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An Improved Assay for Nanomole Amounts of Inorganic Phosphate

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A colorimetric assay for the determination of nanomole amounts of inorganic phosphate is described. The procedure combines a very high molar extinction with color stability and insensitivity to newly released phosphate from labile organophosphates.

For studies on the energetics of active ion transport in various epithelial membrane systems, we needed a dependable ultramicromethod for the determination of inorganic phosphate. The most sensitive colorimetric method, that of Itaya and Ui (1) as modified by Hess and Derr (2), has some serious drawbacks: (a) the optical density of both the blank and the samples increase with time to an objectionable level, and more importantly (b) both ATP and ADP are hydrolyzed in the presence of the reagents. Both the highly acidic conditions and the catalytic effect of molybdate contribute to the hydrolysis of labile organophosphates and result in various phosphate levels being recorded according to the length of time allowed for color development (3,4).

Since our laboratory started to measure ATPase activity routinely, we settled on our slight modification of the Baginski method of P_i determination (5). This method (through the use of a citrate/arsenite mixture added immediately after the molybdate reagent) is relatively sensitive, color stable, and has the advantage of being insensitive to any newly released phosphate (e.g., P_i released by hydrolysis of ATP after stopping the enzyme reaction). For our energetic studies, however, the above mentioned is not sensitive enough to measure P_i in nanomolar amounts. We have successfully modified the "quenching" procedure of Baginski so that it can be used with the Hess and Derr method, giving the latter the color stability and insensitivity to nascent phosphate that it lacks. Furthermore, this method has virtually the same sensitivity as the Hess and Derr method.

MATERIALS AND METHODS

The materials and methods are essentially those of Hess and Derr; all the precautions recommended by those authors were observed. All solutions were made with deionized water.

(1) 0.045% malachite green hydrochloride (MG)[2]
(2) 4.2% ammonium molybdate in 4 N HCl (AM)
(3) Sterox (St), Coleman Instruments
(4) 34% sodium citrate·$2H_2O$ (w/v)
(5) 10 mM KH_2PO_4 (salt was dried several hours at 100°C); appropriate dilutions were made from this stock and used for standards.

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[2] Abbreviations used: MG, 0.045% malachite green hydrochloride; AM, 4.2% ammonium molybdate in 4 N HCl; St, Sterox.

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TABLE 1

ABSORBANCE OF "UNQUENCHED" AND "QUENCHED" BLANKS WITH TIME[a]

Blank	Time (min)				
	0	30	60	90	180
"Unquenched"	0.000	0.026	0.042	0.053	0.083
"Quenched"	0.000	0.000	0.000	0.000	0.000

[a] Tubes were kept at room temperature. Original blank at each interval was read against a freshly prepared blank. Two-milliliter quartz cuvettes with a 1-cm optical path length were used.

The color reagent was prepared from the above solutions as follows:

(a) 3:1 mixture of MG and AM solutions. Mixed at least 20 min and then passed through Whatman No. 5 filter paper (MG/AM).

(b) 100 μl of Sterox added to 5 ml of MG/AM solution (MG/AM/St).

To 50 μl of sample (total of sample and water), 800 μl of the MG/AM/St solution is added and mixed. After 1 min, 100 μl of the citrate solution is added and mixed. This solution can be read immediately at 660 nm in a spectrophotometer. We feel, however, that 30 min at room temperature should be allowed for full color development. The color is stable for at least 4 h; in our studies the samples were routinely read within 2 h. The above procedure represents a doubling of the amount of color reagent (MG/AM/St) used by Hess and Derr so conventional 1- or 2-ml cuvettes may be used. We therefore had 1.96 the volume used by those authors and consequently almost one-half the concentration. When sample sizes are smaller, the whole procedure may be scaled down and measurements made in microcuvettes. Greater amounts of sample (up to 500 μl) can be used without additional color reagent as long as appropriate corrections are made for dilution effects. Blanks contain water, color reagent, and citrate.

In all our attempts to repeat the Hess and Derr method we could get no better than 70% the molar absorptivity (extinction) obtained by those authors. This variability is not uncommon in color reactions involving phosphomolybdate complexes (6). In our assay the addition of citrate reagent did not change the molar absorptivity (80,000) from that obtained without citrate.

RESULTS

Table 1 shows the increase in optical density of both the "unquenched" (without citrate) and "quenched" (with citrate) blanks at 0.5-h intervals over a 3-h period.

Figure 1 shows the increase in optical density of a sample of ATP over a 3-h period in the presence of the color reagent (MG/AM/St) without citrate. From the linear portion of the curve, a hydrolysis rate of 2.70 nmol of ATP per hour can be calculated. The amount of terminal phosphate released after 1 h represents 10.8% of the total ATP originally in the sample. Figure 2 shows that the increase in optical density stops immediately upon the addition of citrate, and the color remains stable for several hours. Figure 3 demonstrates that the linearity of the standard curve is unaffected by citrate. There is a small shift in the absorption maximum from 660 to 650 nm; nevertheless, the sample's absorbance may be read anywhere between 600 and 700 nm. In addition to ATP,

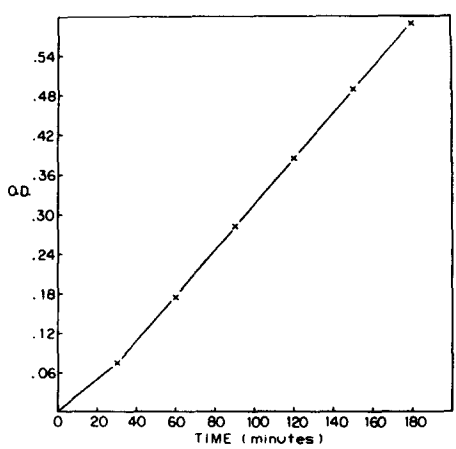

FIG. 1. The hydrolysis of ATP in the presence of the color reagent without citrate. Samples containing 50 μl of 0.5 mM ATP + 800 μl MG/AM/St + 100 μl H_2O were kept at room temperature.

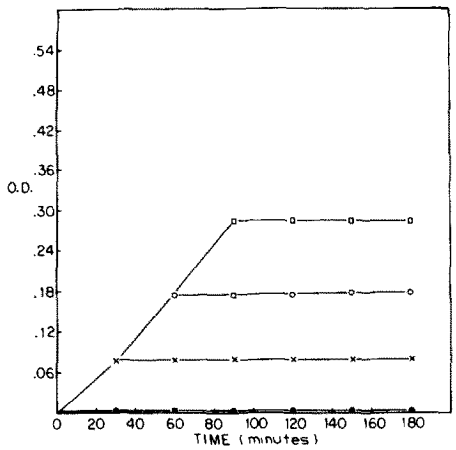

FIG. 2. The hydrolysis of ATP in the presence of the color reagent; interrupted color development by citrate addition at several time intervals. (●) Citrate added immediately; (X) citrate added after 30 min; (○) citrate added after 60 min; (□) citrate added after 90 min.

several other organophosphates were tested as to their lability in the color reagent. Of the group sampled, only ATP and ADP showed any detectable liberation of phosphate in the color mixture (Table 2).

The molybdate-mediated hydrolysis was temperature dependent. The use of citrate could be avoided if the color mixture was kept at 0°C. However, color development is slower and somewhat more erratic than at room temperature. At 0°C, provision must be made to cool the cuvette chamber of the spectrophotometer to prevent condensation on the cuvettes.

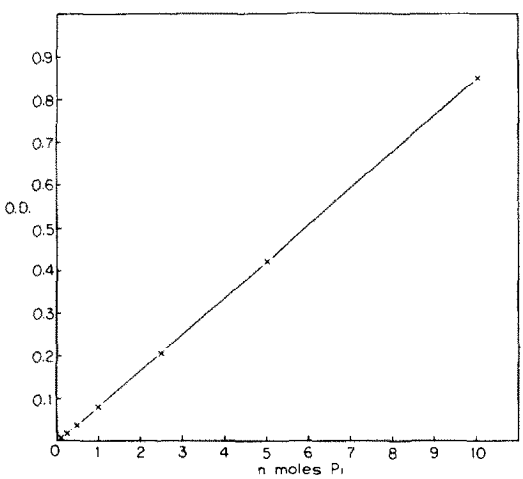

FIG. 3. Standard curve in the presence of citrate.

TABLE 2

RATE OF HYDROLYSIS OF ORGANOPHOSPHATES BY COLOR REAGENT WITHOUT CITRATE[a]

Substance	Nanomoles P_i released/h
ATP	2.70
ADP	0.18
AMP	0.00
Glucose 6-phosphate	0.00
Fructose 6-phosphate	0.00
Fructose 1,6-diphosphate	0.00

[a] All substances were present at a final concentration of 26.32 μM (25 nmol/950 μl). Tubes were incubated at room temperature.

DISCUSSION

The hydrolysis of ATP by the color reagents most commonly employed in the measurement of inorganic phosphate introduces serious measurement errors. These errors can be especially critical when nanomole amounts of inorganic phosphate are measured as with the method of Hess and Derr. By introducing citrate into their mixture, the color reaction is rendered insensitive to nascent phosphate, and hence becomes a stable and reliable measurement of extremely small amounts of inorganic phosphate.

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