CONTINUOUS-FLOW ENZYMATIC DETERMINATION OF CREATININE WITH IMPROVED ON-LINE REMOVAL OF ENDOGENOUS AMMONIA

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SUMMARY

A new continuous-flow automated enzymatic method suitable for the direct determination of creatinine in physiological samples is described. The proposed system utilizes an on-line gas predialysis unit in conjunction with a flow-through enzyme reactor coil and a potentiometric ammonia detector. The enzyme reactor contains immobilized creatinine iminohydrolase (EC 3.5.4.21) which converts creatinine to ammonia and *N*-methylhydantoin. Ammonia liberated from this reaction is detected downstream with the membrane electrode-based detector. The novel gas predialysis unit effectively removes >99.8% of endogenous ammonia (up to 1 mM) present in the sample. Thus, final peak potentials recorded by the electrode detector are directly proportional to the logarithm of creatinine concentrations present. The method is shown to be precise (<3%), selective, and capable of accurately determining creatinine in serum and urine samples containing abnormally high endogenous ammonia levels. Determinations of creatinine in serum samples ($n = 30$) using this new method correlate well with an existing Technicon AutoAnalyzer colorimetric method ($r = 0.996$).

In recent years, various selective enzymes have been coupled with ammonia detection methods to measure important amino acids and metabolites in physiological samples [1—6]. In such assays, ammonia liberated from the enzymatic reaction should be directly proportional to the concentration of analyte initially present in the sample. In reality, problems arise from the background levels of ammonia nitrogen (NH_3—N) found in many biological samples. Indeed, normal blood ammonia levels are generally in the same concentration range as most amino acids and metabolites (10—100 μmol l^{-1}) [7, 8]. Moreover, any delay in analysis of the blood sample will result in large increases in background ammonia levels because of hydrolysis of labile glutamine and aspargine residues in proteins [9]. The problem is further

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compounded when samples are drawn from patients with disease states that result in hyperammonemia (e.g., hepatic coma, Ryes syndrome, etc.), where ammonia can reach levels as high as 1 mmol l^{-1} in fresh serum [10].

Various methods have been proposed to alleviate or correct for the endogenous ammonia problem in blood and particularly urine. Time-consuming manual pretreatment steps including cation-exchange [11, 12] and distillation [13, 14] have been used. Difference measurements in which background ammonia is first determined and then subtracted from the total ammonia present after the enzyme reaction have been proposed for blood analysis [11]. Unfortunately, the accuracy of this latter method decreases when the ratio of endogenous ammonia to analyte levels increases. Recently, several researchers [2, 15—17] have used glutamate dehydrogenase (GLDH) to remove endogenous ammonia enzymatically

$$NH_4^+ + \alpha\text{-ketoglutarate} + NADH \rightarrow NAD^+ + L\text{-glutamate} + H_2O$$
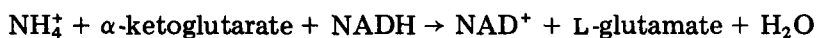
from physiological samples. The method requires the use of NADH or NADPH, and soluble or immobilized GLDH, making the system less attractive for adaptation to high throughput autoanalyzer-type systems. In order to reduce the cost, the amount of NADH used per assay can be lowered; however, this severely limits the concentration of endogenous ammonia which can be tolerated [10].

In 1983, a novel flow-through on-line gas predialyzer was introduced, which was capable of removing approximately 90% of endogenous ammonia in serum and plasma samples [18]. The original concept was demonstrated in an automated system for the determination of asparagine in plasma samples. While such a system could adequately reduce normal background ammonia levels, errors greater than 10% could still occur if samples contained abnormally high ammonia concentrations. In this paper, a simple temperature modification to the original on-line gas predialyzer design is described; it enables greater than 99.8% of the ammonia to be removed from serum and urine samples containing up to 1 mM ammonia. Additionally, this predialyzer is used in conjunction with immobilized creatinine iminohydrolase enzyme (EC 3.5.4.21) to develop an automated system for the direct determination of creatinine in serum and urine samples.

Creatinine levels in blood and urine are the most widely accepted indicators of kidney function. The classical Jaffé colorimetric method is known to suffer many interferences [19]. Consequently, the accuracy and precision requirements of clinical creatinine assays require the development of non-Jaffé methods [20]. Among alternative techniques it is well recognized that enzymatic methods can greatly enhance the selectivity of creatinine determinations [19, 21]. Towards this end, the new system (Fig. 1) contains a tubular enzyme reactor coil to convert creatinine to N-methylhydantoin and ammonia. The ammonia is detected downstream with an improved membrane electrode-based detector [22]. The on-line predialyzer is placed ahead of the enzyme coil where it removes endogenous ammonia from the
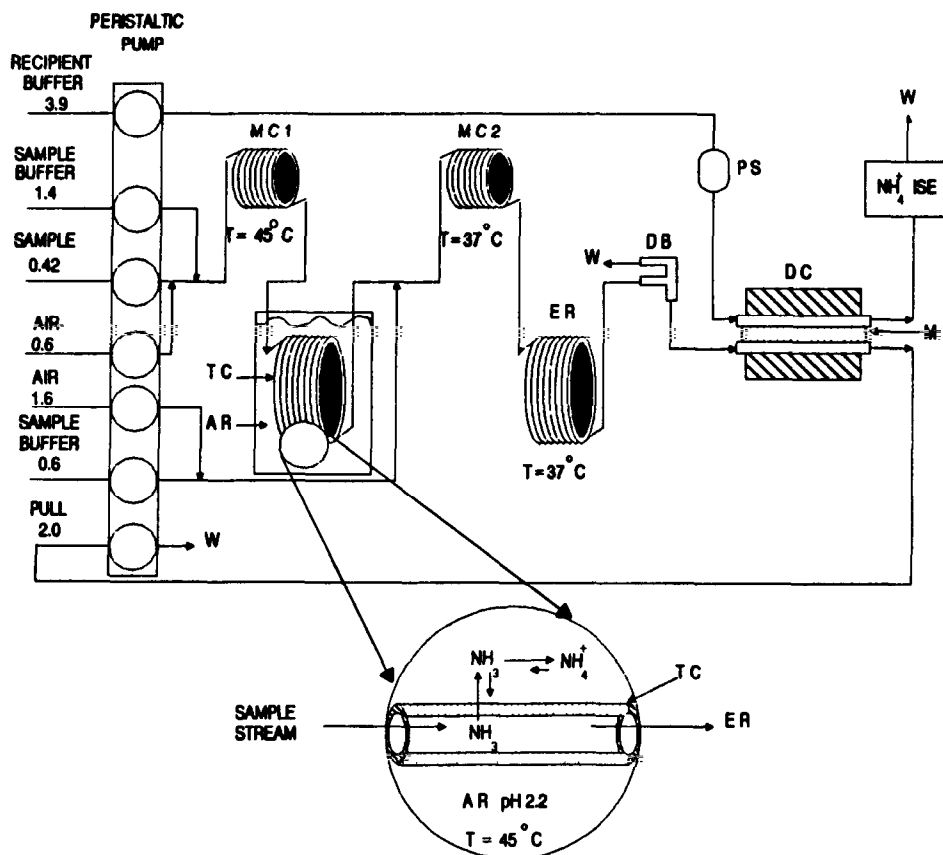
Fig. 1. Flow diagram of automated creatinine system: (MC) mixing coil; (TC) on-line teflon predialyzer coil; (AR) acid reservoir trap; (ER) enzyme reactor coil, (DB) debubbler; (PS) pulse suppressor, (DC) gas dialysis chamber; (M) porous gas permeable membrane; (W) waste. Numbers represent flow-rates in ml min^{-1}.

sample before it enters the enzyme coil. Consequently, detected ammonia is directly proportional to the concentration of creatinine, regardless of the initial ammonia level present in the sample.

EXPERIMENTAL

Apparatus

A schematic diagram of the automated creatinine system is shown in Fig. 1. A Rainin (Woburn, MA) peristaltic pump was used to deliver the sample, reagents and air through the system. A Technicon Sampler II served as the autosampler, and was operated at a throughput of 30 samples per hour, with a 1:2 sample/wash ratio.

The ammonia predialysis unit consisted of a 4.2-m length of teflon gas-permeable tubing [poly(tetrafluoroethylene), 1-mm i.d., 50% porosity, Anspec Company, Ann Arbor, MI]. The tubing was coiled and placed in a 250-ml acid reservoir (0.2 mol l^{-1}, pH 2.2, H_3PO_4, NaOH buffer). The predialyzer was thermostated at temperatures ranging from 25 to 45°C, with optimal ammonia removal being obtained at 45°C. The predialyzer was preceded by a 40-s (1.5 ml) mixing coil that was also thermostated at the predialyzer temperature. The sample residence time in the predialysis coil was 110 s.

The enzyme-reactor coil consisted of creatinine iminohydrolase immobilized on the inner walls of a 1-m length of 1.0-mm i.d. nylon tubing. The enzyme was obtained from G.D.S. Technology (Elkhart, IN) and was immobilized by established procedures [23]. The specific activity of this enzyme coil was evaluated by the method described by Hornby and Noy [24]. The method was applied in the range 3—85 mmol l^{-1} creatinine where the fractional conversion varied, i.e., where the enzyme activity was rate-limiting. The apparent Michaelis-Menten constant, K', was found to be 2.8×10^{-2} mol l^{-1}. The specific activity of the enzyme coil was 0.84 μmol min^{-1} cm^{-1}. When stored as described below (see Reagents) the activity of this enzyme coil remained essentially constant for 1 year and several thousand creatinine samples.

For certain preliminary experiments, commercial enzyme-reactor coils obtained from Farmitalia Carlo Erba (Milan, Italy) were used. The various enzyme reactors were thermostated at 37°C and preceded by a 25-s (1.5 ml) mixing coil maintained at the same temperature. Sample residence times in the enzyme reactor were usually 10—12 s.

The potentiometric ammonia detection system used in this work was similar in design to those described previously [22, 25]. An improvement in response to low levels of ammonia was achieved by using a custom-designed gas dialysis chamber (for details, see [26]) which enhances the efficiency of gas transfer between the sample and recipient streams. The final configuration was further modified to reduce extensive streaming potential oscillations (±1—2 mV) caused by the relatively high recipient buffer flow rate used in this particular work (3.9 ml min^{-1}). This was accomplished by using two Ag/AgCl electrodes, rather than a single reference and stainless steel nipple, in a circuit arrangement analogous to that suggested by van den Winkel et al. [27]. With this change, oscillations in baseline potentials were reduced to ±0.2 mV.

Potentiometric measurements were made with a Fischer Accumet pH Meter (Model 620) and were recorded on a Linear (Irvine, CA) Model 1201 strip-chart recorder. The recorder output of the Fisher pH meter was also monitored by a computer-based data acquisition system. The data acquisition system consisted of a Keithley Model 179 TRMS-A Digital Multimeter with IEEE-488 interface connected to a Commodore Pet Model 8032 microcomputer. Appropriate software was used to detect and record auto-

matically the baseline, peak potentials and ΔE values (peak minus baseline potentials).

Reagents

All chemicals used were analytical-reagent grade. Standard solutions and buffers were prepared with reverse-osmosis deionized water. Buffer concentrations refer to total ionic strength. Reagents were stored in sealed glass containers to minimize absorption of atmospheric ammonia.

The sample stream diluent was 0.1 mol l^{-1} boric acid/sodium hydroxide, pH 9.50, containing 2 mmol l^{-1} EDTA. The recipient stream buffer at the detector was a 0.01 mol l^{-1}, HCl/Tris [tris(hydroxymethyl)aminomethane] buffer, pH 7.50.

Enzyme-reactor coils were stored refrigerated (4°C) between experiments and filled with a 0.1 mol l^{-1} phosphate storage buffer, pH 7.0, containing 0.1% (w/w) EDTA and 0.05% (w/w) sodium azide.

Weekly, a fresh 5×10^{-2} mol l^{-1} creatinine stock solution (prepared in 0.01 mol l^{-1} HCl) was diluted with deionized water to prepare working creatinine standards in the range of 25—5000 μmol l^{-1} (0.28—56.6 mg dl^{-1}).

Procedures

Evaluation of predialyzer unit. Initially, the predialysis unit and enzyme-reactor coil of Fig. 1 were removed so that the ammonia response could be calibrated and the ammonia content of serum and urine samples could be determined. Aqueous ammonium chloride standards (5 –5000 μmol l^{-1}) were sampled and corresponding ΔE values recorded. Following the standards, ammonium chloride-spiked pooled serum (undiluted) and urine samples (1 + 99 diluted) were aspirated into the system and the resulting ΔE values were recorded. The predialysis unit was then reincorporated into the system and the same series of ammonium chloride standards and ammonium chloride-spiked serum and urine samples were analyzed.

The pooled serum used for these experiments was obtained from healthy members of our research group. Reconstituted Fisher Level 1 Control Urine (lot 258-074) was used to prepare the ammonium chloride-spiked urine samples. These latter samples were prepared by diluting (with deionized water) 1 ml of reconstituted urine plus microliter amounts of 0.1 mol l^{-1} ammonium chloride to a final volume of 100 ml. All serum and urine samples were stored frozen (−21°C) between experiments.

Creatinine determinations. The complete flow configuration was used for creatinine determinations (Fig. 1). An initial series of aqueous creatinine standards ranging from 25 to 5000 μmol l^{-1} (0.28—56.6 mg dl^{-1}) were sampled. The potential was then allowed to return to baseline before serum or urine samples were analyzed. Following the serum and/or urine samples, the potential was again allowed to return to baseline before a second series of creatinine standards was sampled. Unknown creatinine levels were determined from a linear least-squares fit of the combined initial and final aqueous calibration data in which peak-height potentials (ΔE) were plotted vs. the logarithm of creatinine concentrations.

RESULTS AND DISCUSSION

The basic operating principles of the proposed creatinine system are straightforward. Samples are pumped from the autosampler, immediately air-segmented, and diluted with an alkaline buffer to convert partially ammonia in the sample to free ammonia gas. The samples then pass through the on-line gas predialyzer. The insert in Fig. 1 shows an expanded view of the predialysis unit and the chemical processes that take place within. As the sample passes through the predialyzer, free ammonia gas in the sample diffuses through the walls of the gas-permeable teflon tubing and is trapped as ammonium ions by the acid reservoir. Thus, as the sample traverses the length of the coiled tubing, the ammonia content of the sample is reduced while the creatinine level remains unchanged. Following the predialyzer, additional air-segmentation and buffer are added to the sample prior to its passage through the enzyme reactor. In the enzyme reactor, immobilized creatinine iminohydrolase catalyzes the following reaction:

creatinine + $H_2O \rightarrow$ N-methylhydantoin + NH_3
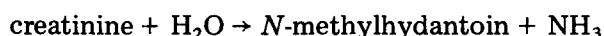
Following the enzyme reactor, the sample stream is debubbled and passed through the flow-through potentiometric ammonia detector. The recorded potential changes (ΔE) are proportional to the logarithm of the creatinine concentration in the sample.

In the design of the final automated arrangement, numerous studies were done to optimize sample ammonia removal and to maximize the response of the system to creatinine. The results of these studies are detailed below.

Optimization of ammonia and creatinine response

Initial experiments with the enzyme reactor coils demonstrated that approximately 50% conversion of creatinine to ammonia could be achieved with a sample-stream diluent pH of 9.50 and a total sample/diluent ratio of 1:5. Consequently, the ammonia detection system was optimized under these conditions. For ammonia response studies, the gas predialyzer and the enzyme reactor were removed and the response was monitored as a function of varying flow rate combinations through the detector gas dialysis chamber. In all, 22 combinations of flow rates for the sample and recipient streams were examined. The flow-rate combination shown in Fig. 1 (3.9 ml min^{-1} recipient; 2.0 ml min^{-1} sample stream in the dialysis chamber) provided a good compromise for reasonably fast detector washout times and nearly optimal potential response for given ammonia concentrations.

Typical calibration curves for aqueous ammonia and creatinine standards obtained by using this combination of flow rates are shown in Fig. 2. The creatinine curve was obtained by adding the enzyme reactor coil to the flow system while still leaving the predialyzer unattached. Response to creatinine paralleled that obtained for ammonia but was shifted to higher concentrations owing to the incomplete conversion of creatinine to ammonia in the
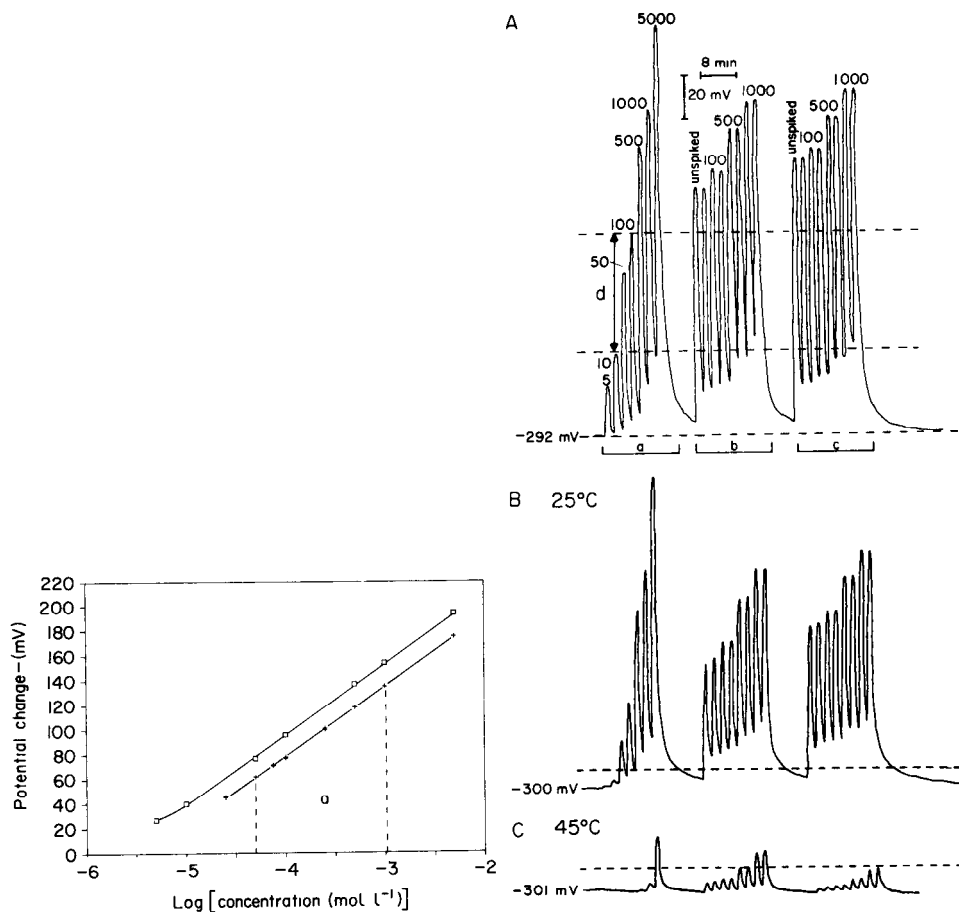
Fig. 2. Typical calibration curves obtained for ammonia (□) and aqueous creatinine standards (+) with the system shown in Fig. 1 without gas predialyzer in place. Range (a) is the clinical range (50—1000 μmol l^{-1}, 0.56—11 mg dl^{-1}).

Fig. 3. Strip-chart recordings illustrating the effectiveness of the on-line gas predialyzer coil in removing endogenous ammonia at two different predialyzer temperatures: (A) without predialyzer in place; (B) with predialyzer maintained at 25°C; (C) with predialyzer maintained at 45°C. Samples were aqueous ammonium chloride standards (a), ammonium chloride-spiked serum (b), and ammonium chloride-spiked (1 + 99) diluted urine (c). The range labeled (d) is the normal range of serum ammonia (10—100 μmol l^{-1}). Numbers above peaks in (A) refer to μmol l^{-1} ammonia in standards or added to serum and urine samples.

reactor. Nevertheless, the creatinine response is nearly Nernstian (58 mV/decade) in the range of 25—5000 μmol l^{-1}, a range which extends well below and above that required for clinical applications (50—1000 μmol l^{-1}). It should be noted that, given the ammonia detection capabilities of the proposed system, creatinine conversion efficiencies as low as 20% can be tolerated while still maintaining calibration linearity in the low end of the clinically important creatinine range.

Optimization of predialyzer efficiency

Hydrochloric acid solutions (1.0 and 0.1 mol l^{-1}), citric acid buffer (0.2 mol l^{-1}, pH 3.2) and phosphoric acid buffer (0.2 mol l^{-1}, pH 2.2) were each studied as potential solutions for the acid-trap reservoir. Though hydrochloric acid was used successfully in the previous predialyzer configuration [18], its use in the current system was not possible. At the elevated predialyzer temperatures used in these studies, hydrogen chloride readily diffused through the walls of the gas-permeable predialyzer coil and caused large changes in the sample stream pH. Of the two non-volatile buffers studied, the phosphoric acid buffer with lower pH (pH 2.2) proved to be the more efficient trapping medium because of the larger pH difference between the sample-stream buffer inside the coil and the external acidic reservoir.

The most critical parameter with regard to the efficiency of on-line ammonia removal is the temperature of the predialysis unit. As shown in Fig. 3, for a given predialysis residence time, dramatic improvements in efficiency can be realized when the temperature of the unit is increased. Figure 3A shows a strip-chart recording obtained when aqueous ammonium chloride standards along with serum and urine samples spiked with varying levels of ammonium chloride were passed through the system without the predialyzer and enzyme reactor in place. Figure 3B and 3C show the response of the system toward the same standards and samples after the predialyzer is added and thermostated at 25°C and 45°C, respectively. The baseline potential of the system also changes upon addition of the predialyzer. This is a result of the removal of trace levels of ammonia in the diluent buffer. Taking this baseline shift into account, removal of ammonia is approximately 90% for all samples when the predialyzer is thermostated at 25°C. In contrast, elevating the predialysis temperature to 45°C results in efficiencies greater than 99.8%, even for serum samples spiked to contain more than 1 mmol l^{-1} ammonia (10 times the highest levels normally found in serum). This dramatic temperature effect may be rationalized in terms of decreasing sample stream viscosity, increasing ammonia diffusion coefficient and decreasing ammonia solubility in the sample stream with increasing predialyzer temperature.

Determinations of creatinine in physiological samples

With the various components optimized, the performance of the complete continuous-flow arrangement was assessed. The response to creatinine in physiological samples (control serum and control urine) was studied with and without the predialyzer in place. Figure 4A shows the tracing obtained for a series of aqueous creatinine standards followed by serum and diluted urine (1 + 99) samples spiked with varying amounts of ammonium chloride. As can be seen, without removal of endogenous ammonia, the "creatinine" response closely follows the levels of ammonium chloride added. When the predialyzer is in place (Fig. 4B), regardless of the amount of ammonium chloride added, the response towards creatinine remains constant and
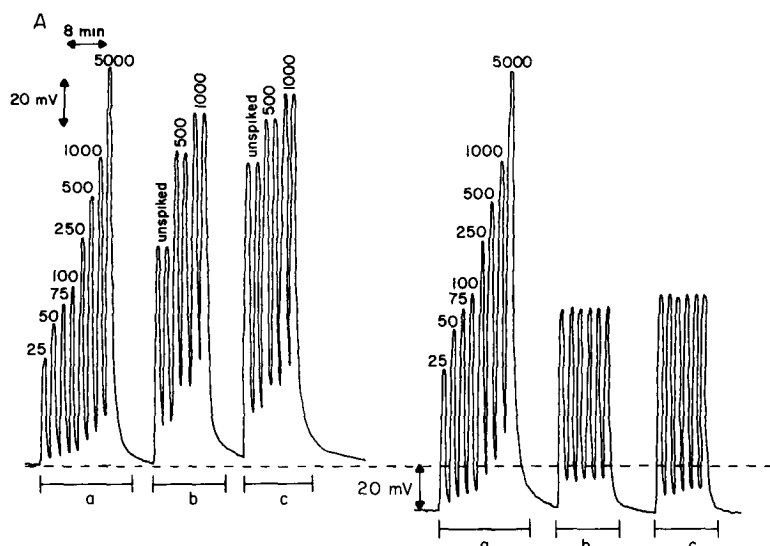
Fig. 4. Strip-chart recording showing the creatinine response of the automated system: (A) without gas predialyzer coil in place; (B) with predialyzer maintained at 45°C. Samples were aqueous creatinine standards (a), ammonium chloride-spiked control serum (b), and ammonium chloride-spiked (1 + 99) diluted control urine (c). The numbers above the creatinine standard peaks indicate μmol l^{-1} creatinine. Those above the serum and urine samples indicate μmol l^{-1} added ammonia. Reported creatinine values for control serum and urine were 92 ± 1 and 106 ± 4 μmol l^{-1}, respectively.

closely correlates with the control values reported for these samples (see legend to Fig. 4). Additionally, it can be seen from Fig. 4 that within-run precision is excellent (s.d. less than ±0.5 mV, corresponding to a concentration reproducibility of 2%) even for samples containing constant creatinine but varying ammonia. In subsequent experiments, between-run precision was evaluated by analyzing 30 different serum samples in each of three successive runs (see Experimental section for calibration and analytical procedures corresponding to a single run). The observed between-run relative standard deviation was 3% or less over the entire concentration range of the samples (0.5—12 mg dl^{-1}; 44—1060 μmol l^{-1}).

Recovery studies were performed on pooled serum and control urine samples to which known amounts of creatinine were added. These samples were further divided into lots which were spiked with varying levels of ammonium chloride. As shown in Tables 1 and 2, added creatinine could be quantitatively recovered regardless of the background of added ammonia.

The proposed creatinine method was further evaluated by conducting a correlation study with the existing Technicon SMAC method number SG4-0011B83 (Jaffé reaction) using unidentifiable serum samples obtained from the University of Michigan Hospital (n = 30). Prior to creatinine determinations, the range of endogenous ammonia in these serum samples was found to be 5.7×10^{-5}—3.3×10^{-4} mol l^{-1}. Figure 5 shows a com-

TABLE 1

Recovery of creatinine added to urine at two background ammonia levels

Creatinine added[a] (mg dl^{-1})	Recovery[b] (%)	
	Endogenous ammonia	1.0 mM ammonia added
1.13	106.7	113.5
2.83	100.9	100.9
5.66	99.3	103.8
11.31	103.7	99.9
Average	102.7 ± 3.3	104.5 ± 6.2

[a]To a (1 + 99) dilution of urine. The unspiked urine creatinine level was 1.21 mg dl^{-1}.
[b]Average of three determinations.

TABLE 2

Recovery of creatinine added to serum at three different background ammonia levels

Creatinine added[a] (mg dl^{-1})	Recovery[b] (%)		
	Endogenous ammonia	0.5 mM NH_3 added	1.0 mM NH_3 added
1.13	93.7	104.2	103.8
2.82	98.5	98.3	102.2
5.60	96.1	102.7	101.8
11.09	95.0	95.9	102.5
Average	95.8 ± 2.0	100.3 ± 3.9	102.6 ± 0.9

[a]Unspiked serum creatinine level was 0.91 mg dl^{-1}. [b]Average of three determinations.

parison of results with the proposed method and the SMAC method for different ammonia levels in the samples. The negative y-intercept is expected in view of the known chromophoric interferences with Jaffé methods [19]. The slope of <1.0 suggests that the efficiency of the enzymatic creatinine conversion and/or the detection of ammonia is decreased somewhat in the serum samples relative to the aqueous standards used in these studies. Thus, before routine clinical use, further characterization of this bias will be required.

Conclusions

These studies demonstrate that the proposed on-line gas predialysis method can be used to remove virtually 100% of normal and elevated levels of endogenous ammonia from physiological samples. This approach offers significant advantages over other ammonia abatement methods in that no additional reagents or secondary enzyme systems are required. In view of
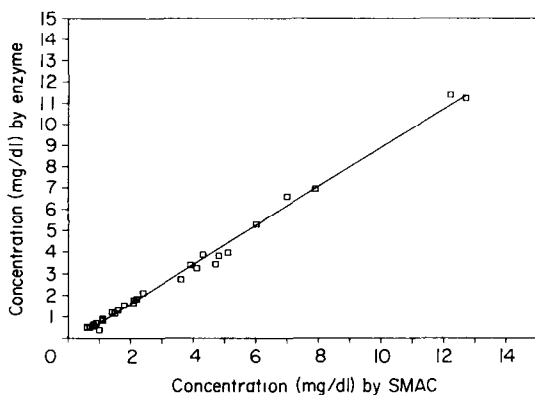
Fig. 5. Results from correlation study between the enzyme-based continuous-flow method and the Technicon SMAC colorimetric method. The SMAC assays were run at the Chemical Pathology Laboratory, University of Michigan Hospital. $N = 30$; slope = 0.91 ± 0.02; intercept = -0.20 ± 0.07; correlation coefficient = 0.9966, standard error of estimate = ± 0.24 mg dl^{-1}.

the large number of selective enzymes which liberate ammonia, the system described here should offer a general approach for the selective determination of a variety of biomolecules. Furthermore, it is likely that this concept could be extended to the removal of endogenous carbon dioxide from biological samples thereby enabling the adaptation of decarboxylating enzymes to the development of analogous bioanalytical systems.

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