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# Nylon Tube-Immobilized Creatinine Iminohydrolase and Glutamate Dehydrogenase in Serum and Urine Creatinine Analysis

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## ABSTRACT

Immobilized enzyme nylon-tube reactors incorporating creatinine iminohyrolase (CI) and glutamate dehydrogenase (GDH) were used to assay creatinine in serum and urine.

Optimum substrate concentrations for the assay were determined.

The reactors were incorporated into a continuous flow system for creatinine analysis. The method was evaluated with respect to linearity, sample interaction, precision, accuracy, and analytical recovery. Comparison studies were carried out with a standard Jaffé method and the effect of interfering substances was investigated.

From the results obtained, it was concluded that the assay was suitable as a simple, reliable, and specific method for serum and urine creatinine determinations.

**Index Entries:** Nylon tube, immobilized enzymes for creatinine analysis on; immobilized enzymes, for creatinine analysis; enzymes, immobilized for creatinine analysis; creatinine, analysis by nylon tube-

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immobilized enzymes; creatinine iminohydrolase, immobilized for creatinine analysis; glutamate dehydrogenase, immobilized for creatinine analysis; serum, creatinine analsyis in; urine, creatinine analysis in.

## INTRODUCTION

The methods most commonly used in clinical laboratories to measure creatinine are based on the Jaffé reaction (*1*). The difficulties of accurately quantifying serum creatinine have been described in a recent review (*2*), in which the authors noted the inaccuracy of both the conventional Jaffé reaction and the modifications designed to improve its specificity. Enzymic methods for the assay of creatinine (*2, 3–11*) involve the use of either the amidohydrolase to convert creatinine to creatine (*3–5*), or the iminohydrolase to convert creatinine to *N*-methylhydantoin and ammonia (*6–11*).

The method described here is an automated, continuous flow technique based on the reaction proposed by Lim (*11*), illustrated in Fig. 1. A similar method of assay has been described by Tanganelli et al. (*8*); endogenous ammonia in serum and urine was removed by pre-incubation of the sample with a solution containing GDH, and creatinine was quantitated by measuring the ammonia produced as a result of subsequent reaction with immobilized CI via a Berthelot reaction. In the proposed method, *both* CI and GDH are immobilized by covalent attachment to the inner surface of nylon tubing.

The concept of immobilized-enzyme nylon tubular reactors was initially introduced by Sundaram and Hornby (*12*). More recently, a comprehensive review on immobilized enzyme tubular reactors in continuous automated analysis has been presented by Pedersen and Horvath (*13*). Immobilized-enzyme analytical systems 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. In addition to these advantages, the method described here is simple and specific. The sample containing creatinine is mixed with the

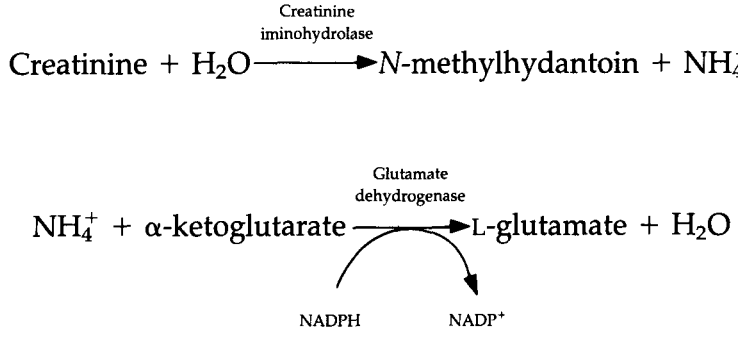


Fig. 1. Reaction scheme for creatinine analysis.

substrate solution in a single reagent stream that is allowed to flow through the enzyme reactors. Creatinine is quantitated by NADPH consumption with a corresponding decrease in absorption of the substrate solution at 340 nm.

## MATERIALS AND METHODS

### *Instrumentation*

1. A Technicon AAII 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 GDH reactors.
3. Vitatron Programmable Analyser, PA800 (Vitatron UK Ltd., Boyn Valley Road, Maidenhead, Berkshire, UK) was used for the analysis of creatinine by the Jaffé method in comparison studies.

### *Reagents*

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

#### *Preparation of the Nylon Tube GDH Reactors*

1. Nylon (type 6) tubing, 1 mm internal diameter (Portex Ltd., Hythe, Kent, UK).
2. Triethyloxonium tetrafluoroborate, 10% w/v in dichloromethane (*14*).
3. Adipic acid dihydrazide (Sigma Chemical Co. Ltd., Fancy Road, Poole, Dorset, UK), 4% w/v in formamide.
4. GDH (Sigma, Ltd.), 290 U/mg, supplied as a solution containing 2 mg in 0.08 mL Tris HCl, pH 8, containing 5 mM EDTA and 0.5% sodium azide. The solution was diluted to 2 mL prior to immobilization.
5. Borate buffer (0.2*M*, pH 8): 0.2*M* boric acid was titrated to pH 8 with 2*M* NaOH.
6. Glutaraldehyde, 25% w/v, electron microscopy grade. A 5% solution in borate buffer was prepared immediately before use.
7. Potassium borohydride, 1% w/v, in borate buffer was prepared immediately before use.
8. Phosphate buffer (0.05*M*, pH 8.0). A volume of 250 mL of 0.2*M* $KH_2PO_4$ was added to 234 mL of 0.2*M* NaOH. The volume was made up to 1 L with deionized water.

*Assay of Creatinine*

1. Sodium chloride, 0.9%.
2. Buffer/substrate: Tris, 43 mM and L(+)-tartaric acid, 7 m*M*, pH 8.0, containing $K_2SO_4$, 23 m*M*, NADPH, 0.20 m*M*, sodium α-ketoglutarate, 10 m*M*. The mixture is stable for at least 1 wk at 2–8°C.
3. Clinibond® CI nylon tube reactor (Farmitalia Carlo Erba, 20090 Rodano, Italy). A blank reactor is also supplied.
4. Creatinine standards.
   (i) Aqueous standards, 100–1000 μ*M*, prepared by making dilutions of a 10 m*M* stock creatinine standard.
   (ii) Serum standards, 76–1000 μ*M*, prepared by adding varying amounts of the 10 m*M* aqueous stock standard to aliquots of a commercially supplied quality control serum.
5. Ammonium sulfate standard, 10 mM, in deionized water, for studies using the GDH reactors.
6. Merck kit for creatinine assay using the Jaffé reaction.

## Preparation of the Immobilized GDH Reactors

1-Meter GDH nylon-tube reactors were prepared using the acid hydrazide derivatives of O-aklylated nylon tubing, in the manner described by Ginman et al. (*15*).

The activity of the reactors was determined in terms of μmol $NADP^+$ formed/min/m. by pumping a solution consisting of 0.1 mL of the 10 m*M* $(NH_4)_2SO_4$ standard and 1.0 mL of buffer/substrate through the reactors at a rate of 0.32 mL/min, and measuring the absorbance of the perfusate and effluent at 340 nm.

## Use of the Reactors for Creatinine Analysis

The manifold was prepared according to the flow diagram shown in Fig. 2, the CI and GDH reactors were inserted, the reagent pumped, and the baseline adjusted on the recorder chart. Serum creatinine standards were sampled, followed by patients' serum samples and controls. Urine samples were diluted 1 in 100 with deionized water prior to assay, and aqueous standards were used for the urine calibration. It was found necessary to use separate standards for serum and urine analyses because of differences in their dialysis rates.

The endogenous ammonia content of the sera and urines was determined by replacing the CI reactor with the blank reactor and re-assaying the samples. A calibration curve was plotted, of test minus blank peak height against creatinine concentration, from which the creatinine content of the patients' samples could be determined.

After the run, the CI reactor was replaced and the system washed through with deionized water for 10 min. The reactors were removed

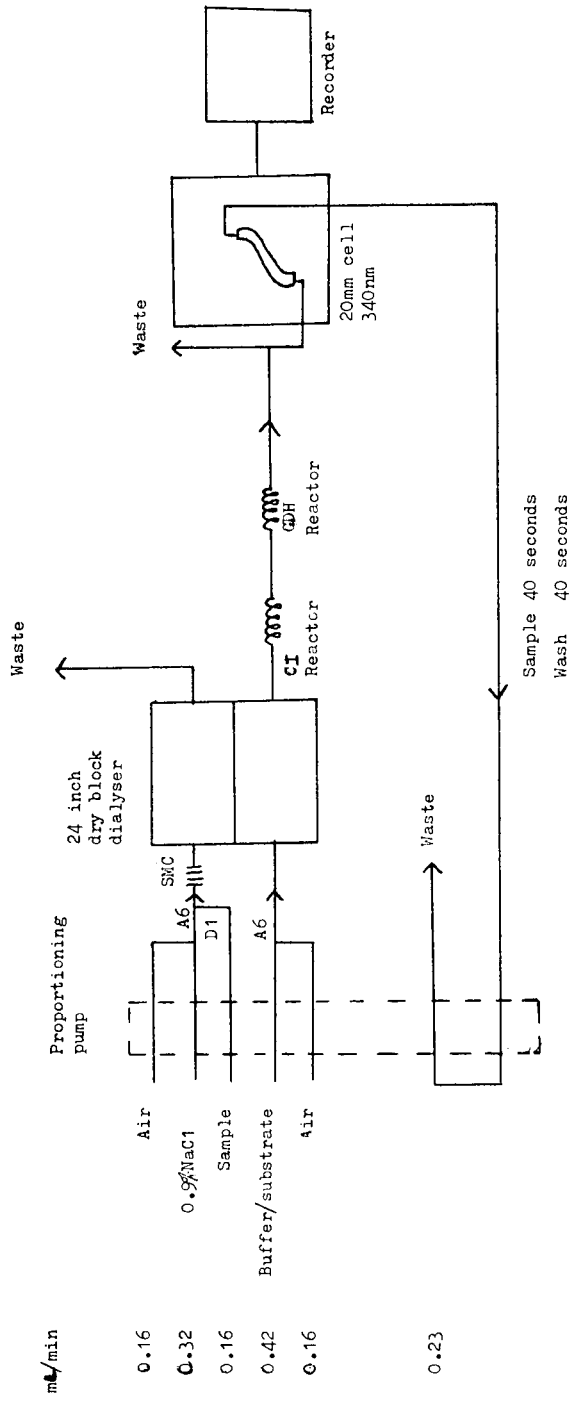


Fig. 2. Flow diagram for the determination of creatinine using the CI and GDH nylon-tube reactors.

and stored at +4°C filled with phosphate buffer. The N9 connecting nipple was replaced before lifting the platen.

## RESULTS

### *Optimization of Substrate Concentrations*

#### *α-Ketoglutarate*

The α-ketoglutarate concentration in the buffer/substrate was varied from 2 to 20 m*M*. The change in absorbance at 340 nm resulting from the passage of the buffer/substrate containing 1 m*M* $NH_4^+$ through the GDH reactor was determined for each different concentration. The results are seen in Fig. 3, which indicates that maximum change in absorbance was obtained using 10 m*M* α-ketoglutarate.

#### *NADPH*

The NADPH concentration in the buffer/substrate was varied from 0.1 to 0.25 m*M* and the change in absorbance at 340 nm determined, as above, for each NADPH concentration. As shown in Fig. 4, maximum change in absorbance was obtained using 0.2 m*M* NADPH. However, when using a 'blank' solution, in which the $NH_4^+$ was replaced by deionized water, it was found that, as the NADPH concentration was in-

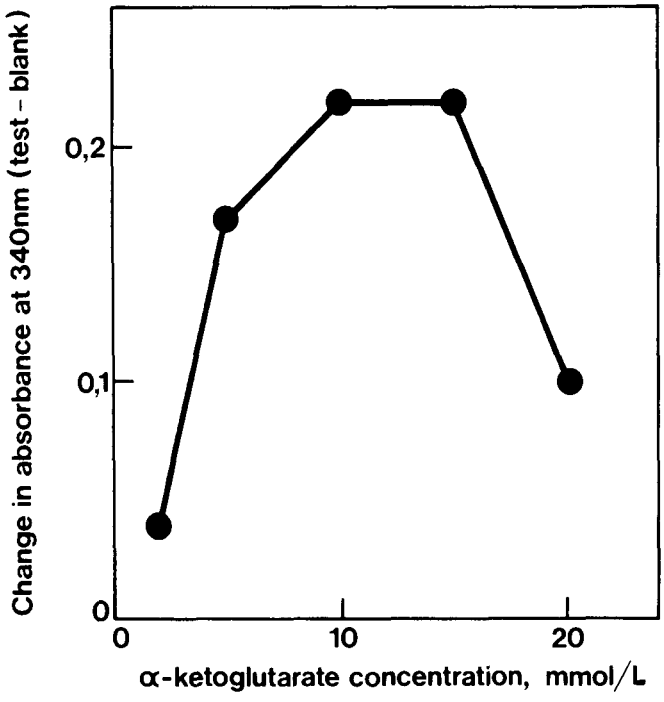


Fig. 3. Effect of α-ketoglutarate concentration on the assay of creatinine using immobilized CI/GDH.

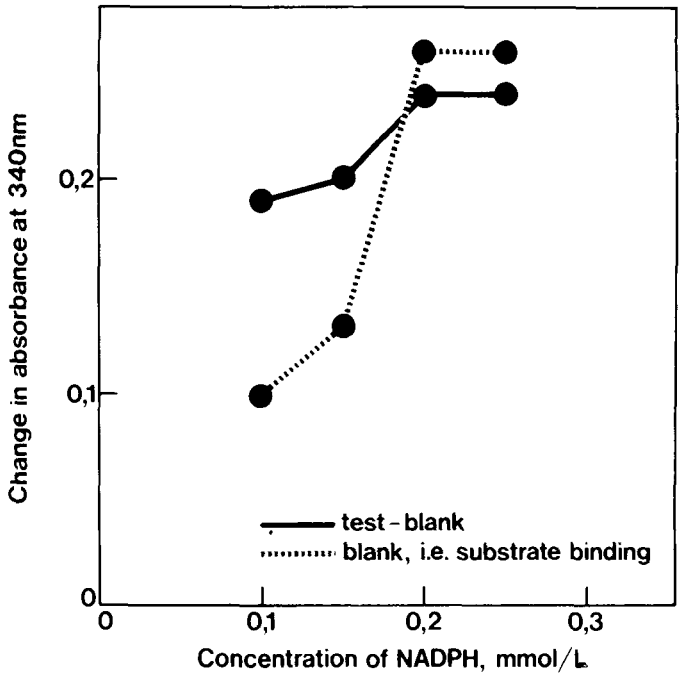


Fig. 4. Effect of NADPH concentration on the assay of creatinine using immobilized CI/GDH.

creased, binding of the negatively charged NADPH molecules to the cationic nylon support was also increased; this is a phenomenon previously described by Noy (*16*), and has also been observed by Ginman et al. (*15*).

## Use of the Reactors in the Continuous Flow Analysis of Creatinine

A typical recorder tracing, obtained during the precision studies, showing serum sample peaks assayed using the system shown in Fig. 2, is illustrated in Fig. 5.

### Linearity

The linearity of the method is shown in Fig. 6, in which sample minus sample blank peak height is plotted against creatinine concentration in μmol/L. The origin offset has previously been observed in methods of analysis using immobilized enzymes (*15*), and has been attributed to diffusion effects (*17*) and NADPH binding (*16*).

### Recovery

The analytical rercovery of the method was determined by adding accurately measured quantities of 10 m*M* creatinine to aliquots of a serum sample previously assayed for creatinine. These samples were then

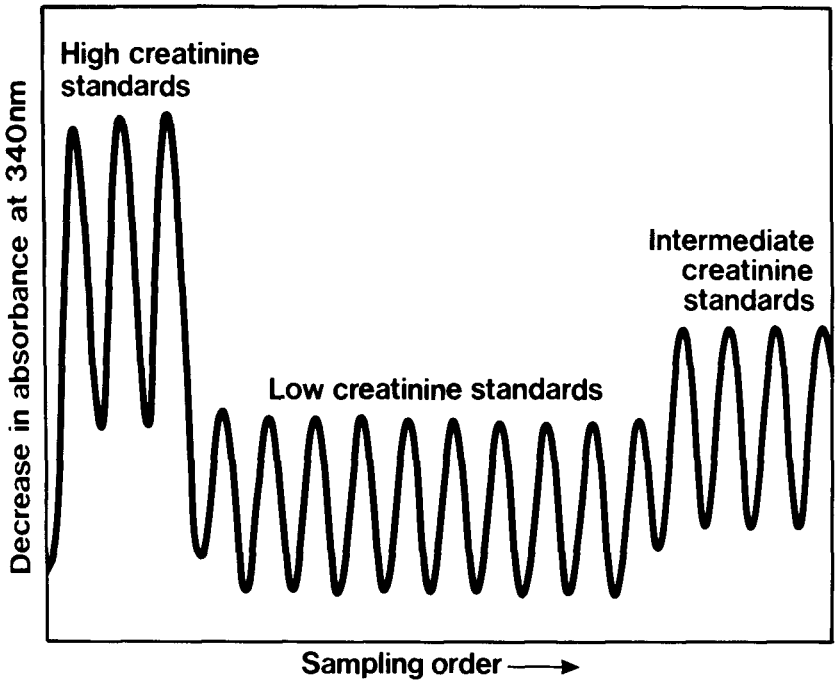


Fig. 5. Recorder tracing obtained using the immobilized enzyme method for creatinine analysis.

reassayed and the recoveries were found to vary from 93 to 99%, as shown in Table 1.

### *Sample Interaction*

The carry-over coefficient was calculated according to the method of Broughton et al. (*18*). When using three high standards (1000 μ*M*) followed by three low standards (76 μ*M*), the carry-over coefficient was found to be 1.7%.

### *Precision*

Within-run precision was determined at three levels using samples with low, intermediate, and high creatinine concentrations, and is shown in Table 2.

Table 3 shows a comparison of the between-batch precision of the immobilized enzyme method with the between-patch precision of the standard Jaffé method. Greater precision was obtained using the immobilized enzyme method.

### *Accuracy*

Various commercially supplied quality control samples were assayed for creatinine by the immobilized enzyme method. These values were compared with the stated assay ranges, and are shown in Table 4.

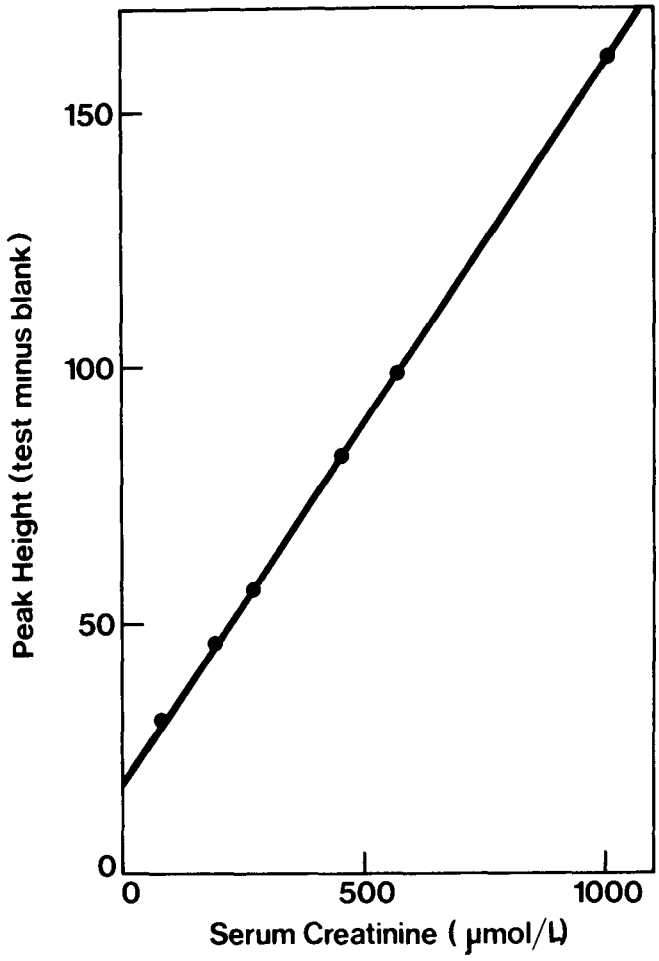


Fig. 6. Graph of peak height (test minus blank) against concentration of serum creatinine standards.

TABLE 1
Analytical Recovery of Added Creatinine from Serum Using the Immobilized Enzyme Method

| Sample | Assayed value, μmol/L | Expected value, μmol/L | Recovery, % |
|---|---|---|---|
| Serum with no added creatinine | 120 | | |
| Serum with 25 μL of 10 m*M* creatinine | 200 | 203 | 99 |
| Serum with 50 μL of 10 m*M* creatinine | 270 | 284 | 95 |
| Serum with 75 μL of 10 m*M* creatinine | 340 | 364 | 93 |
| Serum with 100 μL of 10 m*M* creatinine | 440 | 443 | 99 |

TABLE 2
Within-Run Precision, Performed by Analysis of Samples with Low Intermediate, and High Creatinine Concentration

| Parameter | Low | Intermediate | High |
|---|---|---|---|
| Mean, μmol/L | 80 | 465 | 1000 |
| SD | 0.866 | 0.516 | 0.789 |
| CV, % | 1.519 | 0.559 | 0.450 |
| *n* | 10 | 10 | 10 |

TABLE 3
Between-Batch Precision

| Parameter | Immobilized enzyme method | Jaffé method |
|---|---|---|
| Mean, μmol/L | 113 | 120 |
| SD | 7.5 | 9.4 |
| CV, % | 6.66 | 7.8 |
| *n* | 10 | 10 |

TABLE 4
Accuracy

| Quality control material | Manufacturer's stated mean, μmol/L | Assay range | Creatinine value obtained, μmol/L |
|---|---|---|---|
| Precinorm U | 153 | 125–181 (Enzymatic methods) | 170 |
| Precipath U | 291 | 283–343 (Enzymatic methods) | 300 |
| Wellcome QAC I | 147 | 138–155 (Jaffé method) | 160 |
| Wellcome assayed | 205 | 173–237 (All methods) | 210 |
| Ortho urine control | 9300 | 7500–11,000 (All methods) | 10925 |
| Bistrol urine control | 7500 | 7000–8000 (All methods) | 7500 |

## Comparison Studies

A number of patients' samples were assayed for creatinine by the immobilized enzyme method and by the Jaffé method. The correlation and regression lines for serum and urine samples, by the method of linear least squares, are shown in Figs. 7 and 8. The better correlation ob-

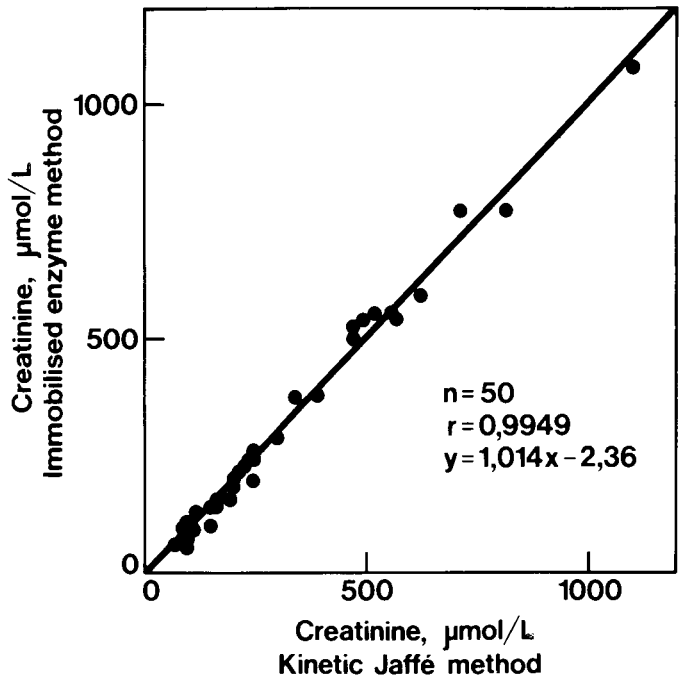


Fig. 7. Correlation and regression line for serum creatinine by the immobilized enzyme method against serum creatinine by the Jaffé method.

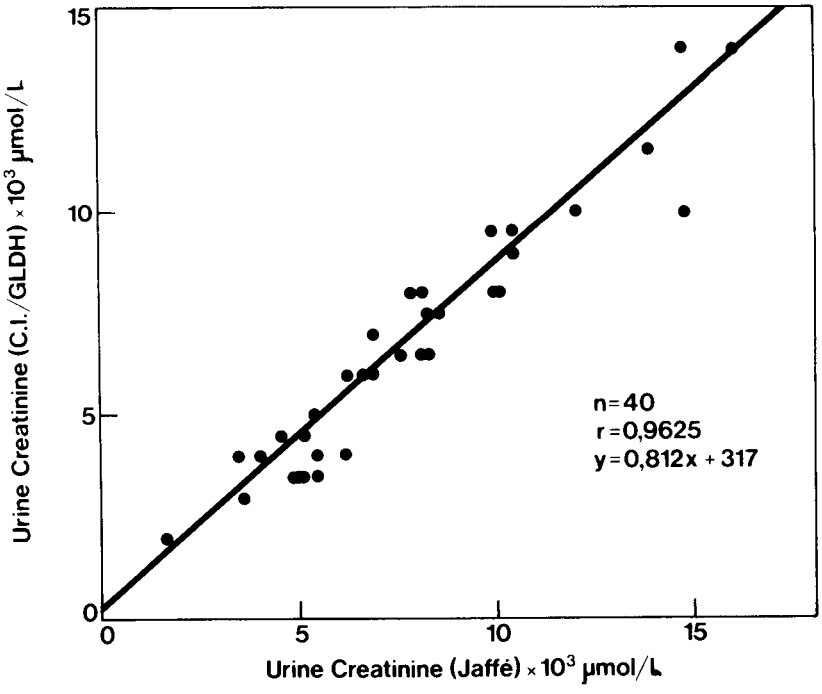


Fig. 8. Correlation and regression line for urinary creatinine by the immobilized enzyme method against serum creatinine by the Jaffé method.

tained for serum creatinine is expected since the 1 in 100 dilution used for urines would exaggerate any inherent errors.

### *Interference Studies*

Potential interferents—metabolites and other substances, including drugs—were added to 5 mL aliquots of a previously assayed serum pool. These aliquots were then re-assayed for creatinine. If the difference in the values obtained with and without the added substance exceeded ±3 SD, then that added substance was considered to be an interferent. In addition, no interference was observed with moderately hemolyzed or lipaemic sera. The results are shown in Table 5.

## DISCUSSION

This paper describes a simple, automated method for the determination of creatinine, showing good accuracy, precision, sensitivity, and specificity. There is negligible baseline drift and the standard peak

TABLE 5
Effect of Interfering Substances

| Interfering substance | No interference up to |
|---|---|
| Glucose | 56 m*M* |
| Bilirubin | 342 μ*M* |
| Urea | 17 m*M* |
| Uric acid | 1.19 m*M* |
| Ascorbic acid | 568 μ*M* |
| Sodium pyruvate | 10 m*M* |
| $NaHCO_3$ | 40 m*M* |
| KCl | 8 m*M* |
| NaCl | 155 m*M* |
| Acetone | 10 m*M* |
| Thymol | 18.7 m*M* |
| Sodium valproate | 6.0 m*M* |
| Paracetamol | 1323 μ*M* |
| Phenobarbitone | 129 μ*M* |
| Amitryptiline hydrochloride | 1.3 μ*M* |
| Acetylsalicylic acid | 3.3 m*M* |
| Carbamazepine | 85 μ*M* |
| Phenytoin | 79 μ*M* |
| Anticoagulants | |
| $K_2EDTA$ | |
| Trisodium citrate | No interference observed using commercially supplied specimen tubes |
| Heparin | |
| Fluoride/oxalate | |

heights are extremely reproducible, thus making repeated analysis of standards unnecessary.

At first, GDH reactors were produced using a less active, nonspecific source of GDH, EC 1.4.1.3, 15.9U/mg, supplied in lyophilized form. The initial activity of the reactors varied from 1 to $10 \times 10^{-3}$ μmol $NADP^+$ formed/min/m. Using the more active and specific source of GDH, EC 1.4.1.4, 290 U/mg, the initial activity of the reactors produced was $41 \times 10^{-3}$ μmol $NADP^+$ formed/min/m. With this greater reactor activity, it was possible to reduce the length of reactor required to give adequate sensitivity to 50 cm, which minimized the carry-over from NADPH binding effects. The operational stability of the GDH reactors is still under investigation; after 3–4 months of continuous usage, their activity has fallen by less than 25%. This has proved to be a result of storage rather than repeated sample analyses and excellent sensitivity is still being achieved in the assay for creatinine. The commercially supplied CI reactor has shown good operational stability over the entire 6-month period of study to date.

In the method described, because of the relatively high levels of endogenous ammonia compared with creatinine in serum and urine, the samples were reassayed in order that the blank values could be subtracted from the test values. An integrated system of assay has been investigated, in which the recipient stream emerging from dialysis was divided into test and blank channels, the blank being electronically subtracted from the test at the colorimeter. There were several disadvantages with this system. Firstly, there was an increase in carry-over; secondly, with the peaks obtained, it was difficult to ensure that both the test and blank reagent streams arrived at the colorimeter flow cells at exactly the same time, particularly when the pump tubes showed signs of wearing; thirdly, the precision was poor. As a result, it was necessary to increase the sampling time to 70 s, the sample volume consequently being increased to 0.37 mL (the original method required a total sample volume of 0.22 mL); in addition, the correlation with the Jaffé method was poor. This was attributed either to the phasing difficulties or to inadequate electronic subtraction of the blank values from the test values. Incorporation of a GDH reactor in the donor stream to remove endogenous ammonia was not possible, since the product of reaction, $NADP^+$, dialyzes into the recipient stream. Reassaying the samples to determine the blank values is not considered a disadvantage in laboratories handling small numbers of samples, but in laboratories with larger workloads, where speed of analysis is important, the blank channel should be run in parallel with the test channel using a single sample uptake split into two streams at the manifold. Additional pump tubes, dialyzer, and GDH reactor are required, together with a dual-channel colorimeter and recorder. The additional GDH reactor would not significantly increase the cost of analysis because of the short length of reactor required and its excellent operational stability.

The use of NADPH instead of NADH as coenzyme in the assay eliminates interference from substrate consumption by serum dehydrogenase (*19*); this, together with the use of CI, the more specific form of GDH and the determination of sample blanks provides a completely specific method for creatinine determination, suitable for use in the Clinical Chemistry Laboratory.

## ACKNOWLEDGMENTS

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