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Research Article

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Osmoprotective Activity for *Escherichia coli* in Mammalian Renal Inner Medulla and Urine

Correlation of Glycine and Proline Betaines and Sorbitol with Response to Osmotic Loads

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Abstract

Escherichia coli are protected against hypertonic NaCl by human urine. We have shown that this is due in part to the presence of glycine betaine and proline betaine. Several investigators have proposed that betaines and sorbitol are concentrated in the cells of the renal inner medulla where they exert a protective role against urea and extracellular osmotic forces. *E. coli* was used in the present studies as an "osmosensor" to detect osmoprotective activity in mammalian tissues. The greatest activity was found in extracts of renal inner medulla and to a lesser extent in the renal outer medulla and cortex of several mammalian species. Liver extracts were more active than other nonrenal tissues. Bacterial osmoprotective activity and concentration of glycine betaine in the renal inner medulla of rabbits were found to correlate closely with urinary osmolarity. Concentrations of sorbitol were found to be also increased in the renal inner medulla during osmotic stress, but this compound is not osmoprotective for *E. coli*. Glycine and proline betaine were recovered in urine of rabbits and were increased in those given high osmotic loads. Only small amounts of proline betaine were recovered in the renal inner medulla. The source from which proline betaine is derived is unknown.

Introduction

All forms of life must adapt to alterations in the osmotic forces of their external environment. Bacteria respond through osmotic tolerance genes triggered by osmosensitive proteins located on the outer membrane (1). One of these responses is active transport or synthesis of osmoprotective molecules. These include glycine betaine, proline, and glutamine (2). They are termed "compatible solutes" because they accumulate intracellularly in the response to external solute concentrations and protect the cell from dehydration. Of these glycine betaine is the most important. It is a zwitterion that is highly soluble in water. Intracellular accumulation of glycine betaine accounts for the resistance of halophilic bacteria to high external osmotic pressure (3). Similar mechanisms of cell volume regulation have evolved in marine algae (4).

In multicellular animals osmoregulation is achieved by

several mechanisms. These include alterations in the amino acid pool in the erythrocytes of the shark and in the muscle fibers of crab *Hemigrapsus edwardsi* (5), accumulation of glycine betaine in cells of *Limulus polyphemus* (6) and proline betaine in the extremely euryhaline mollusc *Elysia chlorotica* (7). The intracellular fluids of urea-rich fish accumulate trimethylamine-N-oxide and glycine betaine (8). Somero (9) has termed this the "counteracting solute" strategy to denote the protection of proteins from perturbation by urea.

Several groups (9–13) have shown that trimethylamine compounds, including glycerophosphoryl choline and glycine betaine, are located in the inner renal medulla of mammalian tissues. Bagnasco et al. (13) have reported that these compounds, as well as sorbitol and inositol, increase in concentration in the inner renal medulla of rats and rabbits during anti-diuresis. They suggested that these substances play a significant role in the maintenance of intracellular osmotic balance. We arrived at a similar hypothesis based on observations that human urine is even more osmoprotective for bacteria than is glycine betaine (14). We have shown that human urine contains relatively large amounts of glycine betaine and proline betaine (15). Because choline is transported into tubular cells where some of it undergoes oxidation to glycine betaine we postulated that glycine betaine may accumulate intracellularly and thereby protect the renal tubular cells from high osmotic forces generated as the glomerular filtrate becomes increasingly concentrated within the tubular lumen. This would provide a potential source for the glycine betaine in the urine (16). *Escherichia coli* may be used as a "sensor" to detect osmoprotective activity in vertebrate tissues. In this report we will show that bacterial osmoprotective activity is particularly abundant in the renal inner medulla of several species of animals. Osmoprotective activity in rabbit renal inner medullae is decreased during diuresis and increased under conditions of dehydration and increased solute load. The activity is associated with accumulation of glycine betaine. These results are in close accord with those of Bagnasco et al. (13) and provide a striking parallel between adaptive mechanisms in microbes and those in more complex forms of life. Accumulation of osmoprotective agents may provide an additional explanation for the remarkable susceptibility of the renal inner medulla to infection (17).

Methods

Animal studies. Tissues were taken immediately after sacrifice of healthy animals of several species. Adult mongrel dogs weighing 12–18 kg were sacrificed by intravenous injection of thiopentone. Male Sprague-Dawley rats weighing ~350 g were sacrificed with carbon dioxide. A pig, cow, and two sheep were killed by exsanguination. One sheep was deprived of water for 3 d before sacrifice and another was given water freely. 26 10-wk-old New Zealand rabbits weighing 2.1–2.4 kg were divided into four groups and housed in separate metabolic cages. All had free access to rabbit chow. The fluid regimens were as

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follows: group A received 2% glucose in its drinking water and subcutaneous injections of 100 ml of 5% glucose twice daily; group B received unrestricted tap water; group C was deprived of water; and group D received 3% NaCl in drinking water. After 3 d on these diets the animals were sacrificed with carbon dioxide. Immediately after death urine was aspirated from the bladder, tissues were removed, washed with normal saline sufficiently to remove blood, and blotted dry. The kidneys were frozen and stored at -40°C immediately after harvesting. The frozen kidneys were bisected along the longitudinal axis. The tip of the inner medulla was grasped with forceps and then amputated at its base with a scalpel. This tissue was termed inner medulla. Cortical tissue was dissected from the outer medullary strip. The remaining tissue was termed outer medulla. All tissue sections were stored at -40°C . The protocol for these studies was approved by the Animal Experimentation Committee of the Ohio State University.

Detection of bacterial osmoprotective activity. The osmosensitive bacterium used in these experiments was a strain of *E. coli* No. 31, which had been isolated from a patient with urinary tract infection. Its response to osmotic conditions has been well defined (14). The organism grows in the presence of up to 0.7 M NaCl in minimal medium and to 0.9 M NaCl in the presence of 1×10^{-3} M of glycine betaine. The minimal growth medium contained glucose, ammonium sulfate, and trisodium citrate as the only sources of carbon and nitrogen (18). pH of the medium was adjusted to 7.0 before use. Bacterial inoculum consisted of 0.1 ml of a 1:1,000 dilution of an overnight culture, grown in minimal medium, into 0.8 ml of minimal medium plus 0.1 ml of sample. This provided about 1×10^5 colony-forming units. The culture was incubated at 37°C , and after 48 h turbidity was determined by measuring the optical density at 400 nm using a spectrophotometer (Spectronic 20, Bausch and Lomb, Rochester, NY).

Measurement of osmoprotective activity in tissues and urine. In experiments with tissues from dogs, rats, cows, and pig, the tissues were homogenized in water (Brinkmann Instruments Co., Westbury, NY). In all subsequent experiments, perchloric acid extracts were used with identical results. Tissues were homogenized in 1 M perchloric acid and centrifuged at 4°C at 1,000 g for 20 min. The supernates were neutralized with 2 M KOH and centrifuged for 10 min. This produced a 1-in-10 dilution of the tissue and urine. Osmoprotective activity was determined as the highest initial twofold dilution of tissue extracts that permitted the test strain of *E. coli* to grow in the presence of 0.9 M NaCl.

Measurement of glycine betaine and proline betaine in tissues and urine. This was performed by the methods described previously (15). Ammonium reineckeate solution was added to an equal volume of sample and alkalinized with sodium triphosphate. After 2 h in an ice bath the precipitate was removed by centrifugation and discarded. The supernatant was acidified with hydrochloric acid. After a further 2 h in an ice bath the reaction mixture was centrifuged and the supernatant was discarded. The sediment containing the reineckeate-betaine complex was extracted with an equal volume ethyl acetate. The phases were then separated by centrifugation. This process was repeated until all of the reineckeate was removed from the aqueous phase. The aqueous phase was chromatographed on a 0.7×8 -cm Dowex cation exchange column. The resin was prepared by washing with 1 N HCl (1 liter) and then with triple-distilled water until the pH of the eluent was 7.0. The column was eluted with 15 ml of distilled water and then 0.1 M NaCl. The biologically active fractions were pooled, lyophilized to dryness, extracted with methanol, dried under nitrogen, and dissolved in water. This was subjected to reverse-phase high-performance liquid chromatography (HPLC) using a 1080 B instrument (Hewlett-Packard Co., Palo Alto, CA) with a semipreparative reverse-phase column (C3, 10 micron, absorbosphere, 25 cm \times 10 mm) and an analytical reverse-phase column (C 18, 5 micron, nucleosil, 25 cm \times 4.6 mm) at room temperature (Alltech Associates, Inc., Deerfield, IL). The mobile phase was pure water (HPLC grade, Burdick & Jackson Laboratories, Inc., Muskegon, MI). A continuous record of ultraviolet (UV) absorbance (205 nm) of eluent was made on a chart recorder. Timed fractions of the eluent from the semipreparative column were evaporated under

vacuum. These were redissolved in water, and glycine betaine and proline betaine were measured quantitatively by analytical reverse-phase chromatography. The identity of the UV light absorbance peaks was made by co-elution with standards of glycine betaine and proline betaine added to the sample and confirmed by bioactivity. Quantitation was made by comparing the UV light absorbance produced with a 10- μl injection of the sample with a standard curve generated by 10- μl injections of standard solutions. The sensitivity of the assay for glycine betaine and proline betaine was $0.5 \mu\text{mol/g}$ of tissue or ml of urine. To increase the sensitivity of the assay, 1.0 ml of urine was extracted from the saline-treated and water-deprived animals, 5 ml from those receiving water freely, and 10 ml from rabbits receiving glucose.

Composition and osmolarity of tissues and urine. Tissues were homogenized (Brinkmann Instruments Co.) in water, and tissue debris was removed by centrifugation. Content of urea and electrolytes were assayed by the Clinical Chemistry Laboratory at Ohio State University Hospitals. Osmolarity was determined by freezing point depression. Sorbitol was assayed in perchloric acid tissue extracts using a commercial kit (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Chemicals. Chemical reagents were of the highest purity available. Glycine betaine and ammonium reineckeate were purchased from Sigma Chemical Co., St. Louis, MO. Proline betaine was synthesized by the method of Cornforth and Henry (19).

Statistical tests. Statistical analysis was done using the SAS computer program (Statistical Analysis System, SAS Institute Inc., Cary, NC). The biological activity in tissue extracts of various animals was analyzed by one-way analysis of variance and differences between sites by the Tukey method. The data from the rabbit tissues were analyzed for dependence and then by two-way analysis of variance with repeated measures. Differences between individual groups of data were assessed by the Tukey-Kramer method. The correlation coefficients were computed using the same program.

Results

Bacterial osmoprotective activity in animal tissues. Aqueous extracts of tissues obtained from dogs, rats, hog, cow, and sheep were tested for the highest dilution that permitted *E. coli* to grow in the presence of 0.9 M NaCl. In control experiments, using minimal medium alone, the organism grew to 0.7 M NaCl. Osmoprotective activity as measured by bacterial growth in 0.9 M NaCl was detected in all tissues. In the kidneys of virtually all the animals there was a progressive increase in activity from the cortex to the outer medulla to the inner medulla (Fig. 1). The greatest activity was found in renal inner medullae of rats. The inner and outer medullae for all

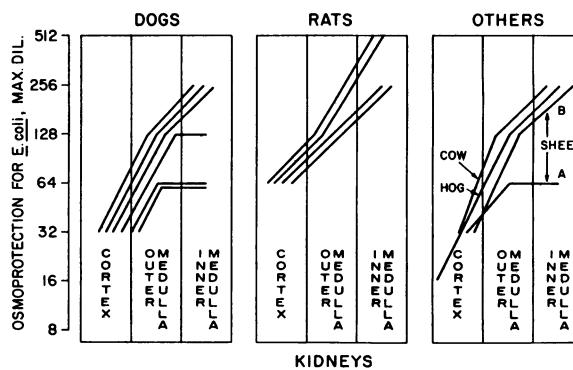


Figure 1. The maximum twofold dilution of aqueous extracts of sections of renal tissues from various animals which permitted growth of *E. coli* in hypertonic NaCl (0.9 M). Sheep A was given water ad lib, whereas sheep B was deprived of drinking water for 3 d.

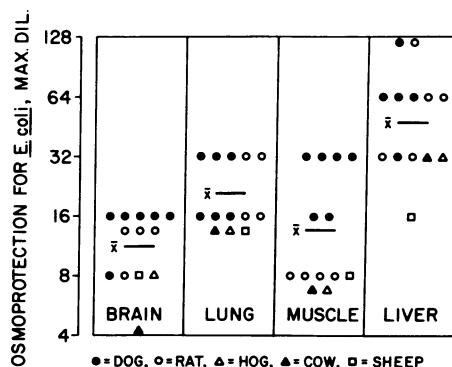


Figure 2. The maximum twofold dilution of aqueous extracts of brain, lung, muscle, and liver from various animals which permitted growth of *E. coli* in hypertonic NaCl (0.9 M).

animals combined were significantly more active than all other tissues ($P < 0.05$ compared with liver, $P < 0.01$ for other tissues including renal cortex).

Extracts of brain, lung, and muscle were found to be protective at dilutions of 1:4–1:32, for *E. coli* grown in 0.9 M NaCl (Fig. 2). Liver extracts were significantly more protective than other nonrenal tissues. The maximum protective dilutions of liver ranged from 1:16 to 1:128 ($P < 0.01$ for liver compared with muscle and brain).

Effect of water deprivation in sheep on osmoprotective activity and concentrations of sorbitol in the kidney. In a preliminary experiment osmoprotective activity was measured in kidney tissues of a sheep maintained on a regular water intake and a sheep deprived of drinking water for 3 d (Fig. 1 and Table I). The osmolality in the inner medulla and urine was higher in the dehydrated animal. This was associated with greater bacterial osmoprotective activity in the outer medulla and inner medulla and higher concentrations of sorbitol in the inner medulla.

Effect of diuresis, fasting, and a salt load on renal osmoprotective activity in rabbit kidney. Groups of rabbits were fed diets which varied in content of water, glucose, and sodium

Table I. Effect of Dehydration on Renal Osmoprotective Activity for *E. coli* and Concentrations of Sorbitol in Various Regions of the Kidney in Sheep

Sheep	Cortex	Outer medulla	Inner medulla	Urine
Water ad lib				
Osmoprotection*	32	64	64	
Sorbitol ($\mu\text{mol/g}$)	1.3	1.3	14.4	
Osmolality (mosmol/kg)	330	460	560	885
Dehydrated [†]				
Osmoprotection*	64	128	256	
Sorbitol ($\mu\text{mol/g}$)	<0.08	0.8	54.1	
Osmolality (mosmol/kg)	500	480	940	2,060

* Reciprocal of maximum dilution which permitted *E. coli* to grow in 0.9 M NaCl. [†]Deprived of water for 3 d.

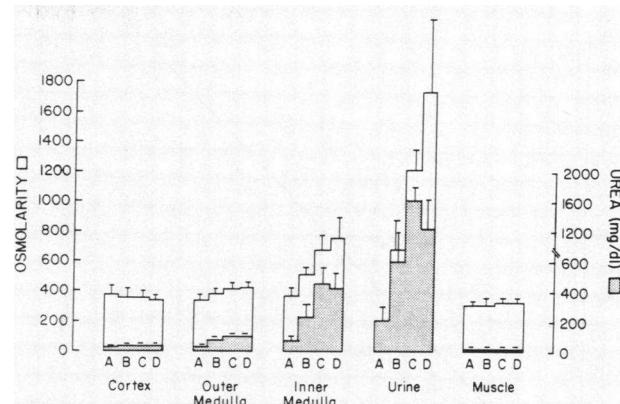


Figure 3. The osmolality and concentration of urea (± 1 SD) in sections of renal tissue, urine, and muscle in rabbits, given various osmotic loads for 3 d. A received 2% glucose in drinking water and subcutaneous injections of 100 ml of 5% glucose twice daily, B received unrestricted tap water, C was deprived of water, and D received 3% NaCl in drinking water. Significant differences in the osmolality were observed in the inner medulla (glucose vs. all other groups, $P < 0.05$; unrestricted water vs. no water and NaCl, $P < 0.05$) and urine (between all groups $P < 0.05$). Significant differences in the urea concentration were observed in the inner medulla (between glucose vs. NaCl, and glucose vs. no water, $P < 0.05$) and the urine (NaCl vs. all other groups and no water vs. all other groups, $P < 0.05$).

chloride. As shown in Fig. 3, as the osmolar load in the diet was progressively increased there was a corresponding graded increase in osmolality in the renal inner medullae and urine. Increase in osmolality was less pronounced in the renal outer medulla. There was no detectable change in the osmolality or concentration of urea in renal cortex or muscle.

Bacterial osmoprotective activity in rabbit kidney, urine, and muscle was examined in each of the groups of rabbits (Fig. 4). A stepwise increase in osmoprotective activity was noted

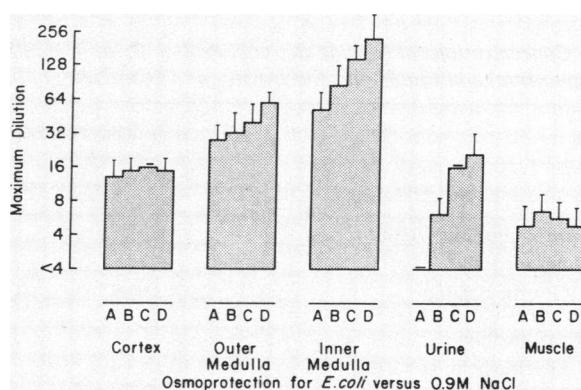


Figure 4. The maximum twofold dilution (± 1 SD) of perchloric acid extracts of sections of renal tissues, from rabbits given various osmotic loads for 3 d, which permitted growth of *E. coli* in hypertonic NaCl (0.9 M). (See Fig. 3 for identification of groups of animals.) There were no significant differences between any of the groups of animals in the renal cortex and muscle. In the outer medulla significant differences were observed between those given glucose vs. NaCl and unrestricted water vs. NaCl, $P < 0.05$. In the inner medulla glucose vs. NaCl, glucose vs. no water, and unrestricted water vs. NaCl, were significantly different, $P < 0.05$. In the urine all groups were different except no water vs. NaCl, $P < 0.05$.

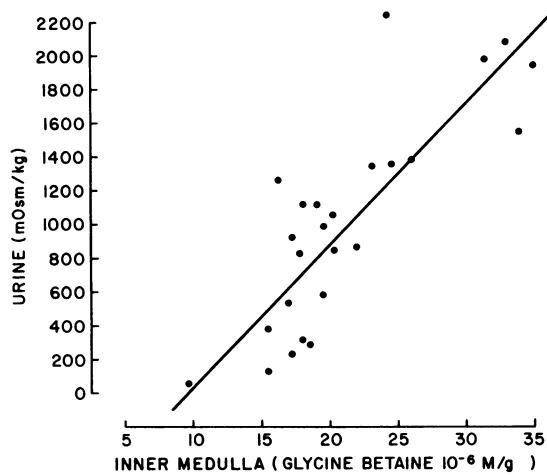


Figure 5. Correlation between urinary osmolarity and concentration of glycine betaine in the renal inner medulla of rabbits given various osmotic loads. The line of regression is $y = 82.9$, $x = 775.1$, $r = 0.79$, $P < 0.001$.

from cortex to outer medulla to inner medulla. A graded increase in osmoprotective activity for *E. coli* was noted in the outer medulla and inner medulla and in the urine in response to the increased osmotic loads. In contrast, there was no alteration in osmoprotective activity in renal cortex or muscle in the same experimental groups.

Effect of different osmotic loads on concentrations of glycine betaine, proline betaine, and sorbitol in rabbit kidney. Glycine betaine was present in the renal outer medulla and in much higher amounts in the inner medulla, but could not be detected at the level of sensitivity of the assay (0.5 $\mu\text{mol/g}$) in the cortex (Fig. 5, Table II). There was a significant graded increase in concentrations of glycine betaine in the renal outer medulla and inner medulla in response to increasing osmotic loads (Table II). Proline betaine was detectable in small

amounts only in the inner medullae of rabbits deprived of water or given 3% NaCl in their drinking water.

Sorbitol could not be detected at the level of the sensitivity of the assays (0.08 $\mu\text{mol/g}$) in the renal cortex or outer medulla of any of the groups of rabbits. Although concentrations of sorbitol were higher in the renal inner medulla of animals deprived of water, this was found not to be significantly different than for other groups (Table II).

Relation between urinary osmolarity and concentration of glycine betaine in renal inner medullae. The concentration of glycine betaine in the renal inner medulla of each of the rabbits was compared with the osmolality of their urines. There was a significant correlation ($r = 0.79$, $P < 0.001$) between urinary osmolarity and concentrations of glycine betaine in the renal inner medulla (Fig. 5).

Urinary excretion of glycine betaine and proline betaine in rabbits in response to osmotic loads. The osmolarity and concentrations of creatinine achieved in the urine of each group of rabbits according to osmotic load is shown in Fig. 6. As expected, the group receiving 3% NaCl exhibited the highest osmolarity, but concentrations of creatinine were similar to those in animals deprived of water. The concentration of proline betaine and glycine betaine in the urine of the rabbits increased in a stepwise manner with increased osmolarity of the urine (Fig. 7). To assess the relation between the concentration of betaines achieved in the urine with urinary osmolarity and concentrations of electrolytes, we performed a series of regression analyses (Table III). There was a significant correlation ($P < 0.001$) between urinary osmolarity and concentrations of sodium and potassium with those of the betaines. When the concentrations of the betaines were expressed as the ratio to creatinine versus osmolarity, a significant correlation ($P < 0.001$) remained for glycine betaine but not for proline betaine. Excretion of proline betaine was more closely correlated with that of creatinine than was glycine betaine. Glycine betaine appeared to be related independently to osmolarity (Table III).

Table II. Concentrations of Glycine Betaine, Proline Betaine, and Sorbitol in the Renal Outer Medulla and Inner Medulla of Rabbits According to Intake of Fluids and Electrolytes and Osmolality of the Inner Medullae and Urine

	Composition of drinking water			
	2% Glucose	Tap water	None	3% NaCl
Compound ($\mu\text{mol/g}$ wet wt)				
Outer medulla*				
Glycine betaine	1.2 \pm 0.9	2.4 \pm 1.3	2.7 \pm 1.2	5.7 \pm 3.4
Inner medulla				
Glycine betaine	16.1 \pm 3.4	18.5 \pm 2.2	20.3 \pm 3.2	29.1 \pm 5.8
Proline betaine	<0.5	<0.5	0.5	0.9 \pm 0.5
Sorbitol	14.4 \pm 4.1	13.6 \pm 5.5	21.0 \pm 8.3	15.6 \pm 5.9
Osmolality (mosmol/kg)				
Inner medulla	376 \pm 53	523 \pm 43	683 \pm 103	761 \pm 141
Urine	212 \pm 106	689 \pm 231	1,208 \pm 129	1,741 \pm 451

* None of the compounds could be detected in the renal cortex. Only glycine betaine was detectable in the renal outer medulla. Significant differences observed for glycine betaine in the renal outer medulla were glucose vs. NaCl ($P < 0.05$), tap water vs. NaCl ($P < 0.05$). In the renal inner medulla, significant differences were glucose vs. NaCl ($P < 0.05$), tap water vs. NaCl ($P < 0.05$), no water vs. NaCl ($P < 0.05$). The concentrations of sorbitol in the renal inner medulla were not significantly different between any of the groups.

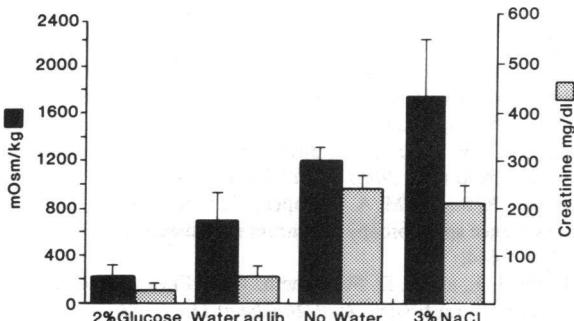


Figure 6. The osmolarity and concentration of creatinine (± 1 SD) in the urine aspirated from the bladder of rabbits after being given various osmotic loads for 3 d. Differences for osmolarity were significant between all groups, $P < 0.05$.

Discussion

This study reveals that extracts of mammalian tissues contain substances that protect *E. coli* against high concentrations of NaCl. The osmoprotective activity was greatest in the renal inner medulla, urine, and, to a lesser extent, in liver. This was associated with the quantitative recovery of glycine betaine and to a lesser extent with proline betaine in tissues and urine. These compounds are known to be osmoprotective for enteric gram-negative bacteria (1, 14). Thus it appears that osmotically stressed *E. coli* may provide a highly sensitive indicator of the presence of betaines in tissues and urine. Because liver is a major site of synthesis of glycine betaine (20) this probably accounts for its osmoprotective activity.

The tissue extracts of renal cortex, muscle, lung, and brain contained substances that protect *E. coli* against high concentrations of NaCl. This was not associated with recovery of glycine betaine or proline betaine. The only sites in which there was a correlation between increased biological activity and recovery of glycine betaine and proline betaine, to a lesser extent, was renal inner medulla and urine. Sorbitol was also found in the inner renal medulla, but it does not possess sufficient osmoprotective activity for *E. coli* to account for biologic

Table III. Correlation between Concentrations of Glycine and Proline Betaines with Osmolarity and Solutes in Urine of Rabbits Given Various Osmotic Loads

Constituent	Glycine betaine	Proline betaine
	r	r
Osmolarity vs. betaine	0.84*	0.90*
Osmolarity vs. betaine/creatinine	0.74*	0.17 [§]
Sodium vs. betaine	0.87*	0.88*
Potassium vs. betaine	0.79*	0.77*
Creatinine vs. betaine	0.55 [‡]	0.78*
Urea vs. betaine	0.74*	0.71*

* $P < 0.001$.

† $P < 0.01$.

‡ $P > 0.05$.

activity in this tissue (14). The differences in biologic activities at various tissue sites may be due to as yet undescribed osmoprotectant effects or to augmentation of the effect of small amounts of glycine betaine by nonspecific substances on growth of bacteria in minimal medium. The "nonspecific" or "background" osmoprotective activity of tissues other than renal medulla were not altered by changes in the osmotic load administered to the rabbits.

As the osmotic pressure in the urine increases there is a corresponding increase in content of glycine betaine in the inner medulla and to a lesser extent in the outer medulla. This is accompanied by increased concentrations of glycine and proline betaine in the urine. These results are in general accord with those of Bagnasco et al. (13), who also demonstrated increased concentrations of glycine betaine in the inner medulla of rabbit and rat kidneys in response to antidiuresis. They did not, however, detect glycine betaine in the urine. They reported increased concentrations of sorbitol in renal tissue during antidiuresis. We noted a concurrent increase in concentrations of sorbitol in the renal papilla of a dehydrated sheep and a trend toward increased content of sorbitol in the renal inner medulla in rabbits deprived of water. The role of proline betaine as a renal osmoprotective compound is much less clear. Only small amounts were detected in the renal inner medulla of dehydrated rabbits and those given a highload of NaCl. It is therefore unlikely that proline betaine has a significant role as an intracellular compatible solute in the rabbit kidney. The source of proline betaine recovered in both the urine of rabbits and man (15) is unknown. It is possible that it may be derived from the diet because it has been isolated from a variety of plants (21). The results of the present experiments support the notion set forth by ourselves and several other groups of investigators (8–10, 12–15) that the renal inner medulla has several mechanisms by which it accumulates osmoprotective compounds. The current concept is that glycine betaine is synthesized in the cortex and accumulates in the inner medulla, whereas sorbitol is synthesized in the inner medulla. These compounds may fill the "osmotic gap" observed by Beck et al. (22) in rat renal inner medullae. Several workers have suggested that betaines may also counteract the toxic effects of urea (8, 9). We propose that they serve to counteract external osmotic forces and in the case of the zwitterion, glycine betaine, may balance external charge forces as

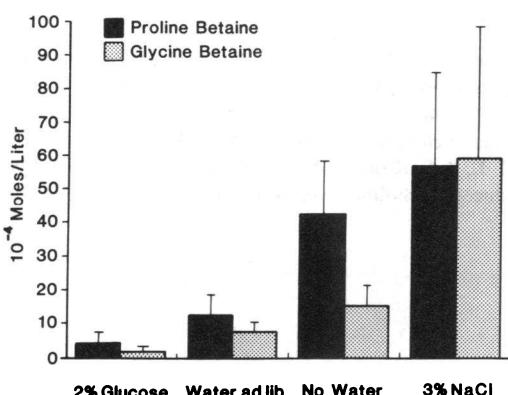


Figure 7. Concentrations of glycine betaine and proline betaine in the urine aspirated from the bladder of rabbits after being given various osmotic loads for 3 d. There were significant differences in glycine betaine between the NaCl and all other groups and proline betaine between glucose vs. no water, glucose vs. NaCl, between unrestricted water vs. no water and unrestricted water vs. NaCl ($P < 0.05$).

well. It is likely that the primary stimulus for the accumulation of compatible solutes is osmotic. We were not able, however, to eliminate urea as the primary stimulus because the saline diuresis did not significantly alter the concentration of urea in the inner medulla.

A clue to the pathogenetic significance of the presence of glycine betaine in the renal inner medulla may be found in the literature published in the 1940s on hemorrhagic kidneys observed in young animals that were rendered choline deficient (23–26). Hartcroft (23) described necrosis of the peripheral cortical tubules and degeneration of the deeper ones. The glomeruli were unaffected. Because choline is believed to be the principle source of glycine betaine in the kidney (16) it seems reasonable to suppose that choline deficiency would lead to glycine betaine deficiency as well.

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