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Enzyme-amplified immunoassays*

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Abstract: The sensitivity of enzyme immunoassays may be enhanced by the use of enzyme-amplification. This technique uses the enzyme label in the immunoassay to provide a trigger substance for a secondary system that can generate a large quantity of coloured product. Two examples of enzyme amplifiers are described, using either a substrate cycle with phosphorylated hexose sugars, or a redox cycle involving the co-enzyme NAD^+. The redox enzyme-amplifier has a detection limit of less than one attomole for the enzyme label, alkaline phosphatase.

The limited dynamic range of enzyme-amplified immunoassays may be overcome by kinetic analysis of the colour development in the enzyme-amplifier, to add at least a further order of magnitude to the range of directly measured analyte concentrations in the immunoassay. This is illustrated in an enzyme-amplified immunoassay for human thyroid stimulating hormone. Amperometric measurement of the enzyme-amplifier provides a method to extend the dynamic range still further and compares favourably with the performance of a gamma counter, a luminometer or a fluorimeter.

Keywords: *Ultrasensitive non-isotopic immunoassay; amplification by enzyme modulation; dynamic range extension by kinetic analysis; amperometric detection of enzyme labels; thyroid stimulating hormone.*

Introduction

In the field of immunoassay in recent years there has been an increasing awareness of the need for a new, highly active label to replace the best of the current radioactive markers. The need is greatest in the labelled antibody (reagent-excess) immunoassays that employ two antibodies to capture the antigen in the now familiar "sandwich" configuration. An elegant analysis [1] has shown that the ultimate sensitivity attainable in such reagent-excess immunoassays can be increased by several orders of magnitude if a label is used that has an "infinite specific activity", thereby avoiding the signal measurement errors inherent in the detection of gamma radiation from radioisotopes. The term "infinite specific activity" can be interpreted simply as the ready detection of the presence of a single labelled entity remaining after the separation of excess labelled antibody. Thus, the design of new immunoassays for substances that are present in particularly low concentration has initiated a search for labels with a high specific activity, and this has led

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to the adoption, so far, of three new systems, based on luminescence (light emission) [2, 3], on fluorescence (light absorption and re-emission) [4, 5] or on enzymes (light absorption by a coloured product) [6, 7].

Enzyme labels are, by definition, catalysts and thereby share with fluorescent labels the useful property of continued signal generation over an extended time period. Their disadvantage to date has been the inherent insensitivity of colorimeters to the minute changes in light absorption in response to the product of the small numbers of enzyme labels remaining after the separation step in the immunoassay.

In this paper the application of enzyme-amplification to enzyme-labelled immunoassays is described. This technique has been used to extend further the potential of enzyme labels in the new area of ultrasensitive immunoassays and has demonstrated that the label can approach the requirement of "infinite specific activity" [8–12]. The use of kinetic analysis of the colour development in the enzyme-amplifier is also described. The application of this technique has contributed to the resolution of the other major disadvantage of enzyme labels, namely that of the limited dynamic range of the colorimeter itself.

Finally, the possibility of amperometric detection of enzyme-amplified immunoassays is briefly discussed, and it is proposed that the future direction of enzyme immunoassay may be towards measurement by electrical rather than colorimetric means.

Experimental

Materials

Diaphorase (NADH: dye oxidoreductase, EC 1.6.4.3), alkaline phosphatase (EC 3.1.3.1), NAD^+ and $NADP^+$ were obtained from Boehringer Corporation, London, UK. Alcohol dehydrogenase (EC 1.1.1.1), *p*-iodonitrotetrazolium violet (INT) and bovine serum albumin, fraction V, were obtained from Sigma Chemical Co., Poole, UK. Purified thyroid stimulating hormone (TSH) (WHO Second International Reference Preparation 80/558) was obtained from the National Institute for Biological Standards and Controls, London, UK. 96-well microplates (Immunoplate II) were obtained from Nunc, Denmark. All other chemicals were of the highest grade available.

Amplifier activity

Alcohol dehydrogenase and diaphorase were dissolved in a buffer containing 20 mM-sodium phosphate pH 7.2, 4% (v/v) ethanol, 0.55 mM-INT violet and 5 mg/ml bovine serum albumin. The concentration of enzymes giving rise to a rate of colour development (at 492 nm) of 0.067 absorbance units per min (at 25°C) with 100 nM-NAD^+ was arbitrarily defined as 1.0 unit/ml.

Amplifier activity as a function of NAD^+ concentration was determined by adding 0.2 ml of amplifier (1.18 units/ml) to 0.1 ml of a solution of NAD^+ in a 50 mM-diethanolamine buffer pH 9.5 containing 1 mM-magnesium chloride, 0.1 mM-zinc chloride and 15 mM-sodium azide. The rate of increase in absorbance at 492 nm was measured by a Titertek Multiskan MCC 340 plate reader interfaced to a BBC Model B microcomputer. Amplifier activity as a function of enzyme concentration was measured in the same way, except that a fixed concentration of NAD^+ (100 mM) was used. For the temperature dependence experiment the volumes of amplifier and NAD^+ (100 nM) were increased to permit measurements to be made in a Varian DMS 90 spectrophotometer.

Detection of alkaline phosphatase

Alkaline phosphatase was diluted into 50 mM-diethanolamine buffer pH 9.5 containing 1 mM-magnesium chloride, 0.1 mM-zinc chloride, 15 mM-sodium azide and 1 mg/ml bovine serum albumin. The enzyme concentration was calculated assuming a specific absorbance of 1.0 for a 0.1% (w/v) solution. In the experiment, 0.010 ml aliquots of the enzyme solution were added to 0.10 ml of 0.1 mM-$NADP^+$ in the same buffer (but without bovine serum albumin) and incubated at 25°C for 3 h before the addition of 0.20 ml of amplifier (6.0 units/ml). The rate of increase in absorbance at 492 nm was measured in a Titertek Multiskan plate reader interfaced to a BBC Model B microcomputer.

Immunoassay for TSH

Reagents were prepared as described by Stanley *et al.* [10]. For the experiment demonstrating the detection limit of the immunoassay (Fig. 8), 0.050 ml of the alkaline phosphate conjugated antibody was added to the antibody coated wells of a 96-well microplate, followed by 0.050 ml of a solution containing different concentrations of TSH. The microplate was incubated for 17 h at 6°C before washing four times with 10 mM-Tris buffer pH 8.0 containing 150 mM-sodium chloride, 0.05% (v/v) Triton detergent and 15 mM-sodium azide. Then 0.10 ml of a solution containing 0.1 mM-$NADP^+$ (in the diethanolamine buffer without bovine serum albumin described above) was added to the wells and the accumulation of NAD^+ was allowed to proceed for 70 min at 25°C before 0.20 ml of amplifier (3.0 units/ml) was added. The rate of increase in absorbance was measured as above.

In the routine assays for TSH the immunoassay was essentially carried out as above, except that 0.075 ml of conjugate and 0.025 ml of sample were used. The immunoincubation time was reduced to 2 h at 25°C and the incubation with $NADP^+$ was reduced to 20 min at 25°C. After the addition of amplifier (1.18 units/ml) the absorbance in each well was measured kinetically for the next 20 min (the absorbance generated in each well was measured at 60-s intervals and the data was captured and stored on a microcomputer). Alternatively the colour development was stopped after 20 min with 0.050 ml of 0.3 M-sulphuric acid and a single end-point measurement was made.

Results and Discussion

Principles of enzyme-amplification

In its simplest form, represented schematically in Fig. 1, the enzyme label in the immunoassay (the primary system) is used to provide a trigger substance for a secondary system that can generate a large quantity of coloured product. The enzyme-amplified immunoassay differs from the conventional type in that the product from the enzyme label need not be measurable in itself, but can instead act catalytically on the secondary system, which remains essentially silent until activated in this way. To use an electronic analogy, the enzyme label in the immunoassay can be regarded as a pre-amplifier, which feeds a signal directly into the secondary system or power amplifier. This provides the immunoassay with great flexibility because the gain of the secondary system can be adjusted readily to suit the required sensitivity.

Biochemical amplifiers suitable for use in enzyme-amplified immunoassays can include the substrate cycles found throughout metabolism, or may employ redox cycles involving reduced and oxidised co-enzymes or co-factors. An enzyme amplifier based on the

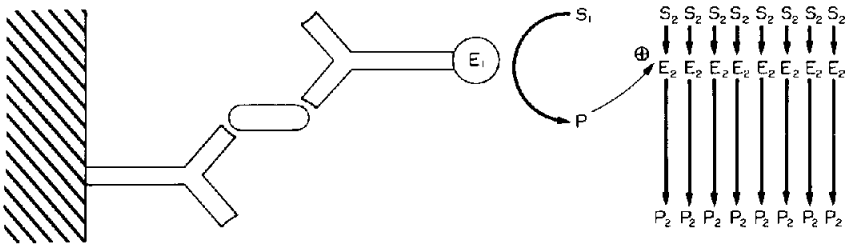
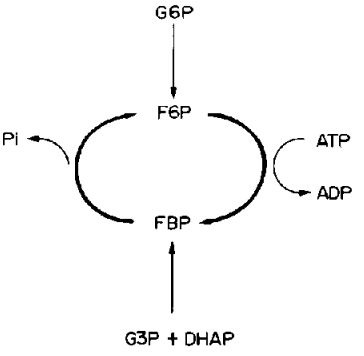


Figure 1
An enzyme-amplified immunoassay for human thyroid stimulating hormone (TSH). The formation of a sandwich is illustrated, involving the antigen and two monoclonal antibodies. One antibody is immobilised to a polystyrene surface, the other is linked to alkaline phosphatase (E_1). S_1 is the primary system substrate, P_1 is the trigger for the secondary system enzymes (E_2), which generate product P_2 from substrate S_2. ⊕ denotes the trigger as a positive effector. [Adapted with permission from *Am. Biotech. Lab.* **3**(3) (1985).]

Figure 2
A substrate cycle as a secondary system amplifier. The primary system enzymes, aldolase or phosphoglucoisomerase, generate the substrate for the "futile cycle" driven by the enzymes phosphofructokinase and fructose-bis-phosphatase. An excess of adenosine triphosphate (ATP) allows catalytic cycling of the substrates fructose-6-phosphate (F6P) and fructose-bis-phosphate (FBP), with the generation of inorganic phospate and ADP as products. G6P is glucose-6-phosphate, G3P is glyceraldehyde-3-phosphate, DHAP is dihydroxyacetone phosphate and ADP is adenosine diphosphate. [Adapted with permission from *Am. Biotech. Lab.* **3**(3) (1985).]



substrate cycle fructose-6-phosphate/fructose-bis-phosphate is illustrated in Fig. 2. An enzyme-amplified immunoassay based on this system would employ either fructose-6-phosphate or fructose bis-phosphate as the trigger for the secondary system, with both compounds acting as catalysts in a substrate cycle driven by the two enzymes phosphofructokinase and fructose-bis-phosphatase. The enzyme labels in the immunoassay would be phosphoglucoisomerase or aldolase, and the phosphate produced by the cycle would be measured colorimetrically. Note that the amplifier is poised by an excess of ATP, but remains inactive until the appropriate substrate is provided by the primary system. A similar substrate cycle has been proposed by Harper and Orengo [13]; in this case the ADP generated by the enzyme-amplifier was assayed in a further series of coupled-enzyme reactions.

An analogous redox amplifier, involving the cycling of redox equivalents, is illustrated in Fig. 3. The trigger in this case is the co-enzyme NAD^+ (nicotinamide adenine dinucleotide) which is generated in the primary system by the dephosphorylation of $NADP^+$ (nicotinamide adenine dinucleotide phosphate), a reaction catalysed readily by the enzyme alkaline phosphatase. In the redox amplifier, the primary system product acts catalytically in a cycle driven by the enzymes alcohol dehydrogenase and diaphorase. The amplifier is poised by an excess of ethanol and INT-violet, but again remains inactive until NAD^+ is generated by the primary system. The product of this redox

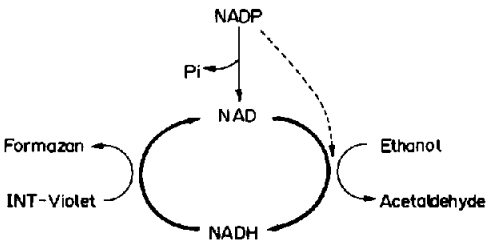


Figure 3
A redox cycle as a secondary system amplifier. The primary system enzyme, alkaline phosphatase, generates the trigger nicotinamide adenine dinucleotide (NAD^+) for the redox cycle driven by the enzymes alcohol dehydrogenase and lipoamide dehydrogenase. An excess of ethanol and INT-violet allows catalytic cycling of reduced and oxidised nicotinamide, with the generation of formazan dye and acetaldehyde as products. [Adapted with permission from *Am. Biotech. Lab.* **3**(3) (1985).]

amplifier is a formazan dye with a powerful absorbance in the visible region of the spectrum.

Biochemical amplifiers can have a poor signal-to-noise ratio. The source of noise is illustrated in Fig. 3 as a dashed line leading from the primary system substrate to the amplifier enzymes. This indicates that these enzymes are not absolutely specific for their intended substrate and may instead use the substrate of the primary system. This will lead to high background measurements, which can only be minimised by the use of amplifier enzymes of the highest specificity. With the right choice of enzymes, however, the primary system product may be cycled with very little interference from high concentrations of the primary system substrate. This is an improvement on the enzymatic cycling assays described earlier [14], which required the destruction of the interfering primary system substrate before cycling could begin. In the example given in Fig. 3 the specificity of alcohol dehydrogenase and diaphorase for NAD^+ over $NADP^+$ is such that a signal-to-noise ratio of 10,000 is attained (defined as the optical absorbance generated by the amplifier when given the same amount of NAD^+ or $NADP^+$).

A rigorous solution to the kinetic equations of the two-enzyme redox amplifier can be proposed [15]. Essentially the velocity of such a cycle is a function of the apparent second order rate constants of the enzymes and their respective concentrations:

$$v_c = \frac{k_1.e_1.k_2.e_2}{k_1.e_1 + k_2.e_2}.[P], \tag{1}$$

where v_c is the velocity of the cycle, $[P]$ is the concentration of the primary system product (or trigger) and k_1 and k_2 are the apparent second order rate constants ($kcat/K_m$) for the enzymes e_1 and e_2.

Since $k.e = V_{max}/K_m$, this equation can be re-written as:

$$v_c = \frac{V_{max1}.V_{max2}}{V_{max1}.K_{m2} + V_{max2}.K_{m1}}.[P]. \tag{2}$$

These relationships show that the velocity of the cycle is proportional to the concentration of the amplifier enzymes and, for low concentrations of P, the cycle rate is proportional to $[P]$. Note, that for high concentrations of P it can be shown that the cycle velocity saturates in a hyperbolic manner, analogous to the Michaelis equation.

It is interesting to compare the relative velocities of the primary and secondary systems in the redox amplifier. The turnover number of alkaline phosphatase with $NADP^+$ is about 60,000 (i.e. 60,000 molecules of the primary system product are produced per min). The velocity of the redox amplifier, driven by the enzymes alcohol dehydrogenase

and diaphorase, is about 30 per min, or, to continue the electronic analogy, the amplifier turns over at about the same speed as a long playing record. Hence, the secondary system is extremely slow in comparison to the primary system, but because each molecule of NAD^+ can generate one molecule of formazan every 2 s, the flux through the secondary system over a 10-min period can be immense.

One further point to note about the redox amplifier is that the velocity of the cycle depends upon the ratio of V_{max} and K_m of both enzymes. Since the temperature dependencies of these two parameters may differ it can be predicted from this relationship that the velocity of the cycle will not change with temperature in the same way as a simple enzyme catalysed reaction would.

Performance characteristics of the redox enzyme-amplifier

In order to propose the use of enzyme-amplifiers in immunoassays it is necessary to show that their performance characteristics are indeed suitable for their intended use as an ultra-sensitive, non-isotopic detection method. The majority of the experimental validation work in this area has been carried out with the redox amplifier [11]. In Fig. 4 the response of this system is shown to be linear with respect to the concentration of NAD^+, from 0 to greater than 100 nM. Hence this enzyme-amplifier can provide a linear cycle velocity over at least a two orders of magnitude variation in the concentration of primary system product. Figure 5 demonstrates that the response of the redox amplifier is linear with respect to enzyme concentration and this offers the possibility of tuning the gain of the amplifier; essentially a "custom" detection system can be made for each individual immunoassay. Figure 6 confirms that the enzyme-amplifier shows significantly less temperature dependence than that expected from a simple enzyme-catalysed reaction.

To demonstrate the potential of this enzyme-amplifier an experiment was carried out to determine the limit of detectability of the enzyme label, alkaline phosphatase. By extending the incubation times to their practical limit and by using an amplifier of very high gain the detection limit (defined as the concentration of alkaline phosphatase which corresponds to an absorbance 2.5 standard deviations from the mean absorbance of six replicates of the zero standard) was 1.1×10^{-20} mol, or about 6600 molecules of alkaline phosphatase (Fig. 7). In a further experiment, this ultrasensitive detection system was employed in a sandwich immunoassay for human thyroid stimulating hormone (TSH). The immunoassay was optimised for the greatest sensitivity by using the highest possible concentration of labelled antibody before significant non-specific binding was encountered. The detection limit for TSH in this assay was 43×10^{-20}, or about 280,000 molecules (Fig. 8). The sensitivity of this immunoassay was limited largely by the relative error in the measurement of the non-specific signal. Note that this experiment was carried out only to show the sensitivity potential of an enzyme-amplified immunoassay and therefore has little relevance to an immunoassay intended for routine use (see next section).

Sensitivity and dynamic range in an enzyme-amplified immunoassay for TSH

The dynamic range of a typical colorimeter, i.e. the region in which a measurement can be made with acceptable accuracy, is limited to two, or at best, three orders of magnitude of variation in the absorbance of a coloured solution. This limits the ability of an enzyme immunoassay, whether of the conventional or enzyme-amplified type, to measure as wide a range of analyte concentration as, say, a similar assay employing a radioactive or

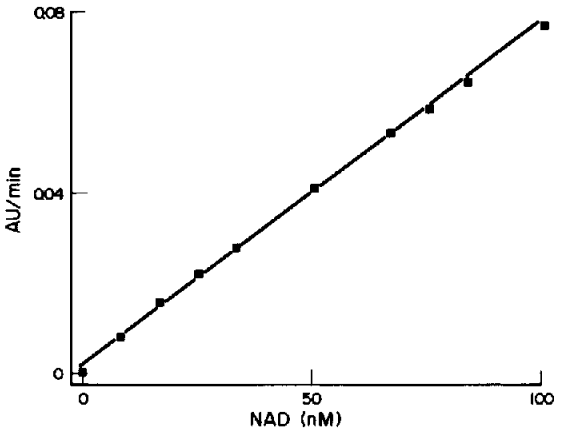


Figure 4
The response of the enzyme-amplifier to NAD^+. The rate of increase in absorbance at 492 nm generated by the amplifier (at 1.18 units/ml) is given for each NAD^+ concentration. Each point represents a single rate measurement made over a 10-min period. [Adapted with permission from *J. Immunol. Meth.* **87** (1985).]

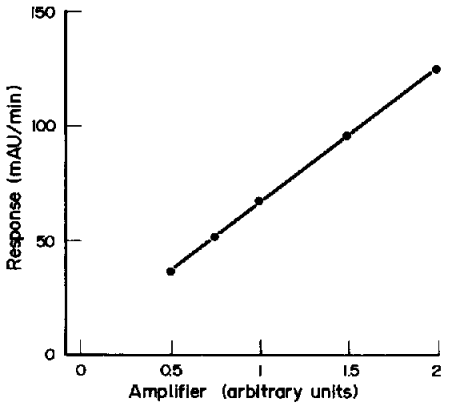


Figure 5
The rate of increase in absorbance generated by the amplifier as a function of enzyme concentration. Duplicate rate measurements (over a 10-min period) were made at each concentration of the amplifier enzymes. The NAD^+ concentration was 100 nM. [Adapted with permission from *J. Immunol. Meth.* **87** (1985).]

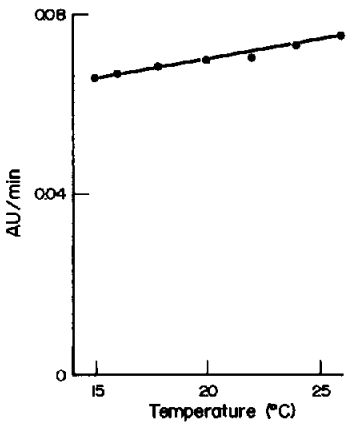


Figure 6
Amplifier activity as a function of temperature. The rate of increase of absorbance over a 3-min measurement period is plotted against the temperature of a thermostatted cell inside a Varian DMS 90 spectrophotometer. The NAD^+ concentration was 100 nM and the amplifier was 1.18 units/ml. [Adapted with permission from *J. Immunol. Meth.* **87** (1985).]

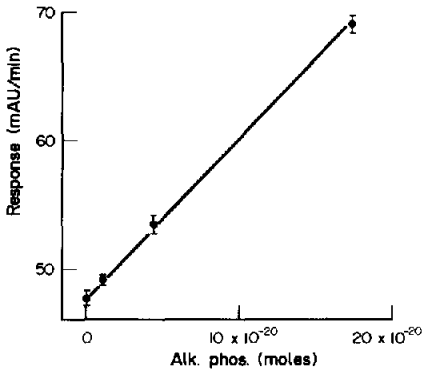


Figure 7
Detection of the enzyme label alkaline phosphatase. Replicate samples (6) of the enzyme were incubated with $NADP^+$ for 3 h before the addition of amplifier (6 units/ml). The rate of increase in absorbance is plotted against the amount of alkaline phosphatase added. The error bars are ±SD. [Adapted with permission from *J. Immunol. Meth.* **87** (1985).]

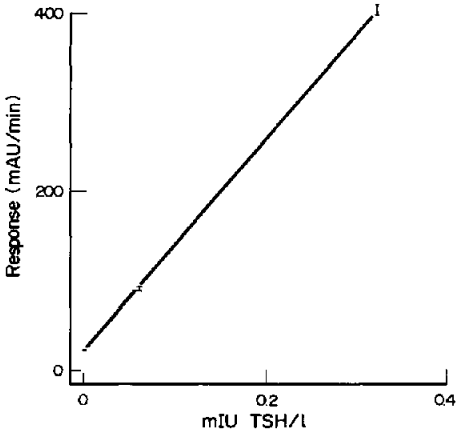


Figure 8
An ultrasensitive enzyme-amplified immunoassay for TSH. The rate of increase in absorbance is plotted against the concentration of TSH in the sample. The error bars represent the mean of six replicates (±SD). [Adapted with permission from *J. Immunol. Meth.* **87** (1985).]

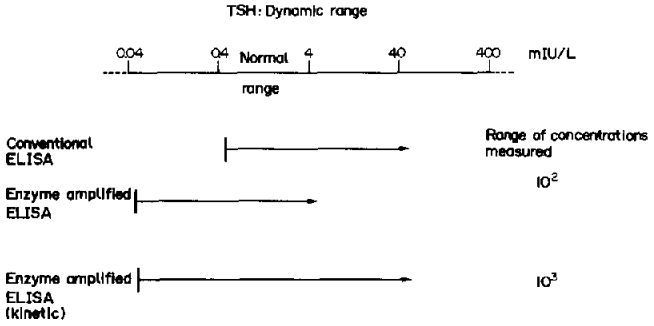


Figure 9
The dynamic range of enzyme immunoassays. The analyte concentration (top) increases from left to right. The ability of each detection method to measure accurately across the physiological range is described by a horizontal line representing the working range of the assay (where the intra-assay SD is less than 15%). The detection methods illustrated are: conventional substrate (p-nitrophenol), end-point enzyme-amplifier or kinetic enzyme-amplifier.

fluorescent label. This problem is particularly pronounced in the case of human TSH where the variation in analyte concentration can be as great as four orders of magnitude, from thyrotoxicosis to hypothyroidism. This is represented schematically in Fig. 9 where the TSH concentration in human serum is shown to range from below 0.04 mIU/L to greater than 400 mIU/L, a range that is impossible to measure accurately with either conventional enzyme or enzyme-amplified "end-point" immunoassays. At best, these assay methods can only provide a "window" located somewhere within the physiological range of this analyte, and further measurements must be made after dilution of the sample. However, with the advent of plate readers that are capable of sampling a 96-well plate on a continuous basis it is now possible to analyse kinetically the colour development in the enzyme-amplifier. The use of kinetic reading can lead to a dynamic range extension of at least a further order of magnitude, thereby permitting a wider range of TSH concentrations to be measured directly (Figs 9 and 10). There are also other advantages to be gained from kinetic reading, such as the improved precision resulting from the multiple measurement of the colour development in each assay well and a reduction in the complexity of the assay owing to the absence of a "stopping" solution.

Amperometric enzyme-amplified immunoassays

In the redox amplifier illustrated in Fig. 3 the tetrazolium salt can be regarded as an accumulator of electric charge; for every turn of the cycle 2 electrons are transferred from ethanol, via NAD^+, to INT-violet. It is, however, possible to replace this chemical charge storage system with an electrode and therefore to access directly the electrons in the redox cycle. This can be done with a mediator such as ferricyanide ($E'_0 = +418$ mV) which will accept electrons from the NAD^+/NADH cycle ($E'_0 = -320$ mV) and transfer them to a platinum electrode held at a suitable potential (Fig. 11). In this way an amperometric enzyme-amplified immunoassay can be constructed with a sensitivity equal to its colorimetric counterpart, but with an even wider dynamic range owing to the

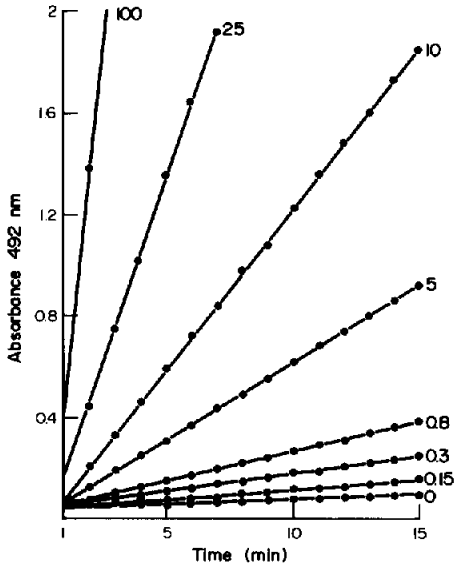


Figure 10
Kinetic measurement of an enzyme-amplified immunoassay for TSH. The absorbance in each well was recorded at 60 s intervals by a Titertek Multiskan MCC 340 microplate reader under the control of a BBC model B microcomputer. Results are shown for TSH calibrators ranging from 0 to 100 mIU/L.

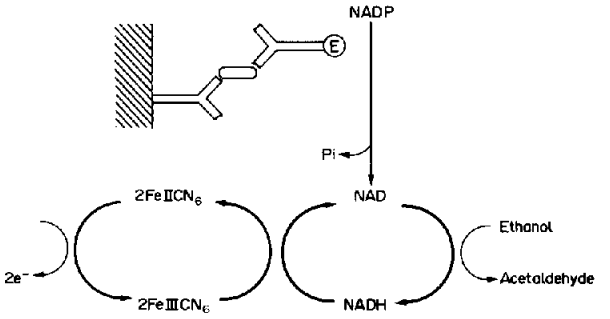


Figure 11
A redox enzyme-amplifier adapted for amperometric detection. Two electrons at each turn of the $NAD^+/NADH^+$ cycle are transferred to a platinum electrode at +450 mV, via a ferricyanide/ferrocyanide mediator cycle.

ability of an electrode to measure currents ranging from picoamps (10^{-12}) to milliamps (10^{-3}) [16]. The use of amperometric detection of enzyme labels in immunoassays has been recognised in other systems [17, 18] and it may provide a means of matching the capabilities of a gamma counter, luminometer or fluorimeter.

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