PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Enzyme Electrodes and Their Application [and Discussion]

F. Scheller, D. Kirstein, L. Kirstein, F. Schubert, U. Wollenberger, B. Olsson, L. Gorton, G. Johansson, W. J. Albery, F. Scheller and J. D. R. Thomas

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Enzyme electrodes and their application

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Starting from the state of the art, principles for improving the analytical characteristics of enzyme electrodes are discussed. Coupling of appropriate amperometric electrode processes with enzyme systems, e.g. urease or aminopeptidases, results in a simplification of operation. Optimal sample frequencies are realized on the basis of enzyme membranes, with both a small characteristic diffusion time and a high enzyme activity, applied in a well-designed sample-processing system. Coupled enzyme reactions of the sequence or competition type are successfully used for extension to new analytes, e.g. inhibitors, cofactors or alternative substrates. Cyclization of the analyte enhances the sensitivity of enzyme electrodes to the nanomolar concentration range. Enzymic anti-interference layers are a tool for improving the sensor specificity. The operational characteristics of enzyme electrodes are thus adaptable to any given analytical problem.

Introduction

Applications for enzymes are not new. Starting about 60 years ago, their relevance as analytical tools in clinical diagnostics, food analysis and pollution control has steadily increased. Frequently cited objections to the routine application of enzymes for analytical purposes have been their limited stability and the difficulty of performing analyses. Therefore invention of 'enzyme test strips' by Free *et al.* in 1956 was a considerable advance. Application of these test strips simplifies the analytical procedure: the sample is simply dropped onto the test strip, and thus dilution or aliquotting steps are avoided. However, this advantage is offset by a rigorous time régime of operational steps. Furthermore, test strips are one-way materials, i.e. immobilization of the enzyme does not result in reusability of the reagents.

Further progress in applying immobilized enzymes in analytical chemistry was made by L. C. Clark and C. Lyons in 1962. Starting from their membrane-covered oxygen electrode, they entrapped an enzyme solution by a semi-permeable membrane in front of the indicator electrode. This step resulted in the reusability of the enzyme and represents the birth of the first biosensor.

The present state of enzyme electrodes

At present, about 50 different substrates and enzyme activities are measured, more or less routinely, by using enzyme reactions. Highly sophisticated reagent strips for the determination of 15 different metabolites and for 10 enzyme activities have been commercialized by international firms (Libeer 1985). In addition to enzyme test strips and enzyme immunoassays,

enzyme electrodes are at the leading edge in analytical application of immobilized biocatalysts. Up to now biosensors for the determination of about 80 different substances, including substrates, inhibitors, activators, cofactors, prosthetic groups, enzyme activities, haptens, antigens and microorganisms, have been described in publications and patents (Scheller *et al.* 1985). Analysers based on enzyme electrodes for the determination of the metabolites glucose, galactose, uric acid, choline, ethanol, lysine and lactate, of the disaccharides sucrose and lactose and of α-amylase activity have been commercialized, starting in 1976 with the Roche Lactate Analyzer 640 (Scheller *et al.* 1985).

The most evident advantage of enzyme electrodes is their negligible enzyme consumption, which is in the range of a few milliunits per sample. In addition to the drastic reduction of reagent costs, an important advantage of amperometric biosensors is the considerable simplification of the measuring devices. They represent the spatial unity of dialyser, enzyme reactor and electrochemical detector, thus minimizing time-consuming transport processes.

Potentials of enzyme electrodes

(*a*) *Simple operation*

Simple sample pretreatment and analyser operation are important preconditions for the general acceptance of a new method. An important diagnostic parameter in kidney diseases and for dialysis control of artificial kidneys is the urea concentration in serum. Urease-based potentiometric sensors are well established in this field (Scheller *et al.* 1986). We developed an amperometric urea sensor based on the pH-dependent anodic oxidation of hydrazine (Kirstein *et al.* 1985). The advantages of this method are a linear calibration curve (compared with the logarithmic response of potentiometric sensors), excellent reproducibility, a throughput of 40 samples per hour and a measuring range of 1–80 mmol l^{-1} in the sample.

The amperometric urea sensor, in combination with the Glukometer, has been successfully applied in urea monitoring of dialysis patients. The figure illustrates the time-dependent decrease of urea concentration during the dialysis treatment. In this way the time of dialysis can be adjusted for the effective removal of urea. This process control results in a considerable decrease in costs and, more importantly, in a reduction of the discomfort caused to dialysis patients.

The simple electrode reaction, i.e. hydrazine oxidation, has been used for yet another method: the measurement of the activity of alanine aminopeptidase. The conventional method uses the respective hydrazide as substrate, and the hydrazine formed is then coupled to a colour-forming reaction. We demonstrated that the hydrazine can be measured directly; the preincubation time was thus reduced by 40%, compared with the original procedure. Correlation between the two methods was good.

(*b*) *Optimal sample frequency*

At present the sample throughput of enzyme-electrode-based analysers is limited to a maximum of about 100 samples per hour. At high enzyme loading, the response of the membrane-covered electrode depends both on the concentration–time profile in the cell compartment and on the characteristic diffusion time, i.e. the internal diffusion.

For our glucose oxidase (GOD) sandwich membrane, which has an enzyme-loading factor of about 1000, a characteristic diffusion time of 24 s was determined (Olsson *et al.* 1986).

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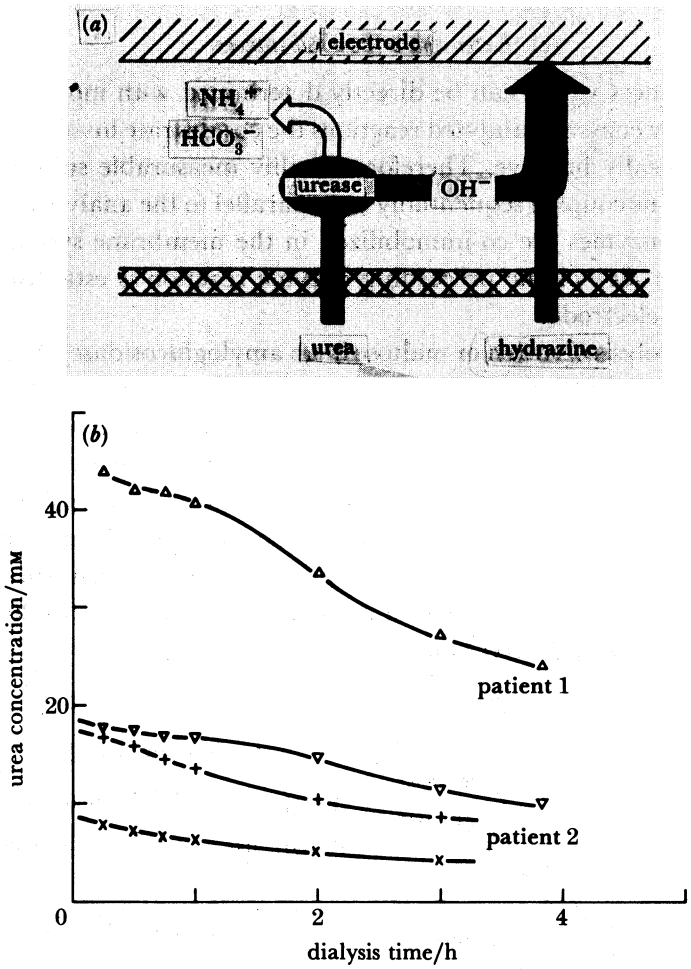

FIGURE 1. (*a*) Schematic diagram of amperometric urea sensor and (*b*) time course of urea concentration in serum and dialysate during dialysis treatment of patients. Symbols: △, +, serum; ▽, ×, dialysate. The current of the anodic hydrazine oxidation depends linearly on the concentration of hydroxyl ions at a potential of 100 mV (measured against a standard calomel electrode): $N_2H_4 + 4OH^- \rightarrow N_2 + 4H_2O + 4e$ (electrode reaction). Thus the OH^- formation in the urease-catalysed reaction is also reflected by a proportional current increase.

Application of the GOD electrode in a stirred measuring cell exhibited a width of the response curve between 20 and 30 s. This resulted in a maximum sample frequency of 120 h^{-1} for the manual glucose analyser. In a flow-injection analysis (FIA) system the width of the response peak at 1 % of the maximum peak height was 12 s. Under these conditions a sample throughput of 300 h^{-1} was possible. The response was linear from the detection limit of 10 μM up to 100 mM glucose. The relative standard deviation for 25 successive injections of 1 mM glucose was 0.5 %. This highly effective FIA system has been extended to the measurement of lactate. Owing to the lower specific activity of lactate oxidase (LOD) (15 units mg^{-1}) as compared with GOD (50 units mg^{-1}) a thicker enzyme layer was applied. 180 lactate samples per hour were measured with good precision and negligible carryover.

It can be concluded that it is always advantageous to use a membrane electrode with a small characteristic diffusion time provided that the enzyme activity is sufficiently high. The full exploitation of the merits of a thin membrane, however, requires a well-designed sample-processing system.

(c) *Extension to new substances*

The number of substances which can be directly determined with monoenzyme electrodes is limited because in many enzyme-catalysed reactions the cosubstrate involved and the product formed are electrochemically inactive. Therefore, readily measurable substances have to be formed in enzyme reactions coupled sequentially or in parallel to the analyte conversion. Either the sequentially acting enzymes are co-immobilized in the membrane system in front of the electrode (Pfeiffer *et al.* 1980) or the preceeding enzyme reaction is established in a reactor upstream of the enzyme electrode.

We combined the hydrolysis of starch or maltose in an amyloglucosidase (AMG) reactor with the detection of glucose by a GOD electrode in a FIA system. The reaction system is complicated by the mutarotation of excess β-glucose formed in the AMG-catalysed reaction. With a residence time between 20 and 60 s in the packed-bed reactor, the steady-state signal for both maltose and Zulkowski starch was independent of the flow rate and it coincided, after addition of mutarotase, with that of equimolar glucose samples. These results indicate complete substrate conversion in the AMG reactor used. The peak response for the different substances depends linearly on the respective concentration over almost three decades. Up to 30 starch samples per hour were measured with high precision.

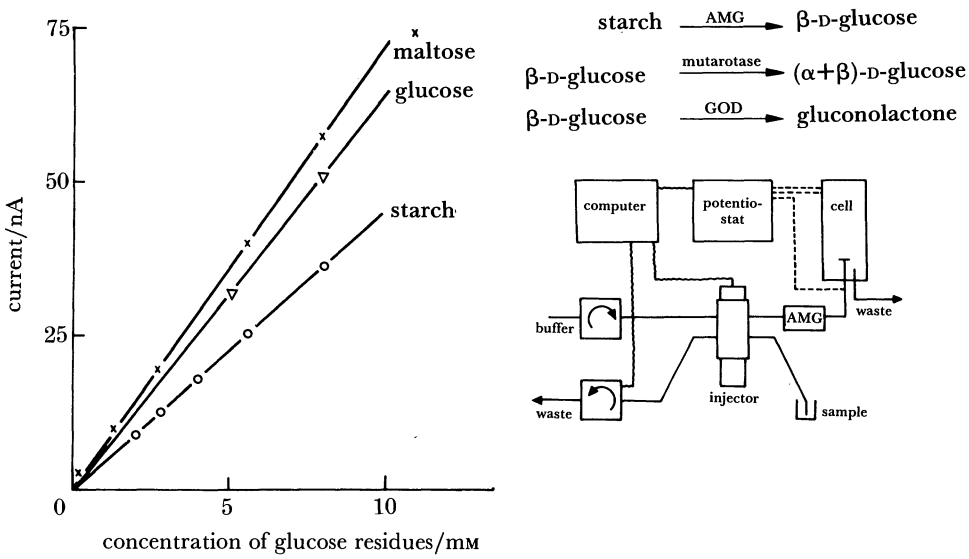

FIGURE 2. FIA system for measurement of glucose, maltose and starch by means of an amyloglucosidase (AMG) reactor and a GOD electrode.

A general problem of sequentially acting enzyme systems, both in the reactor configuration and in the membrane sensor arrangement, is the different sensitivity to the substrates of the respective enzymes, e.g. for glucose, maltose and starch. Even when all substrates are completely converted, differences in the diffusion coefficients influence the response curve. This is a drawback for practical applications when the sum of several substances, e.g. the sugar content, is to be determined.

On the other hand the enzyme-sequence electrode based on lactic dehydrogenase (LDH) and lactic monooxygenase (LMO, decarboxylating) is characterized by identical sensitivity to

both lactate and pyruvate. Obviously the diffusion control by both enzymes and the coincidence of the diffusion coefficients are the basis of this behaviour. Therefore this sensor is appropriate for the sequential determination of lactate and pyruvate, the ratio of which is a highly important diagnostic parameter. The same electrode has been also used for the determination of glutamate pyruvate transaminase in blood serum.

Competitive reactions offer the opportunity to measure other types of substances than ordinary substrates, namely inhibitors, alternative substrates or cofactors which are not directly converted to electroactive products. The principle of competition of two alternative substrates has been used in the measurement of aniline or phenol. Methaemoglobin (MetHb) converts both hydroquinone (HQ) and aniline; however, only the benzoquinone (BQ) formed is electrochemically active at a potential of 0 mV. Therefore, the current of BQ reduction reflects the rate of HQ conversion. At constant HQ concentration, addition of aniline or phenol results in a decreased BQ signal. The current decrease depends linearly on aniline concentration up to 1.0 mM.

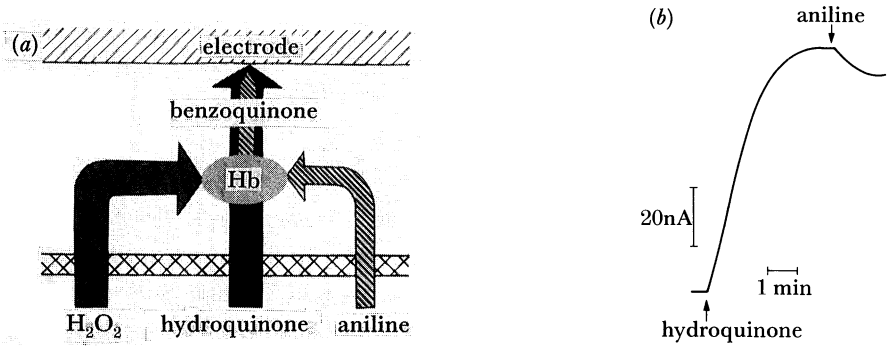

FIGURE 3. Aniline measurement by substrate-competition electrode with immobilised methaemoglobin, (*a*) Schematic diagram; (*b*) time course of sensor output.
Methaemoglobin catalyses the parallel reactions
$2HQ + H_2O_2 \rightarrow 2BQ + 2H_2O$,
aniline $+ H_2O_2 \rightarrow p$-aminophenol $+ H_2O$.
The rate of BQ formation is indicated by the electrode.

TABLE 1. PARALLEL ENZYME REACTION SYSTEMS

reaction type	analyte	enzyme	substrate	indicator
enzyme inhibition	PO_4^{3-}, F^-	acid phosphatase	glucose 6-phosphate	GOD–O_2
substrate competition	fructose	hexokinase	glucose	G6P-DH–PMS
	aniline	MetHb	HQ	BQ
enzyme competition	NAD^+	GOD–GDH	glucose	O_2
	ATP	GOD–hexokinase	glucose	O_2
	bilirubin, aminopyrine	POD–catalase	H_2O_2	O_2

Competition of peroxidase (POD) and catalase for their common substrate, H_2O_2, was used in a sensor for the determination of bilirubin or aminopyrine. The peroxide was either added to the measuing solution or directly generated in the enzyme layer by GOD. In both cases addition of POD substrates resulted in a decrease of the oxygen reduction current caused by H_2O_2 consumption in the substrate conversion (Renneberg *et al.* 1982).

(*d*) *Adapted sensitivity*

If substrate concentrations in the nanomolar range are to be determined, the sensitivity of enzyme electrodes can be enhanced by using substrate amplification. Operational conditions have to be adjusted in such a way that one enzyme catalyses the regeneration of the substrate of the second enzyme. This has been achieved by coupling the respective oxidase and dehydrogenase, and also by using kinases.

TABLE 2. SUBSTRATE AMPLIFICATION SYSTEMS

analyte	enzymes	cosubstrates	indicator reaction	amplification factor
lactate or pyruvate	LOD–LDH	O_2–NADH	O_2	4100
lactate or pyruvate	cytochrome b_2–LDH	$[Fe(CN)_6]^{3-}$, NADH	$[Fe(CN)_6]^{4-}$	10
glucose	GOD–GDH	O_2, NADH	O_2	10
glutamate	GPT–GLDH	alanine, NAD^+	NADH	60
ADP or ATP	PK–HK	PEP, glucose	lactate, O_2	200

Two different amplification systems for lactate have been described: Mizutani *et al.* (1985) obtained a maximal amplification factor for the steady-state current of 250 in a system of LOD–LDH immobilized in polyvinylchloride. On the other hand, no amplification was found with this relatively thick enzyme layer in the differential mode. Using a very thin enzyme layer of coimmobilized LOD and LDH, we obtained an amplification up to a factor of 4100 both in the steady-state and the kinetic (di/dt) measuring régime. However, the reproducibility at this extreme amplification is influenced by the concomitant NADH oxidase activity of the LOD used, the time-dependent decrease of the enzyme activities and the slow washout process of the cofactor. Theoretical considerations demonstrate that the maximum amplification obtained in our system is a realistic value for the parameters – characteristic diffusion time and first-order rate constant – of the enzyme membrane used.

The other lactate amplification system was established in parallel by our group (Schubert *et al.* 1985) and Kulys (Vidziunaite & Kulys 1985) using LDH and cytochrome b_2. For lactate in the linear range, an amplification factor of 10 was achieved by the NADH-dependent recycling method. The detection limit was 0.3 μM. Pyruvate can be determined with almost the same sensitivity.

For signal amplification of the cofactors ATP or ADP we used hexokinase (HK) and pyruvate kinase (PK) which were co-immobilized with LDH and LMO to transduce pyruvate formation into oxygen consumption. In the presence of an excess of glucose and PEP, the nucleoside phosphate is shuttled between the kinases. Pyruvate is formed in a much greater amount than that of the cofactor present in the enzyme layer. The linear region extends up to 6 μM ATP with 200-fold increase of sensitivity as compared with the unamplified signal in absence of glucose.

By combining both amplification systems for ADP and pyruvate, 'double amplification' for ADP should be possible. In this system the excess of pyruvate formed in the kinase cycle enters the second cycle, where it is further amplified. Whenever we were able to demonstrate the

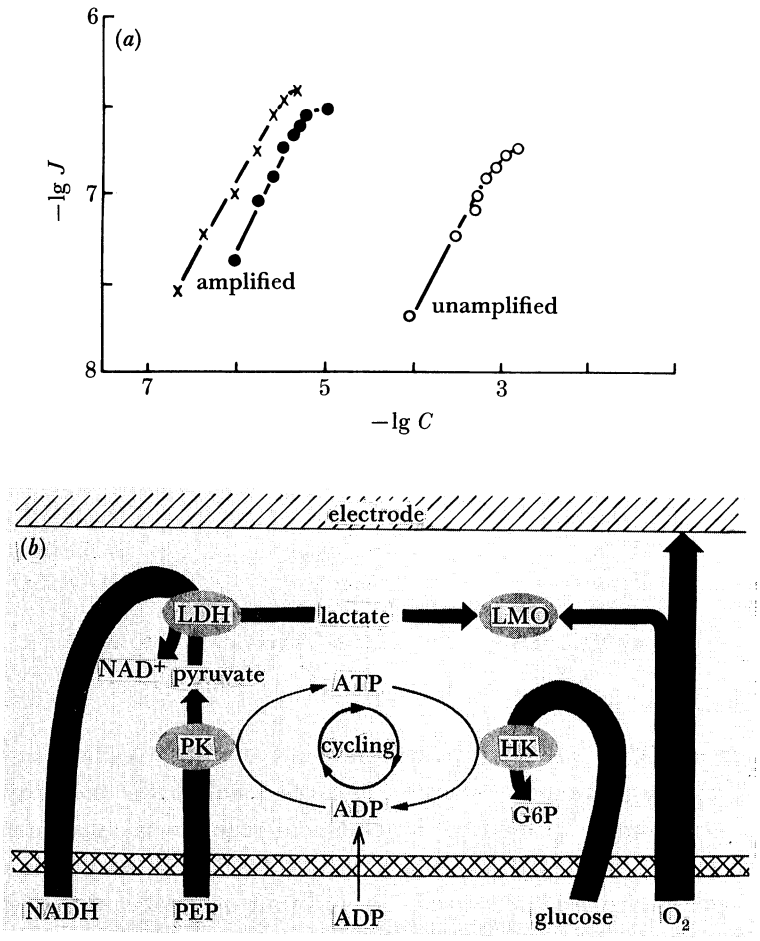

FIGURE 4. Double logarithmic plot of the current–concentration dependences of the pyruvate kinase–hexokinase–lactate dehydrogenase–lactate monooxygenase sensor. Open circles (○) indicate ADP measurements made in the absence of glucose, i.e. the sensor was working as a three-enzyme sequence sensor (PK–LDH–LMO). Other measurements of ADP (●) and ATP (×) in the presence of 1.2 mM glucose, i.e. the PK–HK cycle worked together with the LDH–LMO sequence.

realization of this concept, the reproducibility was bad. The influence of disturbances, discussed for the LOD–LDH cycle, was drastically increased in this highly complex system.

On the other hand, coupling of enzyme reactions also allows one to shift the measuring region of sensors to higher substrate concentrations: the GDH-catalysed reaction of glucose with NAD^+ proceeds in either direction, depending on the concentration of the reagents. Gluconolactone, formed in the GOD-catalysed reaction, is reconverted to glucose in presence of NADH, as is reflected by the amplified glucose signal. However, addition of the oxidized cofactor, NAD^+, favours the forward reaction so that GDH competes with GOD for the common substrate, glucose. Therefore increasing concentrations of NAD^+ result in a decreased oxygen consumption and an increased linear measuring range.

(*e*) *Improved specificity*

Disturbances by substances interfering with the enzymic analyte conversion, such as alternative substrates or inhibitors, may cause serious problems in the practical application of

enzyme sensors. In biosensors based on coupled enzyme reactions the substrate of each particular enzyme will interfere. Loss of specificity of the biosensor may be also introduced at the level of the electrochemical indicator reaction.

We demonstrated that interfering substances, e.g. ascorbic acid, uric acid or endogeneous glucose, which disturb the indicator system, can first be eliminated by an enzyme-catalysed reaction.

Table 3. Anti-interference layers

interferent	eliminating enzyme	analyte	indicator system
Ascorbic acid	ascorbate oxidase	catecholamines	coal electrode
glucose	GOD–catalase	sucrose maltose	invertase–GOD–H_2O_2 amyloglucosidase–GOD–H_2O_2
glucose	hexokinase	sucrose	invertase–GOD–O_2
lactate	LMO	pyruvate	LOD–LDH–O_2
uric acid ascorbic acid	laccase	glucose	GOD–H_2O_2
O_2	GOD–catalase	NAD^+, pyruvate	HDME

If laccase is used, either the interfering substances are directly oxidized by oxygen, as with adrenaline, or a preceding oxidation reaction is carried out in the background solution by ferricyanide. The ferrocyanide formed in the chemical reaction is subsequently oxidized by laccase which is co-immobilized with the respective oxidase. In this way the current signal of glucose–ascorbic acid mixtures is decreased, on addition of ferricyanide into the measuring cell, to the signal relevant to glucose only. The interference caused by ascorbic acid is completely eliminated (Wollenberger *et al.* 1986).

Conclusions

Among biosensors, enzyme electrodes possess the unique property of adaptable functional parameters, depending on the practical situation. They provide the possibility of simple handling in the doctor's office, of high sample throughput in flow analysers and of adapted sensitivity and increased selectivity for trace analysis or *in situ* application.

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Discussion

W. J. Albery, F.R.S. (*Department of Chemistry, Imperial College of Science and Technology, London, U.K.*) I am interested in what determines the amplification factor for substrate cycling. Consider the following simple scheme:

$$\xrightarrow{k_d} A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B \xrightarrow{k_d}$$

In this scheme k_d is a mass-transfer rate constant (cm s^{-1}) describing the supply of A and the removal of B; the rate constants k_1 and k_2 describe the substrate cycling. If we assume that the reaction takes pace in a layer of thickness L then we obtain for the amplification factor, G,

$$G = \frac{(1 + k_1 L/k_d)}{[1 + k_1/(k_2 + k_d/L)]}.$$

It can be seen that significant amplification can only be achieved if Lk_1 is much greater than k_d. Hence this strategy is a means of overcoming unfavourable mass transport and the amplification factor will depend on the mass transport. Does Professor Scheller agree?

F. Scheller. To model the influence of the different parameters, we used a similar reaction–diffusion model; however, the consumption at the electrode of a cosubstrate, e.g. oxygen, and not of A or B, was used for the calculation. On the other hand, a no-flux condition for A and B holds at this boundary. Using this model, we obtained the following approximation for the amplification factor, G:

$$G = \frac{(k_1 k_2 L^2)}{[2_d (k_1 + k_2)]},$$

where d is the coefficient of diffusion of A and B.

The same relation was obtained by Vidziunaite & Kulys (1985). The expression shows that G increases with k_1, k_2 and L but it decreases with mass transport rates. This is quite similar to the conclusions Professor Albery has drawn from his considerations.

J. D. R. Thomas (*Applied Chemistry Department, UWIST, Cardiff, U.K.*). With regard to the mixed-enzyme system of invertase, mutarotase and glucose oxidase in the sensor for the determination of sucrose, can Professor Scheller give details of the immobilizing matrix? Would this be a mixture of the enzymes in a single matrix, or would separate layers of matrix, with an enzyme on each, be used?

F. Scheller. The three-enzyme layer contained all enzymes entrapped in polyurethane on a cellulose membrane. To get a more universal arrangement, we co-immobilized GOD and mutarotase on the same dialysis membrane. This layer was then sandwiched with another membrane containing the immobilized disaccharidase, e.g. invertase or maltase, at the inner face.

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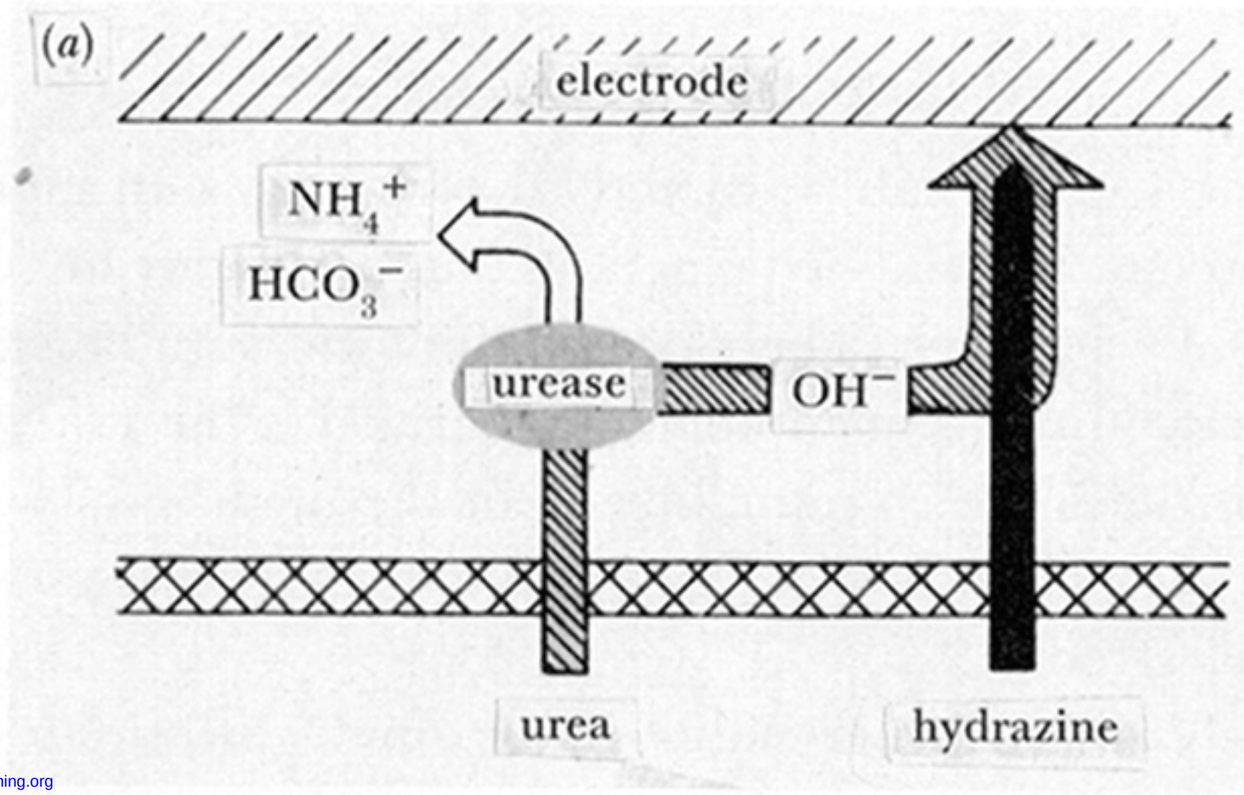

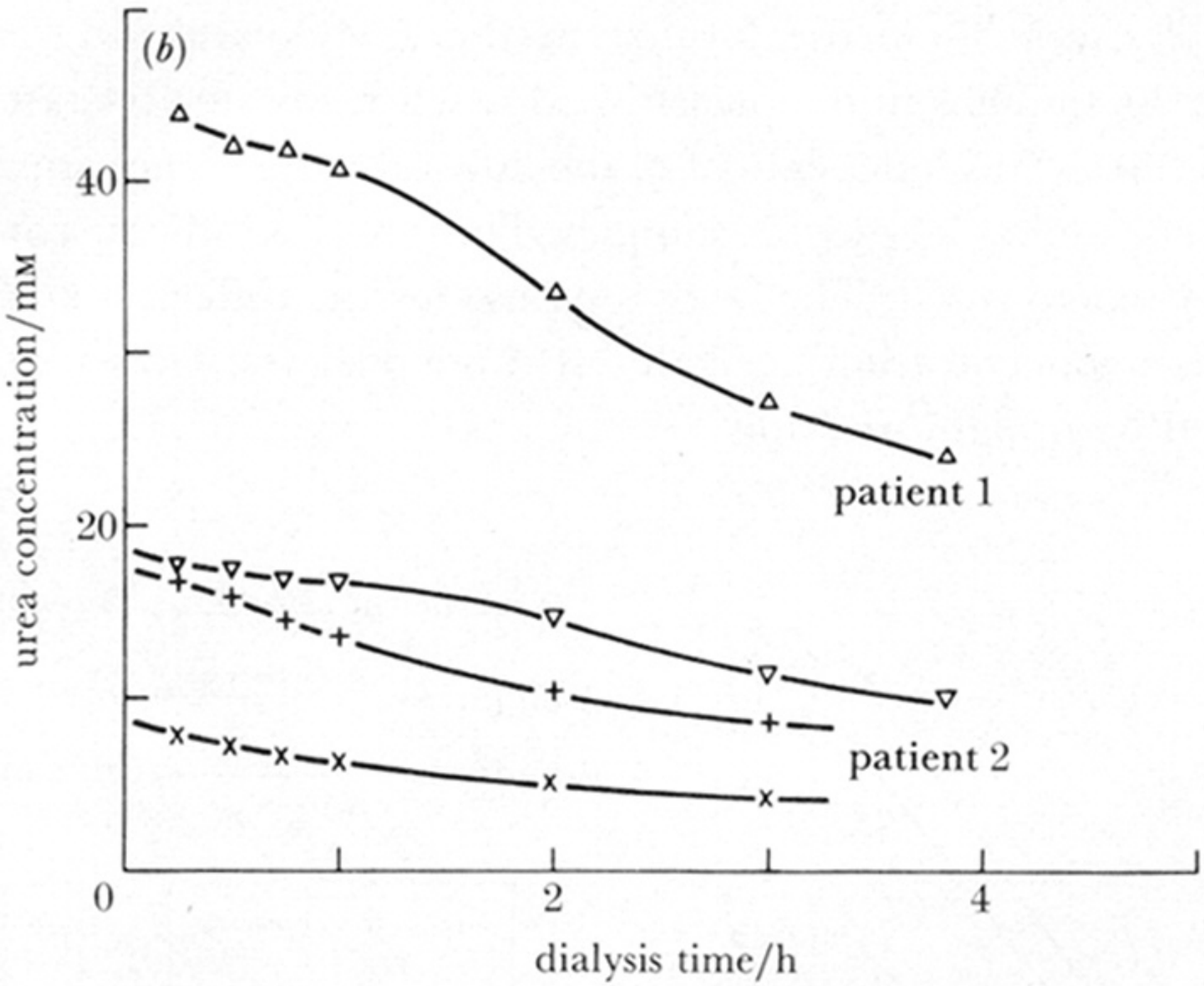

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