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The Use of an Immobilized Enzyme Nylon Tube Reactor Incorporating a Four Enzyme System for Creatinine Analysis

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Abstract

A single immobilized enzyme nylon tube reactor was produced incorporating a four enzyme system for the analysis of creatinine.

The enzyme activity ratios in the coupling solution used to prepare the reactor were found to be of extreme importance in governing the activity of the latter.

The reactor was incorporated into a continuous flow analysis system used to assay creatinine in urine samples and the results were correlated with a manual technique employing the same enzyme system in solution.

The precision, correlation, high specificity, simplicity, and speed of the analysis were concluded to be factors in favor of the method's suitability for urine creatinine determinations.

Index Entries: Immobilized enzyme reactor, for creatinine analysis; reactor, creatinine analysis by immobilized enzyme; nylon tube reactor, immobilized enzyme; enzyme reactor, creatinine analysis using immobilized; creatinine analysis, immobilized enzyme reactor for.

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Introduction

Urinary excretion of creatinine is fairly constant in a given individual (1–2 g/24 h), being independent of diet and representative of the total active muscle mass. In addition to its usefulness as an index of chronic failure of renal excretory function, measurement of creatinine excretion affords a useful check on whether any substantial fraction of a 24-h urine collection has been inadvertently discarded; furthermore, determinations of the excretory ratio of another compound under investigation to that of creatinine is often considered advantageous.

Many methods are available for creatinine analysis, the most widely used analytical procedures being based on the Jaffé reaction (*1*). The disadvantage of this procedure is that it lacks specificity and is subject to interference by many biological and pharmacological compounds (*2–5*). Modifications of the Jaffé method have been devised in an attempt to reduce interference, e.g., kinetic Jaffé assays (*6, 7*), the shortcomings of which have recently been reviewed (*8, 9*), and alternative methods have been developed, such as high performance liquid chromatography (*10, 11*), and coupled enzymatic assays (*12, 13*). Enzymatic methods improve the specificity of the assay, but until recently have been laborious, usually employing manual techniques.

Jaynes et al. (*14*) described an adaptation of the Boehringer Mannheim Diagnostics coupled-enzymatic creatinine assay (Creatinine Enzymatic UV-Method) to a centrifugal analyzer. Sundaram and Igloi (*15*) described a continuous flow method of analysis using the same enzyme system immobilized onto a series of nylon tube reactors.

The concept of coiled nylon tubular reactors containing enzymes immobilized on their inner surfaces was first introduced by Sundaram and Hornby (*16*). Subsequently many methods of analysis have been developed incorporating immobilized enzyme systems; these are less costly than the same enzymatic methods in solution since the enzyme is not lost during the analysis, and often the enzyme shows increased stability on immobilization.

The method described for creatinine analysis by Sundaram and Igloi (*15*) used nylon tube reactors with some or all of the enzymes immobilized, the remaining enzymes being mixed with the substrate solution. Where all the enzymes were immobilized, creatinine amidohydrolase (C) and creatine kinase (CK) were coimmobilized and pyruvate kinase (PK) and lactate dehydrogenase (LDH) were coimmobilized. A single reactor with all four enzymes coimmobilized was not used, and a study of the kinetics of the system only was made, no clinical results being given.

The method described here is a continuous flow technique utilizing the reaction scheme shown in Fig. 1, with all four enzymes coimmobilized in a single 1-meter nylon tube. The sample, containing creatinine, is mixed with the substrate solution in a single reagent stream that is allowed to flow through the enzyme reactor. Creatinine is quantitated by nicotinamide adenine dinucleotide (NADH) consumption with a corresponding decrease in absorption of the substrate solution at 340 nm, determined spectrophotometrically. The method is simple, specific, and quick, analyzing 60 samples/h with a throughput time of approximately 5 min.

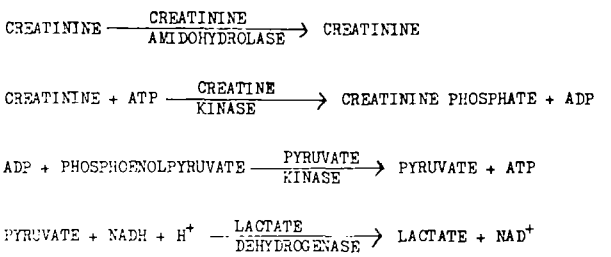

Fig. 1. Reaction scheme for creatinine analysis.

Materials and Methods

Instrumentation

1. A Technicon AA *II* System (Technicon Instruments Co. Ltd., Hamilton Close, Houndmills, Basingstoke, UK) was used for the continuous flow analysis of creatinine.

2. A Pye Unicam SP800 Spectrophotometer (Pye Unicam Ltd., York Street, Cambridge, UK) was used for making absorbance measurements in determining the activity of the nylon tube reactors, and for making absorbance measurements in the estimation of creatinine by the manual enzymatic assay.

Reagents

Chemicals were analytical grade where available, obtained from British Drug Houses Ltd., Poole, Dorset, UK, except where stated.

(a) Preparation of the Nylon Tube Reactors.

1. Nylon (type 6) tubing, 1 mm internal diameter (Portex Ltd., Hythe, Kent, UK).

2. Triethyloxonium tetrafluoroborate, 10% w/v in dichloromethane (*17*).

3. Adipic acid dihydrazide (Sigma Chemical Co. Ltd., Fancy Road, Poole, Dorset, UK), 4% w/v in formamide.

4. Diaminoethane.

(b) Covalent Attachment of the Enzymes.

1. Phosphate buffer (0.05 mol/L, pH 8.0). A volume of 250 ml of 0.2 mol/L KH_2PO_4 were added to 234 mL of 0.2 mol/L NaOH and the volume made up to 1 L with deionized water.

2. All enzymes were supplied by Sigma Ltd. The following preparations were made in phosphate buffer:

(i) Creatinine amidohydrolase (C), 500 U/mL. The solution of the enzyme as supplied in glycerol was concentrated using a Minicon-B15 concentrator (Amicon

Ltd., Amicon House, 2 Kingsway, Woking, Surrey, UK) and the enzyme redissolved in 1 mL of phosphate buffer.

(ii) Creatine kinase (CK), 500 U/mL.

(iii) Pyruvate kinase (PK), 1500 U/mL.

(iv) Lactate dehydrogenase (LDH), 1500 U/mL.

3. Borate buffer (0.2 mol/L, pH 8.5). 0.2 mol/L boric acid was titrated to pH 8.5 with 2 mol/L NaOH.

4. Glutaraldehyde, 25% (w/v), electron microscopy grade. A 5% solution in borate buffer was prepared immediately before use.

5. Potassium borohydride, 1% (w/v) in borate buffer was prepared immediately before use.

(c) Assay of Creatinine

1. Glycylglycine buffer (0.1 mol/L, pH 8.3). A 6.6 g quantity of glycylglycine (Sigma Ltd.,) was added to 288 mL of 0.1 mol/L NaOH and the volume made up to 100 mL with deionized water. The solution was stored at +4°C.

2. Working "substrate" solution, prepared in glycylglycine buffer. Stock solutions, where used, were prepared in glycylglycine buffer, unless stated.

Starts solution	Working concentration, mmol/L
Magnesium chloride, 1 mol/L (aqueous)	0.14
β-NADH (Sigma), 12 mmol/L	0.34
ATP (Sigma), 20 mmol/L	1.13
Phosphenolpyruvate (Sigma), 22 mmol/L	1.24
EDTA disodium salt	5
Potassium sulfate	11.4
Dithioerythriotol (Sigma)	0.85
Creatinine, 10 mmol/L (Aqueous)	1.41

The creatinine was omitted from the reagent when used in the continuous flow system.

3. Boehringer Enzymatic UV method for creatinine analysis (cat. no. 166413, obtained from the Boehringer Corporation Ltd., Bell Lane, Lewes, Sussex, UK)

4. Creatinine Standards. A range of standards, 100–1000 μmol/L were prepared by making dilutions of a 10 mmol/L standard in deionized water.

Preparation of the Immobilized Enzyme Nylon Tube Reactors

Coiled tubing 1 or 2 m in length, was *o*-alkylated, using the method described by Morris et al. (*18*), by filling it with a 10% solution of triethyloxonium tetrafluorotorate (TOTFB). The nylon tubes were incubated for 15 min at +25°C and then washed with dichloromethane. Acid hydrazide and amine-substituted nylon derivatives were prepared by filling the *o*-alkylated tubes with a solution of 4% adipic acid dihydrazide in formamide or diaminoethane, respectively, and incubating the tubes for 90 min at room temperature. The nylon tubes were then washed for 20 h with deionized water, using a flow rate of 1 mL/min.

The enzymes were coupled to the nylon by crosslinking with glutaraldehyde. The tubes were filled with a solution of 5% glutaraldehyde in 0.2*M* borate buffer, pH 8.5, and incubated at room temperature for 15 min. After washing for 5 min with 0.2*M* borate buffer, pH 8.5, the tubes were filled with the enzyme solution that consisted of the four enzymes in a 0.05*M* phosphate buffer, pH 8, and incubated overnight at +4°C.

After the enzyme coupling stage, the reactors were washed with deionized water for 5 min and then perfused with a 1% (w/v) solution of potassium borohydride in borate buffer for approximately 60 min in order to stabilize the covalent attachment of the enzymes by the reduction of the double bond of the Schiff's base (*19*). The tubes were then finally washed with deionized water for approximately 30 min.

Use of the Reactors for Creatinine Analysis

The manifold was prepared according to the flow diagram shown in Fig. 2. The combined enzyme reactor was inserted, the reagent pumped, and the baseline adjusted on the recorder chart. Aqueous creatinine standards were sampled, followed by urine samples and controls in random order, diluted 1 in 30 with deionized water. After the run, the system was washed through with deionized water for 10 min, the enzyme reactor removed, and the N9 nipple replaced before lifting the platen. The enzyme reactor was stored at +4°C after filling with phosphate buffer containing 10 mmol/L EDTA.

Results

Initial Activity of the Reactors Using Different Enzyme Ratios in the Coupling Solution, with Amine and Hydrazide-Substituted Nylon Derivatives

The initial activity of the reactors was determined in terms of μmol NAD^+ formed/min/m by measuring the absorbance at 340 nm of the substrate (containing creatinine) before and after being perfused through the reactors at a rate of 0.32 mL/min. Table 1 shows the activities obtained using reactors prepared from both acid hydrazide- and amine-substituted nylon derivatives, with different enzyme ratios in the coupling solution. The amount of protein bound was determined by measuring the absorbance of the enzyme coupling solution at 280 nm before and after the enzyme coupling stage.

From Table 1, it can be seen that reactors of greatest activity can be obtained using enzyme ratios of C = 1:CK = 1:PK = 5:LDH = 5, from acid hydrazide nylon derivatives, although the amine derivatives appear to bind more protein.

Optimization of the Assay

The assay was optimized in terms of pH and substrate concentrations, and the results are shown in Fig. 3a, 3b, 3c, and 3d. It was felt that the use of higher NADH concentrations would be unnecessary since adequate sensitivity was obtained.

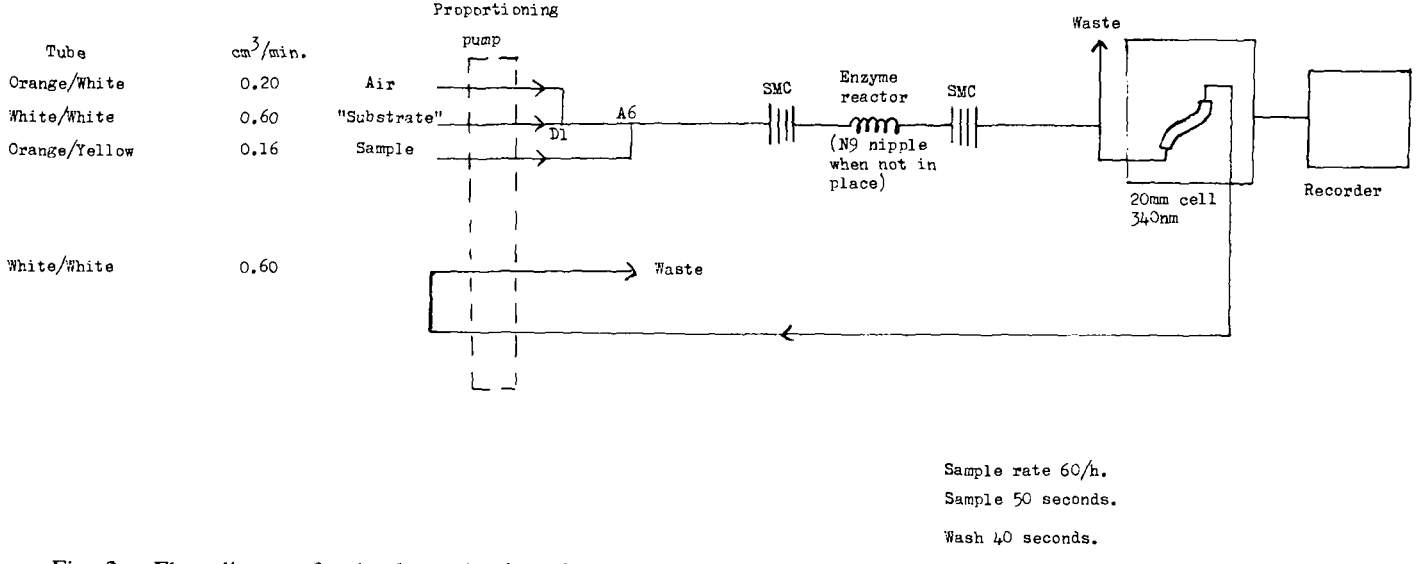

Fig. 2. Flow diagram for the determination of creatinine using a combined C/CK/PK/LDH nylon tube reactor. Air tube: 0.20 cm^3/min; "substrate" tube, 0.60 cm^3/min; sample tube, 0.16 cm^3/min. The sample rate was 60/h, with each sample requiring 50 s, and each wash step 10 s.

Table 1
Initial Activities of Immobilized Enzyme Nylon Tube Reactors Prepared Using Different Enzyme Ratios in the Coupling Solution, from Acid Hydrazide- and Amine-Substituted Nylon Derivatives

Nylon derivative[a]	Enzyme activity ratio in coupling solution, C:CK:PK:LDH,	Total protein content of coupling solution, mg/mL	Protein bound, mg/m	Initial activity, μmol NADH consumed/min/m
AH	1:1:5:5	2.42	0.52	0.041
AH	2:1:1:1	1.77	0.53	0.027
AH	1:1:5:6	3.35	—	0.024
AH	1:1:7:7	2.17	—	0.016
AH	1:1:4:4	3.37	—	0.033
DAE	1:1:5:5	2.42	0.92	0.033
DAE	1:2:3:4	1.91	0.92	0.033
DAE	3:2:5:4	3.35	—	0.031

[a]AH = acid hydrazide-substituted nylon; DAE = amine-substituted nylon.

Use of the Reactors in Continuous Flow Analysis of Creatinine

A typical recorder tracing obtained using a 1 m hydrazide-substituted nylon tube reactor is illustrated in Fig. 4, showing aqueous standard peaks and diluted urine sample peaks, assayed using the system shown in Fig. 2.

Linearity

The linearity of the method is shown in Fig. 5, in which peak height (NADH consumption) is plotted against creatinine concentration in μmol/L. Linearity exists from 100 to 1000 μmol/L. This good response range should enable creatinine levels to be determined readily and with confidence. The origin offset is probably caused by diffusion effects [Sundaram (*15*)], and by NADH binding (*19*). A curvature of the Lineweaver-Burke plot is also shown, and has been attributed by Sundaram (*15*) to diffusion effects.

Recovery

The analytical recovery of the method was determined by adding accurately measured quantities of 10 mmol/L creatinine standard to urine samples that had previously been assayed. These samples were then reassayed and the recoveries were found to range from 98 to 125%. The results are shown in Table 2. The value of 125% probably results from experimental error since single tests only were used and any small errors are magnified by the 1 in 30 dilution employed.

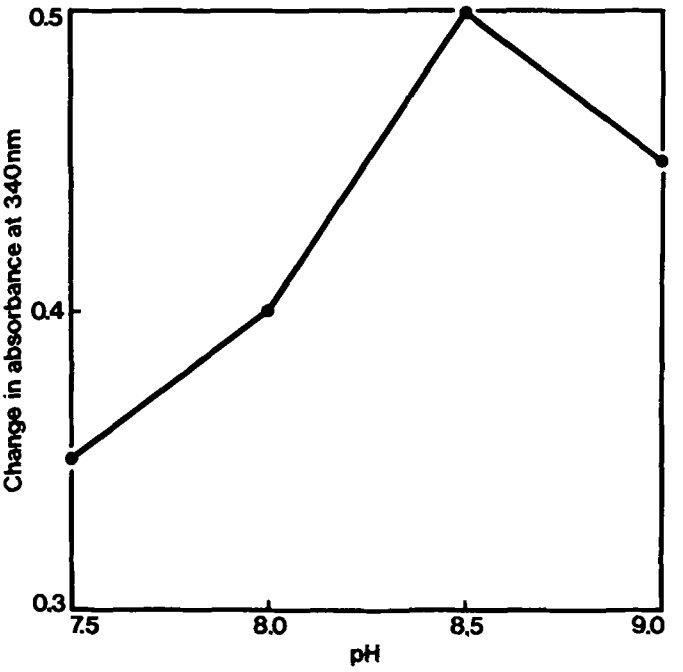

Fig. 3a

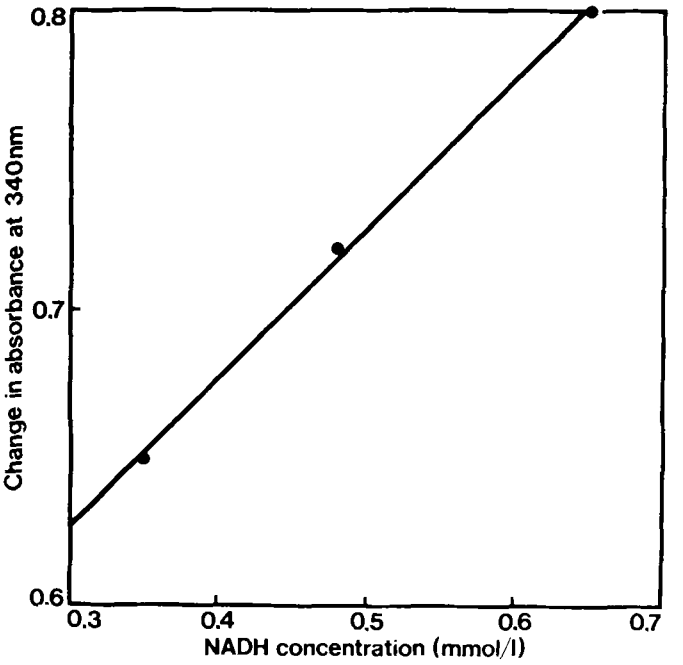

Fig. 3b

Fig. 3a–d. Effect of pH, NADH, ATP, and PEP concentrations on the assay of creatinine using immobilized C/CK/PK/LDH.

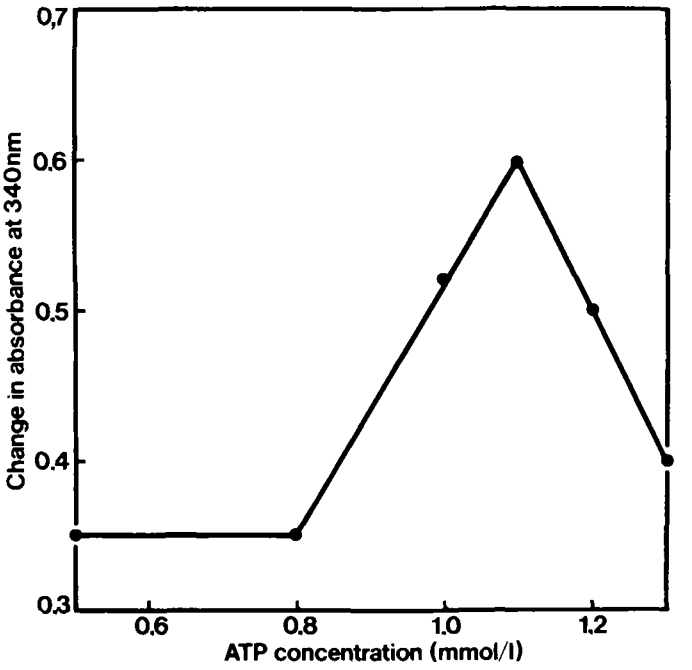
0,7
0.6
0.5
0.4
0.3
Change in absorbance at 340nm
0.6
0.8
1.0
1.2
ATP concentration (mmol/l)

Fig. 3c

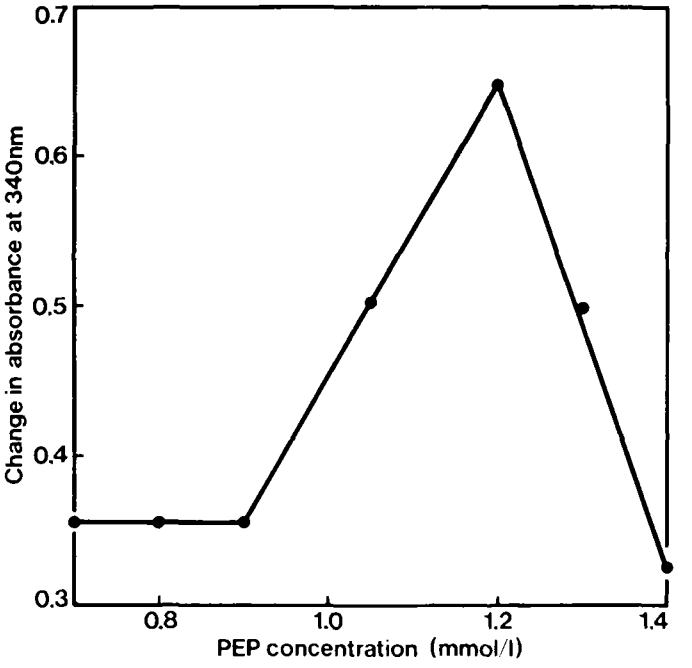
0.7
0.6
0.5
0.4
0.3
Change in absorbance at 340nm
0.8
1.0
1.2
1.4
PEP concentration (mmol/l)

Fig. 3d

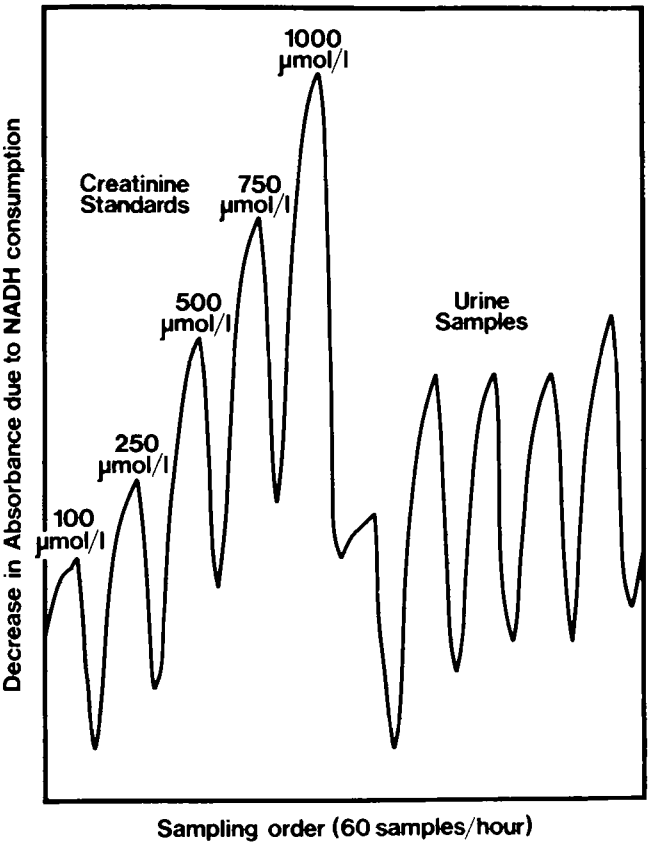

Fig. 4. Recorder tracing obtained using the immobilized enzyme method for creatinine analysis.

Sample Interaction

The carry-over coefficient was calculated according to method of Broughton et al. (*20*). When using three high standards (1000 µmol/L) followed by three low standards (100 µmol/L), the carry-over coefficient was found to be 0.56%.

Precision and Accuracy

Within-run precision was determined at three levels using samples with low, intermediate, and high creatinine concentrations. The results are shown in Table 3.

Between-batch precision and accuracy were determined using a commercially supplied quality control urine. Table 4 summarizes the results. The between-batch coefficient of variation was found to be 3.69% and the urine sample used was within the supplier's stated assayed range.

Comparison Studies

A number of patients' urine samples were assayed for creatinine by the immobilized enzymatic method and the Boehringer enzymatic kit method, which employs

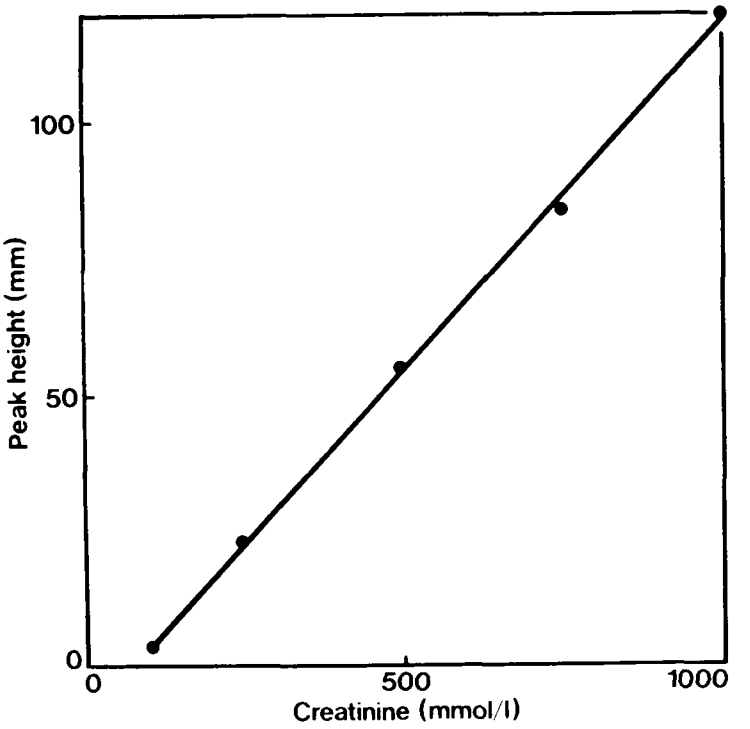

Fig. 5. Graph of peak height vs concentration of aqueous creatinine standards.

Table 2
Analytical Recovery of Added Creatinine from Urine[a] Using the Immobilized Enzyme Method

Creatinine value of urine with no added creatinine, μmol/L	Creatinine value with 0.05 cm^3 of 10 mM creatinine added to a 2 cm^3 aliquot, μmol/L	Expected reading, μmol/L	Recovery, %
3300	6000	4800	125
6300	7800	7800	100
4200	6450	6600	98

[a]All urines were diluted 1 in 30.

Table 3
Within-Run Precision of the Method, Performed by Analysis of Creatinine Samples with Low, Intermediate, and High Creatinine Concentrations

	Low	Intermediate	High
Mean, μmol/L	152.5	371	748
SD	4.25	6.15	5.73
CV, %	2.78	1.66	0.77
n	10	10	10

Table 4
Between-Batch Precision and Accuracy of the Method

Mean, μmol/L	9412
SD	347.1
CV, %	3.69
n	10
Assay range	8000–11,000

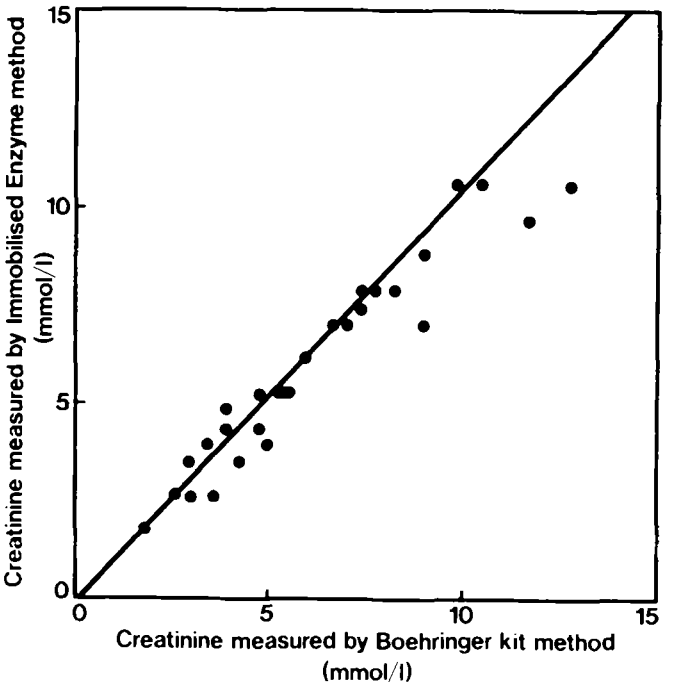

Fig. 6. Correlation and regression line for urinary creatinine by the immobilized enzyme method against urinary creatinine by the Boehringer kit enzymatic method, by method of linear least squares.

the same series of reactions, but is a manual assay in solution requiring sample blanks. The correlation and regression line is shown in Fig. 6.

Discussion

This paper describes a simple, specific, and quick automated method for the determination of creatinine. Reaction takes place readily at room temperature and is complete after the passage of the "substrate" through the nylon tube reactor. This is more efficient than assay in solution, which is laborious, requiring sample blanks and a 30-min incubation time.

The reactors have been found to be stable for approximately 3 weeks when in continuous usage. Ideally, greater stability is desirable and this requires further investigation, but the production of the reactors is not too costly if they are produced as required, storing the previously *o*-alkylated nylon tubes at +4°C and the enzyme coupling solution at −20°C. Lactate dehydrogenase and pyruvate kinase appear to be the first enzymes to deteriorate, creatinine amidohydrolase and creatine kinase being more stable. It is essential to include quality control samples in the run since these will readily show when the reactor begins to lose its activity. Dilutions less than 1 in 20 should not be used because of the presence of inhibitors to lactate dehydrogenase in urine.

There is a diffusion layer that occurs at the interface of the nylon-bound enzyme and the reaction mixture (*21*); this affects the ease of transport of substrate to the active site of the enzymes and thereby establishes a concentration gradient. The presence of this diffusion layer could account for the reduced sensitivity observed at low creatinine concentration. With the amine-substituted nylon derivatives, the higher concentration of bound protein probably causes an increase in thickness of the diffusion layer, making the enzyme appear to be less active. Similar observations have been made by Noy (*19*). In addition, slight carry-over effects are observed with the system, the negatively charged NADH molecules being attracted to the cationic nylon support. Both these effects are reduced, but not entirely eliminated, by increasing the ionic strength of the substrate medium; in this work, this is achieved by the addition of potassium sulfate. Thus it is advisable to sample one or two standards after every 10 patients' samples, with a range of standards at the beginning and end of the run. The actual carry-over coefficient obtained is well within the limits of acceptability suggested by Tonks (*22*).

From the above factors and from the precision, regression line, and correlation obtained with the Boehringer kit method, it was felt that the immobilized enzymatic method would be suitable for the analysis of creatinine in urine.

The use of the immobilized enzymatic method for the analysis of creatinine in serum has proved to be difficult because of the much lower concentration of creatinine in these samples. When dialysis is employed to remove the protein, a difference in dialysis rate is observed between serum and aqueous solutions; also there is a loss in the sensitivity. Further work is currently being carried out in order that the method may be adopted for use with serum samples.

Acknowledgments

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