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EXPERIMENTAL PYELONEPHRITIS

VIII. THE EFFECT OF ACIDIFYING AGENTS ON SUSCEPTIBILITY TO INFECTION†

INTRODUCTION

The experiments to be described were conducted with the aim of testing the suggestion of Beeson and Rowley,[1] that the peculiar susceptibility of the kidney to certain forms of bacterial infection may be dependent on local inactivation of complement by ammonia which is produced in renal tubular cells. This was based on the finding that kidney tissue has a considerably stronger anticomplementary action than other tissues because of inactivation of the fourth component of complement, a component known to be affected by weak concentrations of ammonia. Evidence was obtained that the kidney's anticomplementary action tended to parallel glutaminase activity. It appeared therefore that the hypothesis could be subjected to further test in living animals by determining whether stimulating ammonia formation by administration of acid loads increased susceptibility to bacterial infection in the kidney.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats of comparable age and weight were used as the experimental animals.

Bacterial strains

The strain of *E. coli* used has been maintained in this laboratory for five years and details of its handling have been described previously.[8] The standard infecting dose for animals was 0.5 ml. of a four-hour culture, containing 2-6x10^8 organisms per ml.

A hemolytic coagulase negative *Staphylococcus albus* of low virulence which had been obtained from a human throat was also used. Agar slant cultures were stored at 4° C. At weekly intervals a blood agar plate was inoculated and incubated; a single

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† Supported by a grant from the United States Public Health Service—(E—1850)
A preliminary report of this work was presented at the Henry Ford Hospital, October 1959, at the Symposium, *The Biology of Pyelonephritis* (in press).

Received for publication 3 October 1960.

colony from this was used to inoculate 10 ml. of trypticase soy broth. After 12 hours of incubation at 37° C. the broth contained 2-4x10^7 bacteria per ml. Many experiments with this organism have shown that no observable infection, in the kidneys or elsewhere, follows intravenous injection of 10^8 bacteria in normal animals. For the experiments involving animals with acid loads, the infecting dose was 0.5 ml. of the 12-hour culture, i.e. 1-2x10^7 organisms.

Enumeration of bacteria in organs

Under pentobarbital anesthesia, the abdominal and thoracic cavities were opened and the organs to be examined removed and placed in a Teflon tissue grinder. In the case of kidney or spleen, whole organs were used, whereas with the lung, approximately one gram of tissue was taken. Homogenates were made in 9 ml. of 0.9 per cent saline. Serial ten-fold dilutions were mixed with melted agar, placed in petri dishes, and incubated for 24-48 hours, after which, colony counts were made. Urine for culture was obtained by needle aspiration of the bladder at the time of sacrifice. One drop was streaked on a blood agar plate.

Renal glutaminase

Determinations for this enzyme were performed according to the method of Rector, Seldin, and Copenhaver[18] with the modification that KCN was not used.

Criteria of infection

Kidneys were considered to be infected when they contained 50,000 or more viable microbial units. They frequently showed gross evidence of abscess formation. Urine cultures were considered positive when 10 or more colonies were found on the streak plate. This is equivalent to at least 10^3 to 10^4 colonies per ml. of urine.

Diet and drinking solutions

The diet consisted of Purina Lab Chow pellets. The particular method of feeding will be described in connection with the individual experiments.

Solutions of various salts and amino acids for drinking water were made with tap water. Due to its relative insolubility, DL-methionine solution was prepared by dissolving 4.5 gm. in 100 ml. of hot water. This solution was stored (as was the DL-valine) in the refrigerator. The refrigeration of DL-methionine caused the precipitation of crystals which were then removed by filtration before giving the solution to the rats as drinking water. The precise concentration of DL-methionine in the drinking water probably did not exceed 3.38 gm./100 ml. water, since this is its solubility at 25° C.

Urine pH

An approximation of urine pH was obtained with nitrazene paper.

EXPERIMENTAL

The simplest method of administering acid to rats is to provide ammonium chloride solution in place of drinking water. The ammonium is converted to urea in the liver, leaving the chloride ion to be excreted by the kidney. This is, therefore, in reality a convenient way of administering hydrochloric acid.

It causes increased renal glutaminase activity and augments ammonia production by renal tubular epithelial cells.[8]

The first experiment was conducted with 12 animals drinking 2 per cent NH_4Cl and 6 control animals drinking an equal amount of water. These animals were fed the same amount of chow pellets. The average daily intake of NH_4Cl by the test animals was 8.8 millimoles.

TABLE 1.

	Colonies E. coli		*Gross*	*Urine*	*Blood urea N*	*Serum chloride*
	Left kidney	*Right kidney*	*abscesses*	*culture*	*mg.%*	*m.Eq/L*
	40,000	40,000	0	0		
	>1,000,000*	1,000,000*	+	+		
	>1,000,000*	1,000,000*	±	+		
	70,000*	2,000	0	0		
	30,000	200,000*	0	0		
2%	500	1,000,000*	±	+		
NH_4Cl	80,000*	200,000*	0	+	41	116
	600,000*	400,000*	+	0	35	118
	200,000*	80,000*	+	+	37	118
	400,000*	80,000*	+	+	43	122
	40,000	30,000	0	0	41	120
	50,000*	10,000	0	+	41	123
	3,000	700	0	0		
	400	0	0	0		
Water	0	0	0	0		
	200	600	0	0	48	107
	4,000	>1,000,000*	0	+	43	109
	2,000	1,000	0	0	33	110

* Infection.

The standard inoculum of *E.coli* was injected intravenously into the 18 animals on the third day, and all were killed seven days later. Gross inspection and culture of the kidneys revealed numerous instances of pyelonephritis among the acid-fed rats whereas only 1 of 12 kidneys in the control group contained a significant number of bacteria. Details of these results are shown in Table 1.

The concentration of NH_4Cl in the drinking water was found to be excessive, interfering with food and water consumption. During the period of the experiment weight losses were comparable; the test animals lost an

average of 30 per cent of body weight, as compared with 26 per cent in the pair-fed controls.

Because of the severe impairment of appetite and thirst which resulted from drinking 2 per cent NH_4Cl solution, the concentration of this salt was

TABLE 2.

	Colonies E. coli		*Gross*	*Urine*	*Blood urea N*	*Serum chloride*
	Left kidney	*Right kidney*	*abscesses*	*culture*	*mg.%*	*m.Eq/L*
	0	0	±		23	116
	1,000,000*	>1,000,000*	+		27	113
	10,000	0	0		21	117
	80	0	0		23	114
	0	9,000	±		22	114
1.6%	100	3,000	0		30	110
NH_4Cl	500	10	0	+	17	106
	100	40	0	0		
	40,000	60,000*	0	0	28	107
	400	400,000*	+		23	110
	7,000	>1,000,000*	+	+	22	116
	400,000*	30,000	0	+	19	111
	300	50	0		34	97
	90	0	0		32	98
	30	0	0		24	100
2.3%	0	2,000	0		29	101
$NH_4C_2H_3O_2$	0	0	0		34	102
	20	3,000	0		24	99
	0	0	0	0	15	98
	0	10	0	0	23	98
	0	10	0	0	16	102
	40	10	0	0	20	102
	30	0	0	0	23	103

* Infection.

reduced to 1.6 per cent in subsequent experiments. This was better tolerated and permitted the test animals to more nearly maintain their initial weights for the duration of the experiments.

Comparison of effects of ammonium chloride and ammonium acetate

To rule out an effect of the ammonium ion, an experiment was carried out in which animals drinking 1.6 per cent ammonium chloride were compared

with animals drinking equimolar ammonium acetate. This would supply the same amounts of NH_4^+, but the acetate would not produce systemic acidosis. The animals were comparably fed in groups of 6. *E. coli* were injected intravenously five days after the experiment began and bacteriological examination was carried out nine days later. As shown in Table 2, there were 6 infected kidneys in the NH_4Cl group and none in the ammonium acetate group. The average weight loss of the latter group was 9.3 per cent whereas the NH_4Cl group average weight was the same at the end of the experiment as it was at the start. Of the 5 infected animals, one had gained weight and only one had lost more than 10 per cent of its original body weight.

TABLE 3.

% Loss of body weight	*Number kidneys infected*
10	0/30
15	0/12
20	0/18
35-41 (LD-50)	1/10

The effect of NH_4Cl in increasing the susceptibility of the kidney appeared to be dependent upon the production of systemic acidosis with consequent increased renal NH_3 production rather than upon the administration of NH_4^+ in the diet.

Effect of inanition

Since many rats drinking NH_4Cl lost weight, it was necessary to determine whether inanition alone would effect the susceptibility of the kidney to infection with *E. coli.* A group of rats was therefore underfed and allowed drinking water freely. After four to ten days they were inoculated intravenously with *E. coli* and sacrificed four to seven days later. As can be seen in Table 3, only when an extreme degree of weight loss was achieved did one of the surviving animals develop infection.

Induction of acidosis two days after inoculation of bacteria

It has been demonstrated that *E. coli* persist in the rat kidney for several days after the blood culture has become negative.[8] The following experiment was designed to test whether bacteria persisting in the normal kidney at a time the blood culture would be expected to be negative could produce an infection during acid administration. Two days after the inoculation of 41 normal rats with *E. coli* intravenously, 11 were started on 1.6 per cent

NH_4Cl; the remainder received equimolar solutions of NaH_2PO_4, ammonium acetate, NaCl, or urea.* Four to eight days after the inoculation of organisms bacteriological studies were carried out. None of the 30 control animals had infection in the kidneys or urine, whereas of the 11 rats drinking NH_4Cl, 3 kidneys of 2 rats were heavily infected. This experiment demonstrates that pyelonephritis may occasionally be produced even when NH_4Cl administration is not begun until after the inoculation of *E. coli.*

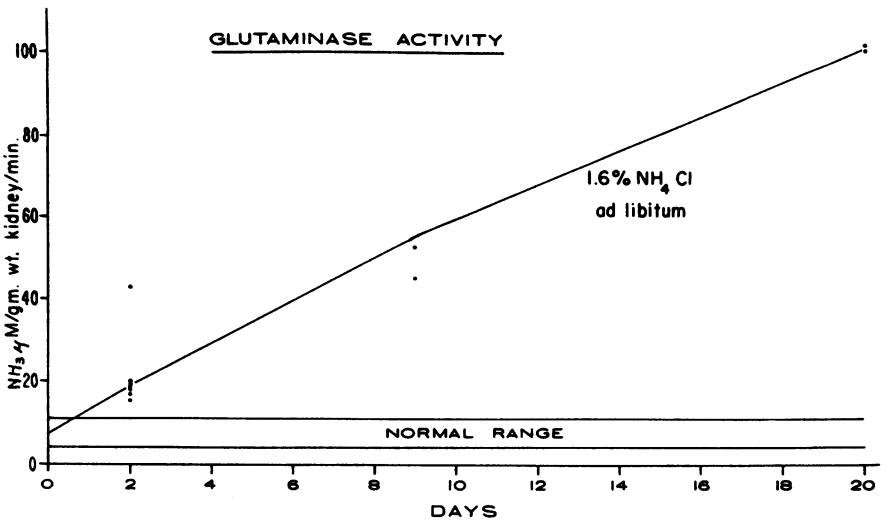

FIG. 1.

Effect of varying duration of acidosis

Administration of NH_4Cl to rats results in a steady rise in glutaminase activity as illustrated in Figure 1. This progressive rise is associated with increasing oral intake of NH_4Cl. One would anticipate then that the incidence of infection would vary according to the duration of acidification. To test this point, 12 rats were given 1.6 per cent NH_4Cl drinking water five days prior to infection. Another group of 12 rats, comparably fed with the first group, drank unlimited amounts of tap water until one day before receiving *E. coli* when they too had 1.6 per cent NH_4Cl substituted for tap

* Although NaH_2PO_4 increases acid excretion, it does not cause a rise in renal glutaminase activity.[16] This salt would be an excellent control to distinguish the effects of acid secretion from those of NH_3 production. Unfortunately, it rapidly produces severe morphological damage to the kidney, thus complicating the interpretation of experiments designed to test susceptibility to infection.

water. Both groups drank NH_4Cl for an additional three days at which time their kidneys were examined. Of the group drinking NH_4Cl for eight days, 6 kidneys from 3 animals were heavily infected. In the group drinking NH_4Cl for four days, no infections occurred.

It appears that the degree of susceptibility to infection induced by NH_4Cl does have some relation to the duration of its administration. Had larger numbers of animals been used, one would have expected to find infections even in the animals receiving NH_4Cl for four days since some susceptibility to infection was demonstrated in a previously described experiment in which NH_4Cl was begun two days after the inoculation of *E. coli.*

Reversal of effect of NH_4Cl by alkalinizing agent

If the effect of NH_4Cl on renal susceptibility to infection is biochemical, and not dependent on structural alteration, this should be reversed by neutralizing the acidosis and abolishing the stimulus to renal production of ammonia. To test this, 54 rats were given 1.6 per cent NH_4Cl as drinking water for seven days. The animals were then divided so that the half which had lost the most weight (18 per cent) were allowed to drink 2.5 per cent (equimolar) sodium bicarbonate, whereas the remainder (11 per cent weight loss) continued drinking NH_4Cl. After 24 hours, all animals were inoculated with *E. coli* and the same drinking solutions continued. The rats drinking $NaHCO_3$ were limited to 40 ml. per rat per day. Five to seven days later bacteriological examination of their tissues and body fluids were performed. The results are shown in Table 4. Among the rats which had been given $NaHCO_3$, 3 of 54 kidneys were infected. In the group which continued drinking NH_4Cl, 22 of 54 kidneys, representing 13 of 27 animals, were infected.

It is apparent from this experiment that the mechanism by which NH_4Cl, increases renal susceptibility to infection is largely nullified by the administration of an alkalinizing agent. The reduction in renal glutaminase activity which follows drinking 2.5 per cent $NaHCO_3$ for 24 hours is shown in Figure 2. It appears that renal susceptibility to infection under the conditions of this experiment varied with renal glutaminase activity.

Effect of NH_4Cl ingestion on the normal kidney

To test for evidence of morphological abnormality in the kidneys of rats drinking NH_4Cl, urinalyses were performed on 12 animals seven and eight days after they had been drinking 1.6 per cent NH_4Cl ad libitum. Evidence of renal injury in the form of abnormal proteinuria, hematuria, pyuria, or cylindruria was not observed. Bacteriological examination failed to reveal

TABLE 4.

	Colonies E. coli		Gross abscesses	Urine culture
	Left kidney	Right kidney		
	200	600		0
	60,000*	50,000*		0
	70,000*	>1,000,000*	+	+
	700	200		0
	0	300		0
	>1,000,000*	>1,000,000*	±	+
	2,000	70,000*		0
	400	40,000		0
	1,000,000*	>1,000,000*		+
	>1,000,000*	>1,000,000*		+
NH_4Cl	420	740		
	90	50		
	0	0		
	34,000	540		0
	0	0		0
	8,000	4,000		0
	0	0		0
	40	30		0
	23,000	>1,000,000*		+
	470	140		+
	>1,000,000*	>1,000,000*		+
	>1,000,000*	>1,000,000*		+
	0	0		0
	10,000	>1,000,000*	+	+
	600	>1,000,000*	+	+
	135,000*	>1,000,000*	+	+
	>1,000,000*	>1,000,000*	+	+
	0	0		0
	0	0		0
	0	0		0
	0	20		0
	400	900		+
	8,000	>1,000,000*		0
	0	20		0
	5,000	20,000		0
NH_4Cl	90	720		0
and	470	5,000		0
$NaHCO_3$	70	170		0
	6,000	7,000		0
	610	940		0
	24,000	3,000		0
	68,000*	13,000		0
	8,0000	120		0
	0	0		0
	0	0		0
	130	150		0
	0	0		0
	0	8,000		+
	70	7,000		+
	970	130,000*		+
	2,000	1,000		0
	0	120		0
	24,000	0		0
	0	0		0

* Infection.

the presence of bacteria in the kidneys or urine. Careful histological examination of all kidneys failed to reveal morphological evidence of renal injury. A group of 10 rats was given 1.6 per cent NH_4Cl as drinking water for three weeks and then changed to 1.0 per cent NH_4Cl which they drank for an additional two and one-half months prior to examination. The kidneys of these animals were sterile, and no abnormalities were seen grossly or microscopically. Insofar as these methods of examination can be relied

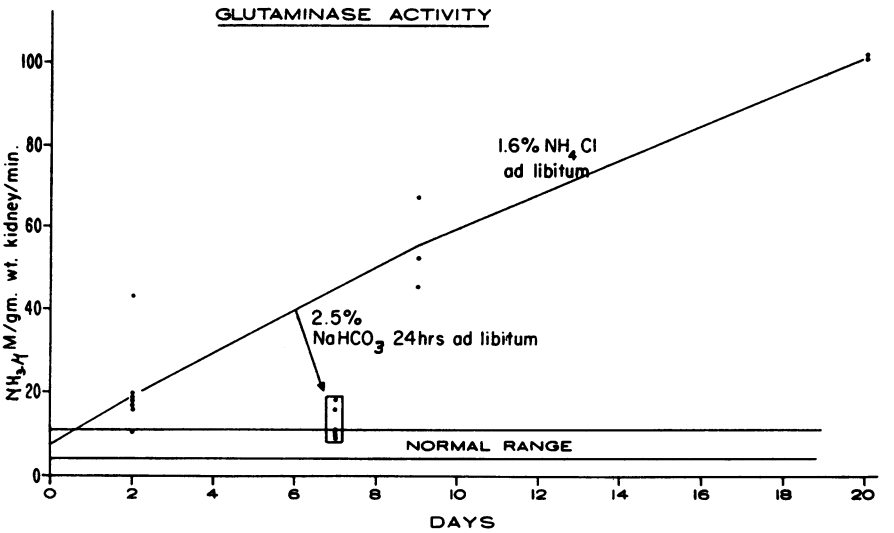

FIG. 2.

upon, there was no evidence that the effect of acid ingestion on susceptibility to infection is secondary to injury of kidney substance.

Maintenance of effect of NH_4Cl by methionine

To test whether acidifying agents other than NH_4Cl were effective in lowering renal resistance to infection, the effects of the amino acids DL-methionine and DL-valine were compared. Methionine produces an acid urine because of the excretion of sulfate.[9] Valine contains the same number of carbon and nitrogen atoms but does not contain sulfur and therefore would not be expected to acidify the urine. Forty-seven rats which had been drinking 1.6 per cent NH_4Cl ad libitum for 11 to 20 days were divided into two groups of 24 and 23 animals. The NH_4Cl drinking water was removed from all the animals and those which had lost weight (average 4 per cent) were given 3.5 per cent DL-valine to drink; those which had not lost weight

TABLE 5

	Colonies E. coli		*Gross abscesses*	*Urine culture*
	Left kidney	*Right kidney*		
	404,000*	>1,000,000*	+	+
	460,000*	100,000*		0
	0	0		0
	1,000	2,500		0
	14,000	62,000*	±	0
	>1,000,000*	1,000		+
	130	0		0
	0	0		0
	>1,000,000*	>1,000,000*	+	0
NH_4Cl	0	0		0
and	0	0		0
Methionine	50	190		0
	148,000*	208,000*		0
	1,000	70		0
	36,000	10,000		0
	0	0		0
	74,000*	62,000*	+	0
	0	0		0
	2,000	3,300	±	0
	0	0		0
	26,000	>1,000,000*	+	+
	1,600	0		0
	1,600	20		0
	6,000	175,000*	+	0
	0	0		0
	4,000	1,000		0
	1,000	1,000		0
	46,000	4,250		0
	0	0		0
	0	0		0
	0	0		0
	0	0		0
NH_4Cl	0	0		+
and	720,000*	1,300	+	0
Valine	0	0		0
	0	40		+
	3,000	13,000	±	0
	1,000	10,000		0
	5,000	186,000*	+	0
	0	0		0
	0	0		0
	0	0		0
	4,000	38,000		0
	1,000	0		0
	0	0		0
	20	0		0
	20	0		0

* Infection.

were given DL-methionine. Twenty-four hours later all animals were inoculated intravenously with *E. coli.* The animals drinking DL-valine were allowed only as much fluid as had been taken by the animals drinking DL-methionine. After five to seven days when each group was 11 per cent below the average starting weight, the animals were examined bacteriologically (See Table 5). Infection was found in 14 kidneys of rats drinking DL-methionine and in 2 kidneys of animals drinking DL-valine.

This result indicates that the acidifying amino acid DL-methionine can maintain the biochemical susceptibility to infection resulting from NH_4Cl ingestion. The amino acid DL-valine, on the other hand, which is not an acidifying agent, seemed to permit the animals to recover their resistance to infection.

Methionine vs. valine

Since methionine is being used in clinical practice to combat urinary tract infection by acidifying the urine, it seemed important to test whether DL-methionine alone could lower renal resistance to infection. Seventeen rats were given this amino acid in their drinking water for 15 days. At the same time 18 similarly fed rats were given equal volumes of drinking water containing 3.5 per cent DL-valine. All were then inoculated with *E. coli* intravenously and the pair-drinking and feeding continued for four to six days, at which time bacteriologic studies were carried out. As can be seen in Table 6, 3 rats drinking DL-methionine were infected whereas none of the animals drinking DL-valine was infected.

It would appear from the last two experiments that acidifying agents other than NH_4Cl can increase renal susceptibility to infection.

Staphylococcus albus

In view of the similarities between coliform and staphylococcal pyelonephritis in animals,[4] the effect of acidification on renal staphylococcal infection was tested. As already stated, the strain chosen for this experiment does not produce pyelonephritis even when 10^8 viable units are injected intravenously. Twenty-two rats were given 1.6 per cent NH_4Cl as drinking water for four to eight days. An equal number of control animals was pair-fed to the experimental group and allowed similar quantities of tap water to drink. All animals were inoculated with 10^7 staphylococci intravenously and examined bacteriologically four to seven days later. The average weight loss of the experimental and control groups at the time of inoculation was 3.9 per cent; at the time of sacrifice the weight losses were 4.6 per cent and 7.5 per cent respectively. Of the 22 rats drinking NH_4Cl,

TABLE 6

	Colonies E. coli		*Gross abscesses*	*Urine culture*	*Urine pH*
	Left kidney	*Right kidney*			
	410	19,000		0	6.0
	580	420		0	
	650	320		0	6.5
	4,000	24,000		0	7.0
	16,000	5,000		0	7.0
	20	20		0	7.5
Valine	0	400		0	7.5
	4,000	7,000		0	6.5
	120	0		0	7.0
	0	0		0	7.0
	0	520		0	6.0
	0	0		0	6.0
	0	0		0	7.0
	0	30		0	6.5
	0	0		0	6.5
	0	20		0	6.5
	0	0		0	6.5
	0	0		0	7.0
	7,000	1,000		0	6.0
	30	0		0	5.5
	4,000	1,280		0	6.0
	4,500	29,000		0	6.0
	0	0		0	5.0
	3,400	1,500		0	5.0
	0	0		0	5.0
Methionine	0	0		0	5.0
	0	0		0	5.0
	0	0		0	5.5
	16,000	450,000*	+	+	5.5
		20,000		0	5.5
	2,000	148,000*	+	+	6.0
	0	0		0	5.5
	0	0		0	5.5
	30,000	154,000*	+	+	6.5
	390	0		0	5.0

* Infection.

there were 7 infected kidneys in 5 animals. None of the control animals was infected.

Pathology of NH_4Cl-induced infections

In order to carry out conclusive bacteriological studies, portions of experimental kidneys were not routinely examined histologically. Since, however, gross abscesses were found frequently, histological sections were prepared from hemisections of these kidneys, the remainder being taken for bacteriological study. The gross lesions were always wedge-shaped streaks of suppuration involving both medulla and cortex. Examples of such lesions are shown in Figures 3 and 4. They resemble those produced by direct inoculation of bacteria into the renal medulla.[5] The non-infected portions of the kidneys appeared normal.

Infection was never observed grossly in other regions of the body such as lung, liver, spleen, or peritoneum. Samples of lung, liver, and spleen cultured during the course of these studies were never found to be infected. Blood cultures were occasionally found to be positive in both experimental and control animals.

DISCUSSION

The preceding experiments have demonstrated that measures designed to increase the activity of the renal ammonia-forming mechanism in the rat bring about an increased susceptibility of the kidney to infection. The effect seemed limited to the kidney and was independent of weight loss. The action of acidifying agents seemed to be chemical, since structural changes in the kidney could not be detected; furthermore, the increased susceptibility was promptly overcome by administration of an alkalinizing agent.

It is often difficult to measure the significance of increased tissue susceptibility to infection since, as is well known, the virulence of the particular bacteria employed is an important factor. For this reason it is important to note that the *E. coli* used in these experiments has not produced pyelonephritis in normal rats,[8] rats acutely depleted of potassium,[3] or rats poisoned with mercuric chloride.[15] This organism has also failed to cause pyelonephritis in rats with grossly visible nephrocalcinosis due to Vitamin D intoxication (400,000 units/d x 4).[6] It is thus not likely that the effect of NH_4Cl was mediated by the increased urinary loss of K and Ca known to occur during acidosis since experiments designed to test the effect of these specific factors were negative.

The gross appearance of the lesions produced during ingestion of acidifying agents is similar to those resulting from direct inoculation of bacteria

FIG. 3. Hemisection of kidney of rat drinking NH_4Cl seven days after intravenous inoculation of *E. coli*. Pale raised streaks of infection occupy more than half of this plane. Each division of ruler equals 1 mm.

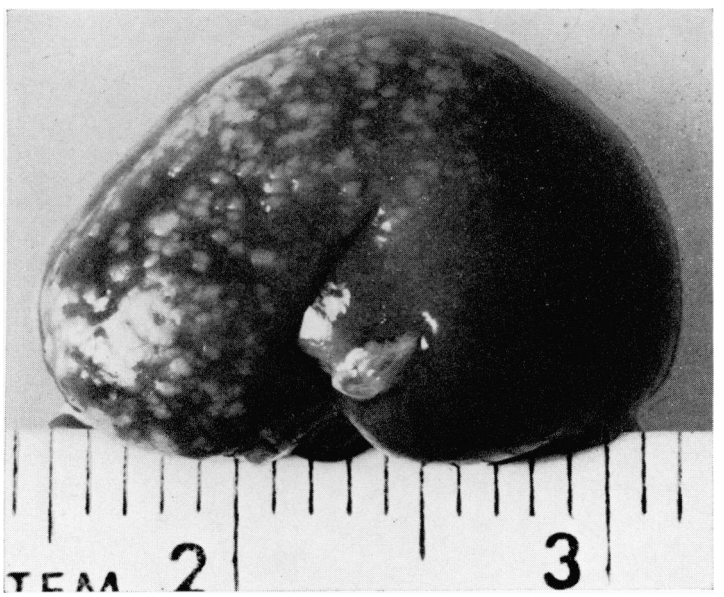

FIG. 4. Surface of kidney shown in Figure 3 showing many abscesses beneath the capsule.

into the renal medulla. It is reasonable to presume that the medulla was the initial site of bacterial multiplication in the present experiments since the number of organisms required to produce infection there is much smaller than the number needed to initiate infection in the cortex.[5] Furthermore, unpublished observations from our laboratory have demonstrated that *E. coli* persisting in the normal kidney following intravenous injection are located almost exclusively in the medulla.[11] These findings point to local factors favorable to bacterial survival and multiplication in the renal medulla. The fact that both micropuncture studies[17] and stop-flow techniques[18] indicate that ammonia enters the urine mainly at the level of the collecting tubule is consistent with the thesis that local complement-inactivation may be one of the factors responsible for this peculiar zone of vulnerability. Since ammonia is highly diffusible, and since under certain conditions an elevated level of ammonia is demonstrable in renal vein blood,[10] it seems reasonable to postulate a comparatively high concentration in the interstitial tissues of the medulla, as well as in the tubular fluid.

The role of complement in natural resistance is not established with any certainty. Although lysis of many Gram-negative bacilli by the combination of complement and antibody is easily demonstrable *in vitro*, it must be kept in mind that some Gram-negative bacilli, including the strain of *E. coli* used in these studies, and all Gram-positive cocci, are resistant to lysis. It is entirely possible, nevertheless, that complement may have an effect on bacteria short of lysis which retards their rate of multiplication or renders them otherwise more susceptible to host defense. There is evidence that complement acts as an opsonin.[14] In a situation such as the one under study here only a slight tipping of the balance between host and parasite would be sufficient to change the result from no infection to infection.

The present experiments do not provide an explanation for the markedly increased renal susceptibility to infection attendant upon ureteral obstruction. Although it has been shown that there is immediate acidification of urine excreted during ureteral obstruction,[12] measurements of glutaminase activity of such kidneys revealed levels that were far below those which in the present experiments were associated with demonstrably increased susceptibility to infection.[7]

SUMMARY

Using *E. coli* and *staphylococcus albus* as test organisms, administration of ammonium chloride or methionine was found to increase the susceptibility of rats to bacterial pyelonephritis.

Susceptibility tended to become greater after several days of acid ingestion. The effect was promptly overcome by alkali administration.

There was no evidence that the altered susceptibility caused by acidifying agents was related to structural alteration of the kidney.

Other tissues did not exhibit increased vulnerability to infection during the period of acid loading.

The findings are consistent with the suggestion that ammonia, formed in the kidney in response to acid stimulus, facilitates local growth of bacteria by interfering with complement activity.

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