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Improved method for estimation of inorganic phosphate: Implications for its application in enzyme assays

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The conventional method of Fiske and Subba Row for the estimation of inorganic phosphate (Pi) is although rapid, but suffers from the disadvantage that the color is unstable and hence the optical density (OD) measurements have to be carried out within a short time span of 8-12 min. This poses a restriction on the number of samples, which can be handled in a batch. Although, modified procedures involving use of alternate reducing agents/or increasing the concentration of H_2SO_4 in conventional method have been subsequently developed, but the problem of color stability could not be solved. In addition, the use of higher concentrations H_2SO_4 has rendered the methods unsuitable in enzyme assays, especially if the acid labile phosphate containing substrates have been used. In the present study, attempts have been made to suitably modify the method to improve the stability of the color and sensitivity and also for its applicability in enzyme assays, especially when acid labile phosphate containing substrates such as ATP is used. We used the higher concentrations (0.625, 0.8 and 1.0 *N*) of H_2SO_4 rather than 0.5 *N* used in the conventional assay procedures. Under these conditions, the reagent blanks do not develop color for up to 24 h, whereas the intensity of the molybdenum blue color in the standard and/or experimental tubes increased with time reaching optimum value at 24 h. Simultaneously, the absorption maximum shifts from 660 nm to 820 nm. The highest concentration of H_2SO_4 (1.0 *N*) is found to be the most effective in the process of color development. The sensitivity of the method is from 1.7 to 2.1 times higher, as compared to the conventional Fiske and Subba Row method for the measurements carried out at the end of 15 min at 820 nm and with the highest concentration of H_2SO_4 (1.0 *N*); the sensitivity increased 4.8-fold at the end of 24 h. Presence of glucose and sucrose (1-10 m*M*), NaCl and KCl (5-100 m*M*), $MgCl_2$ (1-10 m*M*) and BSA (10 to 500 µg per assay tube) do not interfere either with color development or with OD measurements. The extent of ATP hydrolysis is 1.6 to 3.4% for up to 1 h, depending upon the concentration of H_2SO_4 used. Only negligible hydrolysis of G6P is observed under these conditions. These results suggest that the presently modified method is suitable for Pi analysis in the enzyme assays, in the presence of labile phosphate containing substrates.

Keywords: Pi determination, Stabilization, Molybdenum blue color, Improved sensitivity

Fiske & Subba Row[1] first described the quantitative method, widely used for colorimetric estimation of phosphate. The method was based on conversion of inorganic orthophosphate (Pi) to phosphomolybdic acid, followed by the reduction of molybdenum to produce the molybdenum blue species, which had ill-defined absorption characteristics. This method used a mixture of sodium sulfite, sodium bisulfite and 1, 2, 4-aminonaphthol sulfonic acid (ANSA) as the reducing agent. Although, the method was rapid, it suffered from the disadvantage that the color was unstable and hence the optical density (OD) measurements had to be carried out within a short time span of 8-12 min. Subsequently, modifications using reducing agents such as ascorbic acid, hydroquinone, 2, 4-aminophenol, thiosulfate, stannous chloride, hydrazine sulfate etc. were described, however, the problem of color instability persisted[2,3]. Using a mixture of hydrazine sulfate and stannous chloride as reducing agent, although the sensitivity improved for measurements made at 700 nm, the color which developed maximally in 3 min was stable only up to 40 min[3]. Use of high concentration of H_2SO_4 (1.0 *N*) posed additional problem[3].

In a recently described method[4], excess molybdate was removed by adding oxalic acid and the color was extracted in isobutyl alcohol that stabilized the color for 7 h. In another method, phosphomolydic acid was extracted with methyl isobutyl ketone and quantification of molybdenum was done by the

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atomic absorption spectrophotometry[5], rather than by colorimetric method[1]. Methods for determination of phosphorous in the presence of pyrophosphate in soybean and of phosphate released by hydrolysis of nucleic acids using HNO_3 were also reported[6,7], however, no modifications were introduced for Pi determination carried out by the conventional method[1]. In the procedure described by Bartlett[8], stability of the color and sensitivity improved significantly. However, as the color was developed by boiling the samples at 100°C for 7 min in the presence of 1.2 *N* H_2SO_4, the method could not be used in the enzyme assays, especially when a substrate such as ATP which contains labile phosphate groups was used.

It was desirable to develop a method that produced a stable molybdenum blue species and at the same time was rapid, had higher sensitivity and had applicability for the enzyme assays. Keeping these requirements in mind, in the present communication, attempts were made to optimize the assay conditions for the estimation of Pi by developing the color in the presence of higher concentrations of H_2SO_4 (0.625, 0.8 and 1.0 *N*) and monitoring the OD changes over a period of up to 24 h. Corresponding absorption spectra at fixed time intervals were recorded and the values of extinction coefficient (E_M) were computed.

Materials and Methods

Chemicals

D-Glucose 6-phosphate (G6P) was purchased from Sigma Chemical Co., St. Louis, MO, USA. Bovine serum albumin (BSA) fraction V and sodium salt of adenosine 5'-triphosphate (ATP) were obtained from SRL, *Mumbai,* India and 1, 2, 4-aminonaphthol sulfonic acid (ANSA) was purchased from Glaxo Laboratories, (India) Ltd., Mumbai. Sodium dodecyl sulfate (SDS) was obtained from Koch-Light, Colnbrook, England. All other chemicals were of analytical reagent grade and purchased locally.

Procedure for phosphate estimation with varying concentrations of H_2SO_4

Aliquots of standard solution (40 µg Pi/ml) 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 stock solution of H_2SO_4 was added to achieve the final concentration of 0.5, 0.625, 0.8 or 1.0 *N* i.e. to get the final concentration of 0.5 *N*, concentration of stock solution was 4.0 *N*. This was followed by the addition of 0.2 ml of 5% ammonium molybdate solution (freshly prepared in distilled water). The color was developed by the addition of 0.1 ml of reducing agent solution (details given below) with vigorous shaking. OD measurements and recording of absorption spectra were carried out in a Shimadzu model UV 160 A or JASCO model V 530 UV/VIS spectrophotometers.

The triturate of reducing agent was prepared by grinding 1.2 g sodium sulfite, 1.2 g sodium bisulfite and 0.4 g ANSA using a porcelain mortar and pestle and stored in an amber-colored bottle[9]. Reducing reagent solution was prepared fresh prior to use and contained 40 mg triturate/ml of distilled water.

Acid hydrolysis of ATP and G6P

For determination of extent of acid hydrolysis, two phosphate esters were incubated under phosphate estimation conditions in the presence of varying concentrations (0.5, 0.625, 0.8 or 1.0 *N*) of H_2SO_4. In incubation mixture, the final concentrations of ATP were 2.0 and 5.0 m*M*, and that of G6P were 1.0 and 5.0 m*M*. Release of Pi was monitored over a 24 h period. Instability of the color posed a problem, when determinations were carried out in the presence of 0.5 *N* H_2SO_4 by the conventional Fiske and Subba Row method[1]. Thus, G6P and ATP at two above-mentioned concentrations were incubated with 0.5 *N* H_2SO_4 and aliquots were taken at specified time intervals to monitor the amount of Pi released using conventional procedure[1]. The extent of substrate hydrolysis at different time intervals was determined from the amount of Pi released, for which the corresponding values of slope were used (see Fig. 4 and 5).

Effect of sugars, salts and proteins

To examine, if sugars, salts or proteins interfered in the development of color and/or determination of the OD, experiments were carried in the presence of glucose and sucrose (1-10 m*M*), NaCl and KCl (5-100 m*M*), and $MgCl_2$ (1-10 m*M*) in the presence of varying concentrations of H_2SO_4 as detailed above. The interference by proteins was determined by including 10-500 µg BSA/assay tube in the presence of SDS (final concentration, 0.25% w/v). These experiments were performed using 4 µg Pi, which represented the middle of the assay range. Color development was monitored spectrophotometricaly for up to 24 h

Results

Effect of varying H_2SO_4 concentration

In the initial experiments, estimation of Pi was carried out by the conventional Fiske and Subba Row method[1]. The final concentration of H_2SO_4 was 0.5 *N*. Under these conditions, OD readings between 8-15 min were linear with the Pi concentration. However, at the end of 30 min, linearity was lost and the standards as well as reagent blank developed intense blue color, making measurements of the OD of the sample tubes difficult. Thus, further experiments were carried out only with the three higher concentrations (0.625, 0.8 or 1.0 *N*) of H_2SO_4 and the time course of color development was followed for up to 24 h. These data are shown in Fig. 1.

In the presence of higher concentrations (0.625, 0.8 or 1.0 *N*) of H_2SO_4, the color in the reagent blank as well standards were 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. The intensity of the color increased with time in all the sets of experiments and optimum color development was observed in the presence of 1.0 *N* H_2SO_4. Also, magnitude of increase in the OD was higher, when monitored at 820 nm. This suggested that with progress of time there was a shift towards formation of molybdenum blue species which absorbed at 820 nm. To verify this, absorption spectra of standard Pi (4 µg) solution at different time intervals were recorded. It was evident from the absorption spectra (Fig. 2) that although the absorption at 660 and 820 nm increased with the time, increase of absorbance at 820 nm was of greater magnitude. The spectra also confirmed that 1.0 *N* H_2SO_4 was most efficient both in the color development and shift in the absorption maxima.

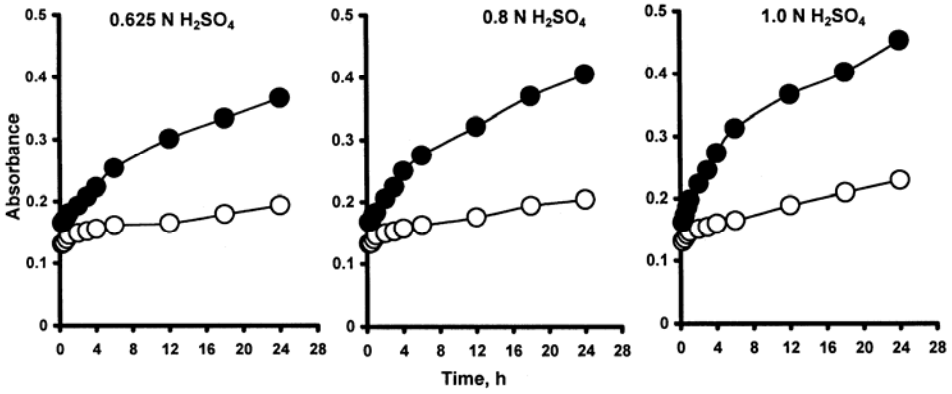

Fig. 1—Time course of color development in Pi assay [Experiments were performed using 4 µg Pi. Concentrations of H_2SO_4 were 0.625, 0.8 and 1.0 *N*. OD measurements at 660 nm and at 820 nm were carried out at specified time periods as indicated. Each data point represented average of 3 independent observations. Variation ranged from 2-3%. OD measurements at 660 nm (-○-) and at 820 nm (-●-)]

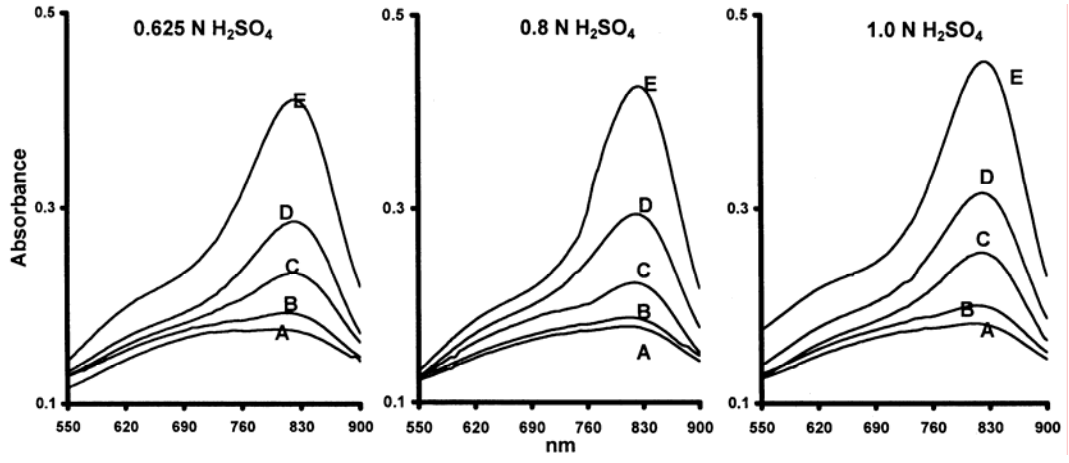

Fig. 2—Absorption spectra depicting the time course of color development in Pi assay [Experiments were performed using 4 µg Pi. Concentrations of H_2SO_4 were 0.625, 0.8 and 1.0 *N*. Absorption spectra were recorded at: A, 15 min; B, 1 h; C, 6 h; D, 12 h; and E, 24 h]

Also, under these conditions, there was a gradual shift in absorption maxima around 820-830 nm region towards higher wavelength. Typical plots depicting this shift in the absorption maxima in the presence of 0.625 and 1.0 *N* H_2SO_4 are shown in Fig. 3; the picture was intermediate for 0.8 *N* H_2SO_4 (data not shown).

The relative improvement in sensitivity of Pi estimation, in terms of a standard curve was monitored at different time intervals. For this purpose, the OD readings were recorded at 660 and 820 nm. These standard curves are shown in Fig. 4. It may be noted that the plots were linear up to the Pi

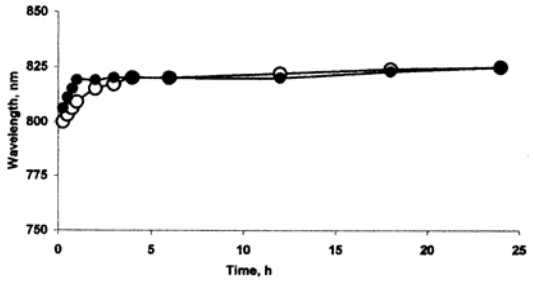

Fig. 3—Time course of shift in absorption maxima in Pi assay [Experiments were performed using 4 µg Pi. Concentrations of H_2SO_4 were 0.625 and 1.0 *N*. Each data point represented average of 3 independent observations. Variation ranged from 2-3%. (-○-), 0.625 *N*; and (-●-) 1.0 *N* H_2SO_4]

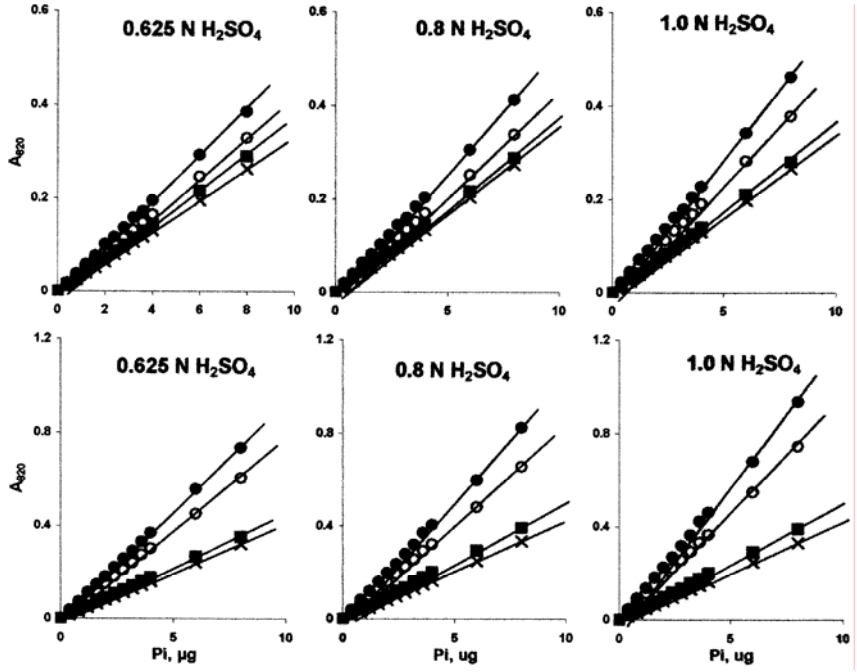

Fig. 4—Standard curves showing Pi concentration-dependent changes in absorbance at different time intervals [Pi concentration was 0-8 µg. Concentrations of H_2SO_4 were 0.625, 0.8 and 1.0 *N*. OD measurements were carried out at 660 and at 820 nm. Each data point represented average of 3 independent observations. Variations ranged from 2-3%. The scales are different for OD at 660 and 820 nm. (-×-), 15 min; (-■-), 1 h; (-○-), 12 h; and (-●-), 24 h]

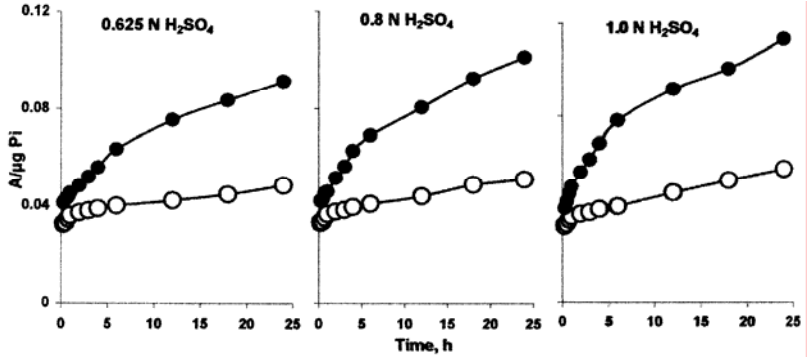

Fig. 5—Plots depicting changes in the slope (OD/µg Pi) with time [Color was developed using 4 µg Pi. Concentrations of H_2SO_4 were 0.625, 0.8 and 1.0 *N*. OD measurements at 660 nm and at 820 nm were carried out at specified time periods as indicated. Each data point represented average of 3 independent observations. Variation ranged from 2-3%. OD measurements at: 660 nm (-○-) and at 820 nm (-●-)]

concentration of 8 µg, under all the experimental conditions. However, the slopes increased with time. The changes in slope with time, monitored at 660 and 820 nm are shown in Fig. 5, which once again emphasized the efficacy of 1.0 *N* H_2SO_4.

The values of molar extinction coefficients (E_M) at different time intervals were computed based on the changes in the slope (Fig. 5) and the results are given in Table 1. Under the experimental conditions outlined above (Fig. 5), the E_M values at 660 or 820 nm did not change appreciably up to 1 h after development of the color and thereafter, the values increased steadily up to 24 h. The values of E_M determined at 660 nm for the three concentrations (0.625, 0.8 or 1.0 *N*) of H_2SO_4 were 1.46, 1.53 and 1.75-times higher, compared to that obtained in the conventional Fiske Subba Row method[1] (E_M = 2914) (Table 1). For measurements at 820 nm, these values (24 h) were 2.23, 2.42 and 2.81-times higher. This was also reflected in about 2-fold increase in the E_M at 820 nm at the end of 24 h, as compared to the corresponding values at 660 nm. Once again, 1 *N* H_2SO_4 was found to be more efficient in the color development.

Effect of H_2SO_4 concentration on substrate hydrolysis

Although the color development in the presence of 1.0 *N* H_2SO_4 (final conc.) was the most efficient in improving the sensitivity, it raised a concern with respect to applicability of the method in enzyme assays, where substrates such as ATP that contain acid labile phosphate was used. Therefore, it was pertinent to check the acid hydrolysis of the substrates under the assay conditions. Hence, the experiments were carried out to monitor hydrolysis of ATP (2 and 5 m*M*) and G6P (1 and 5 m*M*) in the presence of H_2SO_4 (0.5, 0.625, 0.8 and 1.0 *N*). The data for extent of hydrolysis of ATP with 0.5, 0.625 and 1.0 *N* H_2SO_4 are shown in Fig. 6. Results with 0.8 *N* H_2SO_4 showed an intermediate pattern (data not shown). The extent of ATP hydrolysis ranged from 0.08 to 1.51% at the end of 30 min for the three H_2SO_4 concentrations and at the end of 1 and 2 h, these values ranged from 0.4 to 3.4%, and 0.7 to 6.5% respectively. By 12 h, almost an equilibrium point was reached and the extent of hydrolysis ranged from 5.3 to 18.6%, depending upon the concentration of H_2SO_4 (Fig. 6). Hydrolysis of G6P was negligible (0.01-0.29%) under these conditions (data not shown).

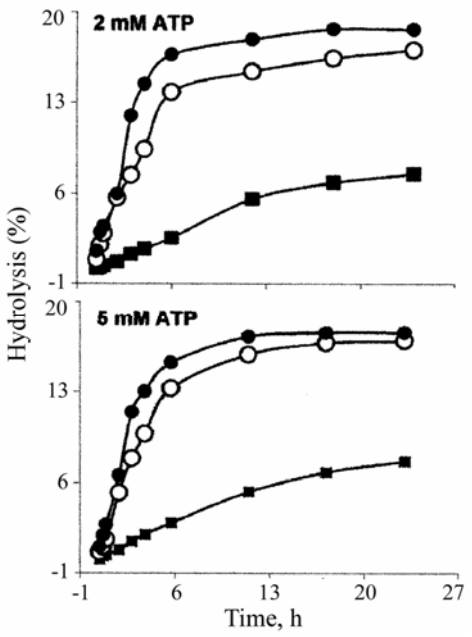

Fig. 6—Time course of ATP hydrolysis [Concentration of ATP was either 2.0 m*M* or 5.0 m*M*. Each data point represented average of 3 independent observations. Variation ranged from 2-3%. H_2SO_4 concentration was (-■-), 0.5; (-○-), 0.625 and (-●-), 1.0 *N*]

Table 1—Comparison of the sensitivity with three different concentrations of H_2SO_4 for determination of phosphorous by the molybdenum blue method

[Experimental details are as given in the text. The values of molar extinction coefficient (E_M) were computed from the data in Fig. 5. Each value represented the average of 3 independent observations. The value of E_M at 660 nm in conventional Fiske and Subba Row method[1] using 0.5 *N* H_2SO_4 at 0.25 h was 2914]

H_2SO_4 conc.	Wavelength (nm)	Molar extinction coefficient (E_M) at different time intervals, h										
		0.25	0.5	0.75	1	2	3	4	6	12	18	24
0.625 *N*	660	4030	4129	4302	4464	4600	4700	4811	4960	5232	5555	6002
	820	5109	5258	5406	5617	5989	6436	6907	7862	9350	10354	11358
0.8 *N*	660	4117	4166	4340	4526	4650	4737	4873	5047	5444	6026	6312
	820	5196	5282	5592	5679	6316	6956	7762	8544	9982	11470	12561
1.0 *N*	660	4067	4204	4402	4551	4687	4786	4948	5096	5853	6510	7105
	820	4997	5344	5754	6138	6932	7614	8469	9697	11371	12474	14049

Effect of sugars, salts and BSA

Inclusion of sugars (1-10 m*M*), salts (1-100 m*M*) or BSA (10-500 µg BSA/assay tube) at the concentrations indicated did not interfere with the color development or measurement of OD. Under these conditions, intensity of color development ranged from 96-103%, compared to the controls, where there was no addition of sugars, salts or BSA (data not shown).

Discussion

With the view of stabilizing the molybdenum blue color in the conventional phosphorus assay and improving the sensitivity, in the present study, we employed higher concentrations of H_2SO_4 than that was used in the conventional procedure[1]. Using the conventional procedure, we got the slope in the range of 0.022-0.025 OD units/µg Pi at 660 nm (averaged 0.0235 OD units/µg Pi; E_M 2914) and the color was stable maximally up to 15 min (data not shown). However, increased concentrations of H_2SO_4 stabilized the color as well as increased the sensitivity (Figs 1-5, Table 1). The sensitivity at 660 nm in the presence of 0.625 *N* H_2SO_4 was from 1.4-1.5 times higher between 15 min to 1 h period, compared to conventional value, and more than doubled at the end of 24 h. In terms of E_M values at 820 nm, the sensitivity was 1.8-1.9 times higher between 15 min to 1 h and increased to a 4 fold higher value at the end of 24 h (Fig. 5, Table 1). With 0.8 *N* H_2SO_4, sensitivity at 660 nm was from 1.4-1.6 times higher from 15 min up to 1 h period and increased to 2.2-fold at the end of the 24 h. For measurements at 820 nm, the sensitivity was 1.8-2.0 times higher between 15 min to 1 h and increased to 4.3-times higher value at the end of 24 h (Fig. 5, Table 1). In the presence of 1.0 *N* H_2SO_4, the sensitivity at 660 nm was 1.4-1.6 times higher between 15 min to 1 h and increased to 2.4-fold higher value at the end of 24 h. When the comparison was made with E_M values at 820 nm, the sensitivity was 1.7-2.1 times higher between 15 min to 1 h period and increased to a 4.8-fold higher at the end of 24 h (Fig. 5, Table 1).

The above results suggested that a further improvement in the sensitivity was achieved, if the measurements were carried out at 820 nm. Besides, the presence of 1.0 *N* H_2SO_4 had a marginal beneficial effect over 0.625 *N* H_2SO_4 at short time intervals i.e. up to 1 h. However, the use of higher concentrations of H_2SO_4 also raised a concern for applicability of the method in enzyme assays, especially where substrates such as ATP was used. Thus, we monitored the acid hydrolysis of ATP under the Pi assay conditions (Fig. 6). As can be noted, the extent of acid hydrolysis of ATP in the presence of 0.625 *N* H_2SO_4 during 15 min to 1 h period ranged from 0.7 to 3.4%. If the enzyme assay was carried out in 0.1 ml system and after terminating the reaction, if one used the entire assay medium for Pi determination in 4.0 ml assay, the background OD due to acid hydrolysis of ATP would get diluted 40-times and thus the substrate blank would increase only negligibly. Thus, the corresponding slope (Fig. 5) could be used to compute the amount of Pi released.

In Chen's[4] method, the color stability was achieved by removing excess molybdate by adding oxalic acid and extracting the color complex in isobutyl alcohol[4]. In the method of Linden *et al*[5], phophomolybdic acid was extracted with methyl isobutyl ketone and the measurement of molybdenum was carried out by atomic absorption spectrophotometry[5]. These procedures rendered the method cumbersome and time-consuming. Compared to these procedures, our method was simple, since no additional steps or extraction of the color complex was required for stabilizing the color and improving sensitivity. Our results also suggested that molybdenum blue absorbing in the region of 820-830 nm is the only stable species of the color.

In conclusion, the results of the present study demonstrated that Pi assays carried out in the presence of 0.625 *N* H_2SO_4 with OD measurements made at 820 nm increased the sensitivity. Further, it was also found that the inclusion of sugars, salts or protein such as BSA did not interfere with color development.

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