Plant tissue hybrid electrode for determination of phosphate and fluoride

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of the spot diameter and decreases the noise due to local irregularities. Second, the extended lens generates an absolutely larger signal at the detector and, in turn, a better signal/noise ratio.

It is somewhat surprising that there is no change in the detection limits observed for α-ionone. The reason for the lack of improvement is not yet clear. One possibility is that sulfuric acid oxidation causes complete decomposition of a small amount of the quinones. As the sensitivity of the measurement increases, the detection limit may be limited by the amount of quinone which undergoes oxidative condensation only.

Our present results demonstrate that it is possible to obtain picogram detection limits with a straightforward modification to the basic photothermal deflection design. Ideally, the probe beam expansion and heating beam diameter should just match the spot diameter. In practice, it will probably be necessary to choose some compromise dimensions, typically 1–2 mm for high-performance thin-layer chromatography. Automatic adjustment of diameters throughout a scan is technically feasible, although not necessarily economically justified.

Significantly, we have found that a delivered power of 15 mW is more than adequate to provide picogram sensitivies. This power level is now available from the inexpensive air-cooled argon ion (488/514 nm) or helium-cadmium (442 nm) lasers which are used in phototypesetters and laser printers. Clearly, a photothermal deflection densitometer can be a cost-effective system for high sensitivity measurements in thin-layer chromatography or electrophoresis. Applications using both techniques will be reported in separate communications.

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Plant Tissue Hybrid Electrode for Determination of Phosphate and Fluoride

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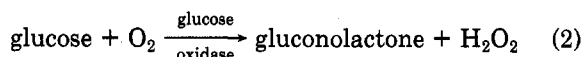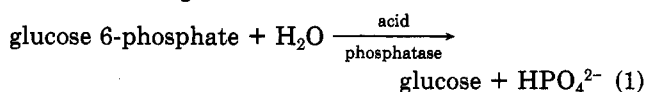
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A biosensor for inorganic phosphate and fluoride has been developed by coupling a potato (*Solanum tuberosum*) tissue slice and immobilized glucose oxidase with a Clark oxygen electrode. Measurement is based on the inhibition by either ion of potato acid phosphatase catalyzed glucose 6-phosphate hydrolysis. The precision is 1.7% and 6.5% and the lower detection limit 2.5×10^{-5} M and 1×10^{-4} M for phosphate and fluoride, respectively. For phosphate determination the hybrid sensor is stable for 28 days or 300 assays. With a higher limit of detection the sensor can be applied in a commercial enzyme electrode based device. Its application for phosphate determination in fertilizer and urine samples is described.

The development of biosensors using higher integrated biocatalytic systems coupled to amperometric or potentiometric indicator electrodes is presently an expanding area of research (*1*, *2*). Such systems comprise cell organelles, mi-

croorganisms, and animal tissue slices. In a few cases also plant tissue slices have been employed (*3–5*). Compared to isolated enzymes these biocatalytic phases often provide an enhanced stability of the biosensor. Another advantage is their cheap and generally simple preparation. The applicability of sensors assembled with such systems can be widely expanded by additional coupling of immobilized isolated enzymes, thus forming hybrid electrodes (*6*, *7*).

A bienzyme electrode with immobilized alkaline phosphatase and glucose oxidase for inorganic phosphate determination based on the inhibition of phosphatase by phosphate ion has been reported (*8*). Using a similar approach the present paper describes a hybrid biosensor based on potato (*Solanum tuberosum*) tissue and immobilized glucose oxidase for the measurement of phosate and fluoride ion. Potato tuber tissue contains high acid phosphatase activity. In the presence of glucose 6-phosphate the biocatalytic reactions at the sensor are the following:

$$\text{glucose 6-phosphate} + \text{H}_2\text{O} \xrightarrow[\text{phosphatase}]{\text{acid}} \text{glucose} + \text{HPO}_4{}^{2-} \quad (1)$$

$$\text{glucose} + \text{O}_2 \xrightarrow[\text{oxidase}]{\text{glucose}} \text{gluconolactone} + \text{H}_2\text{O}_2 \quad (2)$$

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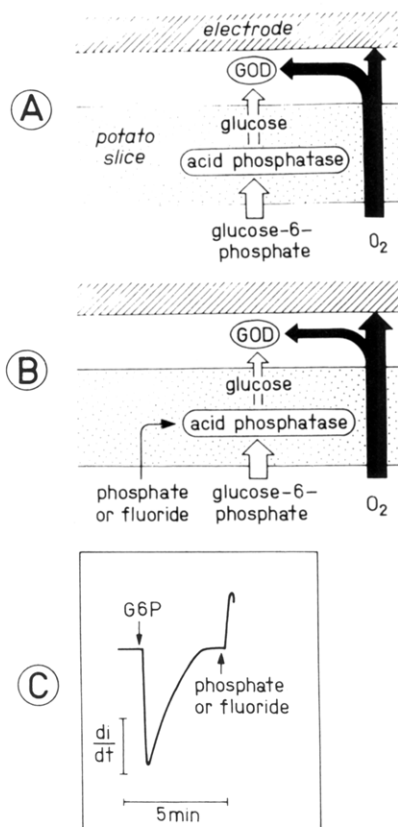
Figure 1. Principle of the hybrid sensor.

The oxygen consumption corresponding to the formation of glucose is sensed electrochemically with a Clark oxygen electrode. Since potato acid phosphatase is inhibited by phosphate and fluoride (9) both substances can be determined with the sensor by their ability to diminish glucose formation.

The applicability of the present sensor in a commercial enzyme electrode based device was tested. Furthermore, phosphate ion was determined in fertilizer and urine samples.

EXPERIMENTAL SECTION

Reagents. Potatoes were purchased from a local vegetable shop. Glucose oxidase (GOD) from *Penicillium notatum* (46 U/mg) was obtained from VEB AWD Dresden. Immobilization of the enzyme in gelatin membranes (gelatin-batch 19002, VEB ORWO Wolfen) was performed as described previously (10). Glucose 6-phosphate was purchased from Boehringer Mannheim. As judged by measurement with the Glukometer (see below) the substance contained no glucose. The phosphate used was KH_2PO_4, the fluoride NaF. All other chemicals were analytical reagent grade and were used without further purification. All the solutions were prepared in doubly distilled water. The buffer solutions were mixtures of sodium citrate and citric acid or imidazole and HCl, respectively, with an ionic strength of 0.1 M and varying pH.

For measurements of phospate in fertilizers the following products were used: Floraphil, Herbaphil (both from VEB Biosanat Erfurt) and S 1 (VEB Mineralstoffgemische Hohberg).

Apparatus. Measurements were carried out either with a polarograph GWP 673 (ZWG Berlin) or with the electrode-based glucose measuring device Glukometer GKM 01 (ZWG Liebenwalde). Unless otherwise stated, the polarographic device was used. The devices were coupled with a recorder. The 2-mL measuring cells were thermostated at 25 °C. A magnetic stirrer assured perfect stirring of the background solution. Clark-type oxygen electrodes (electrolyte: 0.1 M KCl) from Forschungsinstitut Meinsberg (with the polarograph) and from VEB Metra Radebeul (with the Glukometer GKM 01) were used.

Procedures. *Sensor Preparation.* The biocatalytic layer arrangement is shown in Figure 1. A piece of 5 × 5 mm of a GOD enzyme membrane is placed on the high sensitivity polyethylene
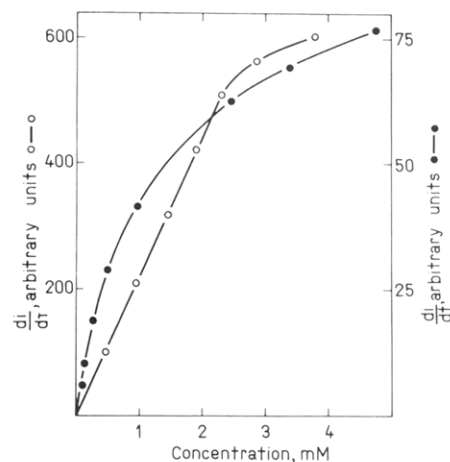

Figure 2. Relationship between the response of the sensor and the concentration of glucose (O) and glucose 6-phosphate (●). Buffer used was 0.1 M sodium citrate/citric acid, pH 6.0.

membrane. A slice of about 0.1 mm thickness is cut from the inner part of a potato tuber by means of a razor blade. One or more pieces measuring 5 × 5 mm of this slice are then placed on the GOD layer and covered with a dialysis membrane (VEB CK Bitterfeld) of 17 μm thickness. This sandwich arrangement is attached to the platinum tip of the Clark oxygen electrode with an O-ring. The prepared hybrid sensor is horizontally arranged in the measuring cell. When not in use the sensor is dipped in the respective buffer at 4 °C.

Measurement. Sensor responses are measured by allowing 2 mL of background solution to equilibrate with atmospheric oxygen and then injecting 20 μL of sample solution. Usually the electrode response is recorded as the first derivative of the current–time curve (kinetic method). Between two determinations the cell is rinsed twice with doubly distilled water. The principle of the measurement is as follows (Figure 1): First, glucose 6-phosphate is added to the background solution, diffuses into the potato layer, and is split to glucose and inorganic phosphate by acid phoshatase. Glucose is oxidized by GOD in the next layer, causing the electrochemically indicated oxygen consumption (Figure 1A). When inhibitor (phosphate or fluoride) is added (Figure 1B), diminishing glucose 6-phosphate hydrolysis and thus glucose formation, a decrease in oxygen consumption, i.e., a relative increase of the electrode signal, is observed (operation mode 1). The magnitude of this increase depends on the inhibitor concentration. The respective kinetic electrode signals are shown in Figure 1C.

Another possible operation mode is the addition of phosphate or fluoride prior to glucose 6-phosphate injection. Then the glucose 6-phosphate signal is smaller compared to that in the absence of inhibitor. This operation mode shall be called "mode 2". Unless otherwise stated mode 1 was performed, being simplified by using glucose 6-phosphate containing buffer.

RESULTS AND DISCUSSION

Glucose Response. The derivative current–concentration dependence of the hybrid sensor with one potato tissue layer for glucose is shown in Figure 2. As expected, the response is linear indicating diffusional limitation. This had been proved in experiments with the GKM 01 using identically prepared GOD membranes (11). Linearity is obtained up to 2.2 mM final concentration.

Glucose 6-Phosphate Response. The response of the sensor with one tissue layer to glucose 6-phospate is considerably lower than that to glucose (Figure 2). Furthermore, the derivative current–concentration dependence is nonlinear over the whole range measured.

Figure 3 shows the effect of increasing the potato tissue thickness on the response time and the sensitivity of the hybrid sensor. Here, the current decrease was measured instead of the derivative current. The tissue thickness was increased by increasing the number of potato tuber slices
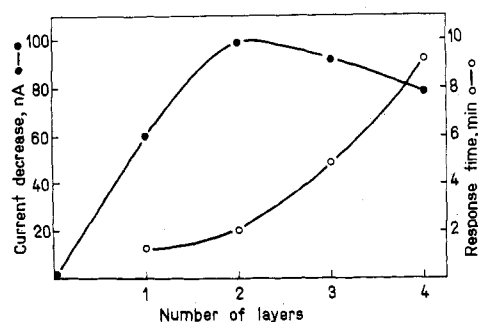
Figure 3. Effect of potato tissue thickness on the sensitivity (●) and the response time (O) of the sensor for glucose 6-phosphate. 1 glucose 6-phosphate glucose-6-phosphate was added to 0.1 M sodium citrate/citric acid, pH 6.0.
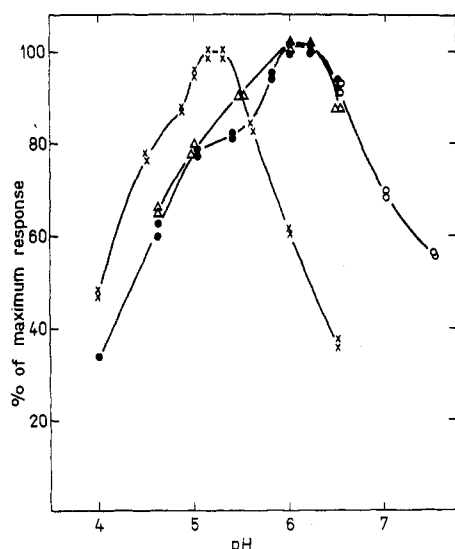

Figure 4. Effect of pH on the response to glucose 6-phosphate (0.5 mM) (△), phosphate (0.78 mM) (O, ●) and fluoride (4.76 mM) (X) of the hybrid sensor. Buffers used were 0.1 M sodium citrate/citric acid from pH 4.0 to 6.5 and 0.1 M imidazole/HCl from pH 6.5 to 7.5. For phosphate and fluoride determination the buffer contained 1.22 mM glucose 6-phosphate.

placed in front of the GOD membrane.

Increasing thickness results in increasing response times up to 9 min with four layers. An increase of the thickness by a factor of about 2 by use of two layers leads to an increase of the stationary current signal for 1 mM glucose 6-phosphate by a factor of 1.65. Further enhancement of the tissue thickness by using more than two layers again brings about a decrease of the current signal. According to theory (12), this behavior indicates that the glucose 6-phosphate response is kinetically controlled to a significant extent when only one tissue layer is applied. In this sensor substrate diffusion is of minor influence. With more than two layers diffusional limitation is effective. The predominance of kinetic control of the sensor with one layer is supported by the nonlinear concentration dependence shown in Figure 2. Since kinetic control is an essential precondition for the determination of an inhibitor, hybrid sensors with only one potato tissue layer were used for all further studies.

The effect of pH on the sensor response to glucose 6-phosphate was studied from pH 4.5 to 6.5 in 0.1 M sodium citrate/citric acid buffer (see Figure 4). The pH optimum between 6.0 and 6.2 is in agreement with literature data for potato acid phosphatase catalyzed hydrolysis of different substrates which range from pH 5.2 to 6.3 (9, 13, 14). Usually glucose 6-phosphate was included in the background solution so that the basic current before inhibitor injection reflects the acid phosphatase activity. When phosphate or fluoride was
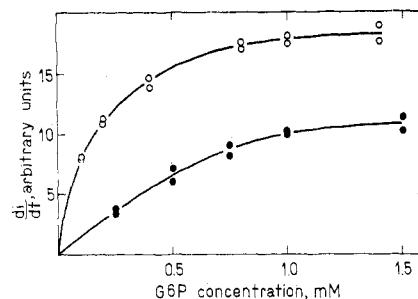

Figure 5. Effect of glucose 6-phosphate concentration on the sensor response to phosphate (O) and fluoride (●). Buffers used were 0.1 M sodium citrate/citric acid, pH 6.0 for phosphate and pH 5.1 for fluoride. [phosphate] = 1 mM; [fluoride] = 4.76 mM; G6P = glucose 6-phosphate.

added to the glucose 6-phosphate containing solution, an increase of the current–time curve was observed. No such increase was detected with only glucose present in the solution indicating that the inhibitors did not affect GOD function.

pH Optimum and Glucose 6-Phosphate Dependence of Inhibitor Determination. The effect of pH on the sensor response to phosphate and fluoride was studied from pH 4.0 to 7.5 and 6.5, respectively, in 0.1 M sodium citrate/citric acid and/or imidazole/HCl buffer containing glucose 6-phosphate. Phosphate or fluoride was added to give final concentrations of 0.78 mM and 5.0 mM, respectively. Sample addition did not affect the pH of the measuring buffer. The results are shown in Figure 4. The optimum pH for fluoride determination is at 5.1 while the highest response to phosphate was measured between pH 6.0 and 6.2. This pH–response profile was reproducible within different preparations of hybrid electrodes. It should be noted that these values do not represent the optimum pH for the enzyme activity but the respective pH values at which the inhibitor is most potent. For the competitive inhibitor (9), phosphate, this value agrees with the pH optimum of the glucose 6-phosphate hydrolysis. The optimal pH of the action of fluoride, which inhibits potato acid phosphatase noncompetitively (9), is different from this optimum.

Figure 5 shows the dependence of the electrode signal for phosphate and fluoride on glucose 6-phosphate concentration in the buffer. The response to phosphate is maximal from 1 mM glucose 6-phosphate upward. For fluoride determination glucose 6-phosphate saturation is reached at slightly higher concentration. All subsequent measurements with operation mode 1 were performed with saturating amounts of the substrate.

Electrode Response to Phosphate and Fluoride. The relationship between the response of the hybrid electrode (di/dt) and the concentration of phosphate is given in Figure 6. Phosphate ion can be determined up to at least 1.25 mM final concentration. The lower detection limit is 50 μM.

The applicability of the hybrid electrode in the commercial device Glukometer GKM 01 was tested. The dependence of the Glukometer digital readout on phosphate concentration is also shown in Figure 6. Compared to the more sensitive polarographic device, the Glukometer, which has been developed for highly active isolated enzyme membranes (15), gives a lower sensitivity with the potato tissue–GOD electrode. However, phosphate concentrations as low as 0.1 mM can be readily determined. Nonetheless all further measurements were carried out with the polarograph.

For an analytical method linearity is desired. This is not provided by the tissue-based hybrid sensor owing to the nature of the reactions involved. Therefore for a calibration curve several different standard solutions must be applied. As suggested previously by Guilbault and Nanjo (8) this can be overcome by using the following approach.
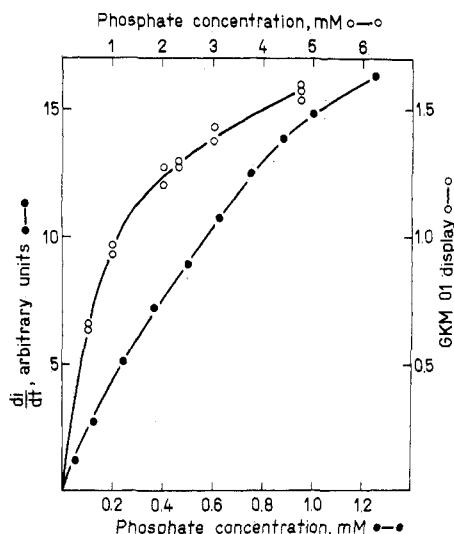
Figure 6. Relationship between the response of the sensor (●) and the digital Glukometer signal (O), respectively, and the concentration of phosphate. Buffer used was 0.1 M sodium citrate/citric acid, pH 6.0, with 1 mM glucose 6-phosphate.
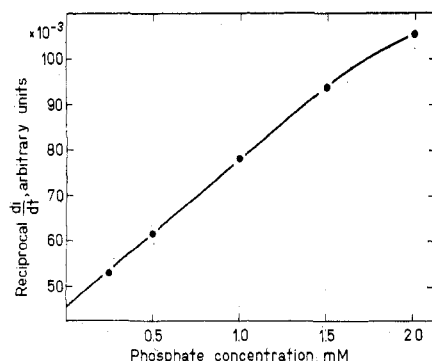

Figure 7. Relationship between the reciprocal response to glucose 6-phosphate of the sensor and the concentration of phosphate. Phosphate samples were added to the measuring buffer (sodium citrate/citric acid, pH 6.0) prior to addition of 0.37 mM glucose 6-phosphate (operation mode 2).

At steady state the Michaelis–Menten equation for competitive inhibition is

$$v = \frac{V_{max}}{1 + \dfrac{K_m}{[S]} + \dfrac{[PO_4]K_m}{[S]K_i}} \quad (3)$$

were [S] is the glucose 6-phosphate concentration and K_i is the inhibitor constant. In the presence of excess phosphate this equation simplifies to

$$v = \frac{1}{[PO_4]} \frac{V_{max}K_i[S]}{K_m} \quad (4)$$

Substrate concentration and V_{max} being constant, the reaction rate, v, will be a linear function of the reciprocal of the inhibitor concentration. Thus, if phosphate is added prior to glucose 6-phosphate addition (mode 2), a linear calibration curve for phosphate can be obtained with kinetic measurement. This is indeed the case, as shown in Figure 7. Proportionality is obtained up to 1.5 mM phosphate. By use of low glucose 6-phosphate concentratons it is possible to determine phosphate concentrations as low as 25 μM with this operation mode. However, an overall time of 9 min for one measurement is needed, which is twice as high as that for mode 1 (see Table I).

Table I. Parameters of the Tissue–GOD Hybrid Sensor[a]

substance	response time, s	measuring time, min	coeff var, % (N)
G6P[b]	25	4	2.2 (12)
KH_2PO_4[c]	30	4	1.7 (17)
NaF[d]	40	6	6.5 (12)

[a] Obtained with 0.1 M sodium citrate/citric acid buffer. [b] G6P, glucose 6-phosphate; pH 6.0; coefficient of variation determined with 1 mM G6P. [c] pH 6.0, with 1 mM G6P; coefficient of variation determined with 0.5 mM KH_2PO_4. [d] pH 5.1, with 1.4 mM G6P; coefficient of variation determined with 2 mM NaF.
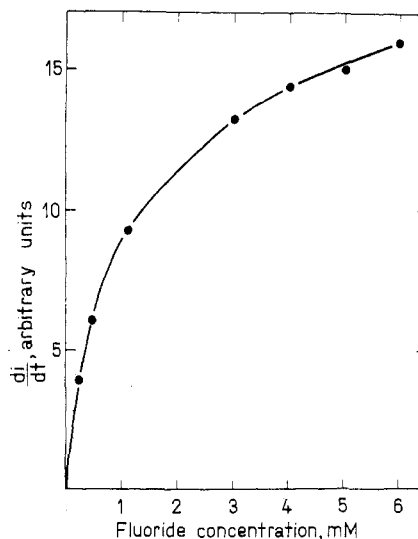

Figure 8. Relationship between the sensor response and the concentration of fluoride. Buffer used was 0.1 M sodium citrate/citric acid, pH 5.1, with 1.4 mM glucose 6-phosphate.

Figure 8 shows the derivative current–concentration curve of the hybrid sensor for fluoride. The sensitivity is considerably lower than that for phosphate (Figure 6). The detection limit is about 0.1 mM. No linearity can be achieved for fluoride by using operation mode 2 as described above. This is due to the noncompetitive inhibition by the substance resulting in a more complicated rate equation.

Measuring Time and Precision. The response time, measuring time, and precision of the hybrid sensor for glucose 6-phosphate and inhibitor measurement are given in Table I. Measuring time is defined as the overall time needed for one determination, including cell rinsing and equilibration. Taking into account the rather high thickness of the tissue slice the response and measuring times are surprisingly low. For any of the three substances at least 10 determinations per hour are possible.

With the exception of fluoride determination an excellent precision is obtained with the hybrid sensor.

Altogether 14 hybrid sensors with one tissue layer have been tested during the course of this study. Variations between different sensors did not exceed 12% for the glucose 6-phosphate as well as the phosphate and fluoride signals.

Interference. Interference studies were performed with respect to other inhibitors disturbing in the phosphate or fluoride measurement. Chloride, bromide, and iodide in concentrations up to 50 mM, which can interfere in ion selective electrode measurements of phosphate or fluoride (16, 17), gave essentially no response. The following oxyacids gave no response when added to give 1 mM final concentration: acetate, carbonate, chlorate, sulfate, oxalate, nitrite. The effect of citrate was tested in 0.1 M imidazole/HCl buffer, pH 6.5.

Table II. Effects of Interfering Ions on the Glucose 6-Phosphate Background Signal

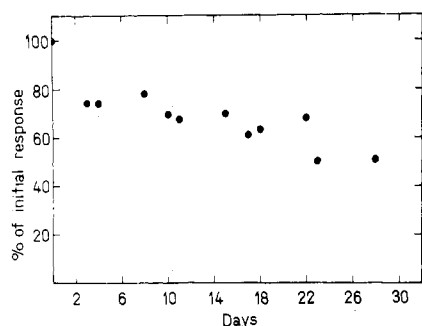| compound	concn, mM	% of rel response
phosphate	1.0	100
nitrate	1.0	8.8
borate	1.0	16.4
tetraborate	0.3[a]	32.0
	1.0	48.2
molybdate	0.3[a]	182.0
	1.0	94.0

[a] Compared to the same phosphate concentration.

Figure 9. Long-term stability of the sensor for phosphate. Conditions are the same as those given in Figure 6. [phosphate] = 1 mM.

The 10 mM citrate did not influence the glucose 6-phosphate background current. Interference by other oxyacids is summarized in Table II. Moderate signals, i.e., inhibition of acid phosphatase, are caused by nitrate, borate, and tetraborate. Molybdate interferes strongly, the effect being higher at low inhibitor concentration. This indicates a lower inhibitor constant as compared to phosphate and is in agreement with results of Sugiura et al. (18). Studying acid phosphatase from sweet potato, these authors found the K_i for molybdate to be 3 orders of magnitude lower than that for phosphate.

In the determination of phosphate in agricultural or physiological samples the interferences by borate, tetraborate and molybdate should be no problem because it is unlikely that any of these ions are present in such samples. However, in water analysis nitrate might cause severe interference.

Owing to the broad substrate specificity of plant acid phosphatase enzymes (e.g., ref 9 and 18) measurements in samples containing organic phosphates will be difficult. These substances might decrease the glucose 6-phosphate background current (or signal, depending on the operation mode used) by competing with glucose 6-phosphate for acid phosphatase.

Glucose, of course, is a potent interfering substance in inhibitor as well as glucose 6-phosphate determination. This can be overcome either by using mode 2 or by applying the recently developed glucose eliminating antiinterference layer (19, 20).

Stability. The long-term stability of the hybrid sensor was studied by testing the response to phosphate in the presence of 1 mM glucose 6-phosphate in the measuring solution. When not in use the sensor was stored in 0.1 M sodium citrate/citric acid buffer, pH 6.0, at 4 °C. Within 4 weeks of operation altogether 300 measurements were carried out. Weekly determined calibration curves were essentially the same as those obtained on the first day up to 0.4 mM phosphate. With higher inhibitor concentrations the response decreased. This is shown for 1 mM phosphate in Figure 9, each point representing the mean of at least eight determinations. Fifty percent of the initial value is reached after 24 days. The apparently higher stability at low inhibitor concentrations might be due to the slight influence of diffusion on the glucose

Table III. Phosphate Determination in Fertilizers

	phosphate content		
sample	chemically,[a] mM	present method mM	%[b]
FL	78.0	63.5	12.5
HE	46.0	44.3	9.9
S 1	51.0	50.3	11.2

[a] According to Fiske and SubbaRow (21). [b] Calculated as H_3PO_4 in the solid substance.

Table IV. Recovery of Phosphate Added to Urine

phosphate added, mM	phosphate recovered, mM	rel error, %
9.9	10.2	2.9
19.2	20.3	5.4
29.1	28.5	−1.7

Table V. Phosphate Determination in Urine Samples

	phosphate, mM	
urine sample	clinical method	present method
1	26.8	27.8
2	22.9	23.0
3	12.8	14.2
4	51.2	56.3

6-phosphate response as shown above.

Phosphate Determination in Fertilizers and Urine. Table III shows the results of phosphate determination in three different fertilizer solutions with the hybrid sensor as compared with the method of Fiske and SubbaRow (21). The fertilizers used were Floraphil (FL), Herbaphil (HE), and S 1.

With the exception of the Floraphil sample the agreement between both methods is quite good. The reason for the difference might be either the presence of some acid phosphatase activator or the interference of a Floraphil constituent in the chemical procedure.

For phosphate determination in urine operation mode 2 was used to circumvent glucose interference. Table IV shows the efficiency of recovery of phosphate ion in urine of the hybrid electrode. Varying amounts of 1 M KH_2PO_4 were added to the urine and the phosphate concentrations in the resulting mixtures were determined.

The hybrid sensor was used to determine phosphate in four samples of undiluted urine. The results (Table V) were compared to those obtained by a clinical laboratory standard method (modified ammonium molybdate method) (22). It should be noted that the clinical laboratory determinations were performed under routine conditions; i.e., no special care was given to these particular samples. From this point of view the results determined with the hybrid sensor are quite good. Relative deviations are between 0 and 10%.

CONCLUSIONS

In the present hybrid biosensor the sequential action of potato acid phosphatase and GOD allows the determination of glucose 6-phosphate. The predominance of kinetic control of these measurements forms the basis of the determination of the acid phosphatase inhibitors, inorganic phosphate, and fluoride, with the sensor. For measurement in river water or serum the sensor is not sensitive enough, but in fertilizers and

urine samples phosphate can be measured with reasonable precision.

Phosphate determination is also possible with an electrode using immobilized isolated phosphatase and GOD (8). However, when working under kinetic limitation, which is a precondition for inhibitor measurements, the usability of this approach is restricted by the relatively low stability of isolated enzymes. In contrast, the native environment of acid phosphatase in the potato tissue provides a high stability allowing several hundreds of samples to be determined with high sensitivity. Another advantage of the hybrid sensor is the great simplicity of biocatalyst preparation. The potato tissue could be replaced by other acid phosphatase rich plant tissue, e.g., from soy bean (23), sweet potato (18), or tobacco leaf (24). Therefore the concept of the hybrid sensor as that of plant tissue based sensors should be of interest expecially in developing countries.

The applicability of the sensor could be expanded by coupling phosphate consuming or producing enzymes. As a first step, two self-contained apyrases (25) have been successfully utilized for ATP measurement. Apyrases split phosphate from ATP, which itself is not hydrolyzed by potato acid phosphatase (9), according to

$$ATP + 2H_2O \rightarrow 5'\text{-}AMP + 2HPO_4^{2-}$$
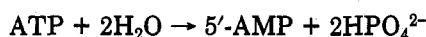
thus generating the acid phosphatase inhibitor directly in the tissue slice. Further investigations in this direction as well as toward optimization of phosphate assay are in progress.

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Effect of Non-Newtonian Solutions on the Behavior of the Thermal Pulse Time-of-Flight Flowmeter

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The thermal pulse time-of-flight liquid flowmeter is shown to calibrate linearly for non-Newtonian fluids which obey the power law model of fluid flow through a tube. Theory is presented which relates the power law parameter, n, to the hydrodynamic cell volume, V_c. Although the calibration is linear for a given polymer solution, the calibration constants differ from those obtained for Newtonian fluids. The results are applied to the use of the flowmeter in GPC. Since n is concentration and molecular weight dependent, V_c can change during a GPC run. The analyses of high molecular weights and large concentrations have the greatest possibility of error, but most polymers used in GPC will not affect the precision of the flowmeter.

In a previous paper (1) the construction and calibration of a thermal pulse time-of-flight flowmeter were described. The basic concept is to heat briefly the liquid flowing through a tube (normally 0.062 in. i.d.) with a thermistor and then detect its arrival at some fixed distance with another thermistor. The next heat pulse is triggered by the detection of the previous pulse. For Newtonian fluids under laminar conditions, the flowmeter was shown to obey eq 1

$$T = V_c/Q + K_c \tag{1}$$

where T is the time interval between heat pulses (s), Q is the flow rate (cm^3/s), V_c is the hydrodynamic cell volume (cm^3), and K_c is a constant, related mainly to detection electronics (s).

Due to the parabolic velocity distribution across the tube, V_c is some fraction (about 0.5) of the geometric cell volume. Since non-Newtonian liquids exhibit different velocity distributions, it was not obvious that non-Newtonian fluids, such as polymer solutions, would calibrate linearly. This work shows under which circumstances linearity holds and applies the results to GPC, the flowmeter's major use to date.

EXPERIMENTAL SECTION

Calibration procedures used were similar to those described previously except that volume was used instead of weight to