Chapter 9

A Simplified Micromethod for Phosphate Determination and Its Application for Inorganic Phosphate analysis and in Enzyme Assays

Introduction

Importance of phosphorous in intermediary metabolism is well recognized and well documented and needs no overemphasis (1,2). Because of its pivotal role, sensitive assay procedure for assay of phosphorous in micro-quantities assumes importance (3). The initial gravimetric / nephelometric methods were changed to colorimetric procedures which were based on the property of orthophosphate to form a complex with molybdic acid (4) It was realized that phosphomolybdate could be reduced to produce a deep blue colored complex called molybdenum blue (5). The first quantitative and reproducible method for colorimetric estimation of phosphate was described by Fiske and Subba Row where 1-amino-2-naphthol-4-sulphonic acid in conjunction with sodium sulphite and sodium metabisulphite was used as the reducing agents (6). Use of several other reducing agents such as hydroquinone, 2,4-diaminophenol, ascorbic acid, thiosulfate, stannous chloride, hydrazine sulfate etc. has since been described (7-12). The basic problem encountered in these methods is that the molybdenum blue color is unstable after a duration of 5 – 10 min depending on the method employed (7-12). Secondly, the acid hydrolysis of labile phosphorous possess a problem of interference and quantitative estimation although alternate procedures have been devised (3). The sensitivity of colorimetric method for phosphate estimation was improved by Bartlett (13), simultaneously the problem of color instability was also solved by this procedure (13). The method requires that the reduction to molybdenum blue be carried out in a boiling water bath for 7 min; this produced stable molybdenum blue species. However, because of the boiling step obviously the method is of little value for enzymatic analysis. Hurst tried to overcome the problem by using a mixture of hydrazine sulfate and stannous chloride as the reducing agent (14). Under this condition the color developed maximally

in 3 min and was stable upto 30 min or so. Also, the nature of molybdenum blue species produced was different from that obtained in the Fiske and Subba Row procedure or Bartlett procedure (14). The intensity of molybdenum blue in Fiske and Subba Row procedure can be monitored over a wavelength range of 600 – 700nm (6). The molybdenum blue produced in Bartlett method shows an absorption maximum at 830 nm whereas the molybdenum blue produced in Hurst method showed an absorption maximum at 700 nm (13,14). Thus obviously depending on the procedure employed molybdenum blue species of variable absorption characteristic and stability seems to be produced (6,13,14).

In the light of the above it was felt that it would be desirable if a sensitive assay procedure is devised which produced a stable molybdenum blue color and at the same time obviated the boiling step as in the case of Bartlett procedure (13) so that the method can be utilized for enzymatic as well as non-enzymatic phosphorous analysis; elimination of boiling step would provide a time saving and less cumbersome device for the color development.

From the literature survey it became apparent that stability of the reducing agents is of primary importance Ascorbic acid and hydrazine sulfate are stable at room temperature Hence it was decided to use a combination of these two reagents in different proportions to optimize the conditions for molybdenum blue color development and to get a stabilized color complex. The details of these experiments for developing a sensitive method for phosphorous estimation with an extended range and its application to enzymatic analysis using labile and stable phosphate esters are described below. The method can be used

most conveniently for inorganic phosphate determination without the necessity of boiling of the samples for color development The details of the same are also given below.

Chemicals

Glucose-6-phosphate (G6P) and bovine serum albumin fraction V (BSA) were purchased from Sigma Chemical Co USA Sodium salt of ATP was obtained from SRL, India. Silica Gel G was from E. Merck, Germany. Hydrazine sulfate and L-ascorbic acid were purchased from Merck, India. All other chemicals were purchased locally and were of analytical-reagent grade.

Experimental

Reducing agents

A mixture of hydrazine sulfate and ascorbic acid in varying proportions was prepared in 1.0 N H_2SO_4 (14) and 0.4 ml of the solution was used for reduction. The contribution to the final normality of H_2SO_4 in the 4.0 ml assay system by the reducing reagents solution is 0.1 N. Under standard assay conditions the reducing agent contained 20 mg each of hydrazine sulfate and ascorbic acid in 1.0 N H_2SO_4.

Assay procedure

Aliquots of solution containing 1- 10 µg of Pi were taken and the volume was made up to 2.4 ml with distilled water. To this 0.8 ml of 3 N H_2SO_4 was added and 0.4 ml of 2.5%

(w/v) ammonium molybdate (prepared in 3N H_2SO_4) was added. The contents were mixed thoroughly and reduction of phosphomolybdic acid was effected by adding 0.4 ml of reducing agent. Thus in the final assay procedure the concentration of H_2SO_4 was 1.0 N.

The variations in assay procedure for optimization of the procedure with respect to normality of H_2SO_4 and the composition of reducing agent mixture are detained in the individual experiments.

In separate experiments the time course of color development was determined under optimized assay conditions.

Enzyme assays

The applicability of the procedure for the determination of phosphohydrolases activities was ascertained using G6P and ATP as the substrates respectively for glucose-6-phosphatase (G6Pase) and Na^+,K^+, ATPase assays. Rat liver microsomes were used as the source of the enzyme (15).

In separate experiments the susceptibility of G6P and ATP to acid hydrolysis was assessed for upto 24 hrs at two different concentrations.

Measurement of G6Pase activity was carried out in the assay system (total volume 0.1 ml) comprising 100 mM sodium acetate, 5mM G6P and Ca. 150 µg of microsomal protein For substrate kinetics studies the concentration of G6P was varied from 0.1mM

to 20 mM The reaction was initiated by the addition of substrate and was carried out at 37°C for 10 min The reaction was terminated by adding 0.05 ml of 5% (w/v) of sodium dodecyl sulfate (SDS) solution Estimation of liberated inorganic phosphate was carried out employing the optimized assay procedure described above. In the presently described micromethod, color development was monitored at the end of 1 hr.

In separate set of experiments the G6Pase activity was also determined by following conventional procedure where the assay volume is 0.3 ml and the phosphate estimation was according to the method of Fiske and Subba Row (16).

Measurement of Na^+,K^+, ATPase activity was carried out in a final assay system (total volume of 0.1ml) comprising 50mM tris-HCl buffer pH 7 4, 120mM NaCl, 10mM KCl and 5mM $MgCl_2$ For substrate kinetics studies the concentration of ATP was varied from 0 1mM to 5 mM. The reaction was carried out at 37°C for 10min. After pre-incubating the enzyme (15-20 μg microsomal protein) for 2-3 min., the reaction was initiated by the addition of ATP. At the end of the incubation period, the reaction was terminated by the addition of 0.05ml 5% (w/v) SDS solution. The liberated inorganic phosphate was estimated by the method described above. In the microprocedure, the optical density measurements were made at the end of 30 min of color development, where contribution of acid hydrolysis of the substrate was minimum (Fig. 5) and about 80% of the optimum color development occurred (Fig. 1).

In separate set of experiments the ATPase activity was determined by following conventional procedure where the assay volume is 0 4 ml and the phosphate estimation was according to the method of Fiske and Subba Row (17).

The data for substrate kinetics were analyzed by the Lineweaver-Burk, Eadie-Hofstee and Eisenthal and Cornish-Bowden methods for the determination of Km and Vmax (18). The values of Km and Vmax obtained by the three methods were in close agreement and were averaged All the kinetics data were computer analyzed employing Sigma plot version 5.0 (19,20)

Protein estimation was by the method of Lowry et al. with bovine serum albumin (BSA) used as the standard (21).

Inorganic phosphate determination

The suitability of the present procedure as a substitute for Bartlett method was ascertained by carrying out analysis of mitochondrial phospholipids. Extraction of total phospholipids using freshly prepared chloroform · methanol (2·1 v/v) mixture was essentially as described previously (22,23). Separation of phospholipid classes by Thin layer chromatography (TLC) was according to the procedure of Skipsky et. al (24). Suitable aliquots of total phospholipids or individual phospholipid spots from TLC plates were subjected to digestion with H_2SO_4 and complete oxidation was insured by treatment with perchloric acid as described in details previously (19,25). Each sample was processed in duplicate

In those samples where phosphorous estimation was carried out by Bartlett method digestion was effected using 0.5 ml of 10N H_2SO_4.

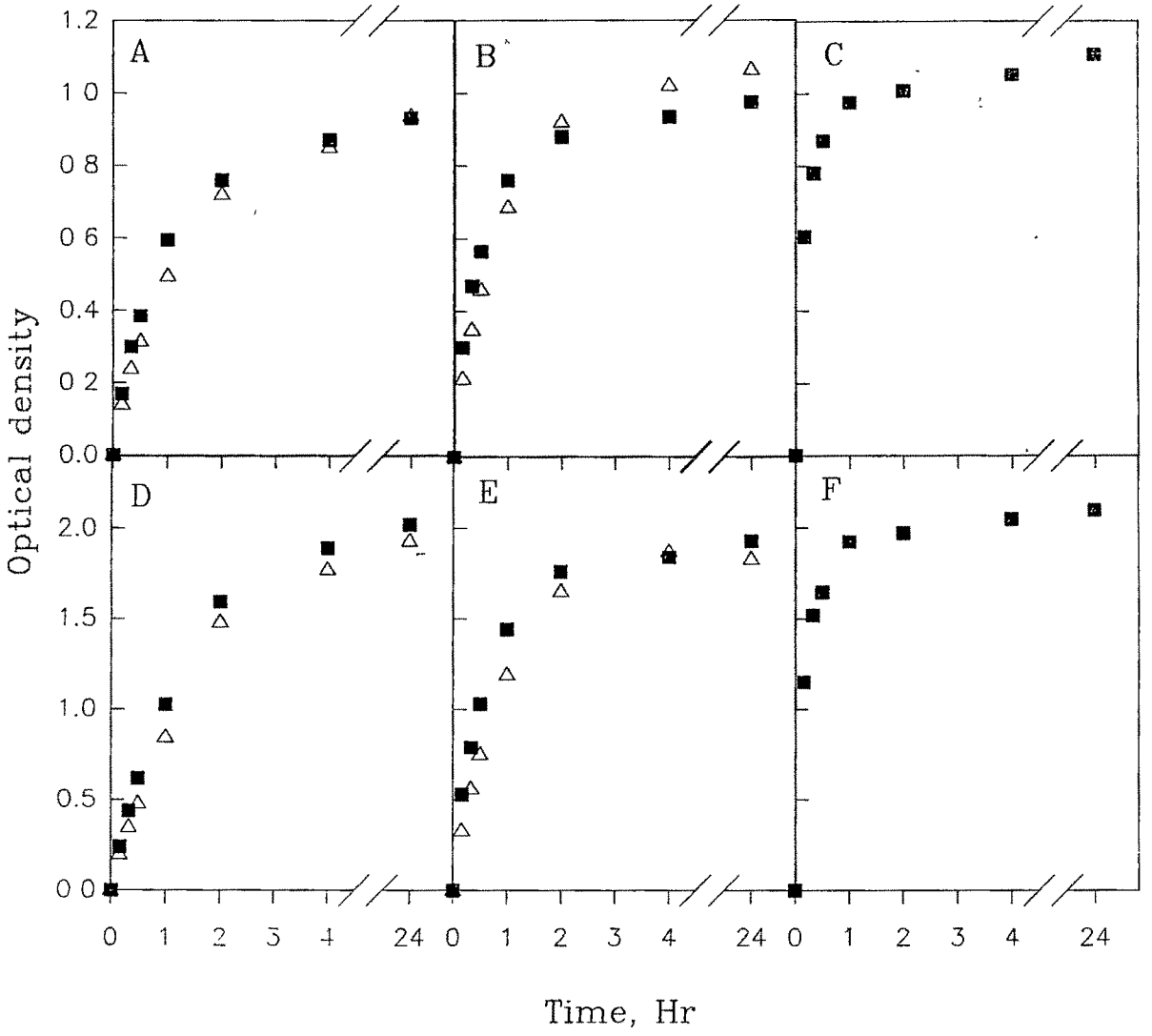
In second set of experiments where the present procedure was used, for phosphorous determination 0 3 ml of 10N H_2SO_4 was used for sample digestion This precaution is necessary in order to maintain the normality of H_2SO_4 in the final color development assay at 1 0 N

Results

In the first set of experiments the effect of varying proportions of hydrazine sulfate and ascorbic acid on the time course of color development was evaluated, using 5 and 10 μg Pi samples The results are shown in Fig.1. From panels A and D (Fig. 1) it can be noted that the addition of reducing agent solution containing hydrazine sulfate and ascorbic acid at 10 and 3 mg respectively/ ml or 20 and 3 mg respectively /ml resulted in a slow rate of color development. Thus by 1 hr time only about 50% of the color development occurred; At 2 hr the color development reached 75-80% and optimum color development was seen at 24 hr. In the next set (Fig. 1, Panels B and E) the concentrations of hydrazine sulfate were the same as in panels A and D, but the concentration of ascorbic acid was raised to 10 mg / ml With these combinations the color developed more rapidly. Thus at the end of 1 hr the color development reached about 60-65% of the optimum value. At the end of 2 hr 85-90 % of the optimum color development occurred. Finally (panel C and F) hydrazine sulfate and ascorbic acid were used at the concentration of 20 mg/ml each and as is evident the color development was very rapid; within 10 min 55-65% of the optimum color developed. By 30 min 75 – 80 % of the optimum color was developed and at the end of 1 hr 95% of the optimum color had developed. Keeping this advantage in mind of rapid color development with the last combination, in all the experiments

Fig 1 The effect of varying concentrations of hydrazine sulfate and ascorbic acid on the course of color development. In the 4.0 ml assay system the Pi concentration was 5μg (Panels A-C) and 10 μg (Panels D-F) and the final concentration of H_2SO_4 and ammonium molybdate were 1.0 N and 0.25% respectively. In A and D, the concentration of hydrazine sulfate and ascorbic acid was 10 mg and 3 mg/ ml of 1.0 N H_2SO_4 (---■--) and 20 mg and 3 mg/ml (---Δ---) respectively. In B and E the concentration of hydrazine sulfate and ascorbic acid were 10 and 10 mg/ ml (---■--) and 20 and 10 mg/ml (---Δ---) respectively. In panels C and F hydrazine sulfate and ascorbic acid were used at the concentration of 20 mg each /ml (---■--). The optical density was measured at 820 nm at various time intervals indicated.

Fig. 1


A
B
C
D
E
F
Optical density
1.2
1.0
0.8
0.6
0.4
0.2
0.0
2.0
1.5
1.0
0.5
0.0
0 1 2 3 4 24
Time, Hr

performed the concentration of hydrazine sulfate and ascorbic acid were kept at 20 mg each / ml of 1N H_2SO_4.

Bartlett has shown that normality of H_2SO_4 is important for color development in the Fiske and Subba Row as well as Bartlett procedures (13). Hurst has shown that in his assay procedure the color was stable over the range of 0.8 – 1.1 N H_2SO_4 (14). We therefore ascertained the effect of the H_2SO_4 concentration on the color development. These data are shown in Fig 2. As can be noted there was hardly any color development in 0.4 N H_2SO_4. In fact, the blank gave much higher color density than the sample tubes. In 0 5 N H_2SO_4 the color development was only about 20% of the optimum value whereas over the range of 0 6 N – 1.0 N H_2SO_4, almost plateau region was seen

The nature of molybdenum blue formed was ascertained in terms of its absorption characteristics. Simultaneously, for the sake of comparison, the spectra of molybdenum blue formed in Fiske and Subba Row and Bartlett methods (6,13) were also recorded using the same concentration of Pi. These spectra are shown in Fig. 3. As is evident the molybdenum blue in Fiske and Subba Row method showed no well defined peak, whereas the molybdenum blue formed in Bartlett and present procedure showed absorption maxima at 820 nm. Also the peak height was higher in the present procedure than that in the Bartlett method.

It is of interest to note here that the molybdenum blue species formed in Fiske and Subba Row method which had ill-defined absorption characteristics (Fig. 3 above) was stable for up to 10 min.(6). The molybdenum blue species in Hurst procedure which absorbs at

Fig 2. Effect of sulphuric acid concentration on the color development. In the 4.0 ml assay system containing 5µg Pi, the concentration of H_2SO_4 was varied as indicated from 0.4 N to 1.0 N. Concentration of ammonium molybdate was 0 25% while reducing agent used were the same as in panels C and F of Fig. 1.

Fig. 2

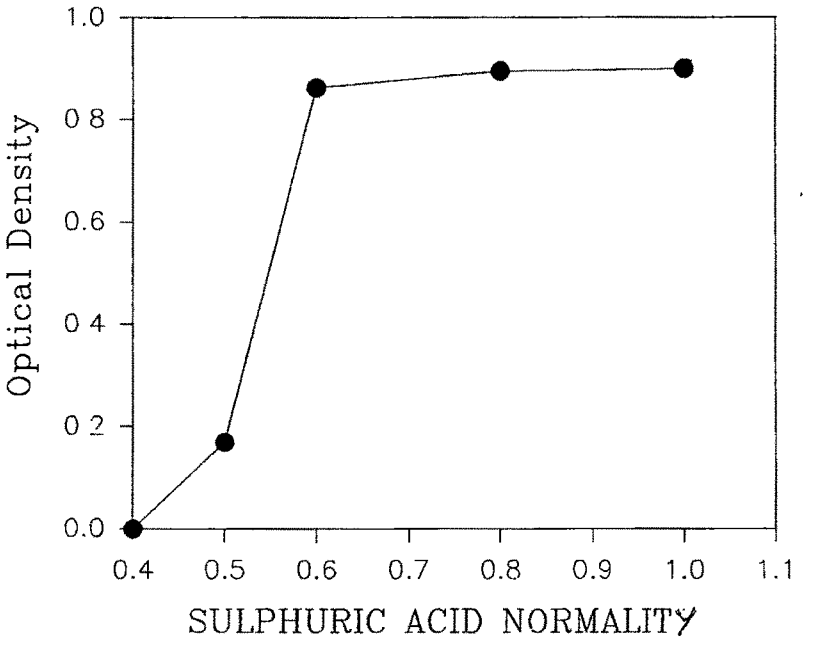
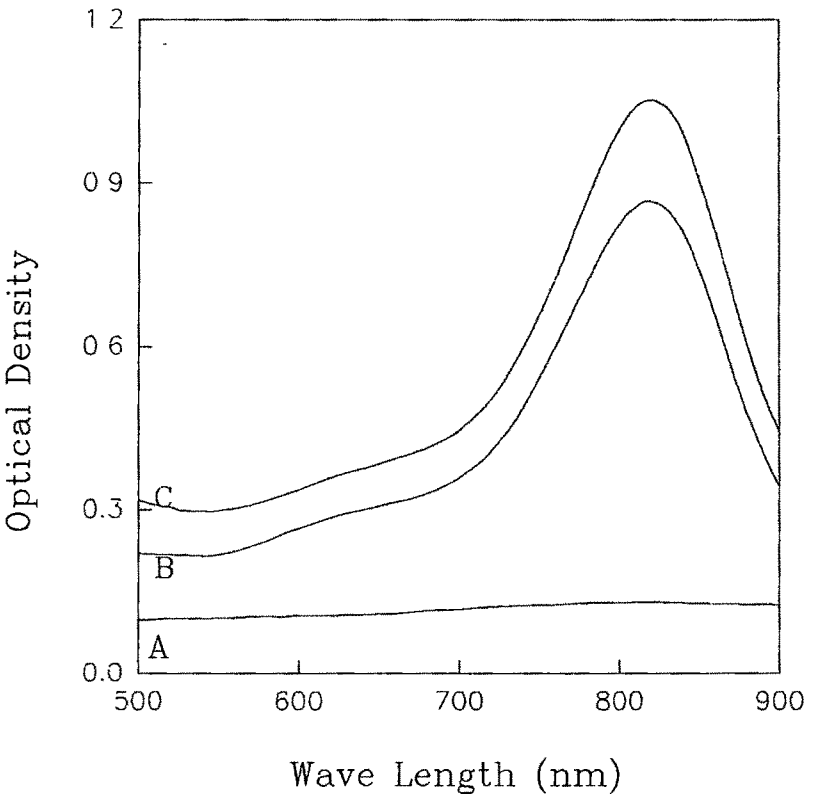

1.0
0.8
0.6
0.4
0.2
0.0
Optical Density
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
SULPHURIC ACID NORMALITY

Fig. 3. Spectra of molybdenum blue produced given by different assay procedures. A, Fiske and Subba Row method B, Bartlett method and C. Present method. In the 4.0 ml assay system the concentration of Pi was 5μg. The spectra was recorded in a shimadzu uv/vis spectrophotometer model 180 over the wave length range of 500 to 900 nm. The optical density measurements were carried out at the end of 24 hr in the present method.

Fig. 3


1 2
0 9
0 6
0.3
0.0
Optical Density
C
B
A
500
600
700
800
900
Wave Length (nm)

700 nm was stable up to 30min (14) whereas the molybdenum blue species produced by Bartlett (13) as well as our procedure which absorbs at 820 nm were stable up to 24 hr. Taken together, the results suggest that the molybdenum blue species with absorption maximum at 820 nm represents the most stable species.

The standard curve for phosphorous estimation according to the present procedure is shown in Fig. 4 As can be noted, not only is the color development linear with concentration of Pi but also that the range for estimation is extended to 10 μg of Pi, which is 2.5 times higher than that in Bartlett procedure (13).

A comparative account of the Molar extinction coefficients of molybdenum blue produced by different procedures is given in Table 1. As can be noted, consistent with the data in Fig. 3 the value of the molar extinction coefficient by the present procedure was much higher signifying improved sensitivity.

The applicability of the present procedure for enzymatic analysis was then evaluated. Prior to the actual enzyme assay we ascertained the extent of acid hydrolysis of the two substrate G6P and ATP for over 24 hr period. The two substrates at two concentrations (2mM and 5mM ATP and 2 and 20 mM G6P final concentration) were incubated under phosphorous assay conditions and the release of inorganic phosphate was monitored over 24 hr period. The results are presented as % hydrolysis of the substrate (Fig. 5) As can be seen G6P was completely stable whereas progressive ATP hydrolysis was seen up to 2 hrs after which a steady state was reached. Thus the extent of acid hydrolysis of ATP at 10,20, 30 and 60 min was 2, 3, 4.5 and 7 % after which the value reached a plateau level of 12.5 %

Fig. 4. The standard curve for Pi estimation by the present procedure. In a 4.0 ml assay system the concentration of Pi was varied from 1μg to 10μg. other experimental conditions were the same as described in panels C and F of Fig. 1 The measurement of optical density was carried out at 820nm at the end of 24 hours of color development.

Fig. 4

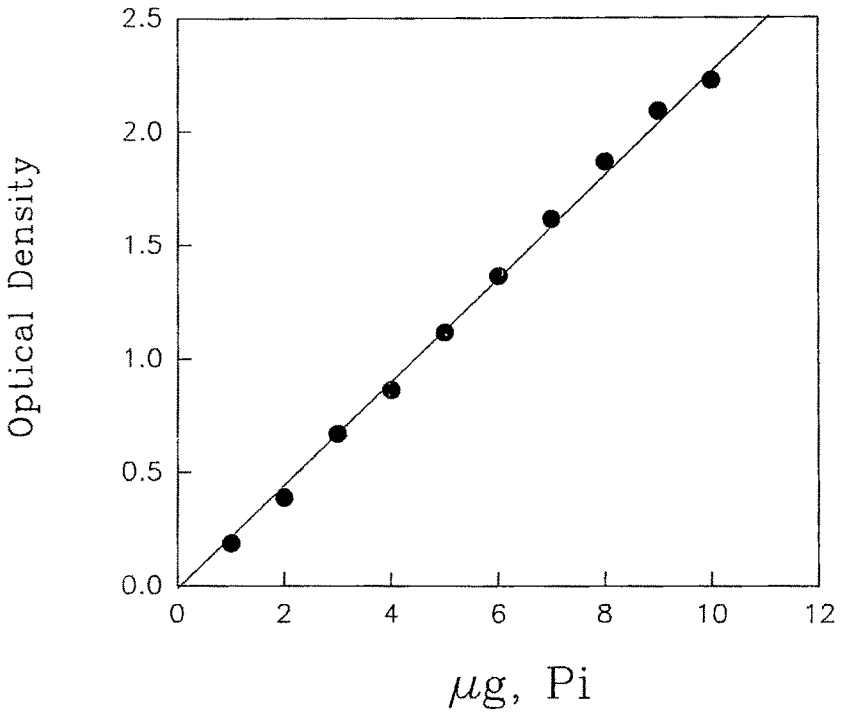
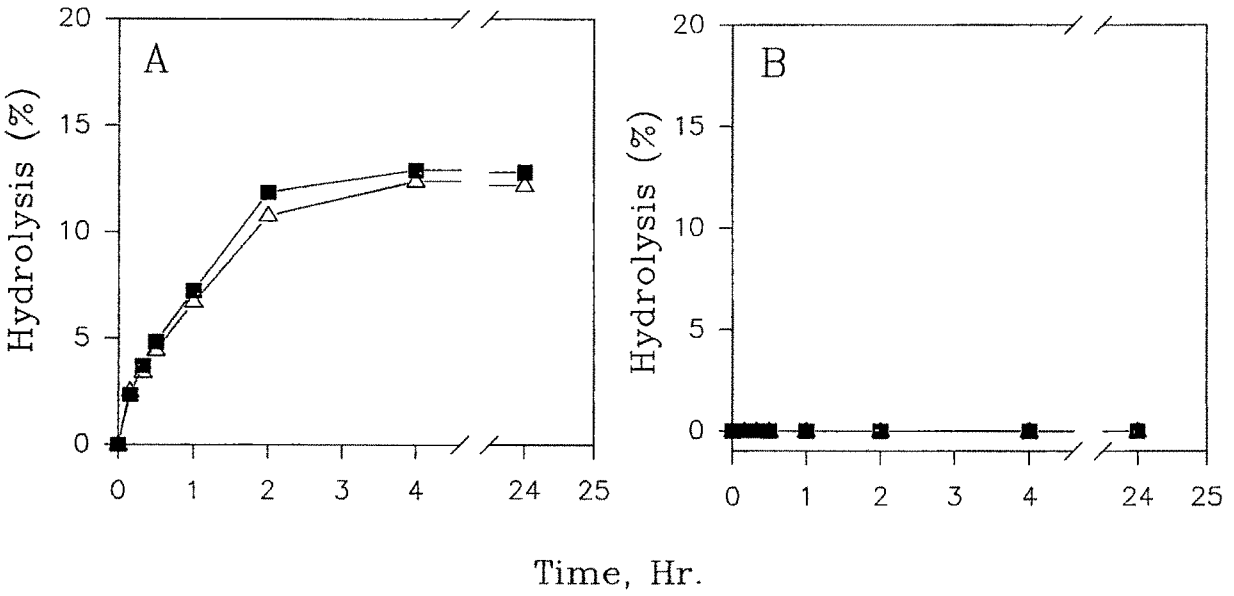


Table 1 Comparison of the sensitivity of the three procedures for the determination of phosphorous by the molybdenum blue method

Method	Reducing agent	Heating period	Molar extinction coefficient	
			700 nm	820 nm
Fiske and Subba Row	Amino-napthol-sulphonic acid	none	3645	4650
Bartlett	Amino-napthol-sulphonic acid	7 min	8903	21452
Present	Hydrazine sulfate and Ascorbic acid	none	11036	26139

Fig. 5. The time course of acid hydrolysis of (A) ATP and (B) G-6-P. ATP (final concentration 2 mM (---□--) and 5 mM(---Δ---)) and G-6-P (final concentrations 2 mM (---■--) and 20 mM(---Δ---)) were incubated with 1N sulfuric acid under the standard Pi assay condition Release of inorganic phosphate was monitored at different time interval upto 24 hr as indicated in the text and in the figure.

Fig. 5



Since it was noted earlier that about 80 % of the optimum color development occurred within 30 min (Fig 1, Panels C and D) it was decided to measure the ATPase reaction by monitoring the color developed at the end of 30 min The rationale for selecting this time interval was that 4 5% hydrolysis of ATP will not add substantially to the background above which the Pi released by enzymatic hydrolysis can be easily picked up. Additionally, the micro-assay procedure employed would substantially reduce the substrate blank.

In the preliminary studies, the activities of the G6Pase and Na^+,K^+, ATPase by following the conventional and the modified micromethod (as described above) were also compared The data (Table 2) show that both the methods give identical results. In the next series of experiments the kinetic properties of the two enzymes were examined.

The typical substrate saturation curves for Na^+, K^+, ATPase by the conventional procedure and by the present method are shown in Fig.6 A and B respectively. The corresponding Eadie-Hofstee plots are shown in Fig 6 C and D respectively. The computed data on Km and Vmax values of the two components of Na^+, K^+, ATPase are given in Table 3 As can be noted the results by the two methods were identical. Also, the values of Km and Vmax by the two methods were consistent with previously reported observation (19).

The substrate saturation curves and corresponding Eadie-Hofstee plots for G6Pase are given in Fig. 7 and the corresponding Km and Vmax values are included in Table 3. Once again the results by the two methods were identical.

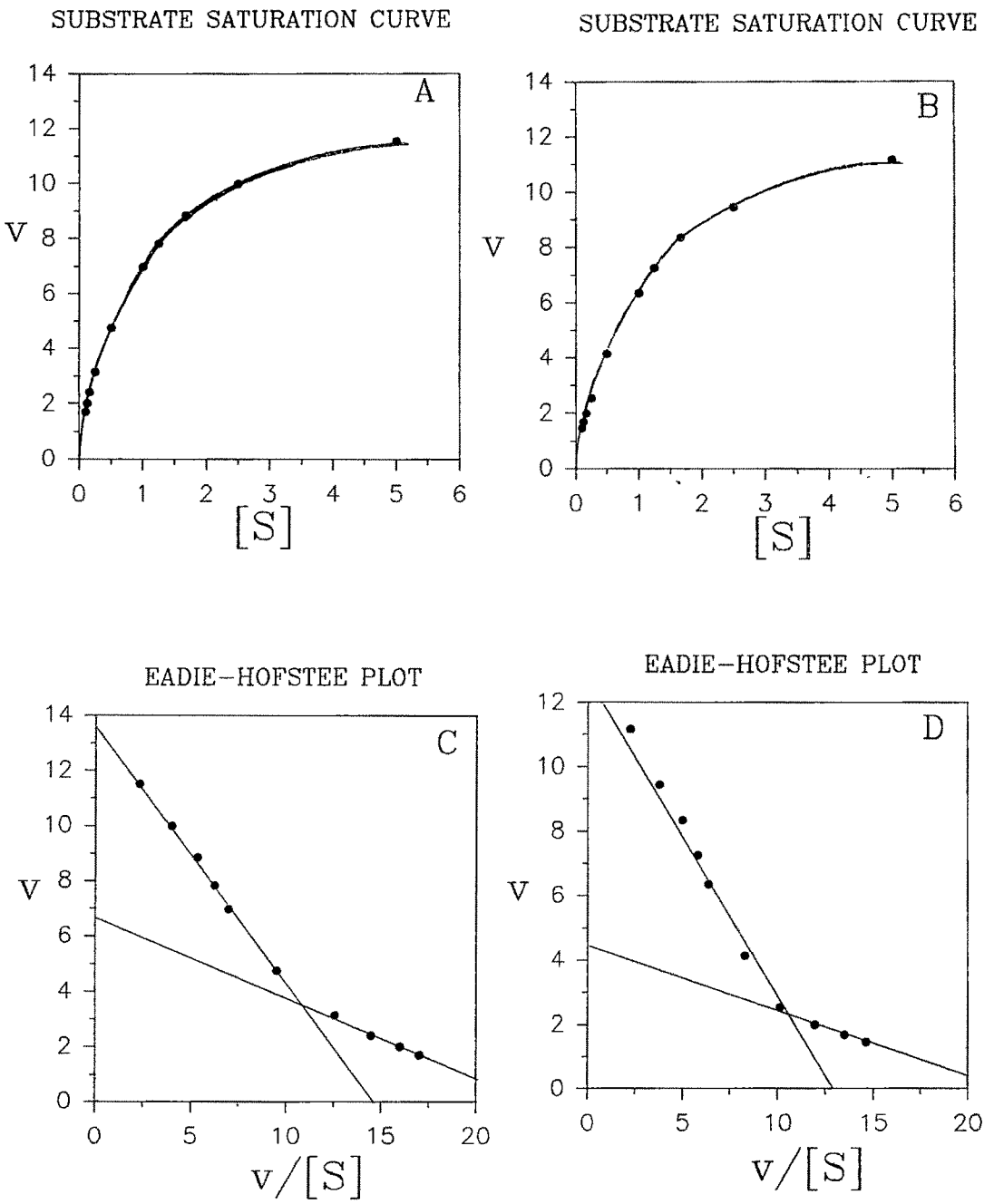
Table 2. Activities of rat liver microsomal glucose-6-phosphatase and Na^+, K^+ ATPase

Enzyme	activity (μmoles of Pi liberated /hr/ mg protein)	
	Conventional method	Present method
Glucose-6-phophatase	7.01±0.18	6.87±0.50
Na^+, K^+ ATPase	8.91±0.57	9.18±1.01

The experimental details are as given in the text. The results are given as mean ± SEM of six independent observations.

Fig.6. Typical substrate saturation curves for rat liver microsomal Na^+, K^+ ATPase, using (A) Conventional procedure and (B) Present method The respective Eadie-Hofstee plots are shown in (C) and (D) Experimental details are as given in the text.

Fig. 6


SUBSTRATE SATURATION CURVE
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SUBSTRATE SATURATION CURVE
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V
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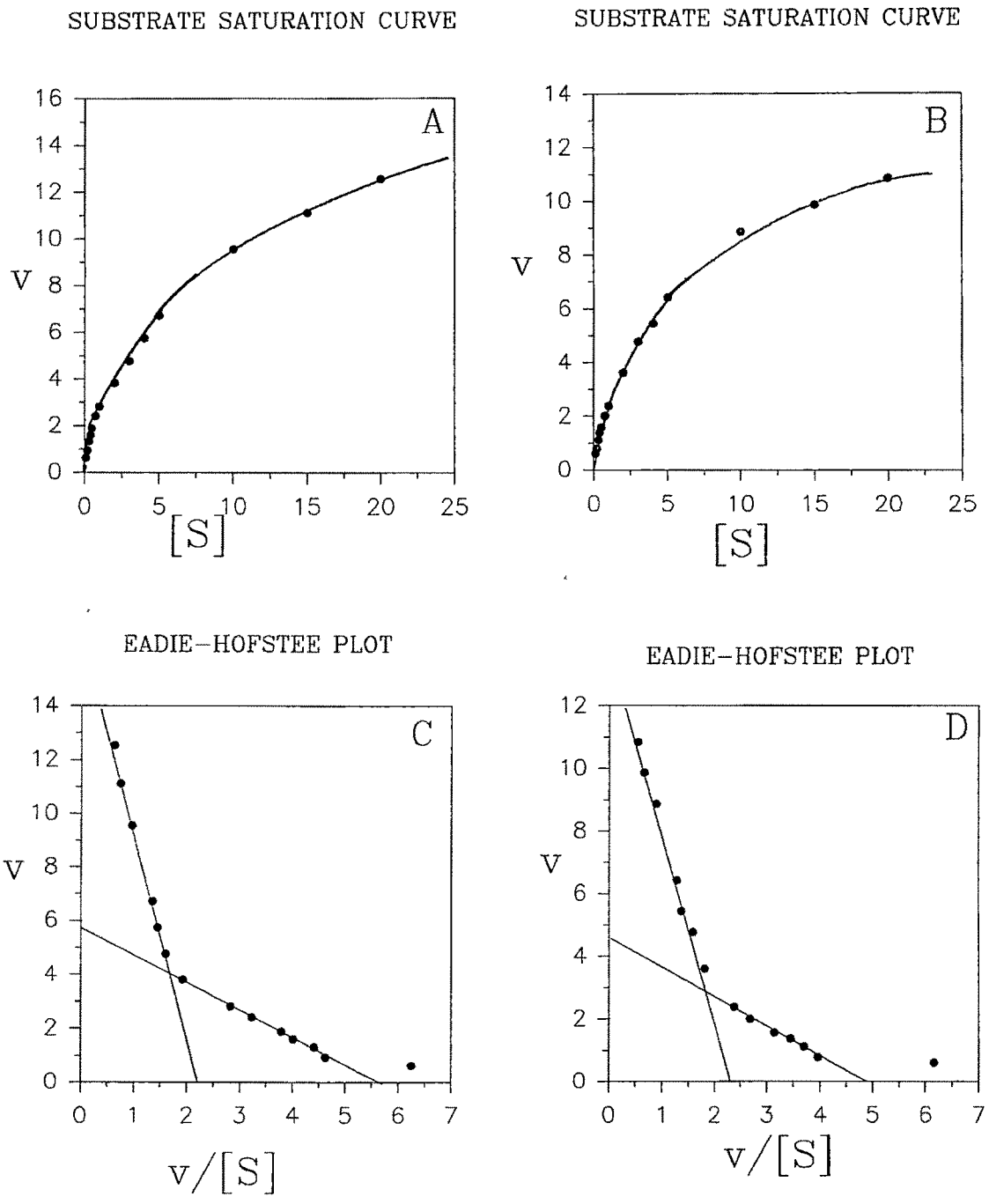
EADIE–HOFSTEE PLOT
C
V
v/[S]
EADIE–HOFSTEE PLOT
D
V
v/[S]

Fig.7. Typical substrate saturation curves for rat liver microsomal glucose-6-phosphatase using (A) Conventional procedure and (B) Present method. The respective Eadie-Hofstee plots are shown in (C) and (D). Experimental details are as given in the text.

Fig. 7


SUBSTRATE SATURATION CURVE
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EADIE–HOFSTEE PLOT
C
V
v/[S]
EADIE–HOFSTEE PLOT
D
V
v/[S]

Table 3. Kinetic attributes of the rat liver microsomal glucose-6-phosphatase and Na^+, K^+ ATPase

Enzyme	Assay procedure	Component I		Component II	
		Km	Vmax	Km	Vmax
G6Pase	Conventional	0.23±0.01	4.64±0 21	0.85±0.15	12.10±0.89
	Present	0.26±0.02	5.08±0.49	0.93±0.14	12.23±0.67
Na^+, K^+ ATPase	Conventional	0.94±0.08	5.90±0.34	5 46±0.37	14.62±0.57
	Present	0.93±0.09	5.42±0.67	5.65±0.47	14.69±0.96

Experimental details are as given in the text.
Km =mM;
Vmax is given as µmoles of Pi liberated /hr/ mg protein
The Km and Vmax values were calculated by three different methods of analysis as described in the text using Sigma Plot version 5.0 and were averaged for calculating the mean ± SEM values.
The results are given as mean ± SEM of the six independent observations.

The applicability of the present method for phospholipid analysis was also checked. As can be noted from the data in Table 4 the values for mitochondrial and microsomal total phospholipids by the two methods were in excellent agreement as were the data for mitochondrial phospholipid composition (Table 5).

Thus the method that was developed seems to be more sensitive with extended concentration range for Pi analysis. Besides the enhanced sensitivity, the method obviates the need for boiling the sample for color development. The procedure produced a stable molybdenum blue color species. The color development followed a rapid course and about 95% of the optimum color development was seen within 1 hr. Even under this condition the extent of hydrolysis of acid labile phosphate was negligible. Nevertheless, for application to phosphohydrolase activity using substrates with acid labile phosphate, 30 min time seems to be a safe period for monitoring color development (Fig. 1, 5 and 6) The additional advantage is that the volume of enzyme assay is reduced by a factor of 3 – 4 thereby reducing the input of materials and sample. Thus the method becomes not only more sensitive but also economical.

Although the present procedure has been standardized for a final assay volume of 4ml, in principle it should be possible to cut down the sample volume to 1.0ml thereby increasing the sensitivity further by 4 times.

The additional advantage of the method is that the reducing agents hydrazine sulfate and ascorbic acid are stable at room temperature as is sulfuric acid. This obviates the need for preparing the reducing reagent mixture in large quantity and taking care for its storage.

Table 4 Total phospholipid content in rat liver mitochondria and microsomes.

	TPL content (μg /mg protein)	
Assay procedure	Mitochondria	Microsomes
Bartlett	177.3±6.50	424.5±31.41
Present	185.4±8.11	415.6±21.04

The experimental details are as given in the text. The results are given as mean ± SEM of 6 independent observations.

Table 5. Phospholipid composition of rat liver mitochondria.

Phospholipid class	Phospholipid composition (% of Total)	
	Bartlett procedure	Present procedure
Lyso	4.45±0.52	4.51±0.61
SPM	5 42±0.33	4.41±0.58
PC	42.13±1.99	43.95±2.50
PI	3.15±0.64	3.35±0.43
PS	3.06±0.54	2.91±0.43
PE	28.58±1.31	17.01±0.98
DPG	15.25±1.03	15.99±0.90

The experimental details are as given in the text. The results are given as mean ± SEM of 6 independent observations.

In conclusion it was felt that the above described method is a versatile micromethod for phosphorous estimation with its applicability for enzymatic and non-enzymatic phosphate estimation. The method is also less cumbersome since the boiling step is eliminated.

References

1. Mc Elory W D and Bently G (Eds). Phosphorous metabolism Vol. 1, The Johns Hopkin press. Baltimore 1951
2. Mc Elory W D and Bently G. (Eds). Phosphorous metabolism Vol. 2, The Johns Hopkin press. Baltimore 1951
3 Lindberg O and Ernster L Determination of organic phosphorous compound by phosphate analysis, In Methods of Biochemical analysis, Vol. III. Ed. David Glick, Interscience publishers Incr, New York, 1956;1-22.
4. Stern , A., Ind, Eng. Chem., Anal. Ed , (1942) 14, 74.
5. Taylor, A E , and Miller C.W J. Biol. Chem.(1914) 18,215.
6. Fiske C H, Subba Row Y. Colorometric determination of phosphorous. J. Biol. Chem. 1925;66: 375-400.
7 Eggleton, P. and Eggleton G. P Biochem. J. (1927) 21,190.
8. Muller, E. Hoppe-Seyler's Z. Physiol. Chem (1935) 237,35.
9. Ammon, R and Hinsberg, K. Hoppe-Seyler's Z. Physiol. Chem. (1936) 239,207
10 Ikeda. N,J Chem Soc Japan Pure Chem Sect (1951) 72,23.
11. Horwitt, B.N J Biol Chem. (1952) 199, 537.
12 Boltz, D.F, and Mellon. G M. Ind, Eng. Chem., Anal. Ed., (1947) 19,873.
13. Bartlett G R. Phosphorous assay in column chromatography. J Biol Chem 1954;234: 466-468.
14 Hurst, R O. The determination of nucleotide phosphorous with a stannous chloride-hydrazine sulphate reagent. Can. J. Biochem. 1964;42:287-292.
15 Lehninger A L Biochemistry, 1st ed, Worth Publishers, New York. 1970.

16. Hepatic microsomal glucose-6-phosphatase of normal and alloxan-diabetic rats. Biochim Biophy. Acta. (1981),657.106-121.

17. Patel H G, Aras R V, Dave K R, Katyare S S. Kinetic attributes of Na^+, K^+ ATPase and lipid/phospholipid profiles of rat and human erythrocyte membrane. Z Naturforsch. 2000;C 55:770-777.

18. Dixon M and Webb E C. Enzymes. Eds. M Dixon, E C Webb, C Thorne Jr., K F Tipton. 3rd edn Longman, London. 1979.

19 Kaushal R, Dave K R, Katyare S S. Paracetamol hepatotoxicity and microsomal function Environ. Toxicol. Pharmacol.1999;1: 67-74.

20. Dave K R, Syal A R, Katyare S S. Tissue cholinesterases. A comparative study of their kinetic properties Z Naturforsch. 2000;C 55:100-108.

21. Lowry O H, Rosebrough N J, Farr A L, Randall R J. Protein measurement with Folin phenol reagent. J. Biol. Chem. 1951;193: 265-275.

22. Folch J, Lees M and Sloane-Stanley G H. A simple method for isolation and purification of total phospholipids from animal tissues. J Biol Chem. 1957;226:497-509.

23 Stahl E Apparatus and general techniques, In: TLC in thin layer chromatography: A Laboratory Handbook, 2nd ed. Ed. E Stahl. Springer-Verlag, New York, 1969;pp. 52-86.

24. Skipski V P, Barclay M, Barclay R K, Fetzer V A, Good J J and Archibald F M. Lipid composition of human serum lipoprotein. Biochem. J. 1967;104: 340-361.

25. Parmar D V, Ahmed G, Khandkar MA and Katyare S S. Mitochondrial ATPase: a target for paracetamol-induced hepatoxicity. Eur. J. Pharmacol. Section. 1995;293: 225-229

Summary

A micromethod for phosphate estimation has been developed which is sensitive, easy, economic and applicable for both enzymatic and non-enzymatic assays. A mixture of hydrazine sulfate and ascorbic acid was used as the reducing agent for developing the molybdenum blue color for which the conditions were standardized. In the 4.0 ml assay system, 0.4 ml of the mixture containing 20mg hydrazine sulfate and 20mg ascorbic acid per ml of 1 0 N H_2SO_4 gave a rapid optimum color development with absorption maximum at 820 nm. The method has a higher range of Pi estimation and high sensitivity. The applicability of the method for assay of microsomal glucose-6-phosphatase and Na^+,K^+, ATPase which use acid stable and acid labile substrates e.g. G6P and ATP respectively was checked in comparison to the conventional procedures. Likewise the applicability for phospholipid analysis in comparison with the conventional method was also checked In both enzymatic and non-enzymatic assays the results by the micromethod gave results identical to those obtained by the conventional methods In general the method is not only rapid, economical and convenient but also has wide applicability