Improved procedures for the determination of lipid phosphorus by malachite green

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Summary We have developed two procedures for the measurement of lipid phosphorus based on interaction between phosphomolybdenum and malachite green. One method, the "micro" assay uses 50–200 μl of $HClO_4$ and has a sensitivity range of 0.01–1.5 μg phosphorus. The second method, the "macro" assay, has a sensitivity range of 0.03–5.0 μg phosphorus with 100–500 μl $HClO_4$. Both assays are very reproducible with day to day standard deviations of less than 6% between triplicates irrespective of the $HClO_4$ content used. At different concentrations of $HClO_4$, each method was successfully used to determine the phosphorus content in phosphatidylethanolamine and sphingomyelin standards that covered the proposed sensitivity ranges. The increased range, sensitivity and greater volumes of $HClO_4$ permitted in the procedures represent significant improvements over existing methods.—**Zhou, X., and G. Arthur.** Improved procedures for the determination of lipid phosphorus by malachite green. *J. Lipid Res.* 1992. **33:** 1233–1236.

Supplementary key words phospholipid quantitation

The digestion of phospholipids followed by estimation of the released inorganic phosphorus is used in quantitating phospholipids. The method of Bartlett (1) and variations thereof (2) are widely used; however, the limited sensitivity (0.5 μg Pi) has led to the development of alternate methods for determining Pi (3–5). Some of these have been adapted for the measurement of lipid phosphorus (5–8) and are based on complex formation of malachite green with phosphomolybdenum and its resultant shift in absorption maximum (8). These methods are sensitive but have a limited range of about 20 nmol Pi (0.62 μg). In addition, the digestion procedures limit the use of some of the methods. The method of Petitou, Tuy, and Rosenfeld (5) uses H_2SO_4 for digestion and cannot be used for direct quantitation of phospholipids on silica gel (9). The low volume of $HClO_4$ (50 μl) used in digestion by Chalvardjian and Rudnicki (6) is inadequate for the complete digestion of samples with large amounts of organic material or for the direct estimation of phospholipids separated on silica gel. $HClO_4$ acid affects color development with malachite green (3, 9) but whether this renders the procedure unsuitable outside the $HClO_4$ concentrations reported is not known. In this communication we report the development of two methods for the determination of lipid phosphorus by malachite green which extends the permissive concentrations of $HClO_4$ as well as the sensitivity and range of comparable procedures.

MATERIALS AND METHODS

Malachite green base, Tween 20, and PE (L-α-cephalin, bovine brain) were obtained from Sigma (St. Louis, Mo). Sphingomyelin (beef brain) was obtained from Serdary (London, Ontario). Ammonium molybdate, $HClO_4$ (70%), and HCl were products of Fisher Scientific. All other chemicals were obtained from Baxter Canlab (Winnipeg).

Preparation of malachite green reagent

A stock solution of malachite green was prepared by the method of Itaya and Ui (3) as modified by Chalvardjian and Rudnicki (6). A 0.2 or 0.4% (w/v) suspension of malachite green base was prepared by vigorously stirring the required amount of the powder in DDW with a magnetic stirrer for at least 30 min. One volume of 4.2% ammonium molybdate in 5 M HCl was added to three volumes of the malachite green suspension. This solution was stirred vigorously for at least 30 min before filtering through Whatman #2 filter paper. This stock solution was stable for 2–3 weeks in the dark at room temperature. A working solution of malachite green was prepared before use by mixing sufficient Tween 20 (1.5%, w/v) with a volume of the malachite green stock solution to give a final concentration of 0.045% Tween 20 (w/v) for the "micro" assay and 0.06% Tween 20 (w/v) for the "macro" assay. After addition of Tween 20 to the malachite green stock solution, the mixture was stirred for at least 30 min before use.

Digestion of lipid samples

Aliquots of lipid extract were pipetted into tubes and the solvents were removed with N_2. Perchloric acid (0.5–1.0 ml) was added to the sample-containing tubes as well as to tubes with no samples. The tubes were covered with Teflon tape and placed in a multi-block heater (Lab-Line) pre-heated to 160°C. The time required for digestion depended on the phospholipid and the quantity of organic material; the digestion was only complete after clearing of the $HClO_4$ in sample-containing tubes. In our experiments all tubes were heated for the same period as the sample that required the longest time to be digested. The tubes were allowed to cool and aliquots were taken for determination of Pi by the procedures described below. The $HClO_4$ in tubes without samples was used for the calibration curve. When determining the lipid Pi content of samples in silica gel, the silica gel with the sample was scraped into tubes and an equal amount of silica gel from a blank lane was also scraped into a tube. The silica gel

Abbreviations: PE, phosphatidylethanolamine; SM, sphingomyelin; DDW, double-distilled water.

was digested with 1 ml $HClO_4$ as described above until the $HClO_4$ was clear. The tubes were cooled and centrifuged to precipitate the silica gel. Aliquots of $HClO_4$ were removed for Pi determination as described below.

Determination of 0.01–1.5 μg phosphorus (micro assay)

Doubled-distilled water and/or aliquots of KH_2PO_4 solution containing known quantities of Pi (0–1.5 μg) were added to 50–200 μl of $HClO_4$ to bring the total volume to 400 μl. After mixing, 2 ml of 0.4% malachite green working solution was added and mixed and the tubes were allowed to stand for at least 20 min. The absorbance (OD) was measured at 660 nm in a Hitachi U-2000 double-beam spectrophotometer with DDW as reference using 1-cm glass cuvettes. In all experiments in this study, comparable blanks with DDW (OD_{blanks}) were run and subtracted from the sample tubes (OD_{sample}) to give the ΔOD used for plotting the calibration curves or determining lipid Pi. Typical absorbance values for blanks in this assay are 0.341, 0.353, and 0.242 for 50, 100, and 200 μl of $HClO_4$, respectively.

Determination of 0.03–5 μg phosphorus (macro assay)

Double-distilled water and/or aliquots of KH_2PO_4 standard solutions were added to 100–500 μl of $HClO_4$ to bring the total volume to 1 ml. After mixing, 5 ml of 0.2% malachite green working solution was added. The solution was mixed and allowed to stand for 20 min. The absorbance was read at 660 nm against DDW as described above. Blanks with DDW were prepared simultaneously and used as described above. Typical absorbance values for blanks in this assay were 0.238, 0.180, and 0.140 for 100, 300, and 500 μl, respectively.

RESULTS AND DISCUSSION

The bases for the development of the micro and macro assays were the procedures described by Chalvardjian and Rudnicki (6) and Itaya and Ui (3), respectively. We investigated the effect of using 0.2, 0.4, and 0.6% malachite green solutions as well as increasing the $HClO_4$ content on the sensitivity and reproducibility of the micro and macro assays. With 50 μl $HClO_4$ and 0.1 μg Pi, a 40% increase in ΔOD was observed when 0.4% malachite green was used for the micro assay compared to 0.2% malachite green solution. The increase in ΔOD was only 7% between 0.4 and 0.6% malachite green. Decreases in ΔOD occurred with increasing $HClO_4$ content. Even though the extent of decrease caused by $HClO_4$ was greater at 0.4% than 0.2% malachite green, the ΔOD was always higher with 0.4% than 0.2% malachite green (data not shown). Consequently, 0.4% malachite green was selected for the micro assay together with a $HClO_4$ content of 50–200 μl (2.1–8.3%). Chalvardjian and Rudnicki (6) used 0.2% malachite green with $HClO_4$ content restricted to 50 μl (2.1%). Considerations similar to those described above led to the selection of 0.2% malachite green for the macro assay along with a $HClO_4$ range of 100–500 μl (1.7–8.3%). A 0.2% malachite green solution was also used by Itaya and Ui (3) with $HClO_4$ concentrations of 2% or less.

The wavelength spectra of blanks and sample were measured from 900 nm to 400 nm. Although the maximum absorption wavelength was at 620 nm, the greatest difference in absorbance between the sample and blanks was between 650 and 660 nm and was not changed by the $HClO_4$ content. We therefore selected 660 nm as the preferred wavelength as did Itaya and Ui (3).

Having established the malachite green concentrations, range of $HClO_4$, and optimum wavelength, we investigated other variables that affected the assay. Detergents were used to stabilize the malachite green/phosphomolybdate complex in the assay. In the methods that formed the basis for the current procedures, Tween 20 was added to the tubes immediately after the addition of the malachite green reagent (3, 6). This protocol does not allow addition of the dye in a batch-wise fashion and also at high Pi concentration there is the increased chance of adherence of complex to the sides of the tubes, which would decrease the absorbance. The addition of detergent prior to the addition of the dye retards the complex formation (3). To circumvent this problem, the detergent was

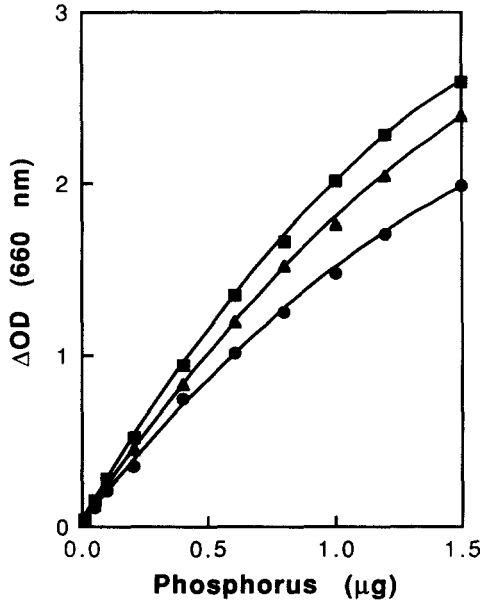

Fig. 1. Calibration curves for the micro assay with different concentrations of $HClO_4$. Calibration curves for the micro assay were determined as described in the Methods section with 50 μl (■), 100 μl (▲), and 200 μl (●) $HClO_4$. Each point is the mean of triplicates from three different experiments. The standard deviations from the mean are less than 6% for points on each line.

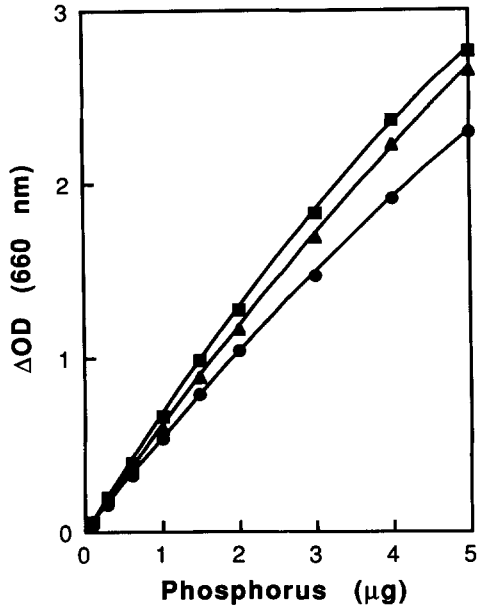

Fig. 2. Calibration curves for the macro assay with different concentrations of $HClO_4$. Calibration curves for the macro assay were determined as described in the Methods section with 100 μl (■), 300 μl (▲), and 500 μl (●) $HClO_4$. Each point represents the mean of triplicates from three different experiments. The standard deviations from the mean are less than 5% for each point on all three lines.

added to the stock dye solution to form a working dye solution as was previously done by other investigators (5, 7, 8). When the results of such assays were compared to assays where the dye and detergent were added separately, the sensitivity of the method was significantly increased (data not shown). This was due to both a lowering of the OD_{blank} and an increase in OD_{sample}, presumably due to better dissolution of the color complex.

The time required for color development was investigated at values encompassing the Pi range for each assay with different concentrations of $HClO_4$. The time required to attain the maximum absorbance ranged from 7 to 15 min. We therefore recommend that, after addition and mixing of working dye solution with the sample, tubes are left to stand for 20 min before the absorbance is read at 660 nm. The stability of the color formed (measured as $OD_{sample} - OD_{blank}$) after this period was monitored periodically. Five h after the initial reading, the values did not vary by more than 8% and 4% for the micro and macro assays, respectively.

After studying the conditions affecting the development of color, we examined the useful range of both the micro and macro assays in the presence of different concentrations of $HClO_4$. **Fig. 1** shows the calibration curves for the micro assay from 0–1.5 μg Pi with three different $HClO_4$ concentrations, 2.08, 4.16, and 8.33% (50, 100, and 200 μl). Deviation from linearity occurred earlier with decreasing $HClO_4$ content. Thus with 50 μl $HClO_4$, deviation occurred at 0.2 μg Pi compared to 0.5 μg Pi with 200 μl $HClO_4$. The sensitivity range for determination of Pi is fourfold over that reported by Chalvardjian and Rudnicki (6) and allows the use of fourfold more $HClO_4$. In **Fig. 2**, standard curves for the macro method at three different concentrations, 1.7, 5.1, and 8.5% (100, 300, and 500 μl) $HClO_4$ with up to 5 μg Pi are displayed. Deviation from linearity was at 3 μg Pi with 300 and 500 μl $HClO_4$, and from 2 μg Pi for assays with 100 μl $HClO_4$. The

TABLE 1. Determination of Pi content in PE and SM standards, by the micro and macro malachite green assays with different volumes of $HClO_4$

	Expected Values	Observed Values		
		50μl $HClO_4$	100 μl $HClO_4$	200 μl $HClO_4$
	μg		μg	
Micro assay				
PE	0.075	0.080 ± 0.004	0.070 ± 0.003	0.073 ± 0.004
SM	0.090	0.096 ± 0.005	0.087 ± 0.004	0.086 ± 0.006
PE	0.450	0.452 ± 0.025	0.445 ± 0.006	0.437 ± 0.025
SM	0.540	0.551 ± 0.026	0.544 ± 0.017	0.555 ± 0.034
PE	0.900	0.924 ± 0.014	0.913 ± 0.038	0.905 ± 0.025
SM	1.080	1.118 ± 0.010	1.118 ± 0.025	1.111 ± 0.037
		100 μl $HClO_4$	300 μl $HClO_4$	500 μl $HClO_4$
			μg	
Macro assay				
PE	0.25	0.240 ± 0.014	0.249 ± 0.012	0.250 ± 0.004
SM	0.30	0.292 ± 0.019	0.299 ± 0.002	0.291 ± 0.006
PE	1.50	1.531 ± 0.660	1.558 ± 0.033	1.531 ± 0.010
SM	1.80	1.776 ± 0.109	1.812 ± 0.046	1.763 ± 0.093
PE	3.00	3.094 ± 0.063	3.214 ± 0.105	3.108 ± 0.026
SM	3.60	3.555 ± 0.072	3.701 ± 0.732	3.603 ± 0.054

Values represent the mean ± SD of triplicates for three different experiments.

results of both assays were very reproducible with standard deviations of 6% or less between points obtained on 3 different days irrespective of the $HClO_4$ content. With both procedures, it is recommended that high $HClO_4$ content should be used in assays with relatively high Pi content in order to obtain absorbance readings that are not beyond the limit of most spectrophotometers.

The reliability of both the micro and macro assays at different concentrations of $HClO_4$ was examined by determining the phosphorus content of PE and SM standards. The phospholipid content of the standards was determined by the method of Bartlett (1) and the values obtained were used to compute the expected values. The results (**Table 1**) indicate that, irrespective of the $HClO_4$ concentration, each method was successful in estimating the Pi content at low, medium, and high levels within the proposed sensitivity range. Even though the Pi range of the macro overlaps that of the micro, differences in the sensitivities of the two procedures suggest different applications for each method. At comparable concentrations of $HClO_4$, a Pi content of 0.1 μg produces a ΔOD change in the micro assay that is fourfold that of the macro (Figs. 1 and 2). Thus the micro assay is the method of choice for studies involving samples of low lipid content or those requiring the monitoring of relatively small changes in lipid composition. One advantage of the above procedures is the wide range of permitted $HClO_4$ concentrations that may be used within the defined limits, provided similar amounts are used for the calibration curve. The procedure is also simple and it allows the determination of the lipid phosphorus content in different aliquots of the same digested sample. Thus, not only does it ensure that some of the chosen aliquots will fall within the range of the assays, but also the same digest can be used for both macro and micro assays. The present procedures should prove useful in the quantitation of phospholipids.

This study was supported by the National Cancer Institute of Canada with funds from the Canadian Cancer Society, and by a Medical Research Council scholarship to G. A.

Manuscript received 6 November 1991 and in revised form 31 March 1992.

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