_4 .

Effect of H_2SO_4 concentration on substrate hydrolysis

While 1.0 N H_2SO_4 proved to be the most efficient in improving the sensitivity, this also raised a concern with respect to applicability of the method in enzyme assays where substrate such as ATP which contains acid labile phosphate is used. Hence it was important to check the acid hydrolysis of the substrate under the assay conditions. Therefore experiments were carried out to monitor hydrolysis of ATP (2 mM and 5 mM) and G6P (1 mM and 5 mM) in the presence of 0.5 N, 0.625 N, 0.8 N and 1.0 N H_2SO_4. Once again the results with 0.8 N H_2SO_4 shoed an intermediate pattern; the points overlap with the 0.625 and 1.0 N H_2SO_4 data and hence are not included.

As can be noted (Fig. 8) extent of ATP hydrolysis ranged from 0.08-1.51 % at the end of 30 min for the 3 H_2SO_4 concentrations used at the end of 1h these values ranged from 0.4 to 3.4 and 0.7 to 6.5 % at the end of 2h. An equilibrium point is reached at the end of 24

Figure 3

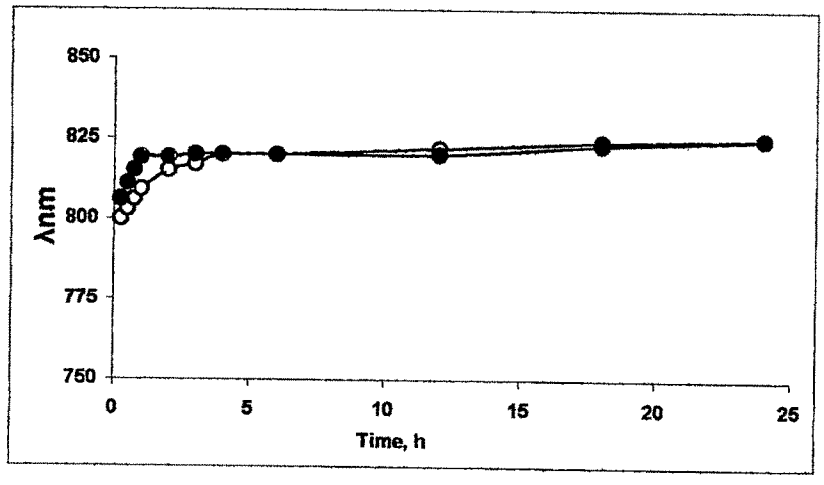


Figure 4

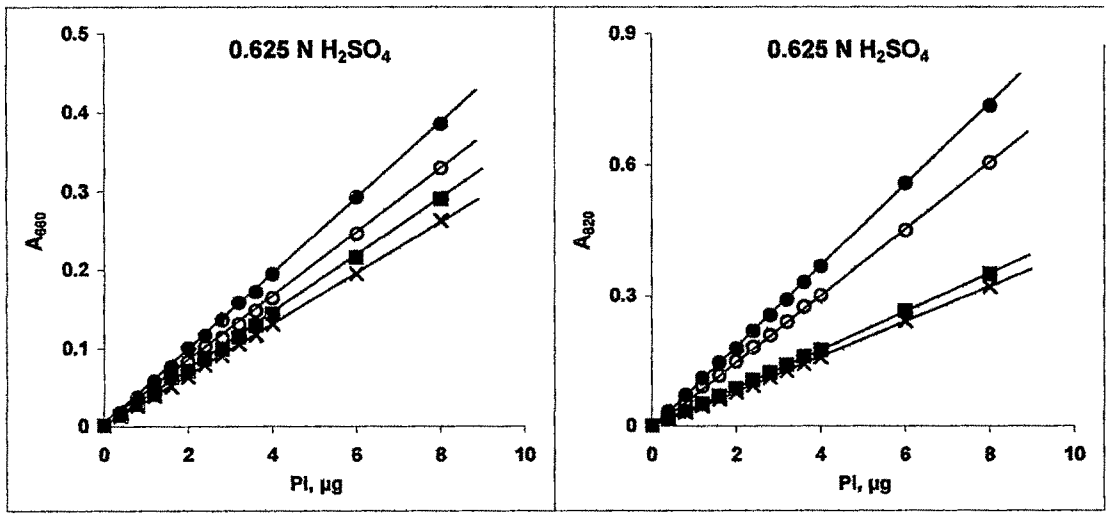


Figure 3. Time course of shift in λmax in Pi assay. Color was developed using 4 μg Pi. Concentrations of H_2SO_4 were 0.625 N and 1.0 N. Each data point represents average of 3 independent observations. Variation ranged from 2-3 %. —○— ,0.625 N H_2SO_4 and —●— , 1.0 N H_2SO_4.

Figure 4. Standard curve showing Pi concentration dependent changes in absorbance at different time intervals. The Pi concentration was 0-8 μg. Concentration of H_2SO_4 was 0.625 N. Each data point represents average of 3 independent observations. Variation ranged from 2-3 %. Note that the scales are different for optical density at 660 nm and 820 nm.

—x—, 15 min; —■—, 1 h; —○—, 12 h and —●—, 24 h.

Figure 5

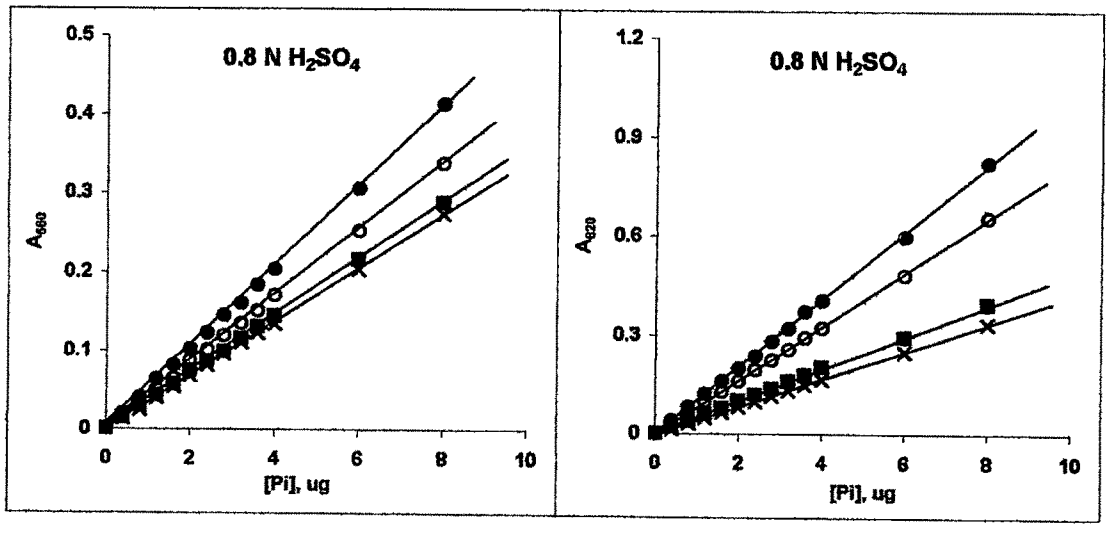


Figure 6

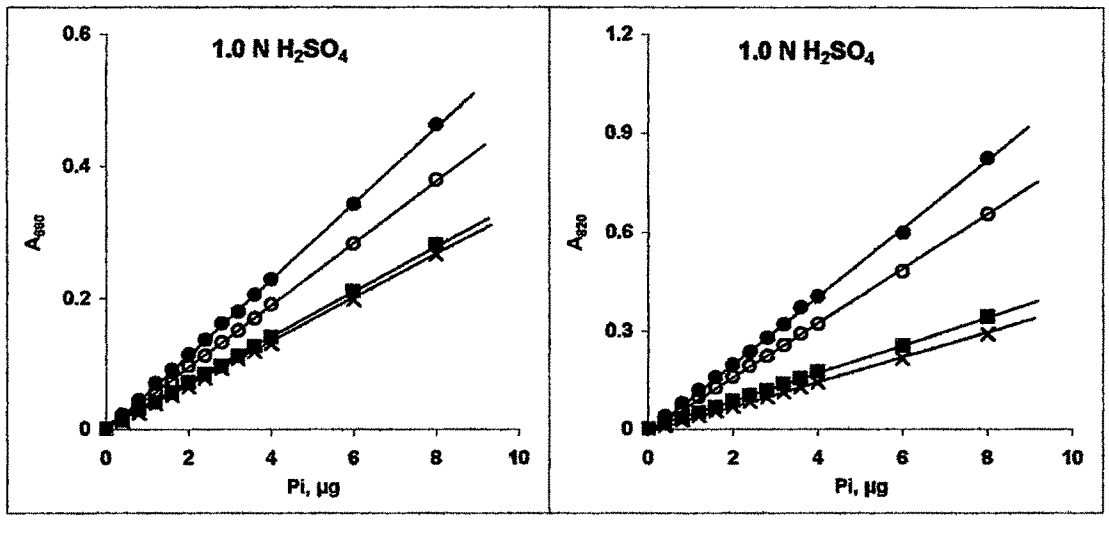


Figure 5. Standard curve showing Pi concentration dependent changes in absorbance at different time intervals. The Pi concentration was 0-8 μg. Concentration of H_2SO_4 was 0.8 N. Each data point represents average of 3 independent observations. Variation ranged from 2-3 %. Note that the scales are different for optical density at 660 nm and 820 nm.

—x—, 15 min; —■—, 1 h; —○—, 12 h and —●—, 24 h.

Figure 6. Standard curve showing Pi concentration dependent changes in absorbance at different time intervals. The Pi concentration was 0-8 μg. Concentration of H_2SO_4 was 1.0 N. Each data point represents average of 3 independent observations. Variation ranged from 2-3 %. Note that the scales are different for optical density at 660 nm and 820 nm.

—x—, 15 min; —■—, 1 h; —○—, 12 h and —●—, 24 h.

Figure 7

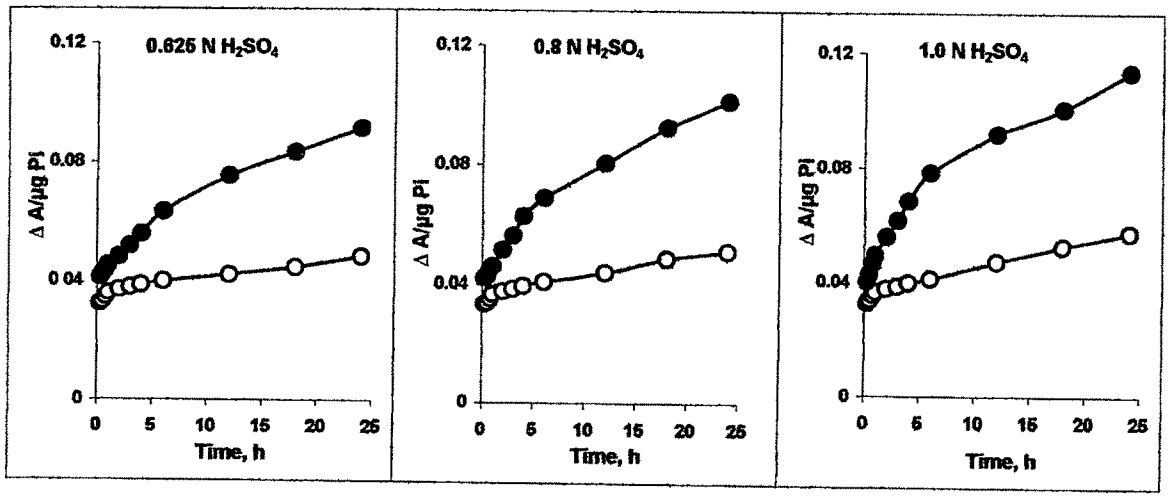


Figure 8

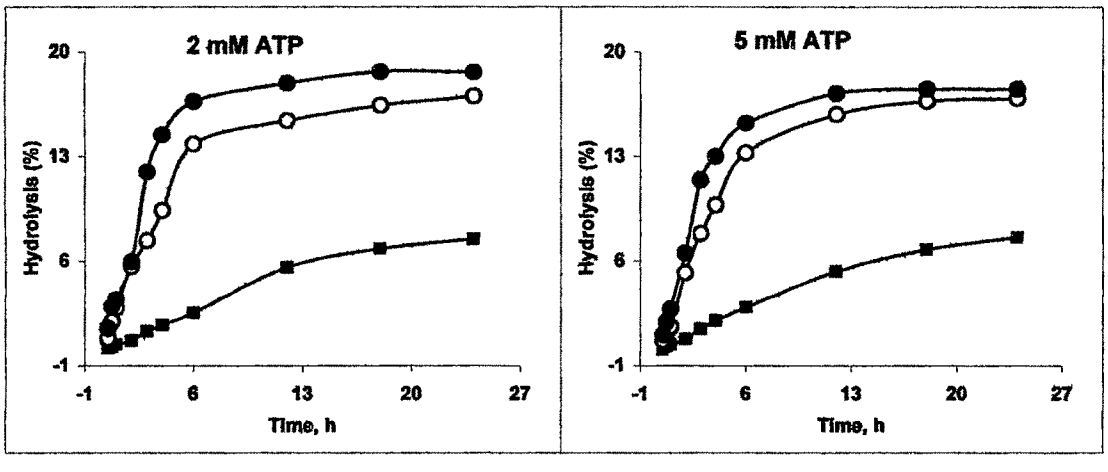


Figure 7. Plots depicting changes in slope with time. Color was developed using 4 μg Pi. Concentration of H_2SO_4 were 0.625 N, 0.8 N and 1.0 N. Optical density measurements at 660 nm and at 820 nm were carried out at specified time periods as indicated. Each data point represents average of 3 independent observations. Variation ranged from 2-3 %.

Optical density measurements at 660 nm, —○— and at 820 nm, —●— .

Figure 8. Time course of ATP hydrolysis. Concentration of ATP was either 2.0 mM or 5.0 mM. Each data point represents average of 3 independent observations. Variation ranged from 2-3 %. H_2SO_4 concentration was —■—, 0.5 N; —○—, 0.625 N and —●—, 1.0 N.

h, where the extent of hydrolysis ranged from 7.5 to 18.6%. Hydrolysis of G 6 P was negligible (0.011-0.287 %) under these conditions (data not shown).

Inclusion of sugars, salts or BSA at the concentrations indicated did not interfere with color development. Intensity of color development ranged from 96-103% under these conditions (data not shown).

Discussion

The present investigations were undertaken with the view of stabilizing the molybdenum blue color in the conventional phosphorus assay and increasing the sensitivity. For this purpose we decided to use higher concentrations of H_2SO_4 (1, 3, 4).

In our hands, employing the conventional procedure (Fiske and Subba Row, 1925) we get the slope in the range of 0.022-0.025 O.D. units/μg Pi at 660 nm and the color is stable maximally up to 15 min (data not shown).

As is evident from the results presented (Fig. 7) in the presence of 0.625 N H_2SO_4, compared to the conventional value, the sensitivity at 660 nm was from 1.4-1.6 times higher between 15 min to 1 h period and almost doubled at the end of 24 h. When the comparison was made with the slopes obtained at 820 nm, the sensitivity was 1.8-2.0 times higher between 15 min to 1 h period and increased fourfold at the end of 24 h.

With the 0.8 N H_2SO_4, the sensitivity at 660 nm was from 1.4-1.6 times higher from 15 min up to 1 h period and almost doubles at the end of the 24 h. on the other hand, at 820 nm the sensitivity was 1.8-2.0 times higher between 15 min to 1 h and increase by 4.5 times at the end of 24 h.

In the presence of 1.0 N H_2SO_4, compared to the conventional value (0.022-0.025 O.D. units/μg Pi), the sensitivity at 660 nm was from 1.4-1.6 times higher between 15 min to 1 h period and almost doubled at the end of 24 h, was 2.5 times higher. When the comparison was made with the slopes obtained at 820 nm, the sensitivity was 1.8-2.2 times higher between 15 min to 1 h period and increased almost fivefold at the end of 24 h.

The results thus suggest that indeed inclusion of higher concentrations of H_2SO_4 helped in improving the sensitivity and stabilizing the color. Also, if the measurements were carried out at 820 nm, there was a further improvement in the sensitivity. It also became apparent that presence of 1.0 N H_2SO_4 had marginal beneficial effect over 0.625 N H_2SO_4 at short term intervals i.e. up to 1h.

Thus the higher concentrations of H_2SO_4 undoubtedly helped the purpose of color stabilization and improving the sensitivity. This in turn can make the method more versatile and increase the applicability such that batch operations using large number of samples are possible. However, this also raised a concern of applicability of the method in enzyme assays where substrates like ATP which contains acid labile Pi. We have

addressed this question by monitoring the acid hydrolysis of ATP under the Pi assay conditions (Fig. 8). As can be noted, the extent of acid hydrolysis during 15 min to 1 h period ranged from 0.7 to 3.4%. If one carried out enzyme assay in 0.1 ml system, and uses the same for Pi determination in 4.0 ml assay the background O.D. due to acid hydrolysis of substrate gets diluted 40 times and thus would increase the substrate blank only negligibly. One can use a corresponding slope (Fig. 7) to compute the amount of Pi released.

In conclusion, our results suggest that Pi assays carried out in the presence of 0.625 N H_2SO_4 with optical density measurements performed at 820 can increase the applicability of the method and make it more versatile. Besides, we have also shown that the inclusion of sugars, salts or protein such as BSA does not interfere with color development thereby making the method adaptable for Pi estimation in enzyme assays.

Summary

A modified method has been developed for estimation of inorganic phosphate (Pi) which involves increasing the concentration of sulfuric acid (H_2SO_4) in the final assay medium. Use of 0.5 N H_2SO_4 was unsuitable since the blanks developed intense blue color at the end of 30 min whereas with higher concentrations of H_2SO_4 (0.625, 0.8 and 1.0 N) the blanks were stabilized for up to 24 h and the intensity of the molybdenum blue color increased with time reaching saturation at about 24 h. Simultaneously, a shift in absorption maximum to 820 nm was noted, highest concentration of H_2SO_4 being most effective in the process. The sensitivity of Pi determination was from 1.5 to 2.2 times higher as compared to the conventional Fiske and Subba Row procedure. Presence of sugars (1-10 mM), NaCl and KCl (5-100 mM), $MgCl_2$ (1-10 mM) or BSA (up to 500 µg) per assay tube did not interfere in color development. The extent of ATP hydrolysis was 1.8 to 3.4 % for up to 1h. Only negligible hydrolysis of G 6P was noted under these conditions. Thus the method is suitable for Pi analysis in enzyme assays in which substrate containing labile phosphate is used. The present modification, thus besides producing stable color and improving sensitivity also allows batch operation of a large number of samples.

References

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