Implication of Creatinine and Gut Flora in the Uremic Syndrome: Induction of “Creatininase” in Colon Contents of the Rat by Dietary Creatinine

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“Creatininase” activity was induced in the colon flora of rats by feeding creatinine. Sarcosine and methylamylamine were tentatively identified as the major products of the enzymatic action. A third (unidentified) compound was observed. These compounds and methylguanidine were labeled in the urine of rats given $^{14}$CH$_3$-creatinine orally. The results suggest that creatinine should not be considered biologically inert in animals, especially in those with decreased renal function.

Additional Keyphrases: $^{14}$CH$_3$-creatinine • methylguanidine • sarcosine • methylamylamine • uremic toxins • ion-exchange chromatography • urea metabolism in hepatic coma

Various nitrogenous compounds found in body fluids of uremic patients have been considered as possible uremic toxins. Recently, attention has focused on methylguanidine and guanidinosuccinate. Although serum creatinine increases to very high concentrations in chronic uremia, it has been used only as an indicator of decreased renal function and has not been suggested as a possible precursor of uremic toxin because it has been considered metabolically inert. This concept bears reinvestigation.

Dubos and Miller (1) first induced in bacteria isolated from soil the ability to degrade creatinine and proposed that this activity be used to measure “true” creatinine. Since then, this enzymic activity has been demonstrated in a number of microorganisms cultured on creatinine. Roche et al. (2) obtained sarcosine and urea from creatinine in cultures of Pseudomonas ovalis, hence the name creatinine aminohydrolase (EC 3.5.3.3). More recently, Van Eyk et al. (3) identified methylguanidine and acetic acid when Pseudomonas stutzeri was incubated with creatinine under aerobic conditions. In anaerobic incubations they found methylguanidine and acetic acid in a ratio of 1:1, urea, and variable amounts of ammonia, but no sarcosine. They concluded that the action of the “creatinase” was not fully understood.

Thus, creatinase can be induced in at least some microorganisms, but the products obtained from its action depend on the incubation conditions and may depend on the specific microorganism. If induction of such activity could be demonstrated in the gastrointestinal tract of an intact animal, it would suggest the possibility that any creatinine, a freely diffusible substance, that enters the lower gastrointestinal tract is catabolized.

In this communication, we describe the induction of creatinase in the colon contents of intact rats fed creatinine and the identification of some products of the enzyme action; also, we draw an analogy between the metabolism of creatinine in uremia and the metabolism of urea in hepatic coma.

Experimental Procedures

Male albino rats (Sprague-Dawley strain, supplied by our institutional colony), 150 to 250 g, were maintained in individual wire-bottomed cages at 21° to 23°C, with food and water freely available. Diets were “Rat Chow” [Rockland Mouse/Rat diet (complete); Teklad, Winfield, Iowa], with or without an added 10 or 20 g of crystalline creatinine (General Biochemicals, 950 Laboratory Park, Chagrin Falls, Ohio) per kilogram.

A 10-fold dilution of homogenate of gut contents or equivalent, isolated by fractional centrifugation, in phosphate buffer (33 mmol/liter, pH 7) with 25 mg of creatinine added per 100 ml was incubated for 1 h at 37°C. The supernatant fluid and upper layer from an initial centrifugation for 3 min at about 117 × g were resuspended in buffer and recentrifuged at 800 × g for 8 min, and the pellet, which consisted mainly of bacteria, was resuspended in phosphate buffer. Incubation was stopped by adding HClO$_4$ to a final concentration of 0.12 mol/liter; this mixture was then filtered and neutralized with K$_2$CO$_3$. The resulting supernatant fluid was either assayed for creatinine by reaction with alkaline picrate (Technicon Auto-Analyser File N-11b, Technicon Instruments Corp., Tarrytown, N. Y. 10591) or chromatographed to identify the products.

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Creatinine-\(^{14}\)CH\(_3\) (Mallinckrodt/Nuclear, P.O. Box 5439, St. Louis, Mo.) was added to some incubation mixtures to aid in the identification of products (this label was chosen because we believe that creatinine was a likely precursor of methylguanidine, a toxic compound that has been implicated in the uremic syndrome (4)). Label position was proved by subjecting the labeled creatinine to van Pilsum's \(\alpha\)-nitrobenzaldehyde procedure (5) for determining creatinine. Of the label, 89% was in the methylguanidine isolated by ion-exchange chromatography of the final reaction mixture, about the same amount we obtained by this chemical method of analysis of unlabeled creatinine; 5% remained in creatinine and 6% was in a third peak, presumably the intermediate compound in this reaction sequence, oxalylmethylguanidine (6). On ion-exchange chromatography of the standard, 99% of the radioactivity traveled as a single symmetrical peak, coincident with that for creatinine.

The ion-exchange chromatographic system used to measure the products was a modification of that described by Durzan (6). We used a Beckman Model 120B amino acid analyzer (Beckman Instruments, Inc., Palo Alto, Calif.) with Beckman resin type 50A and 150A, and the effluent was monitored by reaction with either Sakaguchi reagent or ninhydrin; we added a flow cell in a Model 314E liquid scintillation counter (Packard Instrument Co., 2200 Warrenville Rd., Downers Grove, Ill.) to allow monitoring of compounds containing carbon-14. A complicating factor was the extreme difference in retention times between some very acidic compounds, such as urea, and basic ones, such as methylguanidine. As a result, the most acidic compound(s) has not yet been positively identified. The second ion-exchange chromatography system was the gradient elution system of Piez and Morris (7), as described for physiologic fluids, with an amino acid analyzer (Phoenix Precision Instrument Co., Philadelphia, Pa.).

**Results**

**Experimental conditions.** When creatinine (10 or 20 g/kg of food) was fed to the rats, their large intestinal contents and feces readily degraded creatinine (Figure 1). Specimens from rats fed the control diet did not display this activity. More activity was found in those rats fed the diet richer in creatinine and when the assay incubation was under anaerobic conditions.

The activity could not be demonstrated in contents of cecum, small intestine, liver, kidney, or other tissues from rats fed creatine. The activity was confined to the bacteria-containing portion of the gut contents and was very labile when exposed to air. Attempts to separate the activity from the cells by sonication destroyed the activity. Limited studies indicated that the buffer for maximal activity, either in homogenates of complete contents or in a bacterial fraction isolated by fractional centrifugation, is phosphate buffer (33 mol/liter, pH 7), conditions similar to those used by Dubos and Miller (1). If pH was altered from 6.2 to 7.8, malate buffer substituted, or divalent cations added, activity was not significantly changed.

**Factors affecting induction.** Figure 2 illustrates the induction of enzyme activity with time. Although activity markedly varied among rats at 1 and 2 days, all animals had high enzyme activity at four days and approached the activity observed at longer intervals after 8 days on the diets. Interestingly, the blank tubes had higher alkaline picrate values at the earlier intervals than at day 8, which presumably indicates that some of the dietary creatinine was unaffected during the passage before the enzyme was induced.

From Table 1 it is apparent that proportionately less creatinine was enzymatically altered when the incubation mixture was under air than when it was under nitrogen, and that the decrease was directly reflected in the amount of the compound appearing as peak 3. We have tentatively identified peak 1 as sarcosine by retention times on two ion-exchange columns. It reacted with ninhydrin as does sarcosine, and the 440/570 nm absorbance ratio of the ninhydrin reaction product is similar to that of sarcosine. Peak 2 is unidentified at present. Peak 3 appears to be methylamine; the compound traveled in two ion-exchange chromatogra-
quantitatively recovered (91%, by chemical determination), we assume that it is not an intermediate under these conditions.

**Radiochemical experiments.** We added $^{14}$CH$_3$-creatinine to an incubation mixture and took aliquots at 15, 30, 60, and 90 min. The aliquots were chromatographed and the labeling of the compounds was determined (Figure 3). The products identified as sarcosine and methylamine increased with time. Peak 2 was highest immediately after the labeled creatinine was added to the incubation mixture. These data are consistent with the conclusions that peaks 1 and 3 are end products and peak 2 may be an intermediate.

To determine if similar products would be formed in the intact rat, we fed rats control or creatinine-containing diets for two weeks and then gave them 5 μCi of $^{14}$CH$_3$-creatinine by stomach tube. The urine was collected on solid carbon dioxide for 20 h. Table 2 gives the distribution of label in the fractions, which were determined as before. The data are expressed as percentages of total quantity of radioactivity recovered in the urine specimen; 50 to 65% of the label administered was recovered in these experiments. Most of the radioactivity was recovered within the first 4 hours in unchanged creatinine, traces appeared in peak 2 and in methylguanidine, and about 4% appeared in peak 1. On rechromatography of peak 1 in a more acidic system, it was resolved into sarcosine (30%) and more acidic presarcosine peaks (70%). About 8 and 12% of the label was recovered as methylamine in control and creatinine-treated animals, respectively. If creatinine is catabolized in the intestine of the intact rat as we observed in the in vitro incubation—that is, much greater quantities being metabolized to peak 1 (sarcosine) than to peak 3 (methylamine)—a considerable quantity of the sarcosine may be metabolized by the rat to the more acidic compound found in the urine.

**Discussion**

Catabolism of creatinine by the microflora of the large intestine could be physiologically signifi-

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**Table 1. Labeled Products from Incubations of Colon Contents of Rats Fed Creatinine with $^{14}$CH$_3$-Creatinine: Typical Experiment**

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Incubation atmosphere</th>
<th>Label in ion-exchange fraction, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Standard</td>
<td>N$_2$</td>
<td>0</td>
</tr>
<tr>
<td>A</td>
<td>N$_2$</td>
<td>63</td>
</tr>
<tr>
<td>B</td>
<td>N$_2$</td>
<td>65</td>
</tr>
<tr>
<td>A</td>
<td>Air</td>
<td>60</td>
</tr>
<tr>
<td>Peak 1*</td>
<td>N$_2$</td>
<td>99.2</td>
</tr>
<tr>
<td>Peak 2*</td>
<td>N$_2$</td>
<td>54.6</td>
</tr>
<tr>
<td>Peak 3*</td>
<td>N$_2$</td>
<td>0</td>
</tr>
</tbody>
</table>

* Peaks 1, 2, and 3 were isolated from incubation mixtures and added to fresh incubation mixtures of colon contents.

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**Table 2. Labeled Products in Urine of Rats Given $^{14}$CH$_3$-Creatinine by Stomach Tube**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Label in ion-exchange fraction, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Control</td>
<td>3.7</td>
</tr>
<tr>
<td>2% creatinine</td>
<td>3.7</td>
</tr>
</tbody>
</table>

* Mean value, two rats/treatment; similar distribution of label was observed in collections made in the interval 0 to 16 or 0 to 24 h after the administration.

* Peak "1" resolved to sarcosine (30%) and presarcosine (70%) peaks in a more acidic chromatography system.

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**Fig. 2. Induction of creatininase activity in intestinal contents of rats fed 1 (open circles) or 2 g (solid circles) of creatinine per 100 g of diet. Points are means, with range for four rats.**
significant for two reasons: it would explain the fate of the 55 to 70% of the creatinine that previously was unaccounted for in an anuric individual; and microbial action could lead to formation of toxic products from creatinine that, in the presence of decreased renal function, would be retained.

Creatinine has been considered to be metabolically inert in animals, probably because Mackenzie and du Vigneaud (8) showed that the methyl group did not appear as $^{14}$CO$_2$ when $^{14}$CH$_3$-creatinine was administered orally or parenterally to rats. Also, Bloch and Schoenheimer (9) demonstrated, using $^{14}$N-labeled creatinine, that it was not transformed to creatine and that most of the creatinine given to animals was immediately recovered unchanged in the urine. Serum creatinine has been observed to increase at the predicted rate of about 2 to 3 mg/100 ml/day in acute anuria. However, in chronic anuric uremia, the rate of observed increase is 1 mg/100 ml/day or less, about one-half to two-thirds of the expected creatinine accumulation. This decreased rate of creatinine accumulation and the deficit in urinary excretion become apparent when serum creatinine concentration exceeds 6 mg/100 ml and neither appears to result from a decrease in the mass of total body muscle (10).

Dominguez and Pomerene (11) showed that about 80% of creatinine, administered orally, was absorbed from the small intestine, while creatinine injected intravenously was almost quantitatively recovered in the urine. Goldman (10) found that the daily fecal excretion of creatinine did not exceed 23 mg/day, even in a patient with a serum creatinine concentration of 27.8 mg/100 ml. These data have led some to conclude either that there is an as-yet-undescribed pathway for creatinine catabolism or that creatinine exerts a negative feedback control on creatinine formation. The latter idea is less acceptable, because creatinine is formed nonenzymatically from creatine and creatine phosphate, and there are no additional data to support a feedback concept.

Walser and Bodenlos (12) estimated that at least 25% of the urea formed in normal man diffuses into the gastrointestinal tract, where it is hydrolyzed to ammonia and CO$_2$ by bacterial urease. The ammonia normally is reabsorbed and incorporated by the liver into amino acids for reuse or into the urea cycle. When the liver is diseased, the load of ammonia exceeds the capacity for fixing ammonia, or the ammonia by-passes the liver and the blood ammonia concentration increases. This scheme for the metabolism of ammonia in hepatic coma is supported by much indirect evidence. For example, ammonia production decreases when the character of the microflora has been altered by feeding poorly absorbed antibiotics or lactulose, or by the administration of enemas. Decreasing the protein content of the diet also decreases ammonia production.

The creatinine space is equivalent to the total body water (13), which indicates that creatinine (like urea) is freely diffusible across membranes, although the rate of exchange across at least one membrane, chorioamnion, is somewhat slower than that of urea (14). Therefore, creatinine (like urea) should diffuse into the gut where it might be catabolized. The total quantity of creatinine involved at a normal serum concentration of 1.0 mg/100 ml may be negligible but, when the concentration increases to 15 mg/100 ml, as is observed in uremia, the quantity available for recycling through the gastrointestinal tract becomes significant.

Further, that the suggested phenomenon (excretion into the gastrointestinal tract and induction of catalytic activity) may be a common occurrence is supported by the following observations. Brown et al. (15) recently demonstrated an increase in bacterial ureases in uremic men that should "... fortuitously prevent availability of the enzyme becoming a limiting factor in the recycling of urea nitrogen." Sorensen (16) demonstrated that about 66% of administered uric acid was recovered in the urine of normal persons, whereas in gouty persons, only 46% was recovered; the remainder was metabolized by the microflora of the gastrointestinal tract into allantoin, allantoic acid, urea, and CO$_2$, or was incorporated into the bacteria.

Although the gut has been suggested as the source of toxins by a number of investigators, to our knowledge no one has implicated creatinine as a toxin precursor. The products sarcosine and methyamine are not notably toxic to normal animals. It is to be expected that other products might appear from further catabolism of the initial
catabolites of creatinine in the gut of uremic patients since the patient whose renal function is decreased is in effect nearly a closed system. One cannot extrapolate from the products formed in a normal rat to those presumably formed in an anuric patient with altered nitrogen metabolism, but the results are suggestive. In the uremic patient with an altered nitrogen metabolism, the products of creatinine metabolism may assume physiologic significance. An estimated 500 to 750 mg of creatinine could be catabolized per day. Accumulation of the resulting products would be undesirable if they were toxic or had the ability to alter any metabolic pathway.

References