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FLUOROMETRIC AND ELECTROCHEMICAL METHODS FOR ASSAY OF BIOLOGICAL AND ENVIRONMENTAL SUBSTANCES

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The isolation, purification, and analytical use of enzymes from microbial sources are described: L-lysine decarboxylase from *E. coli*, L-methionine ammonia lyase from *Pseudomonas ovalis*, nitrate reductase from *Chlorella vulgaris*, nitrite reductase from *Azotobacter chroococcum*, sulfate reductase from *Desulvibrio desulficans*, and creatininase from *Pseudomonas*. Both electrochemical, fluorometric, and spectrophotometric methods are proposed for the assay of enzyme activity, and specific, sensitive, fast, and inexpensive methods are described for the assay of 10^{-1}–10^{-5} *M* concentrations of L-lysine, L-methionine, nitrate, nitrite, creatinine, and triglycerides.

INTRODUCTION

Excellent chemical analysis can be performed with enzymes, which are biological catalysts; the real advantages of immobilized enzymes are many in analyses using electrochemical probes or other methods. One advantage of an immobilized enzyme is a pH shift, i.e., the pH optimum can be shifted to that region at which one wants to make a measurement, by choosing the right support for immobilization. Take an enzyme with a narrow pH range of, e.g., 6–8: this can be shifted on immobilization down to the acidic side, or conversely, up to the basic side. The enzymes are furthermore much more stable. In some work we did at Edgewood Arsenal, we actually heated our enzymes to 150°F and brought them back down to room temperature, with very little loss in activity. No soluble enzyme could be treated in this fashion (1).

One advantage that should be pointed out, which is often overlooked in the immobilized enzyme, is that better selectivity can be realized with the enzyme when immobilized: this insolubilized reagent becomes much more selective for an inhibitor, and only the most powerful inhibitor can actually

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attack the enzyme. We demonstrated this several years ago in an immobilized cholinesterase alarm for the assay of organophosphorus compounds in air and water. No other common interferants disturbed the alarm—it responded only to organophosphorus compounds (1).

The stability of the enzyme depends on the type of entrapment. Here again, there is a lot of ambiguous information in the literature in the reporting of immobilization data. Some individuals use a dry storage over a long period of time, and then report a fantastically long lifetime. One should realistically define the immobilization characteristics and the stability of the enzyme in terms of dry storage *and* use storage. The lifetime of most soluble enzymes, except perhaps in the case of some types of glucose oxidase, which are quite stable in the crude form, is generally about one week or 25–50 assays. However, one must realize that there are potential interferences that arise in the use of soluble enzymes, which are not found in the use of an entrapped enzyme. The physically entrapped enzyme lasts about 3–4 weeks, or 50–200 assays. For the chemically bound enzyme, 200–1000 assays is a good range (1). In many cases we, and others, have achieved at least this, although I must point out that there are many enzymes available, bound onto nylon tubes, for example, like the ones Technicon is coming out with for use on SMAC or the Auto Analyzer, the ones Boehringer has been experimenting with, or which Miles is selling under the trade name Catalink, which are very stable. These tubes have been demonstrated for ten thousand assays.

Several analytical techniques can be used to monitor immobilized enzyme catalyzed reactions for routine analysis, spectrophotometry, electrochemistry, and fluorescence, to name but a few (1). In this presentation, I shall cover our recent research in analysis, using the latter two techniques, together with immobilized enzymes, for the assay of amino acids and oxyanions, such as nitrite, nitrate, sulfate, triglycerides, and creatinine.

AMINO ACID ANALYSIS

The two key limiting amino acids in foods are L-lysine and L-methionine. A single, direct assay would allow a simple calculation of the protein quality of foods, instead of using the rat assay procedure.

Our interest, hence, in a food technology program, is to induce and isolate specific amino acid enzymes, which would act on only one of these key amino acids, like lysine, methionine, or tyrosine, and to use a base probe, which is a CO_2 or ammonia electrode, to measure the product of the enzymatic reaction. We have used phenylalanine ammonia lyase, isolated from Wisconsin potatoes. Following irradiation, the enzyme is isolated and immobilized for the very rapid assay of phenylalanine. The enzyme is totally

specific for L-phenylalanine. L-Arginine and L-lysine can be measured with an enzyme from *E. coli*, which has been purified to get the decarboxylase, and thus to obtain total specifity. Histidine and tyrosine were assayed using enzymes from various strains. To prepare the enzyme electrode, one simply smears a glutaraldehyde solution of the amino acid decarboxylase or lyase directly on the tip of a CO_2 or ammonia membrane. This is done in a very thin film, so that no problems with a slow return to baseline are obtained. Thus the amino acid electrode is simply a base CO_2 or ammonia sensor; on top of this CO_2 or NH_3 membrane is placed an enzyme layer. Typical response times are of the order of 1–3 min, and the return to baseline is about 10 min (2).

The main problem with any electrode is that if the enzyme layer is too thick, the return to baseline is slow. We, and others, have shown that one does not have to wait for the complete return to baseline. One can inject the next sample at 3/4 or even 2/3 of the time necessary for return to baseline; this is a matter of only a couple of minutes. However, one can do this only when measuring high substrate concentrations. If low concentrations are to be assayed, then, of course, one would have to wait for a complete return to baseline. Typical calibration curves are linear from 10^{-1} to 5×10^{-5} M for most of the amino acid electrodes; the C.V. is about 2.5%. The arginine electrode is completely specific for L-arginine. Of some 50-odd amino acids tested at first, only L-glutamate interfered. This is a native impurity (enzyme impurity) in the arginine decarboxylase, i.e., the glutamate decarboxylase, which we then separated out by affinity chromatography, making the arginine electrode totally specific. In the L-lysine electrode, arginine was an interference; this is because both enzymes are present in *E. coli*. But this impurity can be removed by purification of the enzyme. Histidine decarboxylase, because it comes from a different source, from which no other amino acid decarboxylase is found, is totally specific for histidine. Likewise, the L-tyrosine electrode produced is totally specific for this essential amino acid.

At the last US–USSR Conference (Tallin, 1977), we reported our success in developing L-lysine electrodes, used to determine the lysine content in standard mixtures containing 20 common amino acids, and also in flour samples spiked with lysine. Excellent results were obtained. After 50 days of use, an L-lysine electrode retained approximately 84% of its initial response after 60 days of use (2).

$$\text{L-lysine} \xrightarrow[\text{decarboxylase}]{\text{lysine}} CO_2 \qquad (1)$$

L-Methionine is one of the essential amino acids in our diet. However, it is also one of the deficient amino acids in most grains. Thus grains are

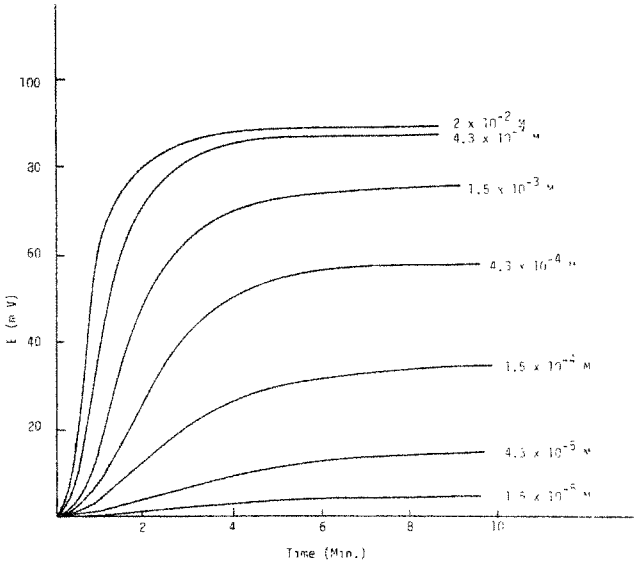

FIG. 1. The response curves of the electrode at different concentrations of methionine.

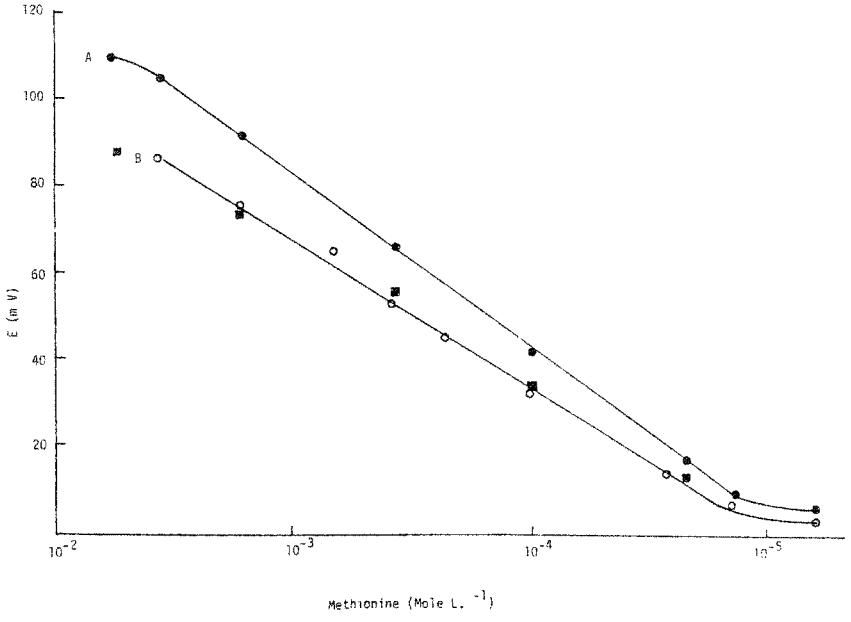

FIG. 2. Calibration curves for the methionine electrode at two different temperatures: $A = 26°C$; and $B = 39°C$. The circles and squares represent the two different electrodes.

routinely screened for L-methionine. A rapid, simple, and specific method for its determination will be useful.

A totally specific methionine sensor has been developed in our laboratory. The sensor was prepared by covalent coupling of the methionine-lyase directly onto an ammonia electrode. The ammonia generated by the enzymatic reaction was measured (3).

$$\text{L-methionine} + H_2O \xrightarrow[\text{(pH 8)+}\\ \text{P-5-P (coenzymes)}]{\text{phosphate buffer}} \text{ketobutyrate} + \text{methyl mercaptan} + \text{ammonia} \qquad (2)$$

The effect of the nature of the buffer solutions, pH, and temperature on the response of the electrode were studied. The response time of the electrode was about 2–3 min in the 10^{-3}–10^{-2} M region (Fig. 1), and the recovery time was about 10 min. The rate of the change of the electrode potential (mV/min) was linearly proportional to the concentration of methionine in the region of 10^{-5}–10^{-2} M as shown in Fig. 2. About 15 min was required for each analysis of a liquid sample.

The enzyme has been successfully purified to such a stage (Table 1 and Fig. 3) that none of the naturally occurring amino acids interfered, with the exception of glutamine, which causes a very slight interference (Table 2). Since glutamine is destroyed during acid hydrolysis of food protein, this

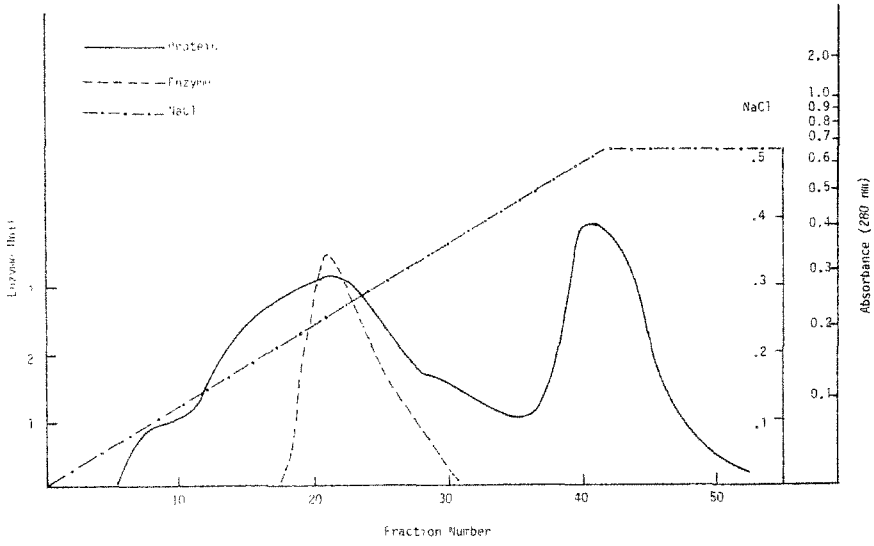

FIG. 3. Gradient column diagram of pooled fractions from Sephadex G-100 separated on DEAE cellulose.

TABLE 1. **Purification of Methionine Ammonia Lyase**

Fraction	Protein (mg/ml)	Specific activity	Fold purification	Yield activity
Crude extract	26.81	0.062	1	100
Heat treatment	4.15	0.13	7	76
$(NH_4)_2SO_4$ fractionation	3.06	0.68	11	50
Sephadex G-150 chromatography	1.09	1.56	25	39
DEAE cellulose	0.37	2.98	48	18

TABLE 2. **Substrate Specificity of the Final Preparation**

Substrate	Relative activity	
	Method 1 (Abs. at 480 nm)	Method 2 (ΔmV/min)
L-Methionine	0.345	21
D-Methionine	0	0
L-Asparagine	0.005	NM[a]
L-Glutamine	0.075	4
L-Threonine	0	0
L-Cysteine	0.002	NM
L-Cystine	0	0
L-Serine	0	0
L-Histidine	0	0
L-Alanine	0	0
L-Arginine	0	0
L-Aspartic acid	0	0
L-Glutamic acid	0	0
L-Glycine	0	0
L-Hydroxyproline	0	0
L-Isoleucine	0	0
L-Lysine	0	0
L-Phenylalanine	0	0
L-Proline	0	0
L-Tryptophan	0	0
L-Tyrosine	0	0
L-Valine	0	0

[a]NM = not measurable.

enzyme, after immobilization on an electrode, can be used to assay the total methionine in food samples without any interferences (4).

ASSAY OF OXYANIONS

Attempts have been made to develop specific, ultrasensitive methods for the assay of oxyanions, such as nitrate, nitrite, and sulfate, using microbial enzymes that catalyze simple one-step reductions:

$$NO_3^- + NADH_2 \xrightarrow[vulgaris]{Chlorella} NO_2^- + NAD + H_2O \quad (3)$$

$$NO_2^- + NADH_2 \xrightarrow[chroococcum]{Azotobacter} NH_3 + H_2O + NAD \quad (4)$$

$$SO_4^{2-} + 4NADH_2 \xrightarrow{Desulvibrio} S^{2-} + 4NAD + 4H_2O \quad (5)$$

Thus the progress of the reaction can be followed by noting the change in absorbance or fluorescence of $NADH_2$, or the change in diffusion current as $NADH_2$ (electroactively oxidized at a Pt or C electrode) is converted to NAD. Thus simple assay procedures could be developed.

In purification of the enzyme from *Chlorella vulgaris* for nitrate assay, the one-step affinity chromatography procedure previously employed for the separation of NO_3^- reductase from *C. vulgaris* was found to be impractical and expensive, since the price of the commercial Sepharose cross-linked blue dextran is still very high, and the enzyme thus purified is very unstable, probably due to the loss of a cofactor that is required for the stability of the enzyme. Conventional methods have been tried, and it was found that the only interferer, NADH oxidase, in the crude extract could be separated by ammonium sulfate fractionation and gel filtration on Sephacryl 200 (Table 3). The preparation is rather pure and much more stable, and it has been used to assay nitrate content in drinking water as well as in food by monitoring the NADH change fluorometrically. From 10^{-4} to 10^{-5} *M* nitrate can be assayed specifically in the presence of most other anions (Fig. 4).

A nitrite reductase, which reduces nitrite to form ammonia in the presence of $NADH_2$, has been isolated from *Azotobacter chroococcum*. The purification scheme used to separate the isolated enzyme is described in Table 4. Some of the characteristics of the isolated and purified enzyme are shown in Table 5. The partially purified preparation has been immobilized directly onto an ammonia gas membrane electrode for the measurement of free NO_2^- in water and food. The electrode responds fairly well at high

TABLE 3. **Purification of Nitrate Reductase Using Sephacryl S-200**

Fraction no.	ΔF/min[a]	Blank
2	4	
3	4	
4	7.5	
5	16	3
6	23	3.5
7	20	2.6
8	15	
9	11	
10	6.5	
11	5	
12	4	

[a] ΔF/min is a measure of the activity of nitrate reductase.

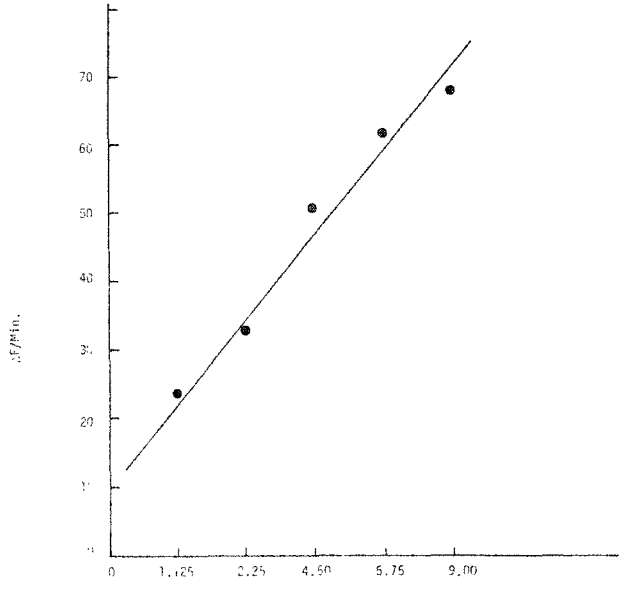

FIG. 4. Calibration curve (sonified enz.).

TABLE 4. **Purification of Nitrate Reductase**

1. High speed centrifugation: 40,000 *g* at 0°C for 1 h
2. Ammonium sulfate fractionation: 0–40% saturation, five-fold purification
3. Sephadex G-25 or Millipore concentrator, to remove ammonium salt
4. Attempted:
   a. Column chromatography: DEAE cellulose; Chelex-100
   b. Gel filtration: Sephadex G-100; Sephadex G-150; Sephacryl S-200;
   c. Affinity chromatography: Sepharose 4B linked blue dextran, *p*-nitroaniline linked to cellulose beads

NO_2^- concentrations, and the response is very specific to NO_2^-. The presence of SO_3^-, SO_4^-, ClO_3^-, ClO_4^-, NO_3^-, and oxalate anions in the mixture does not interfere in the assay at all. However, at nitrite concentrations lower than 10^{-4} *M*, the response is slow, due mostly to the sensitivity of the gas membrane electrode, but partly to the low specific activity of the nitrite reductase used. Efforts are now being made to improve both the sensitivity of the electrode and the specific activity of the enzyme, as well as to develop fluorometric procedures for the assay of nitrite, following the decrease in the $NADH_2$ ($\lambda_{ex} = 365$ nm, $\lambda_{em} = 450$ nm).

TABLE 5. **Characteristics of Nitrite Reductase**

1. pH optimum
   NO_2^- reductase, 8.0
   NADH oxidase, 7.0
2. Stability
   NO_2^- reductase very unstable—lost all activity: < 6 h at room temp.; ~ 1 day at 4°C; ~ 2 day at −20°C; ~ 5 min at 60°C
   Stabilizers
   2 m*M* each: dithiothreitol + glutathione
   No loss in one month at −20°C
   Slight loss at 4°C
   5 m*M* 2-mercaptoethanol
   No significant loss in a week at −20°C
   Half activity lost in 2 weeks
3. K_m and linear range
   $K_m = 3.4 \times 10^{-5}$ *M* ($NaNO_2$)
   Linear range: 3.3×10^{-5}–3.3×10^{-6} *M* NO_2^-
4. Inhibitors
   NO_2^- and NADH inhibition observed
   N_3^- inhibits oxidase, not reductase
   Cu^{2+} inhibits both; Ca^{2+} no effect
5. Specificity study
   No interference: NO_3^-, SO_4^{2-}, SO_3^{2-}, ClO_4^-, ClO_3^-, Pi, Cl^-, oxalate

A sulfate reducing strain was recently isolated from the muds collected from different areas in the Gulf of Mexico. The microorganism could be grown in a slightly modified Starkey's medium, and the crude extract enzyme was found to actively reduce sulfate to sulfide. Attempts are now being made to increase the population of the microorganism and to eliminate the formation of black sulfide precipitate during fermentation. Crude enzyme extract has been used, attempting to develop a colorimetric method for sensitive and specific assay of SO_4^{2-} in solution. Immobilization of the crude extract on the surface of a sulfide electrode is being tried as well.

CREATININE

Determination of serum creatinine is now one of the most important screening tests for evaluating renal function. It has the advantage over the urea determination in evaluating kidney diseases, since it is not affected by high protein diet.

The spectrophotometric methods widely used for routine creatinine determination are based on the Jaffe reaction between creatinine and picric acid in alkaline solution. These methods are rather time consuming and require deproteinization and incubation to develop color, while their most serious defect is nonspecific interferences due to the presence of many other materials in the serum that give a positive Jaffe reaction.

After the successful isolation of a pure creatininase (E.C.3.5.4.2) from *Pseudomonas* by Szulmajster (5), a highly purified enzyme which hydrolyzes creatinine to *N*-methyl hydantoin and ammonia has been introduced by Beckman for enzymatic determination of creatinine. This enzyme has been extensively purified (Fig. 5) by DEAE chromatography, so that only creatininase activity remains.

Because of the higher sensitivity of fluorometric methods, the use of the following coupled enzymatic reactions for fluorometric determination of serum creatinine was investigated:

$$\text{creatinine} + H_2O \xrightarrow[\text{(E.C.3.5.4.2)}]{\text{creatininase}} NH_4^+ + N\text{-methyhydantoin} \tag{6}$$

$$NADH + NH_4^+ + \alpha\text{-ketoglutarate} \xrightarrow[\substack{\text{dehydrogenase}\\ \text{(E.C.1.4.1.3)}}]{\text{glutamate}} NAD^+ + \text{glutamate} + H_2O \tag{7}$$

Owing to its simplicity, speed, and economy, a solid-surface method was employed in an attempt to develop a specific semisolid surface fluorometric pad method for routine blood creatinine analysis. All of the

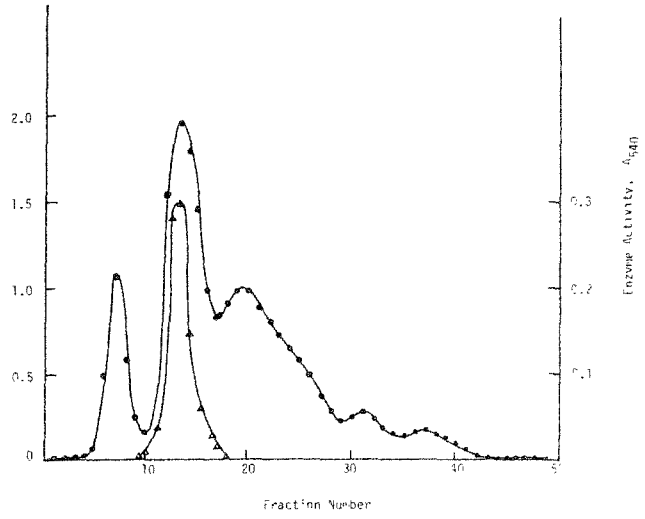

FIG. 5. Purification of creatininase by DEAE chromatography. Enzyme pooled: fractions 10–19.

reagents for this assay are applied on the surface of a silicone-rubber pad; only 45 μl of reagent is necessary with a total sample volume of 85 μl.

The rate of disappearance of NADH fluorescence at 460 nm (excitation wavelength, 340 nm) is monitored and is proportional to serum creatinine concentration. The whole assay uses only 0.41 U of creatininase and takes less than 5 min. The calibration curve is linear from 0 to 8.2 mg creatinine per 100 ml. Thus the method offers a rapid, simple, and inexpensive means for creatinine assay, and the results obtained correlated well with the modified Jaffe method analyzed on a automatic Technicon SMA 12/60 (correlation coefficient 0.998) with a recovery of 97–100% (6).

An immobilized creatinine enzyme electrode was prepared by covalent coupling directly onto the NH_3 sensor by a procedure similar to the work done by Anfalt et al. Up to now, the electrode has been used for 8 months with 200 assays; no significant activity loss has been detected (8). This immobilized enzyme electrode can tolerate temperatures up to 40°C (loss of 15% activity for 50 assays); the rate obtained at 40°C is twice as large as that at room temperature (25.2°C). The calibration curve is linear from 0 to 5 mg%, with a rate change of 12 mV/2 min/2.4×10^{-5} *M*.

The free ammonia in serum is the only interference for this method. After pretreatment with Dowex 50×8 or an enzymatic process, serum creatinine levels above 15 mg/l can be successfully detected using an

electrode. Finally, we immobilized creatininase on alkylamine porous glass beads with the use of glutaldehyde as cross-linked reagent.

A small stirring bar loaded with immobilized enzyme beads was used for fluorometric determination of serum creatinine. The stirring bar has an activity of 0.6 U at 22 cps and 25°C, and was found to be stable for 6 months with more than 150 assays. A larger stirring bar is now being designed for enzymatic measurement of creatinine using an ammonia electrode as sensor. A stirring with an activity 1.5 U per stirring bar was developed. This approach can eliminate the free ammonia interference, thus having the advantage over the creatinine specific enzyme electrode. However, this method is less sensitive to the same concentration of substrate.

TRIGLYCERIDES

Triglycerides, together with phosphatides and cholesterol, constitute the most important lipid fraction of blood. Adipose tissues have the largest triglyceride content, 60–85%. Triglycerides are esters of fatty acids with glycerol.

The determination of triglycerides in plasma or serum is of interest in following the course of biliary obstruction, diabetes mellitus, or nephrosis. The level in serum depends on the age of the individual, ranging from 10–140 mg/dl at ages 50–59 years (9). Elevations in serum lipids, as triglycerides, can be correlated with an increased risk of coronary artery diseases and arteriosclerosis.

The present methods available in determining the triglyceride content involves the following steps:

$$\begin{array}{l} CH_2{-}O{-}COR \\ | \\ HC{-}O{-}COR + 2H_2O \\ | \\ H_2C{-}O{-}COR \end{array} \xrightarrow[\text{or enzymes}]{\text{alkali, 70°C}} \begin{array}{l} H_2COH \\ / \\ HCOH + \text{F.F.A.} \\ \backslash \\ H_2COH \end{array} \qquad (8)$$

$$\text{glycerol} + \text{ATP} \overset{GK}{\rightleftharpoons} \text{glycerol-3-P} + \text{ADP} \qquad (9)$$

$$\text{ADP} + \text{PEP} \overset{PK}{\rightleftharpoons} \text{ATP} + \text{pyruvate} \qquad (10)$$

$$\text{pyruvate} + \text{NADH} + H^+ \overset{LDH}{\rightleftharpoons} \text{lactate} + \text{NAD} \qquad (11)$$

The NADH is followed by the decrease in absorbance at 340 nm, of fluorescence at 365 and 460 nm.

The major problem with this assay is that it is a four-step procedure, requiring four enzymes and three cofactors. This is expensive, requires

considerable reagent preparation, and is subject to "bugs" that are difficult to trace (i.e., which enzyme or cofactor has gone bad if the sequence does not work).

A simple approach for the assay of triglycerides will be use of bacterial lipase (either from *Rhizopus arhizus* or from *Chromobacterium viscusum*) and *glycerol dehydrogenase*, which catalyze the reactions

$$\text{triglycerides} \xrightarrow{\text{lipase}} \text{glycerol} + \text{FFA} \tag{12}$$

$$\text{glycerol} + \text{NAD} \xrightarrow[\text{dehydrogenase}]{\text{glycerol}} \text{NADH} + \text{DHA} + \text{H}^+ \tag{13}$$

The NADH produced is measured either electrochemically, colorimetrically, or fluorometrically (10).

Electrochemically (amperometrically):

$$\text{NADH} + \text{Fe(CN)}_6^{3-} \xrightarrow[\text{diaphorase}]{} \text{NAD} + \text{Fe(CN)}_6^{4-} + \text{H}^+ \tag{14}$$

The ferrocyanide produced is oxidized at a Pt electrode, using a constant voltage of +0.35 V.

Fluorometrically:

$$\text{NADH} + \text{resazurin} \rightarrow \text{resorufin} \; (\lambda_{ex} = 540, \lambda_{em} = 580 \text{ nm}) \tag{15}$$

Colorimetrically:

$$\text{NADH} + \text{INT} \xrightarrow[\text{diaphorase}]{} \text{formazan} + \text{NAD} \tag{16}$$

The absorbance of formazan is monitored from INT (iodonitrotetrazolium) at 500 nm.

The results obtained for both the colorimetric and amperometric methods show a good linearity up to 400 mg% of triglycerides, with excellent precision and correlation to standard clinical procedures. The data can be obtained either using a rate method or a steady state method.

Fluorometric methods are likewise being worked out. No major or significant interference is observed from substances such as ethanol, bilirubin, glucose, uric acid, or ascorbic acid, even when present in abnormal concentrations.

ACKNOWLEDGMENTS

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