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Analytical Methods Section

Phosphate determination in foodstuffs using a plant tissue electrode

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It is well known that phosphate concentration can be determined from the inhibition by this species of acid phosphatase activity.

A suitable plant tissue electrode for the analysis of inorganic phosphate was developed by adding the enzyme glucose oxidase to a slice of *Solanum tuberosum* containing the acid phosphatase enzyme and using an amperometric Clark electrode as indicating sensor.

The biosensor was fully characterized and used for phosphate determination in authentic matrices such as fresh and powdered milk, wine and tomato purée. The results obtained were compared with those obtained using Bartlett's spectrometric method.

INTRODUCTION

A suitable assembly of a valid biosensor for phosphate analysis is a problem affecting an expanding area of research, as phosphate determination is of increasing importance in environmental matters, in relation to eutrophization phenomena, in the biomedical field, as well as in the food and beverage industry for effluent control. Acid (or alkaline) phosphatase enzymes would seem suitable for this purpose. However, it is difficult to design an enzyme sensor based only on the reaction catalysed with the acid (or alkaline) phosphatase since there is no electrochemical difference between substrate and products (Guilbault & Nanjo, 1975). However, Guilbault & Nanjo (1975) showed that a dual enzyme system (i.e. alkaline-phosphatase and glucose oxidase) with an oxygen sensor for electroanalytical measurement, can be profitably used. When the glucose formed from glucose-6-phosphate hydrolysis, catalysed by phosphatase enzyme, comes into contact with the glucose oxidase (GOD), a reaction occurs in which oxygen is consumed and which can be monitored using a Clark electrode. The presence of phosphate inhibits the former reaction, thus lowering glucose formation and oxygen consumption and making phosphate analysis possible. Other authors (Schubert *et al.*, 1984; Linders *et al.* 1985) made hybrid sensors using a potato tissue layer containing acid phosphatase, which was superimposed on another polymeric membrane with immobilized GOD and coupling these two layers with an H_2O_2 or oxygen-indicating electrode. We recently (Campanella *et al.*, 1990) considerably simplified the assembly of the biosensor by allowing the GOD enzyme to diffuse into the potato slice and then superimposing the slice on, and securing it to, a commercial oxygen sensor by means of a dialysis membrane and a rubber O-ring. As the new sensor was available and proved easy to handle we used it for phosphate determination in biological matrices, such as human urine, as well as in several commercial pharmaceutical preparations (Campanella *et al.*, 1990).

This paper describes new analytical applications performed with our sensor in the analysis of phosphate content of several foods or beverages and the analytical comparison, during phosphate determination in foodstuffs, between the data from this electrochemical-biotechnological approach and Bartlett's classical spectrometric method (Bartlett, 1959; Tomassetti *et al.*, 1984).

MATERIALS AND METHODS

Sensor assembly

In outline, the biosensor consists of a Clark commercial oxygen electrode with two superimposed enzymes,

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acid phosphatase (AF) and glucose oxidase, both immobil-ized in the potato slice tissue (Fig. 1), the first natur-ally, the latter by us.

A thin slice of potato tissue (≤0·1 mm) was dipped in an aqueous GOD solution (60 U/ml) and left in the solution at 4°C for 24 h so that the enzyme could diffuse completely into the tissue slice and any traces of glucose present could be completely hydrolysed. The slice was then cut into discs about 0·6–0·8 cm in diameter. This slice and a superimposed dialysis membrane were both secured to the Clark electrode body by means of a rubber O-ring. The sensor was stored at +4°C when not used. Its lifetime was generally longer then 2 weeks, with a decrease in sensitivity of about 30% by 15 days after the assembly. The GOD and AF immobilized activity, measured by the same methods as reported in a previous paper (Mascini *et al.*, 1983), were respectively 2·50 and 0·013 U/mg of plant tissue.

Reagents

Glucose oxidase (E.C. N.1.1.3.4) from *Aspergillus niger*, glucose-6-phosphate (G-6-P) for the enzyme-amperometric measurements and Fiske–Subbarow reagent (Fiske & Subbarow, 1925) containing 1-amino-2-naphthol-4-sulphonic acid for Bartlett's method (Bartlett, 1959), were supplied by Sigma Chemical Co, St Louis (USA). Ammonium molybdate, potassium dihydrogen phosphate, citric acid and sodium citrate, for the buffer solution, perchloric acid and other chemicals, of the highest available purity, were obtained from Carlo Erba, Milan (Italy). Plant tissue of *Solanum tuberosum* containing the acid phosphatase was obtained from potatoes purchased in a local vegetable shop.

Samples

Examined foodstuffs (several fresh milk samples, powdered milk, red wine, tomato purées) were all commercial products, generally sealed in tins or glass containers.

Measurements and apparatus

Amperometric measurements were carried out using a commercial oxygen Orion electrode coupled to an Orion Research Ionalyzer 901 and an Amel model 686 recorder, under magnetic stirring, in a 10 ml glass cell, thermostatted at 25°C by forced water circulation. The buffer used was a 0·1 mol/l sodium citrate–citric acid aqueous solution, at pH 6·0.

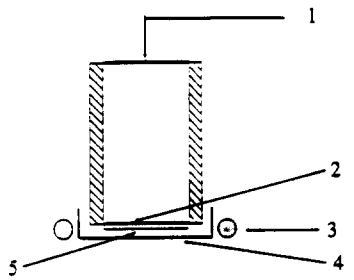

Fig. 1. Assembly of the plant tissue electrode: 1, Oxygen indicating sensor; 2, gaspermeable membrane; 3, O-ring; 4, dialysis membrane; 5, Plant tissue of *Solanum tuberosum*, containing adsorbed GOD.

To obtain the calibration graph the sensor was dipped into 10 ml of the buffer solution and a suitable volume of concentrated solution of G-6-P was added to obtain a $4{\cdot}6 \times 10^{-3}$ mol/l final concentration of G-6-P. On the basis of the two following enzymic reactions:

$$\text{glucose-6-phosphate} + H_2O \xrightarrow{AF} \text{glucose} + H_2PO_4^-$$

$$\text{glucose} + O_2 \xrightarrow{GOD} \text{gluconolactone} + H_2O_2$$

a decrease of the current was observed, due to oxygen consumption, corresponding to the oxidative reaction, induced by GOD, of the glucose produced by G-6-P hydrolysis. This hydrolysis reaction is catalysed by the AF contained in the plant tissue slice. After stabilisation of the current a measured small volume of the sample solution containing phosphate was added to the buffer, under stirring. The phosphate inhibition causes a decrease on the G-6-P hydrolysis reaction rate, lowers the glucose concentration and increases oxygen concentration in the buffer solution. Five minutes after addition, the relative increase of the current, proportional to the increase of the oxygen in solution as a function of the phosphate concentration on the buffer solution, was measured (Fig. 2). The phosphate concentration was evaluated by means of a calibration graph obtained using KH_2PO_4 aqueous standard solutions and the procedure described above. Food samples were analysed, as such (fresh milk) or after being subjected to a mineralization procedure with perchloric acid (Tomassetti *et al.*, 1984) (wine and tomato purée), in the same way, adding the sample, appropriately diluted if necessary, to the buffer solutions containing also G-6-P, placed in the thermostatted cell, and recording the current increase.

Bartlett's spectrometric method (Barlett, 1959) had been previously described and optimized (Tomassetti *et al.*, 1984). Briefly, the samples to be analysed were first fully mineralized with perchloric acid (Tomassetti *et al.*, 1984). Total phosphorus was then determined as inorganic phosphate using Fiske–Subbarow reagent (Fiske & Subbarow, 1925) and reading off the absorbance at 820 nm against a reagent blank. Spectrometric mea-

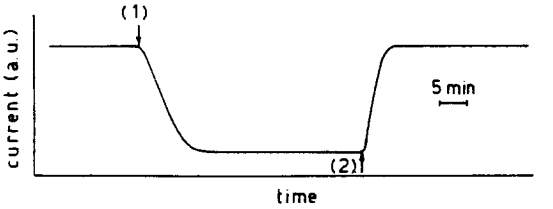

Fig. 2. Behaviour of the response of the plant tissue electrode on addition to the buffer solution, first (1) of glucose-6-phosphate, then (2) phosphate.

surements were performed with a Perkin–Elmer Lambda 5 UV/VIS spectrophotometer and 1·0 cm path-length silica cell.

RESULTS

The experimental working conditions (temperature, pH, buffer, substrate concentration) are summarized in Table 1.

First the correlation between the response of the sensor and the concentration of glucose (Table 2) was investigated. As expected, a good and wide linearity range was found (Fig. 3), indicating that control was actually only diffusional. On the other hand, the sensor response to increasing G-6-P concentrations, as shown in Fig. 4, is clearly non-linear over the whole measured range, as a result of a kinetic control. According to theory (Schubert *et al.*, 1984), this is in fact necessary for the tissue sensor to function correctly in phosphate determination, since kinetic control is an essential precondition for the determination of an inhibitor such as phosphate. Nevertheless the tissue electrode was found useful also for determining G-6-P over a narrow range

Table 1. Main working conditions for the plant tissue electrode

Indicating electrode	Oxygen amperometric sensor
Enzymes	Acid phosphatase, contained in a slice of *Solanum tuberosum* GOD adsorbed on the same plant tissue slice
Substrate	G–6–P (4·6 × 10^{-3} mol/l)
Temperature analysis	25°C
pH	6·0
Buffer	Sodium citrate – citric acid 0·1 mol/l
Working conditions	Steady solution, in a thermostatted 10 ml glass cell, under magnetic stirring

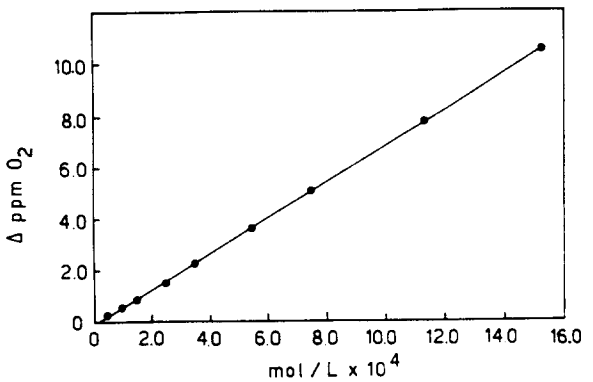

Fig. 3. Glucose calibration graph, by the plant tissue electrode, in the linearity range of response.

in which current versus concentration dependence is fairly linear (Table 3).

In Fig. 5 the correlation between the response of the plant tissue electrode and the phosphate concentration is shown. Of course, owing to the inhibition process involved, dependence is non-linear over the whole measured range. To linearize the response, Guilbault & Nanjo (1975) have shown that the reaction rate should be a linear function of the reciprocal of the inhibitor concentration and proposed another measurement procedure in which phosphate is added prior to G-6-P.

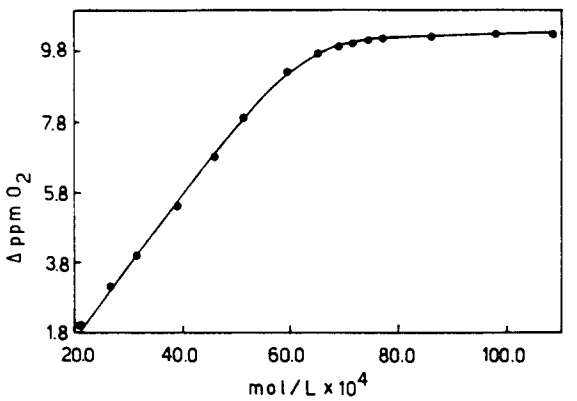

Fig. 4. G-6-P calibration graph by the plant tissue electrode

Table 2. Analytical characterization of the plant tissue electrode, in glucose standard solutions

Response time	<1 min
Lifetime	(10–16) days
Equation of the calibration curve	Y=700X–0·16 (Y=Δppm O_2; X=glucose concentration in mol/l)
Linearity range	(2·5 × 10^{-5} – 1·5 × 10^{-3}) mol/l
Correlation coefficient	0·9999
Minimum detection limit	1·2 × 10^{-5} mol/l
Precision on standard solutions (as RSD%)	1·8

Table 3. Analytical characterization of the plant tissue electrode, in glucose-6-phosphate standard solutions

Response time	<1 min
Lifetime	(10–16) days
Equation of the calibration curve	Y=364X+0·31 (Y=Δppm O_2; X=G-6-P concentration in mol/l)
Linearity range	(9·1 × 10^{-4} – 6·5 × 10^{-3}) mol/l
Correlation coefficient	0·9982
Minimum detection limit	7·2 × 10^{-4} mol/l
Precision on standard solutions (as RSD%)	3·0

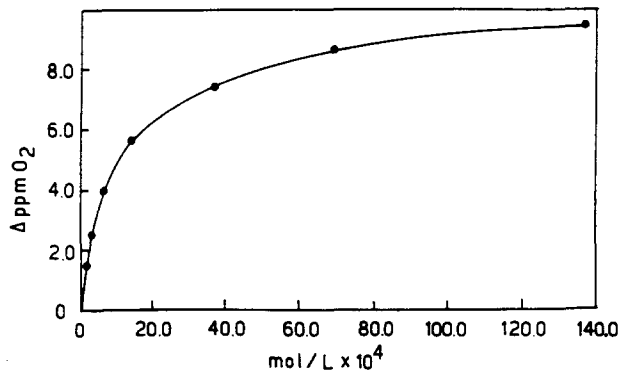

Fig. 5. Phosphate calibration graph, by the plant tissue electrode.

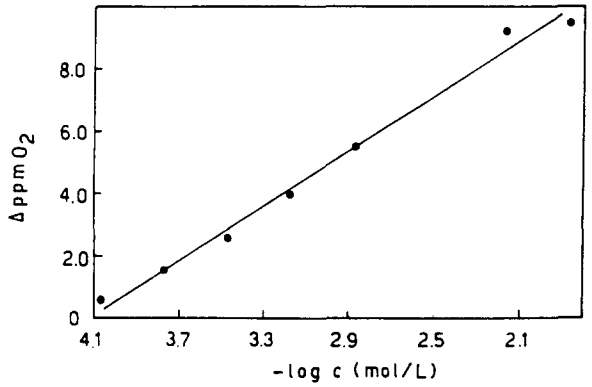

Fig. 6. Phosphate calibration graph (logarithmic plot) by the plant tissue electrode: correlation coefficient = 0·994, linearity range $8{\cdot}5 \times 10^{-5} - 1{\cdot}4 \times 10^{-2}$ mol/l.

Table 4. Analytical characterization of the plant tissue electrode, in phosphate standard solutions

Response time	<5 min
Lifetime	(10–16) days
Decrease in sensitivity after 15 days from its assembly	30%
Equation of the calibration curve	$Y=139X-0{\cdot}22$ (Y=Δppm O_2; X=phosphate concentration in mol/l)
Linearity range	$(8{\cdot}6 \times 10^{-5} - 1{\cdot}4 \times 10^{-3})$ mol/l
Correlation coefficient	0·9998
Minimum detection limit	$6{\cdot}2 \times 10^{-5}$ mol/l
Precision on standard solutions (as pooled SD%)	1·7
Accuracy on standard solutions, as recovery data by standard addition method	(–97·5 – 101·0)%

However, Schubert *et al.* (1984) claimed that in this way, an overall time of 9 min for one measurement is needed. More recently Linders *et al.* (1985) showed that a good straight line can be obtained by plotting current

Table 5. (a) Phosphate determination in commercial food products containing phosphate. Comparison of the results, obtained by plant tissue electrode and by spectrometric Bartlett's method (values (in mmol/l) of mineralized samples, are the mean of four determinations)

Sample nature	Amperometric method (a)	RSD %	Spectrometric method (b)	RSD %	$\frac{b-a}{a}$ %
Tomato purée no. 1	11·5	4·1	10·9	3·5	–5·2
Tomato purée no. 2	13·3	3·7	13·2	3·0	–0·8
Red wine	8·5	2·8	8·2	2·5	–3·5
Powdered milk	26·1	2·0	27·0	1·8	+3·3

(b) Recovery of phosphate, by the standard addition method, in commercial food products, by plant tissue electrode, (values (in mmol/l) of the mineralized samples, are the mean of four determinations)

Sample nature	Phosphate found in the sample	Phosphate added	Total (nominal value) (a)	Total (found value) (b)	$\frac{b-a}{a}$ %
Tomato purée no. 1	11·5 (RSD% = 4·1)	9·6	21·1	21·8 (RSD% = 3·6)	–3·3
Tomato purée no. 2	13·3 (RSD% = 3·7)	9·6	22·9	22·3 (RSD% = 2·3)	–2·7
Red wine	8·5 (RSD% = 2·8)	9·6	18·1	18·6 (RSD% = 2·8)	+2·7
Powdered milk	26·1 (RSD% = 2·0)	9·6	35·7	37·0 (RSD% = 3·1)	+3·6

Table 6. (a) Phosphate determination in fresh milk samples. Comparison of results, obtained by plant tissue electrode and by spectrometric Bartlett's method (values (in mmol/l) and mean of four determinations, were obtained without mineralization pretreatment of samples. For samples no. 6 and 7, also values found after mineralization are given in brackets).

Sample no.	Amperometric method (a)	RSD %	Spectrometric method (b)	RSD %	$\frac{b-a}{a}$ %
1	25·2	1·2	26·0	1·5	+3·2
2	28·0	1·3	27·0	0·8	−3·6
3	19·4	2·6	20·3	2·2	+4·6
4	21·0	1·9	21·7	1·1	+3·3
5	25·0	3·2	24·2	1·9	−3·2
6	32·2 (33·0)	1·3 (1·8)	32·4 (32·4)	1·4 (1·4)	+0·6 (−1·8)
7	28·0 (28·5)	1·2 (2·3)	27·9 (27·9)	1·2 (1·2)	−0·4 (−2·1)

(b) Recovery of phosphate, by the standard addition method, in some fresh milk samples, by plant tissue electrode (values (in mmol/l) are the mean of four determinations)

Sample	Phosphate found in the sample (RSD% ≤ 2·6)	Phosphate added	Total (nominal value) (b)	Total (found value) (a) (RSD% ≤ 3·0)	$\frac{b-a}{a}$ %
2	28·0	9·67	37·7	36·8	−2·4
3	19·4	9·67	29·1	28·6	−1·7
4	21·0	9·67	30·7	30·1	−2·0
5	25·0	9·67	34·7	35·6	+2·5

against the logarithm of concentration. In Fig. 6 the results of the application of this latter suggestion to phosphate measurements obtained using our sensor are shown. It is apparent that the value of the correlation coefficient is not completely satisfactory. On the other hand, we observed that, over a narrower range of about 1·5 decades, the relationship between sensor response and phosphate concentration shows a satisfactory linear correlation (Table 4 and Fig. 7). Therefore, in the present work, we use this less wide calibration graph for phosphate determination in authentic food samples using the direct method.

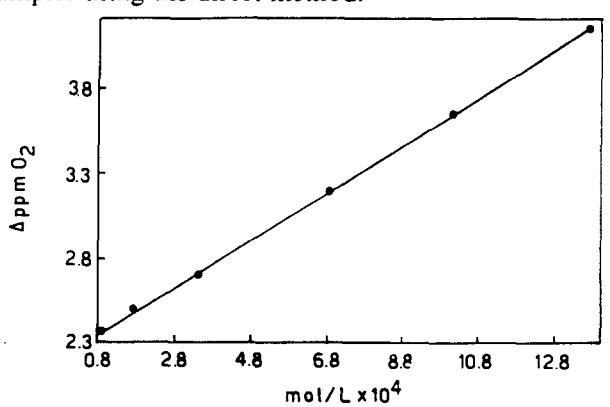

Fig. 7. Phosphate calibration graph by the plant tissue electrode, in the linearity range of response.

The features of the plant tissue electrode (response time, lifetime) and the analytical results of the method (equation of best calibration graph over the linearity range, minimum detection limit, repeatability and accuracy of the measurements) applied to the standard phosphate solutions, are shown in Table 4. The results of the measurements on authentic samples are always compared with those obtained using the spectrometric method. This is done in order to check the new method against a widely accepted one. The results obtained, including precision data (as RSD% values), for the analysis of different food samples containing phosphate, using the plant tissue electrode, are summarized in Tables 5 and 6, respectively, where they are compared with those obtained using Bartlett's spectrometric method. Accuracy was determined, by the recovery data, on adding known amounts of potassium dihydrogen phosphate, to the same samples.

DISCUSSION

The experimental conditions used (shown in Table 1 and reported in the experimental section) are practically the same as those found to be the best by Schubert *et al.* (1984). Only the G-6-P concentration adopted is

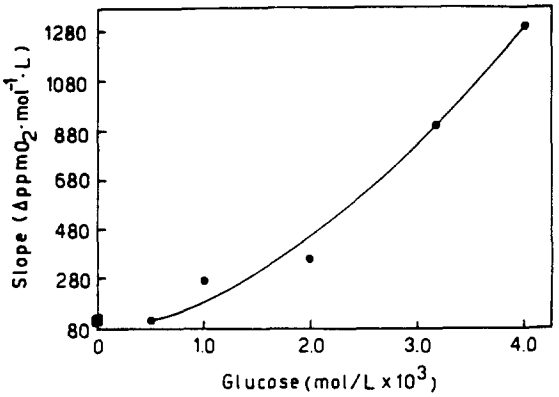

Fig. 8. Glucose interference evaluation. Slope of phosphate calibration graph versus glucose concentration in solution. ■, value of the slope without glucose interference; ●, values of the slope by increasing concentration of the glucose added in solution.

about ten times higher than that used by these authors. This choice can be justified by the research performed by Linders *et al.* (1985), namely that the small observed decrease of sensitivity is balanced by the fact, observed also by us in a previous paper (Campanella *et al.*, 1990), that a greater glucose concentration in solution exerts a somewhat more marked buffering action toward possible interference by the glucose, if the latter is contained in the authentic samples. In fact, as can be seen from Table 7, where data referring to the problem of possible interference from other analytes are shown, glucose is found to be the most interfering species after fluoride. We specially investigated this interference, as glucose is contained in several foodstuffs. Figure 8 shows that the sensor response is unaffected by glucose interference only at glucose concentrations lower than 0·5 mmol/l, i.e. if the level of the interfering glucose in solution is no higher than about $^1/_8$ of the glucose produced by the hydrolysis of G-6-P ($4{\cdot}6 \times 10^{-3}$ mol/l). Obviously, at lower G-6-P concentrations the same levels of any glucose contained in the food samples to be analysed can exert a more marked interference. On the other hand, by adopting our G-6-P concentration level, any glucose present in the examined foodstuffs failed to have a negative effect on sensor response, as is shown by the results of the recovery tests reported in Tables 5 and 6.

Table 7. Plant tissue electrode for phosphate determination. Main interferences

Tested interfering species	
Fluoride	Gave about 100% of interference in the linearity range of the sensor
Nitrate	Interference only at concentration higher than 1 mmol/l
Acetate	No interference at concentration up to 60 mmol/l
Carbonate	No interference at concentration up to 60 mmol/l
Chloride	No interference at concentration up to 60 mmol/l
Sulphate	No interference at concentration up to 60 mmol/l
Oxalate	No interference at concentration up to 60 mmol/l
Glucose	Interference only at concentration higher than 0·5 mmol/l

Another interesting point is the simplicity and the speed with which the plant tissue electrode we proposed can be assembled, as well as its response time, lifetime and enzyme activity. In addition, by using only the plant tissue membrane containing both the needed enzymes, and eliminating other polymeric layers used by other authors (Guilbault & Nanjo, 1975; Schubert *et al.*, 1984; Linders *et al.*, 1985), the diffusion process of both the substrate and the inhibitor is facilitated. This enhances sensor measurements based on the inhibition method as well as reproducibility.

Finally, the analysis of food samples using the new sensor is of considerable interest due to the repeatability of the measurements, as well as the accuracy, both undoubtedly satisfactory (see the recovery data using the standard addition method shown in Table 5 and 6). In addition, comparison with the data obtained by analysing the same samples with Barlett's classical spectrometric method indicates general good agreement. Also in the case of fresh milk samples, the correlation between the two methods is good, although tissue electrode analysis was performed without mineralization pretreatment of the sample (Table 6).

For this purpose, it is important to note that, by using the tissue sensor, it was actually possible to analyse the phosphate content of some food samples, e.g. fresh milk, without any mineralization pretreatment. Indeed, as shown in Table 6, two samples analysed by tissue sensor, with and without mineralization pretreatment, gave about the same results. On the other hand, the full mineralization samples were always needed in Bartlett's method in order to obtain results in agreement with those obtained using the tissue sensor.

ACKNOWLEDGEMENT

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