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THE EFFECT OF ACID AND ALKALINE SALTS ON SOME PATIENTS WITH RHEUMATOID ARTHRITIS

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Low grade edema, which is frequently part of the clinical picture presented by arthritic patients, is often ignored, and its rôle in the disease is not clearly understood. Scull and Pemberton[6] have called attention to this edema or "tissue swelling" and have observed that, with bed rest and a low carbohydrate diet, arthritic patients often experience relief of pain, decrease in joint swelling, and an increase in joint mobility concomitantly with the loss of water from the body.

It has also been demonstrated[1,5] that synovial fluid may be regarded as a dialysate of plasma containing mucin, and that the presence of edema from any cause may be associated with an increase in the volume of synovial fluid.[2] These observations suggest that the volume of synovial fluid of arthritic patients may be related to the volume of the extracellular fluid compartment of the body.

In this study the clinical effects of water loss occurring after diuresis by ammonium chloride, and the clinical effects of water retention resulting from the administration of sodium bicarbonate were observed in 5 patients with rheumatoid arthritis.

Procedure. Five female patients with chronic rheumatoid arthritis were studied. They were placed on a diet containing 150 gm. carbohydrate and 75 gm. protein. The caloric value of the diet was adjusted for each patient by adding sufficient fat so that 40% more than the basal caloric requirement as estimated from the tables of Dubois and modified by Boothby and Sandiford[1] was given. The diet was the same every day for each patient and prepared so that it contained less than 2.5 gm. sodium chloride. Water was not restricted, although the daily water intake was estimated and the urinary output was measured. The patients were weighed daily on a balance accurate to 1 gm. Under these circumstances it can be assumed that changes in body weight reflect water loss or water retention.[3] All patients had been hospitalized for several weeks or months prior to the experiments, and the symptoms resulting from the arthritis remained unimproved. None had menopausal symptoms. The patients received no physiotherapy, and analgesics were administered upon request of the patient during the course of these experiments.

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The patients were examined every morning. The mobility of the affected joints was measured and expressed in terms of degrees of range of motion (the degree of maximum extension minus the degree of maximum flexion) the circumference of the joints was measured. The symptom of pain was evaluated by careful questioning of the patient and noting the frequency with which she requested analgesics.

After control joint measurements were made over a period of 3 to 5 days the patients were given 6 to 8 gm. ammonium chloride daily for varying lengths of time. The ammonium chloride period was followed by the administration of 6 to 8 gm. of sodium bicarbonate for 7 to 10 days, and the same observations were made. The experiment was concluded by repeating the administration of ammonium chloride.

Results. Table 1 shows the detailed data on Patient 1. The remaining 4 patients responded similarly and their summarized data are shown in Table 2. Although there were individual variations in clinical response to the administration of ammonium chloride and sodium bicarbonate, the character of the response was remarkably similar in each case.

Table 1. Data on Patient B. R.*

Date, 1949	Period	Weight in (kg.)	Range of motion (degrees) Right knee	Left knee	Left elbow	Circumference (cm.) Right knee	Left knee	Pain
10-16	Control	48.3	30	40	64	36.4	32.5	+ +
10-19	"	48.0	30	40	63	36.5	32.5	+ +
10-20	NH_4CL	47.9	30	40	65	36.5	32.5	+ +
10-22	"	46.3	40	45	70	35.4	31.0	+
10-24	"	45.4	55	55	70	34.0	31.2	0
10-26	"	44.3	70	60	85	33.0	31.5	0
10-30	"	41.8	85	85	112	32.0	31.5	0
11-3	$NaHCO_3$	44.9	84	90	110	34.4	31.2	0
11-5	"	42.8	70	70	105	33.0	33.0	+ +
11-8	"	45.2	64	65	50	35.0	35.5	+ + +
11-11	NH_4Cl	46.0	50	50	35	35.0	35.5	+ + +
11-13	"	44.5	60	70	50	34.5	35.0	+ +
11-16	"	42.0	80	65	65	33.5	33.5	0
11-22	"	40.8	86	85	70	34.0	32.5	0
11-30	"	42.0	80	82	70	34.0	33.0	0
12-8	"	41.0	80	80	70	34.0	33.0	0
12-9	$NaHCO_3$	44.7	80	80	75	34.0	33.0	0
12-11	"	43.0	50	70	70	34.0	34.5	+ + +
12-15	"	45.4	45	40	50	35.5	35.5	+ + +
12-16	NH_4Cl	46.2	40	42	50	35.5	35.5	+ + +
12-18	"	45.4	55	70	60	35.0	34.0	+
12-22	"	44.7	100	95	75	33.0	33.0	0

* Every other set of determinations has been omitted by editorial request, in order to save space.

In each case the period of ammonium chloride administration was characterized by a significant decrease in body weight which under the conditions of the experiment can be assumed to be mainly due to water loss from the body.[3] Associated with the

loss there was a decrease in joint swelling as measured by the joint circumferences. In Cases 1, 2, and 3 there was also a progressive increase in the mobility of the joints. Cases 4 and 5 showed no significant change in mobility but Case 4 had relatively little impairment of joint mobility initially, and Case 5 had very severe joint destruction with some ankylosis. All patients experienced relief of joint pains during the period of ammonium chloride administration. Relief of pain occurred even though the range of motion was not significantly altered (Patient 5).

TABLE 2. DATA ON 4 PATIENTS

	Control period, 2–5 days	NH_4Cl period, 25–33 days	$NaHCO_3$ period, 7–10 days	NH_4Cl period, 6–8 days
Patient (A. B.):				
Weight (kg.)	80.0	71.2	78.6	75.5
Right shoulder:				
Abduction (deg.)	30	80	65	90
Extension (deg.)	30	100	60	120
Right knee circumference (cm.)	41.5	39.0	41.0	39.0
Left knee circumference (cm.)	40.0	40.0	41.0	39.0
Pain	+ + +	0	+ + +	0
Patient (V. B.):				
Weight (kg.)	31.2	27.7	30.9	28.6
Right shoulder abduction (deg.)	60	90	45	90
Right knee range of motion (deg.)	80	155	112	150
Right knee circumference (cm.)	29.5	28.0	31.0	29.5
Left knee range of motion (deg.)	0	65	65	90
Left knee circumference (cm.)	29.0	25.0	29.0	25.5
Pain	+ + +	0	+ + +	0
Patient (L. S.):				
Weight (kg.)	53.2	51.0	51.0	51.7
Right knee circumference (cm.)	35.0	33.0	35.0	33.0
Left knee circumference (cm.)	35.5	32.0	34.0	31.5
Right ankle circumference (cm.)	26.5	23.0	25.0	25.0
Left ankle circumference (cm.)	26.5	24.0	27.0	27
Pain	0	0	+ + +	
Patient (A. K.):				
Weight (kg.)	59.3	57.9	59.1	57.[illegible]
Right shoulder extension (deg.)	135	170	135	15[illegible]
Right knee circumference (cm.)	37.0	34.5	37.0	35.[illegible]
Left knee circumference (cm.)	36.5	34.0	36.0	34.[illegible]
Right ankle circumference (cm.)	30.0	25.0	30.0	29.[illegible]
Left ankle circumference (cm.)	29.0	26.0	28.0	26.0
Pain	+ +	0	+ + +	0

The period of sodium bicarbonate administration was characterized by an increase in body weight and in joint swelling. Joint mobility was significantly decreased and in some instances joints essentially asymptomatic before the onset of these observations became swollen and painful. Pain was aggravated in each case. Case 4, who had had no pain initially, developed severe pain. Case 5 developed more pain while receiving sodium bicarbonate than she had initially.

Discussion. Under the conditions of these experiments the changes in body weight which were observed can be assumed to

represent mainly either water retention or water loss.[3] It was to be expected, therefore, that the period of ammonium chloride administration should be characterized by a reduction in body weight due to water loss, since the diuretic effect of ammonium chloride has been demonstrated by numerous investigators. Similarly, an increase in body weight following the administration of sodium bicarbonate must be attributed to an increase in the water content of the body. In these cases of rheumatoid arthritis the decrease in body weight and in joint circumference with the administration of ammonium chloride, and the increase in body weight and joint circumference with sodium bicarbonate suggests that the fluid in the joints and in the periarticular tissues, as well as the extracellular fluid volume, is affected by these drugs.

The results of these observations are in general agreement with the observation of Scull and Pemberton[6] that the loss of body water in rheumatoid arthritis is associated with clinical improvement. This would suggest that in rheumatoid arthritis the symptom of joint pain is caused, at least in part, by the swelling of articular and periarticular tissues. Similarly, in the absence of definite bony ankylosis, the immobility of the joints in rheumatoid arthritis may be partly due to this swelling and the pain resulting therefrom. To the extent that this is true, and in the absence of definite joint ankylosis, the reduction of tissue edema may be expected to be associated with clinical improvement of the patient as manifested by relief of pain, decrease in the swelling of the affected joints, and an increase in their range of motion. The fact that these changes are readily reversed with the administration of sodium bicarbonate supports such a view.

Other patients not as carefully studied, and not included in this report, have been tried on this régime and similar changes were not uniformly observed. The response was judged largely on the basis of subjective changes and was not as impressive. Some patients noted no changes at all, some rather insignificant improvement with ammonium chloride, and others were moderately benefited. Sodium bicarbonate administration was more often associated with an increase in the severity of symptoms.

It is not the purpose of this report to recommend ammonium chloride or other diuretics as a therapeutic measure in rheumatoid arthritis. However, the striking clinical changes observed in this small series of patients with alterations in the body water suggest the need of further investigation of the rôle of water in this disease.

Case Reports. CASE 1. B. R., a 44-year-old white female, had had rheumatoid arthritis for 10 years. At the time these observations were made she was bedridden because of multiple joint involvement. There was pain on motion of both shoulder joints, pains in both elbows and limitation of motion of the left elbow. Both wrists were painful and swollen. The hands showed interosseous atrophy and fusiform swelling about the metacarpo-phalangeal and the proximal phalangeal joints. There was

extreme pain on motion of the hip joints, more marked on the right. Both knee joints were swollen, painful, and limited in motion. The right ankle was swollen, painful, and limited in its range of motion. Although many of the joints were swollen, there was no evidence of pitting edema.

Roentgen ray revealed slight loss of joint space in both knees with decalcification of the adjacent bone. The other joints showed marked decalcification of the adjacent bones, some loss of joint space, but no ankylosis.

After control joint measurements were made for 4 days, the patient was given 8 gm. ammonium chloride daily for 13 days (Table 1). Her weight decreased 6 kg. Concomitantly with the decrease in weight there occurred a significant increase in range of motion of the right knee, left knee, left elbow and both hips. The circumference of the right knee decreased significantly. The circumference of the left knee was also decreased, although not to such a marked degree. All joint pains were remarkably relieved. There was a decrease in the swelling of the left wrist and an increase in the mobility of this joint. The patient tolerated the ammonium chloride well, and she felt generally improved.

Following the period of ammonium chloride, the patient was given 8 gm. sodium bicarbonate daily for 8 days. The changes in measurements are shown in Table 1. During this time her weight increased 4.1 kg. There was a complete reversal of the changes noted in the preceding period. The circumference of the left knee which had not changed remarkably under the influence of ammonium chloride now increased significantly. Joint pains which had been relieved by ammonium chloride became severe, the patient complained bitterly and required analgesics.

The alternating periods of ammonium chloride and sodium bicarbonate were repeated. Each time the periods of sodium bicarbonate administration were characterized by an increase in body weight, increase in joint circumferences, decrease in joint mobility, and increase in joint pain. On the other hand, the periods of ammonium chloride administration were characterized by a decrease in body weight, decrease in joint circumference, increase in joint mobility, and relief from joint pains.

Case 2. A. B. was a 38-year-old white female whose arthritis was of 1 year's duration. At the time these observations were made she exhibited limited ability to abduct or extend the right shoulder. There was swelling of both knees and both ankles, and although their mobility was not impaired to passive movement, she was unable to walk because of pain and limitation of active motion. The patient required 45 gr. of acetyl-salicylic acid daily to control her pain, and she was given codein every night to assure a night's sleep. Roentgen rays of the affected joints revealed no ankylosis and no joint destruction.

The patient was given 8 gm. ammonium chloride daily for 22 days. During this time her weight decreased 5.8 kg. The degree of abduction and extension of the right shoulder were significantly increased. Swelling of the right knee and ankles decreased. Joint pains which had been a prominent symptom previously were completely relieved, analgesics were unnecessary and she was able to walk.

Ammonium chloride was then discontinued and 8 gm. sodium bicarbonate were administered daily for 15 days. The patient's weight increased. The swelling of the knees and ankles returned. Pain promptly reappeared and became even more severe.

The final period of ammonium chloride administration (8 days) was again characterized by a loss of weight, increased mobility of the shoulder and diminished circumference of both knees. Joint pains were again completely relieved. This patient was sufficiently improved to be discharged to the outpatient department. Table 2 shows the summarized data on this patient.

Case 3. V. B. was a 28-year-old white female whose illness was of 3 years' duration. At the time of these observations the patient complained of severe pain and stiffness of the cervical, dorsal, and lumbar spine. There was pain and limited abduction of the right shoulder; pain, swelling, and limited motion of the right knee and both ankles; pain, swelling, and fixation at an angle of 90° of the left knee. Roentgen ray examination showed no evidence of bone or joint changes in the cervical, dorsal, or lumbar spine. There was decalcification of the bones of both knees and ankles with sharply etched articular surfaces but no ankylosis.

The patient was given 6 gm. ammonium chloride daily for 26 days. During this time her weight gradually decreased 3.5 kg. The degree of abduction of the right shoulder increased significantly. The range of motion of both knees and ankles increased while the swelling decreased. Pain was remarkably relieved and no medication was needed. Mobility of the spine was not affected although back pain was relieved.

Following this period, 6 gm. sodium bicarbonate were administered daily. As is indicated in Table 2, the patient gained approximately 3.2 kg. in weight. Pain of the joints became a very prominent symptom so that she required codein. The swelling of the joints increased and, in general, the range of motion decreased.

In the final period of ammonium chloride administration the symptoms of pain again disappeared completely. Swelling of the joints decreased. The range of motion of the knees and right shoulder was further increased, and the patient felt considerably better.

Case 4. I. S. was a 48-year-old white female whose rheumatoid arthritis had become progressively worse over a 10-year period. At the time these observations were made, there was swelling of the dorsal surfaces of both hands and of both knees, although mobility was not markedly impaired. Both ankles were swollen and moved with difficulty. Pain of the joints was not a prominent symptom at this time. Roentgen ray revealed marked narrowing of joint spaces in the left hand and knees.

During a 23-day period of ammonium chloride administration the patient's weight decreased 3.2 kg. The circumferences of the knees and ankles decreased significantly, the puffiness of the dorsal surfaces of the hands disappeared and the mobility of the fingers improved.

During the next 10 days the patient received 8 gm. sodium bicarbonate daily and gained 3 kg. in body weight. The circumference of the affected joints increased to approximately their initial size. Joint pain, which had previously not been a prominent symptom, became severe and the patient required codein for its relief. Swelling of the dorsal surfaces of the hands reappeared and the patient complained of numbness of the fingers of both hands.

During the final period the patient received 8 gm. ammonium chloride daily for 7 days. The weight again decreased. The circumferences of the knees and ankles diminished, and joint pain was relieved.

Case 5. A. K., a 47-year-old white female, had had rheumatoid arthritis for 8 years. When these studies were begun, she complained of pain and restriction of motion of both shoulder joints, more marked on the right. Both elbows, wrists and knees were swollen, painful and limited in motion. The ankles and toes were deformed and not movable. Analgesics were continually required.

Roentgen ray revealed destruction of many of the joint spaces with bone erosion and decalcification of all the bones.

For 33 days the patient received 8 gm. ammonium chloride daily and lost 1.4 kg. During this period the degree of extension of the right shoulder increased. The circumference of the right and left knees and the right and left ankles decreased without improvement of joint motion. Joint pains were decreased, and no medication was required.

For the next 7 days the patient received 8 gm. sodium bicarbonate daily. During this time she increased her weight 1.2 kg. The degree of extension of the right shoulder was again decreased to its original value. The circumferences of the knees and ankles again were increased. Edema of the face and of the dorsal surfaces of the hands developed. Joint pains became severe and required analgesics for their control.

For the next 6 days the patient again received 8 gm. ammonium chloride daily. Again her weight decreased, and the circumferences of the knees and ankles were decreased. Edema of the face and hands again disappeared. Joint pains were again relieved, and no analgesics were required.

Summary. Five females with typical rheumatoid arthritis were placed on a fixed diet and were given ammonium chloride and sodium bicarbonate while changes in their body water were estimated. With the loss of water induced by ammonium chloride there was in general a decrease in the pains and joint swelling and an increase in the joint mobility. These changes were reversed by the accumulation of water induced by sodium bicarbonate. It would appear that, in these cases at least, some of the symptoms of rheumatoid arthritis are altered to some degree by changes in the body water.

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AMMONIUM CHLORIDE DECALCIFICATION, AS MODIFIED BY CALCIUM INTAKE: THE RELATION BETWEEN GENERALIZED OSTEOPOROSIS AND OSTITIS FIBROSA

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Plates 41 and 42

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Experimental hyperparathyroidism in young and old animals on high and low calcium intakes results in bone decalcification and secondary fibrosis leading to the production of generalized ostitis fibrosa (1–6). We conceived the mechanism of these changes as follows:

"It may well be that the immediate antecedent of experimental ostitis fibrosa is a condition, perhaps related to the disturbed acid-base equilibrium, which may also be caused by other agents than parathormone. However, it seems essential that the action be continuous, and that the condition which it causes be maintained for long periods without endangering the life of the experimental animal, or a certain minimum of well-being. Parathormone is specific in the sense that it satisfies these requirements" (1).

We recognized, in view of the known effects of parathormone upon the mineral metabolism, that other means of similarly affecting mineral metabolism might produce similar end-results. It was deemed desirable to undertake a quantitative study of the question of experimental bone decalcification, using ammonium chloride to bring about generalized osteoporosis and possibly ostitis fibrosa.

Ammonium chloride has long been known to be an effective agent in producing acidosis, as in its metabolism urea is synthesized from the ammonium radicle and hydrochloric acid is liberated. Ammonium chloride may be administered in considerable quantities for a long time. Haldane demonstrated an increased excretion of bases and phosphates in the experimental acidosis thus produced (7). The

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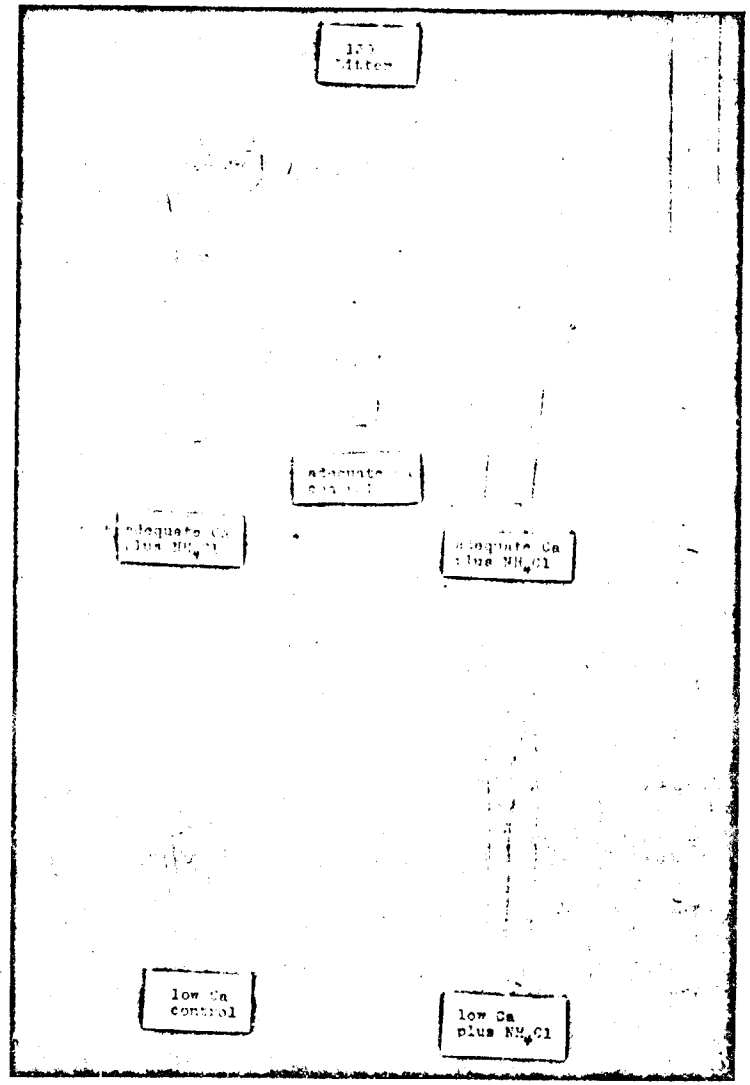

calcium reserves in the bone may be expected to take a considerable part in the neutralization of the acid, and an increased excretion of calcium and phosphorus, especially through the kidneys, has been confirmed experimentally (8).

Increases of serum phosphatase were demonstrated in the course of this study, which were roughly parallel to the degree of bone decalcification (9).

Methods

Four litters of dogs were used, 3, 6, 8, and 18 months old, respectively, at the beginning of the experiment. At the outset the 3 months old puppies averaged about 3 kilos, the 6 months about 8 kilos, the 8 months old dogs about 10 kilos, and the 18 months old dogs about 17 kilos. Each group contained one or more control animals which did not receive ammonium chloride. Two types of control were used, those maintained on a calcium-adequate diet and those on a calcium-inadequate diet. The experiment was continued for 11 weeks.

Diet.—The diet consisted of fresh ground lean horse meat, mixed with 2 per cent cod liver oil and 5 per cent canned tomatoes. This constituted the calcium-poor diet. It was adequate in other respects. The animals receiving the calcium-adequate diet were given a supplement consisting of 2.5 gm. of bone meal and 2.5 gm. calcium lactate (equivalent to about 1 gm. calcium) per kilo of meat mixture. On this diet the control animals grew well and were in excellent condition. The food intake, though measured daily, was given practically *ad libitum*. The animals consumed from 0.5 to 2.5 kilos per day, depending on their size. The youngest dogs on the low calcium diet with ammonium chloride supplement suffered loss of appetite towards the end of the experiment and their food consumption decreased to about 0.5 kilos per day.

Ammonium Chloride Administration.—Ammonium chloride was administered at first in the form of a 1 per cent solution by stomach tube, and later in 1½ per cent and 2 per cent solutions, once a day at the beginning of the experiment, and twice a day when the volume administered would otherwise have been too large to be given at one time. At the beginning of the experiment the amount of ammonium chloride was about 0.08 gm. per kilo of body weight per day, for all the groups. It was increased relatively rapidly in the 18 months old dogs to a final daily dose of 1 gm. per kilo body weight, in the 8 months old dogs to 0.8 gm. per kilo, in the 6 months old dogs to 0.7 gm. per kilo, and in the 3 months old dogs to 0.4 gm. per kilo. The final dose was continued for 17 days before the conclusion of the experiment.

Course of the Experiments

The control animals on the adequate calcium intake continued to grow, including even the 18 months old dogs that had practically completed their growth at the beginning of the experiment. The animals on a low calcium diet without ammonium chloride gained weight more slowly after a time, although their growth in length seemingly continued. On the other hand, the dogs on the low calcium diet receiving ammonium chloride eventually developed anorexia and gained no weight. This was particularly noticeable in the youngest group where gain in weight ceased after the first 2 weeks on the low calcium diet with ammonium chloride. One animal developed deformities and fractures—a young puppy on low calcium diet, receiving ammonium chloride. The ammonium chloride effect was not as pronounced in the older animals. The animals receiving high calcium intakes and the ammonium chloride grew well.

Gross Pathologic Findings

At autopsy the bones of the control dogs receiving the calcium supplement were normal in every respect.

They cut with usual resistance and the cortices were compact. The spongy trabeculae at the ends of the long tubular bones were entirely normal in appearance, and the internal architecture showed the usual arrangement of the trabeculae. The periosteum covering the long tubular bones stripped normally and the articular cartilages were smooth and glistening. The soft tissues showed no abnormalities in the gross, and the intestinal mucosae were intact throughout.

The animals of all age groups on a low calcium intake showed, on the other hand, readily discernible thinning of their bones, on comparison with their controls.

In Fig. 1 it is plainly visible that the cortex of the femur of the dog on the low calcium diet shows marked cortical thinning when compared with its adequate calcium control. Gross lamellation of the cortex with enlargement of the vessel canals is apparent. The general marrow cavity is enlarged. The spongy bone at the lower end of the femur extends over not quite as great a distance as does the spongy bone of the litter mate controls on the high calcium diet. The individual trabeculae are thinner, and this thinning of the trabeculae appears both in the extreme epiphyseal ends of the bone and in the metaphyses. The spongy trabeculae of the diaphysis have practically disappeared. Thus the effect of a low calcium intake is not limited to the spongy bone, but is reflected also in the cortex.

The macroscopic effects of the ammonium chloride treatment were observed in all the age groups, but were most striking in the younger

animals. In all the age groups the gradations of change were found to be strikingly dependent upon the calcium intake. Those dogs receiving an adequate calcium intake and ammonium chloride showed less bone thinning than those receiving a low calcium diet and ammonium chloride.

Examination of Fig. 1 will show that in the 170 litter, the femoral cortex of the dog on a low calcium diet plus ammonium chloride is thinner than that of the dog on an adequate calcium diet plus ammonium chloride but not very much different from the low calcium control. The femoral cortices of both of these animals are definitely lamellated and the spongy bone of both the low calcium control and the low calcium plus ammonium chloride animals, while fairly abundant, consists of much more delicate trabeculae than observed in the metaphyses and epiphyses of the dogs receiving an adequate calcium intake plus ammonium chloride.

In the youngest age group the added effect of ammonium chloride brought out more striking changes than the low calcium diet alone.

In a dog put in the experiment at 3 months of age, a low calcium diet and ammonium chloride led to fractures and deformities. Less striking changes were observed in the litter mate given ammonium chloride and a high calcium diet. These differences are depicted in Fig. 2. The photograph shows a pronounced osteoporosis of the femur of the dog receiving an adequate calcium intake plus ammonium chloride; the cortex is extremely thin, about one-half to one-third that of its control. The spongy bone which is fine and delicate continues into the diaphysis but not as far as in the control; there are no deformities or fractures; the cortex shows definite gross lamellation. The litter mate on a low calcium intake with ammonium chloride suffered extensive fractures of the ribs, femora, and humeri. At autopsy great care had to be exercised in the removal of the bones to prevent further fractures because of the very marked friability. Healing of the fractures with callus proliferation and deformities was observed. The specimen shown in the figure is from the lower end of a macerated femur. The bone was so friable that some of it disintegrated in the process of maceration. Nevertheless the thin tissue-paper-like cortex is noticeable. The narrow deformed marrow cavity was closed in places by internal callus and the spongy trabeculae of the metaphyseal region were so soft and friable that careful handling had to be exercised to prevent the production of artefacts.

This also holds true for the 180 litter, shown in Fig. 3. The cortex of the femur of the dog on a low calcium intake plus ammonium chloride is very much thinner than that of the dog on an adequate calcium intake plus ammonium chloride and even somewhat thinner than the cortex of the low calcium control. The spongy trabeculae are thinner and the intertrabecular spaces wider in the animal on the

low calcium diet plus ammonium chloride than in the one which received an adequate calcium intake plus ammonium chloride. The spongy bone is even more delicate than in the low calcium control.

It was obvious in the gross as well as microscopically that rickets played no part in these experiments. Indeed our experience in this and in other series of tests has shown our meat diet, with the cod liver oil and tomato juice supplement, entirely adequate for growth and for prevention of deficiency diseases.

Microscopic Anatomy

In the study of the ribs, comparative examinations were made of the various animals within the same group. We were careful to make comparisons on the basis of the severity of changes at equal distances from the costochondral junction.

Three of four ribs from each of the dogs were subjected to histologic examination. Generally the 6th, 7th, 8th, and 9th ribs were taken, and a large piece including the costochondral junction was examined. Fixation in Helly's fluid, decalcification in nitric acid, and staining with hematoxylin and eosin.

18 Months Old Group.—There were slight but readily distinguishable differences in the ribs of all the animals within this group, but these were not very marked. The control showed the most compact bone with the narrowest vessel canals and the fewest osteoclasts. The dogs on the adequate calcium intake plus ammonium chloride showed the least deviation from the normal. In them the vessel canals were already moderately enlarged but on the whole quite smooth, and osteoclasts were few in number. The dog on the low calcium diet and that on the low calcium diet plus ammonium chloride showed, in those ribs examined, the greatest change from the normal. Their ribs displayed the most marked enlargement of the vessel canals, and the greatest amount of subperiosteal and subendosteal resorption. It is, however, striking that the ribs in none of the dogs of this group presented marrow fibrosis.

8 Months Old Group.—In this group, the adequate calcium control had the most compact ribs. The histologic examination showed that maturity had not as yet been reached, and that there was active bone formation at the costochondral junctions. Naturally, the vessel canals of the cortex had not taken their final narrow size, though they were smooth walled and there was, except in the region of the costochondral junction, a minimum of subperiosteal and subendosteal resorption. The other two animals in this group showed a slight but definite deviation from the control, and the one on the low calcium diet with the ammonium chloride probably showed this change more than the one on the adequate calcium plus ammonium chloride. The differences between these two animals receiving

ammonium chloride was evident, although not very pronounced. These deviations from the normal consisted of increased size of the vessel canals, some increase of the connective tissue within these enlarged canals, and some increased subperiosteal and subendosteal resorption. In none of the animals of this group was the marrow fibrosed.

6 Months Old Group.—The histologic examination of the ribs showed a distinct separation of the animals into two classes—those whose bones were normal or approached the normal, and those whose bones were resorbed and considerably fibrosed. The calcium intake was the factor that governed the separation of the animals into these two groups.

The control dogs receiving an adequate calcium intake showed the usual minimal transformation changes, limited especially to the vicinity of the costochondral junctions. These are to be expected in growing transforming ribs in normal animals. The cortices of such ribs were more compact as they receded from the costochondral junctions. The vessel canals were of varying widths, but on the whole their walls were smooth and there were a minimal number of osteoclasts. The dogs receiving the adequate calcium diet plus ammonium chloride showed only the slightest histologic deviation from the control. In these the administration of ammonium chloride caused at the most a slight cortical thinning and some slight osteoclast increase in the enlarged Haversian canals. The marrow was not scarred in either these or the control.

The control receiving a low or inadequate calcium intake showed pronounced changes characterized by a pathologic exaggeration of the normal resorptive and transforming phenomena observed in the bones of growing dogs. The rib cortices throughout the sections were thinned to a very pronounced degree; the vessel canals were increased in diameter and filled with cellular connective tissue; the walls of the vessel canals showed many lacunae containing osteoclasts. There was in addition considerable subperiosteal and subendosteal resorption. The marrow was very extensively fibrosed and the intramedullary trabeculae were thinned, deformed, and showed evidences of active resorption. The proliferating cartilage zones were not widened and there was active resorption and fibrosis immediately beneath the growing zones.

The changes in the ribs of those dogs receiving this low or inadequate calcium intake plus ammonium chloride were entirely in the same direction as those observed in the low calcium control. The changes were of the same nature and distribution, and possibly of slightly greater extent.

3 Months Old Group.—In this group the adequate calcium control showed the expected transformation changes. Normal rib ossification was in progress, resulting in the production of a more compact cortex at distances away from the most active zones of transformation (costochondral junctions). The dog receiving adequate calcium and ammonium chloride showed slight but definite resorption in excess of that present in the normal animal. This was characterized by some subperiosteal and subendosteal resorption and some increased connective tissue in the vessel canals with increased osteoclasts within these canals. Furthermore there was a very slight tendency to marrow fibrosis near the costochondral junctions.

The animal receiving the low calcium diet plus ammonium chloride showed very extensive resorptive and fibrotic changes. Every rib examined had fractures and infractions in various stages of healing. Some of the long bones showed fractures and deformities in various stages of healing. The effects of this régime caused scarring of the marrow, thinning of the cortices of the ribs, marked enlargement of the vessel canals with increased connective tissue in them. Numerous osteoclasts were observed.

DISCUSSION

These experiments are in accord with the known fact that the administration of ammonium chloride induces porosis of bones. The age correlation is significantly demonstrated, as the effects of ammonium chloride diminish very materially as soon as the animal passes the young puppy stage. Smaller dosage per kilo sufficed to produce in young puppies very much more marked changes than could be produced by much larger doses in the adult.

The effect of ammonium chloride administration should not be considered, as it sometimes is, independently of the calcium intake or the calcium-phosphorus ratio. In this series of experiments, particularly in all animals 6 months and older, the level of calcium intake was most decisive in causing development or prevention of marked degrees of bone decalcification. In these dogs the differences between the low calcium and adequate calcium groups were frequently more obvious than the differences between dogs on an adequate calcium intake with and without ammonium chloride supplement. In the youngest puppies a definite effect of ammonium chloride in the adequate calcium group was evident, but even here the effect of the calcium withdrawal was considerably more drastic. The effects of administration of ammonium chloride with or without an adequate calcium intake led to gross fractures and deformities in only one of the youngest dogs. This animal received no supplementary calcium.

In none of these animals was there any evidence of rachitoid lesions which Bernhardt and Rabl (10) observed in young rats on a diet deficient in calcium and phosphorus, to which 2 per cent ammonium chloride was added as well as a small and, under the conditions of their experiment, possibly inadequate cod liver oil supplement.

The effects of ammonium chloride in causing an increased excretion of calcium are known. Furthermore the conclusion that the calcium comes from the skeleton is obvious. These facts have prompted certain clinicians to utilize ammonium chloride decalcification as a therapeutic vehicle towards the correction of bone deformities due to rickets (11). The ammonium chloride was generally given in doses of about 0.2 gm. per kilo body weight daily, but supplementary procedures, such as Bier's hyperemia daily for 15 to 20 hours, absolute rest in bed, and the application of constant traction and mechanical correction were used. It has been reported that by such non-surgical procedures some degree of correction of rachitic bow-legs is obtainable at a saving of time. It is more difficult to obtain these effects after the period of active rickets, and while osteoporosis may be obtained with ammonium chloride, it is more difficult to correct a deformity except by a long tedious usage of the auxiliary mechanical methods.

The histologic studies, furthermore, revealed a number of features that are helpful in understanding certain of the pathologic aspects of the general subject of osteoporosis. The feeding of an adequate calcium supplement led to a definite reduction in the rate of decalcification in all of our experiments. The older dogs receiving ammonium chloride and supplementary calcium showed a minimal number of osteoclasts and few Howship's lacunae. With an inadequate calcium intake and ammonium chloride, the ribs of such dogs showed increases in lacunae and osteoclasts and also in subperiosteal and subendosteal resorption. The same features held in the younger age groups whose animals receiving an adequate calcium intake tended to show less active resorption. In these animals also a low calcium intake led to the appearance of more osteoclasts and Howship's lacunae and the associated subperiosteal and subendosteal resorption. In the younger animals calcium insufficiency was found to be distinctly responsible for the rapidity with which the histologic pictures of resorption appeared, and for their severity.

When the decalcification was rapid, such degrees of marrow fibrosis and such extensive bone resorption resulted that the histologic picture could be designated as generalized ostitis fibrosa. The youngest animals in our experiments receiving an inadequate calcium intake plus ammonium chloride developed such lesions, while the older ones on the same régime only developed what could be called simple osteoporosis.

This difference in effect in the various age groups brings up the question of what is to be comprehended by the term ostitis fibrosa, and also the still more important question of relationship to each other of the ostitides fibrosae produced by various procedures. We have previously reported on the production of ostitis fibrosa in guinea pigs and dogs through the administration of parathormone. These lesions are in a broad sense of the same histologic appearance as those that are produced in young actively growing dogs on a low calcium but otherwise adequate diet, although other important differences were noted. The administration of ammonium chloride to a puppy receiving an inadequate calcium diet will lead, when the diet is so protected that none of the vitamin deficiencies will result, to generalized ostitis fibrosa, histologically quite indistinguishable from that which results from an inadequate calcium intake alone. Furthermore, the amounts of calcium adequate for slow growth will, if these puppies are very young and actively growing, produce a histologic picture of ostitis fibrosa, different only in quantitative aspects from that which appears in a rapidly growing puppy on an inadequate calcium intake.

While the ostitides fibrosae produced by the above mentioned procedures lead to basically the same histologic pictures, the gross appearances of the dogs subjected to these varying régimes will be different. Dogs on low calcium intake, receiving parathormone or relatively large doses of ammonium chloride will show severe ostitis fibrosa associated with stunting and deformities. The stunting is due primarily to loss of appetite. It is significant therefore that a severe ostitis fibrosa will develop under the influence of ammonium chloride or parathormone in such animals whose growth has practically stopped. The ostitis fibrosa is here clearly attributable to a drastic decalcification unaided by the demands of rapid growth. In simple calcium deficiency a similar picture of ostitis fibrosa will be produced in an animal in which the calcium deficiency is aggravated not by parathormone nor by ammonium chloride, but by the physiological demands of bone growth.

We must conceive of generalized ostitis fibrosa as a rather inclusive category, embracing not only the clinical generalized form (von Reck-

linghausen's disease), which has a specific parathyroid etiology, and the experimentally produced ostitis fibrosa of similar origin, but also the experimentally produced conditions which are caused by other means leading to very rapid calcium depletion with resulting marked marrow connective tissue proliferation. Thus, either clinically or experimentally, if bone decalcification is very rapid—and this would hold in the young or in the adult—generalized ostitis fibrosa occurs. From this point on there may be modification of both the gross and histologic features of the ostitis fibrosa, due to the special underlying factor inducing the decalcification. Thus it is conceivable that hyperparathyroidism may in the course of the development of ostitis fibrosa favor incidentally very extensive hemorrhage into the marrow, resulting in brown blood cysts and the formation of giant cell tumors. It has been recently shown (12) that generalized ostitis fibrosa may exist without the other classical features of von Recklinghausen's disease (cysts and giant cell tumors) apparently on the basis of a hyperparathyroidism, as at autopsy parathyroid adenomas were found.

On the other hand, a slowly developing decalcification from whatever cause will lead, both in the young and adult, to a less severe lesion which is called osteoporosis, because of the absence of striking fibrosis. The occurrence of osteoporosis in adults under a number of circumstances is well known and one of the most common causes for it is Graves' disease. In this disease there is negative mineral balance, but the patients do not suffer from the effects of diminished calcium intake. The disease is chronic and the decalcification is slow but progressive.

In long tubular bones the same phenomena are observed. Relatively rapid decalcification has been found to occur in the metaphysis—the region of rapid bone growth and metabolism,—and slow decalcification in the diaphysis and epiphysis. Corresponding to these changes are the histologic pictures of ostitis fibrosa and osteoporosis observed in the respective portions of such long bones (6).

The modifying effects of several factors are also illustrated in experimental rickets. It is well known that rickets cannot be produced experimentally in rats by withholding vitamin D unless there is a simultaneous mineral imbalance (disturbance of calcium-phosphorus ratio). In very young rapidly growing rats with curative

vitamin D supplement, but on a low calcium diet, an ostitis fibrosa will develop. On the same régime with 1000 to 2000 rat units of vitamin D, the marrow fibrosis will be essentially prevented and simple osteoporosis will ensue. The same diet with the exclusion of vitamin D will produce rickets.

While these experiments have yielded comparisons of low calcium and high calcium régimes, it is recognized that similar results might have been obtained with a different type of calcium-phosphorus imbalance—namely on low phosphorus diets.

CONCLUSIONS

1. In all age groups the effects of ammonium chloride administration were found to be strikingly dependent upon the calcium intake.
2. Dogs receiving an adequate calcium diet and ammonium chloride showed less decalcification than those receiving a low calcium diet with or without ammonium chloride.
3. In the younger groups the added effect of ammonium chloride to calcium-low diet brought out more striking changes than a low calcium diet alone.
4. When the decalcification was less severe—in the oldest dogs on the low calcium diet with or without ammonium chloride, and in the younger dogs on an adequate calcium intake with ammonium chloride—generalized thinning of the bones without marrow fibrosis resulted (osteoporosis).
5. When the decalcification was rapid and severe—in the youngest dogs on low calcium diet, particularly with ammonium chloride—generalized decalcification and secondary marrow fibrosis resulted (ostitis fibrosa).
6. Generalized ostitis fibrosa is a rather inclusive term and may be applied to the histologic picture which results when clinical or experimental decalcification is rapid, and therefore leads to extensive marrow fibrosis.
7. The special underlying causes of the decalcification may incidentally contribute features to modify the generalized osteoporosis or ostitis fibrosa as in rickets and in von Recklinghausen's disease.

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EXPLANATION OF PLATES

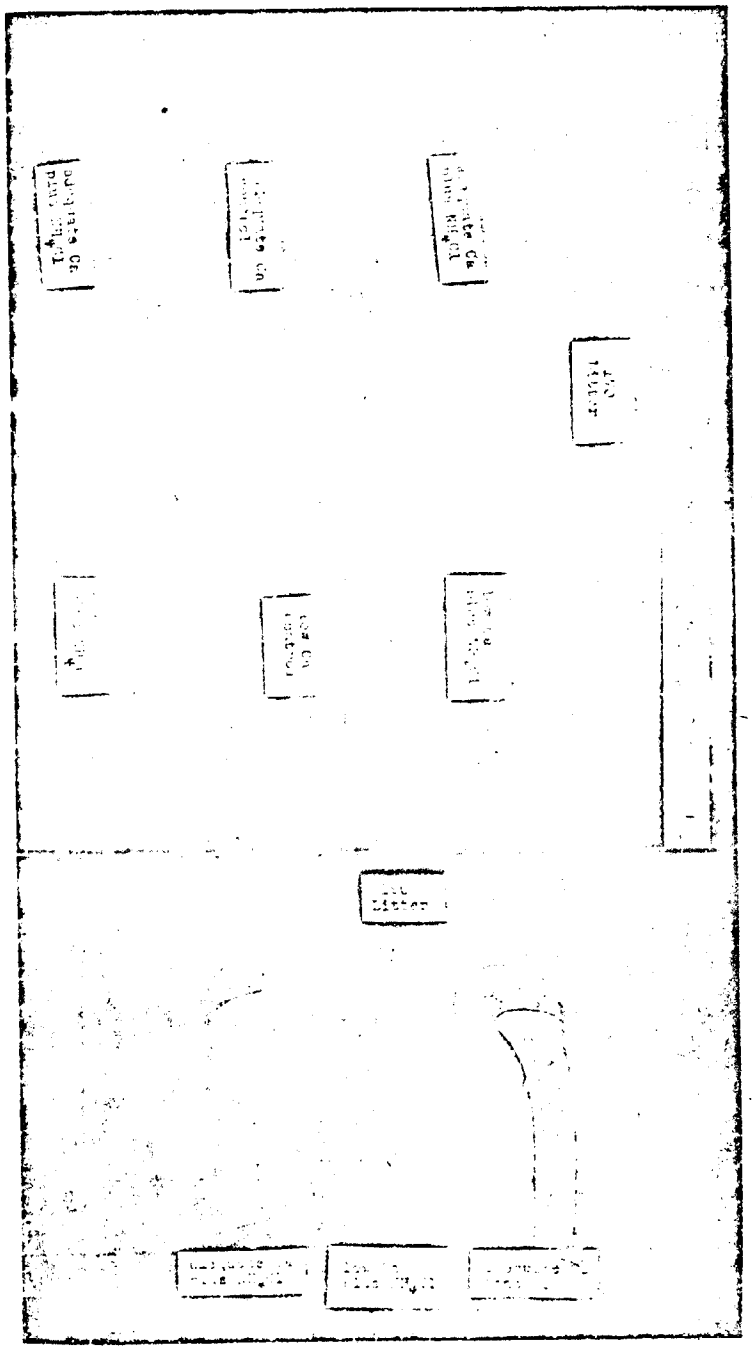
Plate 41

Fig. 1. Photograph of the distal portions of the femora of the 6 months old dogs. Macerated specimens.

Fig. 2. Photographs of the distal portions of the femora of the 3 months old dogs. Macerated specimens.

Plate 42

Fig. 3. Photographs of the distal portions of the femora of the 18 months old dogs. Macerated specimens.



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THE INFLUENCE OF ORALLY ADMINISTERED ALKALI AND ACID ON THE RENAL EXCRETION OF QUINACRINE, CHLOROQUINE AND SANTOQUINE[1]

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Haag and Larson (1) demonstrated in 1942 that in the case of nicotine the extent of urinary excretion of a chemical may be related to the reaction of urine. They emphasized the importance of taking into consideration the dissociation constant of a drug and the relative reabsorbability of the free and dissociated base. Extending these studies to the urinary excretion of quinine in man, Haag, Larson and Schwartz (2) found in 1943 that the urinary output could be doubled by passing from an alkaline to an acid urine and they ascribed the difference to greater resorption of quinine from the urinary tract when the urine is alkaline.

In connection with studying the behavior of newer antimalarial drugs in man, the extent of the influence of urinary acid-base balance upon renal excretion was re-examined. Parallel studies by Emerson and Dole (3) indicated that renal clearance of quinacrine is subject to 100-fold variations due principally to 2 variables, the urinary pH and the renal plasma flow. Work conducted at the same time by the Army Malaria Research Unit at Oxford (4) emphasized the striking parallelism between urinary excretion of quinacrine and ammonia. They found that the ratio of urinary quinacrine to urinary ammonia is directly proportional to the plasma quinacrine concentration. Trager and Hutchinson (5) in 1946 similarly found the urinary excretion of quinacrine to be closely correlated with the urinary excretion of ammonia.

The present investigation was designed to ascertain the effect of oral ingestion of alkali and acid upon the renal excretion of this general type of organic compound. The substances selected for study were quinacrine, chloroquine (SN-7618), and santoquine (SN-6911).

METHODS AND MATERIALS

The patients utilized in this study were from the middle and older age groups. No restriction was placed on the diet or upon water intake during the observations and the patients were permitted the freedom of the ward. All drugs were administered orally and according to a single general dosage pattern. A high initial dose was administered during the first 24 hours and this was followed by the serial administration of smaller maintenance doses. Several days were allowed to elapse for the stabilization of the plasma drug concentration before observations were begun.

Alkali was administered orally in the form of sodium bicarbonate at a total daily dose of 20 grams. This was given in doses of 2.5 grams at 2-hour intervals during the day for 7 doses and once during the night. Acid was administered orally in the form of ammonium chloride at a total daily dose of 8 grams. The dosage schedule was the same as for the bicarbonate, each dose being 1.0 gram.

Plasma and urine estimations were performed by methods developed by Brodie. Quinacrine was determined fluorometrically by the single extraction procedure using ethylene dichloride (6); the alkaline wash was used in the urine determinations but not in the plasma determinations. The ethylene dichloride extract was made acid with trichloracetic acid (Masen [7]). Santoquine was estimated by the colorimetric procedure depending upon a salt formation with methyl orange (Brodie *et al* [8]).

The recently developed specific method (1947) for the analysis of chloroquine (9), involving the use of heptane and alkaline washes, was not yet available at the time of the present study. The procedure used involved the extraction of chloroquine with ethylene dichloride at pH 11, followed by the return of the drug to dilute acid. The acid solution was buffered at pH 9.5 and subjected to ultraviolet irradiation for a 1-hour period. Concentration was estimated by measurement of fluorescence generated by the irradiation. This procedure includes in the measurement interfering degradation products of chloroquine which are found in plasma to the extent of 20 to 50 per cent. However, it is quite specific for urine since only negligible amounts of these degradation products appear in urine.

[1] This work was carried out under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and New York University.

Urine pH was determined in all cases on freshly voided urine samples using a glass electrode. No precautions were taken to maintain CO_2 tensions in the transfer of fluid. Consequently the data on urinary pH must be taken as close approximations rather than precise estimations of the pH of bladder urine. During the period of alkali administration the urinary pH rose to values between 7.5 and 8.0; during the acid period the pH fell to values of 4.0 to 5.0.

RESULTS

Quinacrine

The experimental data obtained on 4 patients receiving maintenance doses of 0.2 and 0.3 grams of quinacrine dihydrochloride are summarized in Table I. The amounts and concentrations noted are expressed in terms of free base. The control values for the renal excretion of quinacrine varied from 1 to 3 per cent of the daily dose. Excretion was reduced to 0.07 to 0.3 per cent of the daily dose during alkalinization and increased to 2 to 6 per cent during acidification. There was in individual cases a 14- to 25-fold increase in going from one state to the other. As is to be expected, such a change is not reflected in the plasma quinacrine concentration since the extent of renal excretion in relation to the oral dose is low in all cases. The plasma level of quinacrine remained roughly constant in 1 patient who was at equilibrium before the observations were begun, and increased as a more or less linear function of time in the 2 who had not as yet achieved an equilibrium plasma quinacrine concentration.

TABLE II

The influence of orally administered alkali and acid on the renal excretion of chloroquine (SN-7618)

Patient	Si.	Wo.	Ke.	Al.	Ch.
Drug intake	200 mgm. per day	100 mgm. per day	400 mgm. per day	400 mgm. per day	400 mgm. per day
	Urinary excretion	Urinary excretion	Urinary excretion	Urinary excretion	Urinary excretion
	milligrams per 24 hours				
Control period	113 104 75	30 105 99	133 84 119	62 110 103	110 60
$NaHCO_3$ *20 grams daily for 3 days*	47 24 41	55 26 17	53 34 37	136 79 61	60 27 30
NH_4Cl *8.0 grams daily for 3 days*	119 188 137	54 75 114	119 208 205	147 192 317	103 80

Chloroquine

The experimental data obtained on 5 patients receiving 0.2 or 0.4 grams of chloroquine daily are summarized in Table II. The daily excretion, in terms of percentage of the daily dose, was high in all patients as compared to quinacrine. This varied in the case of chloroquine, from 15 to 50

TABLE I

The influence of orally administered alkali and acid on the renal excretion of quinacrine

Patient	Wr.		Wo.		Ni.		Na.	
Drug intake	300 mgm. per day		200 mgm. per day		200 mgm. per day		200 mgm. per day	
	Urinary excretion	Plasma level	Urinary excretion	Plasma level	Urinary excretion	Plasma level	Urinary excretion	Plasma level
	mgm. per 24 hrs.	μg. per L.	mgm. per 24 hrs.	μg. per L.	mgm. per 24 hrs.	μg. per L.	mgm. per 24 hrs.	μg. per L.
Control period	2.92 7.47 5.98	81	1.63 3.7	54	1.98 0.808	23 31	7.14 4.38 9.3	50 54
$NaHCO_3$ *20 grams daily for 3 days*	1.32 0.9 0.98		1.73 0.66 0.54	40 50	0.38 0.27 0.18	48 55	2.1 0.79 0.42	87 97
NH_4Cl *8.0 grams daily for 3 days*	5.85 13.6 13.9		6.65 8.25	43	0.18 1.8 3.1	67	5.4 10.3 12.1	102

per cent in the [illegible] cycle; was reduced to 7 to 20 per cent during alkalinization and was increased to 20 to 90 per cent during acidification.

Santoquine

Observations on santoquine were limited to a single individual. However, they were designed to stress the effects of changes in the renal excretion of drug upon the maintenance of the plasma santoquine concentration while on a constant drug intake. Each phase of alkali and acid administration was of 5 days' duration and 2 complete cycles of alkali-acid treatment were obtained (Table III).

TABLE III

The influence of orally administered alkali and acid on the renal excretion of santoquine (SN-6911)

Patient McK.

Drug intake: *400 mgm. per day*

	Urinary excretion	Plasma concentration
	mgm. per 24 hrs.	*micrograms per liter*
Control period	154 107 262 190	410 400
$NaHCO_3$ *12.0 grams daily for 5 days*	36 77 56 82	630
NH_4Cl *6.0 grams daily for 5 days*	90 384 290 219 260	738 685 418
$NaHCO_3$ *20.0 grams daily for 5 days*	109 49 37 60 40	432 775 1010
NH_4Cl *8.0 grams daily for 5 days*	166 181 191 149 183	881 343 324

It should be noted that a large portion of the daily dose of santoquine is excreted in the urine. This was perhaps more in the patient utilized than is usual. During the control period average renal excretion accounted for 41 per cent of the daily dose. This was decreased to an average of 16 per cent during the first alkalinization and increased to an average of 64 per cent during the first acidification. Similar results were obtained during the second cycle.

As is to be expected in such a circumstance, the plasma santoquine concentrations showed a cycle which can be correlated with the intervals of low and high renal excretion. During the alkaline phase of low drug excretion, the plasma level slowly rose; during the acid phase of high excretion, the plasma level fell.

DISCUSSION

It is apparent from the data that the renal excretion of the anti-malarial drugs is dependent upon a series of variables in addition to the concurrent plasma concentration. These variables are of sufficient importance in the determination of the renal drug excretion, that the latter may not be used directly as an approximation method to estimate the general level of the plasma drug concentration.

The refinement introduced by the British workers (4) involving the simultaneous measurement of urinary ammonia and quinacrine does permit of an indirect determination of plasma quinacrine from measurements on urine alone. It appears to us that ammonia excretion may be governed by urinary acid-base balance in a manner similar to the other weak organic bases, such as the anti-malarial drugs. The ammonia measurement provides an index of variation in urinary acid-base balance. By taking the quinacrine concentrations in relationship to ammonia excretion, the Oxford workers were able to eliminate the variable of pH. Treger and Hutchinson (5) seem to ascribe a causal role to ammonia in promotion of urinary excretion; it appears far more likely that ammonia and quinacrine excretions are concomitantly altered in a similar manner by urinary changes in hydrogen ion concentration.

The experimental data are interesting for yet another reason. It must be assumed that at least a portion of the drug eliminated by the kidneys is excreted by a process other than glomerular filtration. This follows from a consideration (in the case of quinacrine) of the extent to which it is bound to the non-diffusible constituents of plasma (some 80 to 90 per cent of the total) and the observed rates of excretion. The mechanism responsible for such tubular excretion as occurs ap-

pears to be quite different from those responsible for the tubular excretion of such substances as phenol red, creatinine, diodrast, etc. The latter manifest considerable stability whereas this does not obtain for the tubular excretion either of quinacrine or of chloroquine and santoquine.

Data are not available with which to define the mechanisms involved though there is sufficient to warrant certain reasonable speculations. It is suggested that a small portion of the excreted anti-malarial drug is filtered at the glomerulus, the amount determined by the glomerular filtration rate, and the concentration of unbound drug in plasma water. A second portion is considered to be added to this in the proximal segment by a process of active tubular excretion similar to the conventional processes considered above. The combined filtered and secreted drug is presented to the distal segment of the nephron in concentrated form due in part to the reabsorption of water in the proximal tubule and loop of Henle. In the distal segment, a process of adjustment of hydrogen ion concentration takes place followed by equilibration of drug across the tubular membrane. The data are in keeping with the assumption that free base is more freely diffusible across the cells of the distal segment than the monoacidic and diacidic salts of the drugs studied. Such being the case there may be expected to be net gain or loss of drug depending upon the relation between the hydrogen ion concentration of the tubular urine and that of peritubular fluid (*ca.* 7. 34). Alkalinization of the tubular urine would increase the proportion of drug in the form of free base and in this way would promote reabsorption; acidification would counteract reabsorption by reducing the proportion of free base. A somewhat similar mechanism has been suggested to cover the renal tubular excretion of neutral red in the amphibian kidney (10).

The observation of Traeger and Hutchinson (5) that the plasma quinacrine level rose soon after oral ingestion of large doses of ammonium chloride, would seem to provide another basis for the altered urinary excretion. The elevation in plasma level observed in their experiment is of brief duration and is in keeping with the increased plasma levels observed in dog experiments (11) in which acute acidosis was produced by CO_2 administration. The plasma quinacrine measurement in the present study revealed no significant changes in level since by design the blood sampling and ammonium chloride administration were timed to elicit only sustained changes. The promotion of urinary excretion of quinacrine by ammonium chloride appears to us not to be secondary to plasma drug elevation but rather a direct result of the acid urine counteracting drug reabsorption in the tubules.

Relatively large changes in excretion rate are expected to be reflected in charges in the plasma drug level on a constant regimen of oral dosage only when the absolute rate of excretion of the substance is high. This phenomenon is brought out strikingly in the patient receiving santoquine. During the acid periods drug excretion amounted to as much as 50 to 75 per cent of the orally administered dosage. The effective dosage was thereby reduced to ½ or ¼ of the actual administered dose. During the alkaline period, the per cent of drug excretion was small, namely 20 per cent of the ingested dose. Under these circumstances, the effective oral dose was as high as 80 per cent of the actual administered dosage.

Quinacrine excretion, though markedly altered by acid-base changes, remains in all circumstances such a small percentage of the orally administered dosage that changes in renal elimination would not be reflected in the maintained plasma level. The changes in drug excretion, from 0.2 per cent during the alkaline period to 4 per cent in the acid period, would not reduce the effective oral dosage to an appreciable extent. The actual measurements on quinacrine plasma levels in the present experiment showed no cyclic fluctuation in acid and alkaline phases as were observed with santoquine.

One therapeutic implication of the above results is obvious. When toxicity is encountered during the chronic ingestion of certain anti-malarial drugs, acidification of the urine by ammonium chloride would promote renal excretion of the drug. Effectiveness of such therapy should be good only with drugs of high excretion rate such as chloroquine and santoquine. With a drug like quinacrine which is excreted in the urine to only a slight degree even upon acidification, such therapy should have little value.

SUMMARY

1. The oral administration of ammonium chloride increases the renal excretion of quinacrine, chloroquine and santoquine in individuals on a constant maintenance dose of the drugs. Sodium bicarbonate given in a similar manner causes a retention of these drugs.

2. Changes in renal excretion are reflected in changes in plasma level only when the absolute rate of excretion of the substance is high.

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Gas-liquid Partition Chromatography: The Separation and Micro-estimation of Ammonia and the Methylamines

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The quantitative micro-analysis of a mixture of ammonia and the methylamines is difficult. A number of colorimetric and other methods exist for the estimation of individual amines, but until recently the only comprehensive scheme (Reay, 1937) depended on the separate analysis of several portions of the mixture after selective precipitation or destruction of one or more of the components—a rather lengthy procedure. Partition chromatography is a promising technique for such a problem and has been used by Fuks & Rappoport (1948) with *n*-butanol-water as the phase pair, on a column of starch mixed with calcium hydroxide. The amines were run as the free bases and were estimated by continuous titration of the effluent from the column. The four amines could be separated from one another but some losses by volatilization occurred during application of the bases to the column. Lagerkvist (1950) separated ammonia and methylamine on a starch column with *n*-propanol-aqueous hydrochloric acid as the solvent system, the positions of the zones being determined by treatment of the eluate with ninhydrin. The recoveries obtained were not stated.

The success which attended the use of gas-liquid partition chromatography in the quantitative micro-analysis of the volatile fatty acids (James & Martin, 1952) suggested its application to the analysis of mixtures of volatile bases. A procedure for the separation of ammonia, methylamine, dimethylamine and trimethylamine is given here; the application of the method to the separation of other aliphatic and of cyclic bases is described in the following paper (James, 1952).

By use of the same general technique as described for the fatty acids, ammonia and the three methylamines may be separated on kieselguhr columns containing a mixture of hendecanol (5-ethylnonan-2-ol) and liquid paraffin as the liquid phase, at a temperature of 78·6°, with only a very slight overlap of the zones of the three methylamines (Fig. 4). The amines emerge from the column in the order of their boiling points (ammonia −33·4°, methylamine −6·5°, trimethylamine 3·5°, dimethylamine 7·4°). It was found that the kieselguhr used as the support for the liquid phase was not inert and adsorbed the amines producing a tail on each zone. Pretreatment of the kieselguhr with methanolic sodium hydroxide prevented most of this adsorption, though enough still occurred to make the zones markedly asymmetric (Fig. 4, curve *B*). No means of overcoming this residual adsorption has yet been found.

The corrected retention volume (V_R^0 as defined by James & Martin, 1952) is a measure of the free energy of association between solute and the liquid phase, both hydrogen bonding and van der Waals forces contributing to this energy of association. The van der Waals forces increase with the number of *N*-methyl groups but each successive group contributes less; the hydrogen-bonding forces vary in a way which in detail is unpredictable. A rough estimate of the van der Waals forces can be obtained from the behaviour of the methylamines on a column having liquid paraffin as the stationary phase, no hydrogen bonding with this solvent being possible (Table 1). The relative hydrogen-bonding power of the methylamines might be expected to run parallel with the order of their basic strengths (dimethyl > methyl > trimethyl > ammonia). Where hydrogen-bonding is possible between solute and solvent (e.g. hendecanol) the order expected from the hydrogen-bonding is different from that expected from van der Waals forces (trimethyl > dimethyl > methyl). The actual order in which the amines will emerge from a column will depend on the relative contributions of the two forces. The factors governing the boiling point sequence are the same as those governing retention-volume sequence in so far as the pure amine constitutes a solvent of properties similar to the liquid phase used on the column. The retention volume of the trimethylamine in a solvent containing hydroxyl groups is greater than would be expected from the boiling point, since hydrogen bonding between the nitrogen atoms in the pure amine is not possible. With hendecanol or hendecanol-15% (v/v) liquid paraffin as the liquid phase,

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hydrogen bonding is important and the amines emerge in the order of the boiling points. When the hendecanol content is reduced (e.g. to 50% hendecanol-liquid paraffin) the amount of hydrogen bonding is decreased and the van der Waals forces exert a greater effect on zone order. The trimethylamine zone now coincides with the dimethylamine zone (Fig. 5, curve *A*). With a liquid phase of paraffin alone the trimethylamine zone comes after the dimethylamine zone, but the difference is insufficient to allow a useful separation on a 4 ft. column (see Table 1), possibly because of the relatively greater adsorption of dimethylamine on the kieselguhr.

In a more polar solvent such as glycerol, the bonding energy between the glycerol molecules is greater than that existing between the glycerol and amine molecules, the effect being analogous to a high internal pressure forcing out the molecules of higher mol.wt. The order of emergence with a liquid phase of glycerol is thus trimethylamine, dimethylamine, methylamine (Fig. 5, curve *B*). Unfortunately, the effect does not delay the emergence of the ammonia zone enough to prevent it overlapping the dimethylamine zone; presumably this is due to the low basic strength of ammonia. Such a column is therefore useless for resolution of all four bases. A similar effect is observed with a liquid phase consisting of DC 550 silicone-10% hendecanol (Table 1).

Where mixtures of only three of the four amines are encountered, advantage can be taken of particular liquid phases. Table 2 shows those recommended for different amine mixtures. As a test of the method the amines occurring free in *Chenopodium vulvaria* L. (stinking goosefoot) have been investigated and the results of Cromwell (1950) have been confirmed, only ammonia and trimethylamine being found (Fig. 6).

EXPERIMENTAL

Preparation of the columns. Celite 545 was size graded, ignited and acid-washed as described by James & Martin (1952). Before use it was treated with 5% (w/v) methanolic NaOH, decanted and the wet kieselguhr oven-dried at 100°. It was stored in a desiccator over solid NaOH. The liquids used as stationary phases were the following: hendecanol (5-ethylnonan-2-ol, supplied by General Metallurgical and Chemical Ltd., 12[illegible] Moorgate, London, E.C. 2), liquid paraffin B.P., DC 5[illegible] silicone (Midland Silicones Ltd.) and glycerol. The liquid phase was added to the kieselguhr in the ratio of 3 g. of liquid phase to 7 g. of kieselguhr and the two thoroughly mixed and packed into a 4 ft. 4 mm.-internal-diameter column of the type described by James & Martin (1952). The column efficiencies are of the order of 650 plates, under the conditions of flow rate and temperature shown in Fig. 4.

Table 1. *Relative retention volumes of the methylamines*

(Volumes are relative to that of NH_3, taken as 1.)

Liquid phase (% v/v)	CH_3NH_2	$(CH_3)_3N$	$(CH_3)_2NH$	Temp. (°)
Hendecanol	3·4	4·2	5·2	78·6
Hendecanol-15% liquid paraffin	3·6	4·6	5·8	78·6
Hendecanol-50% liquid paraffin	4·2	7·5	7·5	65
Liquid paraffin-33% hendecanol	3·8	6·8	6·8	65
Liquid paraffin	1·65	2·7	2·7	78·6
Glycerol	2·5	0·47	Approx. 1	100
Silicone DC550-10% hendecanol	1·33	0·36	0·78	65

Table 2. *Conditions recommended for chromatography of amine mixtures*

Amine mixture	Liquid phase	Temp. (°)
Ammonia, mono-, di- and tri-methylamines	Hendecanol-15% (v/v) liquid paraffin	78·6
Ammonia, mono- and di-methylamines	Hendecanol-50% liquid paraffin	78·6
Ammonia, mono- and tri-methylamines	Hendecanol	78·6
Ammonia, di- and tri-methylamines	Glycerol or silicone DC 550-10% hendecanol	100
Mono, di- and tri-methylamines		65

Burettes. The apparatus used was that described by James & Martin (1952), the titrations being carried out automatically with 0·04N-H_2SO_4 using 0·007% (w/v) aqueous methyl red solution in the titration cell. Whilst the recording burette is a great convenience, any burette capable of delivering known constant volumes gives perfectly satisfactory results. The titration is then carried out by supplying to the titration cell a constant amount of acid, the time required for its neutralization is noted and a further addition of the same amount of acid is made. (More dilute indicator solution is preferable with this method.) This proves to be easier than reading the burette at constant time intervals. A curve of total acid delivered against time is plotted, the height of each step giving the amount of each component. Three types of burette are illustrated in Figs. 1-3. The first (Fig. 1a) consists simply of a small

Erlenmeyer flask, containing the acid, fitted with an inlet and outlet tube. By squeezing the rubber tube fitted to the inlet a drop of acid can be formed on the outlet tube and any number of these drops can be rapidly delivered to the titration cell. The drop size is constant and can be calibrated; the number of drops added to the titration cell to keep the solution near the end point can readily be followed.

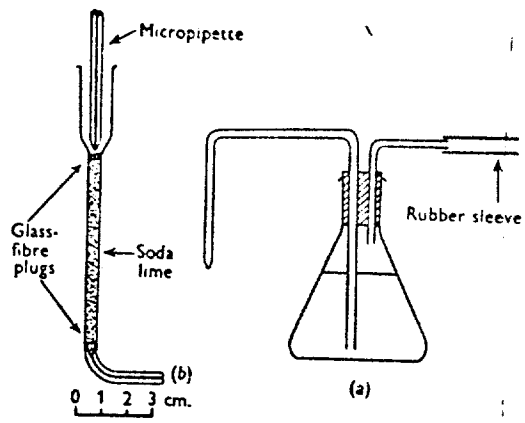

Fig. 1. (*a*) Dropping burette. (*b*) Microcolumn for liberation of the amines from solutions of their salts.

The second (Fig. 2) uses a screw passing through a nut and a cork gland kept in place by sealing wax at the end of the burette. As the screw is rotated by the handle it passes into the burette and the acid is displaced into the titration cell. By counting the number of turns of the screw the total amount of acid delivered is readily ascertained. As glass taps have been found to be unserviceable for long periods because of leakage, the rubber-glass rod valves described by James & Martin (1952) are used. The burette is readily filled by closing the valve leading to the cell and opening the one leading to the acid reservoir, and winding back the screw.

The burette illustrated in Fig. 3 is one which uses the micrometer from an 'Agla' microsyringe (Burroughs Wellcome Ltd.). This type of burette is only suitable when

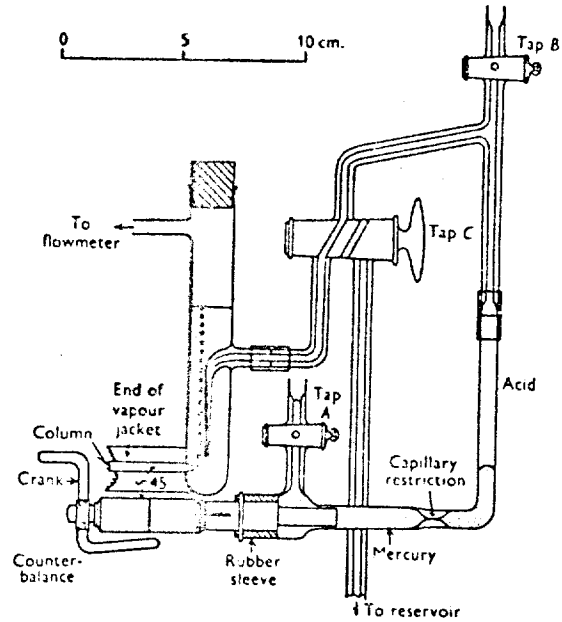

Fig. 3. Micrometer burette and titration cell.

there is a long time interval between the emergence of successive zones. The end of the micrometer barrel is held firmly in the glass tubing by a rubber sleeve. Rotation of the micrometer displaces mercury which in turn forces acid into

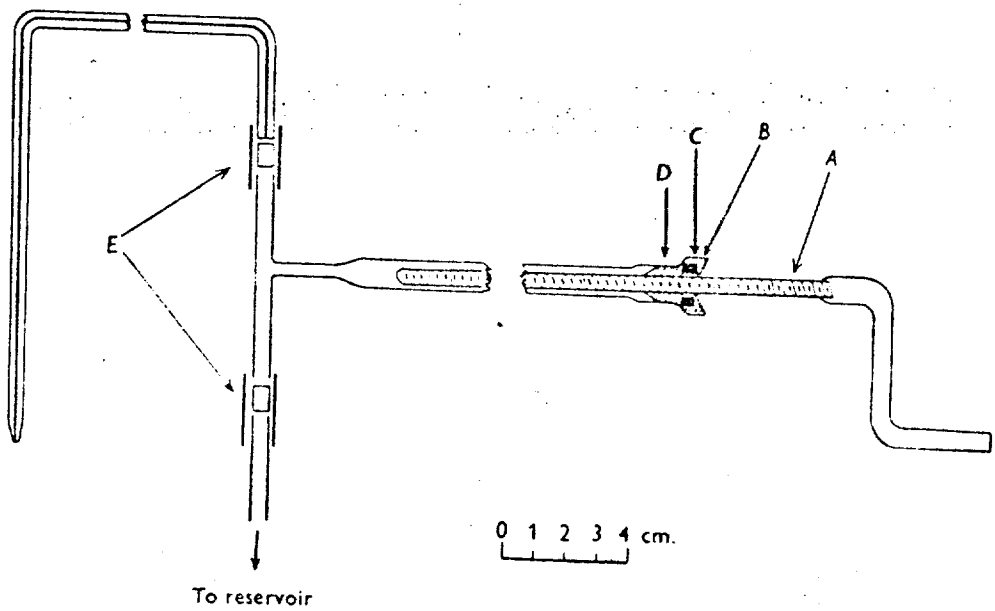

Fig. 2. Screw burette. *A*, screw (2BA thread); *B*, sealing wax; *C*, nut; *D*, cork; *E*, valves.

the titration cell. The taps A and B are not essential, but enable the initial filling of the burette and removal of air to be performed more easily. The mercury is then adjusted to a suitable level by manipulation of tap A. Tap C could perhaps be replaced with advantage by the rubber-glass rod valves previously described. The moving scale on the micrometer is divided into hundredths, and can be estimated to one-tenth of a division; with 0·1 N-acid in the burette, a noticeable change in colour of the indicator is observed for a movement of one- or two-hundredths of a rotation. The scale remains in an almost constant position beneath the titration cell, so that the two may be watched simultaneously

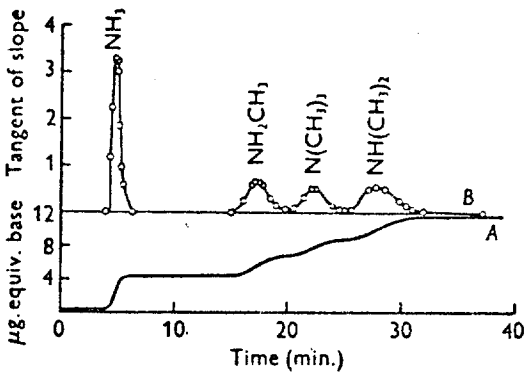

Fig. 4. The separation of ammonia, mono-, tri- and dimethylamines. Curve A: experimental curve; curve B: curve obtained by graphical differentiation of experimental curve, showing slight tailing of zones. Column length, 4 ft.; liquid phase, hendecanol-15% liquid paraffin (v/v); temp., 78·6°; flow rate of nitrogen, 5 ml./min.; nitrogen pressure, 65 mm. Hg.

and readings taken every half min. It is more convenient to operate the burette with the left hand in order to have the right free for recording the results, though the scale marked on the micrometer is then inverted. The burette is filled by reversing tap C and winding back the micrometer.

Liberation of the free bases from the amine salts. Application of the free amine bases in aqueous solution to the column leads to bad separations and so the following technique has been devised.

A small vertical column terminating in a short horizontal capillary (Fig. 1*b*) is filled with dry soda lime (50 mesh) and a very small plug of glass fibre packed on top of the filling. This extraction column is attached by a piece of pressure tubing to the chromatogram held in the vapour jacket, the dead space at the end of the chromatogram tube being filled by a suitable length of loosely fitting glass rod. Approx. 0·8 N solution (total amines) (0·05 ml.) of the amine salts is run on to the column by placing the micropipette on the glass-fibre plug. When the extraction column has taken up the solution, the plug is washed with two lots of 0·025 ml. of distilled water, the N_2 supply is connected and the gas stream started. The wet part of the column is warmed with a microburner until the visible water front has moved almost to the bottom of the column, the whole tube then being hot. The indicator solution is run into the cell and the titration carried out as usual. The recoveries are approximately theoretical, the reproducibility being ±4%.

Operation of the chromatogram and results. The amines were applied to the chromatograms either as solutions of the free bases in ethanol (3 μl. of a 20% solution) or by means of the technique outlined above, starting from a solution of the amine salts.

The four amines were applied singly and in mixtures to the chromatograms. The retention volume of each zone was measured and corrected to zero pressure difference across the column (V_R^0) by the use of the graphs of the relationship between P_1/P_0 and V_R/V_R^0 given by James & Martin (1952).

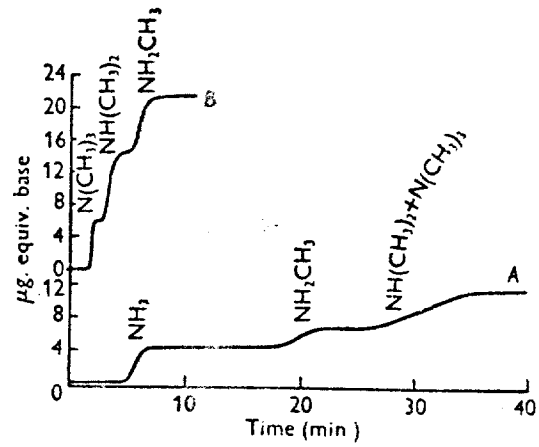

Fig. 5. Curve A: the separation of ammonia and the methylamines showing coincidence of trimethylamine and dimethylamine zones on increasing the paraffin content of the hendecanol to 50%. (Column length, 4 ft.; liquid phase, hendecanol-50% liquid paraffin (v/v); temp., 78·6°; flow rate of nitrogen, 3·6 ml./min.; nitrogen pressure, 65 mm. Hg.) Curve B: the separation of the methylamines showing the effect of a liquid phase having high internal pressure (glycerol). The amines now appear in order of decreasing molecular weight. (Column length, 4 ft.; liquid phase, glycerol; temp., 100°; flow rate of nitrogen, 4 ml./min.; nitrogen pressure, 60 mm. Hg.)

The corrected retention volumes of NH_3 on each type of column used are shown in Table 3. The solvents used in the vapour jacket to give the temperatures quoted are methanol (65°), ethanol (78·6°) and water (100°). Typical analyses of mixed amines are shown in Figs. 4 and 5.

Table 3. *Corrected retention volumes of* NH_3 *with various liquid phases*

Liquid phase (v/v)	Temp. (°)	V_R^0 (ml.)*
Hendecanol	78·6	37·4
Hendecanol-15% liquid paraffin	78·6	23·2
Liquid paraffin-50% hendecanol	65	24·1
Liquid paraffin-33% hendecanol	65	21·0
Liquid paraffin	78·6	8·2
Glycerol	100	15·0
Silicone DC 550-10% hendecanol	65	210

* Defined by James & Martin (1952).

The burette illustrated in Fig. 3 was used with columns run at room temperature, when a few [illegible] taken was much longer than that shown in Fig. 4. The efficiency of the

column under these circumstances is lower (cf. James & Martin, 1952), but is adequate to separate any three of the four amines provided a suitable liquid phase is chosen (Table 3).

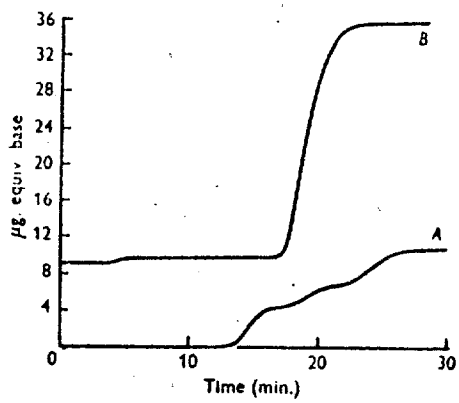

Fig. 6. Analysis of the volatile amines present in *Chenopodium vulvaria* L. Curve *A*: standard mixture of mono-, di- and tri-methylamines. Curve *B*: chromatogram of the amines present in an acid extract of the plant, showing the presence of only ammonia and trimethylamine. (Column length, 4 ft.; liquid phase, hendecanol-15 % liquid paraffin (v/v); temp., 78·5°; flow rate of nitrogen, 5·7 ml./min.; nitrogen pressure, 80 mm. Hg.)

The lower limit of quantity of amine that can be detected with this technique is 0·3 μg. equiv., i.e. 2 μg. of NH_3, 4 μg. of monomethylamine, 7 μg. of dimethylamine and 8 μg. of trimethylamine. The maximum amounts of amines that still allow good separations are 160 μg. of NH_3, 180 μg. of monomethylamine, 180 μg. of dimethylamine and 220 μg. of trimethylamine, using a 4 ft. 4 mm. internal-diameter column.

The estimation of the amines present in Chenopodium vulvaria *L.* The leaves from four plants of *C. vulvaria* L. (approx. 100 g.) were minced in a Waring Blendor and extracted with 0·001 N-HCl (1 L.). The extract was filtered, centrifuged and then made alkaline with NaOH. Air was drawn through the mixture into a wash bottle containing 0·001 N-HCl. At the end of an hour the wash bottle was removed and its contents evaporated almost to dryness. The solution was made up to 2 ml., and 0·1 ml. portions were taken for analysis. The results are shown in Fig. 6.

SUMMARY

1. A technique for the micro-estimation and separation of ammonia and the three methylamines is described.

2. Some factors influencing the relative positions of amines on gas-liquid partition chromatograms are discussed.

3. A method of applying the amines to the columns starting from a solution of the amine salts is described.

4. All the curves illustrated have been obtained by the use of the automatic recording burette described by James & Martin (1952). Three types of manual microburette are now described, together with appropriate titration procedures.

Our thanks are due to Dr B. T. Cromwell for the gift of the stinking goosefoot. Part of this work (by G.H.S.) was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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Renal adaptation during chronic NH_4Cl acidosis in the rat: no role for hyperplasia

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JANICKI, RALPH H. *Renal adataption during chronic NH_4Cl acidosis in the rat: no role for hyperplasia.* Am. J. Physiol. 219(3): 613-618. 1970.—Chronic NH_4Cl acidosis in rats results in renal enlargement and a marked increase in phosphate-dependent glutaminase (PDG). To assess the role of hyperplasia in these processes, the incorporation of tritiated thymidine into renal DNA has been investigated during chronic NH_4Cl acidosis and compared with that seen in the remaining kidney after uninephrectomy. Thymidine incorporation is reduced in acidotic rat kidneys, whereas 2 days after uninephrectomy it is markedly increased. In another experiment, rats have been made acidotic and simultaneously injected with hydroxyurea, an inhibitor of DNA synthesis. Phosphate-dependent glutaminase activity is increased in acidotic rats treated with hydroxyurea to the same extent as in acidotic rats treated with saline. Thus, the data indicate that DNA synthesis and cell division are not an important part of renal enlargement during chronic NH_4Cl acidosis; the existing tubule cells have a remarkable capacity to adapt enzymatically to an acid insult.

glutaminase; mechanism of enzyme adaptation; thymidine incorporation; renal growth; uninephrectomy; hydroxyurea

CHRONIC NH_4CL ACIDOSIS causes renal enlargement[1] in rats (19) and mice (11). After 1 week, renal enlargement resulting from acidosis resembles that seen in the remaining kidney after uninephrectomy (11, 19). However, a temporal study of the two growth stimuli in the mouse has elucidated certain dissimilarities concerning the kinetics of enlargement. After uninephrectomy renal mass increases within 1 or 2 days, whereas during NH_4Cl acidosis the growth response is restrained, and no enlargement is seen for the first 4 days (11).

The mechanism of renal growth after uninephrectomy has been characterized. Within 1 day after contralateral renal extirpation, RNA (20) and protein (2, 3) synthesis have been intensified, and significant changes in subcellular chemistry have developed (9, 11). During the 1st day the enzymes in the DNA synthetic pathway have increased (21), leading to enhanced DNA synthesis (22). A wave of mitotic activity, found predominantly in the proximal tubule cells (28), appears on the 2nd day (22, 28) and is associated with an estimated 6% increase in the total number of renal cells (8).

[1] The words growth and enlargement are used to describe a general increase in size. Cellular hypertrophy refers to an increase in cell size, but not cell number. The term cellular hyperplasia is used only when referring to cell division and an eventual increase in cell number.

Our understanding of the mechanisms involved in renal enlargement during NH_4Cl acidosis is more limited. Although the concentration of renal RNA is slightly elevated during acidosis; protein synthesis during the first 36 hr of NH_4Cl acidosis is unaltered (2). A decrease in the concentration of renal DNA after 1 week of NH_4Cl acidosis, indicating cellular hypertrophy has occurred, has also been reported in the mouse (11).

The role of cell division (cellular hyperplasia) in NH_4Cl-induced kidney growth has remained unexplored. Since significant physiological adaptation occurs in the acidotic rat kidney, it is of interest to know if these adaptations are associated with increases in cell number.

The adaptation of renal phosphate-dependent glutaminase (PDG) during acidosis is of particular interest. In rats PDG is the major enzyme leading to the formation of urine ammonia (6). Ammonia is the major urinary buffer, and its production by the kidney, and eventual excretion, is increased during NH_4Cl acidosis (6, 24, 26). The increase in renal ammonia production in rats (6, 26) and mice (11) is attributed to intensified PDG activity. Moreover, the adaptation of PDG is blocked by protein synthetic inhibitors (5, 11), indicating the synthesis of new enzyme is involved.

To assess whether increased PDG activity during chronic NH_4Cl acidosis is associated with increased cell number, the incorporation of tritiated thymidine (^{3}HTdR) into renal DNA has been investigated. Since no increase in ^{3}HTdR incorporation can be demonstrated, it is unlikely DNA synthesis and eventual cell division is involved. To substantiate this observation, another experiment has been conducted using hydroxyurea, an inhibitor of DNA synthesis (29). Phosphate-dependent glutaminase activity is elevated in acidotic rats injected with hydroxyurea to the same extent as acidotic rats injected with saline. Collating the above results, it is unlikely cell division is involved in renal enlargement during NH_4Cl acidosis in the rat, and the existing population of tubule cells appears to have a remarkable adaptive capacity.

METHODS

Animals

Sprague-Dawley rats weighing 200 g were purchased from Zivic-Miller Laboratories (Allison Park, Pa.) and kept until they weighed between 225 and 275 g. Temperature, humidity, and 12-hr light and dark cycles were

carefully controlled in the animal room. Rats were kept two per cage and fed standard laboratory chow and ordinary tap water ad libitum.

Acidosis

Depending upon experimental requirements, three different procedures were used to induce chronic NH_4Cl acidosis.

Procedure 1. The rats in this study were made acidotic by substituting 0.28 M NH_4Cl for ordinary tap water, according to a previously described procedure of Lotspeich (19).

Procedure 2. The rats in this study were made acidotic by gastric intubation of 1.2 M NH_4Cl (0.1 ml/10 g body wt) twice daily (8 AM and 8 PM). The NH_4Cl load was 24 mmoles/kg per day. A previous study (unpublished data) showed that this method does not interfere with hepatic glycogen content. Presumably, these rats were in a good nutritional state.

Procedure 3. The rats in this study were made acidotic according to a procedure described by Preuss (25). Twelve hours before beginning the experiment, solid food was removed from all the cages, and all the rats received 5.0 ml of 20% dextrose by gastric tube. Thereafter, acidotic rats received twice daily (8 AM and 8 PM) 5.0 ml of 0.6 M NH_4Cl in 20% dextrose, and control rats received 5.0 ml of 20% dextrose. The rats had continued access to ordinary tap water.

Hydroxyurea Treatment

Hydroxyurea (Sigma Chemical Co., St. Louis, Mo.) was dissolved in sterile saline (100 mg/ml), and injected ip at a dose of 1 g/kg. A previous study showed this dose inhibited renal ^{3}HTdR incorporation for 8 hr, without the antimetabolite losing its effectiveness during prolonged administration (10). In this study (Fig. 2), hydroxyurea was administered at 8-hr intervals (8 AM, 4 PM, and 12 PM) for 2 days. As a result of prolonged hydroxyurea treatment, the intestinal epithelium nearly disappeared, substantiating earlier observations (23). The coats of these rats appeared soiled, with some loss of hair. Nevertheless, no rats died as a result of this protocol.

Uninephrectomy

A procedure previously described by Argyris and Trimble (1) was used for the surgical removal of the right kidney.

^{3}HTdR Incorporation

Rats were injected ip with thymidine methyl-^{3}H (3.0 c/mmole) purchased from Schwarz BioResearch, Inc. (Orangeburg, N. J.). Thirty minutes before decapitation and thorough bleeding, each rat received 100 μc in 2.0 ml of saline. Kidneys were excised, stripped of fat, weighed on a Mettler balance, and kept ice cold. A 5% homogenate was prepared in ice-cold distilled water using a loosely fitting motor-driven Teflon pestle. Deoxyribonucleic acid (DNA) was extracted by the Schmidt Thannhauser method described by Leslie (18) and determined by the diphenylamine reaction by Dische (4). A standard curve was prepared from highly purified calf thymus DNA (Sigma Chemical Co.).

Radioactivity was measured in a Packard liquid scintillation spectrometer, model 3380, (Packard Instrument Co., Downers Grove, Ill.) at 5 C. Counting vials contained: 0.1 ml DNA extract in 0.5 N PCA, 1 ml Soluene (Packard Instrument Co.), and 15 ml toluene solution (5.0 g PPO and 0.2 g M_2POPOP in 1,000 ml toluene). Quench corrections were made by the external standard channels ratio method.

Specific activity (SA) was calculated using the formula

$$SA = \frac{\text{dpm}/\mu\text{g DNA}}{100\ \mu\text{c}\ ^3\text{HTdR/g body wt}}$$

Protein Determination

Protein was determined colorimetrically using the biuret procedure described by Layne (17).

Phosphate-Dependent Glutaminase

Whole kidneys were weighed to the nearest milligram, and a 5% homogenate was prepared in ice-cold 0.25 M sucrose. Nuclei and unbroken cells were removed by centrifuging the homogenate at 600 × *g*, for 10 min, at 0–2 C, in a Sorvall superspeed centrifuge (model RC2-B). The supernatant, containing mitochondria (27), was used to determine phosphate-dependent glutaminase.

The reaction was run in ammonia-diffusion bottles. The incubation medium contained: 0.2 ml, 0.05 M Trizma (Sigma Chemical Co.), adjusted to pH 8.0; 0.5 ml, 0.2 M Na_2HPO_4-NaH_2PO_4, at pH 8.0; 0.1 ml of 5% ice-cold homogenate, and 0.1 ml H_2O. After incubation for 10 min, the reaction was initiated by adding 0.1 ml, 0.2 M L-glutamine (Sigma Chemical Co.). Incubations were carried out in air at 37 C, with constant shaking, for 12 min. The reaction was terminated by adding 1.0 ml of saturated K_2CO_3 from a syringe and quickly sealing the vials with a stopper equipped with a glass rod dipped in 5 N H_2SO_4. After diffusion (30 min), ammonia was determined colorimetrically using commercially prepared Nessler's reagent (Fisher Chemical Co., Pittsburgh, Pa.). Each assay was run in duplicate. Simultaneously, an additional vial was run in which 0.2 M Na_2HPO_4-NaH_2PO_4 was replaced with distilled H_2O. The ammonia produced in this vial was subtracted from the amount produced in the presence of Na_2HPO_4-NaH_2PO_4. Thus, endogenous ammonia, ammonia produced from another enzymatic reactions, and the ammonia produced by the nonenzymatic breakdown of L-glutamine was subtracted. Using this procedure, PDG activity was linear over a threefold range of homogenate concentrations.

Protein was determined on an aliquot of the supernatant portion of the homogenate by the biuret method (17). Phosphate-dependent glutaminase activity was calculated as micromoles NH_3 per milligram protein per hour.

Statistics

Fisher's small-sample method of the Student *t* test was used to determine statistical significance. In this report, $P <$

TABLE 1. *Renal wet weight, protein, and DNA after NH_4CL acidosis**

	Experimental Day						
	0	1	2	3	4	5	7
mg Wet wt/100 g body wt	407 ± 14	403 ± 16	410 ± 12	421 ± 11	437 ± 22	435 ± 6	462 ± 12
mean % change		−1	+1	+3	+7	+7	+13
P value		NS	NS	NS	NS	NS	<0.01
mg Protein/g wet wt	179 ± 4	189 ± 4	193 ± 7	197 ± 9	200 ± 3	192 ± 5	200 ± 4
mean % change		+5	+8	+10	+11	+7	+12
P value		<0.05	<0.05	0.05	<0.001	<0.05	<0.001
mg DNA/g wet wt	4.39 ± 0.20	4.70 ± 0.46	4.22 ± 0.14	4.19 ± 0.75	4.47 ± 0.20	4.09 ± 0.21	4.04 ± 0.91
mean % change		+7	−4	−5	−2	−7	−8
P value		NS	NS	NS	NS	NS	NS
mg Protein/mg DNA	40.9 ± 2.0	41.3 ± 3.5	46.3 ± 1.8	46.9 ± 2.2	45.0 ± 2.0	47.1 ± 1.8	49.6 ± 1.3
mean % change		+1	+13	+14	+10	+15	+21
P value		NS	<0.05	<0.05	NS	<0.05	<0.01

Values are the mean ± SEM of 6 rats. NS = not significant. * Rats were made acidotic by substituting 0.28 M NH_4Cl for ordinary tap water, see *procedure 1*.

0.01 was considered significant; however, $P < 0.05$ were included in the Tables 1–3 to assist the reader in recognizing changes which approach statistical significance. All values are presented as the mean ± SEM. Percent change (mean % change) is based on the difference between two means.

RESULTS

In the initial series of experiments, compensatory renal enlargement has been measured in rats made acidotic (*procedure 1*—see METHODS) by substituting 0.28 M NH_4Cl for ordinary tap water (Table 1). A significant increase in renal wet weight (mg wet wt/100 g body wt) develops after 5 days; protein concentration (mg protein/g wet wt) is significantly increased after 4 days. The increased concentration of renal protein in the acidotic rat (Table 1) is not consistent with an earlier report in the acidotic mouse (11), in which no change in protein concentration developed. The concentration of renal DNA (mg DNA/g wet wt) decreases (Table 1); however, it is not statistically significant. Protein/DNA ratios (mg protein/mg DNA) increase during NH_4Cl acidosis; however, no significant change occurs during the initial 5 days.

For the most part, these results confirm an earlier report by Lotspeich (19), which first demonstrated the effect of NH_4Cl acidosis on renal size. In a previous report from this laboratory, the effect of NH_4Cl acidosis on renal enlargement in the mouse has been described on a temporal basis (11). With the exception of increased renal protein concentration noted in the acidotic rat (Table 1), the results of the respective studies are strikingly similar.

The restrained appearance of renal enlargement during NH_4Cl acidosis has been compared with the rapid renal enlargement seen after uninephrectomy (Table 2). One day after uninephrectomy renal wet weight is significantly increased. Renal protein concentration remains unchanged, due, at least in part, to early increases in protein synthesis (2, 3). Despite documented increases in cell number (1, 8, 22, 28), the concentration of renal DNA decreases (Table 2), suggesting cellular hypertrophy is the major mechanism of enlargement. Significantly increased renal protein/DNA ratios tend to substantiate this point (Table 2). For the most part, changes in cellular chemistry induced by uninephrectomy, presented in Table 2, are consistent with previous studies in rats (9) and mice (11).

Although hyperplasia is known to occur in the remaining kidney after uninephrectomy, the role of cell division during NH_4Cl acidosis induced renal growth has remained unclear. To investigate this, the incorporation of tritiated thymidine (^{3}HTdR) into renal DNA has been measured in rats made chronically acidotic by substituting 0.28 M NH_4Cl (*procedure 1*—see METHODS) for ordinary tap water (Fig. 1). For comparison, ^{3}HTdR incorporation has been determined on each of the first 3 days after uninephrectomy.

The incorporation of ^{3}HTdR into renal DNA during chronic NH_4Cl acidosis is significantly decreased on *days 1, 3, 4,* and *5* ($P < 0.01$); and on *day 7*, it approaches statistical significance ($P < 0.05$). This suggests DNA synthesis and eventual cell division is not an important acidosis-induced renal enlargement.

Contrasting, 1 day after uninephrectomy, ^{3}HTdR incorporation is significantly increased ($P < 0.01$). It reaches a peak on *day 2* ($P < 0.001$), and on *day 3* it has returned to normal. This result substantiates previous reports describ-

TABLE 2. *Renal wet weight, protein, and DNA after uninephrectomy*

	Experimental Day			
	Sham	1	2	3
mg Wet wt/100 g body wt	387±5	438±8	441±13	466±15
mean % change		+13	+14	+20
P value		<0.001	<0.01	<0.001
mg Protein/g wet wt	187±4	189±3	187±4	192±4
mean % change		+1	0	+3
P value		NS	NS	NS
mg DNA/g wet wt	4.69±0.12	3.89±0.96	3.97±0.18	3.92±0.17
mean % change		−17	−15	−16
P value		<0.001	<0.01	<0.01
mg Protein/mg DNA	39.9±1.2	48.8±1.2	45.8±1.1	49.3±1.3
mean % change		+22	+15	+23
P value		<0.001	<0.01	<0.001

Values are the means ± SEM of 6 rats.

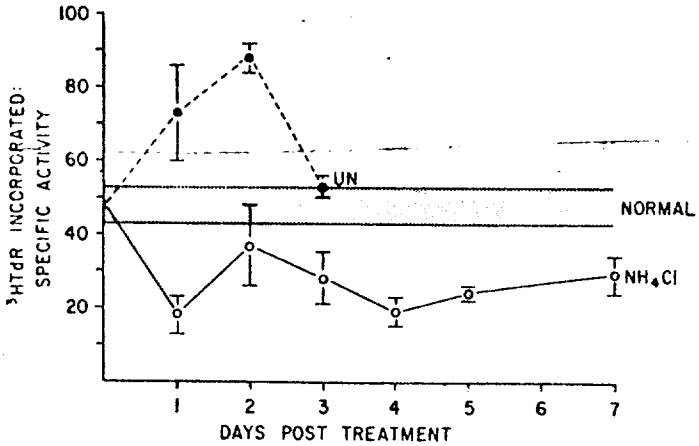

FIG. 1. Incorporation of tritiated thymidine (^{3}HTdR) into renal DNA in acidotic and uninephrectomized rats. Acidosis has been induced by substituting 0.28 M NH_4Cl for ordinary tap water (see *procedure 1*). Data are presented as specific activity (see METHODS). Open (acidotic) and closed (uninephrectomized) circles are the means, and the vertical lines are the standard errors (mean ± SEM). There are 4 rats in each acidotic group and 5 rats in each uninephrectomized group. Shaded area is mean ± SEM of 5 untreated rats.

ing increased mitotic activity 2 days after uninephrectomy (1, 8, 22, 28).

When acidosis is induced by substituting 0.28 M NH_4Cl for ordinary tap water, rats fail to increase their body weights and lose considerable amounts of liver glycogen. Therefore, the question arises whether reduced ^{3}HTdR incorporation in the acidotic rat, noted in Fig. 1, is due to a poor nutritional state. To investigate this, rats have been made acidotic by administering NH_4Cl (24 mmoles/kg per day) by gastric tube (*procedure 2* see METHODS). Meanwhile, these rats continue to consume standard laboratory chow and ordinary tap water ad libitum. Daily increases in body weight and the maintenance of liver glycogen, (unpublished data), indicate these acidotic rats maintain a reasonable nutritional state.

The incorporation of ^{3}HTdR into renal DNA after 2 days of acidosis in these rats is similarly reduced (Table 3). Therefore, this reduction is probably due to the acid load and not to a poor nutritional state. Meanwhile, after 2 days of acidosis, PDG is significantly increased (Table 3).

The data indicate that although renal enlargement ensues during chronic NH_4Cl acidosis, DNA synthesis and cell division do not appear to be involved. Thus, the extensive enzymatic adaptations occurring in the kidney in response to NH_4Cl acidosis (among them an extraordinary increase in PDG) appear to evolve in the existing population of cells. If so, PDG should adapt in acidotic rats in which DNA synthesis (a prerequisite for cell division) is inhibited.

Hydroxyurea has proven to be a useful tool in investigating this. Previous reports indicate hydroxyurea is a specific inhibitor of DNA synthesis while RNA and protein synthesis continue unabated (29). Hydroxyurea inhibits cytidine diphosphate reductase in vitro (16), and this may account for its selective action on DNA synthesis. However, hydroxyurea causes cell damage in the intestinal crypts of mice (23), and continued administration also leads to pronounced anemia in rabbits (15). To avoid complications resulting from disturbed dietary intake as a result of hydroxyurea treatment, the rats in this experiment have been fed by stomach tube. Acidosis has been induced by stomach feeding 5.0 ml of 0.6 M NH_4Cl in 20% dextrose, whereas controls receive 5.0 ml of 20% dextrose (*procedure 3* see METHODS). Hydroxyurea (1 g/kg, ip) is administered every 8 hr throughout the 48-hr experimental period.

After 48 hr, PDG activity in rats receiving NH_4Cl is twice the control value. Phosphate-dependent glutaminase activity is similarly elevated in rats receiving NH_4Cl and hydroxyurea (Fig. 2). The difference between the group receiving NH_4Cl and the group receiving NH_4Cl and hydroxyurea is not significant ($P > 0.1$). The basal level of PDG is unaffected by hydroxyurea treatment. Thus, although renal DNA synthesis has been inhibited, the activity of renal PDG increases during NH_4Cl acidosis.

TABLE 3. *Effect of ammonium chloride acidosis* on renal thymidine incorporation and phosphate-dependent glutaminase*

Treatment	^{3}HTdR Incorporation, SA	Glutaminase, μmoles NH_3/mg protein × hr
Acidotic	32.7 ± 4.3 (7)	8.0 ± 0.5 (6)
Control	51.9 ± 4.2 (7)	5.1 ± 0.5 (6)
Mean % change	−37	+57
P value	0.01	<0.01

Acidotic and control values are means ± SEM with the number of rats per group given in parentheses. SA = specific activity.
* Rats were made acidotic by gastric tube (24 mmoles NH_4Cl/kg per day).

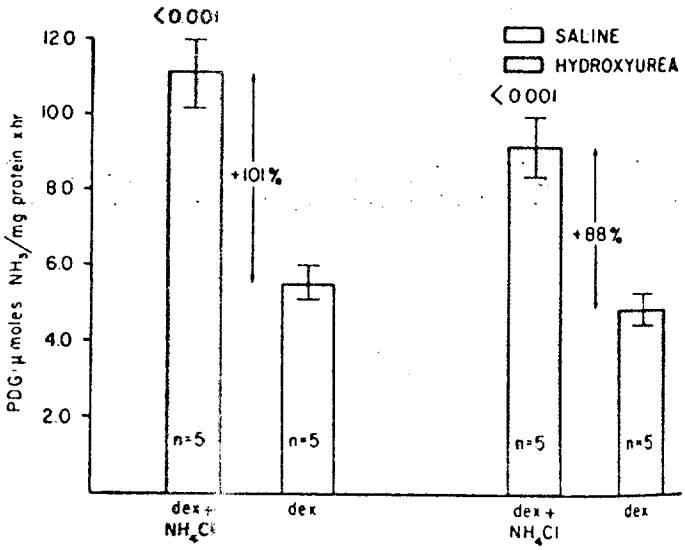

FIG. 2. Adaptations of phosphate-dependent glutaminase (PDG) in rats after 48 hr of NH_4Cl acidosis (24–30 mmoles/kg per day). Acidotic rats have received 5.0 ml 0.6 M NH_4Cl in 20% dextrose, and controls have received 5.0 ml of 20% dextrose. Regimen has been administered twice daily (see *procedure 3*). Shaded bars represent groups of rats similarly handled, which have been injected with hydroxyurea (1g/kg, ip) every 8 hr. Tops of bars represent means while vertical lines represent standard errors (mean ± SEM), with 5 rats in each group. Levels of significance (*P* values) and mean percent changes are indicated. Phosphate-dependent glutaminase activity in acidotic rats is not significantly different from PDG activity in acidotic rats receiving hydroxyurea ($P > 0.1$).

DISCUSSION

Previous studies with rats (19) and mice (11) have demonstrated that renal enlargement ensues during chronic NH_4Cl acidosis. In this communication, using rats, the kinetics of renal enlargement during chronic NH_4Cl acidosis are compared with the enlargement occurring after uninephrectomy. Clearly, the growth responses are dissimilar. Renal enlargement during chronic NH_4Cl acidosis is a gradual process (Table 1), whereas enlargement after uninephrectomy appears much sooner (Table 2). These data are consistent with studies on renal protein synthesis resulting from the respective stimuli. Renal protein synthesis remains near normal during the first 36 hr of NH_4Cl acidosis (2), whereas it is significantly increased during the first day after uninephrectomy (2, 3).

The mechanism of renal enlargement after uninephrectomy has been characterized. Within 1 day DNA synthesis is increased (21, 22), and after 2 days there is a burst of mitotic activity (8, 22, 28), found predominantly in the proximal tubule (22, 28). The results presented in Fig. 1 substantiate these observations.

Whether cell division occurs during NH_4Cl-induced renal enlargement has remained unclear. This is of particular interest, since the acidotic rat kidney is undergoing extensive physiological adaptation. Significant among these adaptations is a marked increase in PDG activity in the rat (6, 19, 26) and the mouse (11), leading to augmented ammoniagenesis (6, 19, 26). Moreover, studies with cycloheximide in the mouse (11) and actinomycin D in the rat (5) have indicated the adaptation of renal PDG during NH_4Cl acidosis involves the synthesis of new enzyme.

Histochemical techniques have indicated PDG is found in the tubule cells of rat kidney, and it is particularly high in the straight portion of the proximal tubule (15). During NH_4Cl acidosis PDG activity is increased, particularly in the convoluted tubules (15).

This laboratory has been interested in determining whether the adaptation in PDG, as well as other adaptations during acidosis (7), occur in the existing population of renal tubule cells. On the cellular level, PDG activity could evolve in two different ways. Figure 3 depicts these mechanisms: *1)* in which cell division is involved, and *2)* in which cell division is absent.

To test this, the incorporation of ^{3}HTdR into renal DNA has been measured during chronic NH_4Cl acidosis induced by two different methods (Fig. 1, Table 3). In both instances, the incorporation of ^{3}HTdR is significantly reduced. Failure to demonstrate an increase in ^{3}HTdR incorporation, similar to that seen in the remaining kidney after uninephrectomy, discounts a role for DNA synthesis and cell division during NH_4Cl-induced renal growth.

Meanwhile, PDG activity has increased (Table 3). If cell division is unimportant for the extensive adaptation of PDG during acidosis, it can be argued that PDG should adaptively increase while cell division is inhibited. Hydroxyurea inhibits DNA synthesis (29) and has been used to test this point. Clearly, PDG activity is similarly induced in acidotic rats treated with hydroxyurea (Fig. 2). This result lends even further credence to the hypothesis that PDG adapts in the existing population of renal tubule cells.

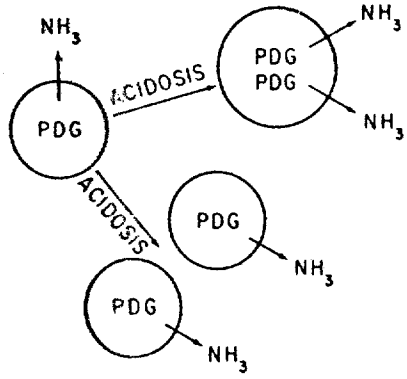

FIG. 3. There are two possible cellular mechanisms by which kidney can increase its phosphate-dependent glutaminase (PDG) activity and enlarge during NH_4Cl acidosis. One involves cell enlargement; other involves cell division. Two lines of evidence: *1)* reduced ^{3}HTdR incorporation into renal DNA and, *2)* ability of PDG to adapt in rats treated with hydroxyurea, to inhibit DNA synthesis, indicate cell division is unimportant for enzymatic adaptation and eventual renal enlargement.

The evidence presented here overwhelmingly discounts any role for hyperplasia in the rat kidney during NH_4Cl-induced acidosis. One must be impressed with the adaptive capacity of the existing population of renal tubule cells to a formidable acid insult.

The stimulus which induced mitotic activity in the remaining kidney after uninephrectomy is still unclear. However, the studies presented here lend some insight into this question. It seems unlikely that renal hyperplasia after uninephrectomy is related to intensified requirements for acid base regulation, since evidence is presented that large acid loads do not induce renal hyperplasia.

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LUNG HISTAMINE AND SUSCEPTIBILITY TO PULMONARY OEDEMA IN THE RAT.

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In a previous paper it was shown that the lung histamine content of rats dying from acute pulmonary oedema induced by ammonium chloride was greatly reduced (Jaques, 1952). It was also noted then that older rats tended to have higher lung histamine values than younger ones. A thiourea derivative, alpha-naphthyl-thiourea (ANTU) is widely used as a rodenticide and kills by producing pulmonary oedema. In field experiments with this rodenticide we had previously observed that of more than fifty brown rats (*R. norvegicus*) which had died from pulmonary oedema none weighed less than 200 g. On the other hand most of the younger animals of the same rat population which were trapped and had their intestinal contents examined for the dyed bait showed no appreciable signs of pulmonary oedema, although they had ingested the compound in amounts sufficient to kill white laboratory rats of an average weight of 250 g. (Jaques, 1948, unpublished). These findings were in agreement with those of Dieke and Richter (1946) who found that adult white rats are much more susceptible to ANTU pulmonary oedema than weanling rats, the LD_{50} with the latter being nearly ten times as great as with adults. In view of these observations the possibility was considered that the relative tolerance of young rats to ANTU and possibly to ammonium chloride might be correlated with low lung histamine levels. Experiments were therefore undertaken to determine the amounts of histamine present in the lungs of rats of different ages and their susceptibility to pulmonary oedema. Ammonium chloride was again chosen as the agent for producing oedema because its quick action facilitates observation, but as a result of some experiments performed with ANTU it is believed that the two do not differ fundamentally in their action on the lung.

METHODS.

Altogether 87 hooded rats weighing from 7 to 388 g. were used. If not stated otherwise, they were males. Three groups of young rats with an average body wt. of about 7, 20 and 60 g. respectively were divided into approximately equal sub-groups. In one sub-group the lung histamine content was determined after bleeding from the carotid under light nembutal anaesthesia. The animals of the other were injected intraperitoneally with

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ammonium chloride (0·8 ml. of a 6 per cent solution per 100 g.) to determine the incidence of pulmonary oedema or the lung histamine. Those rats which did not show signs of pulmonary oedema were either observed for 48 hr., killed at rather short intervals after the injection of ammonium chloride to determine lung weight and lung histamine, or re-injected about 24 hr. after the first injection. Rats surviving a second injection of ammonium chloride were killed 30 min. after the injection and their lung weight and lung histamine determined. Further details are given under Results.

RESULTS.

Histamine Content of the Lungs of Normal Rats and of Rats Injected with Ammonium Chloride.

The results of experiments on rats of different weight and age-groups are summarized in the Table. In the youngest group, aged 1–2 days, the lung histamine of control and of NH_4Cl-treated rats was less than 0·05 μg./g.: none of the treated group showed any increase in lung weight, nor were there signs of respiratory distress, even in 5 further rats injected with twice the standard dose of NH_4Cl and observed for 48 hr. The *second* group, aged 14–16 days, behaved similarly, and in this group also the lung histamine was low (0·1–0·2 μg./g.). None of the 6 treated rats developed pulmonary oedema. The *third* group consisted of rats 51–68 g. in body wt., and was chosen to contain both males (12) and females (8) divided equally between treated and controls. In this group the mean lung histamine in the controls was 0·9 μg./g., and the treated animals showed definite pulmonary oedema. Half of the NH_4Cl group died within $\frac{1}{4}$ hr. with typical oedema, and the other half showed moderate to severe signs of respiratory distress. The survivors were re-injected with the same dose 24 hr. later, and again failed to develop fatal pulmonary oedema. No differences were observed between the males and females in respect of any of the factors examined.

The *fourth* and *fifth* groups consisted of rats >100 g., the only difference

TABLE.—*Lung Weights, Lung Histamine and Paw Histamine of Rats of Different Ages.*

Group.		No.	Body wt. (g.).	Lung wt. (mg./100 g. body wt.).	Lung histamine (μg./g.).	Paw skin histamine (μg./g.).	Pulmonary oedema.
1 (1–2 days)	Controls	5	7 (6·5–7·5)	1294 (1200–1430)	<0·05	38 (35–42) on 3 rats	
	NH_4Cl	10	6·7 (6–7)	1248 (1140–1370)	<0·05	n.d.‡	0/10
2 (14–16 days)	Controls	6	20 (19·2–20·5)	1125 (1057–1200)	0·15 (0·1–0·2)	61 (55–65)	
	NH_4Cl	6	20·2 (19·5–20·5)	1148 (1127–1170)	n.d.	n.d.	0/6
3	Controls	10	59 (53–68)	212* (198–230)	0·9 (0·4–1·4)	62 (60–65) on 3 rats	
	NH_4Cl	10	59 (51–65)	389* (361–420)	n.d.	n.d.	5/10
	Controls	10	150 (125–211)	157† (137–170)	2·55 (1·8–6·5)	56 (50–62) on 2 rats	
	NH_4Cl	10	153 (125–190)	315† (286–383)	1·1 (0·5–1·9) on 7 rats	n.d.	10/10
5	Controls	10	320 (211–388)	117† (101–141)	6·2 (1·5–12·5)	72 (64–78) on 3 rats	
	NH_4Cl	10	274 (190–312)	259† (151–328)	1·7 (0·6–2·8)	n.d.	10/10

Controls were bled under light nembutal anaesthesia. NH_4Cl groups 1 + 2 were sacrificed 30 min. after injection, and groups 3–5 died of pulmonary oedema. Mean figures are given, with the range in parentheses.

* = right lower lobe only.
† = left lower lobe only.
‡ = not done.

between them being that the fifth group contained the heavier rats. All treated animals in these groups died with typical pulmonary oedema, their lungs at autopsy weighing on average twice as much as those of the controls. In the Table are included figures relating to 16 rats studied in the present series together with corresponding figures taken from the previous paper. It will be seen that in the controls the lung histamine content is greater in the heavier group than in the lighter. The increase of lung histamine content with age is illustrated graphically in the Figure, in which the regression of lung histamine (y) upon body weight (x) is given by the equation $y = 0{\cdot}0198\,x - 0{\cdot}26096$. The 5 per cent limits of error to this regression line are shown by the dotted lines ($y \pm 2{\cdot}595$).

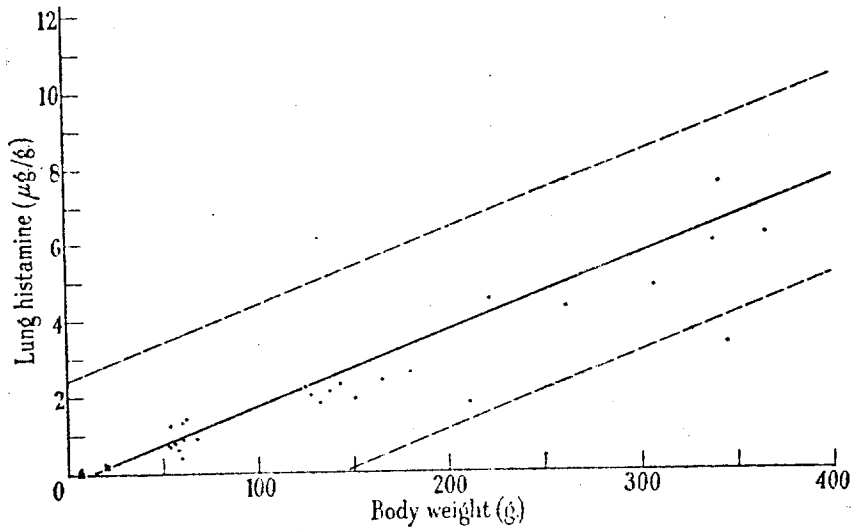

FIGURE.—Relation between body wt. and lung histamine content in rats.

Incidence of pulmonary oedema.

As will be seen from the Table, the incidence of pulmonary oedema was correlated with lung histamine levels. It should be emphasized that in all the animals described in this series as having died from pulmonary oedema, the oedema was sufficient to be reasonably considered by itself to have been the cause of death. Nervous symptoms were uniformly present although they were less in the younger animals, and occasionally an animal died with convulsions, but without pulmonary oedema. This phenomenon was observed by Cameron and Sheikh (1951), who found that pulmonary oedema induced by ammonium chloride is associated with, but independent of, intoxication of the central nervous system, so that certain drugs may completely protect rats from pulmonary oedema yet fail to prevent death with signs of severe nervous disturbance.

Paw skin histamine.

The variation of skin histamine of rats used in the present investigation needs separate consideration here. As can be seen from the Table the paw skin histamine seems to reach adult levels much sooner than lung histamine. In rats of 1 day's age the paw skin histamine was about 38 μg./g. This figure is lower than that

of the heavier rats investigated for which paw skin histamine figures are available. The paw skin of rats of about 20 g. body wt. already contained histamine in amounts which are not significantly different from those of heavier rats.

DISCUSSION.

Tolerance to a pharmacodynamically active agent may be related to various factors such as nonabsorption, rapid elimination, neutralisation or destruction. The findings of the present investigation do not, *a priori*, justify ruling out such factors as being the cause of the peculiar tolerance of young rats to ammonium chloride pulmonary oedema. But this phenomenon is most easily explained by functional peculiarities ("histogenetic" immunity of Behring) the importance of which will be shortly discussed here.

It had been previously observed that when acute pulmonary oedema is induced in rats by means of ammonium chloride the histamine content of the lung drops dramatically (Jaques, 1952). In the present investigation it was found that the lung histamine of rats showed a definite tendency to increase with age. Furthermore, it appeared that when lung histamine was very low, as in baby rats, pulmonary oedema could not be elicited by a standard dose of ammonium chloride and that susceptibility to pulmonary oedema was correlated with critical lung histamine levels. The conclusion therefore seems justified that lung histamine plays a rôle in the pathogenesis of acute pulmonary oedema. Pulmonary oedema induced by ammonium ion is commonly classified as adrenergic (MacKay, Jordan and MacKay, 1949; Cameron and Sheikh, 1951). Intravenous injection of adrenaline also leads to pulmonary oedema in various laboratory animals (Halpern and Cruchaud, 1947*a*, *b*; Halpern and Roux, 1949; Stone and Loew, 1949). The significance of the factors underlying pulmonary oedema calls for further consideration in view of the present findings. Hökfelt (1951) investigating the noradrenaline and adrenaline content of mammalian tissues found that in rats the catechol content of the adrenals increased from very low levels at birth to comparatively high levels in adult rats and also that the relative amount of adrenaline increased with age. Rose and Browne (1941) showed that after adrenalectomy the histamine content of various organs, including the lung, increased, and Rose and Karady (1939) found that after this operation lung histaminase decreased. These observations clearly point to the relationship between adrenaline and lung histamine, and the following reasoning seems to explain the present findings. If ammonium chloride is injected into rats the final outcome will depend on the presence of the two factors adrenaline and histamine. In very young rats the ammonium chloride cannot liberate significant amounts of adrenaline from the adrenals, and furthermore if such amounts were present in the adrenals, they would probably not cause pulmonary oedema because they would reach a lung in which histamine is virtually absent. Tolerance to pulmonary oedema is in this case associated with lung histamine levels which are so low that their displacement by released adrenaline is unable to cause the pulmonary vascular permeability initiating pulmonary oedema.

SUMMARY.

The histamine content of the lungs of rats increases from very low levels at an age of 1 to 2 days ($< 0{\cdot}05$ μg./g.) to comparatively high levels in adult rats (up

to 12·5 μg./g.). The histamine level of the paw skin shows a much less pronounced increase. After injection of a standard dose of ammonium chloride the susceptibility of rats to pulmonary oedema varies according to the amount of histamine present in the lung. When the lung contains very little histamine, as in baby rats, pulmonary oedema does not follow injection of ammonium chloride, and development of pulmonary oedema depends on critical lung histamine levels. The adrenaline-histamine relationship which may underly this phenomenon and the importance of histamine in ammonium pulmonary oedema are discussed.

I am very grateful to my colleagues, Dr. A. Isaacs and Dr. J. H. Humphrey, for help with the English manuscript, and to Miss M. V. Mussett for carrying out the statistical analysis.

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FURTHER INVESTIGATIONS ON EXPERIMENTAL ATHEROSCLEROSIS

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Because blood vessel lesions resembling human atherosclerosis have never been produced experimentally by any means except cholesterol feeding, a good deal of emphasis has been placed on cholesterol as the important etiologic factor in human atherosclerosis also.[1] There are, however, important differences in pathology and pathogenesis between experimental and human atherosclerosis.[2] This, combined with the fact that no one has ever succeeded in producing the experimental disease by cholesterol feeding in any animals but the rabbit and guinea-pig, has led to the growing conviction that there must be some other contributing factor. One possible mechanism is suggested by Duff, who describes a primary injury to the arterial walls in cholesterol-fed rabbits which favors precipitation of lipids in the walls. The nature of the factor causing the injury is, however, still entirely speculative.

For several years considerable work has been done in this laboratory in the hope of demonstrating some such factor. In attempting to explain the primary changes both in human and experimentally produced atherosclerosis, two possibilities were borne in mind: (1) that there was a change in the permeability of the wall of the vessel in the neighborhood of the internal elastica which prevented the normal passage of large molecules such as proteins and lipids from the intima into the media without interfering with the passage of water and crystalloids, and (2) that there was an increased permeability of the intima without other injury to the vessel wall. In both instances there may occur a deposition of relatively large amounts of lipids and of coagulated and

This investigation has been aided by a grant from the Josiah Macy Jr. Foundation.

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1. Anitschkow, N., in Cowdry, E. V.: Arteriosclerosis, New York, The Macmillan Company, 1933, chap. 10.

2. Duff, G. L.: Arch. Path. **20**:81 and 259, 1935.

finally hyalinized protein. It was with these two possibilities in mind that the experiments to be described were planned. Though we shared the growing prejudice against rabbits for this type of work, we felt that feeding cholesterol to rabbits represented the most obvious starting point available at present. The general plan, therefore, was to feed young adult rabbits [3] cholesterol for short periods of time—seldom longer than thirty-one days—and combine other injurious procedures in various ways with the feeding. Since by the method employed [4] feeding cholesterol alone (though it produced a high level of cholesterol in the blood) practically never produced lesions in this short time, it seemed that any effect of the additional procedures should be fairly clearcut. With hypercholesterolemia the conditions appeared favorable for the production of fatty deposits in the aorta if there was damage to the intima resulting in increased permeability.

RABBIT EXPERIMENT 1

The aorta of the rabbit has long been known to be particularly susceptible to a variety of noxious agents. Of these bacteria and their toxins were among the first to be used in the supposed production of arteriosclerosis.[5] The lesions so produced are now generally regarded as either medial or inflammatory unless—as happened in Saltykow's early experiments—the diet also contained cholesterol. There is evidence, however, both in Saltykow's work with staphylococci [6] and in the later experiments of Benson and co-workers with streptococci [7] that bacterial injections may accelerate and intensify the formation of the cholesterol lesions. But there is no recent report of the possible effect of bacterial toxins on the development of these lesions. In experiment 1 of this series a streptococcus toxin was employed for this purpose.

Procedures.—Intravenous injection of streptococcus toxin [8] was combined with cholesterol feeding in six rabbits:

(*a*) Two rabbits were each fed 1 Gm. of cholesterol daily for three weeks in the manner described, and were then given nine increasing intravenous doses of toxin on consecutive days (beginning with 0.1 cc. and increasing to 10 cc.).

3. The rabbits used were chiefly from the stock of gray chinchillas raised on the farm kept by the Animal Care Department of the medical school.

4. One gram of dry cholesterol was mixed with the basic diet three, four or six times per week. The latter consisted of a mixture of whole and rolled oats, with occasional greens.

5. Boinet and Romary: Compt. rend. Soc. de biol. **58**:1153, 1906. Boinet: Arch. de méd. expér. et d'anat. path. **9**:902, 1897. Klotz, O.: Brit. M. J. **2**:1767, 1906. Saltykow, S.: Centralbl. f. allg. Path. u. path. Anat. **19**:321 and 368, 1908.

6. Saltykow, S.: Beitr. z. path. Anat. u. z. allg. Path. **57**:415, 1913.

7. Benson, R. L.; Smith, K. G., and Semenov, H.: Arch. Path. **12**:924, 1931.

8. Dr. Alvin Coburn of the Presbyterian Hospital very kindly supplied the streptococcus toxin. It was made from Dochez' N. Y. 5 strain according to the procedure outlined by Wadsworth (Standard Methods, Baltimore, Williams & Wilkins Company, 1927, pp. 88 and 328).

The last three doses produced shock in both rabbits. Cholesterol feeding was continued, and the rabbits were killed after their last dose of toxin.

(*b*) Four rabbits were each given six increasing doses of toxin (from 0.1 to 6 cc.) on consecutive days and were then started on cholesterol feeding (as described), injections of toxin being continued, at the upper level, for seven days more in two of the rabbits and for sixteen days more in the other two.

(*c*) As controls two rabbits were fed cholesterol alone for eight and nineteen days, respectively.

Result: No fatty lesions were produced in the aortas of the rabbits in any of these groups. Though the time over which the injections of toxin were given was not very long, it corresponded roughly with that of the average acute streptococcus infection. In infants fatty plaques are sometimes found in the intima of the aorta after even a shorter period of severe infection.[9]

RABBIT EXPERIMENT 2

The question of the effects of abnormal diets on the blood vessels has been argued extensively since Ignatowski[10] first observed atherosclerotic changes in rabbits fed protein foods like meat, eggs and milk. Though the chief injurious factor in these diets has now been definitely established to be their cholesterol content, it is by no means so certain that such abnormal diets do not produce in rabbits other metabolic disturbances which might have a primary effect in injuring the wall of the aorta, thereby favoring precipitation of cholesterol and fats. The possible effect of a continued acid-forming diet[11] on an animal unaccustomed to it was brought out by Nuzum.[12] His rabbits, which acquired extensive arteriosclerosis on a diet of liver, had shown the carbon dioxide in the blood lowered and the urine acid for a period of months. An attempt was made in experiment 2 to produce this effect directly by using an acid-forming salt like ammonium chloride (NH_4Cl).[13]

Procedures.—(*a*) Eight rabbits were given from 30 to 50 cc. of 2 per cent ammonium chloride solution by stomach tube daily and from 3 to 4 Gm. of cholesterol per week for four weeks.

(*b*) Nine rabbits were given ammonium chloride alone in the same daily doses for four weeks.

The serum carbon dioxide was in general lowered 20 to 30 per cent after a week of ammonium chloride feeding, but in most cases tended to return to normal by the end of the month.

Result: No fatty lesions were found in the aortas of these rabbits.

9. Zeek, P.: Arch. Path. **10**:417, 1930.
10. Ignatowski, A.: Arch. de med. exper. et d'anat. path. **20**:1, 1908.
11. Newburgh, L. H., and Clarkson, S.: Arch. Int. Med. **31**:653, 1923.
12. Nuzum, F. R.; Seegal, B.; Garland, R., and Osborne, M.: Arch. Int. Med. **37**:733, 1926.
13. Koehler, A. E.: J. Biol. Chem. **72**:99, 1927.

RABBIT EXPERIMENT 3

Experiment 3 was planned with a view to reproducing the early fatty lesions so frequently found in children suffering from acute infections with high fever.[14]

Procedures.—(*a*) Three rabbits were subjected to artificial fever and cholesterol feeding. They were placed in an incubator at from 44 to 45 C. (111.2 to 113 F.) for one hour twice daily, during which time their temperatures would rise to from 107 to 110 F. (normal from 101 to 102 F.) to fall rapidly back to normal within one-half hour after they were taken out of the incubator. One rabbit was fed 1 Gm. of cholesterol daily for twenty-nine days and was then given fever for seven days and fever in addition to cholesterol for ten days more.

(*b*) As controls two rabbits were fed cholesterol alone for fifteen and thirty-four days.

Results: No fatty lesions were found in the aortas of these animals.

RABBIT EXPERIMENTS 4, 5 AND 6

The choice of procedures in experiments 4, 5 and 6 was determined chiefly by their possible effect on intimal permeability. If, in the pathogenesis of arteriosclerosis there is, as we have postulated, a differential change in the permeability of the inner and outer layers of the wall of the aorta—that is, if the permeability of the intima is increased while that of the rest of the wall remains normal—then this condition, particularly if combined with an increased lipid content in the tissue lymph passing through the wall from the lumen of the vessel, should favor deposition of the lipid at the natural barrier between the two parts of differing permeability. Morphologically, the demarcation of the early fatty plaque by the internal elastic membrane strongly suggests that some such factor must come into play.

In experiments 4 and 5 peptone injections and anaphylactic shock were used because, like histamine, they increase capillary permeability.[15] Experiment 6 was suggested by the work of Chini,[16] who in dye diffusion experiments showed that when uric acid was injected intraperitoneally along with congo red the subcutaneous and periarticular tissues became much more deeply colored than when dye alone was injected. The same was true for subcutaneous injections, and Chini concluded that uric acid also increases capillary permeability. It was hoped that a similar effect on intimal permeability might be achieved by using these substances and that the increased permeability might be manifested by an accelerated and more extensive production of cholesterol lesions in the experimental animals.

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14. Ophüls, W., in Cowdry, E. V.: Arteriosclerosis, New York, The Macmillan Company, 1933, chap. 9. Zeek.[9]

15. Sollmann, T.: A Manual of Pharmacology, ed. 3, Philadelphia, W. B. Saunders Company, 1930, p. 475.

16. Chini, V.: J. Exper. Med. **53**:1, 1931.

Procedures in Experiment 4.—(*a*) Six rabbits were given daily intravenous injections of peptone solution (0.2 cc. per kilogram of a 10 per cent solution of a standard commercial preparation) and were fed cholesterol (from 3 to 4 Gm. per week) for one month. The procedure was repeated on six more rabbits a year later.

(*b*) Six controls were given intravenous injections of peptone alone for one month.

(*c*) Ten controls were fed cholesterol alone (from 3 to 4 Gm. per week) for one month.

Result: Three of the first six rabbits receiving cholesterol and peptone and one of the four controls receiving cholesterol alone showed a few early fatty plaques in the arch of the aorta. When the experiment was repeated the next year, however, none of the rabbits, either in the experimental group or in the control groups, showed any lesions.

Procedure in Experiment 5.—Anaphylactic shock was combined with cholesterol feeding in three rabbits. These animals were sensitized by two series of four daily intravenous injections of 0.5 cc. of horse serum, with a three day rest period between the two series. Shock was produced twelve and nineteen days after their last sensitizing dose. Cholesterol feeding (4 Gm. per week) was started at the beginning of the second week and was continued throughout the period of production of shock.

Result: No fatty intimal lesions were found in the aortas of these rabbits.

Procedures in Experiment 6.—(*a*) Intravenous injection of uric acid was combined with cholesterol feeding in three rabbits. (The uric acid was prepared according to Koehler.[17] The dosage was 12 mg. per kilogram on the first day, 24 mg on the second and 48 mg. per kilogram from the third day to the end of the experiment.) Each rabbit was fed 1 Gm. of cholesterol daily for one week before the injections were begun and throughout the period of administration of uric acid. One received four doses, and two received eleven daily doses of the uric acid.

(*b*) As controls two rabbits were fed cholesterol alone for twenty-eight days.[18]

Result: No fatty lesions were found in the aortas of these animals.

Of all these procedures the only one that gave any suggestive results was that of combined cholesterol feeding and peptone injection. Three of the first six rabbits showed a few early intimal fatty lesions in the arch of the aorta as compared with one of the four controls fed cholesterol alone for the same time. However, since these results could not be repeated the next year, it seems reasonable to conclude only that the lesions in the first experiment were due to better absorption of the cholesterol in both groups.

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17. Koehler, A. E.: J. Biol. Chem. **60**:721, 1924.

18. In the six groups of rabbits sixteen controls were fed cholesterol alone for periods varying from eight to thirty-four days, and all of them may justifiably be considered as controls for any one experimental group.

It may be of interest to note here the incidence of spontaneous arteriosclerosis in these groups of rabbits. Of a total of sixty-six rabbits, twenty-two showed the well known medial degeneration of the aorta, and it was about equally distributed between the control and experimental groups.

CAT EXPERIMENTS

In the course of this work with rabbits similar experiments were performed on cats. The fact that vascular lesions cannot be produced in cats by feeding of cholesterol alone [19] indicates that it is difficult to disturb the ordinarily adequate cholesterol metabolism of these animals. Accordingly it was felt that a relatively long exposure to the added injurious agents would be necessary in order to duplicate results produced in rabbits in a shorter time. Most of the cat experiments, therefore, have been long chronic ones.

Procedures in Group 1.—(*a*) Two cats were given intravenous injections of peptone and fed cholesterol. Each cat was given 0.2 cc. of 10 per cent peptone solution per kilogram intravenously and 10 cc. of a cholesterol emulsion (equal to 1 Gm. of cholesterol) in its food daily for eighteen and one-half or twenty-two weeks, respectively. (The emulsion was made by grinding 10 Gm. of cholesterol with 50 cc. of 5 per cent acacia solution in a mortar, adding 50 cc. of peanut oil and mixing till homogeneous.)

(*b*) As controls two cats were fed the stock laboratory diet (cooked meat and vegetable stew).

Result: At autopsy, no changes were found in the aortas.

Procedures in Group 2.—(*a*) In four cats intravenous injections of histamine were combined with cholesterol feeding. Each cat was given 20 cc. of a cholesterol emulsion (equal to 2 Gm. of cholesterol) by stomach tube daily for nine days; then, while cholesterol feeding was continued, each was given a daily intravenous dose of 0.02 cc. of histamine per kilogram (of a solution made by dissolving 1 mg. of histamine in 5 cc. of water) for five, eight, thirteen or sixteen days more, respectively.

(*b*) As controls four cats were given intravenous injections of histamine alone. Two cats received five and six injections, respectively, and two received thirteen and fourteen injections, respectively, on consecutive days. (The dose was the same as that described.)

(*c*) Two cats were given intravenous injections of histamine and fed cholesterol for a long period. The animals were each given 10 cc. of cholesterol emulsion (equal to 1 Gm. of cholesterol) in their food and intravenous injections of histamine as described in (*a*) daily for twenty-two weeks.

Result: The last two cats had increased blood cholesterol, approximately twice the normal, but showed no lesions in the aorta. The cat

19. (*a*) Cirio, L.: Virchows Arch. f. path. Anat. **269**:739, 1928. (*b*) Yuasa, D.: Beitr. z. path. Anat. u. z. allg. Path. **80**:570, 1928.

which received both histamine and cholesterol for thirteen days showed a few small areas of intimal wrinkling and thickening. In the remaining cats the aorta was normal.

Procedures in Group 3.—(*a*) Six cats were fed cholesterol and ammonium chloride. Each cat was fed from 1 to 2 Gm. of ammonium chloride mixed with its food (kibbled bread and salmon, or chopped meat with added calcium gluconate and sodium chloride [NaCl]) daily for five months, and then the same amount of ammonium chloride plus 1 Gm. of cholesterol dissolved in 3 Gm. of butter daily for from eight weeks to ten months.

(*b*) As controls five cats were fed ammonium chloride alone. From 1 to 2 Gm. of this salt was mixed with the food of each daily for from seven weeks to fifteen months. (It was impossible to produce a continued acidosis in cats with ammonium chloride. If given by stomach tube the salt was invariably vomited at once, and when mixed with the food, as it was finally given, it produced only a transitory and slight lowering of the blood carbon dioxide.)

(*c*) As controls three cats were fed the stock laboratory diet.

Result: Two of the cats fed ammonium chloride and cholesterol showed vaguely outlined small yellowish-gray areas in the arch of the aorta, but similar areas were also found in two of the three control cats.

The viscera of the cats fed cholesterol were generally very fatty, and in the long-continued experiments the blood cholesterol was somewhat elevated, in two animals up to twice the normal amount. No significant vascular lesions, however, were present in any of the cats with the exception of one in each of groups *2a* and *3a*. The aorta of one cat in group *2a* contained several small foci of medial degeneration with fibrosis and calcification. In the thoracic aorta of one cat in group *3a* there was present a small opaque gray spot which was found on microscopic examination to represent a thick, fibrous intimal plaque overlying a zone of necrosis in the outer third of the media. In several cats in both the control and experimental groups small areas of intimal wrinkling and thickening with splitting of elastic lamellae were noted. No fatty plaques were found in any of the animals. These experiments, therefore, seem to be only further confirmation of the work of Cirio[19a] and Yuasa,[19b] who found it impossible by feeding cholesterol to produce lesions in the blood vessels of cats comparable to human arteriosclerosis. Nor does administration of a substance like histamine, known to increase the permeability of the vessels, or an excessively acid diet appear to have a predisposing effect.

The absence of a suggestive lead in any of these experiments was disappointing and leaves the nature of the initial change or injury which might favor deposition of lipoids in the intima of the aorta still unknown.

SUMMARY

An unsuccessful attempt has been made to accelerate and increase the development of cholesterol lesions in the aorta of the rabbit by the following injurious procedures added to cholesterol feeding: (1) intravenous injections of streptococcus toxin, (2) ammonium chloride feeding, (3) production of artificial fever, (4) intravenous injections of peptone, (5) induction of anaphylactic shock and (6) intravenous injections of uric acid.

Similarly, no effect on the blood vessels of cats could be demonstrated when they were fed cholesterol and in addition were treated with (1) peptone or (2) histamine intravenously or fed (3) ammonium chloride.

Folia. Haemt.
48:323-329, 1932

Studies in the Physiology of the Eosinophil.

III. The effect of the intraperitoneal injection of ammonium chloride on the white blood cells of the guinea-pig[1]).

By

A. Izard Josey and John S. Lawrence[2]).

With 2 figures.

Introduction.

In the first paper (4) of this series acidosis was mentioned as one possible common denominator in some of the conditions known to be associated with an eosinopenia. The purpose of this presentation is to show the effect of one of the well known acid producing chemicals on the white blood cell picture of the guinea-pig. In brief, the data show an early and marked diminution in the number of the eosinophils and lymphocytes in the peripheral blood associated with a moderate neutrophilic leucocytosis.

Methods.

Guinea-pigs were used for the experiments reported in this paper. Detailed white blood cell studies were made on ten of these animals (numbers 1, 3, 4, 5, 6, 7, 8, 9, 10 and 11). The same general methods of procedure were used as in the two previous papers in this series. Ammonium chloride was injected intraperitoneally into each of the animals. In most instances 0.5 to 0.6 cc. of a saturated aqueous solution was used but in one instance (animal number 11) 0.7 cc. of the saturated solution was diluted to 10 cc. with distilled water before injection. Two of the animals (numbers 9 and 10) received similarly diluted solutions after recovery from the effect of an injection of the saturated solution. In addition, there were four animals (numbers 12, 14, 15, and 16) which were given the drug in the more dilute form. The animals weighed 450 to 750 gms. The amount of salt given was approximately 0.35 gm. per kilogram of body weight. Following the introduction of the salt intraperitoneally, white blood cell studies were made

[1]) From the Department of Medicine, University of Rochester, School of Medicine and Dentistry.

[2]) This investigation was aided in part by a grant from the Josiah Macy Jr. Foundation.

at frequent intervals during the first 24 hours and after that at less frequent intervals. Of the ten animals studied in particular detail as to the white blood cells, two were investigated at 2 to 3 hours, six at 4 to 5 hours, seven at 6 hours, nine at 8 to 9 hours, ten at 10 to 11 hours, and three at 13 to 14 hours after injection. Eight of these animals had white blood cell counts on the day following injection and five on the second and third days after treatment. During the day of the injection the animals were kept in the laboratory. They were allowed water, but no food was given until 5 or more hours after the drug was injected.

Five of the animals (numbers 4, 5, 6, 7, and 8) were observed at frequent intervals during a 10 to 14 hour period on one of the control days. During this day they were kept under the same conditions as they were when the drug was injected. In other words, they were observed throughout a day in the laboratory for any daily fluctuations of the white blood cells which they might have had.

Four of the guinea-pigs (numbers 13, 14, 15, and 16) were killed with ether and subjected to a postmortem examination. Sections from the various tissues were fixed and stained with haematoxylin and eosin. These were examined with reference to their eosinophil content. One of these animals was killed at each of the following intervals after injection of ammonium chloride: 6, 8, 12, and 24 hours. Sufficient white blood cell studies were made on these animals to establish their normal control values and the changes which were present at the time of autopsy. In every instance there was a definite and marked diminution in the number of the eosinophils in the peripheral blood at the time of autopsy.

Results.

Figure 1 represents the average percentage deviation from the normal average control values of the various white blood cells in the ten guinea-pigs that had white blood cell determinations at frequent intervals after the intraperitoneal injection of ammonium chloride. Reference to this figure shows that the total number of white blood cells was increased temporarily and to a slight extent, the maximum average increase being 44 per cent at 2 to 3 hours after injection. There was no deviation from the control values after the 4 to 5 hour interval. The absolute number of the neutrophils was much more strikingly increased, the maximum average increase being 159 per cent in the 4 to 5 hour period. The number of the neutrophils had returned to normal at the end of 24 hours. The absolute number of the eosinophils showed a marked and steady diminution following the injection of ammonium chloride until the maximum average diminution of 76 per cent was reached at the end of 13 to 14 hours. Following this, there was a grad

increase until the normal average was reached at the end of 48 hours. The total number of the lymphocytes rapidly diminished until the maximum average decrease of 58 per cent was reached at the end of 4 to 5 hours. Their number remained diminished until after 10 hours, after which it gradually returned to normal. The absolute number of the monocytes increased rapidly and markedly until there was a maximum average increase of 138 per cent at the end of 4 to 5 hours. Following this, there was a sudden return to normal at the end of 8 hours. There was a slight percentage increase at the end of 24 hours and again at the end of 72 hours. Comparison of the average percentage deviation for the various white blood cell elements with the actual values obtained for the individual animals revealed the fact that the composite curves were a true representation of what had happened to

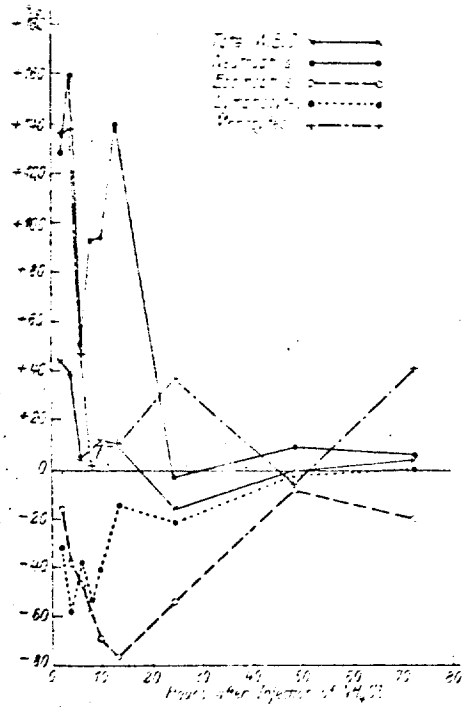

Fig. 1. The average percentage variations from the normal control level of the various white blood cells following the intraperitoneal injection of ammonium chloride solution.

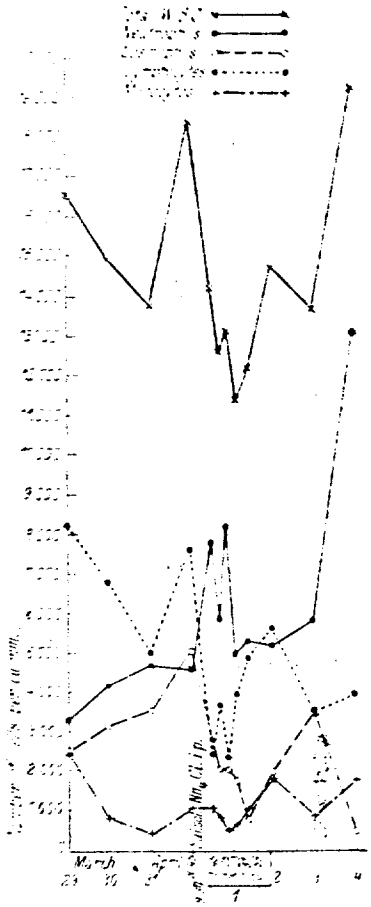

Fig. 2. The variations in the absolute number of the various white blood cells in guinea-pig number 9 following the intraperitoneal injection of a dilute solution of ammonium chloride.

the white blood cells of the individual animals except where the monocytes were concerned. Figure 2 shows the variations in the white blood cells of a typical animal (number 9).

The response as regards the monocytes was inconstant. There was an actual increase in the number of these cells in each of the two animals studied at the end of 2 to 3 hours. However, two animals are insufficient to establish this as an accurate representation. Three of the six animals whose blood was studied at the end of 4 to 5 hours showed an appreciable increase in the total number of these cells. The greatest increase was shown by animal number 1, the average control value being 1307 per cu.mm., and the average value at the end of 4 to 5 hours after injection being 4573 per cu.mm. Three of the animals of this group showed no changes of note in the number of the monocytes. The average control number of monocytes for the group was 898 per cu.mm. as compared with 2140 per cu.mm. after injection. The average increase of 47 per cent in the 6 hour interval is not a true representation of the facts as only one animal (number 4) showed an actual increase in the number of the monocytes. The average control figure for the monocytes in the seven animals of this group was 925 per cu.mm. as compared with 1357 per cu.mm. after injection. Further, the percentage increase shown for these cells at 24 and 48 hours is not a real increase as the values for the animals as a whole were essentially normal at these intervals.

The five animals on which frequent blood counts were made during one of the control days showed minor changes in the number of the white blood cells but no variations that were comparable to those occurring following the injection of ammonium chloride except in one instance (animal 3). This animal showed a greater diminution in the total number of eosinophils and lymphocytes during the control days than it did following the injection of ammonium chloride. No cause for this type of response was found.

One animal (number 2) which was not included in the group for which a composite average was given, showed no characteristic response as regards the lymphocytes and the eosinophils following the injection of 0.3 cc. of a saturated aqueous solution of ammonium chloride but later responded in a typical way when 0.4 cc. of a saturated aqueous solution was given intraperitoneally.

The same white blood cell response was elicited when a dilute solution was given as when the more concentrated saturated solution was injected. Animals reinjected with the drug following recovery showed the same type of response as they did with the first injection.

Comparison of the figures obtained by counting the number of eosinophils in various high power fields of the different tissues of four animals autopsied at intervals of 6 to 24 hours after injection

showed no evidence whatever of any local accumulation of these cells. In fact, there was evidence in general of a slight depreciation in the number of the tissue eosinophils except in the bone marrow where their number remained about the same.

Examination of the peritoneal fluid of four of the animals (numbers 16, 14, 15, and 13) at 4, 8, 12, and 24 hours respectively after the injection of ammonium chloride failed to reveal any accumulation of either the eosinophils or the lymphocytes. The eosinophils varied between less than 1 per cent and 20 per cent. The lymphocytes varied between 2 per cent and 16 per cent. There was a distinct increase in the neutrophils and the monocytes, the maximum values being 74 per cent and 50 per cent respectively. Smears from the omentum likewise failed to show any increased number of eosinophils or lymphocytes.

Discussion.

The data presented in this paper show conclusively that there is a marked diminution in the number of eosinophils and lymphocytes within a few hours after the injection of ammonium chloride intraperitoneally in the guinea-pig. There is also a well defined neutrophilic leucocytosis, and, possibly, a monocytosis as well. These changes in the white blood cell picture are temporary and have entirely cleared up at the end of forty-eight hours.

The factor responsible for this picture is problematical. The first thing that comes to one's mind is that acidosis per se has produced the changes reported. While massive doses of ammonium chloride were given, yet it may be that true acidosis did not occur. No determinations of the pH of the blood of our animals were made. However, it does not seem that acidosis in itself can be considered as the explanation of our results since we have obtained comparable changes in the blood following the intraperitoneal injection of an alkaline producing substance, namely, sodium bicarbonate (6). It is possible, of course, that there is an optimum pH at which the eosinophils and the lymphocytes are present in the blood in greatest numbers. In this connection it should be stated that we obtained more striking changes in the white blood cell picture after the injection of ammonium chloride intraperitoneally in the guinea-pig than did FÖLDES and SHERMAN (2). These investigators produced an acidosis in rabbits and reported slight increases in the neutrophils without any characteristic changes in the total white blood cell count. All of their observations were made, however, within the first seventy minutes after the production of acidosis whereas most of our observations were made at later intervals. HEINSHEIMER's (3) results following a similar procedure in rabbits are more in accord with ours insofar as the neutrophils and the lymphocytes are concerned. His figures for eosinophils indicate a slight in-

crease following the production of acidosis and are thus in contrast to our findings in the guinea pig.

A second explanation that may be considered is peritoneal irritation. This is suggested by the fact that all of the animals showed definite signs of this being present. It is further substantiated by the fact that a similar response was obtained in animal number 11 when a 0.25 per cent solution of sodium chloride was injected intraperitoneally. However, the failure to get a different type of response when a very dilute solution of ammonium chloride was injected argues against peritoneal irritation being the main factor in causing the blood changes.

A third consideration is that the results are due solely to a "salt action". This hypothesis is made more plausible by the fact that acid, basic, and neutral substances have produced similar blood changes. However, one cannot correlate the effects of starvation and bacterial infection with such an hypothesis.

A fourth hypothesis is that some substance is produced as a result of tissue autolysis that causes these changes in the blood picture. It seems logical to assume that tissue autolysis probably has occurred in all of the animals treated in various ways in this series of investigations, and, for the present, we think this the most plausible explanation of the phenomenon.

As to the mechanism involved in the disappearance of the lymphocytes and the eosinophils from the peripheral blood, we can make no definite statements. The possibilities involved have been considered in an earlier paper (4). No evidence has been obtained from a study of microscopic sections of an accumulation locally of these cells.

We feel that the marked neutrophilic leucocytosis is probably due to stimulation of the neutrophilic areas of the bone marrow. We regret that SCHILLING indices have not been determined on these animals as they might answer this question definitely.

It seems to us that the monocytosis shown by some of these animals is suggestive but too inconstant to be considered as a characteristic reaction.

Comparison of the composite curves for the white blood cells in this series of animals with those for the animals infected with bacteria (5) reveals a shift of the whole reaction to the left (that is, an earlier reaction), a less pronounced neutrophilic leucocytosis and a more rapid return to normal.

It is of interest to compare our results with those of BEARD and BEARD (1) following the intravenous injection of a 1 per cent solution of sodium chloride. Most of the observations made by these investigators were within the first two and a half hours after the injection of the salt. Since we made no observations before two hours, no comparison can be made of the findings during this interval. However

data at two to three hours after the injection of ammonium chloride are in very close agreement with those of BEARD and BEARD at this time. Hence, it seems that sodium chloride intravenously in the rabbit produces the same white blood cell changes at the end of two hours as does ammonium chloride intraperitoneally in the guinea-pig. Whether ammonium chloride caused an early leucopenia in our guinea-pigs cannot be stated on the basis of our data.

We wish to emphasize the close relationship between the eosinophilic and the lymphocytic responses in this group of animals. While the curves for the variations in these two groups of cells are not exactly parallel, yet they so closely follow one another as to be most suggestive. This was noted in the animals subjected to starvation (4) and also in those given bacteria intraperitoneally (5).

Conclusions.

1. The intraperitoneal injection of ammonium chloride has resulted in the following changes in the white blood cells of the guinea-pig:
   a) Slight and transient increase in the total number of the white blood cells.
   b) Well defined neutrophilic leucocytosis.
   c) Marked diminution in the absolute number of the eosinophils and the lymphocytes.
   d) Inconstant responses on the part of the monocytes.

2. The similarity of the type of response in the eosinophil and the lymphocyte has been emphasized.

3. Theoretical consideration of the causes of these responses have been given. Tissue autolysis has been considered the most plausible theoretical possibility.

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THE EFFECT OF AMMONIUM CHLORIDE INDUCED MATERNAL ACIDOSIS ON THE HUMAN FETUS AT TERM: II. ELECTROLYTES*

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Administration of ammonium chloride to pregnant women at term readily induces metabolic acidosis. This is associated with a distinct hypoxia, hypercapnia and lowering of the pH in the fetus[5]. The present report is concerned with the distribution of plasma electrolytes in both mother and fetus in normal pregnancy and under the influence of ammonium chloride induced acidosis.

Materials and Methods. Blood was obtained without stasis, in oiled, heparinized syringes, from pregnant patients in the last few weeks of pregnancy, and from them and their infants at the time of birth. Nine of these patients received 6 gm. of ammonium chloride a day for from 5 to 23 days prior to delivery, and 2 others received 4.3 gm. of uncoated ammonium chloride early in labor. The activity and diet of the patients were not controlled. All deliveries were accomplished under local anesthesia. Details of these procedures have been described previously[5].

Blood samples were kept in the original syringes, immersed in ice water, until determinations of pH, hemoglobin and blood gases were completed. Whole blood samples for pyruvate determination had been withdrawn on delivery of the samples to the laboratory. The blood was then centrifuged and the plasma drawn off. Suitable dilutions for study of sodium and potassium, protein and chloride were made at once and the remaining plasma stored for later examination.

Sodium and potassium were determined by flame photometry, employing standards which contained appropriate concentrations of both sodium and potassium respectively. These standards were prepared gravimetrically and checked against those of another laboratory. Measurements were made against a standard curve prepared each day, and a constant slit width was maintained for each day's run. Calcium and magnesium combined were measured by the method of Sobel and Hanok[16], using chelation with disodium ethylene diamine to titrate against an indicator system employing eriochrome black T. Chloride was determined by a modification of the methods of King and Bain[8] and of Stiff[17]. Chloride was used in a silver iodate system using potassium iodide to release iodine. The iodine equivalent to 75 mEq./L. of chloride was then bleached out by thiosulphate and the remainder measured in a spectrophotometer at 420 mμ. Protein was measured by a modification of the bromsulphalein method suggested by Nayyar and Glick[10], in which BSP is precipitated by protein and the excess BSP measured spectrophotometrically. Lactate was measured by a modification of the Barker and Summerson technique[1]. Phosphate was measured by the method of Taussky and Shorr[18]. Pyruvate was measured by the method of Friedemann[3,4]. All measurements were made in duplicate. Details of these methods have been published elsewhere[7].

Results. The distribution of plasma electrolytes in the peripheral vein blood of 25 normally pregnant women

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[illegible] in Table 1 and the first column of Table 4. In these same blood samples (plus one more whose plasma was lost in processing) the pH was 7.37 ± 0.02, pCO_2 37.2 ± 3.5 mm. Hg, hemoglobin concentration 11.5 ± 1.2 gm. % and oxygen saturation 70 ± 19%.

The electrolyte concentrations in the peripheral vein blood of 11 women at the time of delivery, and in the umbilical vessels of their newborns, are reported in Table 2 and column 2 of Table 4. Blood was drawn from the umbilical cord after completion of delivery and before respiration had begun. Two umbilical artery blood samples were insufficient in volume for study. The maternal pH, hemoglobin and oxygen saturation were essentially the same as for the group not in labor. The pCO_2 was 34.1 ± 4.8 mm. Hg, which is probably related to hyperventilation associated with bearing down efforts. Umbilical vein values for this normal control group are: pH [illegible] ± 0.05, pCO_2 41.6 ± 6.8, Hgb. 15.[illegible] ± 0.8 and O_2 saturation 62 ± 13%.

It will be noted that the only [illegible]ternal values which differ in the g[illegible] at the time of delivery from the group prior to labor are sodium, bicarbon[illegible] and lactate, and that none of these [illegible] significantly different. The total anions and cations are essentially equal in both groups.

TABLE 1.—PLASMA ELECTROLYTES AND STANDARD DEVIATIONS (MEQ/L.) IN PERIPHERAL VEIN BLOOD OF 25 NORMAL WOMEN IN THE 39TH WEEK OF PREGNANCY

	Na	*K*	*Ca + Mg*	*Cl*	*HCO_3*	*Protein*	*Lactate*	*Phosphate*
Mean	137.7	4.2	5.4	103.7	20.6	20.0	1.5	1.9
S.D.	2.7	0.5	0.4	4.3	2.0	1.7	0.8	0.2

TABLE 2.—PLASMA ELECTROLYTES AND STANDARD DEVIATIONS (MEQ./L.) IN BLOOD OF 11 NORMAL PATIENTS AND THEIR NEWBORNS AT DELIVERY AT TERM

	Na	*K*	*Ca + Mg*	*Cl*	*HCO_3*	*Protein*	*Lactate*	*Phosphate*
Maternal vein mean	140.5	4.2	5.2	103.8	19.1	19.7	2.3	1.8
S.D.	4.0	0.8	0.3	3.3	2.5	1.9	1.7	0.3
Umbilical vein mean	140.4	4.8	5.5	103.9	21.6	17.7	2.4	2.8
S.D.	4.2	1.4	0.3	3.6	2.6	1.8	1.4	0.4
Umbilical artery mean	133.6	6.5	5.3	104.4	23.8	17.4	2.7	2.6
S.D.	5.8	3.3	0.2	3.7	2.4	1.7	1.5	0.4

In the normal fetus, plasma sodium concentration is equal to that in the maternal peripheral vein. The potassium and calcium and magnesium concentrations are consistently, but in the data not significantly, higher than maternal values. The total fetal cations tend to exceed maternal slightly. Chloride concentrations equal maternal values. The total protein concentration is lower than maternal and lactate [illegible] phosphate higher. The bicarbonate concentration is higher than maternal [illegible] higher in the umbilical artery than [illegible]

umbilical vein blood. This corresponds with the pH of 7.28 ± 0.07 and CO_2 of 52.9 ± 8.5 found in umbilical artery blood. The total fetal anions also slightly exceed maternal totals and equal the fetal cations.

Electrolyte concentrations in blood obtained from patients experiencing the effects of ammonium chloride are shown in Table 3 and the third column of Table 4. The blood samples were obtained under conditions identical with those in the control group. However, the only blood samples subjected to gas and electrolyte analysis in the entire group of 80 patients who received ammonium chloride and who delivered while under its influence were those with extreme changes in pH in the umbilical cord blood. There in the group of 11 patients reported here. Plasma for electrolyte determination was not available for one umbilical vein and 2 umbilical artery samples.

The mean pH for these 11 mothers was 7.28 ± 0.04, as compared with 7.31 for the entire group of treated patients and 7.37 for the untreated controls. The corresponding fetal umbilical vein pH's were 7.14, 7.24 and 7.34. The maternal-fetal gradient in the selected 11 cases is thus 0.14 pH units as contrasted with 0.03 for the controls. The gradient for the entire group of patients given ammonium chloride is 0.07 pH units, which indicates that the selection emphasizes but does not produce the observed results. The difference in pH between umbilical vein and umbilical artery blood is 0.06 pH units for the selected and control groups and 0.07 pH units for the entire ammonium chloride treated group.

TABLE 3.—PLASMA ELECTROLYTES AND STANDARD DEVIATIONS (mEq./L.) IN BLOOD OF 11 MOTHERS RECEIVING AMMONIUM CHLORIDE AND THEIR NEWBORNS AT DELIVERY AT TERM

	Na	*K*	*Ca* + *Mg*	*Cl*	*HCO₃*	*Protein*	*Lactate*	*Phosphate*
Maternal vein mean	137.8	4.2	5.3	104.6	16.8	20.5	2.9	1.9
S.D.	3.0	0.7	0.4	3.3	2.0	2.3	1.5	0.3
Umbilical vein mean	137.0	5.6	5.7	105.1	18.8	19.6	4.7	3.1
S.D.	2.6	1.2	0.4	3.5	2.9	1.8	2.8	0.4
Umbilical artery mean	138.4	5.5	5.6	105.3	20.2	18.1	5.5	3.1
S.D.	3.5	1.3	0.4	2.9	2.8	1.5	3.2	0.4

TABLE 4.—TOTAL OF DETERMINED ANIONS AND CATIONS AND STANDARD DEVIATIONS (mEq./L.)

	Prior to labor		*At delivery*			
			Normal patients		*Ammonium chloride treated*	
	Cations	*Anions*	*Cations*	*Anions*	*Cations*	*Anions*
Maternal vein	147.3±2.9	147.7±4.1	149.8±3.5	146.8±4.6	147.3±3.4	146.5±3.8
Umbilical vein			150.7±3.6	148.4±4.8	148.2±2.8	151.1±3.2
Umbilical artery			150.3±3.8	149.7±3.9	149.6±3.6	152.3±2.7

The [illegible] in the [illegible] patients was [illegible] compared to 3[illegible] in the controls. In their infants, umbilical vein pCO_2 was 57.6 ± 14.0 and oxygen saturation 15 ± 19% as compared to 41.6 ± 6.8 and 62 ± 13% respectively.

In regard to the electrolytes, the pattern of distribution is essentially the same as that observed in the normal controls, except for the elevated lactate and reduced bicarbonate values. By the t test, the pt (normal control compared with treated group) in regard to bicarbonate is: maternal vein 0.026, umbilical vein 0.027 and umbilical artery 0.007. For lactate the corresponding values are 0.285, 0.049 and 0.051. This excess of lactate in the fetal blood results in an excess of determined anions of almost 3 mEq./L., and suggests the presence of an undetermined cation in fetal blood under these conditions. The lower sodium concentrations in the treated group are of borderline statistical significance. In a few trials we were not able to demonstrate sufficient ammonium ion to account for this, but these results were not conclusive. The induced acidosis is manifest more strikingly in the fetus than in the mother in this group as would be expected in part from the method of selection on the basis of degree of pH depression.

Although pyruvate was determined, it was not present in significant concentrations in either maternal or fetal blood, from the standpoint of electrolyte distributions, nor were large differences noted among the groups of patients.

Discussion. The distribution of electrolytes in mother and fetus has been studied for many years, most extensively in the 1930's and 1940's. The literature has been reviewed by Smith[15]. It is limited in two ways. Only infrequently have maternal and fetal blood been studied simultaneously, and then usually in reference to but a few electrolytes at a time. In many instances infant blood samples obtained in the first few hours of life, rather than samples from the functioning umbilical cord, have been used as representative of *in utero* conditions. The excellent studies of Graham *et al.*[8] on neonatal electrolyte homeostasis have as their baseline such immediate neonatal values rather than fetal values.

Scapaticci, Passaro and Carboni[16] have reported on electrolyte concentrations in maternal and umbilical cord blood in 8 cases. However, they did not determine all the electrolytes on which they report in all cases. Where blood was insufficient in amount, they assumed values for potassium, calcium and magnesium and assumed equality of anions and cations to estimate bicarbonate, chloride or organic acid concentrations.

Rogala and Troszynski[12] have recently described sodium and potassium concentrations in maternal and fetal plasma slightly higher than Newman's and ours.

Newman[11] has reported electrolyte studies on maternal and umbilical cord blood in 21 cases. A comparison of his data and ours indicates very close agreement in regard to total cations, although Newman's values for calcium and magnesium, which he measured separately, exceed ours. In both studies fetal cations and anions slightly exceed maternal. In regard to anions, the values for chloride, bicarbonate and phosphate correspond closely. Our protein values are considerably higher than Newman's for both mother and fetus. This difference is almost certainly methodologic. The [illegible] found in Newman's study [illegible] ours, therefore, stress in addition the protein difference, we have determined [illegible]

[illegible] these values appear to be a reasonable approximation of physiologic electrolyte concentrations in cord blood.

The pCO_2 and HCO_3 concentration in the venous blood of the normal mother reflect a mild compensated respiratory alkalosis which increases with the hyperventilation of late labor. Maternal pH is maintained at a normal level, and electrolyte distributions are unremarkable.

In general, the normal fetus appears to be in a state of uncompensated respiratory acidosis[5]. With the placenta and the mother, with her own homeostatic mechanisms, available to compensate in part for this, fetal electrolyte patterns at least at term are much like those of the mother. The consistently higher fetal potassium and phosphate values may be related to similar elevations observed in experimental respiratory acidosis in the respiring animal before renal compensation can occur (Miller[9]). The suggestion is that the placenta is somewhat limited in its function as a kidney in regard to some of the electrolytes. In regard to CO_2 movement, it is not clear whether the placenta is limited in its permeability or the fetal red cell is limited by its lack of carbonic anhydrase.

Dancis, Worth and Schneidau have reported the effect of ammonium chloride acidosis on the pH, carbon dioxide content and chloride of the blood of pregnant rabbits and their fetuses[2]. Their results are essentially the same as ours.

The imposition on the mother and fetus of ammonium chloride acidosis results in a decrease in pH in both and a rise in pCO_2 in the fetus[2], a borderline decrease in sodium in both and significant changes in [illegible] and lactate concentrations in both. Although they are not statistically significant, there are also increases in potassium and phosphate concentrations in the fetus, but not in the mother. Summarized in terms of adult metabolism, there is an increase in the severity of a respiratory type of acidosis in the fetus. In addition, some of the stigmata of metabolic acidosis, such as an elevated lactate concentration, are present. Some of these changes function to depress the oxygen affinity of hemoglobin, and hypoxia, at least insofar as it can be estimated from oxygen saturation, is present.

It may be questioned whether terms like respiratory acidosis are appropriate for use in this connection, since placental "respiration" does not appear to include the possibilities of compensation that the lung affords. Changes in ventilation can alter pCO_2 rapidly to adjust for metabolic deviations. Although the low pCO_2 of the maternal blood in the intervillous space and the large flow of that blood may afford considerable latitude for the diffusion of CO_2 from the fetal blood, the gradient is in no way comparable to that between pulmonary blood and air. Further, there are no known mechanisms for increasing fetal blood flow through the placenta in the human. If they exist, the absence of carbonic anhydrase from the fetal red cell may nevertheless be a limiting condition for CO_2 elimination. With this in mind, the acidotic alterations in the blood of the fetus can be referred to as respiratory in type by analogy only.

As we noted previously, the marked biochemical changes in the fetus associated with induced maternal acidosis are not reflected in any difficulties in the neonatal period in the infants we have observed. The elevated fetal lactate concentrations are compatible with [illegible] that the [illegible] and [illegible] the pathways of [illegible] to adjust to [illegible] [illegible] some [illegible]

nary ventilation is available, after birth, the infant can resume aerobic metabolism. The electrolyte abnormalities are minimal enough to lie well within the capacities of the kidney of the newborn.

Summary. Maternal acidosis induced by ammonium chloride administered to pregnant women at term produces in their fetuses decrease in pH, oxygen saturation and bicarbonate, and a rise in pCO_2 and lactate concentration. Both mother and fetus appear to lose sodium but this change is not statistically significant. A cation deficit appears in our electrolyte balances for which we cannot entirely account.

The data suggest that the mild respiratory type of acidosis ordinarily observed in the normal fetus can be severely aggravated by inducing maternal acidosis, and that the placental-fetal system is limited in its ability to eliminate CO_2 as well as the electrolytes which are consequently increased.

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SUMMARIO IN INTERLINGUA

Le Effecto De Acidosis Materne, Inducite Per Chloruro De Ammonium, Super Le Feto Human Al Termino: II. Electrolytos

Acidosis materne inducite per le administration de chloruro de ammonium a gravidas al termino produce in lor fetos reductiones de pH, de saturation oxygenic, e de bicarbonato e augmentos de pCO_2 e del concentration de lactato. Le matre e le feto pare perder natrium, sed iste effecto non es statisticamente significative. Un deficit cationic se monstra in nostre balancias de electrolyto sed nos non ha un explication satisfactori pro iste phenomeno.

Le datos indica que le leve typo respiratori de acidosis observate ordinarimente in fetos normal pote esser aggravate seriemente per le induction de acidosis materne e que le systema placento-fetal es limitate in su capacitate de eliminar CO_2 e etiam electrolytos, con le consequentia que le concentrationes de istos es augmentate.

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THE TOXICITY OF INTRAVENOUS AMMONIUM COMPOUNDS

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Following Haldane's[5] demonstration in 1921 that the cation (NH_4^+) of ammonium chloride is metabolized in the body to urea thus freeing an equivalent amount of anion (Cl^-), this salt has been used both orally and intravenously as an acidifying agent in the treatment of clinical alkalosis. Its successful intravenous use in one patient was first reported by McCann[8] in 1922 and in another in 1925 by Youmans and Greene[13]. After Gamble and Ross[3] pointed out that this salt may cause sodium depletion and dehydration, enthusiasm for its intravenous use lessened. These workers advised that therapy consist simply of presenting the kidney with adequate amounts of fluid and sodium chloride on the physiologic presumption that the selective excretion of the cation (Na^+) would compensate for the alkalosis. However, the rationale of this procedure may be questioned since it postulates a normally functioning kidney, while actually renal damage or functional insufficiency may occur in alkalosis (Nicol[10], 1940). In 1943 Zintel, Rhoads and Ravdin[15] resumed the use of intravenous ammonium chloride and reported the successful treatment of 7 patients in alkalosis. Several subsequent reports indicate that the infusion of this salt may be a valuable therapeutic measure[2,11,12].

In view of the increasing clinical use of parenterally administered ammonium chloride, it would seem worth while to review our knowledge of its pharmacologic effects and toxicity.

Pharmacologic Effects. As early as 1893 Marfori[7] described the principal effects produced by injected ammonium chloride in animals as twitches, tremors progressing to tetany, violent convulsions, opisthotonus, irregular respiration, salivation, emesis, somnolence and lassitude. Meneguzzi[9] reported in 1912 that if ammonium chloride injection was not immediately fatal, the body functions recover rapidly from the effects. Recently (1946), Brassfield *et al.*[1] showed that intravenous ammonium chloride produced an immediate increase in respiration, fall in blood pressure, and slowing of the heart. More pertinent was the report in the same year by Forbes and Erganian[2] who supplemented their clinical studies with some experimental results. They found that 20 to 30 cc. of 0.89% ammonium chloride per kilogram body weight could be injected into dogs and rabbits producing only symptoms of excessive salivation, hyperpnea and drowsiness, but that a *more rapid rate of injection* produced stupor and convulsions. Koenig and Koenig[3] reported in 1948 that intravenous ammonium chloride can produce pulmonary edema. Ammonium compounds are also reported to have a weak curarizing action on skeletal

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70 minutes after the earliest definite evidence of toxicity, considered usually as the onset of twitchings of the facial muscles or fasciculations of the tongue. In 4 dogs, however, the typical warning signs of toxicity did not appear. In one instance the first evidence of toxicity was a tonic convulsion, followed immediately by death. Another animal showed some tendency to gasping res-

TABLE 1.—INTRAVENOUS INJECTION OF VARIOUS AMMONIUM SALTS IN 5% GLUCOSE SOLUTION GIVEN TO DOGS

AMMONIUM SALT GIVEN	WEIGHT OF DOG (kg.)	RATE OF ADMINISTRATION ml./min. (aver.)	RATE OF ADMINISTRATION mEq./min. (aver.)	INJECTION RATE mEq./kg./min.	ONSET OF TOXIC SIGNS (mins.)	IRe/IRc
2% NH_4Cl	14.1	3.88	1.45	0.103	63	2.29
	10.9	3.39	1.27	0.116	63	2.58
	21.4	7.67	2.86	0.133	45	2.69
	10.9	3.61	1.35	0.124	31	2.76
	6.4	3.19	1.19	0.143	64	3.18
	7.3	2.95	1.10	0.151	32	3.36
	17.7	8.43	3.15	0.178	17	3.96
	7.7	3.98	1.49	0.193	42	4.29
	8.2	4.25	1.59	0.194	36	4.30
	10.9	5.83	2.17	0.199	31*	4.42
	12.5	7.33	2.74	0.219	18	4.87
	12.9	7.69	2.88	0.223	14	4.96
	7.2	4.67	1.74	0.242	11	5.37
	6.7	5.26	1.97	0.292	8	6.48
NH_4Cl used clinically in man.	70.0	8.34	3.12	0.045	60	
2.88% Ammonium Acetate	10.5	5.52	2.06	0.196	50	4.35
	11.4	6.06	2.27	0.199	39	4.42
2.0-2.5% Ammonium Carbonate	13.3**	3.84	1.68	0.127	57	2.82
	10.8***	4.90	1.71	0.159	38	3.54
2.96% Ammonium Bicarbonate	10.8	3.30	1.23	0.114	27	2.53
	13.0	5.30	1.98	0.152	40	2.94
	13.6	4.87	1.82	0.133	66	2.95
	6.3	2.28	0.85	0.137	50	3.03
	10.0	3.66	1.37	0.137	24	3.04
	10.9	4.05	1.52	0.138	30	3.06
	7.2	2.79	1.03	0.143	20*	3.17
	13.3	5.65	2.12	0.159	25	3.54
	13.6	6.13	2.29	0.168	30	3.73
	13.3	6.00	2.24	0.169	50	3.77
	5.9	2.74	1.03	0.173	28	3.82
	6.8	3.30	1.23	0.182	15	4.05
	10.9	5.72	2.14	0.197	18	4.37
	8.9	5.21	1.95	0.219	27	4.87

* Dog died at that time

** 2.5%

*** 2.0%

The columns reading from left to right show in order the weight of the dog in kg., the average rate of infusion of the ammonium salt in ml. per min., the average rate of infusion in mEq. per min., the average rate of infusion of the ammonium compound in mEq. per kg. per min., and the length of time from start of infusion to onset of unmistakable toxic signs. The data listed under the quotient IRe/IRc in the last column is the Injection Rate in that part of experiment expressed in mEq. per kg. body weight per min. (IRe) for Injection Rate experimental over the Injection Rate used clinically by Zuckerman and Kaufman[1] Sellers and Kast[12] recalculated to the same units (IRc) for Injection Rate. Clinical value amounts to 0.045 mEq. per kg. per min.

piration with a little trembling at the end of inspiration, and then quietly ceased breathing. The third animal showed some respiratory irregularity and then died of respiratory arrest without muscular twitchings or other warning signs. The fourth atypical dog showed several gasping respirations immediately followed by 2 successive tonic convulsions, vomited, and died of respiratory failure, possibly from aspirated vomitus.

The data for the onset of toxicity in all 32 dogs are summarized in Table 1. These data show that definite toxicity was observed with rates of infusion as low as 2.3 times that which had been used clinically, while a rate of 6.48 times produced toxic signs in 8 minutes.

Fig. 1 shows the time of onset of toxicity in minutes plotted against the average rate of infusion in milliequivalents of ammonia per minute. It will be observed that the curve is roughly hyperbolic, and that no consistent difference is observed between the several ammonium compounds used.

Table 2 lists the carbon dioxide combining power of the blood, when ammonium salts were injected, before injection, at the onset of definite toxic signs, and at or within 1 to 2 minutes of death. Ammonium chloride consistently caused a fall in carbon dioxide combining power; ammonium bicarbonate usually caused a slight rise, and ammonium acetate no consistent change in the 2 cases studied. The sequence of toxic events, however, was the same regardless of the direction or amount of change in carbon dioxide combining power.

Discussion. The results shown in Table 1 indicate that the appearance of toxic signs from ammonium chloride administration are related to the rate of administration rather than the total

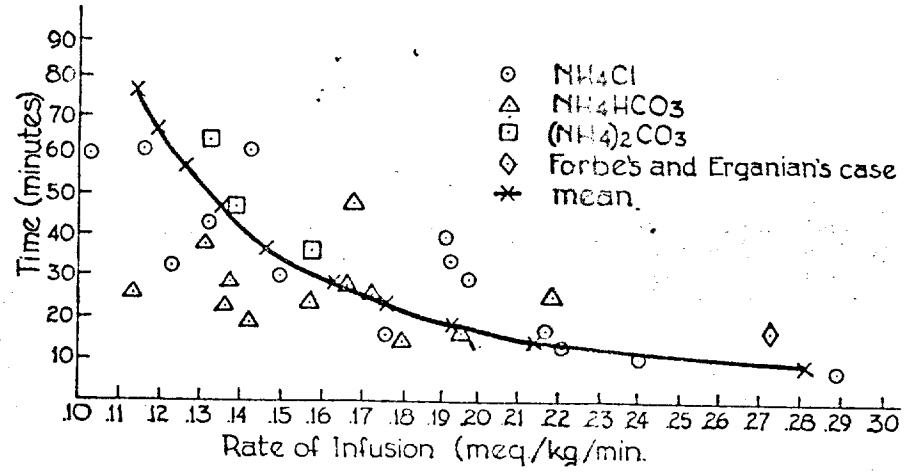

FIG. 1.—Relationship between rate of infusion and time to onset of toxicity.

TABLE 2.—EFFECT OF INJECTION OF AMMONIUM SALTS ON CARBON DIOXIDE COMBINING POWER OF BLOOD

Ammonium Compound Used	Before Injection Vols. %	Onset Toxicity Vols. %	At Death Vols. %
Ammonium Chloride	56	*	4.0
	52.5	48.5	11
	40.5	21.5	18
	46.5	31	27
	41.8	36	21.6
Ammonium Carbonate	50	*	63
	53	61.	53.5
	*	44	56
	39	53	46
	*	*	50.1
Ammonium Acetate	53	45	46.5
	43	47	49

* No determination made because of technical error.

amount injected. The influence of the rate of injection on toxicity was suggested in the earlier experiments of Meneguzzi[9] who found that animals recovered rapidly from the effects of ammonium chloride if the injection was not immediately fatal and Forbes and Erganian[2] who reported that more rapid rates of injection were more likely to produce severe toxic symptoms. Also, this is probably the reason for the appearance of toxic symptoms reported by Forbes and Erganian in the infant who received 30 cc. per kg. intravenously of 0.897 ammonium chloride within 18 minutes. We have recomputed the data given for the rate and amount of salt injected into this infant and find that it amounts to 0.276 milliequivalents per kg. per minute. This is entirely consistent with the rate and time of administration which produced toxic signs in our experiments on dogs (column 5, Table 1 and Fig. 1). This comparison would suggest that our data on dogs may with reasonable safety be applied to human beings. If so, the IRe/IRc ratio listed in Table 1 is seen to be uncomfortably low in some cases. Thus, rates of administration as low as 2.29 times that used clinically could produce toxic signs in patients. This is significant in view of the ease with which an intravenous infusion may, if not constantly attended, flow into the vein in half or less the intended time. It would therefore seem wise to reduce the concentration and, or, the volume per hour, in order to decrease the risk of producing toxic reactions in patients. The occasional death of an animal in our studies with no warning signs re-emphasizes the importance of maintaining a wide safety margin.

Probably because the therapeutic value of ammonium chloride depends largely on its acidifying action, attention has been focused upon this action as the cause of toxicity. Forbes and Erganian attributed the toxic signs in their patient to a too rapid injection with extensive fall in serum pH. Since, however, we have been able to produce identical toxic signs with ammonium compounds which are acidifying, alkalizing, or neutral in their physiologic reactions, it is more likely that the toxicity is due to the amonium ion *per se*, and not to changes in serum pH. In the experiments where we determined carbon dioxide combining power (Table 2), toxic signs and even death occurred without significant changes from the normal carbon dioxide combining power.

Another possibility which should be considered is that the toxicity of ammonium compounds might depend upon the relative fractions existing as NH_4^+ and as NH_3 or NH_4OH. In aqueous solution there is considerable variation in the amount of hydrolysis from moderate in ammonium chloride to considerable in ammonium acetate and bicarbonate. However, at the approximate pH of blood the amount of $NH_3 + NH_4OH$ would always be small relative to NH_4^+ regardless of the variation of pH within the physiological limits.

It might be argued that the anesthetic used (sodium pentobarbital) would interfere with the translation of our results to unanesthetized patients. However, the close quantitative comparison between our results and the single toxic reaction observed by Forbes and Erganian would indicate that there has been little interference by the anesthesia. Furthermore, we have consistently noticed a tendency for the anesthesia to become lighter after administration of ammonium ions was commenced, as evidenced by return of deep tendon reflexes and of the wink reflex. This would suggest, as has been previously reported, that ammonium ions have a central stimulant effect, and that if anesthesia exerted any

influence on the toxicity of ammonium compounds, it would be in the direction of protection rather than enhancement.

The hyperbolic form of the mean curve in Fig. 1 suggests that there is a slow rate of infusion of ammonium compounds which can be tolerated for long periods of time, probably because it is below the rate at which the liver can successfully convert all of the ammonium ion to urea. Conversely there is apparently a minimum time of several minutes which must elapse before toxic signs develop, no matter how high the rate. There is, however, a clear correlation between rate of administration of ammonium ion and the appearance of toxicity. No apparent correlation exists between total amount given and toxicity.

We believe, therefore, that the safety of any clinical procedure using ammonium chloride intravenously depends upon the rate at which it is administered rather than the total amount used. This is a significant fact in clinical practice, as the value of ammonium chloride either as an acidifying agent or as diuretic depends upon the conversion of the ammonium ion to urea, freeing the chloride ion. Consequently, exceeding the maximum rate at which this conversion can be accomplished does not increase either the rapidity or effectiveness of therapeutic action. The therapeutic effects of ammonium chloride are a function of the total amount administered and not of the rate. Conversely, the toxicity is a function of the rate and not of the total amount. Hence, clinical safety without impaired efficiency may be gained by the slow administration of proper amounts of ammonium chloride.

Summary and Conclusion. 1. Intravenously injected ammonium chloride, though a valuable therapeutic agent, has definite toxic potentialities.

2. Occurrence of toxicity is dependent upon rate of intravenous administration, and virtually independent of total amount administered. Therapeutic value, on the other hand, depends upon amount administered regardless of rate. Therefore, slow rates of administration are safer without loss of effectiveness.

3. Toxic reactions to ammonium chloride are due to the ammonium ion and not to the acidifying effect of the compound, since the same effects may be produced by the carbonate or acetate salt without accompanying acidosis.

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Experimental studies on urea formation from ammonium carbonate in vivo and in vitro.

By

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I.

Urea synthesis in vitro through organic ferments.

Introduction

KNIERIEM (1874) was first to demonstrate that ammonium introduced into the animal organism leaves the latter as urea. Said fact was later confirmed by numerous authors (SALKOWSKI, MUNK, EPSTEIN, POULSON,MARFORI,etc.). In humans, an increase of the urea in the urine after the introduction of ammonium salts was confirmed by CONANDA. SCHMIEDEBERG believed it likely that the nitrogenous substances provided with NH_2-NH_2 in the animal organism decompose into ammonium and that the urea is presumably formed from the ammonium carbonate. Liver blood supply tests with ammonium salts carried out by SCHRODER, SCOENDORFF, LOFFLER, among others, supplied evidence on the formation by liver cells of the urea from ammoniacal compounds. SALOMON and BUNGE obtained similar results. RICHET, GOTTLIEB and SCHWARTZ showed that the urea content of a liver mash digested at 39 to 40°C increases. Furthermore, RICHET reports that a watery liver extract still has an urea-forming capacity after precipitation with alcohol. SPITZER repeated RICHET's tests and obtained negative results. LOEWI contends that the urea-like substance, soluble in ether-alcohol, is not an urea at all. .

According to these tests the fermentative formation of urea from various parent substances is conjectured, but so far no urea-forming ferment has been found--except for arginase--during urea formation from arginine. Therefore the purpose of present work is the detection of a ferment which is capable of forming urea from ammonium carbonate.

A.

Description of ferment preparations from the liver.

A tubular needle is tied into the V. jugularis of a dog, connected by a rubber tube with an approximate 5-liter capacity flask, containing RING solution heated to 37°C. A tubular needle is inroduced into the central part of a prepared A. carotis, which is connected with a rubber tube and through which the blood is slowly withdrawn. From the V. jugularis the RING solution flows in under low pressure, and the tissue of the dog organism is gradually flushed through with the aid of (the dog's) own heart function. After heart arrest flushing is repeated under high pressure, until the tissue is theoretically and practically completely free of blood. The liver is subsequently detached from the surrounding tissue, crushed in the mortar until a fine-ground liver mash results; in a wide and flat bowl, ether is poured over the mash, which is then dried as fast as possible at low temperature in the desiccator. The dried liver mash is once more crushed in the mortar into a fine powder and is subsequently sifted. The powder obtained as indicated is stored at a dark and cool location, in a disinfected brown flask.

Test a.

2 larger test tubes contain 1 g of powdered liver each. One of them is filled with 20.0 ccm aq.dest. (distilled water), the other with the same volume of ammoniae, and a small thin glass tube is inserted into each of the two test tubes, which is linked to the KIPP apparatus. Carbon dioxide is slowly transferred into both test tubes through the small glass tubes with the aid of a regulating valve. The test tubes are submerged in a water bath at 37°C, and the urea quantity found in the content of both test tubes is determined according to the NAKAJIMA-MARUOKA method*). which is based on the SCHIFF reaction. A much larger quantity of urea is found in the test tube which contains ammoniae than in the other, as evident from Table 1.

*) See translation of Footnote on p. 3

TABLE I

Nummer der Reagenzgläser	I.	II.	I.	II.
Aq. dest. (ccm)	0	20.0	0	20.0
Aq. Ammoniac (ccm)	20.0	0	20.0	0
Leberpulver (g)	0.1	0.1	0.1	0.1
Zeitdauer der CO_2 einführung (Stunden)	5	5	7	7
Ü-Stickstoff (mg. in 100.0 ccm)	10.0	2.0	7.5	2.0
Harnstoff (mg. in 100.0 ccm)	21.4	4.28	16.06	4.28

1) Number of test tubes.
2) Aq.dest.(ccm) - distilled water
3) Ammonium solution (ccm)
4) Powdered liver (g)
5) Duration of the CO_2 introduction (hours)
6) Ü-nitrogen (mg in 100.0 ccm)
7) Urea (mg in 100.0 ccm)

FOOTNOTE (p. 235 of original text)
*) In 1920 NAKAJIMA and MAROUKA published a new specification method for blood urine, which was based on the SCHIFF reaction. The urease method was not adequate for my in vitro test on account of the ample ammonium content. Said new method was therefore preferred, whereby I modified to some extent the thinning- and volume ratios of the reagents,etc., whenever necessary. Since the new method is not yet generally known, it will be briefly described below:

Reagents. 1. Standard solutions: pure urea is dissolved in re-distilled water which is saturated with chloroform,under rising pressure, for example: 10/5 mg urea in 100.0 ccm aq.dest.,15/5 urea in 100.0 ccm aq.dest.,etc

2. Pure furfural is re-distilled in the atmosphere of the hydrogen gas, and 1.0 ccm of the latter is sealed into, and stored in an ampulla which is also filled with hydrogen.

3. Tin chloride solution: 30% hydrochloric acid is saturated with metallic tin . A fresh mixture with the following ratio is prepared for each test: concentrated hydrocholiric acid 1.0-2.0; tin chloride solution 1,0; aq.dest. 1.0. The material for specification suffices only with 0.15 ccm, whereby accurate measurement with a micro-pipette is necessary. Liquefaction with the triple volume of aq.dest. follows; 0,15 ccm of a 1.5% ammonium acetate solution is added; the albumen is eliminated by means of vigorous agitation and centrifugalization. 0,1 of said centrifugate is transferred with the aid of a micro-pipette into a small test tube which is 5 cm thick and 10 cm long and which stands before a background of frosted glass. Each of the next small test tubes is filled with 0.1 ccm of the standard solution No. 10, 15, etc. Then 0.075 ccm of furfural is filled into each small test tube. Subsequently, 0.4 ccm of a tin chloride solution is added. Immediate agitation follows, after which the solution clears.

The first reaction shows blue color, then after 5 to ten minutes it becomes purple and purple-red after 15 to 20 minutes; this red color is subjected to colorimetric comparison. After 30 minutes the solution becomes black and cloudy as a result of precipitation.

Test b.

When implementing the first procedure, the transit of the CO-gas from the KIPP apparatus encounters considerable difficulties. We therefore used processed (ready-made) ammonium carbonate for the tests listed below. A series of smaller test tubes is filled with 1.0 ccm aq.dest. each which contains increasing doses of ammonium carbonate. For control purposes a test tube is filled with 1.0 ccm of freshly distilled water. A specific amount of a freshly prepared 10% watery extract of the pulverized liver is added to each test tube; the test tubes are subsequently submerged in a water bath at 37°C. After 1 to 20 hours the SCHIFF reaction is stronger in those test tubes which contain more ammonium carbonate, as compared to those which contain less and as compared to the control (tube).

TABLE II.

Nummer der Reagenzgläser	1	2	3	4	5
2 Gehalt des kohlensauren Ammons (%)	1.0	0.5	0.25	0.03625	0
3 10-%iges Wasserextrakt vom Leberpulver (ccm)	1	1	1	1	1
(nach 2 Stunden bei 37°C im Wasserbad)					
5 Schiffsche Reaktion	+++	++	++	+	+

1) Number of test tubes
2) Ammonium carbonate content (%)
3) 10% water extract of pulverized liver (ccm)
4) (after 2 hours, at 37°C in water bath)
5) SCHIFF reaction

TABLE III.

Nummer der Reagenzgläser	1	2	3	4
Gehalt des kohlensauren Ammons (%)	0.25	0.145	0.0725	0
10-%iges Wasserextrakt vom Leberpulver (ccm)	0.5	0.5	0.5	0.5
(nach 20 Stunden bei 37°C im Wasserbad)				
Schiffsche Reaktion	++	++	++	+

1) to 5): translation as for Table II.

Test c.

A water extract of pulverized liver was dialyzed during 24 hours through colloid membrane, for the purpose of obtaining an urea-free ferment extract. We were unable to free the extract completely from the urea. The water extract is still quite effective (actinic) after the dialysis procedure.

TABLE IV.

Nummer der Reagenzgläser	1	2	3	4	5
Gehalt des kohlensauren Ammons (%)	0.5	0.25	0.175	0.0875	0
Dialysiertes 10-%iges Wasserextrakt vom Leberpulver (ccm)	0.5	0.5	0.5	0.5	0.5
(nach 20 Stunden bei 37°C im Wasserbad)					
Schiffsche Reaktion	+++	+++	++	+	+

1) Number of test tubes
2) Ammonium carbonate content (%)
3) Dialysed 10% water extract of pulverized liver (ccm)
4) (after 20 hours, at 37°C in water bath)
5) SCHIFF reaction.

Test d.

For the purpose of removing the urea from the pulverized liver with another method, the latter was extracted for 24 hours with absolute alcohol, and filtered. Subsequently the residue was once more extracted with a 10-fold volume of aq.dest. during 24 hours. Using this water extract which was not entirely free from urea, I tested with the previously indicated method. The difference between tests and controls is more conspicuous here than it was for the preceding test series.

TABLE V.

Nummer der Reagenzgläser	1	2	3	4
Gehalt des kohlensauren Ammons (%)	0.25	0.175	0.0875	0
Mit Alkohol behandeltes Wasserextrakt vom Leberpulver (ccm)	1.0	1.0	1.0	1.0
(nach 24 Stunden bei 37°C im Wasserbad)				
Schiffsche Reaktion	++++	+++	++	+

1) Number of test tubes
2) Ammonium carbonate content (%)
3) Water extract of pulverized liver treated with alcohol (ccm)
4) (after 24 hours, at 37°C in water bath)
5) SCHIFF reaction.

Test e.

A series of small test tubes: each is filled with a 1.0 solution of the ammonium carbonate in increasing doses. One of the test tubes contains 1.0 ccm of freshly distilled water for control purposes. A drop of the extract prepared as indicated above is introduced into all these test tubes. After an incubation period of 24 hours in the incubator at 37°C, the intensity of the SCHIFF reaction is compared. The intensity of the color reaction decreases proportionately to the increasing urea content and the difference at both ends becomes quite evident.

TABLE IV.

Nummer der Reagenzgläser	1	2	....	13	14	15
Gehalt des kohlensauren Ammons (%)	0.5	0.4	....	0.02	0.01	0
Mit Alkohol behandeltes Wasserextrakt vom Leberpulver (Tropfen)	1	1	....	1	1	1
(nach 24 Stunden bei 37°C im Wasserbad)						
Schiffsche Reaktion	++++ stärker	>		schwächer	+ ganz spur	+ ganz spur

1) Number of test tubes
2) Ammonium carbonate content (%)
3) Water extract of pulverized liver treated with alcohol (drops)
4) (after 24 hours, at 37° in water bath)
5) SCHIFF reaction
6) stronger
7) weaker
8) complete
9) trace

Test f.

Furthermore, the effectiveness of the liver extract was investigated after heat reaction. The higher the temperature is to which the liver extract is exposed, the weaker the effect of the latter will be on the ammonium carbonate; after the boiling of the water extract the urea-forming capacity vanishes completely. The following method was applied: a series of small test tubes is filled with extract which was not heated; another series (of test tubes) is filled with the boiled extract, and is treated as indicated above. In the first series of test tubes the SCHIFF reaction shows a definite difference between tests and controls, while (said reaction) is entirely lacking when boiled extract is used.

TABLE VII.

1 Nummer der Reagenzgläser	1	2	3	1	2	3
2 Gehalt des kohlensauren Ammons (%)	0.25	0.145	0	0.25	0.145	0
3 gekochtes 10-%iges mit Alkohol behandeltes Wasserextrakt vom Leberpulver (ccm)	1.0	1.0	1.0	0	0	0
4 Nicht gekochtes 10-%iges mit Alkohol behandeltes Wasserextrakt vom Leberpulver (ccm)	0	0	0	1.0	1.0	1.0
5 (2 Stunden bei 37°C im Wasserbad)						
6 Schiffsche Reaktion	+	+	+	+++	++	+
(nach 24 Stunden bei 37°C im Wasserbad)						
Schiffsche Reaktion	+	+	+	+++	++	+
9 Harnstoff (bestimmt nach Nakajima-Maruokascher Methode)	3.21	3.21	3.21	5.35	4.24	2.68

1) Number of test tubes
2) Ammonium carbonate content (%)
3) Boiled 10% water extract of pulverized liver, treated with alcohol (ccm)
4) Not boiled 10% water extract of pulverized liver, treated with alcohol (ccm)
5) (2 hours in water bath at 37°C)
6) SCHIFF reaction
7) (after 24 hours in water bath at 37°C)
8) SCHIFF reaction
9) Urea (determined according to the NAKAJIMA-MARUOKA method).

Test g.

5 g of pulverized liver with a 50.0 ccm equal mixture of water and glycerin is vigorously agitated for 2 hours in the agitator. After standing for 24 hours in a refrigerator the glycerin-water extract is filtered through gauze and the filtrate is stored in the refrigerator in a well-sealed flask. Said glycerin-water extract has an urea-forming capacity, as does the water extract, and can be preserved (stabilized) much better than the water extract.

TABLE VIII.

Nummer der Reagenzgläser	1	2	....	13	14	15
Gehalt des kohlensauren Ammons (%)	0.5	0.4	....	0.02	0.01	0
10-%iges Glycerinwasserextrakt vom Leberpulver (Tropfen)	1	1	....	1	1	1
(nach 24 Stunden bei 37°C im Wasserbad)						
Schiffsche Reaktion	+++	+++			+	+
Harnstoff (bestimmt nach Nakajima-Maruokascher Methode) (mg. in 100.0 ccm)	0.535				fast	negativ

1) Number of test tubes
2) Ammonium carbonate content (%)
3) 10% glycerine-water extract of pulverized liver (drops)
4) (after 24 hours in water bath at 37°
5) SCHIFF reaction
6) Urea (determined according to the NAKAJI-MARUOKA method) (mg in 100.0 c
7) nearly
8) negative

TABLE IX

1 Nummer der Reagenzgläser	1	2	....	13	14	15
2 Gehalt des kohlensauren Ammons (%)	0.5	0.4	....	0.02	0.01	0
3 10-%iges Glyzerinwasserextrakt vom Leberpulver (ccm)	0.5	0.5	....	0.5	0.5	0.5
4 (nach 24 Stunden bei 37°C im Wasserbad)						
Schiffsche Reaktion	+++	+++	>	+	+	+
Harnstoff (bestimmt nach Nakajima-Maruokascher Methode) (mg. in 100.0 ccm)	1.005					0.535

1) to 6): see translation of text for Table VIII)

Test h.

On this occasion I gave preference to a physiological saline solution as solvent of the ammonium carbonate, so as to obtain conditions as similar as possible to natural conditions in the animal organism. Otherwise, I proceeded as indicated above. With this method, the result was the same as the result obtained from preceding test, or it was somewhat more evident.

TABLE X.

1 Nummer der Reagenzgläser	1	2	....	13	14	15
2 Gehalt des kohlensauren Ammons in phys. Kochsalzlösung (%)	0.5	0.4	....	0.02	0.01	0
3 10-%iges Glycerinwasserextrakt vom Leberpulver (Tropfen)	3	3	....	3	3	0
4 (nach 24 Stunden bei 37°C im Wasserbad)						
Schiffsche Reaktion	+++		>		++	+
Harnstoff (bestimmt nach Nakajima-Maruokascher Methode) (mg. in 100.0 ccm)	2.675					0.535

1) Number of test tubes
2) Ammonium carbonate content in the phys. saline solution (%)
3) 10% glycerine-water extract of pulverized liver (drops)
4) (after 24 hours in water bath at 37°C)
5) SCHIFF reaction
6) Urea (determined accordi to the NAKAJIMA-MARUOKA method (mg in 100.0 ccm)

The above test series establish the fact that the water- and the water-glycerine extract of pulverized liver has the capacity to form urea from ammonium carbonate in vitro; this occurs through some fermentative process, because the water extract becomes inactive after heat application.

B

The idea that an auxiliary factor, such as possibly a co-enzyme or activator of the urea-forming liver ferments, might be present during urea formation from ammonium carbonate, promted me to undertake the following test.

A powder is prepared from spleen, pancreas and muscle with the method used for the preparation of pulverized liver. All this powder is then agitated for 2 hours in the agitator together with a 10-fold volume of glycerine and aq.dest.āā ; after standing for 20 hours in the refrigerator, it is filtered through gauze.

2 series of small test tubes are submerged at 37°C in a water bath. Each test tube is filled (as during the last mentioned procedure) with 1.0 ccm physiological saline solution with decreasing doses of ammonium carbonate; one test tube of each series is filled for control purposes with 1.0 ccm freshly prepared physiological saline solution. Into each test tube of the first series three drops each of a 10% glycerine-water pulverized liver extract and three drops each of a 10% glycerine-water pancreas powder extract are introduced, and into each of the second series of tubes six drops of a 10% glycerine-water pulverized liver extract only are introduced. After 24 hours the definition (definite evidence) of the difference in the SCHIFF reaction between tests and control tests is compared for both sries.

TABLE XI. Liver and pancreas.

Nummer der Reagenzgläser	1	2	3	1	2	3
Gehalt des kohlensauren Ammons in phys. Kochsalzlösung (%)	0.5	0.25	0	0.5	0.25	0
10-%iges Glycerinwasserextrakt vom Leberpulver (Tropfen)	3	3	3	6	6	6
10-%iges Glycerinwasserextrakt vom Pankreaspulver (Tropfen)	3	3	3	0	0	0
(nach 5 Stunden bei 37°C im Wasserbad)						
Schiffsche Reaktion	+++	++	+	+++	++	+
(nach 24 Stunden bei 37°C im Wasserbad)						
Schiffsche Reaktion	+++	++	+	+++	++	+

Die Untersuchung mit Leber- und Milzextrakt war etwas undeutlicher als mit Leberextrakt.

1) Number of test tubes
2) Ammonium carbonate content in the phsy. saline solution (%)
3) 10% glycerine-water pulverized liver extract (drops)
4) 10% glycerine-water extract of pulverized pancreas (drops)
5) (after 5 hours in water bath at 37
6) SCHIFF reaction
7) (after 24 hours in water bath at
8) SCHIFF reaction

The test results with liver and pancreas extract proved to be somewhat less well-defined than with liver extract.

TABLE XII. Liver and spleen.

Nummer der Reagenzgläser	1	2	3	1	2	3
Gehalt des kohlensauren Ammons in phys. Kochsalzlösung (%)	0.5	0.25	0	0.5	0.25	0
10-%iges Glycerinwasserextrakt vom Leberpulver (Tropfen)	3	3	3	6	6	6
10-%iges Glycerinwasserextrakt vom Milzpulver (Tropfen)	3	3	3	0	0	0
(nach 5 Stunden bei 37°C im Wasserbad)						
Schiffsche Reaktion	++	+	+	+++	++	+
(nach 24 Stunden bei 37°C im Wasserbad)						
Schiffsche Reaktion	++	+	+	+++	++	+

Indem ich mit Muskel in gleicher Weise untersuchte, fand ich keinen Unterschied zwischen beiden Reihen.

1), 2),3),5),6),7) and 8): see translation for Table XI.
4) 10% glycerine-water pulverized spleen extract (drops)

I found no difference between the two series when testing with muscle (powder, using the same procedure.]

TABLE XIII. Liver and muscle.

Nummer der Reagenzgläser	1	2	3	1	2	3
Gehalt des kohlensauren Ammons in phys. Kochsalzlösung (%)	0.5	0.25	0	0.5	0.25	0
10-%iges Glycerinwasserextrakt vom Leberpulver (Tropfen)	3	3	3	6	6	6
10-%iges Glycerinwasserextrakt vom Muskelpulver (Tropfen)	3	3	3	0	0	0
(nach 5 Stunden bei 37°C. im Wasserbad)						
Schiffsche Reaktion	+++	++	+	+++	++	+
(nach 24 Stunden bei 37°C im Wasserbad)						
Schiffsche Reaktion	+++	++	+	+++	++	+

1),2),3),5),6),7) and 8): see translation for Tables XI and XII.
4) 10% glycerine-water pulverized muscle powder extract (drops).

With this method, I was unable to detect a co-enzyme or activator anywhere.

C.

How should the function of the urea formation from ammonium be localized? Or is it a general function of all organic tissues? Numerous works were published to date on the solution of said problem.

After elimination of the liver from the circulation through the ECK fistula or through the simultaneous ligature of the A.hepatica, deactivation or, if possible, total extirpation of the same, decrease of the urea and increase of ammonium was found in most cases (see SCHRODER, HAHN and NENCKI, v.MEISTER, LIEBLEIN, MAGNAMINI, NENCKI, PAWLOW and ZALESKI, SALASKIN and ZALESKI, etc.). During various liver ailments, decreased urea elimination was clinically observed (FRERICHS, MURCHINSON, BROUARDEL, PARKER, CHORCOT, CHAUFFARD, KELCH and others), and increased ammonium excretion was clinically found (HALLEVORDEN, STADELMANN, FAWITZKY). WEINTRAUD and MUNZER, however could thereby not detect any decrease of the urea excretion. KAUFAMANN and GOTTLIEB report that the urea content of the liver exceeds the urea content of the blood. MOLESCHOTT found plenty of urea in the muscle of a frog the liver of which had been removed. Ample amounts of urea were found in various organic tissues by STADLER and FRERICHS (rays and sharks) and by Gad ANDERSEN (various animals: dogs, rats, etc.). V. V. SCHRODER reached the same conclusion, but he attributed the fact to sluggish kidney function. Numerous authors also reported on the ample urea content of the muscle tissue (NENCKI and KOWALSKI, PICARD, DEMANT, HAYCRAFT, GREHAUT, and QUINQUARD, etc.).

Further resreach is therefore of interest concerning the question whether urea is formed from ammonium carbonate in other tissues besides the liver; this idea led me to the following investigation.

While testing with the above-mentioned method the 10% glycerine-water extracts of spleen-, pancreas- and muscle powder, I found that pancreas- and muscle extracts have an urea-forming capacity, and that heating inactivates said capacity.

TABLE XIV (Spleen)

1 Nummer der Reagenzgläser	1	2	3	1	2	3
2 Gehalt des kohlensauren Ammons in phys. Kochsalzlösung (%)	0.5	0.25	0	0.5	0.25	0
3 10-%iges Glycerinwasserextrakt vom Milzpulver (Tropfen)	3	3	3	0	0	0
4 Gekochtes 10-%iges Glycerinwasserextrakt vom Milzpulver (Tropfen)	0	0	0	3	3	3
5 (nach 24 Stunden bei 37°C im Wasserbad)						
6 Schiffsche Reaktion	+	+	+	+	+	+

1) Number of test tubes
2) Ammonium carbonate content in phys.saline solution (%)
3) 10% glycerine-water spleen powder extract (drops)
4) Boiled 10% glycerine-water spleen powder extract (drops)
5) (after 24 hours in water bath at 37°C)
6) SCHIFF reaction

TABLE XV. (Pancreas)

1 Nummer der Reagenzgläser	1	2	3	1	2	3
2 Gehalt des kohlensauren Ammons in phys. Kochsalzlösung (%)	0.5	0.25	0	0.5	0.25	0
3 10-%iges Glycerinwasserextrakt vom Pankreaspulver (Tropfen)	3	3	3	0	0	0
4 Gekochtes 10-%iges Glycerinwasserextrakt vom Pankreaspulver (Tropfen)	0	0	0	3	3	3
(nach 24 Stunden bei 37°C im Wasserbad)						
6 Schiffsche Reaktion	+++	++	+	+	+	+

1),2),5) and 6): see translation for Table XIV.
3) 10% glycerine-water extract of pancreas powder (drops)
4) Boiled 10% glycerine-water extract of pancreas powder (drops)

TABLE XVI. (Muscle)

1 Nummer der Reagenzgläser	1	2	3	1	2	3
2 Gehalt des kohlensauren Ammons in phys. Kochsalzlösung (%)	0.5	0.25	0	0.5	0.25	0
3 10-%iges Glycerinwasserextrakt vom Muskelpulver (Tropfen)	3	3	3	0	0	0
4 Gekochtes 10-%iges Glycerinwasserextrakt vom Muskelpulver (Tropfen)	0	0	0	3	3	3
5 (nach 24 Stunden bei 37°C im Wasserbad)						
6 Schiffsche Reaktion	+++	++	+	+	+	+

1), 2), 5) and 6): see translation for Tables XIV and XV.

3) 10% glycerine-water extract of muscle powder (drops)
4) Boiled 10% glycerine-water extract of muscle powder (drops).

II.
Urea formation in vivo from ammonium carbonate.

It is highly probable that urea is formed fermentatively from ammonium carbonate in the liver and in other organs (pancreas, muscle,etc). The following study concerns the mechanism of the urea formation from ammonium carbonate in the animal organism. I used rabbits as test animals for this purpose, because blood can be withdrawn conveniently. TASHIRO describes as "fixation-hyper-carbamidaemia" a phenomenon which is very similar to the fixation hyperglycaemia, i.e. when the rabbit is tied down the urea content of the rabbit's blood increases gradually. Therefore I avoided the shackling of the test animal entirely during my tests. The rabbit was put into a box the somewhat slanting lid of which has a top opening through which the head and part of the rabbit's neck emerges, so that it can sit calmly in its customary squatting position.
The urea level in the blood of unfettered rabbits during the test period was studied for control purposes. After fasting for approximately 12 hours, blood is taken from the ear vein of the tested rabbit while it sits calmly, in intervals of one hour, and the blood urea content is determined according to BAHLMAN's micro-method. The apparatus used for the determination was modified by Dr. ITATSU in our Laboratory, so that the sodium carbonicum solution and the caprilalcohol can be filled into the test tube without opening its lid (stopper). I preferred the urease of Squibb & Sons, New York, as ferment for the test. The urea level showed a constant value during the investigation, as indicated on Table I.

TABLE I.

Nummer des Kaninchens		II	III	IV	V	VI	VII
Zeit							
	Harnstoff (g/l)						
0	0.73	0.39	0.39	0.37	0.25	0.39	0.32
30'	0.36						
1st.	0.45	0.38	0.31	0.41	0.31	0.39	0.32
2	0.42	0.35	0.30	0.39	0.32	0.38	0.36
3	0.45	0.38	0.30	0.44	0.27	0.37	0.33
4	—	0.41	0.31	0.41	0.27	0.35	0.32
5	0.40	0.37	0.32	0.44	—	0.36	0.34
6	0.40	0.38	0.33	0.39	0.29	0.35	0.33
7	0.46	0.38	0.28	0.37	0.27	0.36	0.35

Introduction of the ammonium carbonate per os.

After determining the blood urea with the above method in rabbits which had fasted for 12 hours, a freshly prepared solution of 2.5 g/dl (2.5 g in 100.0 ccm) ammonium carbonicum, 10.0 ccm per kg of rabbit body weight, was introduced into the stomach with the aid of an esophagus probe, after which the urea content of the rabbit's blood was once more determined per hour. After 1 to 3 hours the urea level rises definitely and in most cases returns to normal values after 7 hours, as shown on Table II. The increase of the blood urea proves that the urea is formed from ammonium carbonate in the rabbit organism.

TABLE II.

number of rabbits

Nummer des Kaninchens	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
Zeit Time					UREA							
					Harnstoff (g/l)							
0	0.62	0.17	0.18	0.20	0.24	0.72	0.52	0.35	0.19	0.35	0.32	0.22
15'	—	0.30	0.15	0.30	0.27	0.88	0.63	0.32	0.29	0.43	0.36	0.24
30'	0.68	0.30	0.26	0.34	—	0.75	0.57	0.38	0.23	0.48	0.41	0.30
1st	0.81	0.65	0.30	0.35	0.33	0.81	0.63	0.32	0.38	0.46	0.35	0.32
2	0.80	0.33	—	0.36	0.34	0.81	0.63	0.32	0.33	0.47	0.35	0.34
3	1.19	0.29	0.41	0.36	0.27	0.77	0.50	0.56	0.34	0.62	0.38	0.36
4	0.71	0.31	0.32		0.27	—	0.56	0.48	0.34	0.45	—	0.31
5	0.74	0.29			0.29	0.70	0.56	0.39	0.36	0.46	0.32	0.33
6	—	—			0.28		—	0.25	0.35	0.32	0.33	0.33
7	0.74	0.31			0.28		0.47	0.23	0.36	0.42	0.33	0.31

Involvement of the reticuloendothelial system in the formation of the urea.

Since the time when KUPPFER first described the so-called star cells in 1876, numerous works have been pulished by authors dealing with the histological field (KYONO, UNO, NAGAWO, BOEMER, PUTZELT,etc.).In the field of serology numerous endothelium blocking tests were also implemente with colloidal substances, coloring agents, and ink , namely for the purpose of blocking the reticuloendothelial system regarding the formation of antigens (MURATA, OKADA,BIELING, KATO, SIEGMON,KOBAYASHI, SHIOTSU, GAY & CLARK, STANDENATH, SAKUMA, AMAKO, OMURANA, among others. ITO and KATO showed that malfunction of the reticuloendothelia can result when ink is intravenously injected, and that the reticuloendothelia, in particular KUPPFER's star cells, have a specific function in sugar metabolism. The question now arises whether the reticuloendothelial system, in particular the star cell of the liver, is involved at all in the urea synthesis? The following steps were taken to answer the above question: I preferred the intravenous injection of ink for blocking the reticuloendothelium. The ink is prepared with aseptic precautions as follows: good Chinese ink is rubbed for about 10 minutes on an ink stone (Suzuri) with approximetely 40.0 ccm of a physiological saline solution; filtering follows. The sterile solution prepared as indicated is intravenously injected into a well-fed rabbit in the amount of about 20.0 ccm; the animal is then subjected to fasting. The urea content of the blood is determined approximately 12 hours later, and a thin esophagus probe is used for introducing into the stomach 10.0 ccm per kg of rabbit body weight of a freshly prepared ammonium carbonate solution. The hourly determination of the blood urea follows. It becomes evident thereby--at variance from Table II--that no definite rising of the blood urea level occurs in the course of said test series (see Table III). In each case a histological control was carried out after the test to ascertain that the reticudoendothelia, in particular KUPPFER's star cells,were blocked with fine coal particles. The absorption of the ink particles inhibits the urea-forming function of the reticuloendothelial system and in particular of the liver star cells, to such an extent that the introduction of ammonium carbonate will no longer raise the urea level of the blood, as it does in a normal animal.

Table III.

Nummer des Kaninchens	I	II	III	IV	V	VI
	Harnstoff g/l.					
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	—	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
5	[illegible]	—	[illegible]	[illegible]	[illegible]	[illegible]
6	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

a.

Urea formation during partial liver elimination.

Despite the fact that the urea is formed from ammonium carbonate in various organs besides the liver, it cannot be doubted that the principal location of the origin is the liver. It was attempted therefore to observe the formation of urea after the partial extirpation of the liver, with and without the introduction of ammonium carbonate.

Test with partial liver extirpation without ammonium carbonate supply.

A rabbit which fasted for about [illegible] hours is tied to the operating table in supine position and the abdominal wall is opened at the linea alba. Several lobes of the liver on the right side are firmly ligated, and is even resected in some cases, and the abdomen is immediately closed. [illegible] operation is carried out under strict aseptic conditions. [illegible] however, is thereby applied, so as to avoid its side-effect on the metabolism. The keenly determination of the urea content in the blood follows. As shown on Table IV, the urea level rises during the [illegible] in most cases.

TABLE IV.

number of rabbits

Nomen des Kaninchens / Zeit Time	I	II	III	IV	V	VI
			Harnstoff (g/l)			
0	0.28	0.82	0.18	0.80	0.27	0.28
1	0.29	0.88	0.17	0.82	0.88	0.26
2	0.28	0.81	0.22	0.88	0.83	0.27
3	0.28	0.84	0.28	0.87	0.89	0.28
4	0.25	0.42	0.25	0.47	0.46	0.41
5		0.82	0.82			0.82
6			0.88			0.88
7			0.42			0.40

b.

<u>With oral administration of ammonium carbonate in the rabbit, during partial liver elimination.</u>

A freshly prepared 2.5 g/dl ammonium carbonate solution (quantity: 10.0 ccm per kg of body weight) was introduced with a thin esophagus probe into the stomach of a rabbit the liver of which had been partially resected or ligated; the hourly determination of the urea content in the blood followed. An evident rising of the urea level in the blood was likewise found during these test series; therefore the difference between said tests and the control series with administration of ammonium carbonate was no longer well-defined. It follows that the elimination of half of the liver has a moderately conspicuous effect on the urea formation.

TABLE V.

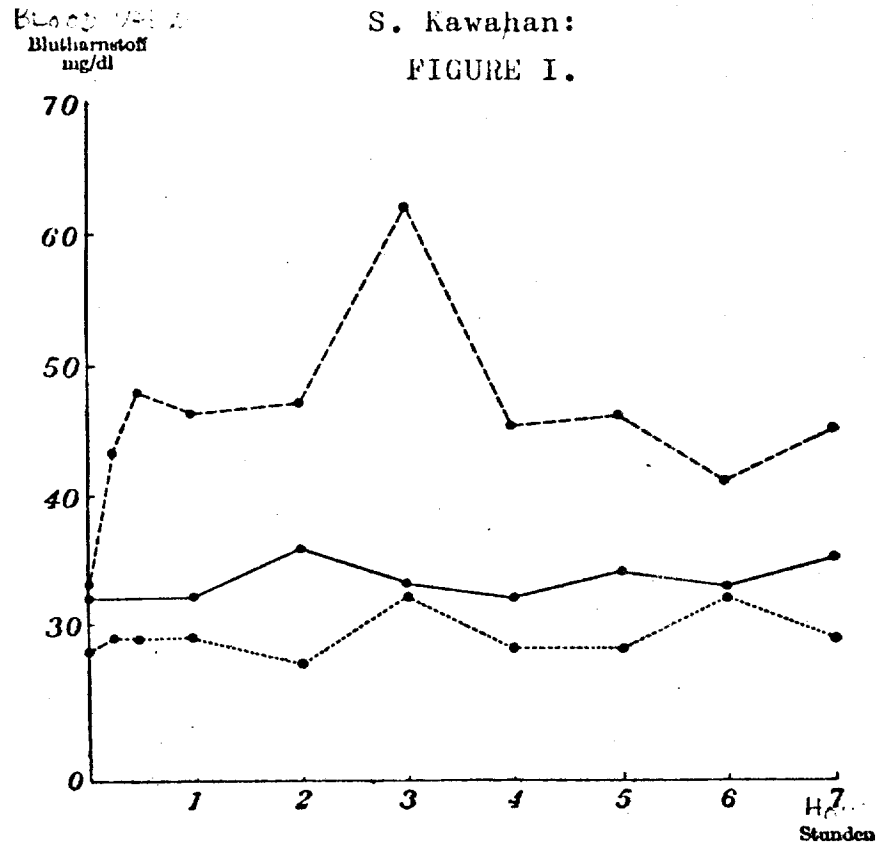
Number of rabbits

Nummer des Kaninchens	I	II	III	IV	V	VI
Zeit						
			Harnstoff (g/l)			
0	0.25	0.30	0.31	0.28	0.32	0.27
15	0.26	0.24	0.37	0.30	0.28	0.26
30	0.28	0.30	0.40	0.35	0.33	0.27
1 St.	0.30	0.26	0.32	0.28	0.37	0.27
2	0.33	0.32	0.43	0.23	0.35	0.32
3	0.29	0.32	0.51	0.33	0.35	0.28
4	0.31	0.32	0.48	0.39	0.43	0.33
5	0.31	0.33	0.47	0.33	0.50	0.37
6	0.35	0.32	0.60	0.38	—	0.29
7	0.36	0.33	0.55	0.35	0.55	0.36

Figure I shows the control test with intact rabbits and the test with the ammonium carbonate solution, with and without ink injection; Figure II shows the test without administration of the ammonium carbonate solution, using rabbits the liver of which was eliminated. (Text garbled. Translator).

S. Kawahan:

FIGURE I.

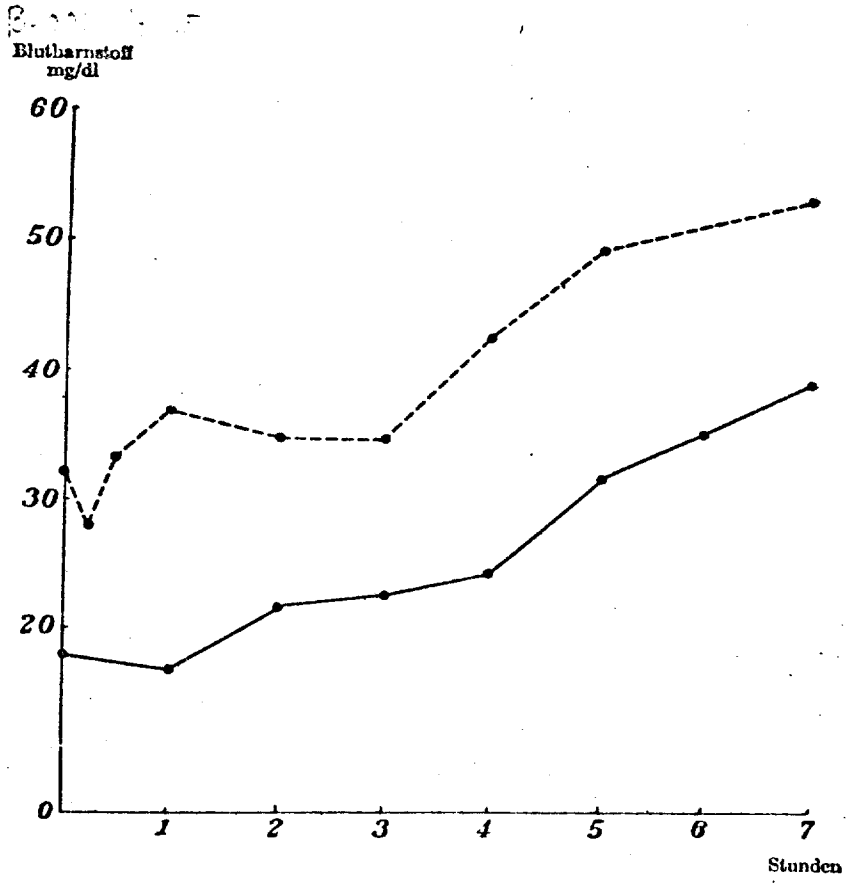


_____ Control in normal test animal.

- - - - -Supplied ammonium carbonate

. . . . Ink injection and supplied ammonium carbonate.

FIGURE II.



——————— Control with liver elimination.

- - - - - - - - Liver elimination and supply of ammonium carbonate.

III.

Urea formation from ammonium carbonate during B-avitaminosis and fasting.

A.

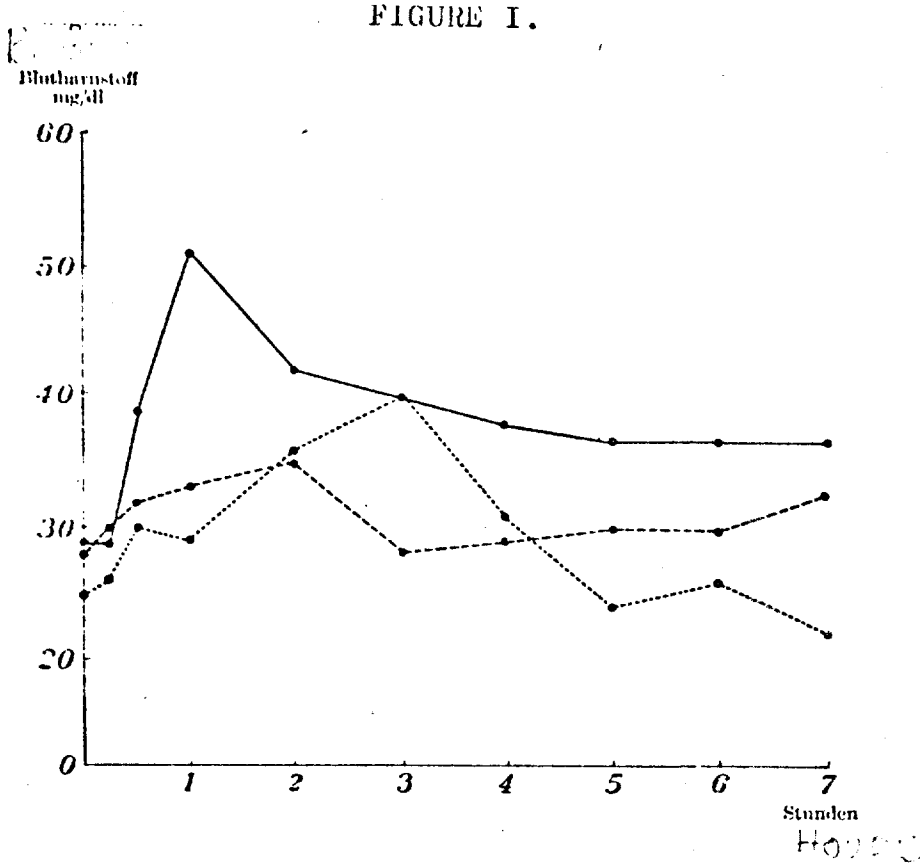
Urea formation during B-avitaminosis in the rabbit.

According to Part I of present work it was shown in vitro as highly probable that urea is formed from ammonium carbonate by fermentative processes. It will now be shown what occurs when the fermentative action in the living organism is reduced. Vitamin B deficiency was selected as a comparable condition, since previous tests demonstrated a decrease of various ferments under such conditions. Thus UJIHARA found reduced body diastase and blood trypsin, SHIMAZONO and TAKAYAMA and NAKANISHI observed reduced blood catalase in in cases of beri-beri and B-avitaminosis of humans, and the "polished rice disease" of birds; SAKAMOTO, NISHOTA, HOSOYA and SERITA reported the decrease of blood lipase in beri-beri cases, and B-avitaminosis in dogs; TAKAKUSU observed a dicrease of ptyalin in the beri-beri saliva of dogs, and HAMADA found decrease of diastase, trypsin and lipase in the duodenal fluid of beri-beri patients. ORAWA, HIRUMA, TAMURA and TANCUCHI and MURATA, KUMAGAYA, KOMIYA, KATSURA and SAITO demonstrated earlier that rabbits can contract a beri-beri - like disease when fed with polished rice, unpolished rice or with the latter diet plus straw. ASAI could obtain symptoms of pure B-avitaminosis in rabbits by feeding them with SHIMOZANO's A-type ready-made (factory) diet and "Tofu"*); it has been established earlier by SHIMAZONO that these foodstuffs are deficient in vitamin B and albumen. On this occasion, I likewise used rabbits as test animals; they received an alternate diet of A, B and C-type ready-made (factory) food which SHIMAZONO had found deficient in vitamin B and albumen after thorough research work. The rabbits also received "Tofu" to compensate for the missing albumen. The body weight of the rabbits decreases rapidly (after 1 month of test diet, in most cases). Some were subject to paralysis of their rear extremities (after 1 month of test diet, in most cases). Two of them recovered completely after receiving vitamin B, and some of them survived for a prolonged period of time after the onset of the paralysis, but no recovery took place. The urea content is determined only after the oral administration of ammonium carbonate, on an hourly basis.

*) Processed food made of beans; . . . (illegible word) albumen.

Subsequently the animal is fed for some time with the above-mentioned food free from vitamin B, and similar tests are carried out repeatedly during the test feeding period. The rise of the urea level in the blood is considerably less after the oral feeding of ammonium carbonate during the vitamin-B deficiency stage than during the control period, and it is entirely absent in some cases. However, as evident according to tests No. II and IV, the rise again becomes more conspicuous after the recovery of the animals following supply of vitamin B. This is no longer definitely demonstrable during the stage of advanced emaciation because the urea content of the blood increases considerably as a result of body albumen decomposition and retention of the same. Since it has already been shown that residual nitrogen and urea retention occurs in the course of beri-beri and "polished rice disease" in rabbits (ARAMI, HASAI, HAYASHI and OSAWA and KUSUNOKI), it cannot be doubted that a lesser or missing rise of the urea level after administration of ammonium carbonate during the B-avitaminosis stage does not result from the fact that the kidney become permeable for urea; it results instead from the reduced capacity of the organism to form urea synthetically. It has accordingly been established that the capacity of urea formation from ammonium carbonate is reduced during the B-avitaminosis stage; it returns to normal after vitamin B has been supplied. In cases when blood sugar was additionally determined, a slight hyperglycemia was evident during the stage when the urea-forming capacity is reduced. . SHIMAZONO and ASAI proved the fact that a slight hypeglycemia occurs in rabbits during B-avitaminosis. For data see Table 1 ; Figure I shows as an example the blood urea level curves under No. II, so as to offer an obvious demonstration of the condition.

FIGURE I.



——— Before test feeding

- - - - During test feeding

. . . . After recovery

Table I

Nummer des Kaninchens	I		II				III			IV			V				VI			VII		VIII		IX		X	
Datum	16/I 1926	2/II	20/I	3/II	10/III	26/IV	26/I	5.II	4/III	27/I	12/II	1/III	29/I	10/II	3/III	10/V	1/II	11/II	8/III	17/III	7/IV	18/II	6.IV	24/III	14.IV	23/III	19/IV
Körpergewicht	1800	1650	2310	2450	2290	2500	1500	1680	1300	2130	2100	1410	2000	2140	1940	1900	1550	1380	1370	1970	1750	1800	1630	2030	2810	2120	2200
Ab- u. Zunahme des Körpergewicht	—	−150	—	+110	−50	+160	—	+180	−200	—	−30	−720	—	+140	−60	−100	—	−170	−180	—	−220	—	−110	—	−120	—	+80
Zeit																											
Harnstoff (g/l)																											
0	0.35	0.39	0.29	0.29	0.28	0.25	0.37	0.36	0.91	0.31	0.34	1.25	0.33	0.31	0.41	0.31	0.24	0.38	0.32	0.39	0.37	0.41	0.40	0.21	0.35	0.42	0.36
15'	0.26	0.43	0.29	0.28	0.30	0.23	—	—	0.99	0.31	0.34	1.35	0.33	0.29	0.34	0.33	0.26	0.45	0.34	0.48	0.36	0.45	0.40	0.23	0.37	0.38	0.35
30'	0.38	—	0.39	0.37	0.32	0.30	0.44	0.38	0.96	0.38	0.40	1.27	0.37	0.31	0.35	0.34	0.30	0.45	0.30	0.53	0.43	0.53	0.41	0.29	0.39	0.35	0.35
1	0.47	0.50	0.51	0.38	0.33	0.29	0.50	0.40	1.03	0.43	0.36	1.30	0.45	0.30	0.41	0.40	0.30	0.55	0.28	0.66	0.45	0.57	0.40	0.29	0.40	0.51	0.37
2	0.48	0.40	0.42	0.41	0.35	0.36	0.48	0.36	1.04	0.45	0.45	1.32	0.47	0.31	0.40	0.39	0.40	—	0.35	0.55	0.45	0.49	0.43	0.31	0.45	0.50	0.40
3	0.40	0.45	0.40	0.38	0.28	0.40	0.48	0.39	1.03	0.40	0.39	1.34	0.49	0.35	0.40	0.35	0.32	0.56	0.40	0.52	0.45	0.46	0.42	0.26	0.44	0.44	0.37
4	0.37	—	0.38	0.32	0.31	0.31	0.47	0.41	—	0.31	0.37		0.40	0.40	0.43	0.39	0.28		0.32	0.51	0.44	0.41	0.41	0.25	0.41	0.42	0.39
5	0.41	0.45	0.37	0.32	0.32	0.24	0.45	0.41	1.05	0.36	0.38		0.33	0.34	0.33	0.35	0.27		0.32	0.53	0.45	0.43	0.41	0.25	0.39	0.40	0.38
6	0.38	0.45	0.37	0.34	0.32	0.26	0.41	0.43	1.12	0.36	0.41		0.34	0.34	0.45	0.38	0.23		0.36	0.42	0.45	0.43	0.41	0.27	—	—	0.40
7	0.33	0.45	0.37	0.31	0.35	0.24	0.38	0.40	1.28	0.33	0.42		0.34	0.39	0.42	0.38	0.25		0.34	0.41	0.42	0.41	0.40	0.22	0.40	0.33	0.39
Blutzucker																				0.088	0.103	0.100	0.130	0.076		0.094	

1) Number of rabbits
2) Date
3) Body weight
4) Increase and decrease of body weight
5) Time
6) Urea (g/l)
7) Blood sugar

B.

Urea formation in fasting rabbits.

When the urea-forming function from ammonium carbonate is reduced during the B-avitaminosis of the rabbit, the body weight of the rabbit which receives a diet deficient in vitamin B will decrease simultaneously. The question must therefore be raised whether said lessening of the urea formation is caused by pure B-avitaminosis, or whether hunger is somehow involved in the process as well. For the purpose of answering the above question, control tests with a series of fasting rabbits became necessary. 2.5 g/dl of a freshly prepared ammonium carbonate solution (quantity: 10.0 ccm per kg of body weight) was introduced into the stomach of well-fed rabbits with the aid of the esophagus probe; the hourly determination of the urea level in the blood followed. The rabbit is fed daily with 100.0 g of "Tofu-kara" only. The rabbit's body weight diminishes gradually from day to day. When said body weight decrease becomes clearly evident, the urea is determined after administration of ammonium carbonate. However, the urea level of the blood increased during the fasting stage and it is therefore difficult to decide whether or not the urea increases after the oral supply of ammonium carbonate. But it is certain that during the initial stage of the fasting the urea formation did not decrease at all. During the later fasting stage a rather high blood urea value is detected, as during B-avitaminosis. When ammonium carbonate is now supplied per os, the urea level of the blood continues to rise.

TABLE II.

(For translation of Items 1) to 6) see Table I, p. 24 of translation)

1 Nummer des des Kaninchens	I		II		III		IV			V		VI	
Datum	21/IV 1926	28/IV	20/IV	14/V	5/V	17/V	18/V	25/V	31/V	26/V	2/VI	1/VI	7/VI
2 Körpergewicht	2150	2000	2070	1800	1950	1860	2360	2100	1590	2050	1900	2250	2050
4 Ab- u. Zunahme des Körpergewichts	—	−150	—	+130	—	−90	—	−260	−770	—	−150	—	−200
5 Zeit													
	Harnstoff (g/l)												
0	0.35	0.80	0.40	0.47	0.30	0.29	0.31	0.41	0.93	0.40	0.72	0.27	0.55
15'	0.35	0.82	0.41	0.47	0.30	0.30	0.33	0.45	0.89	0.45	0.76	0.34	0.57
30'	0.43	0.83	0.41	0.58	0.33	0.34	0.43	0.50	0.91	0.46	0.78	0.33	0.67
1st.	0.45	0.82	0.40	0.56	0.44	0.43	0.44	0.57	0.95	0.50	0.84	0.41	0.70
2	0.48	.83	0.43	0.60	0.44	0.39	0.47	0.49	0.98	0.49	0.91	0.37	0.64
3	0.47	0.97	0.41	0.60	0.30	0.41	0.40	0.43	1.03	0.47	—	0.32	0.62
4	0.47	0.91	0.43	0.60	0.38	0.41	0.42	0.48	0.93	0.48	0.94	0.35	0.64
5	0.45	0.92	0.41	0.61	0.30	0.41	0.40	0.48	0.99	0.45	0.93	0.36	0.62
6	0.44	0.85	0.40	0.53	0.32	0.37	0.33	0.43	0.99	0.45	0.87	0.33	0.62
7	0.39	0.86	0.40	0.52	0.32	0.39	0.37	0.41	0.99	0.42	0.88	0.34	0.62

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Tissue Levels of Some Poisoning Agents Less Frequently Encountered*

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The literature is replete with information regarding blood, urine and tissue levels of the most common agents involved in accidental or suicidal death, namely barbituric acid derivatives, analgesics and carbon monoxide. Despite all this information, questions still frequently arise as to what really is a lethal level. What then of the agents that are less frequently encountered? There is certainly a great deal of information to be desired to assist the pathologist, medical examiner or physician when one of these substances is involved. Naturally, there are many factors which can influence tissue levels of noxious agents but the cases to be described in this paper are selected only on the basis of the poisoning agent involved. No attempt has been made to screen out patients with other extensive pathology; these cases represent "routine" poisoning cases where the agent involved was the underlying cause of death. The cases to be described are not all acute episodes, but the causative agent in each case was established beyond a reasonable doubt.

The purpose of such a presentation is to introduce into the literature this laboratory's experience with biological levels of less commonly used materials. The cases represent only a

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small part of the analytical toxicology of medical examiner cases over the past few years. No attempt is made to give extensive clinical or pathological data, indeed in some cases, no such data were available. In some instances, more than one case of a certain agent will be demonstrated in order to show the variability of tissue levels dependent probably in part upon the rate and the amount ingested.

I. ETHYLENE GLYCOL

It is generally conceded that the use of ethylene glycol can be blamed on the fact that a transient exhilaration is produced with symptoms similar to alcoholic intoxication and further ingestion of this substance (while in the intoxicated state) is generally lethal.

The variability of tissue levels has been previously reported together with the methodology used (1), and this paper will serve to produce additional evidence of this fact, as well as an interesting sequel to one of the described cases.

Case #1: L. T., male, age 40, found in a semicomatose state, described as obviously inebriated. This patient was taken to a hospital where after five hours he developed convulsions and died. No history was ever received on this patient. Postmortem examination revealed typical oxalate crystals in the kidney sections. Toxicological examination showed large amounts of ethylene glycol (see Table I) and small amounts of methanol.

Case #2: A. S., female, age 56, admitted to the hospital in a drowsy, lethargic state, with shortness of breath and with a history of drinking for two days prior to admission. Tentative diagnosis on admission was alcoholism plus acute pyelonephritis. Her condition progressively declined and she expired 18 hours after admission. Postmortem examination revealed granular scarring of the kidney cortex, pale parenchyma with irregular areas of hemorrhage over the pyramids. Toxicological analysis revealed presence of ethylene glycol (see Table I).

Case #3: R. C., male, age 15, went out to a party where he allegedly consumed "blue vodka." He came home at about 2 a. m. Three hours later he awoke, vomiting and breathing hard, and he expired one hour later at home. His mouth contained a frothy fluid. Gastric contents were aspirated into the trachea and bronchi. Analysis of brain only showed the presence of ethylene glycol (see Table I).

TABLE I

Case	Approx. Hrs. After Ingestion	Kidney mgs. 100 gms.	Liver mgs. 100 gms.	Brain mgs. 100 gms.
#1 R. T.	5	1131	1512	
#2 A. S.	48	93	310	
#3 R. C.	4			196

An adjunct to Case #3 may be noted here. A friend of R. C., a male, L. M., age 18, following this same party, was admitted to another hospital in a stuporous condition with severe vomiting, stomach pains and acute renal failure. He underwent hemodialysis during the first 24 hours and again five and eight days later. During his hospital stay, his condition alternately improved and deteriorated. He had periods of convulsions, periods of speech difficulty, and a confused mental status. Following intensive care, he was discharged after 21 days. His urinary oxalic acid excretion was normal 9 days after admission. Nine days after discharge, he was readmitted with acute bacterial endocarditis of the aortic valve and expired several hours later. His kidney sections showed hyperemia, extensive tubular damage with "crystals of [illegible] structure" to those from a known case of ethylene glycol poisoning. The brain showed ruptured mycotic aneurysm of the right middle cerebral artery with embolic occlusion of one of the branches. No ethylene glycol was found. Death occurred approximately four and [illegible] after apparent ingestion.

II. MERCURY

Acute mercury poisoning has apparently become less frequent despite the fact that such compounds are still easily available. This type of poisoning is so characteristic in its various phases that gross examination at autopsy can be almost conclusive. However, since death is usually delayed, some variability is encountered on that basis as well as being dependent upon the amount and type of mercury salt ingested.

Case #1: H. W., female, age 18, ingested 1.5 grams of bichloride of mercury which was estimated by the extraction of mercury as the dithizonate following reflux digestion (2). The patient was admitted to the hospital suffering from a violent gastroenteritis with bloody emesis and diarrhea. Her condition deteriorated slowly despite peritoneal dialysis and intensive dimercaprol therapy and she expired on the 8th day after admission following complete renal shutdown. Postmortem examination revealed the usual extensive ulceration of the entire colon and part of the small intestine. The kidneys were swollen with grayish cortex, with some tubular cell destruction. The pharynx, esophagus and stomach displayed varying degrees of corrosion. Toxicological data are shown in Table II.

TABLE II

Case	Tissue	Hg. mgs.*
H. W.	Blood	0.08
	Brain	0.11
	Colon	0.31
	Lung	0.56
	Jejunum	0.56
	Liver	0.72
	Kidney	1.67

III. SODIUM FLUORIDE

The use of this compound as an insecticide, as a chemical reagent, and its use in dentistry makes it generally available to many people. The two cases described here seem quite similar, in that both were brought into the hospital emergency room and died shortly thereafter of cardiac arrest. Corrosion of mouth, pharynx, stomach was noted in both cases, and other pathological findings were typical. Tissues were analyzed by the method of Rowley and Farrah and detailed findings are outlined in Table III.

TABLE III

Case	Stomach Contents	Brain	Liver	Kidney	Blood	Urine	Esoph. Stomach Wall
E. H. M. 60	4.6	2.3	4.1	3.2	1.6	11.1	
L. D. F. 54	4.7	0.4	3.2	2.8			1.9	0.9

* Quantities are expressed in mgs. NaF per 100 gms. or mls.

IV. CYANIDE

The availability of cyanide compounds as insecticides can, in the wrong hands, produce tragic consequences, as this case illustrates.

Case #7: P. H., female, age 50. This woman was found dead in a hospital bed at 6 a. m. after supposedly spending a "peaceful night." She had been attended previously at 2 a. m. and was reported sleeping well. The following morning after her death was discovered, a can of ant poison containing [illegible] was found with a [illegible] in a paper cup with some water and a spoon. There appeared to be no [illegible] manifestations, no [illegible] [illegible] showed some [illegible] [illegible] the [illegible]. It was concluded that a [illegible] [illegible] [illegible] [illegible] [illegible]. Cyanide was determined by a [illegible] [illegible] [illegible] [illegible] outlined in Table IV.

TABLE IV

Case	Tissue	CN mgs.
P. H.	Blood	[illegible]
	[illegible]	[illegible]
	[illegible]	[illegible]
	[illegible]	[illegible]
	[illegible]	[illegible]

V. AMMONIUM HYDROXIDE

[illegible] available. The odor of ammonia in the [illegible] is generally conceded to be sufficient proof of [illegible]. However, it is considered to be of advantage if at least some proof of presence of ammonia is obtained.

Case #8: F. C., [illegible] age [illegible], was found [illegible] was analyzed and found to contain 2 [illegible] NH_3. [illegible] was found in blood and brain. The stomach contents had the typical ammoniacal odor and autopsy disclosed an hemorrhagic esophago-gastro-duodenoenteritis. Ammonia nitrogen levels were done by a modified Conway Technique (6) and the levels calculated as NH_3 are shown in Table V.

TABLE V

Case	Tissue	NH
F. S.	Gastric	15.3 mgs. total
	Blood	3.3 mgs. %

SUMMARY

A group of subjects exposed to compounds less frequently involved in accidental or suicidal deaths is reported with the tissue levels found after autopsy. No attempt has been made to correlate these levels to lethal doses of these compounds.

It is hoped that this paper will stimulate other investigators to publish similar data which are much needed in respect to the less frequently encountered causes of death from poison.

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Studies on the metabolism of L(+)-glutamic acid.

1.Report: general effects and blood mechanism modifications, in particular of the ketoglutaric acids in the plasma, after the administration of l(+)-glutamic acid in vivo.

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Little is known about the behavior of glutamic acid, introduced per os or parenterally. While it is known that it belongs to the glucoplastic amino-acids and that it is unconditionally transferred by means of transamination into the citric acid cycle and is excreted in paired form in the urine, the most important question still remained unanswered; it was raised by the studies of CHRISTENSEN, STERICHER and ALBINGER[1] and concerns the effect of glutamic acid, supplied in excess, on the quantitative behavior and the reaction of other amino acids in the living organism. Since the problem is physiologically most important in view of the constant, standard quantitative ratio of the amino acids, we undertook tests to study the fate of parenterally administered glutamic acid and the shifts in the metabolism of other metabolites originating from protein substances. The tolerance of rats for injected glutamic acid was tested in the course of preliminary tests.

Methods.

Glutamic acid solutions: The poorly soluble L(+)-glutamic acid was suspended in water, dissolved with soda liquor or with ammonium hydroxide at pH 7,0, and thinned to obtain a content amounting to 10 or 20%, calculated for glutamic acid; then monosodiumglutaminate or monoammoniumglutaminate is substantially present.

pH measurement: blood was taken by means of heart puncture and was immediately determined in the glass electrode.

Microbiological determination of the glutamic acid:
Blood from the ear vein was immediately centrifuged; protein was eliminated from 1 to 3 ml serum according to HILL and BARGELM[7] by thinning the latter with 1,4 ml water per ml; then it was mixed with 0,35 ml 0,6 -n H_2SO_4 and 0,25 ml 10% sodium-wolframate solution. Following the microbiological determination with lactobacillus arabinosus 17-5, the clear filtrate was reduced to pH 6.8 with soda liquor. The determination was performed according to the data of SNELL[8a].

Enzymatic determination of the pyruvic *) acid and of the α-ketoglutaric acid: protein was eliminated from 3 to 6 ml serum with the same volume of 6% perchloric acid. With small quantities of ketoglutaric acid, as for example in the plasma after fasting, peroxide-free ether was used for the extraction; in case of larger quantities, for example after the administration of glutamic acid, no extraction was needed because the interfering substances had not increased correspondingl The ether extract was mixed with water and the ether was evaporated The resulting watery solution, analogously to the non-extracted prot free serum filtrate, was reduced to pH 6,8 with 2 KOH, and at 366 the decrease of the extinction was measured after adding ammonium acetate, n/20 triethanolamine buffer (pH 7,4), DPNH and ferments (cristallized lactic acid dehydrogenase and cristallized glutamic acid dehydrogenase).

Determination of the free ammonium: in the blood: 1,0 ml of fresh blood taken from the ear vein is filled into pipettes, together with 1,0 ml saturared potassiumcarbonate solution and 2,0 ml 0,01-n H_2SO_4, using the diffusion bowls of CONWAY. After a diffusion period of 120 minutes, the ammonium is photomatrically determined in 1,5 ml of the fluid from the inner circle of the NESSLER reagent.

Behavior of rats after the injection of sodium glutaminate, ammonium glutaminate and ammonium chloride.

Shifts of the blood-pH

Intraveous injections of L(+)-glutamic acid can cause nausea and vomiting in humans and dogs. Rats and especially mice are known to be less sensitive. The toxic dose is considered to be 100 to 129 mg/kg of body weight. PFEIFFER[3b] was able to administer more than 2 g/kg glutamic acid orally as well as parenterally to guinea pigs and mice without evidence of any toxic symptoms, described as trembling or partial paralyses.

In the course of further tests we applied neutralized glutamic acid intraperitoneally in increasing concentration to rats with a body weight of 100 to 150 g. When using glutamic acid neutralized with soda liquor it became evident, after the injection of at least 2,1 g/kg corresponding to 14,5 mmol/kg, that following a latency period the respiration accelerated considerably and motor debility increased. 15 to 35 minutes after the injection the animals became agitated, frequently as a consequence of external stimuli. They either began to tremble or jumped suddenly and impetously. The convulsive stage finally began with clonic jerks, 23 to 72 minutes after the injection. The entire reflex muscle system strongly contracted in a violent tetanus, whereby the legs were maximally extended. In case of prolonged duration this convulsive stage end in death. But it mostly relaxed, with spasmodic clonic convulsions. A stage of exhaustion followed each convulsion; this frequently lasted for a few minutes only. Tonic tetanic stretching occurred subsequently; either spontaneously or triggered by sensible stimuli; the stretching often lasted for one hour.

*) Brenztraubensäure

When more than 3,6 g/kg, corresponding to 25 m mol/kg of sodium glutaminate was applied, more than 50% of the rats died within 100 minutes in convulsions; in sporadic cases death occurred only after 12 hours (Table 1). The intraperitoneal injection of glutamic acid neutralized with ammonium hydroxide caused, already after 2 to 4 minutes, an intensified agitation and convulsions, i.e. 0,09 g/kg corresponding to 6,5 m mol/kg sufficed for generating the intoxication aspect. It is characterized, besides consvulsions, by the typical KUSSMAN respiration which frequently begins even before convulsive seizures and is followed by a coma. Said symptoms indicate that the metabolic situation is acidotic. When using an ammonium glutaminate volume which is only slightly above the convulsion-causing dose, namely: 1,0 g/kg, corresponding to 7,0 m mol/kg, 50% of the animals died during convulsions or in acidotic coma (Table 1).

Table 1. Toleration of glutamic acid in rats.

[illegible]	Krämpfe erregende Dosis [1]	50% Letalität [2]
Natriumglutaminat	14,5 mMol/kg	25 mMol/kg
Ammoniumglutaminat	6,5 mMol/kg	7 mMol/kg
Ammoniumchlorid	7,0 mMol/kg	8 mMol/kg

1) Convulsion-causing dose
2) 50% mortality

Since the aspect of the intoxication after intraperitoneal administration of the salts of glutamic acid largely resembles the aspect of an ammonia intoxication, ammonium chloride was administered to rats for comparison purposes. After the intraperitoneal injection of at least 7 m mol/kg of ammonium chloride, deeper breathing occurred within 5 to 10 minutes, together with violent stretching convulsions. Already with 8 m mol/kg more than 50% of the rats died, mostly in the course of the first spastic seizure (Table 1). While the convulsion is clinically most conspicuous following th sodium glutaminate injection, after the application of ammonium glutaminate symptoms occur which specifically indicate the presence of acidosis. Regarding [illegible] effect and regarding the mortality rate, ammonium glutaminate is somewhat less toxic than ammonium chloride.

Disorders which occur after ammonium glutamate injection (Kussman respiration and coma) indicating acidosis are related the behavior of blood pH; 10 minutes after intraperitoneal injection of sodium glutamate normal values were found; but after the injection of ammonium glutamate, as after ammonium chloride, the reaction shifted towards acidity to an extent which clearly showed the correlation with the administered dose (Table 2).

To clarify the question whether these highly lethal gluconic acid dosages affect the carbohydrate metabolism, in particular considering an eventual hypoglycemic involvement during spastic conditions, we tested 10 rats (SPRAGUE-DAWLEY) for blood sugar in heart blood sub finem after the intraperitoneal injection of 50 m mol/kg Na-glutaminate in the form of a 20% solution. The convulsions occurred between 40 and 80 minutes post injectionem. The blood sugar values were average at 90 mg%, with a variation range of 72 to 120 mg%.

Table 2. pH of the blood , 15 minutes after the intraperitoneal injection, in rats; measurement with the glass elctrode.

Ammoniumchlorid		Ammoniumglutaminat		Natriumglutaminat	
mMol/kg	pH	mMol/kg	pH	mMol/kg	pH
4,7	7,36	5,5	7,04	12,1	7,29
4,9	7,10	6,4	7,10	21,6	7,48
8,0	7,06	10,9	6,54 (Agone)	27,9	7,30
8,4	6,92 (Agone)	11,6	6,58 (Agone)		
9,2	6,66 (Agone)				

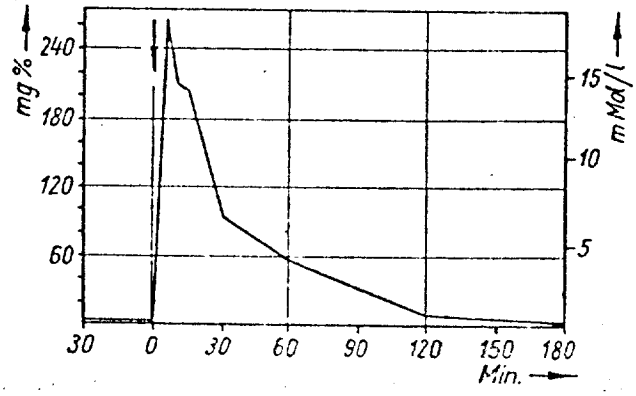

Illustration 1. l(+)-glutamic acid in the serum after intrevenous injection of 2,0 mol/kg of neutralized glutamic acid: rabbits.

The blood level of the glutamic acid

Sound rabbits with a body weight of 3 to 3,5 kg which were kept fasting for at least 15 hours prior to the start of the test were used to study the blood level modifications of the free glutamic acid after perenteral injection. 300 mg/kg (2,0 m mol/kg) sodiumglutaminate was intravenously injected and blood was taken 30 minutes earlier as well as in specific intervals after the injection from the other ear, for the purpose of microbiological specification. The glutamic acid concentration increased after the injection from a blood level of 1,35 mg%, corresponding to 0,092 m mol/l to 270 mg%, corresponding to 18,4 m mol/l; after 30 minutes, more than 50% of the supplied volume had again vanished from the serum, and 3 hours later the approximate initial value was once more attained (III. 1).

Investigations concerning the movements of the amino-nitrogen and concerning the carbon structure of the injected glutamic acid.

The effect of L(+)-glutamic acid on the citric acid cycle was investigated in the course of additional tests by determining the α ketoglutaric acid content of the serum parallely with the determination of the glutamic acid. 300 mg/kg, corresponding to 2,0 m mol/kg of sodium glutaminate was intravenously administered to sound rabbits of 3 to 3,5 kg body weight. Imediately after the intravenous injection the ketoglutamic acid content increased considerably; the maximum was reached already after 5 to 6 minutes; it was 10 to 13 times above its initial value (III.2.). When 500 mg/kg of sodiumglutaminate was intraperitoneally applied, said content increased up to the 20-fold volume of its intial value. Exactly as in case of the glutamic acid concentration, the ketoglutaric acid in the serum decreased gradually and reached its starting point again after 120 to 180 minutes. As standard values we found 0,195 - 0,325 mg% ketoglutaric acid in the serum of rabbits. The maximal increase after intravenous administration of 300 mg/kg sodiumglutaminate was 3,18 mg%, the minimal increase was 1,90 mg%. After parenteral administration of 500 mg/kg sodiumglutaminate the highest value was 4,75 mg%, and the lowest was 4,30 mg%. Furthermore the increase of the α ketoglutaric acid concentration in the serum shows a well-defined correlation with the values of the supplied glutamic acid.

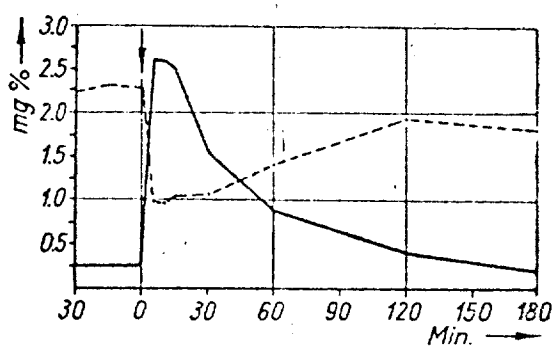

Illustration 2. Pyruvic acid content of the serum (- - - -) and ketoglutaric acid content of the serum (____); intravenous injection of 2,0 m mol/kg of neutralized glutamic acid; rabbits (3500 g).

It had to be assumed that the ketoglutaric acid originated from the supplied glutamic acid. Then, however, the fate of the amino-group had to be clarified. A transamination into other ketoacids seemed to be the most acceptable supposition. Therefore the behavior of the Brenz racemic acid in the serum was investigated. The increase of the ketoglutaric acid did in fact occur in each case simultaneously with a decrease of the pyruvic acid in the serum.

We found the following intial values for pyruvic acid in the rabbit serum: 1,80 to 2,10%, corresponding to 0,2 to 0,35μ mol/l. After glutamic acid injections the values decrease to 0,260 mg%, corresponding to 0,03μ mol/l. In most cases the lowest pyruvic acid values were measured at the same time after the injection with the highest α ketoglutaric acid concentration; occasionally a further decrease of the pyruvic acid in the serum occurred 5 to 15 minutes later. 60 to 90 minutes after the decline of the α ketoglutaric acid to the initial value the pyruvic acid likewise reached its original position in the serum; but in some cases the former level was not reached even 4 hours later.

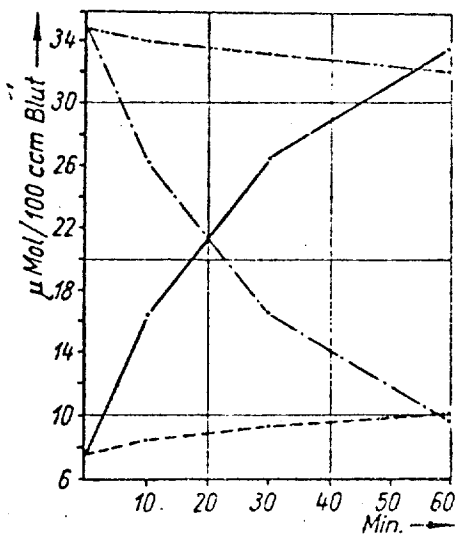

Illustration 3. Shifts of the α ketoglutaric acid and of the pyruvic acid during tests in vitro with rabbit blood; aerob. 37°. Blood without additive: α ketoglutaric acid in μ mol/100 ml (- - -); pyruvic acid in mol/100 ml (-..-); blood (20 ml) after addition of 50 mg sodiumglutaminate: α ketoglutaric acid μ mol/100 ml (-); pyruvic acid in μ mol/100 ml (-.-.).

1) blood)

When the molar ratio is calculated for the increase of the α ketoglutaric acid in the serum proportionately to the decrease of the pyruvic acid, it becomes evident that the decrease of the pyruvic acid corresponds to 85-90% of the α ketoglutaric acid concentration increase.

This rapid and almost quantitative correlation [illegible] the question: to what extent does the blood itself cause the shift of the normal ketoacid equilibrium after the glutamic acid injection?

During the following tests rabbit blood, freshly taken from the ear vein, was defibrinated by means of agitation with glass beads; 20 ml of the same without additives and 20 ml after addition of 50 mg sodiumglutaminate was agitated in a water bath in the presence of air at 37°. Before inserting the thermostats, after 10, 20 and 30 minutes, samples were taken to determine the ketoglutaric acid and the Brenz racemic acid. A distinct increase of ketoglutaric acid, which reaches its maximum only after 60 min. and is accompanied by a corresponding decrease of pyruvic acid, is shown here also (Ill. 3). The molar changes are 93-96% quantitative in this in vitro test; they are similar to the glutamic acid changes, corresponding almost completely to the amount of added glutamic acid (Tab. 3). Evidently the blood itself is capable, to a not inconsiderable extent, of the transamination.

Tab. 3. α-Ketoglutaric acid and pyruvic acid in defibrinated rabbit blood after agitation in a water bath at 37°.

I. Blut ohne Zusatz

	(1) Ketoglutarsäure		(2) Brenztraubensäure		Zunahme (3) Ketoglutarsäure	Abnahme (4) Brenztraubensäure	(5) Ausbeute in
	mg%	μMol	mg%	μMol	μMol	μMol	%
(6) Ausgangswert:	1,10	7,5	3,06	34,8			
n. 10 Min.	1,23	8,4	2,99	34,0	+ 0,9	- 0,8	89
n. 30 Min.	1,36	9,3	2,93	33,3	+ 1,8	- 1,5	88,5
n. 60 Min.	1,48	10,1	2,84	32,3	+ 2,6	- 2,5	96

II. Nach Zusatz von 50 mg Natriumglutaminat zu 20 ml Blut

	(1) Ketoglutarsäure		(2) Brenztraubensäure		Zunahme (3) Ketoglutarsäure	Abnahme (4) Brenztraubensäure	(5) Ausbeute in
	mg%	μMol	mg%	μMol	μMol	μMol	%
(6) Ausgangswert:	1,10	7,5	3,06	34,8			
n. 10 Min.	2,41	16,5	2,23	26,4	+ 9,0	- 8,4	93,5
n. 30 Min.	3,89	26,5	1,46	16,6	+ 19,0	- 18,2	95,5
n. 60 Min.	4,93	33,7	0,86	9,7	+ 26,2	- 25,1	96

1. Blood without supplement.
2. After a supplement of 50 mg sodium glutaminate + . 20 ml blood

Key: 1. ketoglutaric acid; 2. pyruvic acid; 3. increase in ketoglutaric acid; 4. decrease in pyruvic acid; 5. yield; 6. final value.

This study shows absolutely that the amino groups of glutamic acid do not appear as free ammonium ions in the blood. After intravenous injection of 1.0 g sodium glutaminate in a 3500 g rabbit, all of the NH_3 - values of the serum remained in the range of the respective fasting values; no NH_3 - values went outside our limits of 0.12 - 0.36 mg%. Also, at the time of the maximal glutamic acid or keto-glutaric acid contant or of the pyruvic acid-minimum, the NH_3 - content did not rise. If one gives an equimolar quantity, in comparison to the glutamic acid, of ammonium chloride intravenously (100 mg/kg), then there arrives with a content of 13 mg% free ammonia in the blood no decrease in the pyruvic acid concentration in the serum (Ill. 4). This speaks more in favor of a transamination than of a secondary amination of the pyruvic acid in conjunction with an oxidative desamination of the glutamic acid.

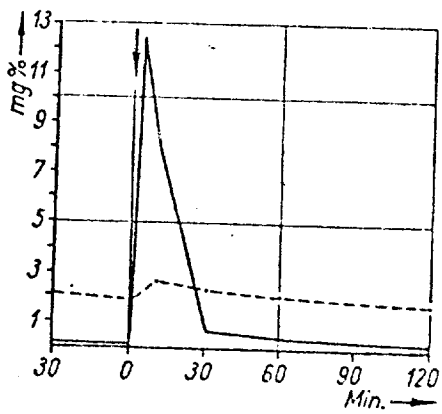

Illustration 4. Shift of the ammonia (—) and pyruvic acid (. . .) in the blood of rabbits; intravenous injection of 1,8 m mol/kg of ammonium-chloride.

Discussion of the results.

Injected glutamic acid is decomposed in the [illegible] with comparative speed; after a rapid rising of the blood [illegible] content does as a rule return to normal values [illegible] minutes. This fact, microbiologically verified [illegible], could be confirmed by [illegible] with colorimetric evidence of glutamic acid in man. The ability of the organism to utilize glutamic acid in metabolism depends not only [illegible] alimentary functional condition but for example also [illegible] the speed of the injection, as emphasized by [illegible][5]. [illegible] part why very different values can be stated for [illegible] as to reconcile the various authors.

According to TIGERMAN, SAC VICAR[7] a glutamine increase is found in the tissues after the simultaneous [illegible] administration of of ammonium and glutamic acid; glutaminase [illegible] metabolizes glutami rapidly and well from the two simultaneously [illegible] compounds: it is well-tolerated. But this utilization [illegible] apply to glutamic acid injected parenterally: [illegible] does not improve the intoxication aspect of ammonium [illegible] in reverse, ammonium does not improve the [illegible] of the glutamic acid; ammoniumglutaminate [illegible] more toxic than sodium glutaminate.

Ammoniumchloride and ammoniumglutaminate cause strong acidosis with very similar symptoms. According to WINTERSTEIN and GORHAN[9] ammoniumchloride-acidosis can be ascribed to tissue alkalosis; the liuqor, for example, becomes alkaline, whereby the NH_3 molecule is adopted as transfer mode of basic valences in the blood into the liquor. While the detoxicating effect of the glutaminase is minor, the involvement of glutamine in the binding and transfer of the ammonia is assured. Possibly it might be the uncharged glutamine here as well which migrates into the liquor and which is responsible for the binding and detoxication of the ammonia and for its transportation. A similar mechanism might be the basis of the intoxication symptoms after glutaminate applications.

It was found during long-term balance tests and it was recently confirmed in the course of tests with isotope-marked glutamic acid that amino-nitrogen finally transforms into urea and that from the carbon structure of the tricarbon-acid cycle acids and glucose can be formed. It could be demonstrated by our tests which is the primary fate of the glutamic acid. Free ammonia does not occur in the blood; on the other hand, ketoglutaric acid forms immediately while the pyruvic acid simultaneously decreases. Trasamination is probably at issue, which occurs predominantly in the liver[11]; but it could be shown here that an important role can likewise be ascribed to the entire blood during the performance of the transamination reactions.

We thank Professor Dr. F. BÜCHER and Dr. G. PFLEIDERER making available the cryst. lactic acid dehydrogenase, Prof. Dr. K. WALLENFELS for cryst. glutamic acid dehydrogenase and Assistant Professor Dr. F. BRANDHOFF for Lactobacillus arabinosus 17-5, for the purpose of glutamic acid specifications and for handing over their methodological rules.

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SECTION MEETINGS

CLEVELAND	
Western Reserve University	February 11, 1949
ILLINOIS	
Northwestern University Medical School	February 1, 1949
NEW YORK	
New York Academy of Medicine	January 5, 1949
Cornell University Medical College	February 23, 1949
SOUTHERN	
Medical College of Alabama	February 5, 1949

16931

Production of Acute Pulmonary Edema by Ammonium Salts.*

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From Department of Anatomy, School of Medicine, University of Pennsylvania, Philadelphia.

During the investigation of another problem,[1] in which guinea pigs were administered ammonium chloride by gavage, small overdoses of this chemical were found to result in pulmonary edema after a short interval.

In guinea pigs so treated, acute pulmonary edema appeared to be a consistent cause of death.[2,3] It was therefore considered worthwhile to experiment further with the thought of utilizing ammonium salts as a tool for a subsequent investigation of the pathogenesis of acute pulmonary edema.

Methods. Guinea pigs and white rats of both sexes were principally used. Six cats and 5 rabbits also were studied. Early in these studies, complete autopsies were done but when it became apparent that changes were limited to the chest, autopsy studies were restricted to the thoracic viscera.

Microscopic studies were carried out on all lungs in which the pathological diagnosis was in doubt, and also in a series of animals in which it was desired to study the histological changes of the grossly abnormal lungs. The lungs were fixed in one of 3 ways: by vascular

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† Presented to the Graduate School of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

1 Windle, W. F., Koenig, H., and Jensen, A. V., *Arch. Neurol. and Psychiat.*, 1946, **56**, 428.

2 Koenig, H., and Koenig, R., *Fed. Proc.*, 1948, **7**, 67.

3 Koenig, H., and Koenig, R., [illegible] 1948, **100**, 52.

perfusion[1] of 10% formalin in isotonic saline solution with 2.4% gum acacia added; by intratracheal injection of 10% formalin in isotonic saline solution; or by ligating and excising a pulmonary lobe, immersing it in 10% formalin in isotonic saline, and agitating it constantly on a shaking device for 24-48 hours. Immersion fixation of an entire lobe gave the best results for the demonstration of edema exudate. All lung sections were stained with hematoxylin and eosin.

A 6% solution of ammonium chloride in water (weight in volume) was used at all times with the exception of the animals administered other salts of ammonium to determine the specificity of the cation with regard to the lung changes. Control animals were treated like the experimental animals except for the administration of ammonium chloride. These animals were also anesthetized with pentobarbital ("Veterinary Nembutal") just before removal of the lungs. Further details of procedure will be given in the appropriate sections to follow.

Results. A. Dosage and routes of administration. Guinea pig. Most of our experience has been with this animal. We have seen typical pulmonary alterations with ammonium chloride given by the gavage, subcutaneous, intramuscular, intraperitoneal and intravenous routes of administration. The dose of ammonium chloride depended upon its mode of introduction. The slower the rate of absorption, the larger the dose had to be. Thus, a dose which would produce acute pulmonary edema by gavage would, when injected into the peritoneal cavity, usually kill the animal before sufficient time had elapsed for the evolution of these changes. Ammonium chloride could be successfully injected into the vein only by slow drip. Another factor of importance was the weight of the animal; the usual direct relationship between body weight and dosage appeared to apply.

The gavage and intraperitoneal routes were utilized almost exclusively because the results were more dependable. By stomach tube, the effective dose was 0.09 to 0.12 g/100 g of body weight; intraperitoneally, it was 0.05 to 0.07 g 100 g of body weight. Thirty of the 40 experimental guinea pigs succumbed to acute pulmonary edema. Wherever lung changes failed to appear, it could be attributed to one of two causes. If the amount of ammonium chloride was too great, or if it was too rapidly absorbed, death resulted from cardiac failure, or more frequently from respiratory failure, before the appearance of lung changes. On the other hand, if the dose was inadequate, or if absorption appeared retarded, the lung changes were not seen and other manifestations of toxicity were often completely absent. Our experience suggested that a definite concentration of the ammonium ion has to be attained and maintained in the blood for a period of at least 10 or 15 minutes for the development of pulmonary edema.

Rat. Ammonium chloride solution was administered to this animal by stomach tube and by intraperitoneal injection. The latter route proved more convenient and reliable. The effective dose for the intraperitoneal route was 0.04 g 100 g (0.66 cc of a 6% solution). Administration resulted in acute pulmonary edema in all of 10 rats.

Cat. The cat proved to be more resistant to the pulmonary edema produced by ammonium chloride than the guinea pig or the rat. Vomiting and salivation were a common occurrence with toxic doses of ammonium chloride given intraperitoneally or by gavage, but care was taken to prevent aspiration. In later experiments, the trachea was cannulated following local infiltration with 1% procaine in order to circumvent these difficulties. Of the 6 cats used, pulmonary edema occurred in 3 animals. The animals in the latter group weighed 1950-2750 g each. Two were given 35 cc of a 6% solution of ammonium chloride intraperitoneally. A third was given [illegible] of a 6% solution of ammonium chloride [illegible] peritoneally in 4 divided doses over a period of 2 hours.

Rabbit. In none of 5 animals did acute pulmonary edema occur after ammonium chloride was given intraperitoneally or by gavage in doses great enough to [illegible] symptoms culminating in death.

[1] Koenig, H., Groat, R. A., and Windle, W. F., *Stain Technology*, 1945, **20**, 13.

B. Specificity of the ammonium ion. In order to determine the specificity of the ammonium moiety of ammonium chloride in this phenomenon, 6 guinea pigs, each weighing about 350 g, were used. One was given 6 cc of 20% ammonium nitrate, a second was given 7 cc of 6.4% ammonium acetate, a third 7 cc of 10% ammonium bromide, a fourth 7 cc of 6% ammonium chloride, and a fifth 7 cc of 7.4% ammonium sulfate. The 4 latter solutions were roughly equivalent with respect to the concentration of ammonium. A sixth animal was given 7 cc of water to serve as a control. Gavage administration was used in these experiments. The first 5 animals all succumbed to acute pulmonary edema. The lungs of the control were normal.

Guinea pigs given the standard dose of ammonium chloride by gavage showed a blood pH of 6.8 to 7.2, 15 to 30 minutes later. Two mechanisms are involved in the production of this acidosis, acid hydrolysis of ammonium salts, and the conversion of ammonia into urea. The first mechanism does not operate with an ammonium salt of a weak acid, such as ammonium acetate. However, guinea pigs and cats rendered even more severely acidotic by gavage administration of a sodium lactate buffer, or a dilute solution of hydrochloric acid, never showed pulmonary edema. It is evident, therefore, that the ammonium ion rather than the acidosis is the primary factor in the pathogenesis of this form of lung edema.

C. Syndrome of ammonium intoxication. In the guinea pig and rat, the characteristic sequence of events transpired in 15 to 60 minutes, taking less than 30 minutes in most of the animals. A considerable increase in rate and depth of respiration occurred several minutes after the administration of ammonium chloride. Weakness and difficulty in locomotion soon appeared; at about the same time, hyperexcitability for tactile, auditory and painful stimuli was noted. Shortly thereafter, muscle fasciculations appeared over most of the body and could be best seen in the tongue. These usually preceded the onset of one or more generalized tonic convulsions. Occasionally the convulsions appeared before the fasciculations. Coma quickly supervened, the corneal reflex was absent and no stimulus however great would bring about a response. The respirations were by this time greatly reduced in frequency and were deep and gasping in character. Stridor was frequently heard. A serosanguineous, or rarely a serous, fluid exuded from the nostrils or mouth before death. Occasionally, no fasciculations or convulsions occurred, or perhaps only a terminal convulsion; yet acute pulmonary edema was present at autopsy.

The above description held also for the cat, with several exceptions. The dose required was greater and the time interval before lung edema appeared was longer, varying from one to 3 hours. Sialorrhea usually occurred and vomiting was common. This animal appeared to be on the whole more resistant to ammonium pulmonary edema than were the rodents. Another notable difference was the character of the edema fluid issuing from the upper respiratory tract. In the cat, the fluid was never blood-tinged, whereas, in the rat and guinea pig, blood-tinged edema fluid was the rule, with very few exceptions.

The effects of toxic doses of ammonium would appear to be chiefly on the central nervous system, at least until pulmonary edema supervenes. They were manifested by muscle fasciculations, tonic convulsions and

FIG. 1.

Photograph of gross specimens. On the left are the trachea and lungs from a rat that died 60 minutes after an intraperitoneal injection of ammonium chloride. The lungs are swollen and present pleural surfaces which are discolored by areas of hemorrhage and congestion. At the right are the trachea and lungs from a control rat.

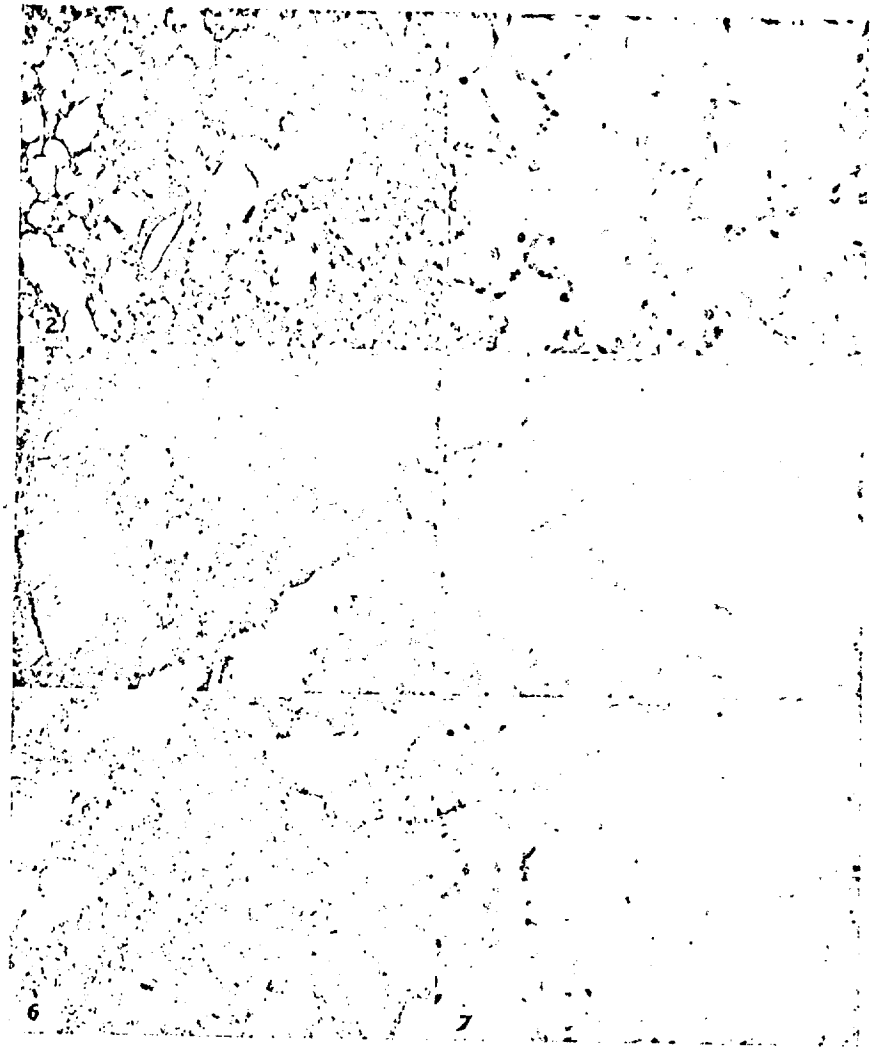

FIG. 2. Photomicrograph of a typical area from a section of lung from a guinea pig which succumbed to acute pulmonary edema 30 minutes after gavage administration of ammonium chloride. Many of the alveoli are filled with a homogeneous precipitate of edema fluid (e). × 64.

FIG. 3. Photomicrograph, at higher magnification, of part of the area shown in Fig. 2 illustrating the homogeneous edema exudate which contains many air bubbles. Hemorrhage is not a prominent feature here, but occasional small groups of extravasated red blood cells are present in the alveoli. × 263.

FIG. 4. Photomicrograph illustrating a typical area of section of lung [illegible] which died 25 minutes after an intraperitoneal injection of ammonium chloride. Many of the alveoli in this field are filled with a homogeneous eosinophilic exudate in which air bubbles and occasional blood cells are included. A branch of the pulmonary artery in the lower left hand field is surrounded by a wide "cellar" of edema exudate containing many red blood cells. × 64.

FIG. 5. Photomicrograph at higher magnification of a portion of the field shown in Fig. 4. The edema exudate and the dilatation and intense congestion of the alveolar capillaries are prominent features. × 263.

FIG. 6. Photomicrograph of a section taken from the lung of a cat which died of acute pulmonary edema 2½ hours after the intraperitoneal injection of ammonium chloride. A fibrillar exudate fills many of the alveoli. A number of [illegible] are seen dilated and filled with blood cells. × 64.

FIG. 7. Photomicrograph at higher magnification of part of a single alveolus in the field shown in Fig. 6. Many fibrillar strands are in the edema exudate. A small bubble (a) of air still remains in the center of the alveolus, another smaller one is at the side. Occasional macrophages (septal cells(s)), and leucocytes (l) appear in what was formerly the alveolar space. Capillaries in the alveolar septa are turgid with blood cells. × 263.

dyspnea. The fasciculations suggested repetitive discharging of primary motor neurons of the brain stem and spinal cord. The tonic convulsions indicated a greatly heightened reflex excitability of the spinal cord. The dyspnea which occurred early had a two-fold origin. Acidosis was an important factor, which probably stimulated respiration reflexly through the chemoreceptors[5] and through an increase in CO_2 tension in the blood which resulted from a depression in pH. In addition, ammonium is a direct respiratory stimulant;[6] we noted dyspnea with ammonium acetate, a salt which produces no immediate depression of blood pH.

D. Anatomical changes. Gross pathology found at necropsy was observed only in the chest and upper respiratory tract. The nasal cavity, larynx, trachea and bronchi were filled with a frothy fluid, usually blood-tinged in the guinea pig and rat, but serous in the cat. These passageways were otherwise unobstructed. Pleural fluid was never seen. The lungs were increased in size (Fig. 1), collapse being prevented by the fluid in the lung parenchyma. The pleural surface of the lungs was always discolored; in some animals there was only a petechial distribution of deep red spots, in others a massive hemorrhagic involvement, but an appearance intermediate between these extremes was more common. The changes showed no predilection for lobes or parts of lobes. On section, the lung was deep red in many places and a frothy serosanguineous fluid could be readily expressed. Congestion of blood vessels and parenchyma was marked. The right atrium, right ventricle and the venae cavae entering the heart were usually moderately dilated, but the left ventricle and left atrium appeared normal.

Histological alterations were limited to the lung and the brain.‡ By far the most important change was the massive pulmonary edema with variable degrees of hemorrhage. The alveolar spaces and smaller air passages were filled with a precipitated pale eosinophilic material in which air bubbles and red blood cells were often included. This edema exudate was usually homogeneous in the guinea pig (Figs. 2 and 3) and rat (Figs. 4 and 5) when the lung was fixed by total immersion, but was distinctly fibrillar in the cat. Interstitial edema was not a prominent finding except in the rat where periarterial "collaring" or "cuffing" of edema exudate was always present. Hemorrhage into the alveoli and the interstitial tissues was always present, but varied greatly in extent and degree. Vascular congestion was intense and involved not only the larger vessels, but also the capillaries. In the cat (Fig. 6 and 7) it was not unusual to find alveolar capillaries that were seven or eight red blood cell diameters in width and filled with densely packed blood cells.

Discussion. The lung changes produced by toxic doses of ammonium salts appear to have escaped notice. Underhill and Kapsinow[7] determined the minimum lethal dose for a series of ammonium salts in white rats, producing death within three hours. They concluded that the toxicity of these compounds, with a few exceptions, varied directly with the ammonium content of the compound. However, these investigators did not report the presence of pulmonary edema, nor did they state the cause of death in the animals studied. Chenoweth and his colleagues[8] studied the toxicity of ammonium gluconate

[5] Schmidt, C. F., Macleod's Physiology in Modern Medicine, Philip Bard, C. V. Mosby Co., St. Louis, 9th edition, 1941, 561, 567.

[6] Breckfield, C. R., Hansen, E. T., and Gesell, R., *Exp. Proc.*, 1946, **5**, 10.

‡ Pathological changes have been seen in the brain and are described elsewhere.

[7] Underhill, F. P., and Kapsinow, R., *J. Biol. Chem.*, 1922, **54**, 141.

[8] Chenoweth, M. B., Civin, H., Salemon, C., Cohen, M., and Gold, H., *J. Lab. and Clin. Med.*, 1941, **26**, 1574.

which was given intravenously to cats, but they did not record the presence of pulmonary edema at autopsy.

It is in the clinical literature that one finds a hint of this toxic effect of ammonium salts. Fazekas[9] described 2 cases of suicide resulting from the ingestion of ammonium chloride. One woman died 10 hours afterward and autopsy revealed, among other findings, an advanced pulmonary edema. The brain changes which were described were characteristic of anoxia. The amount of ammonium chloride ingested was unknown.

Abnormal increases of ammonium in the blood are occasionally encountered in disease. McNeil and Levy[10] found that this occurred most often in conjunction with acute disease processes involving the liver. In view of the important role the liver normally plays in the transformation of ammonium to urea, this is not surprising. While there is apparently little danger of ammonium intoxication with the usual therapeutic doses of ammonium salts, the possibility exists that large doses, such as are occasionally given to cardiac patients to promote diuresis (up to 40 g a day)[11] in the presence of an hepatic insufficiency, may result in a blood concentration of ammonium great enough to cause toxic manifestations—even pulmonary edema. Such an occurrence would ordinarily be imputed to the cardiac disorder rather than the ammonium.

Summary. (1) Ammonium salts in toxic doses produced an acute pulmonary edema in the rat, guinea pig and cat. The cat was more refractory to this effect than the rat and guinea pig. The rabbit did not appear to be susceptible to ammonium lung edema. (2) While a number of routes of administration of ammonium chloride were found effective for the production of acute pulmonary edema, the gavage route (0.09-0.15 g per 100 g of body weight) was most dependable for the guinea pig, and the intraperitoneal route (0.04 g per 100 g of body weight) for the rat. (3) The ammonium ion was found to be the agent responsible for the lung edema. Many ammonium salts produced this effect. Acidosis was ruled out as the primary factor, because acidosis produced in other ways did not result in lung edema. (4) The syndrome of ammonium intoxication consisted chiefly of dyspnea, muscle fasciculations and convulsions, terminating in an early acute pulmonary edema. (5) Gross changes at autopsy were limited to the thoracic cavity. They consisted of pulmonary edema and congestion with variable degrees of hemorrhage and moderate dilatation of the right ventricle, right atrium, and entering venae cavae. The microscopic appearance of the lungs was one of edema, congestion and hemorrhage.

[9] v. Fazekas, G. I., *Deutsch. Ztschrft. f.d. ges. gericht. Med.*, 1934, **23**, 225.

[10] McNeil, H. L., and Levy, M. D., *J. Lab. and Clin. Med.*, 1917, **3**, 18.

[11] Keith, N., quoted by Sollmann, T., A Manual of Pharmacology, W. B. Saunders, Co., 7th edition, 1948, 776.

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Control of Urine pH and Its Effect on Sulfaethidole Excretion in Humans

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A technique is described whereby urine can be excreted by human subjects, at a pH near 5.0 or 8.0, with a variation of not more than 0.1 or 0.2 pH unit over periods of at least 15 hours. The significance of controlled urinary pH in humans with respect to the excretion kinetics of a weak acid, sulfaethidole, has been determined. The harmonic mean $t_{1/2}$ for SETD at a controlled urinary pH of 5.0 is 11.4 hours; the $t_{1/2}$ for this drug at a controlled urinary pH of 8.0 is 4.2 hours.

THIS STUDY was undertaken to establish a technique whereby urine would be voided at a constant pH over a protracted time. Using this technique, the elimination of sulfaethidole (SETD) from human subjects was studied to determine the influence of controlled urinary pH on its excretion kinetics. That the elimination processes of some drugs are influenced by administration of agents which affect the acidity or alkalinity of the urine is well known. For example, Smith, Gleason, Stoll, and Ogorzalek (1) and others (2-6) have demonstrated that renal

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clearance of salicylates is increased when sodium bicarbonate or other alkalinizing agents are employed to increase pH of the urine. Similar results have been shown by Waddell and Butler (7) and by Mollaret, Rapin, Pocidalo, and Monsallier (8, 9) for phenobarbital, by Waddell and Butler (10) for 5,5-dimethyl-2,4-oxazolidinedione, by Gutman, *et al.* (11), for phenylbutazone analogs, and by Peterson, Finland, and Ballou (12) for sulfadiazine. It is generally accepted in clinical practice that the excretion process of chemotherapeutic sulfonamides can be hastened by administration of sodium bicarbonate. Also, it is well known that the elimination of many basic drugs is hastened by agents which acidify the urine (13–18).

The influence of urinary pH on the elimination of a drug such as SETD can be attributed to the pH dependence of renal tubular transfer of the drug. A large portion of SETD in the human body is contained in extracellular fluids, of which the blood is a large component. Blood passing through the nephron of the kidney is filtered of many of its components. The capillary networks of the glomeruli act as filter beds for the plasma, whereby most of the nonprotein substances filter passively through the glomeruli into the renal tubules. Many of the normal components of the glomerular filtrate are, in part, reabsorbed into the circulation from the tubules; absorption is effected by both active and passive processes, depending upon the substance in question. In addition, tubules may actively and passively secrete substances from the blood into the tubular fluid.

Drugs may be filtered through the glomeruli, secreted into the tubules, and reabsorbed from the tubules in ways similar to those which apply to normal blood components; however, knowledge of the active or passive nature of drug secretion and reabsorption at the renal tubule level is limited. For many drugs it is thought that passive diffusion may be more important than active transport in their excretion and reabsorption at the renal tubule level.

A difference in pH between plasma and tubular fluid can result in a considerable difference in the fraction of undissociated weakly acidic or weakly basic drug in these two fluids. In the absence of a specific transport mechanism it is usually the undissociated form of the drug which most readily diffuses between the two fluid compartments. The concentration gradient of the nonionized drug across the cellular barrier separating plasma from tubular fluid is, therefore, considered to be of primary importance in passive transport. Though it is difficult to determine the pH of fluid at sites in the tubule intermediate of the glomeruli and the bladder, the pH of the beginning and terminal processes can be readily determined by measuring plasma and urine pH. Plasma pH in humans is generally very constant, near 7.4, whereas urine pH may vary through a range of about 4.5 to 8.5 under different natural or experimental conditions.

Milne, Scribner, and Crawford (19) have shown that the pH of the tubular fluid and the pKa and partition coefficient of the drug would be expected to influence the renal tubular transfer of many weak acids and bases, just as such factors influence the absorption and secretion of drugs in the gastrointestinal tract (20–24). It was thought that by devising a technique for maintaining urine pH constant at several pH values, a convenient method would become available for assessing quantitatively, in kinetic terms, the effect of pH on the excretion of drugs. Since SETD is a weak acid which is eliminated virtually completely *via* the urinary route, and methodology for the kinetic elimination studies has been established (25–28), this appeared to be a suitable drug for such study.

EXPERIMENTAL

Human Studies. Twelve healthy young adult white male human volunteers were employed in the excretion trials of this study. For most trials, food and fluid intake were not controlled.

Ammonium chloride and sodium bicarbonate were used, in the manner previously reported (29), to maintain the desired acidic or alkaline condition of the urine throughout the studies. Normally, a 3- to 4-Gm. dose of one of these agents was given in tablet form for 2 or 3 hours, until measurement of urine pH indicated that pH values of 5 or 8 had been attained. Smaller doses of these agents were administered, usually each hour, to maintain the pH at the desired value. The pH of the urine was monitored, usually every hour, to determine whether succeeding doses should be increased or decreased to maintain a constant pH.

After the desired urine pH was attained, a single 2-Gm. dose of powdered SETD suspended in water was administered orally. Samples of urine were collected, usually at hourly intervals, for at least 10 hours. Volumes of urine voided, pH, and free SETD excreted were measured in a total of 16 trials at a urinary pH near 8, and in 17 trials at a urinary pH near 5. All subjects were used in at least one trial at each of the two urinary pH values. One subject was studied to determine the effect of induced diuresis on the excretion profile. Another variation involved an excretion profile study with one subject where, in a single trial, the urine was maintained first at an acid pH and then at an alkaline pH.

Analytical Methods. Urine pH measurements were made with a Beckman model G pH meter

TABLE I.—ILLUSTRATION OF TYPICAL DOSAGE REGIMENS OF SODIUM BICARBONATE AND AMMONIUM CHLORIDE REQUIRED TO ATTAIN AND MAINTAIN CONSTANT URINARY pH IN HUMAN SUBJECTS

	Dosage of Ammonium Chloride, Gm.	Time, hr.	Urinary pH	Dosage of Sodium Bicarbonate, Gm.	Time, hr.	Urinary pH
Subject A	4.0	−1.25	...	4.2	−2.00	...
	...	−0.25	5.25	4.2	−1.00	...
	3.0	0	5.15	3.0	0	7.80
	2.0	1.17	5.07	1.2	1.00	8.10
	1.0	2.17	5.07	1.2	2.00	8.10
	1.0	3.33	5.03	1.2	3.00	7.95
	1.0	4.17	4.97	3.0	4.00	7.60
	1.0	5.00	5.00	3.0	5.00	7.90
	1.0	6.00	5.02	1.8	6.00	8.10
	1.0	7.00	5.03	1.8	7.00	8.10
	...	8.00	5.09	1.8	8.00	8.00
	...	9.33	4.87	1.8	9.00	8.10
	...	10.00	4.82	1.8	10.00	8.10
	...	12.00	4.82	...	11.00	8.00
Subject B	4.0	−1.00	...	4.2	−3.00	
	3.0	0	...	4.2	−2.00	...
	1.0	1.00	5.30	4.2	−1.00	...
	1.0	2.00	5.30	4.2	0	7.50
	1.0	3.50	5.30	3.0	1.00	8.05
	1.0	4.00	5.30	3.0	2.00	8.00
	1.0	5.00	5.20	3.0	3.00	8.00
	1.0	6.00	5.10	1.2	4.00	8.10
	1.0	7.00	5.20	1.2	5.00	8.10
	1.0	8.00	5.30	1.2	6.00	8.00
	1.0	9.00	5.30	1.2	7.00	8.00
	...	10.00	5.30	1.8	8.00	8.00
	...	...	...	1.8	9.00	7.95
	...	...	...	...	9.50	7.85

Each urine sample, when collected, was rapidly brought to room temperature and the pH was determined immediately.

Urine samples were assayed for free SETD using a modification of the Frisk macro method (30). Spectrophotometric measurements of the final colored solutions, involving use of the Bratton-Marshall reagent (31), were made with a Beckman model B spectrophotometer, at a wavelength of 550 mμ.

RESULTS

Control of Urine pH.—Typical dosage regimens and the resulting urine pH are presented in Table I. Subjects were somewhat variable with regard to the quantities of ammonium chloride or sodium bicarbonate required to attain and maintain a constant pH; however, after gaining experience with this technique, and by monitoring the urine pH at about hourly intervals, it was a relatively easy matter to maintain urine pH within ±0.2 units over periods of at least 15 hours. The occasional incidence of nausea from the large doses of sodium bicarbonate and ammonium chloride was the principal difficulty encountered in employing this technique.

While good control of urine pH can be attained near pH 5 or pH 8, it was more difficult to maintain constant pH at intermediate values. It was concluded that this could not be accomplished conveniently to the degree of constancy desired for the excretion studies; therefore, studies on the SETD excretion profile were limited to values near pH 5 and pH 8.

Excretion Studies. Typical data for the SETD excretion profile studies under conditions of constant pH are presented in Tables II and III. Since it has been shown previously (26, 27, 32) that oral doses of SETD are virtually completely absorbed, almost totally excreted in the urine, and that virtually all of the drug in the urine is measurable by use of the Bratton-Marshall reagent, interpretation of the excretion data was not difficult. As shown previously for this drug, rate of elimination can be expressed as

$$dW/dt = -k_b W \qquad \text{(Eq. 1)}$$

where W is the amount of drug remaining in the body at any time, t, or W = (dose − amount excreted), and k_b is the specific velocity constant for disappearance of drug from the body. Since

$$\log W = -(k_b/2.303)t + \text{constant} \qquad \text{(Eq. 2)}$$

k_b can be determined from the slope of the straight line resulting from a plot of log W vs. time (27). The biologic half life, $t_{1/2}$, is determined for each trial from $t_{1/2} = 0.693/k_b$.

Plots of logarithm (mg) of unexcreted drug vs. time, for single subjects, gave straight line plots after 1 or 2 hours following administration. Table IV illustrates the biologic half life values obtained from inspection of the exponential plots of the urinary excretion data and the average urine pH as observed for each trial. Average acid urine pH values for subjects in 17 trials ranged from pH 4.5 to pH 5.3. When two trials are eliminated from the summary data, 15 trials fall within the pH range 4.8 to 5.2. The harmonic mean for $t_{1/2}$ values observed in the 15 trials at pH 5 ± 0.2 is 11.1 hours with fiducial limits (26) of 9.9 to 13.5 hours. This corresponds to an arithmetic average k_b of 0.061 ± 0.004 hr.$^{-1}$ where ± designates standard error.

Average alkaline urine pH values for subjects in 13

TABLE II.—TYPICAL DATA FOR URINARY EXCRETION OF SETD,[a] URINE MAINTAINED AT pH 5

Dosage of Ammonium Chloride, Gm.	Hours	Urinary pH	Urine Volume, ml.	SETD per Sample, Mg.	Total Unexcreted SETD, mg.
4.0	−3.33	...	...	...	...
...	−1.83	5.3	...	...	...
1.0	0	...	...	...	...
1.0	1.33	5.1	248	22.3	1978
1.0	2.75	5.1	235	87.0	1891
1.0	3.75	5.1	114	124.3	1766
1.0	4.92	5.0	108	140.8	1626
...	6.08	5.0	119	132.1	1494
1.0	7.08	5.0	80	88.0	1406
1.0	8.08	5.1	100	90.0	1316
0.5	10.08	4.9	192	151.7	1164
...	11.08	4.8	149	82.0	1082
...	12.08	4.9	78	60.8	1021
...	13.23	5.0	57	51.3	970
0.5	14.08	5.0	50	37.0	933
...	15.08	4.9	75	45.6	887
...	17.75	...	391	104.0	783
...	23.00	...	946	200.6	582
...	25.59	...	489	86.1	497

[a] SETD, 2 Gm., administered orally at zero hour.

TABLE III.—TYPICAL DATA FOR URINARY EXCRETION OF SETD,[a] URINE MAINTAINED AT pH 8

Dosage of Sodium Bicarbonate, Gm.	Hours	Urinary pH	Urinary Volume, ml.	SETD per Sample, mg.	Total Unexcreted SETD, mg.
4.2	−2.75	...	...	...	...
4.2	−1.50	6.4	...	...	...
3.6	−0.25	8.1	...	...	...
0.6	0	8.6	...	...	...
3.6	1.00	8.1	70	294.0	1706
3.0	2.00	8.1	62	235.6	1470
3.6	3.08	8.1	95	247.0	1223
3.0	4.08	8.1	113	180.8	1043
3.0	5.08	8.1	99	142.6	900
2.4	6.08	8.1	86	123.9	776
2.4	7.66	8.1	130	179.4	597
...	8.75	8.0	100	108.0	489
3.0	9.25	8.0	44	44.0	445
...	10.59	8.1	130	75.4	369
...	11.59	8.0	105	39.9	329
...	13.59	8.0	130	85.8	244
...	14.83	7.6	158	63.2	180
...	19.75	...	580	118.9	62

[a] SETD, 2 Gm., administered orally at zero hour.

trials ranged from pH 7.4 to pH 8.1. When the three trials at the lowest pH are excluded, 10 trials are observed to fall within the average pH range of 7.9 to 8.1. The harmonic mean for $t_{1/2}$ values in the 10 trials at pH 8 ± 0.1 is 4.2 hours, with fiducial limits of 3.7 to 4.8. Average k_b is 0.165 ± 0.009 $hr.^{-1}$

Figure 1 is a presentation of the composite urinary excretion data from which Table IV values for $t_{1/2}$ were determined. It is a rather dramatic illustration of the adherence of the urinary excretion process, of many trials, to first order kinetics in the time range studied. In addition, it gives a clear visual illustration of the dependence of the process upon the magnitude of the equilibrium urine pH. Figure 2 also illustrates this very well where no abrupt change in the slope of the excretion curve occurs when urine, being collected during a single trial, was suddenly changed from a pH of 5 to a pH of 8.

Though previous data had supported the opinion that, in the presence of adequate urine flow, elimination kinetics of SETD is relatively independent of urine volume (27), in the early phase of this study experimental verification of this point seemed desirable. During the course of an excretion study, a single subject who had consumed approximately 100 ml. of water each hour was suddenly changed to a regimen where he consumed almost [illegible] ml. per hour for several hours. Data presented in Fig. 3 indicate that SETD excretion was essentially unchanged even by tenfold or greater increase in urine volume, so long as precautions were taken to maintain constant urine pH.

DISCUSSION

The fact that urine pH influences [illegible] of SETD to the degree indicated in Table IV indicates that renal tubular transport [illegible] plays an important role in the [illegible] pH. Approximately [illegible]

TABLE IV.—BIOLOGIC HALF-LIFE FOR SETD IN HUMAN SUBJECTS AS A FUNCTION OF URINARY pH

Acid Range		Basic Range	
Av. Urine pH	$t_{1/2}$, hr.	Av. Urine pH	$t_{1/2}$, hr.
4.5	14.2	7.4	6.6
4.8	12.5	7.5	4.7
4.8	12.6	7.8	5.4
4.8	8.9	7.9	5.4
4.8	19.8	7.9	3.7
4.8	12.5	8.0	3.3
4.8	10.0	8.0	5.5
4.9	21.3	8.0	4.4
4.9	8.9	8.0	4.1
4.9	10.7	8.0	3.7
5.0	20.1	8.1	5.3
5.0	11.4	8.1	4.1
5.0	10.3	8.1	3.9
5.1	8.0	...	...
5.1	10.4	...	...
5.2	9.2	...	...
5.3	10.6	...	...
For 15 trials within pH range 4.8 to 5.2, Av. $t_{1/2}$, 11.4 hr.[a]		For 10 trials within pH range 7.9 to 8.1, Av. $t_{1/2}$, 4.2 hr.[a]	

[a] Harmonic mean $t_{1/2}$.

bound (33) and, therefore, only a small fraction of the total SETD is cleared in any single pass of fluids through the glomeruli. While the concentration of diffusible drug in the glomerular filtrate must be low, a condition favoring diffusion of SETD back into general circulation might be created through water reabsorption in the proximal tubule, thus increasing the concentration of drug in tubular fluid over that of unbound drug in the plasma. Furthermore, a decrease in pH of tubular fluid would favor reabsorption of drug by increasing the concentration of the more readily diffusible undissociated form of the drug.

The pKa of SETD, determined by potentiometric titration, is about 5.5, and the fraction of SETD which is undissociated is given by

$$(H^+)/[(H^+) + Ka]$$

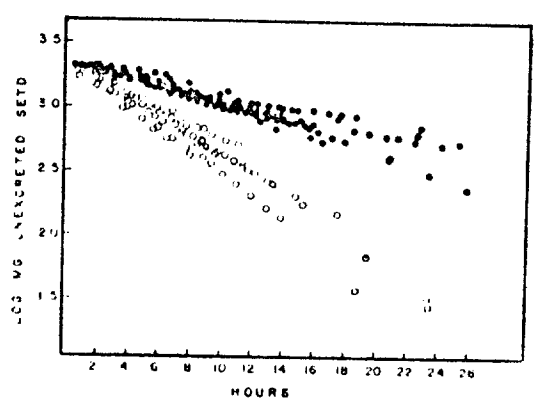

Fig. 1.—Exponential plot of the composite urinary excretion data for sulfaethidole following single 2-Gm. oral doses to 12 subjects in 30 trials. Darkened circles indicate data at constant urine pH near 5 and open circles indicate data where urine pH was maintained near 8 as described in the text.

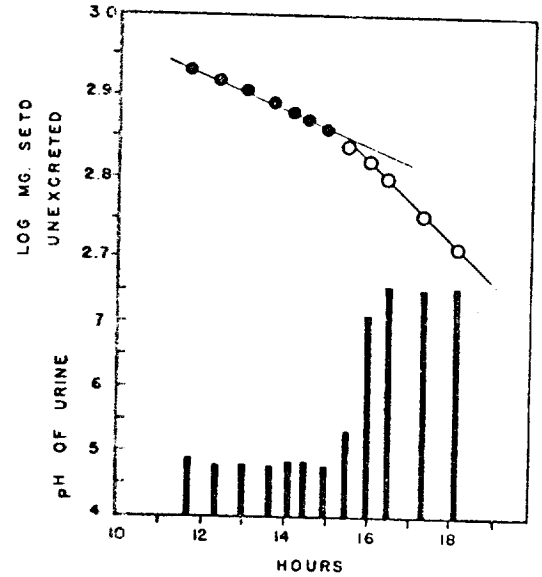

Fig. 2.—Exponential plot of SETD urinary excretion data for a single human subject after a 2-Gm. oral dose. The figure illustrates the abrupt change in slope of the exponential process when urine pH is changed from about pH 5 to pH 8.

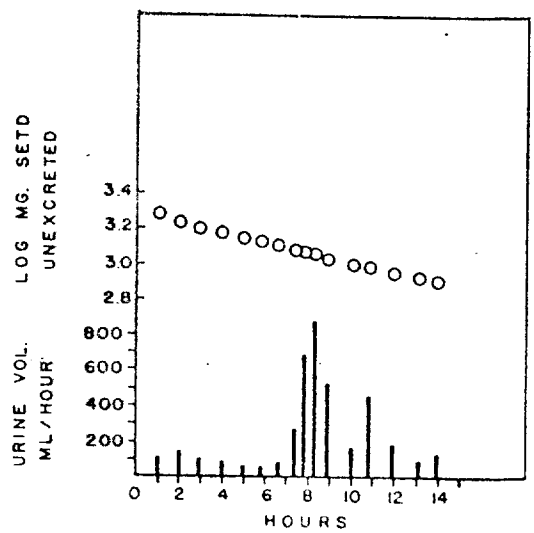

Fig. 3.—Exponential plot of SETD urinary excretion data for a single human subject after a 2-Gm. oral dose when urine pH was maintained near 8 but where the rate at which urine was voided [illegible] appreciably. The figure illustrates [illegible] excretion rate is relatively independent of urine volume voided.

The fraction of SETD [illegible] urine under condition [illegible] plasma, pH [illegible] urine, pH 8.0, [illegible] pH 5.0, [illegible] SETD [illegible]

Although the pH of tubular fluid [illegible] sorption of SETD [illegible] tain that under condition [illegible] the [illegible]

would be alkaline when urine pH was maintained at pH 8 and acid when urine was maintained at pH 5. The longer biologic half-life for SETD under conditions of acid urine suggests that reabsorption of the drug from tubular fluids is favored by higher concentrations of undissociated drug. This concept is similar to those concerning gastrointestinal absorption which have seen considerable discussion in recent literature by Schanker, Brodie, Hogben, *et al.* (20, 21). Milne, Scribner, Crawford (19), Gutman, *et al.* (11), and others have developed such thinking regarding factors influencing net drug elimination *via* the urinary route.

Though the pKa of SETD and other acidic and basic drugs apparently plays an important role in the mechanism of drug elimination, and though controlled urinary pH can exert a profound effect on the rate of net drug elimination, the intrinsic lipid solubility of the undissociated drug, concomitant administration of other drugs, and other factors must also be important in influencing the net elimination rate. It would seem important to obtain a clearer understanding of the mechanism of drug excretion by the kidney, and of the interrelationships of the physical-chemical properties of drugs and the mechanisms which come into play in body elimination of drugs. Such knowledge, coupled with additional knowledge of other body mechanisms for eliminating drugs and drug activity, has a bearing on the intelligent design of drugs and their dosage forms for controlling their duration of action.

The results of this study supplement and support, in a number of respects, previously reported findings concerning SETD excretion kinetics (25–28, 30, 32). In previous studies without benefit of controlled urinary pH, and using both blood concentrations and urinary excretion data, the $t_{1/2}$ for SETD in human subjects was shown to be about 8.4 hours (27). This value is about midway between the values of 4.2 and 11.4 hours, at controlled urine pH of 8 and 5, respectively, determined in this study. These results also give further reinforcement to the opinion that the $t_{1/2}$ values of drugs determined experimentally in human subjects are not to be construed as fixed values. Rather, they are subject to variation by a number of factors about which much additional data are necessary for a better understanding of elimination mechanisms and kinetics.

SUMMARY

1. A technique has been developed to permit control of the pH of urine being excreted by human subjects to ± 0.1 pH unit, in the vicinity of pH 5 or pH 8, over periods of at least 15 hours.

2. Constant urinary pH in the vicinity of pH 5.0 can be attained by administering several grams of ammonium chloride each hour until the desired pH has been attained, and then increasing or decreasing the maintenance dose as indicated by hourly monitoring of urine pH. Constant urinary pH in the vicinity of pH 8.0 can be attained by employing sodium bicarbonate in a similar fashion.

3. Modification of urine pH in human subjects has a profound effect on the rate of elimination of sulfaethidole from the body.

4. The harmonic mean $t_{1/2}$ for SETD is 11.4 hours when urine pH is maintained at pH 5.0; the harmonic mean $t_{1/2}$ is only 4.2 hours when urine pH is maintained at pH 8.0.

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SOME ASPECTS OF CITRIC ACID METABOLISM*

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In addition to its presence in milk (9) and urine (1) citric acid has, within the past few years, been found in many other body fluids including sweat (12), cerebrospinal fluid (2), aqueous humor (7), follicular fluid (14), and blood (3). Apparently, saliva is the only body fluid tested in which citrate has not been detected.[1]

Although citric acid appears to be so generally distributed and is excreted by the normal individual in amounts from 0.2 to 1.0 gm. daily (15), its rôle in metabolism is obscure. Tolerance studies have led to confusing results because of the interfering action of the large amounts of alkali present in the citrates used (sodium citrate contains practically as much alkali per gm. as sodium bicarbonate). The fact that citric acid forms a soluble, slightly ionized salt with calcium has suggested that it may be related to calcium metabolism (5), but Greenberg and Greenberg (6) find little good evidence that such a slightly ionized combination is present in the blood. In 1931, Östberg (15), on the basis of evidence which showed that the amount of citric acid excreted varies directly with the pH of the urine, suggested that citric acid is primarily a part of the acid-base regulatory mechanism and that it is synthesized to neutralize excess alkali just as ammonia is synthesized to neutralize excess acid. On the other hand, the large amounts of citrate excreted in fasting and on ketogenic diets (4) indicate that citrate metabolism must be controlled by other factors in addition to acid-base relationships.

In the present work, the influence of acid-base relationships and

* A report of this work was presented before the American Society of Biological Chemists at Cincinnati, April 10, 1933 (*J. Biol. Chem.*, 100, lxi (1933)).

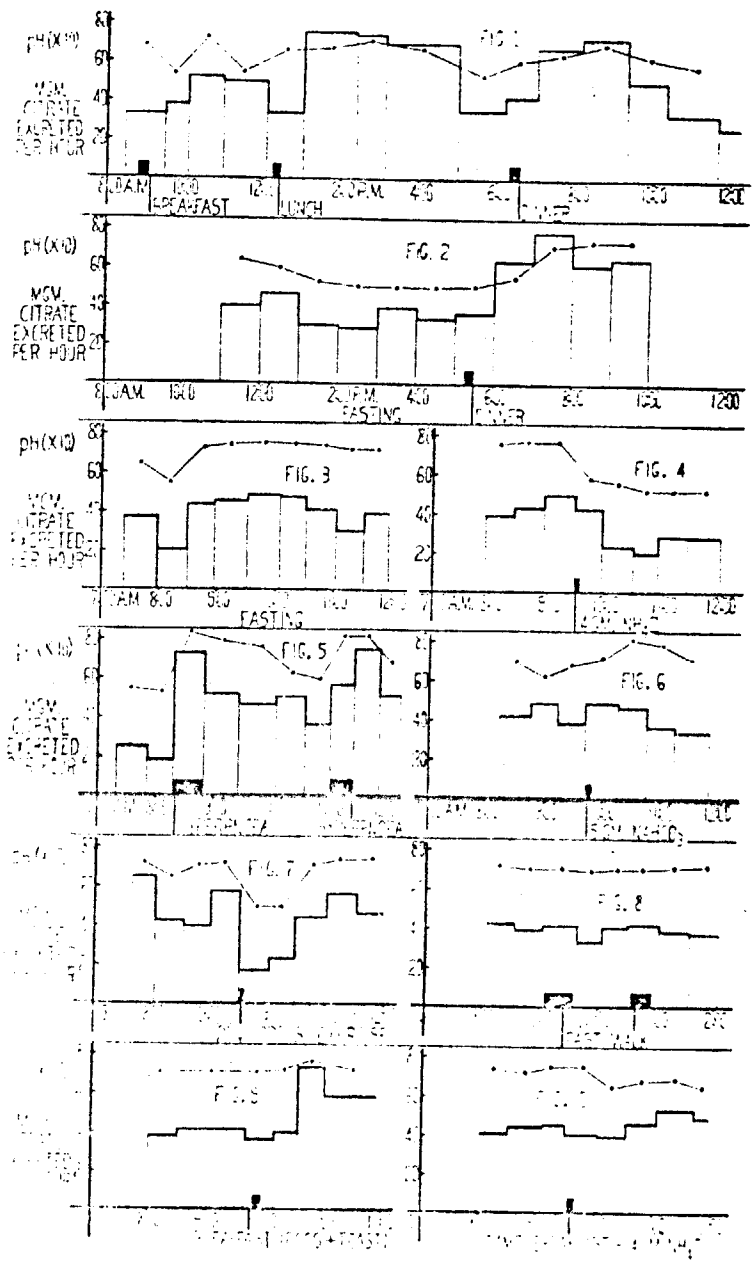
[1] The methylene blue-enzyme method failed to show even a trace.

of dietary factors was investigated by following blood citrate concentration and urinary excretion of citric acid after exercise and after the administration of food, sodium bicarbonate, ammonium chloride, and citric acid itself. The pentabromoacetone method[2] was used for the determination of citrate in urine, the Thunberg methylene blue method[3] for the analysis of blood.

Analysis of urines collected in half hour periods throughout the day from a human subject on a mixed diet revealed that the rate of citrate excretion is directly related to the ingestion of food and

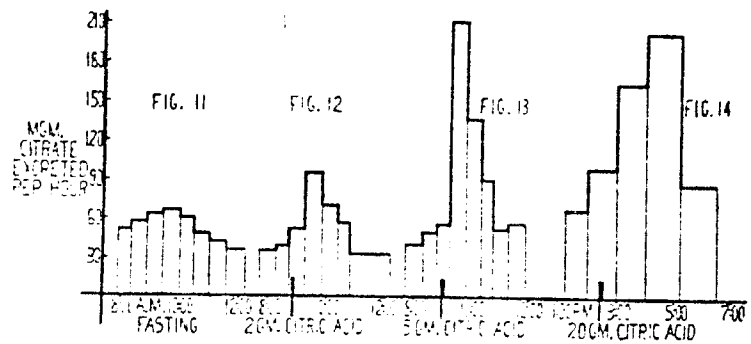
[2] Numerous modifications were introduced into the original Amberg-McClure method (1). The urine sample (50 cc., or smaller amounts diluted to that volume) to which 10 cc. of sulfuric acid (1:1) and 3 cc. of 37.5 per cent potassium bromide solution are added, is heated on a water bath to 52–54° (the limit of 50–55° is rather too wide (10)); sufficient 5 per cent potassium permanganate is added immediately to give a precipitate of manganese dioxide; this is usually 15 cc., but more must be used when lactic acid or other easily oxidized substances are present. An excess of acidified concentrated ferrous sulfate solution is added to reduce the precipitated manganese dioxide and the flask is allowed to cool overnight in the ice box (8). The solution is filtered cold through a Gooch crucible and washed with several small portions of water. The crucible is dried in a sulfuric acid desiccator for 24 hours, weighed, heated at 105° for 10 hours, weighed again, and the difference in weight taken as the weight of pentabromoacetone (13). The amount of citric acid (plus 1 molecule of water) present in the original sample is calculated from the formula, $1.05\ (0.464\ P + 0.019\ S)$, where P is the weight of pentabromoacetone in mg., 0.019 is a solubility factor, and S is the volume of the discarded filtrate (8). Control experiments showed that preliminary treatment with bromine and charcoal (13) did not change the citrate values obtained on normal human urine. Duplicate analyses on standard solutions and on urine samples checked consistently within a few per cent.

[3] Certain modifications of Thunberg's method (17) have already been described (11). Seeds (usually 1 gm. of peeled seeds to 9 cc. of 0.87 per cent K_2HPO_4) were mechanically ground, vigorously, for a period of ½ hour, then centrifuged at high speed for an hour, and allowed to stand at room temperature for at least another hour before use. This procedure produced an extract which maintained a fairly constant activity for 3 hours at room temperature, or longer if kept cold, with very little change in the "critical value." The interfering action of inorganic salts, especially calcium, in the estimation of very small amounts of citrate as found in blood, was minimized by the use of the extract thus prepared; furthermore the use of 1:60,000 methylene blue instead of 1:30,000, as suggested by Thunberg, permitted the precise determination of as little as 2 mg. of citrate per 100 cc. of blood with no interference on the part of the serum salts.



FIGS. 1 TO 10. The relation between urinary pH and citrate excretion

that it appears to parallel the pH of the urine during these short periods as well as in the 24 hour periods reported by Östberg. Following each meal, notwithstanding the absence of food known to contain citrate, such as milk and citrus-fruits, there was a marked increase in the rate of citric acid excretion along with an increase in the pH of the urine as shown in Fig. 1. In Fig. 2 when no breakfast or lunch was eaten both the pH and rate of excretion of citrate remained low until dinner; then they both increased. The experiments recorded in Figs. 3 to 6 were designed to elucidate the relation between urinary pH and citrate excretion. Fig. 3 shows the rate of citrate excretion during a morning's fast. The ingestion of 4 gm. of ammonium chloride (Fig. 4)



FIGS. 11 TO 14. Influence of ingestion of citric acid on citrate excretion

during a similar fasting period caused a decrease in both the pH of the urine and the rate of citrate excretion. Two periods of voluntary hyperpnea and the accompanying alkalosis (Fig. 5) caused marked increases in the rate of citrate excretion. Administration of 5 gm. of sodium bicarbonate (Fig. 6) proved insufficient to cause a significant change in citrate excretion although it increased the pH of the urine. The depressing action of ammonium chloride and the accelerating action of voluntary hyperpnea indicate that the mechanism which controls the output of citrate is flexible like that for ammonia production and that citrate may take an active part in maintaining the proper acid-base equilibria.

Strenuous exercise caused a marked decrease in the rate of citrate excretion (Fig. 7) but two periods of mild exercise (walking, Fig. 8) apparently had no effect. The marked decrease following

strenuous exercise may be related to the accumulation of large amounts of lactic acid and the consequent greater acidity of the urine.

These almost parallel changes in citrate excretion and pH of urine produced by alkalinizing or acidifying measures suggest that the increased citrate output following meals might be simply a reflection of the alkaline tide. However, ingestion of food must act also in another way, inasmuch as sodium bicarbonate produced a more alkaline urine than eggs and toast (Fig. 9) but caused little change in citrate output. Furthermore, when ammonium chloride was added to a breakfast of eggs and toast (Fig. 10) there

TABLE I

Influence of Fasting and Ingestion of $NaHCO_3$ on Citric Content of Rabbit Blood

Day	Time	Procedure	Alkali reserve	Blood citrate
			vol. per cent	*mg. per cent*
1	2.30 p.m.	Food removed		
	3.30 "	Heart puncture	44.9	13.8
2	10.30 a.m.	" "	51.3	4.7
	2.30 p.m.	0.75 gm. $NaHCO_3$ per kilo		
	9.30 "	Heart puncture	61.7	5.0
3	10.30 a.m.	" "	62.0	3.3
4	10.40 "	" "	57.9	3.1
	10.50 "	1.5 gm. $NaHCO_3$ per kilo		
	4.10 p.m.	Heart puncture	82.0	4.7

was still an increase in the rate of citrate excretion in spite of a lowered pH of the urine. Since 50 gm. of glucose caused little change in citrate output, the increase following eggs and toast which is not referable to alkaline tide may be associated with the metabolism of fat or protein. Boothby and Adams (4) suggested that citrate excretion is particularly related to fat and protein metabolism when they found that large amounts of citric acid were excreted at times when the R.Q. indicated that only fat and protein were being oxidized.

Citrate excretion could scarcely be influenced by unsuspected traces of citrate in food. In Figs. 11 to 14, 2 gm. of citric acid taken during a fasting period increased the urinary citrate by less

than 30 mg., 5 gm. by less than 125 mg., and 20 gm. by less than 350 mg. In each case, from 1.5 to 2.5 per cent of the ingested acid escaped oxidation. In a 40 day experiment in which the subjects took a standardized weighed diet and in which 24 hour urine samples were analyzed, similar increases were noted after the ingestion of citric acid; in some cases these were followed by a compensatory decrease in excretion on the following day. Such a compensatory decrease occurring within the same 24 hour period may explain why Östberg in numerous instances missed the increased citrate excretion after the ingestion of citric acid.

TABLE II

Influence of Ingestion of $NaHCO_3$ and NH_4Cl on Blood Citrate Concentration

Subject	Time	Procedure	Alkali reserve	Blood citrate
			vol. per cent	*mg. per cent*
Rabbit B	10.00 a.m.	Food removed		
	11.15 "	Heart puncture	48.8	7.6
	11.40 "	1.25 gm. $NaHCO_3$ per kilo		
	2.25 p.m.	Heart puncture	75.5	7.6
	5.30 "	" "	88.3	7.1
Rabbit C	10.00 a.m.	Food removed		
	11.35 "	Heart puncture	64.0	7.8
	11.50 "	0.75 gm. NH_4Cl per kilo		
	2.40 p.m.	Heart puncture	39.6	5.8
	5.45 "	" "	39.2	3.9

In order to visualize more clearly the events in citric acid metabolism, an attempt was made to correlate the changes in urinary excretion of citric acid with the citric acid content of human and rabbit blood, under the action of the same variables. In a typical experiment on a well fed rabbit (Table I) the blood citrate was 13.8 mg. per 100 cc. A 20 hour fast reduced this value to 4.7 mg. Administration of 0.75 gm. of sodium bicarbonate by stomach tube resulted after 7 hours in a very slight increase of blood citrate, to 5 mg. Although the total carbonic acid remained at about 60 volumes per cent, further fasting caused the blood citrate to decline to 3.1 mg. A second administration of bicarbonate raised the total carbonic acid to 82 volumes per cent, the blood citrate to 4.7 mg. per cent. In another experiment (Table II) sodium bi-

carbonate was administered to a well fed rabbit immediately after food had been removed; within 7 hours, in spite of a high carbon dioxide-combining power, the blood citrate decreased rather than increased. Another fasting rabbit was given ammonium chloride. The combined action of fasting and acid served to reduce the blood citrate to 3.9 mg. within 7 hours. Fasting thus decreased the blood citrate of rabbits regardless of acid or alkaline conditions and the changes produced by administration of acid or base were

TABLE III

Citrate Content of Blood Taken from Various Blood Vessels of the Rabbit

Subject	Time	Procedure	Blood citrate
			mg. per cent
Rabbit D	2.40 p.m.	1.0 gm. $NaHCO_3$ per kilo	
	4.15 "	Amytal anesthesia	
	5.50-6.20 p.m.	Blood taken from	
		Portal vein	15.0
		Hepatic "	15.0
		Renal "	12.0
		Hepatic "	15.0
		Heart	15.0
Rabbit E	1.12 p.m.	1.0 gm. citric acid per kilo	
	1.30 "	Amytal anesthesia	
	2.37-2.49 p.m.	Blood taken from	
		Heart	21.0
		Lower vena cava	22.0
		Portal vein	25.0
		Hepatic "	25.0
		Heart	23.0

small in comparison with the large decrease caused by fasting. This is in agreement with the observations on human urine showing that alimentary processes as well as acid-base relationships are concerned in citric acid production.

In an attempt to localize citrate production and metabolism in particular organs of the body, a number of experiments were performed in which the citrate content of blood taken from various veins was compared with that taken from the heart. Rabbit D (Table III) was given 1.0 gm. of sodium bicarbonate per kilo by stomach tube and anesthetized with sodium amytal. Blood

samples taken from the portal and hepatic veins and from the heart all contained 15 mg. per 100 cc.; no detectable changes in citrate content occurred in passing through the liver or the intestinal circulation. Blood taken from the renal vein contained only 12 mg., indicating that the kidney was removing citrate from the general circulation. After a similar experiment in which blood leaving the kidney was found to contain more citrate than that taken from the heart, Östberg (15) suggested that citrate is made in the kidney. His analyses may have been unreliable because his data show that inorganic salts interfered with his citrate determinations. In another experiment (Rabbit E) samples of blood

TABLE IV

Influence of Exercise and Food on Citrate Content of Human Blood

Time	Procedure	Blood citrate
		mg. per cent
12.10 p.m.	Mixed lunch	
3.10 "	Blood taken from arm	2.8
3.16 "	2 minutes strenuous exercise	
3.25 "	Blood taken from arm	2.8
5.00 "	" " " "	2.4
9.48 a.m.	5 gm. $NaHCO_3$ (fasting)	
11.40 "	Blood taken from right arm after vigorous working of fist	2.0
11.50 "	Blood taken from resting left arm	2.0

taken after the administration of citric acid showed slightly larger amounts of citrate present in the portal and hepatic veins than in the rest of the circulation, probably due to absorption from the intestine, but, as with Rabbit D, the values do not differ sufficiently to relate any organ to citrate metabolism. They rather suggest, as does also the discovery of a citric acid dehydrogenase in muscle (18), that all active tissue may be involved.

Finally, Table IV shows the influence of exercise and of food on the citrate content of human blood. A sample taken from the arm 3 hours after lunch contained 2.8 mg. of citric acid per 100 cc., a second sample taken 10 minutes after a 2 minute period of strenuous exercise which markedly lowered the citrate content of the urine, contained the same amount. The citrate content of a third

sample, taken an hour and a half later, had dropped to 2.4 mg. per 100 cc. The following morning, the subject ate no breakfast but took 5 gm. of sodium bicarbonate. A sample of blood taken 2 hours later from the right arm after the muscles of the forearm had been thoroughly tired out by opening and closing the fist, contained the same amount of citrate as a second control sample taken from the resting left arm, 2 mg. per 100 cc. Apparently, the rapid decrease in urine citrate due to exercise is not a reflection of a marked change in blood citrate. Sensitive as the enzyme method is, at levels as low as 2 mg. per 100 cc., it has too large a limit of error to detect with certainty the decrease in blood citrate necessary to account for the lower rate of excretion by the kidney. The changes in the blood citrate from 2.8 mg. per 100 cc. shortly after lunch to 2.4 mg. just before dinner and 2.0 mg. during a morning's fast suggest that urinary citrate following meals may reflect the concentration of citric acid in the blood, but there is as yet no information as to what the nature of the kidney threshold for citrate may be. Perhaps it is subject to variations similar to those of uric acid (16).

SUMMARY

Alkalosis and acidosis produced in rabbits by sodium bicarbonate and ammonium chloride administration increased and decreased respectively the serum citrate concentration; inanition produced a marked decrease.

The citric acid content of human urine increased slightly after each meal irrespective of the presence of citric acid in the food. Alkalosis produced by hyperpnea increased citrate excretion; vigorous exercise and the ingestion of ammonium chloride decreased it. Evidence is presented to show that the variations following the intake of food cannot be explained solely on the basis of acid-base relationships.

Citric acid is rapidly but not completely oxidized by the human organism; when given in doses of 2 to 20 gm., 1.5 to 2.5 per cent escaped oxidation and was excreted in the urine.

Analyses of rabbit blood drawn from various parts of the body failed to demonstrate a relation between any particular tissue and the oxidation of citric acid. Blood from the renal vein contained less citric acid than did blood from the renal artery.

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Toxicology: Experimental factors which modify the acute poisioning of mice with ammonium chloride (NH_4Cl).

Pierre Lallouette, Gisele Bourderon, and Jean-Claude Beguin, presented by Clement Bressou.

The acute toxicity of mice with NH_4Cl appears to be linked to the diffusion velocity of the poison in the organism. Factors which increase the diffusion velocity shorten the survival time, whereas those which produce slower diffusion establish, with an equal dose, a lag in the appearance of the symptoms and, in certain cases, the survival of the animals.

In general, symptoms of poisioning caused by NH_4Cl appear rapidly after injection. After a period of extreme agitation for several minutes, the animal becomes calm, remains prostrated, its body disturbed by convulsions, its breathing dyspneic. Then an intense convulsive crisis occurrs generally fatal, sometimes accompanied by hemorrhages. Sometimes the animal survives the first crisis, and after a period of prostration, dies in the course of it or else in crises following.

It is necessary, to observe reproducible results, to utilize a preference for animals from the same stock, the same age, and in good sanitary condition. Fasting aggravates acute poisioning and certain epizootic diseases can change it.

As a result of our experiments, actually supported by 2500 mice, we have observed in addition, that for one equal dose of NH_4Cl injected subcutaneously, the survival times were variable as a function of factors influencing the diffusion of the toxin in the organism.

With our experimental equipment, for mice of equal weight of 20g. $\pm$ 1, the injection under the fold of the groin with 0.5 ml. of an NH_4Cl solution (equals 1.50g/kg.) causes the death of 100% of the animals. Under

the same conditions, the injection of 1.25 g/kg. produces 75 to 80% mortality; 1 g/kg. corresponds practically to the LD_{50} and 0.50 g/kg. does not produce death. For lower doses than the LD_{100}, the appearance of symptoms of poisioning is a little slower. When the animal does not appear to be in a convulsive crisis after 60 min. death rarely happens and we were not able to bring about a slow lethal dose.

For a 1.50 g/kg. dose injected s.c. in a volumn of 0.5 ml., corresponding to a solution of 6% NH_4Cl, one observed the death of all the animals in the experiment with a mean survival time between 10 and 12 min. This amount of poison administered by the same means in a volume of 1 ml. allowed us to witness an extension of mean survival time, and the same dose, in 2 ml. the survival of more than half of the animals.

In the other part, injection under the skin of the back generally provoked a more rapid death than injection under the fold of the groin.

When one injects the poison in 0.5 ml. in 15 to 20 sec. the mean survival time is around 50% longer than the one recorded for injections done in 2 sec.

If the animals were not separated, they fought during the agitation phase, which accelerated the appearance of convulsive crises. Blows on the cage or violent noises produced the same phenomenon. In contrast, handling the animals in an isolated laboratory, their division in individual cages protected from too bright a light, permitted prolonged survival time.

In anesthetizing the animals with chloroform, we observed even with the injection of doses equal to twice that of the LD_{100} - a survival of several hours. Moreover, the animals seem to die from poisioning caused by the anesthetic, because controls not injected died practically in the same periods of time.

Therefore the survival time appeared to have been directly influenced by the diffusion velocity of the NH_4Cl. These observations seem to confirm

the hypothesis that there exists a threshold for the elimination of poison by the organism. This phonomenon is particularly important for evaluating the activity of certain substances which oppose the poisioning induced by NH_4Cl. If a substance studied were to reduce the movement of the animals, it would be difficult to validly interpret its effectiveness.

TOXICOLOGY AND APPLIED PHARMACOLOGY 17, 795–803 (1970)

Ammonia Toxicity in Rats: Protection by α-Methylglutamic Acid[1]

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Ammonia Toxicity in Rats: Protection by α-Methylglutamic Acid. LAMAR, CARLOS, JR. (1970). *Toxicol. Appl. Pharmacol.* 17, 795–803. The administration of α-methylglutamic acid (MGA) in doses of 6 mmoles/kg 1 hour prior to the administration of 11 mmoles/kg of ammonium chloride (LD90) protected almost all animals from the toxic effects of ammonia. Studies performed show that MGA did not significantly increase urea synthesis nor the excretion of ammonia in the urine. It was shown that although MGA can serve as a substrate for glutamine synthetase activity the detoxification was apparently not mediated through the formation of methylglutamine. The possible usefulness of this agent in treating hyperammonia syndromes is discussed.

Intermediates of the urea cycle used to protect from ammonia toxicity in patients with liver disease have given disappointing results (Reynolds *et al.*, 1958), This is in spite of experimental effectiveness in lowering the toxicity of exogenously administered amino acids and ammonia (Gullino *et al.*, 1956; Greenstein *et al.*, 1956; du Ruisseau *et al.*, 1956). *N*-Acetyl glutamate stimulates the synthesis of carbamyl phosphate, the precursor of ornithine (Allen and Jones, 1966), and this compound, as well as other *N*-acetyl derivatives, has been shown to lower serum ammonia and protect animals from ammonia administration (Chiosa *et al.*, 1965). An acceptable explanation for the dichotomy between experimental findings and therapeutic results is that in a diseased liver the capability of urea formation is diminished (White *et al.*, 1955). Chiosa *et al.* (1965) reported that α-methyl-DL-glutamate (MGA) pretreatment increased the survival from lethal doses of ammonium chloride (NH_4Cl) in rats significantly, while glutamate, aspartic acid, and α-ketoglutarate pretreatment did not. He postulated that the ability of MGA to detoxify ammonia was related to α-methylglutamine (MGN) formation (personal communication). No information related to the metabolic effects of MGA has appeared in the literature. Since the protection by MGA did not apparently involve reactions known to be impaired in cirrhosis, an investigation of its effects *in vitro* and *in vivo* was undertaken.

Several important aspects of MGA metabolism are known. Glutamine synthetase (GS) is active stereospecifically toward α-methylglutamic acid producing α-methylglutamine (Lichenstein *et al.*, 1953; Kagan *et al.*, 1965). MGA inhibits dog kidney glutaminase, but not glutamic acid dehydrogenase (Lichenstein *et al.*, 1953). Roberts (1953) has shown that mouse brain glutamic decarboxylase is inhibited by MGA and that

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the inhibition becomes progressively more noncompetitive in nature upon preincubation.

This report is concerned with an attempt to elucidate the manner by which MGA protects rats from lethal ammonia intoxication.

MATERIALS AND METHOD

The investigation involves the use of methionine sulfoximine (MSO) a known inhibitor of glutamine synthetase. The protective action of MGA is studied in animals pretreated with MSO and in nephrectomized animals. Serum and urinary levels of ammonia, urea, and total amino acids are determined by conventional methods. Thin-layer chromatography is used to attempt a quantitation of the amino acids excreted under various experimental conditions.

Tris(hydroxymethyl)aminomethane (Tris), ATP, NADH, glutamohydroxamate, and α-DL-methylglutamic acid was obtained from Sigma Pharmaceuticals, St. Louis, Missouri. Glutamic dehydrogenase and L-methionine-DL-sulfoximine and crystalline amino acids were purchased from Calbiochem, Los Angeles, California. All other reagents were of the highest purity available.

GS assay. Fasted male Holtzman rats (100–120 g) were used throughout the experiments. At appropriate times the animals were decapitated and the liver and brain removed, cooled in ice, weighed, and homogenized in 9 volumes of 0.001 M Tris (pH 7.4) by means of a glass homogenizer and tight-fitting Teflon plunger. GS activity was assayed using glutamic acid and neutralized hydroxylamine as substrates as previously described (Sellinger and deBalbian Verster, 1962). A unit of enzyme refers to the formation of 1 μmole of glutamohydroxamate per hr per ml of homogenate. Proteins were determined by method of Lowry *et al.* (1951).

Drug administration. All injections were administered ip. MGA at a dose of 5 mmoles/kg was always given 1 hr prior to the injection of 11 mmoles/kg of ammonium chloride (7.3 mmoles/kg was used in metabolic experiments). When MSO was given, a subconvulsive dose of 200 mg/kg was administered 3 hr prior to the administration of the MGA. Animals killed by ammonia were decapitated immediately. Those surviving were sacrificed 1 hr after the administration of the ammonium chloride.

Bilateral nephrectomies were performed on the afternoon prior to the experiment, at which time there was no noticeable effect of surgery nor from uremia. The LD90 dose of ammonium chloride was carefully determined for anephric rats and found to be considerably less than in intact animals: 7.1 *vs.* 11 mmoles/kg.

Urine collections. Plastic metabolic cages equipped to eliminate urine contamination with feces were used. Animals were fasted for 18 hr and were placed in cages after the administration of the ammonium chloride. Urine collection was carried out for 4 hr.

Animals received MGA in 0.45 M NaCl or NaCl alone in volumes of 10 ml/kg. Ammonium chloride was given at a reduced dose of 7.3 mmoles/kg to allow survival. It was injected in volumes of 5 ml/kg, or this volume of NaCl was given alone to controls.

Determination of urea, ammonia, and amino acids in urine. Aliquots of urine were placed in the outside well (Clarkson and Ferraio, 1969) of modified Conway diffusion flasks provided with a rubber stopper. 4 N H_2SO_4 was placed in the center well. One ml

of 1 M borate buffer (pH 10.0) was injected in the outside well, and diffusion was allowed at 37°C for 3 hr. Ammonia was determined by the Nessler's reaction on an aliquot of the inside well. The ninhydrin reaction was carried out by the modified (Clarkson and Ferraio, 1969) method of Rubinstein and Pryce (1959) on an aliquot of urine in the outside well after diffusion. Urea was determined by Hycel method (Hycel, Inc., Box 36329, Houston, Texas 77036).

Determination of urea, ammonia, and amino acids in plasma. The determination of plasma urea, ammonia, and amino acids was done on blood collected in cooled tubes containing 0.1 ml of heparin solution (10,000 U/ml) following decapitation. Animals were sacrificed 15 min after the administration of ammonium chloride (7.3 mmoles/kg) or NaCl. One group receiving ammonium chloride had been pretreated with MGA (5 mmoles/kg). The plasma was removed following centrifugation and always maintained at the temperature of melting ice. A portion was rendered protein free with 15% perchloric acid, and after neutralization with KOH the clear supernatant was added to modified Conway flasks containing sulfuric acid in the center well. One ml of borate buffer, 1 M pH 10, was added through the rubber stopper and diffusion was allowed for 3 hr. The outside well was removed, and the amino acids remaining were assayed by a ninhydrin method (Clarkson and Ferraio, 1969).

Urea was determined in duplicate by the Hycel method.

Ammonia was determined by adding 0.1 ml of plasma to a 1-ml cuvette containing 100 mM Tris buffer pH 7.4, and 20 mM α-ketoglutarate, 1.3 μM NADH, and 0.2 mg of L-glutamic dehydrogenase. The reaction was monitored at 37°C in a Gilford Model 2000 multiple-sample absorbance unit equipped with a recorder. The reaction rate for the initial 2 min was used in calculations. Appropriate blanks and a control curve employing 0.02, 0.04, and 0.08 μmole of ammonia were run before and after the samples. The results by this method were somewhat higher than by diffusion, but variations from control were the same.

Chromatographic separation of urinary amino acids. Thin-layer chromatography was performed on glass plates prepared with cellulose powder. Two μg of urine was spotted, and the plates were run in pyridine–butanol–water (30:20:10) as the first solvent and aqueous phenol (80%) in the second dimension. Control plates of 0.05 μmole of known amino acids were used to identify the amino acids in urine and determine their concentrations. The plates were dried and were developed with ninhydrin (0.2% in alcohol).

The ninhydrin spots were removed quantitatively 24 hr after spraying and were eluted into a solution of 0.1% $CuSO_4$ in 75% ethanol; the OD was determined at 509 nm.

The quantities of MGA and methylglutamine in some of the urine specimens were such that very small quantities of urine could be applied to avoid overloading. Therefore only amino acids present in urine in concentrations over 1 mmole ml were visible.

RESULTS

The Effects of MSO in Vitro

As previously reported (Lichenstein *et al.*, 1953) MGA may serve as substrate in the GS reaction, and the formation of methylglutamine from this reaction has been demonstrated by Kagan *et al.* (1965) and Kagan and Meister (1966).

The results of a study of the affinity of rat cerebral GS for MGA and the effects on this reaction of MSO, a known inhibitor of GS (substrate: glutamate) are demonstrated in Fig. 1, where kinetic studies using both L-glutamate and α-methyl-DL-glutamic acid alone and in the presence of MSO are shown. Judging from the K_m values, glutamate ($K_m = 2.2 \times 10^{-3}$ M) is the more specific substrate (DL-MGA, $K_m = 4.4 \times 10^{-3}$ M). The results of the experimentally determined K_m compare favorably with those reported by Kagan and Meister (1966) for L-glutamate (3.3×10^{-3} M) and L-MGA (6.4×10^{-3} M). Their data support the concept that glutamate is a more specific substrate for GS. It can

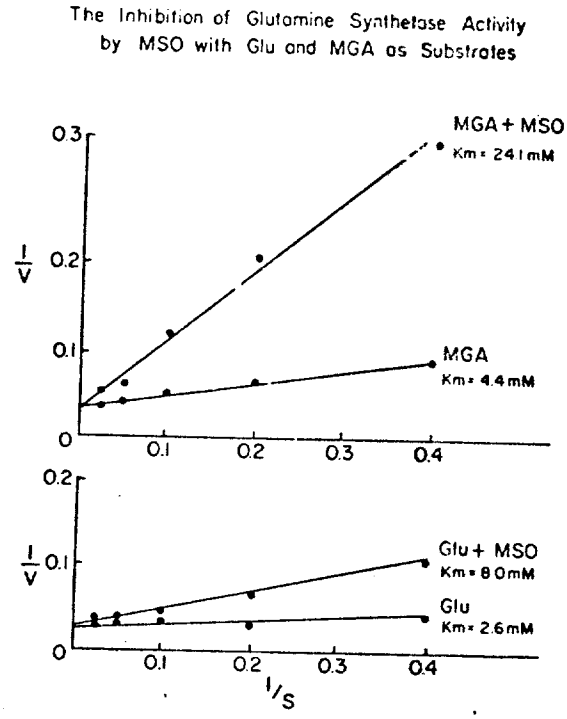

FIG. 1. Lineweaver-Burke plots of rat cerebral glutamine synthetase activity with glutamate or MGA as substrate, both in the presence and the absence of 1 mmole MSO. 1/S is the reciprocal of the substrate concentrations in μmoles/ml. Velocity is expressed as μmoles of glutamohydroxymate synthesized by 1 ml of a 10% homogenate (w/v) per hour.

be seen that MSO inhibits the reaction apparently in a competitive manner and to a far greater extent when MGA is the substrate.

Although the nature of the inhibition appears to be competitive here and as reported by others (Sellinger and Weiler, 1963), careful study has shown that it is partially competitive and partially irreversible even *in vitro* (Ronzio *et al.*, 1969; Rowe *et al.*, 1969). The *in vivo* inhibition is entirely irreversible (Lamar and Sellinger, 1965).

The protection from ammonia toxicity by the preadministration of MGA as reported by Chiosa *et al.* (1965) was confirmed. Most animals pretreated with MGA (5 mmoles/kg) showed no effects from the administration of ammonium chloride in dosages (11 mmoles/kg) that were lethal within 15 min to 93% of unprotected animals. It was remarkable to see the lack of violent behavior in the protected animals.

To test the possibility that α-methyl glutamate was acting to eliminate ammonia through the formation of α-methylglutamine, subconvulsive doses of MSO, sufficiently high to inhibit the GS reaction, were administered. Accordingly, 6 groups of 5 rats were treated with (1) saline, (2) MGA (5 mmoles/kg), (3) MGA 1 hr prior to NH_4Cl, (4) DL-MSO (12 mmoles/kg[2]—one-half convulsive dose) 4 hr prior to NH_4Cl, (5) MSO 3 hr prior to MGA and followed in 1 hr by NH_4Cl, and (6) with NH_4Cl alone. The animals not dead after 2 hr were sacrificed, and GS activity was determined on the livers and brains of all animals. The results are tabulated in Table 1. It can be noted that MSO administration inhibited GS activity in the brain and liver to 20% and 5% of control, respectively. It is of significance that MSO administration did not reduce the protective capabilities of MGA. Assuming that the inhibition of the GS reaction effectively prevented the formation of methylglutamine, the explanation of the protection of ammonia toxicity by MGA required a search for another mechanism.

TABLE 1

RATS DYING AFTER ADMINISTRATION OF NH_4Cl, MSO, and MGA IN VARIOUS COMBINATIONS, AND GLUTAMINE SYNTHETASE ACTIVITY IN THEIR TISSUES

Dose	Died[a] total	% Surviving	GS activity[b] Liver ±SE	GS activity[b] Brain ±SE
Control	0/15	100	21 ± 1.5	16.5 ± 0.3
MGA (6 mmoles/kg)	0/10	100	19.7 ± 2	16.5 ± 0.3
NH_4Cl (11 mmoles/kg)	14/15	7	21 ± 3.5	17.5 ± 0.3
MSO (11 mmoles/kg)	0/14	100	1.8 ± 0.1	3.0 ± 0.1
MGA/NH_4Cl	2/18	89	24.8 ± 2.2	16.6 ± 0.4
MSO/MGA/NH_4Cl	3/25	80	1.5 ± 0.4	2.3 ± 0.4
MSO/NH_4Cl[c]	9/10	10	1.5 ± 0.1	4.0 ± 0.3

[a] Died/total refers to the number of rats dying of total used in experiment.

[b] A unit is that amount of enzyme necessary to form 1 μmole of glutamohydroxymate per hr wet g of tissue.

[c] At a dose of NH_4Cl of 8.6 mmoles/kg, we observed 50% improvement of survival.

In order to study the role of the kidney, a total of 37 rats were subjected to bilateral nephrectomies the afternoon prior to the experiment. The LD90 of ammonium chloride for anephric rats was estimated to be 7.1 mmoles/kg, compared to the LD90 for intact animals of approximately 11 mmoles/kg. The remaining 13 nephrectomized animals were pretreated with MGA, and all survived the administration of 7.1 mmoles/kg of ammonium chloride and showed few side effects. Although the renal system mediated some protection from ammonia intoxication, the action of MGA was apparently not related to an enhancement of this mechanism.

The next procedure entailed the collection and study of urine excreted under various experimental conditions. This was accomplished with the use of individual plastic metabolic cages. Each group contained a minimum of 6 rats. The dosage of NH_4Cl was lowered to 7.3 mmoles/kg to permit the survival of the rats during the 4-hr experimental period. Even at this dosage the animals became violently ill, but all survived.

[2] MSO administration at this dose produces few gross abnormalities.

Table 2 shows the results of this experiment. The animals receiving ammonia alone had a greater urine volume and a greater total output of ammonia and urea, but not of amino acids. However, in the group receiving MGA and ammonium chloride, the amount of ammonia excreted above the control was very small.

TABLE 2

URINARY EXCRETION OF AMMONIA, AMINO ACIDS, UREA, AND PROTEIN IN CONTROL ANIMALS AND RATS TREATED WITH NH_4Cl AND MGA + NH_4Cl (VALUE ± SE)

	Control	NH_4Cl (7 mmoles/kg)	MGA (6 mmoles/kg)	MGA plus NH_4Cl
Ammonia (μmoles/ml), total[a]	61 ± 18	405 ± 20.0	27 ± 3.2	97 ± 15
Ninhydrin-reacting substance after diffusion (μmoles/ml),[b] total[a]	2.7 ± 1.0	6.5 ± 2.1	31 ± 4	30 ± 3.3
Urea (μmoles/ml), total[a]	720 ± 116	2140 ± 145	390 ± 35	510 ± 68
Protein (mg total)	1.9	4.2	1.5	1.3
Volume	1.5	4.7	0.8	1.1

[a] Total refers to total amount recovered in urine over a 4-hr period.
[b] After diffusion refers to the amount remaining after urine was alkalinized and a 3-hr diffusion period allowed. See text for complete explanation.

In rats given MGA alone, ammonia and urea excretion fell below control levels. However, when MGA is administered alone or combined with ammonia, the excretion of amino acids is increased 15-fold over control values and 5-fold over the group given ammonia alone. It is of interest to note that the diuretic effect of ammonia was completely reversed by MGA administration.

In a repeated experiment identical groups of rats were compared, but a group that received MSO 3 hr prior to receiving MGA plus NH_4Cl was also included. The urine was chromatographed on thin-layer cellulose plates as discussed in the Materials and Methods section. The results are shown in Table 3. It is apparent that the major portion

TABLE 3

CONCENTRATION OF SOME URINARY AMINO ACIDS FROM RATS UNDER VARIOUS CONDITIONS AS DETERMINED[a] BY THIN-LAYER CHROMATOGRAPHY

Urinary amino acids: (μmoles/ml glycine equivalents)	Treatment group				
	Control	NH_4Cl	MGA	MGA NH_4Cl	MSO MGA NH_4Cl
Taurine	12.5	2.1	9.0	4.7	3.7
MGA	—	—	14.7	35	37
Methylglutamine	—	—	16.0	28	1.2
Unidentified	—	—	—	—	44
Urine volume[b]	0.6	5.0	0.9	2.0	1.5

[a] Two μg of urine was spotted and chromatographed; the amino acids were identified and eluted, and concentrations were compared to the known.
[b] Volume of urine collected over a 4-hr period.

of amino acids excreted when MGA is given are MGA and methylglutamine. It can also be noted that, when MSO is administered, very little methylglutamine is excreted in the urine.

That MGA probably does not lower serum ammonia by stimulating urea synthesis was shown by the decreased urea excreted in MGA-pretreated animals compared to control. Nevertheless the levels of plasma urea, ammonia, and total amino acids were determined on blood collected by decapitation of the animals 15 min after the administration of ammonia. The plasma values of urea, ammonia, and ninhydrin reacting substances can be seen in Table 4. Ammonia administration elevates the

TABLE 4

CONCENTRATION OF UREA, AMMONIA, AND AMINO ACIDS IN PLASMA FROM CONTROL RATS AND RATS RECEIVING NH_4Cl OR MGA AND NH_4Cl

Treatment group	Plasma		
	Urea (mmg/100 ml ±SE)	Ammonia (μg/100 ml ±SE)	Amino acids (μmoles/ml ±SE)
Control	10.0 ± 0.3	190 ± 40	8.0 ± 0.2
NH_4Cl (7 mmoles/kg)	15.2 ± 1.9	480 ± 100	7.9 ± 0.6
MGA (11 mmoles/kg) + NH_4Cl	17.5 ± 1.0	260 ± 20	6.6 ± 0.5

plasma urea level, but MGA pretreatment does not change the level significantly. However, it can be noted that MGA prevents the plasma ammonia rise that is seen in unprotected animals. Plasma amino acid levels are apparently lowered by MGA administration. The possibility that NH_4Cl causes death within 15 min by precipitating a sudden acidosis, and that MGA acts as a buffering agent, was largely eliminated by findings that the administration of equimolar concentrations of HCl does not do so.

DISCUSSION

The significance of this report lies in the presentation of DL-α-methylglutamic acid as a protective agent in ammonia toxicity. The possibility of implementing MGA in the management of hyperammonia of different sources prompted an investigation of its metabolic effects.

Initially the suggestion of Chiosa that MGA was protecting animals from ammonia toxicity through the formation of methylglutamine was examined (personal communication). In the light of previous work by Warren and Schenker (1964), Laha and Kochar (1964), and Schenker *et al.* (1967), this explanation seemed unlikely. These workers demonstrated that the toxic event caused by ammonia was probably glutamine formation, which in turn could lead to ATP and α-ketoglutarate depletion. Accordingly, it has been shown here that MGA protects animals from ammonia toxicity even when glutamine and methylglutamine synthesis is prevented.

Since data presented indicated that MGA administration lowers blood ammonia

levels, an MGA-mediated stimulation of urea synthesis was examined. This possibility was lessened by the demonstration that MGA-protected animals did not excrete more urea than controls and that serum urea levels were not significantly elevated. Additionally, MGA administration did not increase ammonia excretion in the urine.

The manner in which MGA exerts its effects remains largely unanswered Katunuma *et al.* (1966) have shown that the oxidation of isocitrate to α-ketoglutarate is very sensitive to ammonia inhibition and that ammonia activates a pathway that oxidizes DPNH and TPNH to nicotinamide. They propose that ammonia results in ATP depletion by inhibiting the citric acid cycle and simultaneously by lowering the availability of agents capable of reducing the cytochrome oxidase system.

One alternate mechanism that is currently under study is that MGA acts by activating or stabilizing glutamic dehydrogenase, an enzyme that could lower ammonia levels by causing a synthesis of glutamic acid.

The potential usefulness of MGA in the therapy of hyperammonia syndromes warrants continued research in this area.

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THE EFFECT OF ORAL AMMONIUM CHLORIDE ON THE URINARY EXCRETION OF CALCIUM, MAGNESIUM AND SODIUM IN MAN

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IT is generally accepted that the development of renal calculi is favoured by the presence of hypercalciuria (Flocks, 1939; Hodgkinson and Pyrah, 1958; Maurice and Henneman, 1964), and that systemic acidosis, whether due to an acid ash diet (Farquharson, *et al.*, 1931), renal tubular acidosis (Albright, *et al.*, 1946) or the prolonged ingestion of ammonium chloride (Martin and Jones, 1961; Parfitt *et al.*, 1964; Lemann *et al.*, 1967) may cause an increase in the urine calcium.

In a recent study of 265 patients with urinary calculus disease (Lavan, *et al.*, in preparation) renal tubular acidosis was present in at least 10 instances, and it is therefore apparent that a complete investigation of patients with renal stones should include a screening test for renal tubular acidosis. The most commonly used method for such a screening is that proposed by Wrong and Davies (1959) during which a single dose of ammonium chloride is administered orally.

The present study was undertaken in order to discover the effect of such an acid load on the urinary calcium, magnesium and sodium a) on the day of the acid load and b) on the day following such a test.

Patients and Methods

Twenty-four persons (18 male and 6 female) whose ages ranged from 26 to 69 years were studied. Twenty-one were hospital in-patients with a variety of diseases and 3 were members of the hospital staff. No selection was exercised in the choice of subjects apart from the requirements that all be fully ambulant and that no alkaline mixtures be administered at any time during the test. The group included 2 persons with urinary calculi (both normocalciuric), 2 subjects without a history of calculi but with a urine calcium in excess of 300 mg. per 24 hours, and 18 patients who were being treated for various conditions such as myocardial infarction, obstructive airways disease or neurological complaints. The remaining 2 subjects were considered normal in all respects.

Apart from 2 patients who had been receiving 5 mg. Bendrofluazide daily for some months, no relevant medication was being taken. No alteration was made to the diuretic therapy in these 2 patients and no dietary restrictions were imposed during the test.

Serum calcium and urea nitrogen were estimated in all subjects before the test was commenced. Twenty-four hour urine samples were obtained on 3 consecutive days, the collection commencing before breakfast each day. All cases in which there was any doubt regarding the completeness of the sample were rejected. Ammonium chloride capsules (0.1G per Kg weight) were administered orally with breakfast on the second day. Each patient therefore acted as his own control, Day 1 representing base line values, Day 2 values on the day of the acid load, and Day 3 those obtained on the day after the acid load.

Urine acidity was estimated by a glass electrode pH meter 4 hours after the

administration of ammonium chloride, all samples used for estimation of the urine pH being returned to the 24 hour specimen. Adequate acidification for the purposes of the test was considered to have occurred in all cases, urinary pH having reached 5.3 or lower in 22 of the 24 subjects, and pH 5.6 in the other 2. Serum and urine calcium were estimated by means of an atomic absorption spectrophotometer (Techtron, Model AA-100) using a diluent containing 0. 1N HCL, 0. 1N KCL and 6% Butanol. The normal mean and standard deviation for serum calcium by this method is 8.9 mg. per 100 ml. ± 0.35 mg. per 100 ml. Blood urea nitrogen estimations were carried out with the Technicon AutoAnalyzer according to the method of Marsh et al. (1957). The mean value for normal persons using this method is 15.0 mg. per 100 ml. (± 3.5 mg. per 100 ml.). Urine magnesium was also estimated by atomic absorption, with the same diluent as described for calcium, and the Technicon AutoAnalyzer (Technicon Corporation, 1963) was used in the estimation of urinary sodium.

Serum calcium and urea nitrogen values were within 3 standard deviations of the normal mean in all subjects.

Results

In 21 subjects (16 males and 5 females) higher 24 hour urine calcium values were obtained on Day 2 than on Day 1. This increase in urine calcium on the day of the acid load was found to be statistically significant at the 0.1% level (Table I). By Day 3, the urine calcium had returned to below the base line in 7 of the 22 subjects on whom data were available. In the other 15 (10 males and 5 females) however, the urine calcium values were still significantly raised (Table I) and in 4 of these the urine calcium was at its highest level on this day.

TABLE I

Mean values showing the effect of a single dose of ammonium chloride on urinary calcium, magnesium and sodium.

24 hour Urine	Number Studied	Mean Values (mg or mEq per 24 hrs.)	Mean Increases from Day 1 (mg or mEq per 24 hrs.)	p Values for Mean Increase*	Mean Urine Volume (ml/24 hrs.)
CALCIUM (mg/24 hrs.)					
Day 1	24	147.4	—	—	1568.1
Day 2	24	208.0	+60.6	$p<0.001$	1846.7
Day 3	22	172.4	+23.8	$p<0.05$	1751.4
MAGNESIUM (mg/24 hrs.)					
Day 1	24	90.8	—	—	1568.1
Day 2	24	110.6	+19.8	$p<0.001$	1846.7
Day 3	22	99.4	+7.6	$p<0.2$	1751.4
SODIUM (mEq/24 hrs.)					
Day 1	17	149.5	—	—	1564.7
Day 2	17	181.3	+31.8	<0.05	1834.7
Day 3	14	135.2	−17.3	$p<0.4$	1832.1

* Mean increase was evaluated by Students t test using the equation : $t = (d-0)/(s/\sqrt{n})$, where d and s are the mean and standard deviation of the individual increases from Day 1. (Snedecor, 1956).

Similar effects were observed on urinary magnesium and sodium (Tables 1 and 2) but the effect on sodium excretion was of shorter duration. Thus, in half the subjects tested, the increase in sodium excretion which had been observed on Day 2 had disappeared by Day 3. A significant positive 225

TABLE II

The effect of a single dose of ammonium chloride on urinary calcium, magnesium and sodium.

	Number of patients in whom values on Day 2 exceeded values on Day 1	Number of patients in whom values on day 3 exceeded values on Days 1	Number of patients in whom values on Day 3 exceeded values on Days 1 and 2
URINE CALCIUM	21/24 (87.5%)	15/22 (68.2%)	4/22 (18.2%)
URINE MAGNESIUM	20/24 (83.3%)	17/22 (77.3%)	6/22 (27.3%)
URINE SODIUM	12/17 (70.6%)	7/14 (50.0%)	2/13 (15.4%)

linear correlation was found to exist between the excretion of calcium and magnesium throughout the test and between that of magnesium and sodium on the day of the "acid load" (Table 3).

TABLE III

The significance of the linear correlation between the excretion of calcium, magnesium and sodium following a single dose of ammonium chloride.

	Ca - Mg	Ca—Na	Mg—Na
DAY 1	$r=0.5045$ $p<0.05$	$r=0.0632$ $p>0.1$	$r=0.4369$ $p<0.1$
DAY 2	$r=0.6121$ $p<0.01$	$r=0.3265$ $p<0.1$	$r=0.5444$ $p<0.05$
DAY 3	$r=0.5395$ $p<0.01$	$r=0.2523$ $p<0.1$	$r=0.4874$ $p<0.1$

Discussion

Many workers have demonstrated an increase in urine calcium following the prolonged administration of ammonium chloride (Farquharson, *et al.*, 1931; Martin and Jones, 1961; Lemann *et al.*, 1967) but a review of the medical literature has yielded only one report concerning the effect of a single oral dose of ammonium chloride on such excretion (Martin and Golovsky, 1966). These workers concluded from a study of 5 patients with renal calculi and 5 normal subjects that the administration of an ammonium chloride load as used in the present study, caused no significant rise in the urine calcium during the day of such administration. However, it was not stated that the two 24 hour estimations were consecutive, a point which is of great significance in view of the possible effect of variations in diet on the

urine calcium (Knapp, 1947; Peacock, *et al.*, 1967). Moreover, although the difference was not statistically significant, higher urine calcium values were in fact found by Martin and Golovsky following acid load in 7 out of the 10 of their subjects.

Several theories have been proposed to explain the increased urine calcium in subjects who are acidotic. Albright, *et al.* (1946) maintained that in tubular acidosis "calcium being a base, will appear in increased amounts in the urine" whereas Epstein (1960) considered that the urinary calcium excretion of metabolic acidosis was the result of a primary dissolution of bone rather than the requirement for calcium as a urinary buffer. Walser and Robinson (1963) suggested that the augmented urinary calcium excretion in acute acidosis was directly related to, and dependent on a concomitant increase in urinary sodium excretion. On the basis of an increased urine calcium in spite of a reduction in the calculated filtered load of this substance, Lemann, *et al.* (1967) considered the calcium excretion in cases of metabolic acidosis to be the result of a direct effect of such acidosis on the metabolic processes within the renal tubular cells. Finally, it is possible that the increased urine calcium in acidosis results from the increased ultrafilterable fraction of calcium that is found with reduction of blood pH. (Myers, 1962).

Similarities between the renal tubular handling of calcium and sodium (Lassiter, *et al.*, 1963, Parfitt, 1969) and calcium and magnesium (Samiy, *et al.*, 1960) have been demonstrated, and it has been claimed that these ions may compete for a common tubular reabsorptive pathway (Walser, 1961; Alcock and MacIntyre, 1962). In a study of 72 patients with renal calculus disease it was found that hypercalciurics excreted significantly more magnesium in the urine than normocalciurics who were matched for age, sex, serum calcium and blood urea nitrogen (Lavan, *et al.*, in preparation). This finding and the present investigation support the hypothesis that an interrelation exists between the reabsorption of calcium and magnesium by the renal tubules. However, the poor correlation observed between calcium and sodium excretion in this study suggests that the increased urinary calcium excretion found in acidotic subjects is not dependent on the increase in urinary sodium excretion.

It is concluded that whilst the mechanism of the calciuria, magnesiuria, and natriuria in acidotic subjects remain unresolved, the interrelationship between the renal excretion of calcium and magnesium has been further demonstrated. It is to be emphasised that prolonged acidosis is not essential to increase the urinary excretion of these substances significantly.

Summary

The effect of a single oral dose of ammonium chloride on urinary calcium, magnesium and sodium, has been studied.

A significant increase in the urine values of all three cations was observed on the day of the ammonium chloride administration. A significant increase in urine calcium persisted on the next day. A significant positive correlation was observed between urinary calcium and urinary magnesium on the 3 days of the test.

In order that falsely elevated values should not be obtained, twenty-four hour urine calcium estimations should not be carried out for at least 48 hours following an ammonium chloride acid load.

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A Comparison of the Effects of Glucose Ingestion and NH_4Cl Acidosis on Urinary Calcium and Magnesium Excretion in Man

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ABSTRACT Both glucose ingestion and NH_4Cl acidosis have been reported to augment urinary calcium ($U_{Ca}V$) and magnesium ($U_{Mg}V$) excretion. Both also cause acidification of the urine and an increase in renal acid excretion. To examine whether a common mechanism of action was involved, the effects of glucose ingestion and NH_4Cl acidosis on $U_{Ca}V$ and $U_{Mg}V$ were tested in the same subjects. Glucose ingestion caused significant increases in both $U_{Ca}V$ and $U_{Mg}V$. During stable NH_4Cl acidosis, $U_{Ca}V$ increased significantly, while $U_{Mg}V$ was unaffected. When a glucose load was given during acidosis, the separate effects of acidosis and glucose on $U_{Ca}V$ were additive, whereas $U_{Mg}V$ increased less than observed during normal acid-base balance. Although renal acid excretion increased and the urine was acidified after glucose in the normal steady state, when glucose was administered during NH_4Cl acidosis urine pH rose and there was no change in renal acid excretion. We concluded that NH_4Cl acidosis and glucose ingestion reduce the renal tubular reabsorption of magnesium and (or) calcium, but they act through separate mechanisms.

INTRODUCTION

The ingestion of food (1) or any of a variety of rapidly metabolizable substrates (2) causes a sharp increase in urinary calcium and magnesium excretion. Because this occurs without detectable changes in the glomerular filtration rate (2, 3) or in the concentrations of calcium and magnesium in serum ultrafiltrates (3), it appears to be due to diminished renal tubular reabsorption of calcium and magnesium. We noted that the urine was sharply acidified following glucose ingestion and renal net acid excretion (titratable acid + ammonium − bicarbonate) increased significantly in parallel with the increases in urinary calcium and magnesium excretion (4). We had observed previously that renal tubular calcium reabsorption was inhibited during stable experimental metabolic acidosis and that the increments in urinary calcium excretion during acidosis were closely correlated with simultaneous increments in urinary ammonium excretion (5). Increases in urinary magnesium excretion have also been found in some (6, 7) but not all (8, 9) studies of experimental metabolic acidosis. We speculated about whether glucose ingestion and chronic metabolic acidosis might inhibit renal tubular divalent cation reabsorption by some common mechanism. To test this, renal clearances of ultrafilterable calcium and magnesium were carried out in the same subjects before and after glucose ingestion and after the induction of stable NH_4Cl acidosis. The effects of glucose ingestion were also examined during induced acidosis to test for saturation of a common mechanism.

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METHODS

We carried out renal clearance studies in seven healthy men before and after inducing metabolic acidosis. All subjects were hospitalized in the Marquette School of Medicine Clinical Research Center. The subjects ate constant diet throughout their hospitalization which provided approximately 25 mmoles of calcium and 15 mmoles of magnesium per day. The diets were well accepted and consumed completely each day. After 4–6 days of adaptation to the diet 24 hr urine specimens were [illegible] for 4 days. Six of the subjects then had their [illegible] studies performed after which metabolic acidosis was induced by gradually increasing oral doses of NH_4Cl [illegible] days and then a constant daily dose [illegible] days. [illegible] specimens were collected throughout the period of NH_4Cl loading. In one subject, the [illegible] clearance study was performed at the [illegible]

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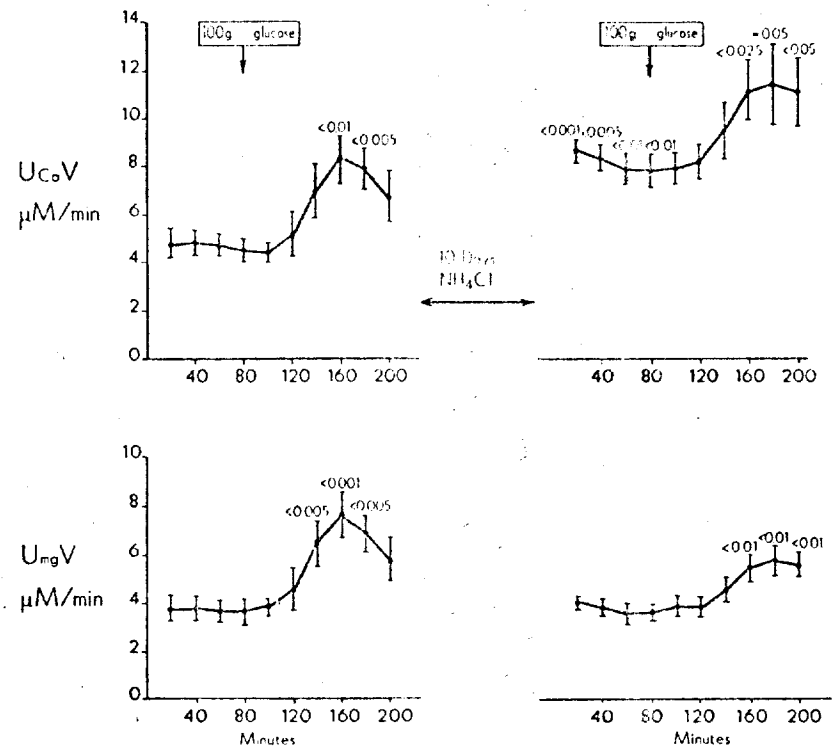

FIGURE 1 Effects of metabolic acidosis and glucose ingestion on urinary calcium ($U_{Ca}V$) and magnesium ($U_{Mg}V$) excretion.

of acid loading and a second clearance study was done 16 days after NH_4Cl administration was stopped. The results in this subject did not differ from those in the other six, indicating that the order of study did not influence the results.

Each clearance study began at 7:00 a.m., after an overnight fast. The subject drank 1 liter of water to initiate a diuresis and drank water every 20 min thereafter to replace urinary losses. After collection of a timed urine specimen and a blood specimen for inulin blank determination, a priming dose of inulin was injected and a sustaining infusion of inulin in isotonic saline was begun at 3 ml/min. After a 45 min equilibration period, four 20 min control periods were obtained.

Glucose (100 g) was then administered orally, and six 20 min experimental periods were observed. The subjects were allowed to sit or stand to void, but remained recumbent at other times during the clearance studies.

The effects of metabolic acidosis on urinary calcium and magnesium excretion were evaluated both by observing changes in the daily urinary content of calcium and magnesium after beginning NH_4Cl loading and by comparing the clearance periods prior to glucose administration in the normal steady state to those during stable metabolic acidosis. The effects of glucose ingestion on blood and urine composition during the normal steady state were compared to those observed during stable metabolic acidosis.

Each subject served as his own control. Estimates of variance throughout the text are presented as the group mean, plus or minus one standard error of the mean.

All other clearance procedures and analytical and statistical techniques employed in our laboratory have been described (5).

RESULTS

Reproducibility of measurements during the control periods were satisfactory. The coefficient of variation for the critical parameters of calcium and magnesium filtration and excretion for the individual subjects averaged: inulin clearance ±3.5%; serum ultrafilterable calcium concentration ±1.1%; serum ultrafilterable magnesium concentration ±1.1%; rate of calcium filtration ±4.2%; rate of magnesium filtration ±3.8%; $U_{Ca}V$ ±6.5%; $U_{Mg}V$ ±7.0%.

The changes in urinary calcium ($U_{Ca}V$) and magnesium ($U_{Mg}V$) excretion during the clearance studies are shown in Fig. 1. In this and all subsequent figures, the data plotted are the mean values for the seven subjects. The vertical lines through the means indicate plus or minus one standard error of the mean. During the normal steady state (left hand portion of the figure),

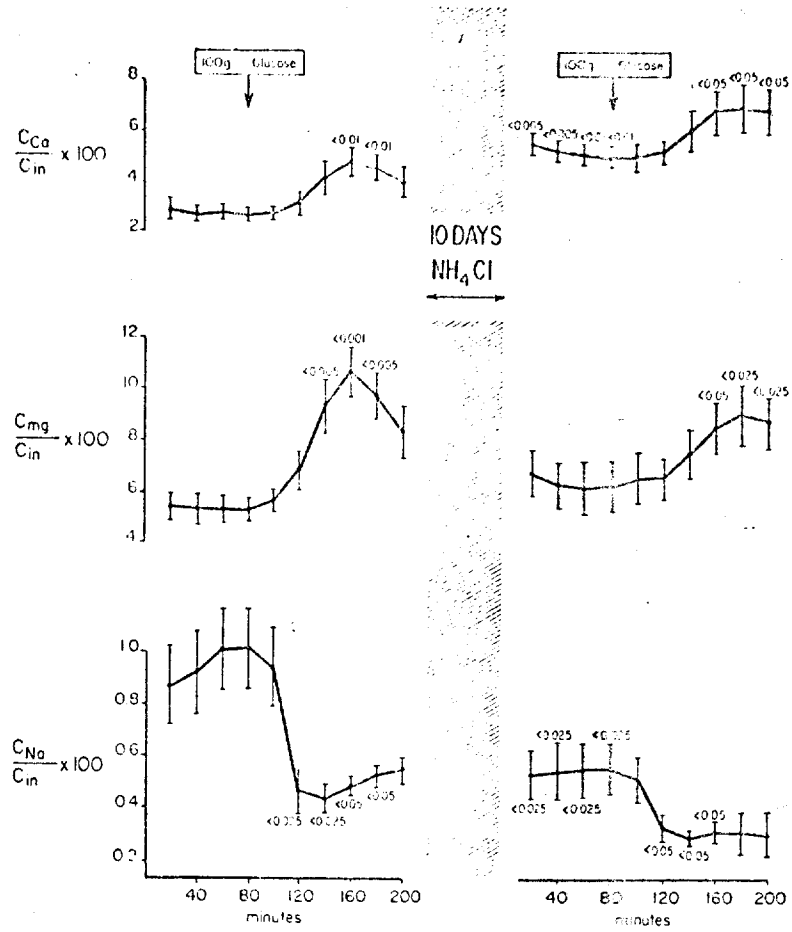

FIGURE 2 Effects of metabolic acidosis and glucose ingestion on fractional calcium, magnesium, and sodium excretion rates. See text.

the subjects' serum bicarbonate concentrations averaged 26.5 ±0.9 mEq/liter. Before glucose ingestion, $U_{Ca}V$ and $U_{Mg}V$ were steady, averaging 4.69 ±0.51 and 3.69 ±0.48 μmoles/min, respectively. After glucose ingestion, both $U_{Ca}V$ and $U_{Mg}V$ increased significantly. The right-hand portion of the figure presents the results of identical clearance studies performed after 10 days of NH_4Cl loading, when the subjects were mildly acidotic (serum bicarbonate concentrations averaging 20.3 ±0.8 mEq/liter). The effects of induced stable metabolic acidosis on $U_{Ca}V$ and $U_{Mg}V$ can be seen by comparing the four base line periods before glucose ingestion during acidosis to the same periods in the normal steady state. As shown, acidosis caused a significant increase in $U_{Ca}V$, averaging +3.50 ±0.7 μmoles/min, but had no effect on $U_{Mg}V$. Glucose ingestion during acidosis was followed by a further significant increase in $U_{Ca}V$ and a significant increase in $U_{Mg}V$.

For the group, there were no significant changes in inulin clearance rates, UF_{Ca}, UF_{Mg}, or in the calculated rates of filtration of calcium or magnesium after glucose ingestion. During acidosis, inulin clearance rates, UF_{Ca}, and UF_{Mg} tended to fall and there were reductions in the calculated rates of filtration of both calcium (−4 ±3 μmoles/min), and of magnesium (−6 ±2 μmoles/min). Since $U_{Mg}V$ did not fall during induced acidosis (Fig. 1), despite a fall in the rate of filtration of magnesium, acidosis may have partially inhibited tubular magnesium reabsorption. Clearly, however, the effect on tubular calcium reabsorption was much greater.

When changes in filtered loads and urinary excretion rates of calcium and magnesium for each subject were compared during all phases of the experiments, some correlation was found for both calcium (r = +0.51) and magnesium (r = +0.45). However, in over one-half of the experimental periods (63 of 112 for calcium and 64 of 112 for magnesium) a change in tubular reabsorption could be inferred, since urinary excretion rate

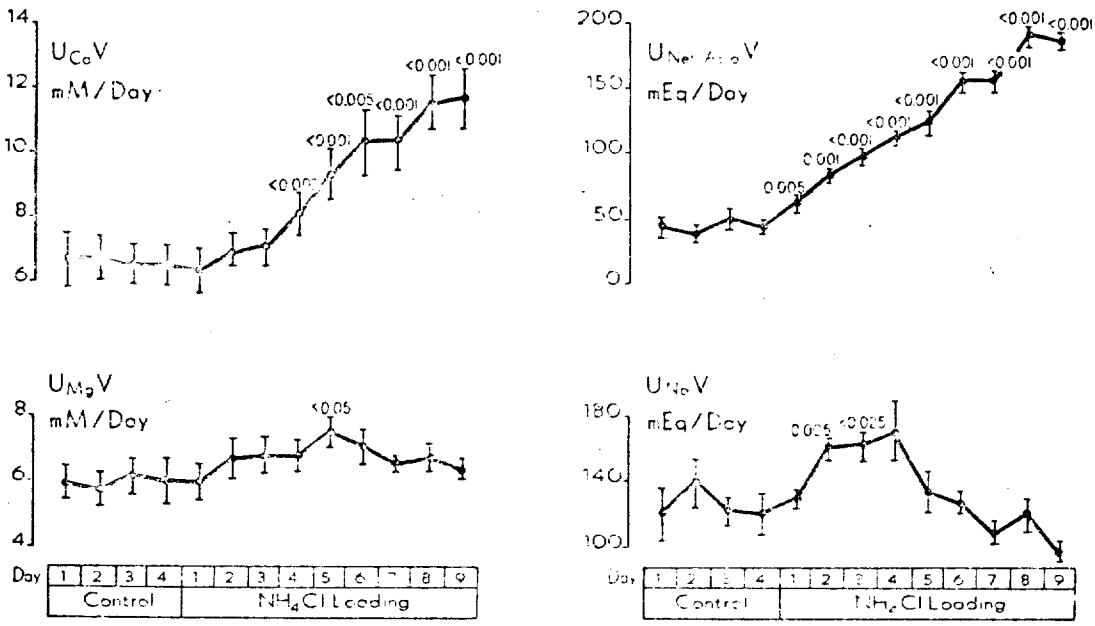

FIGURE 3 The effects of NH_4Cl administration on 24-hr urine contents of calcium, magnesium, net acid, and sodium.

increased more or fell less than did the filtered load, or increased despite a fall in the filtered load.

Fig. 2 indicates that the fractional excretion rates of calcium and magnesium [clearances of calcium (C_{Ca}) or magnesium (C_{Mg}) divided by inulin clearance (C_{In}) × 100] were altered during the experiments in

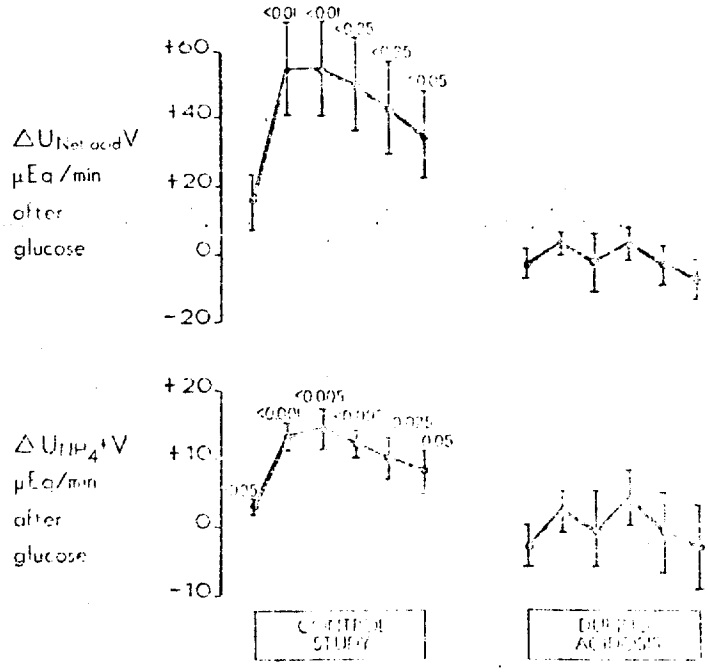

FIGURE 4 The changes from control in urine net acid and phosphate excretion following glucose injection in the normal steady state ("control study") and during metabolic acidosis.

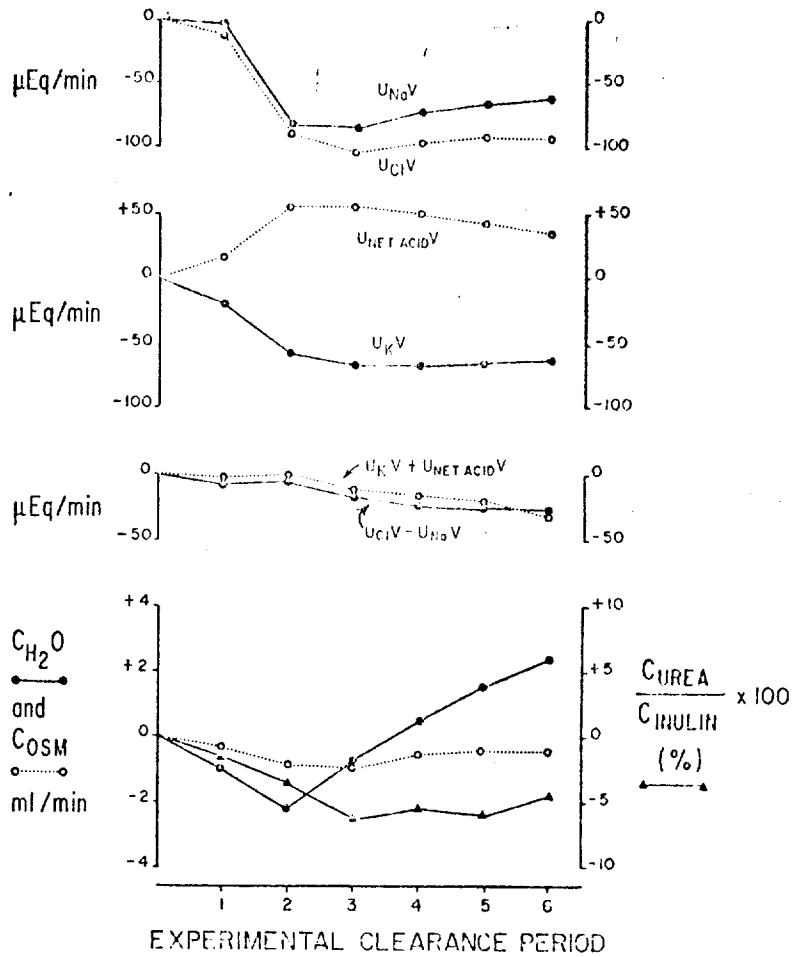

FIGURE 5 Mean changes from control after glucose ingestion in parameters relevant to segmental tubular sodium reabsorption. See text.

the same manner as $U_{Ca}V$ and $U_{Mg}V$. The lower one-third of Fig. 2 shows the simultaneous changes in fractional sodium excretion. Fractional sodium excretion fell significantly both after glucose ingestion and during induced metabolic acidosis. Since there were no significant changes in inulin clearances, in serum sodium concentrations, or in the calculated rates of filtration of sodium throughout the experiments, this must have resulted from an increase in tubular sodium reabsorption.

Increases in calcium but not magnesium excretion were also found in the 24-hr urine collections during NH_4Cl loading, as shown in Fig. 3. During the four control days, $U_{Ca}V$ averaged 6.40 ± 0.58 mmoles/day and $U_{Mg}V$ averaged 5.98 ± 0.48 mmoles/day. When acid loading was begun, $U_{Ca}V$ rose promptly and progressively, while $U_{Mg}V$ was increased significantly only on the 5th day of NH_4Cl administration. The upper right hand portion of Fig. 3 shows that net renal acid excretion ($U_{NET\ ACID}V$) increased *pari passu* with the increases in $U_{Ca}V$. Sodium excretion ($U_{Na}V$) rose transiently when NH_4Cl loading was begun, but thereafter fell progressively to [illegible] control rates.

Fig. 4 shows the changes in renal [illegible] and ammonium excretion that followed glucose [illegible] in the clearance studies. In the normal steady state ("control study") both $U_{NET\ ACID}V$ and $U_{NH_4}V$ [illegible] significantly after glucose and (not shown) urine pH fell significantly. During induced acidosis, [illegible] glucose ingestion had no effect on the already [illegible] rates of [illegible] and $U_{NH_4}V$ and (not shown) urine pH actually rose.

Other changes in blo[illegible] ring after glucose [illegible] of NH_4Cl acidosis have been [illegible] confirmed in the [illegible].

Fig. 5 presents some [illegible] data regarding the changes in $U_{Na}V$ which [illegible] ingestion [illegible]

min = 1.53 + 0.17 $\Delta U_{organic\ acids}V$ μEq/min; $r = +0.75$; $P < 0.001$).

DISCUSSION

The mechanism by which glucose ingestion augments urinary calcium and magnesium excretion has not been established. Previous investigators have concluded that this is the result of diminished net tubular reabsorption of calcium and magnesium (2, 3). More recently, this has been verified by demonstrating that glucose ingestion augmented $U_{Ca}V$ and $U_{Mg}V$ significantly, even when their rates of filtration were significantly reduced by having normal subjects stand after ingesting a glucose load (13). A distal site of action has been suggested because of an associated fall in urinary potassium excretion (2). Because urinary net acid excretion increases and the urine is acidified after glucose ingestion (4), we have drawn an analogy to the impaired net tubular calcium reabsorption previously demonstrated during stable NH_4Cl acidosis (5) and speculated that a common mechanism might be involved. The present studies appear to refute that possibility.

The effects of glucose ingestion and stable NH_4Cl acidosis were compared in the same subjects. Glucose ingestion caused significant and equivalent increases in $U_{Ca}V$ and $U_{Mg}V$. During stable NH_4Cl acidosis, $U_{Ca}V$ was significantly increased, but $U_{Mg}V$ was unaffected. Both glucose ingestion and metabolic acidosis appeared to act by diminishing the net tubular reabsorption of magnesium and (or) calcium. Although some correlation between changes in rates of filtration and excretion was evident throughout the studies, in over one-half of the individual clearance periods the observed changes in $U_{Ca}V$ and $U_{Mg}V$ could not be explained by measured changes in their filtered loads. Some impairment of net tubular magnesium reabsorption during induced acidosis was implied by unaltered rates of $U_{Mg}V$ despite a fall in the filtered load of magnesium. However, the rate of filtration of calcium fell comparably, despite which $U_{Ca}V$ increased significantly.

When a glucose load was administered during induced acidosis, the individual effects of glucose and acidosis on $U_{Ca}V$ were additive. No evidence to suggest competition for or saturation of a common mechanism was found. By contrast, glucose ingestion during acidosis had a slightly but significantly lesser effect on $U_{Mg}V$ than that found in the normal steady state. Finally, glucose ingestion during acidosis augmented $U_{Ca}V$ and $U_{Mg}V$ without altering the already high rates of renal acid excretion and despite increases in urine pH.

The close correlation between increments in urinary sodium, calcium, and magnesium excretion which are found during osmotic diuresis (14-18) or after the administration of drugs which inhibit tubular sodium reabsorption (19-21) have led to the suggestion that these cations might share a common tubular transport mechanism (22). Walser (23) has also pointed out that a nonspecific relationship between sodium reabsorption and calcium and magnesium reabsorption in proximal, freely water-permeable tubular segments would be expected, since sodium (with water) reabsorption would tend to increase calcium and magnesium concentrations in tubular fluid, facilitating their reabsorption. After glucose ingestion, however, net tubular sodium reabsorption increased. The simultaneous reductions in $U_{Na}V$, $U_{Cl}V$, $C_{Ca/o}$, $C_{Mg/o}$, fractional urea excretion, and $V/GFR \times 100$ after glucose suggest increased sodium and water reabsorption in the proximal tubules. Hoffman, Martino, Wahl, and Arky (12) have carried out similar experiments and found evidence suggesting increased proximal tubular reabsorption of sodium and water when subjects ingested sucrose after a 3 day fast. In contrast to our studies, they did not find changes in fractional proximal sodium and water reabsorption when sucrose was ingested after a shorter (overnight) period of fasting. Distal sodium reabsorption, as estimated by changes in the sum of K^+ and H^+ excretion, fell after glucose ingestion. Although studies of the acute effects of parathyroid extract (24) and of adrenal mineralocorticoids (25) suggest strongly that specific and separate mechanisms are present in the distal tubule for sodium reabsorption and for calcium and magnesium reabsorption, a nonspecific change in distal tubular energy metabolism after glucose ingestion could account for our findings.

The correlation between increments in $U_{Ca}V$ or $U_{Mg}V$ and the simultaneous increments in urinary organic acid excretion after glucose ingestion is intriguing. We did not measure serum organic acid concentrations. The modified Palmer-Van Slyke titration (5) used to determine total urinary organic acids is crude and provides no information about the identity of the organic acids titrated. Thus, we cannot state whether $U_{Ca}V$ increased because of increases in serum levels and filtered loads or because of changes in tubular reabsorption and (or) secretion. Although an increase in urinary organic acid content could decrease calcium and magnesium reabsorption by complex formation, this seems unlikely because of the high urine flow rates and [illegible] urines present throughout the [illegible]. If the increased quantities of organic [illegible] after glucose arose within the tubular [illegible] a change in tubular cell energy [illegible] for the reduced reabsorption of calcium and magnesium. Additional studies will be required to support or eliminate these possibilities.

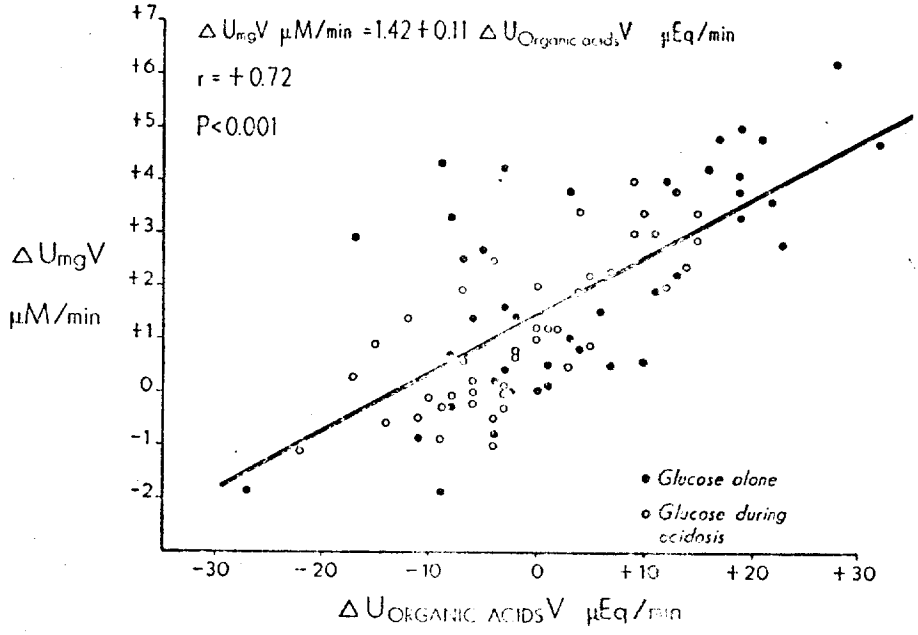

FIGURE 6 The correlation between increments above control in urinary magnesium and total organic acid excretion rates after glucose ingestion.

ing the normal steady state. The upper portion of Fig. 5 shows that, for the group, $U_{Na}V$ and $U_{Cl}V$ fell together during the first two experimental periods. Thereafter, $U_{Cl}V$ remained low, while $U_{Na}V$ tended to rise slightly back toward control rates. $U_{Organic\ acids}V$ rose, while U_KV fell. As shown in the center of the figure, the increasing discrepancy between $U_{Cl}V$ and $U_{Na}V$ appeared to be due to diminishing sodium reabsorption at the distal cation exchange site, since the sum of $U_{Organic\ acids}V$ and U_KV fell progressively. In the lowest portion of the figure, changes in free water clearance (C_{H_2O}) and osmolar clearance (C_{osm}) are presented along with changes in fractional excretion of urea ($C_{urea}/C_{In} \times 100$).

The subjects had been in brisk water diuresis (urine osmolalities averaging 80 ±6 mOsm/liter) for at least 2 hr before ingesting glucose, and "urea exhaltation" (13) had subsided to stable values. Urine osmolalities remained at these low levels after glucose ingestion except for a transient increase (to levels of 102 and [illegible] mOsm/liter, respectively) in two subjects during the second experimental period. We have thus assumed that subjects were in near maximal water diuresis and that the ingestion of the hypertonic glucose solution did not cause any major or sustained increases in the secretion of antidiuretic hormone. Under these circumstances, it has been suggested that back diffusion of urea and of water in distal tubular segments is minimal and that changes in fractional urea excretion (11) and in the fraction of filtered water excreted ($V/GFR \times 100$) (12) reflect primarily fluid delivery from proximal, relatively water-impermeable tubular segments. As shown in Fig. 5, C_{osm} fell slightly after glucose ingestion, while C_{H_2O} fell initially and then rose above control rates. The fractional excretion of urea fell in five of the seven studies and, on the average, remained below control rates throughout the studies. Considering all experimental periods in all subjects, this change was significant, averaging −3.8 ±1.1% ($P < 0.025$). Not shown in the figure, $V/GFR \times 100$ also fell after glucose ingestion, but to a lesser degree, averaging, for all periods, −1.1 ±0.3% ($P < 0.05$).

As in earlier studies of glucose induced augmentation of $U_{Na}V$ and $U_{Cl}V$ (2, 3) no significant change in urinary organic acid excretion were found. However, an analysis of the present data indicated that while urinary organic acid excretion varied in a completely random manner after induction of acidosis, it tended to increase consistently but to a variable degree after glucose ingestion. Moreover, the magnitude of the increases in $U_{Cl}V$, $U_{Na}V$, and $U_{Organic\ acids}V$ after glucose ingestion appeared to be related. Fig. 6 presents a plot of the increments in $U_{Na}V$ after glucose ingestion as a function of the simultaneous increments in $U_{Mg}V$ after glucose ingestion as a function of the simultaneous increments in $U_{Organic\ acids}V$ for each of the subjects. Good correlation was found ($r = +0.72$; $P < 0.001$). Equally good correlation was found between changes in $U_{Na}V$ and $U_{Organic\ acids}V$ after glucose ($\Delta U_{Na}V$ μmoles/

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BIOASSAY OF DIURETICS

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Although many studies have been made of the mechanism of diuresis, there has been no systematic quantitative study done on diuretics. Such an investigation should include a determination of the dose-action relationship of various diuretics by a reliable method, and an estimation of their relative potency with reference to a suitable standard. To be of practical use in future studies, the method should be simple, accurate and applicable to a variety of substances. A method using rats for the estimation of anti-diuretic potency has been described by Burn (1). This method, or a slight modification of it, has been used for diuretic assays by Marx (2), Herre (3), Jaretzky and Neuwald (4), and Vollmer (5). Controversial results reported in the last two citations clearly demonstrate the inadequacy for this purpose of the anti-diuretic assay method, which is devised for the measurement of a decrease in urinary excretion and requires a normally high excretion of urine. For the measurement of an increase, it is desirable to have the normal urinary excretion at a minimum.

In this paper a method suitable for diuretic assays will be described, and some results of its application to the study of several diuretics, most of which are in common use, will be presented.

METHOD. The procedure to be described for the determination of diuretic potency of drugs differs from Burn's in four important points: (1) the amount of liquid fed is reduced to half of that used in the antidiuretic method (i.e., 25 cc./kgm. instead of 50 cc./kgm.); (2) isotonic sodium chloride solution rather than tap water is used for feeding; (3) the number of rats used for each dose is increased from four to eight; and finally (4) the total volume of excretion during a definite period of time, rather than the time necessary for the rate of excretion to reach a maximum, is taken as the measured variable. As a result of these modifications, basal urinary excretion is reduced to a minimum, and variations among groups are reduced considerably.

Healthy male Sherman rats weighing 140 to 240 grams were fasted for eighteen hours previous to the experiment, and were then given the substance to be tested in proportion to their weight. When administered orally, the substance was dissolved or suspended in isotonic sodium chloride solution at approximately body temperature, and fed to the animals through a stomach tube. After the feeding, the animals were placed in sub-groups of four in special metabolism cages (Fig. 1), and remained there without food or water for the duration of the experiment. Two sub-groups were used for each dose. At the end of a definite period of time, the animals were taken out of the cages and the total volume of urine excreted by each group was measured. For all of the substances investigated, except bismuth potassium tartrate and salyrgan, a period of five hours was found to be adequate, as indicated by a great decrease in the rate of excretion during the fifth hour. Since, in the process of feeding, the animals lose all their urine, it was considered important at the end of the experiment to expel the urine from the bladders by pressing at the base of the tail.

In most experiments five groups of approximately equal weight were used. One group, as a control, was fed normal saline solution. In most cases two groups were fed suitable

doses of urea, and the remaining groups were given varying doses of the substance to be tested. The volume of urine excreted during five hours was in each case expressed as per cent of the liquid administered.

This percentage gives a measure of *urinary excretion* independent of group weight, and may be used in the calculation of *diuretic action*. The ratio $\frac{\text{Urinary excretion in test group}}{\text{Urinary excretion in control}}$ could be used as a measure of diuretic action for a given dose. In practice, however, it was found necessary to compare test groups receiving a new preparation with parallel test groups receiving a standard drug. This reduces the variability materially. Since the same control group appears in the estimation of relative potency only as a constant term which would cancel out, it is simpler to use the percentage of urinary excretion for such estimates. The control then serves as a check on experimental conditions as in the assay of Vitamin A, but does not enter into the calculation of the relative potency. *Diuretic activity* is the diuretic potency of a substance referred to that of the standard, urea, the activity of which is taken equal to one. Urea was chosen as the standard diuretic because

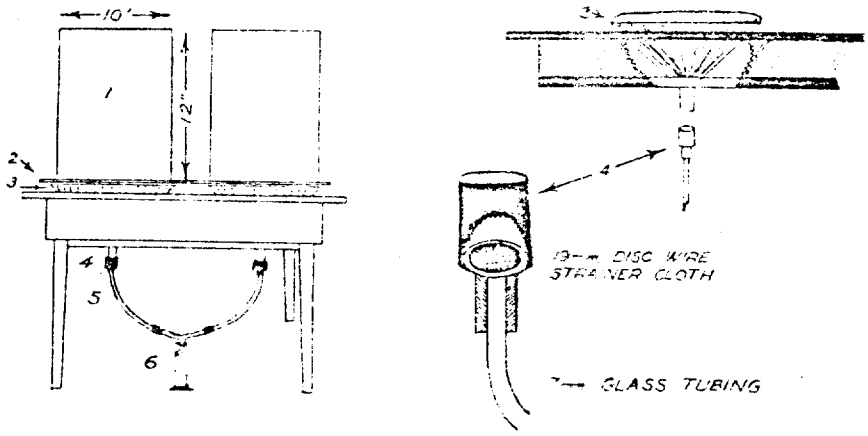

FIG. 1. SKETCH OF THE METABOLISM CAGE

(1) Glass cylinders, 10 inches in diameter and 12 inches high; (2) circular wire screen; (3) rib tunnel, 1 gallon capacity; (4) vacuum stopper with one screen inset; (5) glass tubing with "Y" joint; (6) volumetric cylinder.

it is stable *in vivo* as well as *in vitro*, a relatively weak diuretic and nontoxic in very high doses.

Bismuth potassium tartrate was injected intraperitoneally in doses varying from 2.5 to 50 mg./kg., and the proper amount of normal saline was administered orally following the injections. Salyrgan was injected in doses varying from 6 to 30 mg./kg.; and then normal saline or 1% ammonium chloride in saline (4.7 mM/kg.) was fed. Ammonium chloride was chosen for its well known potentiation of salyrgan diuresis (6). When it was used, determinations of diuretic action were made with reference to the urinary excretion of the group receiving ammonium chloride alone. With these two substances it was found necessary to keep the animals on test for approximately twenty-four hours.

In each instance several experiments were done with three or four different doses of the substance to determine the type of dose-action curve to be obtained. Within a fairly large range of doses, most of the substances yielded an almost linear relationship between log dose and log effect. In several instances the curve was approximately linear only within the range of very low doses. The best fitting straight line for each set of data was calculated from the regression equation (1).

$$y = \bar{y} + b(x - \bar{x})$$

where $\bar{y}$ is the mean value of y (log effect), $\bar{x}$ the mean value of x (log dose) and b the regression coefficient determined from the equation

$$b = \frac{\Sigma y(x - \bar{x})}{\Sigma(x - \bar{x})^2} \qquad \text{(II)}$$

In subsequent experiments the effect of two or more doses of the substance was compared with that of one or more doses of urea. The dose-action curves of the substance and of urea

TABLE 1

Variability of average excretion among groups on same day

Control rats, fed 25 cc. [illegible] gm. of 0.9% NaCl solution

RATS PER GROUP	EXPER. DATE	NO. OF GROUPS									MEAN
4	9-19	4	[illegible]	23.8	32.3	32.1					30.7
	9-20	8	29.5	[illegible]	30.5	33.2	40.8	32.9	19.6	21.2	27.4
	10-8	8	[illegible]	[illegible]	[illegible]	22.4	14.3	7.2	[illegible]	11.1	18.1
	67 male exper.	2[illegible]									
Average											31.7
8	12-29	2	23.0	29.7							26.4
	12-31	5	30.[illegible]	23.3	43.5	39.8	45.8				36.9
	1-2										
	1-5	5	37.2	29.3	32.0	35.0	39.4				32.5
	1-7	3	[illegible]	72.4	47.2						[illegible]
	1-11	2	33.4	29.6							31.5
	1-15	3	33.[illegible]	37.4	28.3						32.5
Average											31.6

Analysis of Variance

RATS PER GROUP		DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE	[illegible]
4	between days	[illegible]	[illegible]	22[illegible].46	2.595
	within days	84	72[illegible]	[illegible]	
8	between days	5	8[illegible].83	17[illegible].67	1.85
	within days	14	[illegible].78	37.[illegible]	

were plotted together. When [illegible] the distance along the abscissa between the [illegible] gives the logarithm of the [illegible]

Results. *Controls.* In the description of the method, special emphasis was placed on the necessity for using a sufficient number of rats in each group, and comparing the urinary excretion of the test groups with that of the control group of the same day, to ascertain that diuresis is obtained. The [illegible] recorded in Table 1 give the comparison of the urinary excretion for groups on the same day

and on *different* days. The analysis of variance shows that the variation between days is significantly greater than between groups tested on the same day. As would be expected, the variance within days for groups of 8 rats is about one-half of that for groups of 4 rats.

Since the animals are fed a considerable amount of liquid at one time, it was of great importance to choose a liquid which is normally retained in the body to a large extent throughout the duration of the experiment, and which does not obscure the action of diuretics. Isotonic sodium chloride was found to fulfill these requirements. Substitution of Ringer's solution for saline does not significantly alter the value of urinary excretion. Substitution of tap water, however, more than doubles it (Table 2).

Urea. Since urea was chosen as the standard, it was more thoroughly studied than any other substance. The results obtained are summarized in Tables 3–5. Eight curves, each with three or four points, were obtained on different days using 8-rat groups. The data are given in Table 3. The variation in the effect

TABLE 2

Effect of substituting Ringer's solution on tap water for saline solution on urinary excretion

Groups of eight rats each

Experiment date	Urinary excretion						Average	
	Ringer's solution			Tap water			Ringer's solution	Tap water
1/7/42	54.1	[illegible]	12.5	50.5	52.4	47.2		
1/11/42	[illegible]	25.6		[illegible]	29.6			
1/15/42	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	Saline			Tap water			Saline	Tap water
[illegible]/19/41	59.9	[illegible]		[illegible]	[illegible]			
12/9/41	[illegible]	[illegible]		[illegible]	[illegible]		[illegible]	[illegible]

expected for each day [illegible] greater than that in the slope of the dosage effect curves, when both are compared with their respective errors. This difference in [illegible] susceptibility has been adjusted so as to bring the mean responses [illegible] for each individual curve on the same line, Fig. 2, with a slope of b = .7005. This value agrees well with that obtained for 55 curves, even based on only 2 points (b = .7200, Table 4 second row). The individual curves are relatively stable in slope within an [illegible] variance than that of individual readings about 7 of the 8 curves in Table 3. One curve, that on 11/30/42, was excessively variable. The mean dose in 65 experiments is 0.710 ± .026. An experiment was done with four different doses of urea on 4-rat groups, using the Latin Square arrangement, and the results were analyzed by Yates' method (7, Table 5). The variation due to the slope of the curve (b = .718) was greatest, that due to the difference in days [illegible] times as large as the error, but differences between [illegible] groups [illegible] straight line were less than the experimental error.

Sodium, Potassium and Ammonium Salts. Of the five salts studied, potassium nitrate, potassium acetate and ammonium chloride are in common use as di-

TABLE 3

Dose-action curves from single experiments with 3 or 4 doses of urea; dose in millimoles of urea per kilogram of rat

DATE	PER CENT EXCRETION IN LOGARITHMS						SLOPE b	STANDARD DEVIATION ABOUT LINE s	LOG EFFECT AT MEAN LOG-DOSE OF 1.4028
	Saline control	With log dose of urea							
		1.097	1.274	1.398	1.574	1.699			
11- 5-41	1.617	1.769	1.867	1.898	2.064		.595	.0328	1.946
12-16-41	1.698	1.812		1.995	2.163	2.222	.703	.0236	2.021
12-17-41	1.643	1.855		2.037	2.152	2.288	.697	.0282	2.056
3-30-42	1.428	1.762*	1.815†	1.912			.848	.0232	1.893
11-27-42	1.732	1.655		1.785	1.949	2.179	.821	.0883	1.865
11-30-42	1.405	1.767		1.731	1.996	2.155	.643	.1328	1.885
12- 1-42	1.555	1.718		1.830	2.036	2.191	.776	.0724	1.916
12- 2-42	1.396	1.805		1.957	2.054	2.192	.615	.0401	1.975
Combined value							.7005	.0683	1.9446

* Log-dose = 1.223.
† Log-dose = 1.318.

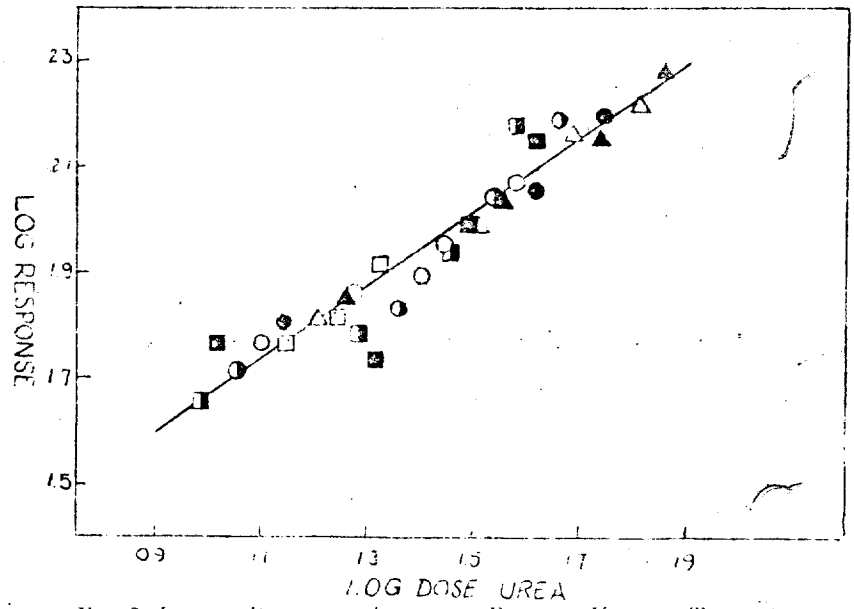

FIG. 2. DOSAGE RESPONSE CURVE FOR DATA ON UREA IN TABLE 3

The experiments on different days have been adjusted to an equal susceptibility for plotting by adding a constant term to the log doses for each day. Each day's results are shown by a different symbol.

ureties, but not sodium nitrate or sodium acetate. The original data for potassium and sodium nitrate are given in Table 6, with the log-ratio of potencies (M)

TABLE 4

Analysis of variance between slopes of dose action curves for urea in Table 3 and of 55 curves with 2 doses, not shown in detail; eight-rat groups on each dose

VARIATION DUE TO	DEGREES OF FREEDOM	MEAN SQUARES
Combined slope, all 63 curves of b = .71048	1	1.34948
Difference between 2-dose and multiple-dose slopes	1	.00028
Difference in slopes of individual curves	61	.00168
Scatter of observations about 7 of 8 separate curves in Table 3	13	.00267
Scatter of observations about curve for 11-30-42	2	.01763

TABLE 5

Experiment with 4-rat groups arranged in a Latin square as a test of sources of variation; highly variable controls omitted in analysis of variance by Yates' method

DATE	DIURETIC RESPONSE IN LOG OF % EXCRETION					TOTAL	SLOPE b
12/8	d 2.075	a 1.790	o (1.612)	b 1.902	c 1.989	7.756	.604
12/14	c 2.093	o (1.899)	b 2.037	d 2.135	a 1.821	8.086	.645
12/18	o (1.882)	c 1.853	d 2.150	a 1.597	b 1.982	7.582	1.019
12/23	a 1.954	b 2.088	c 2.022	o (1.814)	d 2.157	8.221	.364
12/28	b 1.738	d 2.094	a 1.655	c 1.930	o 1.213	7.417	.960
Total	7.860	7.825	7.864	7.564	7.949	39.062	Mean .718

Dose of urea

		SYMBOL			
		a	b	c	d
			[illegible]		
		1.097	1.253	1.398	1.574
Diuretic response in log of % excretion					
Total	8.360	8.817	9.747	9.887	10.611
Mean	1.6720	1.7634	1.9494	1.9774	2.1222

Analysis of variance

VARIATION DUE TO	DEGREES OF FREEDOM	MEAN SQUARE	F
Difference between groups of rats	4	.00534	.60
Difference between days of test	4	.02512	2.82
Slope of regression line	1	.31361	35.24
Curvature about line	2	.00616	.69
Error	8	.00890	1.00

computed for each assay. The data were first analyzed for the parallelism of their dose-response curves with the results shown in Table 6a. The small variance with few degrees of freedom about the individual curves is apparently not characteristic of the variation between curves. Although the 5 groups in Table 6 differ more in slope than the curves comprising the groups, the difference is not significant. Therefore, all observations have been combined to obtain a standard

TABLE 6

Dose-action curves of potassium nitrate and sodium nitrate and their activities relative to urea

M is log-ratio of potency relative to urea; M' that relative to KNO_3

GROUP	DRUG	LOG	LOG EXCRETION ON 10-1	10-2	11-1	11-11-41
		mM./kg.				
1	KNO_3	.297			1.682	
		.393		1.731		1.770
		.598			1.798	
		.694		1.989		1.945
		.773			1.961	
		.787	2.013			
		.870				2.061
		1.092	2.266			
	Urea	1.319	1.863	1.828	1.863	1.845
	Control		1.580	1.561	1.625	1.633
	M		.722	.815	.702	.766
			12-9	12-10	12-11-41	
3	KNO_3	.393	1.618	1.493	1.808	
		.870	2.066	2.048	2.106	
	Control		1.428	1.386	1.644	
			11-2	11-3	12-1-42	
4	KNO_3	.430		1.705	1.742	
		.570	1.709			
		.787		2.066	1.938	
		.870	1.936			
	Urea	1.097	1.636	1.791	1.810	
		1.398	1.886	2.012	1.903	
	Control		1.303	1.687	1.742	
	M		.604	.589	.588	

GROUP	DRUG	LOG	LOG EXCRETION ON 12-1	12-2	12-3-41
		mM./kg.			
2	$NaNO_3$	.301	1.610		
		.469		1.677	1.735
		.582			1.820
		.602	1.794		
		.770	1.933	2.030	2.000
		.946		2.196	2.163
	Urea	1.319	1.904	1.904	
	Control		1.486	1.584	1.539
	M		.606	.670	
			12-9	12-10	12-11-41
	$NaNO_3$	.389	1.595	1.553	1.598
		.866	1.839	2.035	2.106
	M'		−.114	.033	−.126
			12-9	12-11	12-11-42
5	$NaNO_3$	.469	1.733		
		.645		1.724	1.761
		.770	1.871		
		.946		1.957	2.022
	Urea	1.097	1.843	1.731	1.815
		1.398	2.028	1.931	1.987
	Control		1.695	1.632	1.694
	M		.462	.462	.440

deviation of s = .0616, a combined slope of b = 0.8053 and λ = .0765. The log-ratio of potencies and its error is computed for each day with the combined slope. In this way the results are more consistent than when computed from the slopes for each group of tests (Table 7). The potencies of both KNO_3 and $NaNO_3$ relative to urea shift significantly between groups 1-2 and 4-5 in Table 6, but retain the same relation to each other throughout all tests as shown in the right-hand column of Table 7.

The dose-response curve of potassium acetate has the same slope as that of urea, so that its relative potency (avg. 3.35) is independent of the level of urinary excretion at which it is measured (Table 8). The dose-response curves of sodium acetate are, however, significantly steeper (b = 1.1191) than the corresponding curves of urea and potassium acetate (average b = 0.6085). In this case, therefore, it is necessary to compute the relative potency from the data in Tables 9 and 10 at a specified level of log % excretion which is taken at 1.895. At higher levels of excretion, the relative potency of Na acetate would be greater relative to urea than at lower levels. In the later experiments it was significantly less than in the first tests in 1941.

TABLE 6a

Analysis of variance for parallelism of dose-response curves in Table 6

VARIATION	DEGREES OF FREEDOM	VARIANCE	
Between slopes of dif. groups	4	.00599	
Between slopes within groups on dif. days	11	.00352	s^2 = .003794
Between slopes of dif. drugs on same day	9	.00316	
About 3 and 4 dose curves	6	.00056	

TABLE 7

Mean log-ratio of potencies based on Table 6

UREA AS STANDARD				POTASSIUM NITRATE AS STANDARD			
Group	Diuretic	M ± s_m	Relative potency	Groups	Diuretic	M ± s_m	Relative potency
1	KNO_3	.751 ± .046	5.63	1–2	$NaNO_3$	−.113 ± .078	.77
4	KNO_3	.594 ± .044	3.92	3	$NaNO_3$	−.069 ± .044	.85
2	$NaNO_3$	.638 ± .063	4.35	4–5	$NaNO_3$	−.139 ± .063	.73
5	$NaNO_3$	.455 ± .044	2.85				

TABLE 8

Dose-action curves of potassium acetate and its potency relative to urea

DRUG	Log	LOG % EXCRETION						MEAN POTENCY
		3-8	11-3-41	12-21	12-2-2	12-29	12-30-42	
	mM/kg							
K acetate	.582	1.706	1.723	1.774	1.927	1.609	1.772	
	.805	1.951						
	.883		1.899	1.923	2.038	1.916	2.002	3.35
	1.059		1.981					
Urea	1.097			1.668	1.888	1.784	1.813	
	1.319	1.940	1.817					1.00
	1.398			2.005	1.945	1.945	1.985	

The dose-response curve of ammonium chloride has the same slope (b = .8085, s = .0642) as that for urea (b = .6191, s = .1416), so that its relative potency, 2.7, is independent of the level of urinary excretion at which it is measured (Table 11).

Xanthine Diuretics. With theophylline and theobromine, an approximately linear relationship between log dose and log action is obtained only in the lower range of doses, "overdosage" producing a decrease in diuretic action. With caffeine, the measurable dose range is still more restricted. Calculation of the

TABLE 9

Dose-action curves of sodium acetate and its activity relative to urea and to potassium acetate

DRUG	LOG	LOG % EXCRETION ON										
		10-6	11-6	11-26	11-27	11-28	12-4	12-5	12-8	12-12-41	1-6	1-7-43
		1st group					2nd group				3d group	
	mM/kg											
Na acetate	.662										1.557	
	.711						1.728	1.584	1.671	1.809		
	.741		1.611			1.766						1.530
	.866			1.915	1.930	1.940						
	.964	1.865			2.088	2.062					1.944	
	1.042		1.951	2.084	2.152							1.871
	1.063						2.017	2.032	2.000	2.161		
	1.167			2.253								
	1.220		2.118									
	1.265	2.152										
K acetate	.707						1.972	1.856	1.900	1.856		
	1.059						2.145	2.088	2.024	2.141		
Urea	1.097										1.856	1.671
	1.319	1.807	1.858	1.931	1.870	1.948						
	1.398										1.975	1.918

Mean activity of Na acetate

relative to urea	(1st group)	2.762 ± .260
relative to K acetate	(2nd group)	.785 ± .045
relative to urea	(3d group)	1.854 ± .106

diuretic activities is, therefore, possible only approximately. They are: theobromine, 7.2; caffeine, 32; and theophylline, 115. Theobromine in higher doses was fed in suspension, and in such doses it proved after 24 hours to be definitely toxic. With theophylline and caffeine, the doses could be increased to the desired extent, and the effect of overdosage could be studied in entirety. The highest dose used of theophylline produced a decrease in urinary excretion below that of the controls. Even with the lowest doses of theophylline and caffeine, there were definite manifestations of central excitation, but no such signs could be noticed with theobromine.

Bismuth Potassium Tartrate. The preparation used, with a Bi content of 62%, has a diuretic activity of approximately 219. High doses produce a decrease in urinary excretion, but even with diuretically active doses, albuminuria and glycosuria are observed.

Salyrgan. Salyrgan was tested alone and in combination with ammonium chloride. The results, given in figure 3, show salyrgan to be a potent diuretic

TABLE 10

Dose-action curves of sodium acetate and its activity relative to urea on 4-rat groups in the Latin Square arrangement

Since the analysis showed the differences between days relatively unimportant the results were handled as a randomized block.

GROUP	Dose-action				
	Saline control	Acetate		Urea	
		[illegible]	[illegible]	[illegible]	[illegible]
2	1.683	1.495	2.062	1.664	1.743
3	1.515	1.718	2.066	1.651	1.804
4	1.511	1.745	1.969	1.830	2.063
5	1.511	1.750	2.099	1.946	1.931
6	1.367	1.715	2.139	1.964	1.818
7	1.185	1.629	1.949	1.794	1.931
9	1.750	1.643	2.146	1.828	2.017
10	1.448	1.772	2.109	1.963	1.965
Total	11.970	13.477	16.519	14.244	15.392
Mean	1.4963	1.6846	2.0649	1.7764	1.9428

Relative potency = 1.982 ± 101.

Analysis of variance

Groups	7	[illegible]	2.73
Controls vs. treated	1	.843210	111.27
Acetate vs. urea	1	.007200	.96
Combined slope	1	.532803	70.27
Parallelism	1	.118959	15.66
Groups × control vs. treated	7	.028670	3.77
Error	21	.007596	1.00

within a very narrow range of doses. A slight overdose produces a definite decrease in urinary excretion. The active range is seemingly narrower without ammonium chloride and the optimum dose slightly higher. When ammonium chloride was used with salyrgan, in several instances the diuretic effect could be observed after five hours, and in nearly all instances, after ten hours. Without ammonium chloride, it took somewhat longer for the diuretic effect of salyrgan to become manifest. Within the range of diuretic doses, albuminuria was observed

in all instances and glycosuria in samples collected after ten hours. In the lower dose range, linear relationship was again obtained between the logarithm of the

TABLE 11

Dose action curves of ammonium chloride and its activity relative to urea

DRUG	LOG	LOG % EXCRETION ON								MEAN ACTIVITY
		2-23	3-2	3-20	2-23	3-24-42	1-14	1-14	1-18-43	
	mM/kg.									
NH_4Cl	.670	1.646								
	.711								1.647	
	.846			1.696	1.461	1.557	1.850	1.833		2.7
	.971	1.932	1.826	1.718						
	1.012								1.936	
	1.147		2.040	1.959	1.720	1.625	2.[illegible]	2.065		
Urea	1.097						1.734	1.735	1.724	
	1.319	1.838								1.0
	1.398						1.987	2.929	1.833	

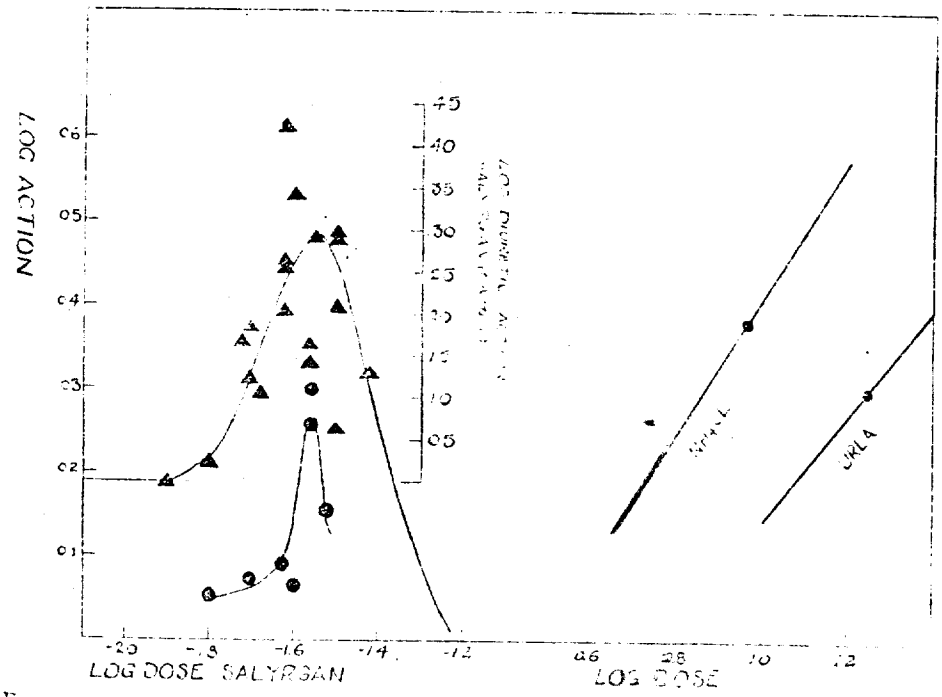

FIG. 3. DOSE-ACTION CURVES OF AMMONIUM CHLORIDE, SALYRGAN, AND SALYRGAN WITH 1.67 mM/KG. AMMONIUM CHLORIDE, OVER A WIDE DOSE RANGE.
●, salyrgan; ▲, salyrgan with NH_4Cl.

dose and the logarithm of action. The diuretic activity was found to be about 400.

Biuret. A number of substances which were considered likely to show some diuretic activity were studied. One of these is biuret which has a formula closely related to that of urea and is similar to it in some respects.[1] The original data obtained with low doses are given in Table 12. With higher doses necessitating a concentration greater than 5 per cent, biuret had to be fed in suspension. Due to its very steep solubility curve, it was practically impossible to make a homogenous suspension of biuret at the proper feeding temperature. Furthermore, when a concentration of 4 per cent or higher was used, biuret crystallized in the excreted urine at room temperature, clogging various parts of the metabolism cage. Therefore, the results obtained only with lower doses of biuret could be used for the calculation of the dose-action curve. This has the same slope ($b = .5507$, $s = .0597$) as that of urea ($b = .5631$, $s = .0814$). The mean potency of biuret relative to urea is 1.4.

DISCUSSION. The method described in this paper for assaying diuretics is significantly different from Burn's method for antidiuretics. It has proved to be applicable to the study of all types of diuretics in common use, and gives not only

TABLE 12

Dose-action curves of biuret and its activity relative to urea

DRUG	DOSE	LOG EXCRETION							MEAN ACTIVITY
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	
	mM/kg.								
Biuret	.779	1.634		1.733	1.641	1.566	1.765	1.696	
	.858		1.708	1.711					
	.983		1.844	1.847					1.40
	1.089	1.953	1.859	1.903	1.838	1.789	1.879	1.874	
Urea	1.097				1.796	1.795	1.786	1.837	
	1.319	1.953							1.00
	1.248		[illegible]	[illegible]	1.888	[illegible]	[illegible]	2.047	

an accurate estimate of the diuretic potency, but also some information concerning the therapeutic range and the toxic effects of the substance being studied. The limitations of this method, as of most bioassays on animals, arise from the fact that the results of assays on normal animals have to be compared with those on human patients. There is no question that the water-salt balance in the tissues of the normal rat is quite different from that in the tissues of the edematous patient. Nevertheless, it is remarkable that the sequence of activities of known diuretics is almost the same with the rat as it is with the human being: urea, ammonium chloride, potassium acetate, potassium nitrate, theobromine, theophylline, bismuth lactate and salyrgan (Table 13). Caffeine, due to its very strong side effects, cannot be properly placed in this sequence. The relative potencies of the xanthine diuretics determined on rats, however, are in agreement with those determined by Myers on rabbits (8).

Though active in very small doses, the xanthine diuretics are to be capable of

[1] Like urea, biuret will hemolyze erythrocytes in an isotonic solution (0.5 gm. per cent) of concentration [illegible] [illegible]

producing as intense a diuresis in normal rats as the other substances studied. With high doses of urea or any of the neutral salts, it is possible to obtain within five hours a urinary excretion approximately twice the amount of the liquid administered. Likewise, with salyrgan, a urinary excretion of approximately the same magnitude may be obtained within the period of its activity. But with the xanthine diuretics, the amount of urine excreted within five hours is never significantly greater, and most frequently less than the amount fed. The shape and the scattering of the points of the dose-action curves, especially in the case of caffeine, suggest that there is a complex mechanism of xanthine diuresis and that a physiological limiting factor (e.g., the antidiuretic hormone) plays an important

TABLE 13

Comparison of diuretic activities from the dose-action curves and from human therapeutic doses

SUBSTANCE	AVERAGE SINGLE HUMAN DOSE			RAT DOSE FOR DIURETIC ACTION 1.5–2.5	DIURETIC ACTIVITY		RATIO RAT DOSE / HUMAN DOSE
					Human	Rat	
	gm	gm/kg	mM/kg	mM/kg			
Urea	20–40	0.3–0.6	5–10	12–25	1	1	2.5
Biuret						1.4	
Sodium acetate						2.0	
Potassium acetate	15–30	0.23–0.46	2.4–4.8	4–8	2.1	3.4	1.7
Sodium nitrate						2.9	
Potassium nitrate	8–13	0.13–0.20	1.3–2.0	2.5–6.0	4	3.9	2.5
Ammonium chloride	10–20	0.15–0.30	2.8–5.6	4.6–8.9	2	2.7	1.6
Theobromine	0.5–0.6	7.7–9.2	0.043–0.051	0.7–4.4	150	7.2	20
Caffeine	0.4	1.5	0.008	1.1–3.1	625	14–32	12–50
Theophylline	0.15–0.20	2.3–3.0	0.013–0.017	0.07–0.16	380	115	5–10
Salyrgan		2.4	0.004–0.008	0.025	1250	abt. 400	5
Bismuth Na (K) tartrate	0.03–0.09	0.5–1.5	0.0017–0.005	0.025–0.075	2000–3000	220	15

role; consequently these diuretics have variable effects on different species and even on different individuals.

Urea, biuret and the salts, with the possible exception of ammonium chloride, showed no toxic effects within the range of doses used. Consequently, the dose-action curves of these substances are linear within this range. The xanthine diuretics produced definite toxic effects in higher doses. The dose-action curves of these substances over a wide range of doses, showed some diuresis, and then a decline. The toxic effects of salyrgan are much more pronounced, and the range of diuretically active doses is much narrower for salyrgan than for the xanthine diuretics.

The following procedure is recommended for assaying new diuretics: Male rats weighing 140–240 gm. are fasted and deprived of water for 18 hours prior to the

experiment. Preliminary experiments are done using five groups of 8 rats each on one day. The control group are fed 25 cc./kgm. 0.9% sodium chloride, containing no drug, and the remaining four groups are given geometrically increasing doses of the substance being tested. Thus the right dose range is determined in very few experiments. For the estimation of potency, five groups of 8 rats are used on one day. One group receives no diuretic, two receive two suitable doses of urea (e.g., 0.75 and 1.5 g./kgm.), and the remaining two receive two doses of the substance chosen so that their actions will fall within the same range as those of urea. The slope, position and the distance between the two regression lines are then calculated. Three or four such experiments will produce enough data for a sufficiently accurate estimate of the mean activity (potency) relative to urea of the substance.

SUMMARY

1. A method using white rats has been devised for the bioassay of diuretics. It has been tested experimentally and statistically and applied to the study of various substances. Urea with an activity of 1 was taken as the standard diuretic.

2. The dose-action curves (log dose in millimols per kilogram body weight, plotted against log diuretic action) of urea, various salts, and salyrgan were found to be straight lines of similar slopes within a suitable range of doses. From these the diuretic activities of the substances were calculated. The dose-action curves of the xanthine derivatives were found to be complex, seemingly limited by a physiological factor.

3. Biuret, a urea derivative, assayed in this way, showed a diuretic activity of 1.4.

4. The following diuretic activities were determined from the dose-action curves: salyrgan, 400; bismuth potassium tartrate, 219; theophylline, 115; caffeine, 32; theobromine, 7.2; potassium nitrate, 3.9; sodium nitrate, 2.9; potassium acetate, 3.4; sodium acetate, 2.0; ammonium chloride, 2.7; and urea, 1.0.

5. Relatively large doses of the salts and urea removed much more water than that which was administered, whereas such doses of the xanthine diuretics, Bi tartrate and of salyrgan not only failed to produce an increase in diuretic action, but in most instances produced a decrease.

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Renal hypertrophy in metabolic acidosis and its relation to ammonia excretion[1]

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LOTSPEICH, WILLIAM D. *Renal hypertrophy in metabolic acidosis and its relation to ammonia excretion.* Am. J. Physiol. 208(6): 1135-1142. 1965.—The chronic ingestion of NH_4Cl causes a true growth of the rat kidney. This involves a symmetrical increase in wet weight, dry weight, and nitrogen of the kidney tissue. Total DNA and RNA are also elevated, indicating that both cell numbers and cell size have increased. The renal hypertrophy in chronic metabolic acidosis is the same order of magnitude as that seen in the remaining kidney after unilateral nephrectomy. The renal growth-stimulating effects of acidosis and unilateral nephrectomy are additive. In the acidotic rat the renal hypertrophy that occurs after unilateral nephrectomy can maintain a high rate of ammonia excretion without any further change in renal glutaminase activity of the remaining kidney. Thus in certain species the stimulation of renal growth may play a part in the increasing capacity to excrete ammonia during sustained metabolic acidosis.

glutaminase RNA, DNA unilateral nephrectomy
acid-base balance

During sustained metabolic acidosis man and certain lower animals show a progressive increase in the capacity to excrete ammonia in the urine. This process may continue until practically the entire load of acid anions is excreted as the ammonium salt (6). As this happens the rate of sodium excretion declines, and there is a progressive improvement in sodium conservation as acidosis continues. The nature of the mechanisms underlying this adaptation in ammonia excretion has been of considerable interest to physiologists, but is not yet well understood. The renal extraction of glutamine, the major source of urinary ammonia (16), is increased in acidosis (5, 13, 16). Whether this can account for the change in ammonia excretion, or coincides temporally with it, has not been determined. Kidney slices (2) and homogenates from chronically acidotic rats and guinea pigs (4, 11) have been shown to produce more ammonia in vitro from glutamine than similar tissue preparations from normal or alkalotic animals. These changes have been interpreted in terms of induced adaptations in one or more glutaminase enzymes (11). In the dog, however, which shows par excellence the developing change in ammonia excretion in acidosis, no change can be demonstrated in the rate of ammonia formation from glutamine by surviving kidney tissue in vitro (8). Since renal glutaminase activity in the dog is considerably higher than in the rat (10) it may not "need" to increase to meet the ammonia excretion demands of acidosis. Thus an induced increase in glutaminase enzymes is probably not the whole explanation for the animal's increasing capacity to excrete an acid load as the ammonium salt.

For this reason we decided to look at other aspects of renal metabolism during experimentally induced metabolic acidosis. It was reasoned that perhaps the glutaminase changes that had been observed, particularly in the rat, might represent one manifestation of a more general increase in rate of protein synthesis in the kidney. If this were so, then the adaptation in ammonia excretion could be explained simply in terms of increased renal tissue mass. By the same token the total glutaminase activity of acidotic kidney tissue should be even greater if the acidosis occurred in an animal whose kidney was undergoing rapid regeneration, as after unilateral nephrectomy.

For these reasons it was decided to begin our study by comparing the effects of acidosis on ammonia-producing capacity of kidney tissue from rats with two kidneys—regenerating at a normal rate—with that from animals with one remaining kidney undergoing rapid regeneration after unilateral nephrectomy. These experiments have shown that chronic metabolic acidosis in the rat causes a true renal hypertrophy of about the same magnitude as that seen after unilateral nephrectomy. Furthermore, when the rat with one remaining kidney is made acidotic, the degree of hypertrophy is even greater than with either acidosis or unilateral nephrectomy alone. These results support the idea that renal growth may play a role—along with enzyme changes and increased glutamine extraction—in producing the

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adaptive increase in ammonium excretion seen in chronic acidosis.

METHODS

All experiments were carried out on female albino rats of the Holtzman strain weighing between 200 and 250 g. They were housed in individual cages kept in a constant-temperature room at 74 F, and fed Purina laboratory chow ad libitum. Metabolic acidosis was produced by substituting 0.28 M NH_4Cl for the drinking water given the controls. All animals drank ad libitum, but records of volume were kept, and only those animals which had drunk nearly equivalent amounts of water or NH_4Cl were selected for use. Both control and NH_4Cl animals were maintained on their respective regimens for 7 days.

For measurement of ammonia excretion, rats were transferred for a 4-hr period each morning, at the same time, into individual metabolism cages that had been previously scrubbed and washed down with distilled water. No food or drinking bottles were present. The bladder was emptied by suprapubic pressure, 5 ml of tap water were given by stomach tube, and the urine collection begun. At the end of the collection period the residual bladder urine was again expressed by suprapubic pressure, and the cage bottom washed with a known volume of distilled water. Urine and washings were collected in calibrated centrifuge tubes. Ammonia was determined on the diluted urine by the Conway microdiffusion method (1).

Unilateral nephrectomy was carried out under ether anesthesia through a midline incision over the lumbar spine. The adrenal gland was left in place.

For in vitro measurement of renal tissue ammonia production and oxygen consumption, rats were killed by decapitation, exsanguinated, and one or both kidneys removed. The organ was rapidly decapsulated, divided longitudinally, and the medulla cut out with fine scissors. The cortex was quickly weighed on a torsion balance, then placed in ice-cold 0.154 M KCl. The tissue was then homogenized in the cold room at 2 C in a Potter-Elvehjem motor-driven homogenizer with a Teflon pestle in approximately 50 ml ice-cold 0.154 M KCl. This homogenate was then centrifuged in the cold room for 1 hr at approximately 7,500 × *g* in a Sorvall angle-head centrifuge. At the end of this time the supernatant fluid was decanted. This eliminated the endogenous substrates in the tissue. The pellet was then resuspended in a volume of 0.154 M KCl to give a tissue concentration of 100 mg/ml. Without added substrate, Mg^{++} ions, and adenine nucleotide these homogenates have no measurable respiration. By adding back these cofactors and specific substrates one can study more carefully delineated tissue reactions than in the whole uncentrifuged homogenates.

For the incubation period 1 ml of this homogenate was placed in iced Warburg flasks previously charged with $MgCl_2$, 10 μmoles; ADP, 4.2 μmoles; inorganic phosphate, 200 μmoles as sodium phosphate buffer 0.2 M pH 7.4; and glutamine, 100 μmoles.[2] Final volume of flask contents was 3 ml. The center well contained 0.2 ml 2 N NaOH and a filter-paper wick for CO_2 absorption. Flasks were placed on their manometers, gassed during shaking for 3 min with 100% O_2 in the thermostatic bath at 37 C. After temperature equilibration for 10 min, incubation was continued. Three consecutive oxygen consumption readings were taken, and at 30 min the flasks were placed in cracked ice. Flask contents were removed and centrifuged in the cold room for 10 min. Ammonia was determined on an aliquot of the clear supernatant fluid by the Conway microdiffusion method. The amount of ammonia liberated from protein, in the absence of added glutamine, was negligible in these homogenate supernatants. Nitrogen was determined on a 1-ml sample of the original homogenate, after digestion, by micro-Kjeldahl distillation and titration. Oxygen consumption and

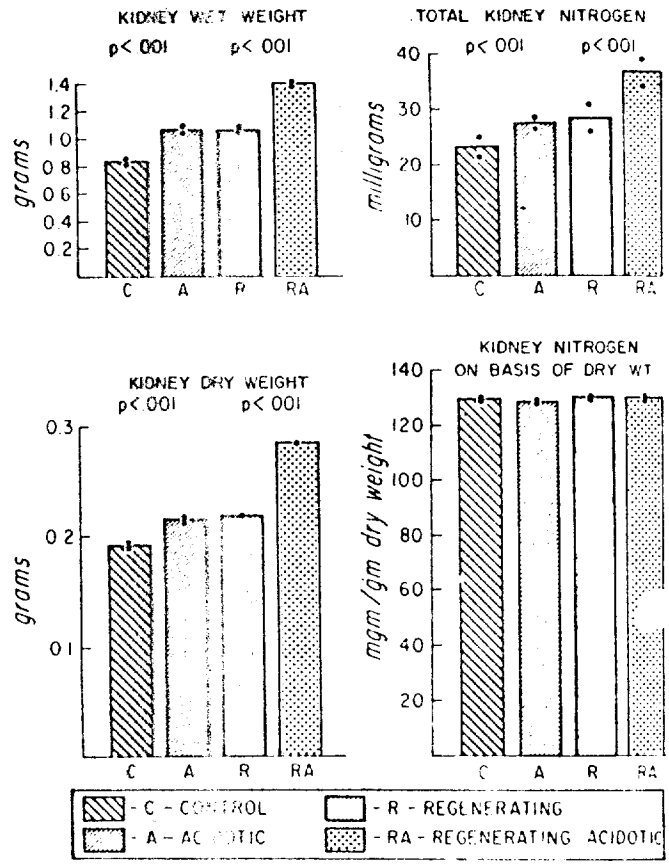

FIG. 1. Wet weight, dry weight, and total nitrogen values for kidneys from 4 groups of rats as indicated at bottom of figure. "Regenerating" refers to remaining kidney after unilateral nephrectomy. Each bar represents mean of observations on 9 animals. Black dots define standard error of the mean. *P* values are over the two bars that they compare.

[2] Preliminary studies showed that 200 μmoles flask [illegible] phosphate at pH 7.4 was optimal for both oxygen consumption and ammonia production from glutamine. The addition of [illegible] μmoles glutamine to each flask was well above a rate-limiting concentration in tissue from both normal and acidotic rats.

TABLE 1. *Experiment testing specificity of ammonium chloride-induced growth of rat kidney*

Solutions	Wet Wt, mg	P	Dry Wt, mg	P
Water	0.784±0.048		0.182±0.010	
Ammonium chloride	0.892±0.020	<0.01	0.204±0.004	<0.01
Sodium chloride	0.849±0.022	<0.05	0.192±0.004	<0.05
Sodium bicarbonate	0.800±0.024	<0.5	0.176±0.004	<0.3
Ammonium citrate	0.798±0.022	<0.5	0.183±0.004	<0.5

Values are means ± SE. *P* values are all related to the water-drinking group. There were 6 animals in each group. All solutions were 0.28 M.

ammonia production rates were then expressed as microliters and microequivalents, respectively, per milligram tissue nitrogen per hour.

Kidney weights, nitrogen, and nucleic acids were always determined on one kidney of intact rats, or on the remaining one of unilaterally nephrectomized animals. For dry-weight determinations kidneys were divided longitudinally, placed in weighed aluminum-foil pans, and dried to constant weight at 85 C in a vacuum oven for 20 hr. Tissue weights were measured on a torsion balance, and total nitrogen determined on the dried tissue, after digestion, by the micro-Kjeldahl method.

DNA and RNA were determined in perchloric acid filtrates of kidney cortex by the Schmidt-Tannhauser-Schneider technique (3). All steps in the procedure up to the final readings were carried out in the cold room at 4 C. Final readings were made in perchloric acid solutions in the Beckman spectrophotometer. DNA was read at 268 mμ and compared with standards made from dried calf thymus DNA. RNA was read at 260 mμ and compared with standards made from dried yeast RNA. (Pure calf thymus DNA was kindly given by Dr. Ernest Kay of our Department of Biochemistry, and pure yeast RNA was obtained from Sigma Biochemical Co., St. Louis, Mo.)

RESULTS

Effects of acidosis and unilateral nephrectomy on kidney growth. These effects are shown in Fig. 1. Each bar in the histogram represents the mean of the observations on nine animals with the standard error of the mean shown as the black dots. All observations were made on the 7th day after start of NH_4Cl or unilateral nephrectomy, or both. We note from the first two bars of each plot (C and A) that acidosis alone caused a highly significant increase in kidney wet weight, dry weight, and total nitrogen. This change was quantitatively similar to that produced by unilateral nephrectomy (bar R). When unilaterally nephrectomized rats were made acidotic in addition, their remaining kidney grew even larger (bar RA). The effects of the two stimuli apparently potentiate one another; with both there is a significantly greater increase in wet weight, dry weight, and total nitrogen than with either acidosis or unilateral nephrectomy alone. These changes represent true growth. In each case the kidney nitrogen was the same when expressed on the basis of dry weight. Thus new tissue protein was synthesized, as a result of these stimuli, in proportion to other cell constituents. The increased weight was not just an increase in water, or fat, but a true new growth.

We had to ask at this point whether this kidney growth was solely the result of NH_4Cl ingestion, with its attendant acidosis, whether it is a specific effect of the ammonium ion, or whether it might also be related to the more nonspecific intake of nitrogen and/or an increased load of solute. To test these possibilities, the experiments shown in Table 1 and Figs. 2 and 3 were performed.

In the experiment shown in Table 1 an attempt was made to test the three variables of acidosis, solute load, and the specificity of the ammonium ion as they affect kidney growth. All solutions were isosmolar (0.28 M) with the NH_4Cl given the rats to produce acidosis. It will be noted that the usual significant increase in wet and dry weight was seen in the NH_4Cl group. Those drinking NaCl also showed a significant, but less marked, increase in kidney wet and dry weight. We have noted this same phenomenon in three additional experiments. It should be noted that the NaCl group drank three or four times as much solution each day as the NH_4Cl group. Thus solute load, in the case of NaCl, does cause some renal hypertrophy, but not as much as NH_4Cl ingested in much smaller amounts. Neither $NaHCO_3$ nor ammonium citrate caused significant renal growth in this experiment.

Next, experiments like those shown in Figs. 2 and 3 were performed. Three groups of rats were compared: one drinking 0.28 M NaCl served as controls; another drank 0.28 M NH_4Cl, and a third a urea solution containing nitrogen in a concentration equivalent to that in the 0.28 M NH_4Cl. Figure 2 shows that only NH_4Cl drinking produced acidosis. Arterial blood pH was determined on aortic blood drawn anaerobically from these animals under light ether anesthesia. All samples from NH_4Cl-drinking rats were significantly below those from urea and NaCl groups. In Fig. 3, NaCl and NH_4Cl groups both had larger kidneys containing more nitrogen than the urea group that drank volumes of solution equivalent to those on NH_4Cl. Therefore it is clear that nitrogen alone in the NH_4Cl is not the cause of the growth. Here, as in Table 1, an equivalent load of 0.28 M NaCl produced kidneys not quite as large as those of the NH_4Cl group. In addition to these results, it can be reported that rats who have lived in an atmosphere of 8 to 7% CO_2 for 7 days and thus have sustained a chronic respiratory acidosis do not show a significant change in renal wet or dry weight or nitrogen. Therefore, the renal hypertrophy seen in chronic NH_4Cl ingestion is apparently predominantly a response to the ingestion of NH_4Cl, with a possible less specific component of solute load also playing a role. It should be

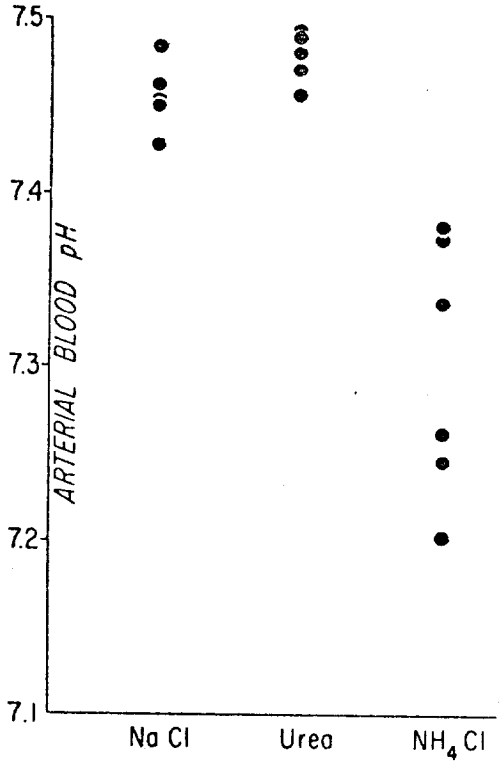

FIG. 2. Effect of NaCl, NH_4Cl, and urea on arterial blood pH. Urea-drinking animals ingested an amount of nitrogen equivalent to that consumed as NH_4Cl by that group. NaCl and NH_4Cl drinking solutions were both 0.28 M.

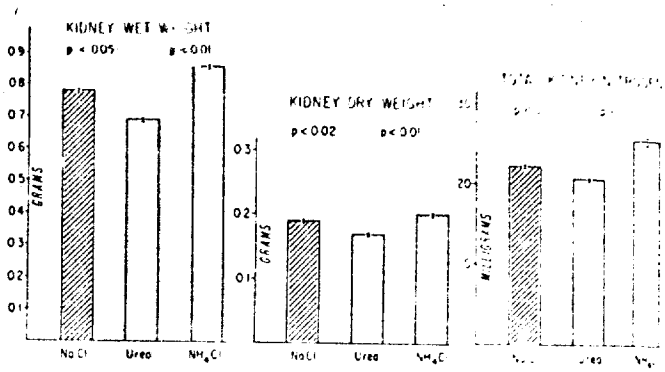

FIG. 3. Effect of NaCl, NH_4Cl, and urea ingestion on kidney wet weight, dry weight, and total nitrogen. Since there was no difference in these values between H_2O- and urea-drinking groups, values for NH_4Cl and NaCl groups are compared to those for the urea group, and P values refer to this comparison. Bars represent mean of 6 animals in each group, with black dots defining standard error of the mean.

pointed out that there is a good deal of quantitative variability in these experiments from group to group of rats. The qualitative differences reported have been repeatedly observed, but the quantitative values for the parameters measured may vary considerably from group to group of rats delivered from week to week. For this reason the importance of including a control group of the same shipment of rats in each experiment must be emphasized.

Nucleic acids. In an attempt to define more precisely the nature of the kidney growth seen in acidosis, and after unilateral nephrectomy, measurements were made of DNA and RNA in the kidneys of both types of animals. The results of these studies are shown in Table 2. With acidosis we saw the usual increase in kidney weight. At the same time total RNA and DNA increased significantly. After unilateral nephrectomy, kidney weight was also up, and both DNA and RNA were increased even more. When the unilaterally nephrectomized rat was made acidotic, wet weight and total DNA and RNA were still higher. Thus the total amount of both these nucleic acids in the whole kidney parallels the changes in organ weight and total nitrogen. This supports the conclusion that in both chronic NH_4Cl acidosis and after unilateral nephrectomy there is true tissue growth involving formation of new cells.

The data on concentration of these two nucleic acids indicate that there is also increase in size of individual cells. Although DNA content increases in each of the groups of rats, its concentration in the tissue decreases. With acidosis alone, although total RNA content increases, RNA concentration in the kidney is unchanged. This could mean either a proportionate increase in RNA and cytoplasmic mass, or an increase in the number of cells each containing equivalent amounts of RNA. With unilateral nephrectomy, and even more so when this was combined with acidosis, both RNA content and concentration in the kidney rose significantly, indicating that in these conditions cytoplasmic RNA increased even more than its non-RNA constituents.

Thus both acidosis and unilateral nephrectomy stimulate kidney growth. In both situations there is stimulation of cell division and increase in size of individual cells. On the basis of the increment in DNA per gram increase in wet weight, the changes are similar in both types of stimulated growth. The additive effects of both acidosis and unilateral nephrectomy in stimulating kidney growth are well illustrated in these nucleic acid data, as they are in the weight and nitrogen observations shown in Fig. 1.

In vitro studies. Homogenates were made, as described above, from kidneys of these four groups of rats. They were incubated with glutamine and phosphate buffer pH 7.4—optimal conditions for glutaminase I activity—and the oxygen consumption and ammonia formation of the tissue observed. The results of these studies are graphically shown in Fig. 4. The right-hand side of the figure shows in the first two bars the increased capacity of isolated homogenized kidney from acidotic rats to produce ammonia from glutamine. This has been repeatedly observed by other workers (4, 11). The third bar shows the ammonia produced by the homogenate of remaining kidney 7 days after unilateral nephrectomy. There is a slight increase over the control, but it is not statistically significant. Tissue from unilaterally nephrectomized-acidotic rats (last bar) shows about the same percent increase in ammonia formation as that from animals with two kidneys. Therefore we can not say that the remaining kidney tissue after unilateral

TABLE 2. *Effects of chronic metabolic acidosis and unilateral nephrectomy on wet weight and nucleic acid content of rat kidney*

Experimental Condition	Wet Wt			DNA						RNA					
	g	t	P	Total, mg	t	P	mg/g Wet wt	t	P	Total, mg	t	P	mg/g Wet wt	t	P
Control	0.787 ±.01			4.33 ±0.13			5.48 ±0.09			6.30 ±0.19			8.02 ±0.11		
Acidosis	0.934 ±.07	4.09	<0.01	4.92 ±0.10	3.71	<0.02	5.30 ±0.14	1.08	<0.4	7.53 ±0.19	4.58	<0.01	8.08 ±0.12	0.37	<0.8
Uninephrectomy	1.009 ±.05	7.95	<0.001	5.18 ±0.10	5.35	<0.01	5.15 ±0.06	3.05	<0.05	8.64 ±0.16	9.41	<0.001	8.57 ±0.21	2.39	<0.1
Uninephrectomy + acidosis	1.258 ±.02	18.1	<0.001	5.41 ±0.09	6.90	<0.001	4.30 ±0.06	10.9	<0.001	11.09 ±0.13	20.7	<0.001	8.81 ±0.11	5.29	<0.01

Values are means of each group ± SE. In each group N = 6. Statistical differences are all related to control observations.

nephrectomy has greater glutaminase activity, nor is it more stimulated by NH_4Cl than in animals with two kidneys regenerating at a normal rate.

On the left-hand side of the figure are shown oxygen consumption rates in these same kidney homogenates in the presence of glutamine and an optimal concentration of phosphate. Since previous studies of this sort have been done without reported measurements of oxygen consumption rates (4, 9, 11), these observations are of interest. In kidney homogenates from acidotic rats the rate of oxygen consumption, with glutamine, is significantly higher. Neither the rate nor its increase with acidosis is significantly different in remaining kidney tissue from unilaterally nephrectomized rats.

The time course of this increased oxygen consumption parallels the development of the increased ammonia formation by kidney tissue in acidosis. This is shown in Fig. 5. Each dot represents the mean of two observations on kidney homogenates made from acidotic rats on each of 8 days after the start of NH_4Cl ingestion. The correlation between these two functions as seen in this figure has been observed in two additional confirming experiments. The steepened slope of the curve around the 5th day has no apparent significance, since it was not a regularly observed phenomenon.

We next asked about the basis for this increased aerobic oxidation of glutamine in "acidotic" tissue. More ammonia is being formed from the amide of glutamine, but this is an anaerobic process. At the same time, however, more glutamic acid is being formed by this deamidation, and the oxidation of this glutamic acid is aerobic, so that perhaps the increased oxygen consumption in the acidotic tissue is simply the result of more glutamic acid having been generated from glutamine. This and other speculations prompted the experiment shown in Fig. 6. Here homogenates from acidotic rats were incubated in 100% oxygen with three different and equivalent levels of glutamine, or its two main products: glutamic and α-ketoglutaric acids. Rate of oxygen consumption was measured. Since we had used 100 μmoles of glutamine (well above a rate-limiting concentration), this was chosen as the highest amount of each substrate added. It is evident that with glutamine and α-ketoglutarate, oxygen consumption was optimal down to the lowest level of substrate tested (25 μmoles/flask). But with glutamate, oxygen consumption fell off as substrate concentration was reduced. Thus at 100 μmoles of glutamine, more glutamic acid would be formed in the acidotic tissue and thus oxygen consumption rate would increase.

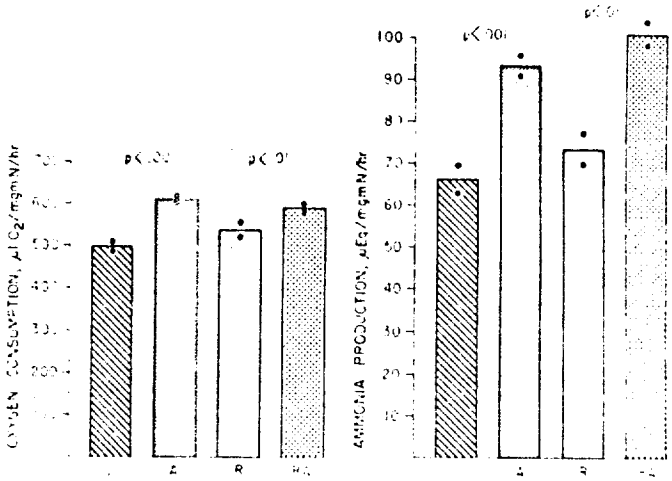

FIG. 4. Effect of acidosis and rapid regeneration after unilateral nephrectomy on glutamine oxidation and ammonia production from glutamine in rat kidney cortex centrifuged homogenates. Height of bars represents mean of 9 observations. Black dots define standard error of mean. P values are over the two bars they compare. Letters referring to bars are as in Fig. 1.

There is another interesting aspect of these data, and that is the very high aerobic oxidation rate of glutamine in relation to glutamic acid, and to a lesser extent α-ketoglutaric. In the assimilation of glutamine, both the deamidation of its omega amide group and transamination of its alpha amino group are anaerobic. Thus, when glutamine is incubated with viable tissue in the presence of ATP and Mg^{++} ions, one would perhaps expect its aerobic oxidation rate to equal that of its oxidizable products: glutamic or α-ketoglutaric acids. Yet, as we see here, its rate of oxidation is considerably higher than either of these at all substrate levels studied. This suggests that the aerobic oxidation of glutamine is

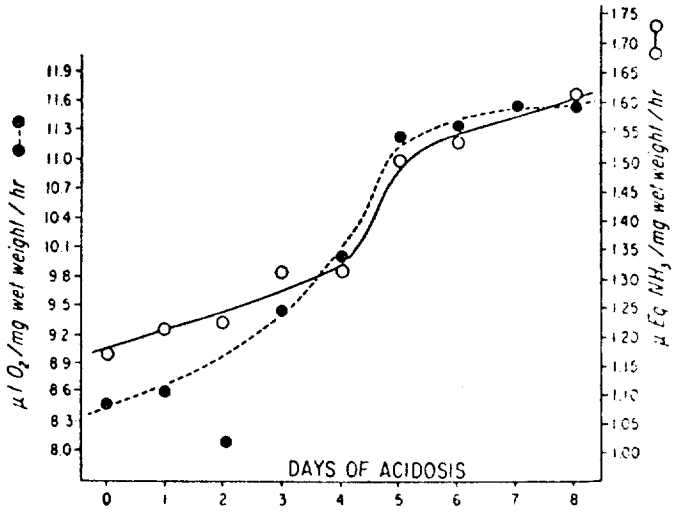

FIG. 5. Experiment showing parallel temporal development of change in oxygen consumption with glutamine and ammonia production from glutamine in centrifuged homogenates of kidney cortex from rats drinking 0.28 M NH_4Cl.

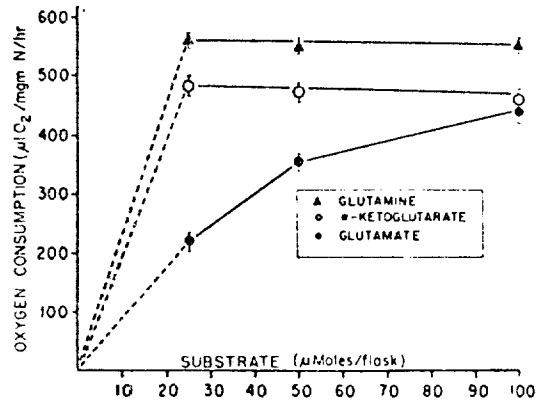

FIG. 6. Oxygen consumption with equivalent levels of glutamine, sodium glutamate, and sodium α-ketoglutarate in centrifuged homogenates of kidney cortex from 7-day acidotic rats. Each point represents mean of 7 rats, with standard errors indicated. In each experiment the 3 substrates were run with the same homogenate.

qualitatively different from either of its major oxidizable products, and may involve enzymes and/or pathways different from the known ones for glutamic or α-ketoglutaric acids.

Perhaps there is oxidative deamination of glutamine, utilizing the NADP-linked glutamic dehydrogenase system. This raises the possibility that some ammonia produced in the kidney from glutamine might come from the α-amino group under normal aerobic circumstances. Experiments in our laboratory make this an unlikely possibility, however. When glutamine containing 95 atoms percent excess N^{15} in the amide group is used in our aerobic homogenate system described here, the free ammonia formed contains 90 atoms percent excess N^{15}, indicating that most of it has come from the amide group.[3] Alternatively glutamine may represent an "active" form of glutamate or α-ketoglutarate, and have a greater affinity or specificity for the assimilative mechanisms used by it and its metabolites. Glutamine could also permeate the mitochondrial membrane more easily than the other two metabolites, and thus have a higher rate of oxidation. These data emphasize the need for more study of the aerobic oxidative metabolism of glutamine in mammalian tissues.

Glutaminase increase and renal hypertrophy in regulation of ammonia excretion in chronic acidosis. Since we have shown that the kidney hypertrophies in chronic metabolic acidosis, it was logical to ask at this point about the role of this process in the expanding capacity of the kidney to excrete ammonia in chronic acidosis. To answer this question, in part, two experiments were performed with similar results. One of these is shown graphically in Fig. 7. After several days acclimatization to diet and surroundings, all animals drank tap water and control measurements of ammonia excretion were made. Then the rats were divided into two groups of six each. One was allowed to drink 0.28 M NaCl, and the other drank 0.28 M NH_4Cl. All rats were kept on these regimens for 5 days during which there were daily urine collection periods of 4 hr at the same time each morning for measurement of ammonia excretion. The figure shows that among the NaCl drinkers there was no significant change in ammonia excretion. However, the NH_4Cl drinkers developed a greatly increased rate that had reached a plateau by the 3rd day. By the 5th day on NH_4Cl, we know that the renal glutaminase activity has increased markedly and that the kidneys have undergone significant growth. Therefore when these changes in the two kidneys of the acidotic rats were fully developed, both control and acidotic rats were unilaterally nephrectomized. Then all animals were continued on their same regimens, with daily measurements of ammonia excretion made as before. During this period we would expect all remaining kidneys to hypertrophy, those on NH_4Cl to an even greater extent (Fig. 2 and Table 1). We would not, however, expect any significant further increase in renal glutaminase enzymes (Fig. 4). Thus any regained capacity of the remaining kidneys to excrete ammonia after unilateral nephrectomy should result from increased renal tissue mass, or some function associated with it, rather than further induction of glutaminase activity. The data show that the control nonacidotic rats showed no significant change in their rate of ammonia excretion. The acidotic rats, after unilateral nephrectomy, showed very little change in ammonia excretion; indeed, by 2 days after operation they had completely regained their prenephrectomy capacity to excrete ammonia. This regained capacity followed the time course of the renal hypertrophy in such unilaterally nephrectomized animals where significant compensatory hypertrophy has already occurred by 36–48 hr.[4]

[3] This experiment was performed by Dr. Daniel O'Donovan in our laboratory, and I am grateful for his permission to quote it.

[4] These experiments were performed by a medical student, Mr. Tunde Solola, during tenure of a summer fellow [illegible] tory.

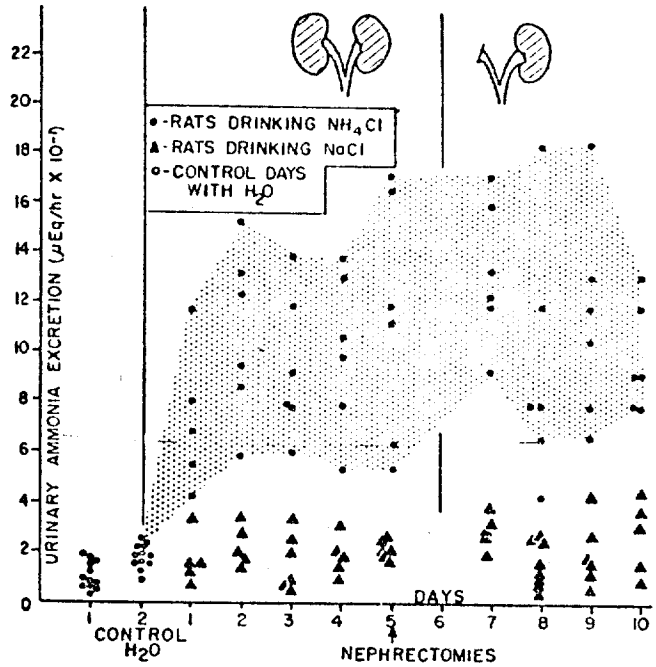

FIG. 7. Comparison of urinary ammonia excretion by rats drinking 0.28 M NaCl, or 0.28 M NH_4Cl before and after unilateral nephrectomy. Each dot represents value measured on 1 rat each day. Stippled envelopes enclose range of points in the two main groups.

The even greater speed of this change in the kidney is underlined by the observation we have made that RNA content is already significantly increased in the remaining kidney by 6 hr after unilateral nephrectomy, and in both kidneys of the acidotic rat by 6 hr after the intragastric administration of 5 ml of 0.6 M NH_4Cl. Thus, simple increase in renal tissue mass, or some as yet unidentified metabolic function of it, might contribute to an increased excretion of ammonia under the stimulus of acidosis. It should be noted that further reduction of urine pH in these animals could have facilitated ammonia excretion and thus accounted for this maintained high rate of ammonia excretion. Urine pH measurements were not made. However, it seems unlikely that a significant enough further drop in urine pH would have occurred in these rats after they had already been on NH_4Cl for 5 days. What relative contributions urine pH, renal hypertrophy, and enzyme induction make to the over-all change in ammonia excretion cannot at present be stated quantitatively from these data.

DISCUSSION

The mechanism of renal hypertrophy following unilateral nephrectomy is still not well understood. Perhaps removal of one kidney either releases a growth-stimulating factor for the remaining kidney, or reduces below a critical level the circulating concentration of some factor that normally inhibits kidney growth. This hypertrophy involves both new cell formation and enlargement of existing cells (12). The synthesis of new DNA accompanying the increased mitosis in compensatory renal hypertrophy has been observed by Simpson (14) using P^{32} incorporation into DNA. We have been able to confirm this observation in our laboratory. Threlfall et al. (15) have also observed a significant increase in DNA content of the remaining kidney at 48 hr, and the experiments reported here confirm this finding, although there is considerable quantitative variation in the results from experiment to experiment.

It is not the purpose of this paper to present a comprehensive review of compensatory renal hypertrophy; much work needs to be done before we have any real understanding of this remarkable process. Rather I wish only to record here the observed renal hypertrophy that occurs in chronic NH_4Cl acidosis, to point out its relation to compensatory hypertrophy after unilateral nephrectomy, and discuss its possible relation to the regulation of ammonia production and excretion by the kidney. I also want to emphasize that this hypertrophy is a true growth of the kidney, involving synthesis of new DNA, RNA, and tissue protein, and further that the growth-stimulating effects of NH_4Cl acidosis and unilateral nephrectomy are additive. Although both DNA and RNA increase in both types of renal growth, there are definite differences in their patterns of increase, indicating differences in the relative rates of increase in cell numbers and cell size in the two circumstances. These differences in pattern of nucleic acid change and the additive effects of acidosis and unilateral nephrectomy on total RNA, DNA, and protein synthesis indicate that the two stimuli affect the renal cellular machinery for protein synthesis in an additive way.

The experiments shown in Fig. 7 show that renal hypertrophy, or some function of it, without any change in glutaminase activity, can significantly affect rate of ammonia excretion. It is possible that the continued high rate of ammonia excretion by the remaining kidney after uninephrectomy resulted from a further stimulation of its ammonia-producing capacity by a relative increase in the acid load presented to it as NH_4Cl. Perhaps in the dog, where no change in renal glutaminase is observed in acidosis (8), there is already enough basal enzyme activity to meet the demands for increased ammonia production and excretion in acidosis. Pitts implies this in his recent review (7). Dog kidney glutaminase activity is considerably higher than that of the rat under normal circumstances (10), and therefore these enzymes may not be saturated with glutamine in normal animals with their prevailing rates of renal glutamine extraction. In metabolic acidosis, when the kidney extracts more glutamine from its arterial blood, these enzymes could therefore produce more ammonia from it without any induced enzyme change. Conversely there could be an increase in total kidney mass of the type seen here in the rat, without any change in enzyme concentration per unit mass of tissue. Either circumstance would result in an increasing capacity to excrete ammonia without any observable change in tissue glutaminase concentration. Thus [illegible] of enzyme saturation in the normal state, induced [illegible]

change, and induced renal growth may all be factors underlying the expanding capacity to excrete ammonia in acidosis. In different species these several variables, and perhaps others as well, may operate to relatively different extents in producing the over-all change in ammonia excretion.

We are grateful to Dr. Ernest Kay, Department of Biochemistry, who was of great assistance in teaching us the methods for nucleic acid analysis. It is a pleasure also to give particular thanks to Gerald Gordon, a student in the Co-op program at Antioch College, who participated in the early part of these studies, and to Rosa Maria Dies for her excellent technical assistance throughout.

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show some differences in the recovery periods, with values rising more slowly when insulin was injected after 6 days of food deprivation. This is in accord with the experimental findings of McCormick (19).

Results of sensitivity tests done on 2 dogs in nonacidotic and acidotic states are presented in figures 3 and 4, with graphs representing concentrations of glucose in whole blood, and of potassium and inorganic phosphorus in the plasma after intravenous injections of standard doses of insulin. In each animal the duration of the perfusions for inducing acidosis was 68 hours. At the time of the second test the serum pH values in both were less than 7.0 and the CO_2 content of the serum less than 6 mEq/liter. Both charts show a sharp drop in concentration of inorganic phosphorus and potassium accompanying the fall in blood sugar after insulin in the nonacidotic animals, and little or no fall in these values after insulin when the animals were severely acidotic. *Dog 8* (fig. 3) showed an unusual degree of diuresis during the development of acidosis, which might account for the drop in concentration of inorganic P from 4 mg. in the normal state to 2 mg./100 cc. in the acidotic state (before insulin). In this animal, correction of the state of acidosis by the intravenous administration of sodium bicarbonate was followed promptly by a considerable decrease in the concentration of both inorganic phosphorus and potassium in the plasma (not shown here). Further studies on effects of the administration of alkali to the acidotic animals will be reported in a later communication.

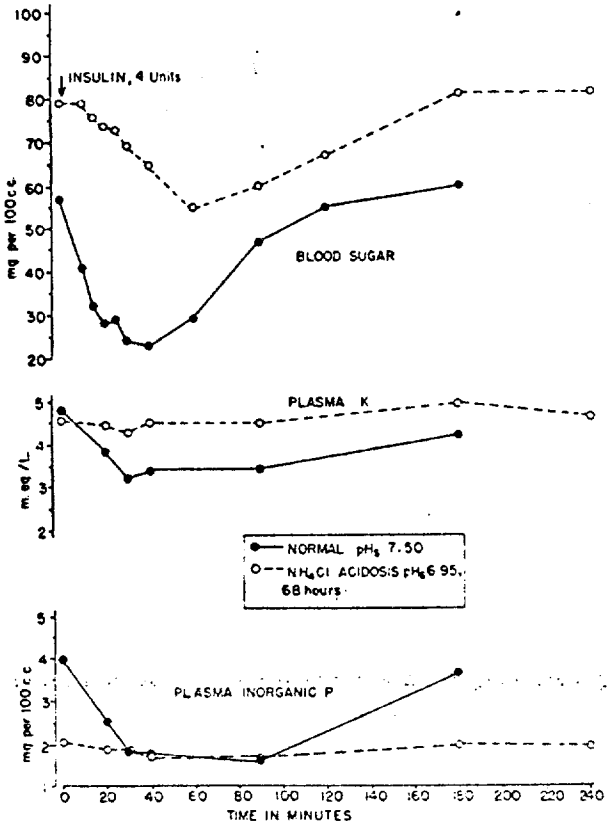

Fig. 3. INSULIN SENSITIVITY tests on *dog 8*, in nonacidotic state and in acidosis induced by intravenous perfusion of ammonium chloride.

Data from a series of such insulin sensitivity tests on 6 dogs in normal (fasting) state and in acidosis are listed in table 1. In the several experiments the duration of the perfusions of ammonium chloride before the second test was from 24 to 68 hours. In each instance the serum pH was found to be below 7.10 before and just after the second test, with the serum CO_2 content below 7 mEq/liter. Qualitative tests for ketones in the urine were consistently negative.

Data on blood sugar concentrations recorded in the tests made in normal and acidotic states were analyzed by the method of covariance (20). The calculations indicated a probability less than 1 in 100 ($P<.01$) that differences between the 2 sets of data could have occurred by chance.

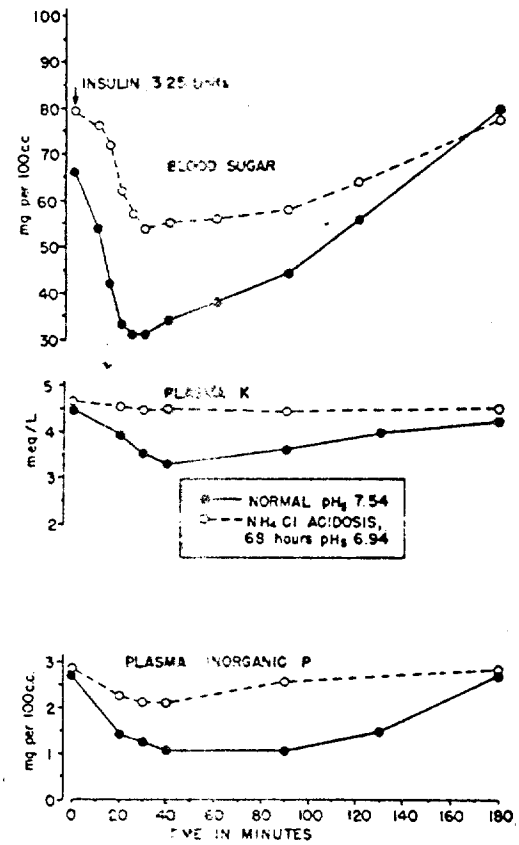

Fig. 4. Insulin sensitivity tests on *dog 9*, in nonacidotic state and in acidosis induced by intravenous perfusion of ammonium chloride.

Discussion

Various investigators (21–24) have observed that the administration of either glucose or insulin produced transient sharp decreases in the concentrations of potassium and inorganic phosphorus in the blood plasma. Fenn in 1939 (25) found that increased storage of glycogen in rat livers was accompanied by concomitant deposition of potassium and phosphate. In 1949 Levine (26) showed that the movement of glucose into cells was expedited by insulin, and that a fall in level of serum inorganic P accompanied the entrance of glucose and other hexoses into the body tissues. In normal animals, closely parallel changes in concentrations of glucose and of potassium and inorganic P in the blood plasma that follow the administration of insulin appear to be dependent upon [illegible] mechanisms of cellular metabolism. Since standard doses of insulin in acidotic animals provoked less fall of glucose and little or no change in concentrations of potassium and inorganic P in the plasma, it may be presumed that those same mechanisms are inhibited under conditions of acidosis.

Many factors are involved in the homeostatic control of relatively fixed levels of potassium and inorganic P in the blood plasma in normal and pathologic states. Changing values for concentrations of potassium and inorganic P in the plasma do not mean that exchanges between cells and plasma are closely balanced, or that changing rates of renal excretion nicely clear the plasma to maintain these elements at a fixed level. In states of acidosis increased cellular catabolism leads to the liberation of potassium and phosphorus into the plasma and, consequently, to greatly increased urinary excretion of potassium and phosphates—if renal function is not impaired. The increased phosphaturia comes in large part from the breakdown of labile stores of organic phosphates in tissue cells, a breakdown that is most easily

TABLE 1. INSULIN TOLERANCE TESTS ON DOGS IN FASTING STATE, NONACIDOTIC (NORMAL) AND ACIDOTIC

		TIME AFTER INSULIN, MINUTES	0	10	15	20	25	30	40	60	90	120	180
Dog 8	pH[1] 7.50	Glucose[2]	57	41	32	28	29	24	23	29	47	55	60
		K[3]	4.8			3.8		3.2	3.4		3.5		4.2
		P[4]	4.0			2.5		1.8	1.8		1.6		2.7
	pH 6.95	Glucose	79	79	76	74	73	69	65	55	60	67	81
		K	4.6			4.4		4.3	4.5		4.5		5.0
		P	2.1			1.9		1.9	1.7		1.7		2.0
Dog 9	pH 7.54	Glucose	66	54	42	33	31	31	34	38	44	56	79
		K	4.4			3.9		3.5	3.3		3.6		4.2
		P	2.7			1.4		1.3	1.1		1.1		2.7
	pH 6.94	Glucose	79	76	72	62	57	54	55	56	58	64	77
		K	4.7			4.5		4.5	4.5		4.4		4.5
		P	2.8			2.2		2.1	2.1		2.5		2.8
Dog 10	pH 7.52	Glucose	76	56	41	36	40	44	37	37	55	73	90
		K	5.0			4.0		3.4	3.3		3.3		4.5
		P	3.4			2.9		2.1	1.8		2.3		3.2
	pH 7.10	Glucose	95	85	78	72	69	71	70	69	64	63	73
		K	5.9			5.2		4.7	5.0		5.2		[illegible]
		P	5.6			4.8		4.8	4.5		4.8		[illegible]
Dog 11	pH 7.54	Glucose	78	56	49	36	54	54	42	44	67	74	82
		K	4.4			3.6		3.3	3.4		3.5		[illegible]
		P	3.8			2.2		1.7	1.8		2.0		3.8
	pH 7.08	Glucose	76	71	67	61	61	60	60	53	52	53	55
		K	4.7			4.4		4.4	3.9		4.4		[illegible]
		P	3.4			3.0		2.9	2.7		2.7		[illegible]
Dog 4	pH 7.49	Glucose	73	59	53	43	34	44	37	35	45	57	[illegible]
		K	5.6						3.6				[illegible]
		P	4.3						2.1				[illegible]
	pH 7.05	Glucose	112	103	95	90	81	81	80	78	83	88	[illegible]
		K	7.1			5.4		5.1	5.3		5.6		[illegible]
		P	3.9			4.0		4.1	[illegible]		[illegible]		[illegible]
Dog 5	pH 7.60	Glucose	71	61	45	39	45	44	[illegible]	39	45	[illegible]	[illegible]
	pH 7.00	Glucose	105	100	101	87	93	94	86	82	77	73	[illegible]

[1] pH of serum during the test period. [2] Blood sugar (glucose) values, mg./100 cc. of whole [illegible]
[3] Plasma K, mEq./liter. [4] Plasma inorganic P, mg./100 cc.

demonstrable in a lowered concentration of organic acid-soluble P in the blood cells, which may serve in this respect as an index of changes occurring in other organs. In each of the experiments here reported the concentrations of organic acid-soluble P in the blood cells were determined and were found markedly reduced [illegible]

to be reported later), in accord with earlier observations on ammonium chloride acidosis in man and animals (27, 28).

Labile phosphoric esters of the blood and tissue cells (ATP, ADP, hexosephosphates, phosphoglycerate) play essential roles as intermediary compounds in carbohydrate metabolism, in the synthesis and storage of glycogen and in processes of mobilization, transport and utilization of carbohydrate in the tissues. By experiments done on blood cells *in vitro* it has been shown that the enzymatic reactions involved in the turnover of these substances are strongly influenced by changes in pH. Synthesis of organic acid-soluble phosphates in the cells during glycolysis is favored by pH values of 7.4 and above, while pH values below 7.3 lead to decomposition of the organic phosphates and liberation of inorganic P from the cells (29). Inhibition of insulin action in states of acidosis may be plausibly ascribed primarily to effects of low pH on enzyme systems, interfering with the synthesis of these esters. Inhibitory effects may also result from increased decomposition of these esters, or from lack of intermediary substrates for phosphorylation and glycogen formation when labile stores of phosphorus and potassium in the body tissues are depleted. Some evidence of the latter effect may be found in the fact that inhibition of insulin action appeared to be greater in the dogs with acidosis of long duration, 68 hours or more, than in dogs with acidosis of short duration, 24 hours or less, the shorter periods presumably involving less depletion of intracellular materials.

Increased destruction of insulin may be suggested as another possible cause for its apparently decreased effectiveness during states of acidosis. It is conceivable that insulin-destroying enzymes (30) may be either more active or present in increased concentration in the body tissues in acidotic states than under normal conditions, but no evidence is available to support such an hypothesis.

Summary and Conclusions

To determine the effects of a nonketogenic type of acidosis on the action of insulin, insulin sensitivity tests were made with 6 nondiabetic fasted dogs in normal states and in states of severe acidosis induced by slow intravenous perfusion of ammonium chloride.

During the development of acidosis in the fasted animals, concentrations of sugar in the blood tended to rise slightly, with maximal increases around 35 mg/100 cc above the fasting level. Standard doses of insulin administered intravenously in severely acidotic dogs produced less fall in the concentration of blood sugar, with slower rate of fall and slower recovery to initial levels in the 3 hour test period, than in the same dogs in nonacidotic states. Levels of plasma potassium and inorganic phosphorus tended to change very little or to remain stationary after injections of insulin in the acidotic dogs; whereas in nonacidotic states changes in concentration of plasma potassium and inorganic phosphorus (both sharply decreasing and then returning to initial levels) paralleled closely the changes of blood sugar induced by insulin.

Conditions of acidosis probably inhibit the action of insulin by interfering with processes of phosphorylation that are involved in the cellular uptake of sugar, potassium and phosphorus from the plasma.

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Effects of Ammonium Chloride Acidosis on Glucose Tolerance in Dogs[1]

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In a previous report (1) we have shown that the blood sugar lowering action of insulin in dogs is inhibited by states of severe acidosis induced by intravenous perfusions of ammonium chloride solution; also, that levels of potassium and inorganic phosphorus in the blood plasma, which normally fall and rise in parallel with changes of blood sugar during periods of insulin action and recovery therefrom, tend to remain stationary after insulin injections in acidotic animals. While the mechanisms involved in such inhibition of insulin effects are not well understood, it seems likely that acidosis per se interferes with enzymatic processes of phosphorylation that are involved in the concomitant cellular uptake of glucose, potassium and phosphorus from extracellular fluids. As an extension of those experiments, the studies reported here deal with parallel determinations of blood sugar, plasma potassium and inorganic P during glucose-tolerance tests done on dogs in nonacidotic and acidotic states.

Procedures

Mongrel male dogs, previously fed a standard diet of commercial dog food and horse meat, were fasted 3 days before each test, with water allowed *ad libitum*. For the glucose-tolerance tests, a sterile 50 per cent solution of dextrose in distilled water was administered intravenously in amounts to give 0.5 gm. of dextrose/kg. of body weight in 4 dogs and 1.75 gm. in 1 dog. The injections were completed within periods of 2 to 3 minutes. Methods of chemical analysis were the same as previously cited (1). Concentrations of sugar in whole blood, potassium and inorganic P in plasma, were determined just before and at standard intervals during 3 hours after the injections of glucose. At least a week elapsed before tests were repeated on the same animals.

Acidosis was induced by continuous intravenous perfusions of sterile M/6 solution of ammonium chloride in distilled water during periods of from 46 to 72 hours, as previously described (1). For following the development of acidosis, determinations of the CO_2 content and pH of the blood serum were made at suitable intervals. Tests of glucose tolerance in acidotic states were made when serum pH values reached

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TABLE 1. GLUCOSE-TOLERANCE TESTS ON FASTING DOGS IN NONACIDOTIC (NORMAL) AND ACIDOTIC STATES

DOG	STATE	GLUCOSE INJECTED, GM/KG.	ANALYSES	MINUTES AFTER GLUCOSE INJECTION					
				0	5	30	60	120	180
12	Normal pH, 7.45	1.75	Glucose	77		289	134	81	81
			K	4.80		4.56	4.35	4.86	4.56
			P	2.82		1.59	1.30	1.71	2.29
	Acidotic pH, 6.93	1.75	Glucose	110		464	362	348	315
			K	7.83		8.52	8.70	9.00	7.59
			P	7.58		6.80	6.76	6.54	6.76
13	Normal pH, 7.52	0.5	Glucose	83	174	157	125	94	74
			K	4.35		4.20	4.26	4.44	4.71
			P	3.80		2.77	3.24	3.80	4.01
	Acidotic pH, 7.07	0.5	Glucose	114	215	197	188	145	122
			K	4.95		4.50	4.41	4.05	4.08
			P	3.55		3.01	3.22	3.54	3.76
14	Normal pH, 7.54	0.5	Glucose	63	205	142	104	68	65
			K	4.32		4.38	4.32	4.38	4.38
			P	3.07		2.26	2.15	2.73	3.14
	Acidotic pH, 7.08	0.5	Glucose	82	184	152	132	111	93
			K	5.70		5.70	5.76	5.64	5.76
			P	3.79		3.49	3.64	3.64	3.81
15	Normal pH, 7.50	0.5	Glucose	67	184	145	103	79	73
			K	4.59		4.05	4.05	4.08	4.23
			P	2.91		1.60	1.32	1.90	2.70
	Acidotic pH, 6.94	0.5	Glucose	97	229	194	181	139	99
			K	5.07		5.10	5.16	5.10	4.74
			P	2.60		2.09	1.77	2.23	2.34
16	Normal pH, 7.45	0.5	Glucose	72		162	142	100	90
			K	4.02		4.35	4.41	4.05	4.08
			P	3.04		2.38	2.20	2.40	2.72
	Acidotic pH, 6.92	0.5	Glucose	157		235	210	104	103
			K	6.00		6.54	6.87	6.87	6.87
			P	3.72		4.06	3.51	3.50	3.46

Analyses: blood sugar (glucose) values, mg/100 cc. of whole blood; plasma K, mEq/liter; plasma inorganic P, mg/100 cc.

7.1 to 6.9. Perfusions of ammonium chloride were continued at a slow rate during the tests and then terminated. The animals all recovered with no apparent deleterious aftereffects.

Results

Table 1 presents data from glucose-tolerance tests done on 5 dogs in normal (fasting) states, with serum pH values around 7.50, and in acidosis, with values for serum pH 7.08 or less and serum CO_2 content less than 7.0 mEq liter. Initial blood sugar levels were uniformly higher in acidotic than in the nonacidotic states. After intravenous injections of glucose the blood sugar values rose in most instances higher in the acidotic than in the nonacidotic dogs, and in all instances fell more slowly during the 3-hour test periods. The blood sugar always returned to initial levels within 2 hours in nonacidotic dogs, but not in acidotic dogs.

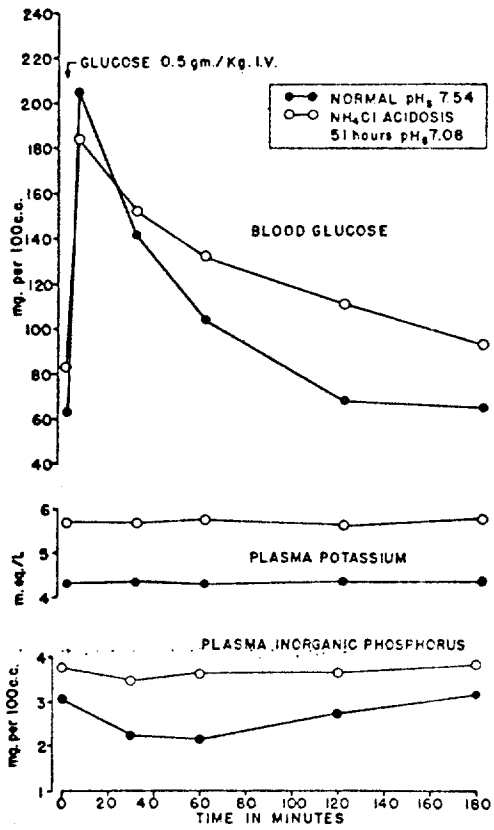


Fig. 1. Glucose-tolerance tests on *dog 14* in nonacidotic state and in acidosis induced by ammonium chloride.

In most of the tests done in nonacidotic states, concentrations of inorganic P in the blood plasma fell immediately following the injections of glucose and rose again within the test period: changes of potassium levels tended to follow this pattern but were of lesser magnitude. In acidotic states, in most instances the levels of inorganic P fell less or tended to remain stationary and plasma-K levels changed but little if at all; the latter changes were not constant and not uniformly similar in pattern. Two slightly different patterns of these changes are shown in figures 1 and 2, representing data from tests done on *dogs 14* and *15*.

For closer comparison and evaluation of differences between changes of blood sugar in tests listed in table 1, the formula of Greville (2) was applied to each set of data. For the interpretation of glucose-tolerance test data of this sort, Greville developed the equation $\log (y - y_0) = kt + C$, wherein y_0 represents the initial blood sugar, y the blood sugar at a given time t after the injection of glucose, and k and C are constants. When values $\log (y - y_0)$ are plotted against t a straight line may be drawn through the points and a regression coefficient may be calculated to represent the sugar-disappearance rate. Figures 3 and 4 illustrate applications of this formula to data from tests on *dogs 14* and *15*, with linear regression lines representing rates of disappearance of sugar from the blood in normal and acidotic states. For statistical evaluation, regression coefficients were thus calculated for each set of data in table 1; tests (3) applied to the dif-

ferences between the paired coefficients indicated clearly that the sugar disappearance rates were significantly ($P < .001$) less in acidotic than in nonacidotic states.

Discussion

Deuel and Gulick have reported (4), on the basis of findings not in accord with results of the present studies, that ammonium chloride acidosis did not affect glucose tolerance in dogs. The apparently conflicting results very likely may be explained by differences in experimental conditions and techniques. Possibly, more severe acidosis developed in the dogs tested in our study. Deuel and Gulick did not report serum pH values in the animals used in their experiments.

The finding of diminished rates of cellular uptake of intravenously injected glucose during states of severe acidosis is in accord with results of our earlier studies demonstrating decreased effectiveness of insulin in acidotic states. Evidence gathered in those studies indicated also that acidosis inhibited the concomitant cellular uptake of potassium and inorganic phosphorus, which seems to be a part of insulin effect. In the present experiments no significant differences were observed in patterns of serum-potassium levels during the glucose-tolerance tests in normal and in acidotic states. Changes of inorganic phosphorus were, however, less in acidotic than in normal states. In normal states usually only a very slight decrease in concentration of plasma potassium after glucose injections was demonstrable. It seems likely that the stimulus to mechanisms that involve cellular uptake of potassium and phosphorus concomitantly with sugar is less after intravenous injection of glucose than after insulin; if so, inhibition of that stimulus in acidotic states would be less apparent in these experiments than in those dealing with insulin effects.

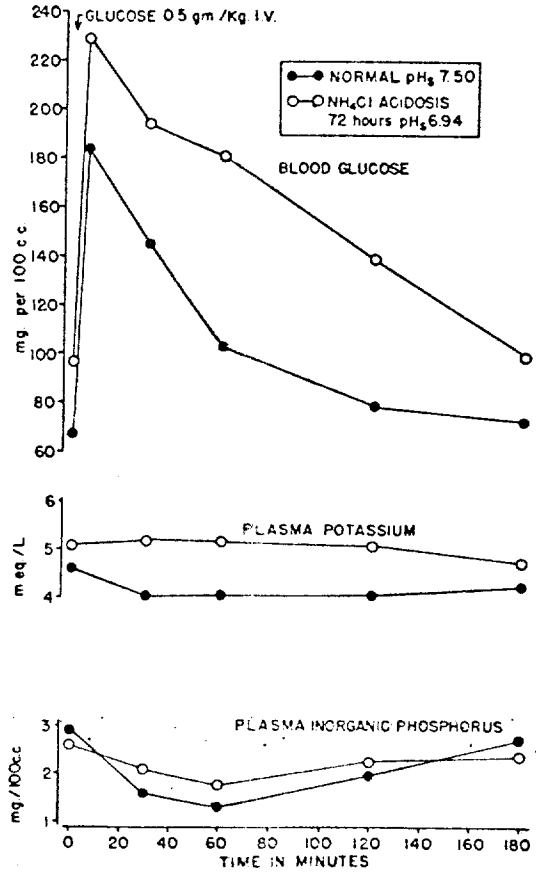
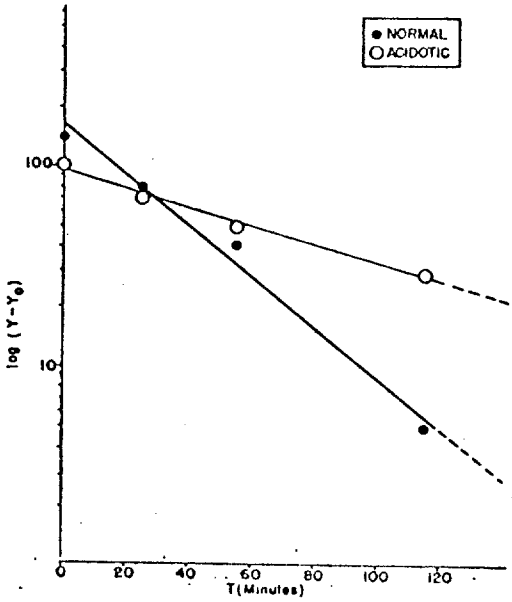


Fig. 2. Glucose-tolerance on *dog 15* in nonacidotic state and in acidosis induced by ammonium chloride.

Summary

Intravenous glucose-tolerance tests with parallel determinations of blood sugar, plasma potassium and inorganic phosphorus were done on dogs in normal (fasting)

states and in states of severe acidosis induced by intravenous perfusions of ammonium chloride solution. Following injections of glucose, the rate of disappearance of sugar from the blood was slower in acidotic than in normal states. The plasma inorganic phosphorus levels in normal states decreased and rose with the rise and fall of blood sugar but in acidosis changed less or remained stationary. Changes of plasma potassium did not follow a consistent pattern.



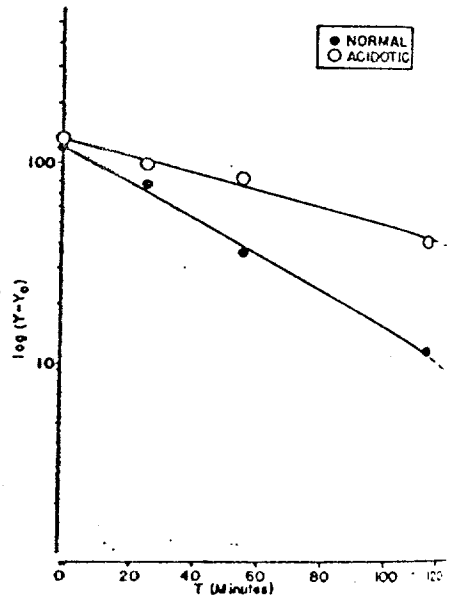


Fig. 3 (*left*). GLUCOSE-TOLERANCE on *dog 14* (see fig. 1). Glucose disappearance rates represented by linear regression lines derived from Greville's equation (2).

Fig. 4 (*right*). GLUCOSE-TOLERANCE TESTS on *dog 15* (see fig. 2). Glucose disappearance rate represented by linear regression lines derived from Greville's equation (2).

The findings are in accord with our previous report that states of acidosis inhibit cellular uptake of sugar, probably by inhibiting processes of phosphorylation.

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The Influence of Various Chemicals and Vitamin Deficiencies on the Excretion of Glucuronic Acid in the Rat

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Introduction

Certain aliphatic alcohols, aldehydes, ketones, terpenes, phenols, hydrocarbons, certain amines, aromatic acids, and many heterocyclic nitrogen compounds are detoxified by conjugation with glucuronic acid (1, 2, 3, 4). Many of the compounds thus detoxified are medicinal agents (5, 6, 7). It has been suggested that glucuronic acid might be used clinically to decrease the toxicity of sulfonamides (8) but the difficulty of preparing glucuronic acid in any degree of purity precludes the practical application of this procedure. It was therefore deemed advisable to seek a simpler chemical which would increase the formation of glucuronic acid in the body.

Procedure

Glucuronic acid (GA) content of the urine was determined by the use of naphthoresorcinol according to the technique of Maughan, *et al.* (9). As pointed out by Bueding (10) limited accuracy is achieved in applying any naphthoresorcinol method for GA. Some substances (glucose, etc.) inhibit the color development; others (mucic acid) tend to increase it. To circumvent this lack of specificity in method, the positive experiments were checked using the method of Quick (11) and the newer method of Bueding (12). It is felt by the present authors that the napthoresorcinol method can be safely applied to a dilute medium such as urine. This is in accord with the reports of other groups (13, 14, 15, 10). Meyer, *et al.* (15) stated, "None of the methods available gave accurate results for uronic acids in native fluids, with the possible exception of normal urine, since carbohydrates and other compounds containing carbonyl groups as well as proteins and their decomposition products interfered." In the experimental studies here reported, if, for example, mucic acid was being tested for its effect on GA excretion, a check was made using a solution containing an amount of the mucic acid equal

to the amount of GA determined. If at this concentration, the mucic acid gave a significant color, the series was discarded. Glucurone, prepared by the method of Williams (16) was used as the standard for all determinations.

All other chemicals unless specified were chemically pure reagent grade. The animals were housed in metabolism cages and urine samples collected under toluene for 24 hour periods after the oral administration of the chemical under test. Glass wool and special filtering devices prevented contamination of the urine samples.

TABLE I

The Effect of Various Compounds on Glucuronic Acid Excretion in the Rat

(Values in mg./kg. of rat for a 24 hr. period)

Compound	Dosage	Glucuronic acid excretion					
Control	*g./kg.*	*80 mg. kg. each.*	*Av. value for 15 sets of 12 rats Max. 95.2 mg./kg., Min. 73.4 mg./kg.*				
Dihydroxyacetone	4	129.0	104.5				
Glycerine	4	168.0	153.0	111.0	135.0		
Lactic Acid	2	117.0	98.0	119.0	140.0	127.0	91.0
Ca Glycerophosphate	4	109.0	60.0	44.0			
Pyruvic Acid	2	24	37	58	127	27	
Acetic Acid	2	55	105	81	118	58	
Glycolic Acid	2	65	101	85			
Na Ethyl Oxalacetate	2	62	92	73	11	96	
Tartaric Acid	2	129	53	41			
Succinic Acid	4	104	156				
Malic Acid	4	231	178				
Fumaric Acid	4	69	83				
Citric Acid	6	86	99	80	98		
Saccharic Acid	4	132	125				
Glucurone	4	94	105				
Ca Phytate	4	58					
Glycerine + Malic Acid	4 ea.	106					
Adenylic Acid + Malic Acid	0.5 & 4	210					
Adenylic Acid	0.5	134	158				
Nucleic Acid	4.0	92	108				

Each value represents the average for 10 sets of 2 rats in each set.

Results

Table I presents the results obtained with carbon compounds. Included in this table are adenylic acid and nucleic acid.

Dihydroxyacetone, glycerine, and lactic, succinic, saccharic, malic, and adenylic acids increase GA excretion in the urine. Pyruvic acid decreased excretion of GA. It is believed that acetic acid, calcium gly-

cerophosphate, glycolic acid, sodium ethyl oxalacetate, tartaric acid, and calcium phytate also decreased GA excretion, but variability of results prevents a final statement. Fumaric acid, citric acid, glucurone, and nucleic acid had little effect.

Of the amino acids tested, histidine, glycine, and alanine decreased GA excretion. Tyrosine and cysteine increased the excretion of GA. Cystine had no effect. Table II presents the results obtained. The results with ammonium chloride and sodium bicarbonate are included to show the effect of disturbance in alkaline acid balance.

TABLE II

The Effect of Various Amino Acids on Glucuronic Acid Excretion in the Rat

(Values in mg./kg. of rat for a 24 hour period)

Compound	Dosage	Glucuronic acid excretion				
	g./kg.					
Histidine	2	56	76	54	74	
Cystine	4	94	63	94		
Glycine	8	73	35	60	48	51
Tyrosine	4	159*	130	151	190	141
Cysteine Hydrochloride	4	128	123			
Alanine	4	48	61			
Ammonium Chloride	2	46	61	55		
Sodium Bicarbonate	2	51	80	58	51	

* Rats receiving stock diet + 10% *l*-tyrosine.
Each value represents the average for 10 sets of 2 rats in each set.

With the exception of riboflavin deficiency, all the vitamin deficiency states resulted in a marked decrease in the excretion of glucuronic acid. Table III lists the results and includes a value showing the effect of 48 hour starvation on the excretion of the acid. All values presented are for rats taken in a stage of a given vitamin deficiency before they became moribund and while their food consumption was still adequate to prevent the intervention of the starvation factor.

With the sulfonamides, the results were variable. In acute experiments all tended to increased GA excretion excepting sulfapyridine. Sulfaguanidine was tried in a chronic experiment in which it constituted 2% of the diet. Under these conditions the rats after two months on the diet showed a marked drop in the excretion of the organic sugar acid.

TABLE III

The Effect of Various Vitamin Deficiencies on Glucuronic Acid Excretion

(Values expressed in mg./kg. of rat for a 24 hr. period)

Deficiency	Urinary glucuronic acid content					
Pantothenate	92	24	54			
B_1 Thiamine	29.9	45.8				
B_2 Riboflavin	114	39	77	107	103	127
D	21.9	33.8	48	105		
A	43.3	80	27			
E	2.3	17.7	15.2	44		
B_6 Pyridoxin	38	33				
Starved 48 hrs	20					

Each value represents the average of 10 sets of 2 rats each.

TABLE IV

The Effect of Various Sulfonamides on Glucuronic Acid Excretion in the Rat

(Values in mg./kg. of rat for a 24 hour period)

Sulfonamide	Dosage	Added compound	Dosage	Glucuronic acid in urine				
	g./kg.		*g./kg.*					
Sulfanilamide	2				83	118	84	110
Sulfanilamide	2	Pyruvic Acid	4	76				
Sulfanilamide	1	Glucurone	2	80	198			
Sulfanilamide	2	Lactic Acid	4	75				
Sulfanilamide	2	Sodium Acetate	4	37				
Sulfanilamide	2	Malic Acid	4	94				
Sulfapyridine	4			59	31	28	34	
Na Sulfathiazole	1			210	134			
Sulfadiazine	6			97	60			
Sulfaguanidine	2% diet, 2 months			11.5	28.8			

Each value represents average for 10 sets of 2 rats each.

Tests conducted with sulfanilamide plus a compound known to produce an effect, positive or negative, on the excretion of GA showed that the sulfonamide tended to return all values to a more nearly normal figure.

Discussion

The objective of this work and of a companion effort (17) was to determine methods by means of which decreased acetylation and increased glucuronic acid formation could be brought about. The hope was that certain compounds might force the detoxication, particularly of sulfanilamide, away from acetylation and into combination with glucuronic acid. The literature contains ample evidence of the probability of this concept.

Our results *in vivo* in rats confirm those of Lipschitz and Bueding (18) *in vitro* using rat liver, in that dihydroxyacetone and lactic acid increased the formation of GA; but they differ in that we did not observe an increase following the administration of pyruvic acid, actually finding decreased excretion of GA. Quick (19) reported that lactic acid decreased GA formation in dogs, a point of dissimilarity with our findings in the rat. His result with glycolic acid is confirmed by our work. Another point of dissimilarity is in our observation that alanine decreased GA excretion, which would be expected if alanine were converted into pyruvic acid by oxidative deamination. Here, our results are in disagreement with both Lipschitz and Bueding (18) and Quick (19). Adeline (20) found that glycine, alanine, arginine, tyrosine, and histidine increased the output of glucuronic acid. In our experience alanine, glycine, and histidine decreased glucuronic acid formation, while tyrosine and cysteine increased its formation. It is probable that all seeming discrepancies observed are merely the result of dosage and species differences.

The sequence of potency with regard to stimulation of GA formation is in the order of decreasing effect: glycerine, dihydroxyacetone, and lactic acid. It is therefore suggested that the three carbon unit precursor sequence is from dihydroxyacetone phosphate to glycerophosphate and finally to phosphoglyceric aldehyde, which then condenses to a six carbon unit.

The phosphorylation of the precursors of GA was suggested by Lipschitz and Bueding (18). This work is corroborated by our observation that adenylic acid increased GA excretion. It is suggested that the high phosphate potential of adenylic acid aids in the phosphorylation of the three carbon unit precursor of GA. Further, this concept is strengthened by our observations that certain dicarboxylic acids, particularly succinic and malic, markedly increase the excretion of GA.

Kalckar (21) demonstrated in experiments with kidney extracts that the oxidation of dicarboxylic acids such as succinic, fumaric, and malic, gives rise to vigorous phosphorylation of various substrates. From the more recent work of Colowick, *et al.* (22) the conclusion is drawn that the generation of energy-rich phosphate originates with the dehydrogenation step from succinic to fumaric acid. It is therefore suggested that succinic and malic acids increase GA excretion in the rat by facilitating the phosphorylation of its three carbon unit precursor.

In riboflavin-deficient rats, the GA excretion is somewhat increased, this constituting the exception to the general rule of decreased excretion in various vitamin deficiency states. Riboflavin is involved in many enzyme systems, among these the dehydrogenation of malic acid in muscle, of lactic acid in muscle, etc. This might lead to increased concentrations of certain four carbon dicarboxylic acids and of certain three carbon units which lead to increased GA formation. The lactic acid oxidase system would be defective and prevent lactic acid, a compound stimulating GA production, from being oxidized to pyruvic acid, a compound inhibiting its formation.

It is much more difficult to explain the results seen with the other vitamin states. Liver pathology observed in rats in some deficiencies may explain defective GA synthesis.

Scudi and Robinson (23) have reported that sulfanilamide, in contrast to sulfapyridine and sulfathiazole, does not stimulate GA output. The work emphasizes the high percentage of sulfapyridine, some 40% of which is found in the form of a highly soluble glucuronide. This work and that of Weber, *et al.* (24) conclusively established the fact that sulfapyridine in the dog is followed by the appearance in the urine of a glucuronide of a hydroxy derivative of sulfapyridine. In our experiments, sulfapyridine clearly depressed GA excretion. It should be noted again that our experimental animals were rats, whereas Scudi and Robinson (23) and Weber, *et al.* (24) used dogs. Sulfanilamide, in our experience, increased to a slight degree the excretion of GA; sulfathiazole increased it to a marked degree; sulfadiazine had little if any effect; and sulfaguanidine fed chronically resulted in a profound decrease.

Summary

Dihydroxyacetone, glycerine, lactic acid, succinic acid, malic acid, and adenylic acid markedly increase glucuronic acid excretion in the rat. Of the amino acids tested, only tyrosine and cysteine had a positive

effect. With the exception of riboflavin deficiency in which there is an increase in glucuronic acid excretion, all the other deficiency states showed decreased excretion of this acid. The effect of sulfonamides on the excretion of glucuronic acid by the rat was studied.

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The effect of ammonium chloride and sodium bicarbonate on the urinary excretion of magnesium, calcium, and phosphate

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Relatively few studies have been made in the control subject on the effect of ammonium chloride or sodium bicarbonate on the urinary excretion of magnesium, calcium, and phosphate. This paper reports such a study in 6 control subjects.

I. Material and methods

Six healthy ambulatory subjects, who were between the ages of 23 and 37 years, and who ranged in weight between 112 and 190 pounds, participated in the study (3 male medical students, 1 female medical student, 1 medical student's wife, and 1 female laboratory technician). They thoroughly understood the importance of the test, as to careful 24-hour collections of urine, and diet control. These subjects ate the same self-selected diet for the 3 periods of 5 days each of the experiment and collected 24-hour samples of urine during the same period. No preservative was added to the urine when the subjects were taking ammonium chloride. Thymol was used as a preservative during the control period and when sodium bicarbonate was taken. Samples of blood were drawn on the third day of each 5-day period. The 3 periods of study were as follows and in the sequence listed, except in one subject (R. J.) in whom periods 1 and 2 were reversed: (1) control diet (5 days); (2) control diet plus 8 Gm. of $NaHCO_3$ (95.2 mEq. of sodium) daily (5 days); (3) control diet plus 8 Gm. of NH_4Cl (plain) (151.2 mEq. of calcium) daily (5 days). During the 2 days, or more in a few instances, between periods the subject ate any diet desired.

Levels of serum magnesium were determined by the method of Simonsen and associates,[1] and magnesium in the urine, by a modification of this method; serum and urinary calcium, by the method of Clark and Collip[2]; and serum and urinary phosphate, by the method of Simonsen and associates.[3] Levels of serum bicarbonate were determined by the Van Slyke titration method. Urine pH was determined by the Beckman pH meter.

II. Results

The urine of 3 subjects was made more acid (pH 4.8-4.9) and the levels of serum bicarbonate fell below control values during ingestion of ammonium chloride. During the ingestion of sodium bicarbonate the urine pH became more alkaline (7.2-8.3), and there was a slight rise in the levels of serum bicarbonate, in all but one subject.

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Table I. Effect of NH_4Cl on urinary excretion of magnesium, calcium, and phosphate

Subject, sex, age	Magnesium: Control period (mEq./5 days)	Magnesium: NH_4Cl* period (mEq./5 days)	Magnesium: Difference (mEq.)	Calcium: Control period (mEq./5 days)	Calcium: NH_4Cl* period (mEq./5 days)	Calcium: Difference (mEq.)	Phosphate: Control period (mM./5 days)	Phosphate: NH_4Cl* period (mM./5 days)	Phosphate: Difference (mM.)
J.A. M, 24	52.54	58.71	+6.17	45.77	70.11	+24.34	147.8	165.8	+18
E.W. M, 23	85.45	91.00	+5.55	67.41	85.26	+17.85	207.6	198.7	−8.9
D.H. M, 31	53.19	56.40	+3.21	76.25	101.56	+25.31	115.45	160.1	+44.65
S.H. F, 28	33.05	35.01	+1.96	32.35	58.85	+26.50	115.41	132.95	+17.54
L.M. F, 30	40.96	46.08	+5.12	43.52	61.3	+17.78	111.05	138.2	+24.15
R.J. F, 37	26.80	34.75	+7.95	24.30	49.7	+25.40	113.0	156.9	+43.9
Mean			+4.99 ± 0.87† p < .01 > .001			+22.86 ± 5.4† p < .01 > .001			+18.2 ± 6.9† p = .05

*Eight grams of NH_4Cl per day by mouth.
†Standard error of the mean

Table II. Effect of $NaHCO_3$ on urinary excretion of magnesium, calcium, and phosphate

Subject*	Magnesium: Control period (mEq./5 days)	Magnesium: $NaHCO_3$† period (mEq./5 days)	Magnesium: Difference (mEq.)	Calcium: Control period (mEq./5 days)	Calcium: $NaHCO_3$† period (mEq./5 days)	Calcium: Difference (mEq.)	Phosphate: Control period (mM./5 days)	Phosphate: $NaHCO_3$† period (mM./5 days)	Phosphate: Difference (mM.)
J.A.	52.54	47.47	−5.07	45.77	42.95	−2.82	147.8	130.9	−16.9
E.W.	85.45	79.66	−5.79	67.41	62.75	−4.66	207.6	194.3	−13.3
D.H.	53.19	54.76	+1.57	76.25	70.10	−6.15	115.45	116.9	−28.55
S.H.	33.05	29.52	−3.53	32.35	28.46	−3.89	115.41	97.3	−18.11
L.M.	40.96	35.87	−5.09	43.52	41.40	−2.12	111.05	117.23	+3.18
R.J.	26.80	29.7	+2.90	24.3	20.3	−4.0	113.0	103.8	−9.2
Mean			−2.5 ± 1.5‡ p < .2 > .1			−3.81 ± 0.61‡ p < .01 > .001			−13.8 ± 4.3‡ p < .05 > .02

*See Table I for ages and sex.
†Eight grams of $NaHCO_3$ per day by mouth.
‡Standard error of the mean.

above the control levels, but not above the normal for this determination.

Table I documents the effect of ingestion of 8 Gm. of ammonium chloride per day for 5 days on the urinary excretion of magnesium, calcium, and phosphate in each subject as compared to the control period. The range of urinary electrolyte excretion of the different subjects is seen to vary widely in the control period. Despite this there was a mean increase in urinary magnesium (+4.99 mEq.), calcium (+22.86 mEq.), and phosphate (+18.2 mEq.), which was statistically significant during ingestion of ammonium chloride.

Table II gives the results of ingestion of sodium bicarbonate on the urinary excretion of these same ions. Four of the 6

*Table III. Review of changes in urinary electrolyte excretion with ingestion of NH_4Cl (from the literature)**

Investigator and year	*Number of subjects*	*NH_4Cl (Gm.)*	*Increase over control electrolyte excretion*			*Type of Comparison*
			Ca (mEq.)	*Mg (mEq.)*	*PO_4 (mM.)*	
Wiley et al.,[5] 1933	1	5.2	22	8	11	Total increase during 6 days on NH_4Cl over 6 days of control period
Tibbetts and Aub,[6] 1937	1 1	12.18 12.18	7 28	8.3 48	 27	Total increase during 6 days on NH_4Cl over 6 days of control period
Martin et al., (This study, 1959)	6	8	22.86	4.99	18.2	Total increase (mean for group) during 5 days on NH_4Cl over 5 days of control period
Keith et al.,[7] 1926	1	10.5	5.5	1.7	.3	1 day on control .3 days on NH_4Cl. Figures given are maximum 24-hour increase while on NH_4Cl
Sartorius and Pitts,[8] 1949	1 1	15 10.5	23.8	3.7	 19	Maximum 24-hour increase on NH_4Cl over mean 24-hour excretion of 5-day control period
Martin et al. (This study, 1959)	6	8	7.11†	3.45†	8.37†	Maximum 24-hour increase in electrolyte excretion on NH_4Cl over mean 24-hour excretion of 5-day control period (mean for group)†

*Rearranged and calculated to compare with our results.
†All highly significant mean changes.

subjects showed a decrease in urinary excretion of magnesium, whereas 2 showed a rise as compared to the control period. The mean change was not statistically significant. All subjects showed a decrease in the urinary excretion of calcium, with a mean decrease of 3.8 mEq., which was a significant mean change. All but one subject showed a decrease in urinary excretion of phosphate, but the mean decrease was not highly significant.

No significant changes in the values for serum magnesium or calcium, as determined on the third day of each test period, were found. Some minor to moderate variations in the levels of serum phosphate occurred in a few subjects, but without any definite pattern.

Discussion

Presumably, healthy subjects are in magnesium, calcium, and phosphate balance, and excrete any excess of these ions ingested. If each subject ate the same diet in each of the test periods, although different for each subject, change in the excretion of electrolyte should represent the effect of the drug taken, and not the amount of dietary electrolyte.

Thus, this project was designed to see not only whether there were changes in the urinary excretion of magnesium, calcium, and phosphate after ingestion of ammonium chloride or sodium bicarbonate, but whether there was an average or mean response to these drugs, despite variation of age, sex, weight, and intake of electrolytes. This was done purposely, since acidifying or alkalinizing agents are given to patients of different age, sex, and weight and on diets which differ as to the content of magnesium, calcium, and phosphate.

The results clearly show an increased urinary excretion of magnesium, calcium, and phosphate in all subjects after acidifi

tion of the urine with ammonium chloride. Furthermore, there was a mean response for the group, which was statistically significant, despite variations in sex, weight, age, and urinary output of electrolytes during the control period.

Only four studies (6 control subjects) have previously been reported[5-8] on the effect of ammonium chloride on the urinary excretion of magnesium, calcium, and phosphate. Table III summarizes this data. The material from the literature, where possible, has been rearranged and recalculated, to compare with our data, although amounts of ammonium chloride and periods of study were not always similar. Increases in the urinary output of magnesium, calcium, and phosphate during therapy with ammonium chloride was shown in all studies. The amount of these increases varied from our results in some instances. In part this may be due to differences in the amount of drug used and the period and design of the study.

Jabir and associates[9] reported acute increases in the excretion of magnesium after ingestion of ammonium chloride, but the design of his experiment does not allow comparison with our results.

Although the effects of acidifying salts on the excretion of calcium is well known, little attention has been paid to their effect on excretion of magnesium in urine.

Previous studies from our laboratory[10] have shown a fall in the level of serum magnesium and marked increases in the output of magnesium in the urine in patients with congestive heart failure who were receiving prolonged diuretic treatment with ammonium chloride and Mercuhydrin. The present study documents increased urinary output of magnesium in control subjects after the administration of ammonium chloride. The possibility of depletion of body magnesium with repeated and prolonged use of ammonium chloride, particularly if the dietary intake of magnesium is low, is thus a possibility, particularly if more potent diuretics, such as the mercurials or chlorothiazide, follow the use of ammonium chloride. The effects of depletion of magnesium in the experimental animal are well known. The studies of Flink[11] have delineated the clinical picture of magnesium deficiency.

Only one other study in one subject[5] has dealt with the effect of sodium bicarbonate on the urinary excretion of magnesium, calcium, and phosphate. In this study, changes in urinary electrolytes during 6 days of ingestion of 100 mEq. of $NaHCO_3$ over a 6-day control period were as follows: decrease in calcium of 7.5 mEq.; increase in magnesium of 7 mEq.; increase in phosphate of 1 mM. Ingestion of sodium bicarbonate in our study caused a decreased urinary excretion of magnesium in 4 subjects, of calcium in 6 subjects, and of phosphate in 5 subjects. The mean change was highly significant only for calcium (−3.81 mEq.).

Summary

Six normal subjects showed mean or average increases, which were statistically significant, in urinary output of magnesium (+4.99 mEq.), calcium (+22.86 mEq.), and phosphate (+18.2 mM.) during 5 days of ingestion of ammonium chloride, as compared to a 5-day control period. This occurred despite differences in the intake of electrolytes, age, sex, or weight. The possible significance of the loss of magnesium during diuretic therapy was discussed.

During ingestion of sodium bicarbonate there was a mean decrease over the control period which was statistically significant only for urinary output of calcium (−3.81 mEq.).

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Further Studies on the Cariostatic Effect of Organic and Inorganic Phosphates

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The results obtained by other investigators[1-7] and the experiments previously conducted in this laboratory[8-12] have indicated that a significant inhibition of experimental dental caries is brought about by dietary phosphorus supplements. In most instances this inhibitory result has been obtained with an inorganic phosphate. In two studies an organic phosphorus compound (i.e., sodium phytate) in the diet of the white rat was highly cariostatic,[12] but it has been reported non-cariostatic by one other laboratory.[13] As observed in the albino hamster, sodium phytate as well as dibasic sodium phosphate was not cariostatic.[11]

Speculation regarding the mechanism of the cariostatic property of a phosphorus compound has led to a comparison of ortho-, meta-, and pyrophosphates[7, 15] and of soluble versus insoluble phosphates,[10] as well as to experiments pertinent to the associative effect of other dietary minerals, particularly sodium chloride,[1, 10, 16] and to the relative availability of a phosphorus supplement while in the oral cavity.[17, 18] Whether these supplemental phosphates affect oral tooth surfaces, react otherwise within the oral cavity, or modify some systemic processes remains speculative. The studies of this report compared the cariostatic action of different ortho- and meta-phosphates and evaluated the caries-inhibitory effect of several organic phosphorus compounds.

EXPERIMENTAL METHODS

Following the general plan of previous studies,[12] these testing regimens utilized, at weaning age, 30–40 Sprague-Dawley white rats in each control and test group. The rats in control and test groups were littermates. Housed two per screen-bottom cage, the rats ate and drank ad libitum and were on experiment usually 60 days; in one study 90 days. After sacrifice, the heads were autoclaved to facilitate removal of soft tissue. The lower molar teeth were examined for dental caries as previously described.[9] The cariogenic diets employed and their percentage composition are shown in Tables 1 and 2. All diets contained as major ingredients 18.0 per cent glucose and whole-wheat flour or dry salt-free bread. The bread was purchased from a local bakery and was prepared according to the following formula: 13–14 lbs. of regular bread flour, 12 oz. shortening, 12 oz. skim milk powder, 12 oz. sugar, 1 lb. of yeast, and 1 gal. of water. The ash, calcium, phosphorus, and protein analysis of the diets are also presented in Tables 1 and 2. Several inorganic phosphates* were studied as dietary ingredients.

* These phosphates and information concerning their properties and uses were supplied by the Victor Chemical Works, Chicago, Illinois: (1) $(NH_4)_2HPO_4$ diammonium orthophosphate, very soluble. (2) $Ca(H_2PO_4)_2 \cdot H_2O$ monocalcium phosphate, monohydrate, Reagent P xx. Approximate solubility 3 per cent or less. Its solubility is complicated because the product undergoes hydrolysis and rearrangement to yield phosphoric acid and dicalcium phosphate. It is used as a leavening acid in

RESULTS

The essential data pertinent to the dental caries results of these studies are presented in Tables 1 and 2. Table 3 summarizes the reductions in caries incidence and

biscuit, cake, and pancake mixes, baking powder, and self-rising flours. (5) $Ca_3(PO_4)_2$ precipitated basic calcium phosphate, extremely insoluble. (4) Victamide an ammonium P_2O_5 complex, very soluble; a sequestering agent for metallic ions such as calcium and magnesium; a product of Victor Chemical Works, Chicago, Illinois. (5) Na_2PO_3 sodium metaphosphate, very insoluble, may yield about 0.5 per cent P_2O_5 in solution. (6) $Na_6P_6O_{18}$ sodium hexametaphosphate, infinitely soluble, active sequestering agent. (7) K_2PO_3 potassium metaphosphate, insoluble, may yield 3.0–5.0 per cent P_2O_5 in solution.

Potassium metaphosphate is a family of compounds with varying chain lengths and in all cases they may be considered as linear and hence will have some chelating properties. The sodium metaphosphates are quite different in nature and in this case the formula $(NaPO_3)_n$ may be considered as a general description. In those cases where n is either 3 or 4 (sodium trimetaphosphate or sodium tetrametaphosphate), such compounds will be cyclic in nature and have little or no chelating ability. When n is 6,

TABLE 1

DIET COMPOSITION AND ANALYSIS, EFFECT OF INORGANIC PHOSPHATE SUPPLEMENTS ON DENTAL CARIES IN WHITE RATS

Diet No.	311	315	316	317	318	300	301	302	303	304
	Study A					Study B				
Whole wheat flour	78.75	77.00	77.00	77.00	77.50	77.75	76.35	76.17	76.17	75.[illegible]
Cerelose	17.00	18.00	18.00	18.00	18.00	18.00	18.00	18.00	18.00	18.00
Liver powder	2.00	2.00	2.00	2.00	2.00					
Calcium carbonate	1.25	1.25	1.25	1.25	0.55	1.25	1.25	1.25	1.25	1.25
L-lysine·HCl						1.00	1.00	1.00	1.00	1.00
Vitamin mix (NBC)						2.00	2.00	2.00	2.00	2.00
$(KPO_3)_n$		1.75								1.75
$(NH_4)_2HPO_4$			1.75							
$(NaPO_3)_n$				1.75				1.58		
$CaCl_2H_2PO_4 \cdot H_2O$					1.75					
Na_2PO_3									1.58	
Victamide							1.10			
	Analysis of Diet									
Ash	2.25	3.98	2.96	3.95	3.97	1.96	2.53	3.49	3.65	4.13
Calcium	0.58	0.51	0.55	0.53	0.55	0.58	0.51	0.54	0.54	0.55
Phosphorus	0.29	0.56	0.61	0.71	0.70	0.30	0.61	0.63	0.64	0.63
Calcium phosphorus ratio	1.79	0.91	1.09	0.75	0.78	1.93	0.84	0.86	0.84	0.87
Protein (N×6.25)	14.38	14.38	16.25	13.75	14.38	14.38	14.38	13.75	12.50	13.13
	Dental Caries Incidence									
No. of rats	31	38	37	38	33	34	32	30	35	39
Av. daily gain (gm.)	1.1	1.2	1.6	1.1	1.1	1.3	1.4	2.1	2.0	2.[illegible]
Rats with caries (per cent)	82.35	81.57	87.83	26.31	20.63	91.17	81.81	70.00	82.13	71.[illegible]
Carious lower molar (per rat)	2.93	2.00	0.65	0.66	0.57	3.56	2.66	1.30	0.45	2.[illegible]
Severity score (per rat)	7.76	3.40	1.02	1.15	1.[illegible]	8.47	5.54	2.43	0.60	[illegible]

severity scores produced by the various phosphates. Inhibition of caries was shown by a reduction in caries incidence (per cent of rats having dental caries), in the number of lower carious molar teeth per rat, and in the caries severity score. With the excep-

this compound is generally considered to be the sodium hexametaphosphate of commerce, although actually the compound itself is a rather large family of complex phosphates—the chain length 6 being only one of the group. This latter compound, however, is linear and has excellent chelating properties.

The organic phosphates were as follows: (1) Phytin—the calcium, magnesium salt of phytic acid (inosital hexaphosphoric acid) contained 14.79 per cent total phosphorus; very slightly soluble; a product of Delta Chemical Company, New York, New York. (2) Calcium phytate—calcium salt of phytic acid, total phosphorus 23.10 per cent; very insoluble; a product of Nutritional Biochemicals Company, Cleveland, Ohio. (3) Sodium phytate—sodium salt of phytic acid; total phosphorus 21.20 per cent, slowly soluble to approximately 70 per cent at 25° C., pronounced sequestering properties; a product of the Corn Products Refining Company, New York, New York. (4) β-glycerol phosphate—the disodium pentahydrate $Na_2 \cdot C_3H_5(OH)_2 \cdot PO_4 \cdot 5H_2O$ was used; 11.02 per cent total phosphorus; soluble in the cold to form a 10 per cent solution; a product of Calbiochemistry, Los Angeles, California.

TABLE 2

DIET COMPOSITION AND ANALYSIS: EFFECT OF ORGANIC AND INORGANIC PHOSPHORUS SUPPLEMENTS ON DENTAL CARIES IN WHITE RATS

Diet No.	408	409	410	411	412	413	414	415
	Study C					Study D		
Dry salt free bread	73.13	73.13	73.13	73.13	69.63	69.63	69.63	69.63
Cerelose	18.00	18.00	18.00	18.00	18.00	18.00	18.00	18.00
Cornstarch	2.50	0.00	0.74	0.43	6.00	2.62	2.91	1.46
L-lysine·HCl	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Vitamin mix (NBC)	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
$CaCO_3$	1.37	1.37	1.37	1.37	1.37	1.37	1.37	1.37
NaCl	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Sodium phytate		2.50						
$NH_4 \cdot HPO_4$			1.76					
Ca_3PO_4				2.07				
Phytin						3.38		
Calcium phytate							3.69	
Beta glycerol phosphate								4.54
	Analysis of Diets, Per Cent							
Ash	3.61	4.65	4.12	3.42	2.76	3.69	3.97	3.42
Calcium	0.64	0.64	0.63	1.45	0.62	0.89	0.91	0.85
Phosphorus	0.14	0.54	0.52	0.51	0.12	0.61	0.61	0.61
Calcium-phosphorus ratio	4.57	1.19	1.21	2.65	5.16	1.46	1.51	1.40
Protein (N×6.25)	12.89	14.06	14.06	12.50	12.93	13.81	13.31	12.68
	Dental Caries Experience							
No. of rats	37	37	33	37	35	38	39	39
Av. daily gain	1.4	2.1	2.1	2.1	1.1	1.8	1.9	1.8
Rats with caries (per cent)	97.4	17.9	16.2	59.5	100.0	57.9	30.8	12.8
Carious lower molar (per rat)	4.2	0.6	0.6	1.8	3.8	1.5	0.7	0.5
Severity score (per rat)	16.5	1.6	1.1	4.8	9.9	2.3	1.1	1.3

tion of the ammonium complex (Study B, Table 1) and $(KPO_3)_n$ (Study A, Table 1), the caries inhibition by the inorganic phosphates was remarkably consistent and significant. Study A permitted a comparison of two soluble phosphates $(NH_4)_2HPO_4$ and $Ca(HPO_4)_2 \cdot H_2O$ diets 346 and 348) in which the water-soluble phosphorus was 0.48 and 0.47 per cent, respectively. In diets 345 and 347 the phosphates $(KPO_3)_n$ and $(NaPO_3)_n$ were compared. They provided 0.12 and 0.11 per cent soluble phosphorus, respectively. The results gave no evidence that solubility affected the cariostatic action. Inhibition by $(NH_4)_2HPO_4$ (Studies A and C) confirmed previous results,[10] as did the results with $Ca(HPO_4)_2H_2O$ (Study A) and $Ca_3(PO_4)_2$ (Study C) when

TABLE 3

PER CENT REDUCTION IN CARIES INCIDENCE AND SEVERITY SCORE DUE TO DIETARY PHOSPHATE SUPPLEMENTS

Diet No.	Phosphorus Supplement and Per Cent in Diet		Phosphorus Content of Diet: Total Per Cent	Phosphorus Content of Diet: Added by Supplement Per Cent	Caries Reduction: In Incidence Per Cent	Caries Reduction: In Severity Score Per Cent
410	$(NH_4)_2HPO_4$	1.76	0.52	0.38	83.4	93.3
346	$(NH_4)_2HPO_4$	1.75	.61	.32	54.1	87.2
348	$Ca(H_2PO_4)_2 \cdot H_2O$	1.75	.70	.41	75.0	79.5
411	$Ca_3(PO_4)_2$	2.07	.51	.37	38.9	70.9
391	Victamide	1.40	.61	.31	10.3	31.8
347	$Na(PO_3)_n$	1.75	.71	.42	70.1	85.9
392	$Na(PO_3)_n$	1.58	.63	.32	23.2	71.8
393	$Na(PO_3)_n$	1.58	.64	.34	64.5	92.9
345	$K(PO_3)_n$	1.75	.56	.27	1.1	56.4
394	$K(PO_3)_n$	1.97	.61	.31	18.4	54.1
413	Phytin	3.38	.61	.49	42.1	76.8
414	Ca phytate	3.09	.61	.49	59.2	88.9
409	Na phytate	2.50	.54	.40	81.7	90.3
	Na phytate*	1.41	.34	.24	100.0	100.0
	Na phytate*	1.41	.42	.32	54.0	80.8
415	Na-β glycerol-PO_4	4.54	0.61	0.49	87.2	86.9

* See Ref. 12

NaCl was present in the diet. Previously, $Ca_3(PO_4)_2$ had not been cariostatic in the absence of dietary NaCl.[11]

The sodium metaphosphates, $(NaPO_3)_n$ (Studies A and B), and sodium hexametaphosphate, $Na(PO_3)_6$ (Study B), were somewhat more cariostatic than the potassium metaphosphates (Studies A and B). The percentage reduction in caries due to these phosphates is shown in Table 3. It is not known whether these differences were due to the sodium versus the potassium moiety. As mentioned above, relative solubility of these inorganic phosphates apparently was not a factor in their cariostatic effect, since $Na(PO_3)_n$ is quite insoluble and $K(PO_3)_n$ is very soluble. With remarkable consistency, particularly as indicated by a marked reduction in the caries severity score, the organic phosphates (Studies C and D) were highly cariostatic. The result with sodium phytate confirmed previous results.[12] Calcium phytate, phytin, β-glycerophosphate, and sodium phytate reduced caries severity scores 88.9, 76.8, 86.9, and

90.3 per cent, respectively (Table 3). These additional data emphasize further the extensive caries inhibition that may be associated with organic phosphorus compounds, at least under these experimental conditions. There was no evidence that variations in solubility influenced the cariostatic action of these organic phosphates as studied under these experimental conditions.

DISCUSSION

These particular test diets, as previously reported,[12] again produced extensive dental caries in white rats. Although most effective in developing smooth-surface caries on lower buccal areas, the lingual surfaces, as well as the upper molars, are involved when caries becomes especially rampant. This type of smooth-surface caries was illustrated in a previous publication.[19] It should be noted that these teeth were not sectioned for diagnosis of incipient small lesions in the deep sulci of the fissures. Occlusal fissure caries, therefore, was discernible and was recorded only when it had developed to a somewhat advanced state of severity so as to be seen microscopically. Only lower molars were diagnosed for caries.

The cause of the cariogenic effect of these diets has not been resolved. The 18 per cent glucose component would not appear to be a major cariogenic factor, although the bread component might provide up to 5–6 per cent of sugar. The protein content of the diets varied from 12.5 to 14.5 per cent. The phosphorus content was low and inadequate for normal growth. It is nonetheless uncertain what role a protein[20] or a phosphorus deficiency in these diets may have played as a factor of cariogenicity. The diet used in Study A was lysine-deficient also. Previously it has been shown that a lysine supplement corrected the cariogenic effect of lysine-deficient skim-milk powder diets.[21] A lysine deficiency was not involved, however, in test Diets 390, 408, and 412 that were supplemented with 1.0 per cent lysine.

The deficiency of lysine in all the diets used in Study A would account for a similar low rate of growth in all the rats in Study A. While this growth rate remained below normal in all groups, it may be noted that the rats receiving the phosphate supplements had a significantly lower caries experience than the control rats on Diet 344, which was not supplemented. It cannot be claimed, therefore, that an improved rate of growth due to the phosphate supplements (excepting the ammonium P_2O_5 complex, as occurred in Studies B, C, and D) was the cause of the cariostatic action of the phosphate supplements. An increased rate of growth does not appear to be responsible for a cariostatic effect of these supplemented diets. The cariogenic test diets, as well as the phosphorus-supplemented diets, contained an adequate level of calcium.

In previous reports[6, 11, 12] possible mechanisms of the cariostatic effect of phosphates have been discussed at some length. Our own early investigations with inorganic phosphates have indicated strongly that availability of the inorganic phosphate in the oral cavity was a determining factor of the cariostatic action. This hypothesis became seemingly less tenable when a cariostatic effect of sodium phytate was observed,[12] and the evidence in this report regarding the cariostatic effect of phytin, sodium phytate, calcium phytate, and β-glycerol phosphate would seem to call for further emphasis on the relation of phosphorus supplements to systemic factors affecting caries etiology. Apparently differences in solubility of the organic phosphates as well as of the inorganic metaphosphates did not affect their anticaries action. As pre-

viously mentioned,[12] this situation has disturbing experimental complications because (1) phytate may be hydrolyzed in the lower intestine and its phosphorus become nutritively available and (2) the rat can be extremely coprophagic, so that its fecal excretion (recirculated through the oral cavity) becomes an important factor in evaluating phenomena occurring in the oral cavity.

Thus further speculation awaits additional studies on the mechanism by which phosphorus may or may not inhibit experimental dental caries. The data do not show a clear-cut distinction between ortho- and metaphosphates and their cariostatic effect. The matter of solubility also remains unresolved. The situation may be enlightened by these studies insofar as they seem to establish an anticaries role of certain organic phosphorus compounds. The phosphorus anticaries mechanism thus should be resolved in light of a cariogenic effect of both organic and inorganic sources of supplemental phosphorus—with uncertain availability within the oral cavity. In a previous report[12] it was pointed out that Jenkins, Forster, Speirs, and Kleinberg[22] have suggested organic phosphates, including phytin, as the cariostatic factor in raw cereal foods, such as brown flour, and in seed hulls. This hypothesis may gain some support from these additional experimental data, pertinent to organic phosphates.

SUMMARY

Experimental dental caries in white rats was significantly inhibited by three orthophosphates—i.e., $Ca(H_2PO_4) \cdot H_2O$, $Ca_3(PO_4)_2$, and $(NH_4)_2HPO_4$—and by two metaphosphates—i.e., $Na(PO_3)_n$ and $Na(PO_3)_6$. The results with the orthophosphates confirmed previous studies. An ammonium P_2O_5 complex and $(KPO_3)_n$ did not significantly reduce caries incidence. They were less effective in reducing caries severity scores than the other inorganic phosphates tested in these experiments. As shown particularly by the caries severity scores and by a reduction in caries incidence, the organic phosphates that were uniformly cariostatic to a significant extent were the following: phytin, sodium phytate, calcium phytate, and β-glycerol phosphate. There is at present no explanation as to the mechanism of this experimental cariostatic effect of both inorganic and organic phosphates.

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248. Cerebral Oxidative Metabolism in Ammonia Toxicity

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Introduced by Edward Rabe

Although the association of coma and other neurological states with increased levels of blood and brain ammonia has been repeatedly observed, the mechanism of ammonia toxicity has not yet been completely explained. In these studies the effects of NH_4Cl on oxygen consumption and substrate utilization have been studied in brain slices and brain mitochondria of the cat. In cerebral cortex slices the addition of 10 mM results in a 16% decrease in oxygen consumption and a marked increase in the utilization of glucose and the production of lactic acid.

The effect of ammonia on mitochondrial metabolism depends on the substrate being utilized. When pyruvic acid or α-ketoglutaric acid is substrate, 15 mM NH_4Cl produces a 30%-40% decrease in oxygen consumption. When succinic acid, glutamic acid, or γ-aminobutyric acid is the substrate, no such decrease is observed.

Studies with pyruvic acid-1-C^{14} as substrate indicate that the decrease in oxygen consumption is equivalent to the decrease in substrate utilization. The addition of cofactors of the pyruvic acid oxidase system or the addition of [illegible] of the citric acid cycle do not [illegible] inhibitory effects of ammonia.

These [illegible] primary toxic effect of ammonia on cerebral oxidative [illegible] interference with [illegible] of pyruvic and [illegible] acids.

Invest. Ophthamol.
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Interrelations of the blood-aqueous potential and acetazolamide in the rabbit

James E. Miller

The intravenous administration of 100 mg. per kilogram of acetazolamide produced a 0.9 mv. abrupt fall in the 7 mv. resting potential, and was followed by a 1.4 mv. rise that persisted for more than 20 minutes. This alteration was not influenced by the previous injection (50 minutes) of 100 μg per kilogram of strophanthin K or nephrectomy (18 hours). One hundred milligrams per kilogram of ammonium chloride produced a 3.7 mv. rise in 37 seconds that lasted 25 minutes. If this injection was followed by acetazolamide in 2 minutes, the changes from the carbonic anhydrase inhibition could be noted, but the rise in positivity was prolonged for 37 minutes. The administration of 225 mg. per kilogram of 0.6M sodium bicarbonate lowered the gradient 1.5 mv. with a return to base line at 18 minutes. An infusion of 535 mg. per kilogram for 90 minutes, however, did not alter the gradient or the response to acetazolamide in spite of the plasma bicarbonate being elevated to 29 mg. per cent. Sodium fluoroacetate (0.25 mg. per kilogram) reduced the potential 1.8 mv., and the increase of positivity due to acetazolamide was maintained for the duration of the experiment in 5 of the 6 rabbits.

It has been noted previously[1] that a 7 mv. resting potential exists between the blood and aqueous, with the aqueous positive. This potential was altered by the intravenous administration of acetazolamide, which produced an abrupt 0.9 mv. fall followed by a 1.4 mv. rise that decreased over 20 minutes (Fig. 1).

Further investigation has shown that it is possible to influence the effect of acetazolamide by alterations in the acid-base balance. In addition, other metabolic inhibitors, such as fluoroacetate or strophanthin, were found to alter the potential gradient between aqueous and blood.

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Method

The preparation of the rabbits and the recording system have been described previously.[1] All of the agents, with the exception of sodium bicarbonate ($NaHCO_3$), were administered via the ear vein in less than 1 minute through a polyethylene cannula with an attached syringe. In the $NaHCO_3$ infusion experiment, a Harvard Infusion Pump was used to deliver 0.4 ml. per minute, and the anterior chamber electrode was inserted after completion of the injection. In all other determinations the potential was recorded prior to the injection. One hundred milligrams per kilogram of sodium acetazolamide (30 mg. per milliliter) was given in all experiments. In the group with nephrectomies, both kidneys were removed by lumbar incisions 18 hours prior to the determination.

Results

Strophanthin K. In 6 animals 100 μg of strophanthin K. (4.0 ml.) per kilogram was administered following the insertion

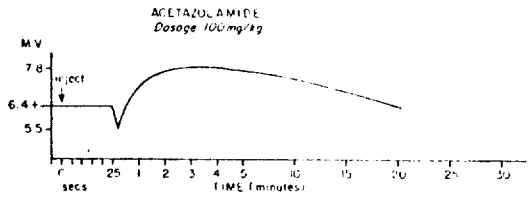

Fig. 1. A 0.9 mv. decrease in potential occurred 25 seconds after administration of acetazolamide followed by a 1.4 mv. increase at 4 minutes.

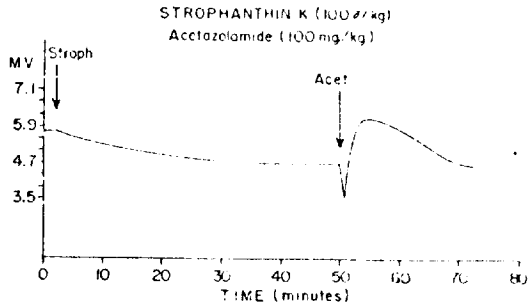

Fig. 2. The administration of strophanthin K was accompanied by a 1.1 mv. decrease over a 30 minute interval. The effects produced by acetazolamide were not altered.

of the electrode into the anterior chamber, and a continuous recording was made for 50 minutes. The average resting potential was 5.8 mv. (standard deviation [S.D.] 1.6). In 4 of the 6 animals a gradual decline of 1.1 mv. (S.D. 0.5) was observed. In the remaining 2 animals there was no change from the preinjection potential. Following acetazolamide, the usual occurrences were noted in all rabbits. The fall measured 1.2 mv. (S.D. 0.3) and the rise 1.5 mv. (S.D. 0.5) with the time of maximum rise averaging 4.6 minutes (Fig. 2).

In all animals the intraocular pressure decrease following strophanthin was measured by Schiotz tonometer. Preinjection tension determinations were 3.1 scale units (S.D. 0.5) and 7.8 scale units (S.D. 1.1) after strophanthin. In 3 animals a further 2.3 scale-unit decline in pressure was measured after acetazolamide. One of the remainder had a 2.0 scale-unit rise in pressure, and the other 2 did not change.

Nephrectomy. Nephrectomy 18 hours prior to the experiments did not change

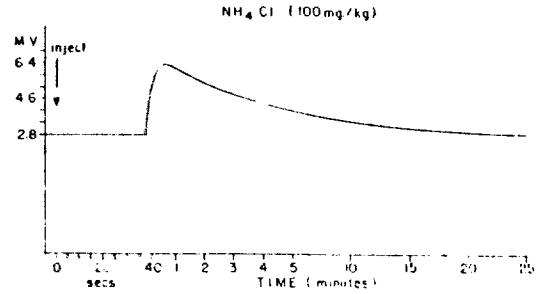

Fig. 3. Ammonium chloride elevated the resting potential 3.7 mv.

the potential or its response to acetazolamide in 6 animals. The preinjection potential gradient was 7.4 mv. (S.D. 4.6). A 1.1 mv. fall (S.D. 3.5) was noted 21 seconds after the administration of acetazolamide, and the subsequent rise was 1.5 mv. (S.D. 0.5). This occurred 4.6 minutes after the injection. The response was almost identical with that in the normal rabbit.

Ammonium chloride. Acidosis was induced in 6 rabbits by the administration of 100 mg. per kilogram of a 3 per cent ammonium chloride solution (30 mg. per milliliter) injected intravenously over a 1 minute interval. There was a 3.7 mv. rise (S.D. 0.7) from the 2.8 mv. base line (S.D. 1.3) which occurred 37 seconds after the injection. The mean return to the base line occurred in 25 minutes (Fig. 3).

In 6 additional animals acetazolamide was given 2 minutes after the injection of ammonium chloride. The alterations caused by acetazolamide appeared to be superimposed upon the ammonium chloride response. However, the rise in positivity was prolonged for an additional 12 minutes (Fig. 4).

The intraocular tension was determined in the undisturbed eye by a Schiotz tonometer prior to the injection of ammonium chloride. The mean value was 4.6 scale units with a 5.5 Gm. weight. Thirty minutes after the injection, it was 10.8 scale units. In the animals given the combination of ammonium chloride and acetazolamide the preinjection tension was 3.6

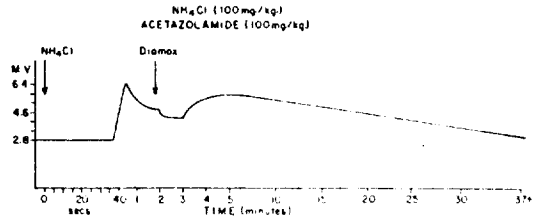

Fig. 4. The combination of acetazolamide and ammonium chloride prolonged the increase in positivity for more than 37 minutes.

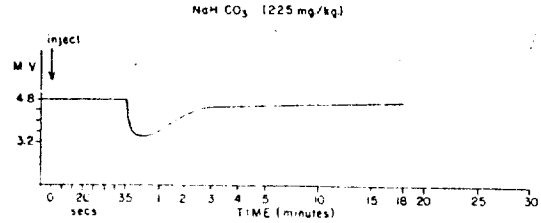

Fig. 5. A 1.5 mv. decrease in 35 seconds followed the injection of sodium bicarbonate.

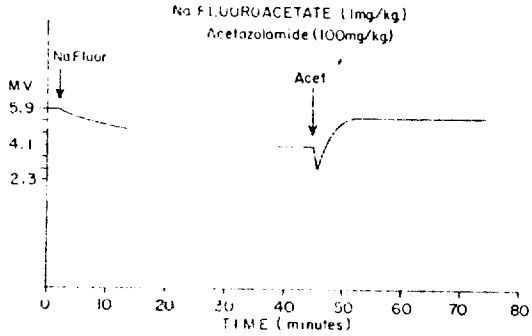

Fig. 6. A gradual decrease of 1.8 mv. was noted over a 45 minute interval. The increase in positivity from acetazolamide was prolonged for the remainder of the tracing.

scale units and the postinjection tension 8.5 scale units.

It appeared that both ammonium chloride and acetazolamide increased the positivity of the anterior chamber with reference to blood and lowered intraocular tension. The combination of the two compounds prolonged the increase in potential but was not additive.

Sodium bicarbonate. Transient alkalosis was produced in 6 animals by giving 225 mg. per kilogram of 0.6M sodium bicarbonate (50 mg. per milliliter). Before injection the potential was 4.8 mv. A 1.5 mv. fall (S.D. 1.7) occurred in 36 seconds and lasted 18 minutes (Fig. 5). Schiotz determinations with 5.5 Gm. weight averaged 3.0 scale units before the experiment and 5.8 scale units after.

In 14 animals 225 mg. per kilogram of 0.6M sodium bicarbonate was infused at the rate of 0.4 ml. per minute. At the completion of the infusion the blood-aqueous potential was found to average 6.8 mv. (S.D. 1.4). Acetazolamide produced a 1.4 mv. decrease (S.D. 0.5) and a 1.2 mv. rise (S.D. 0.5). The increase in the potential gradient lasted for an average of 13.6 minutes.

The plasma pH and sodium bicarbonate were determined in 7 of the infused animals; the pH measured 7.71 at 25° C. (S.D. 0.1), and the plasma bicarbonate 29.4 mg. per cent (S.D. 2.0). Schiotz determinations with a 5.5 Gm. weight averaged 2.9 scale units before infusion, 3.5 after infusion, and 5.5 after acetazolamide. Alkalosis did not block the effect of acetazolamide, either by its alteration of the blood-aqueous potential or decrease in intraocular tension.

E. Sodium fluoroacetate. Sodium fluoroacetate at a dose of 0.25 mg. per kilogram (0.5 ml.) was injected and the potential was recorded for 45 minutes. A gradual decline of 1.8 mv. (S.D. 0.2) was observed over the 45 minute interval. Following acetazolamide, the response was altered in that the increase in positivity of 2.3 mv. was maintained for the remainder of the experiment in 5 of 6 rabbits (Fig. 6). The initial fall did not appear to be altered in that it measured 1.4 mv. (S.D. 0.36). Sodium fluoroacetate decreased the normal blood-aqueous potential and altered the response to acetazolamide by prolonging the increase in positivity.

Discussion

It appears that the resting potential of 7 mv. between blood and aqueous is partially dependent upon sodium-potassium flux

since there was a decrease of 1.1 mv. in the potential following inhibition by strophanthin K. This was similar to a 4.4 mv. decline obtained when a higher local concentration of strophanthin G was administered through the lingual artery.[2] With the latter technique a 60 per cent decrease in aqueous flow was also measured. The potential changes following administration of acetazolamide were not altered by strophanthin, indicating its independence from sodium-potassium interchange. Thus, it appears that the resting potential and the alterations produced by acetazolamide are related to aqueous formation, but involve either separate steps in the same process or separate transport systems.

The most prevalent hypothesis of the action of acetazolamide in reducing aqueous secretion seems to be its influence upon intracellular buffering capacity.[3, 4] By its inhibition of carbonic anhydrase, the production of bicarbonate ions is decreased and leads to a metabolic acidosis. This decrease is at least partially a result of its local action on the eye since intracarotid infusion of acetazolamide has been noted to lower intraocular pressure initially on the infused side.[5] There was further suggestion that the action of acetazolamide occurred on a local basis by the normal potential pattern obtained in animals after nephrectomy.

A similar effect to acetazolamide was produced on aqueous secretion by systemic acidosis.[6] However, the total effect of the agent may be due to a combination of both systemic and metabolic acidosis since the blood pH was found to decrease 0.1 of a unit 5 minutes after the injection of 100 mg. per kilogram of acetazolamide.[7] This interval coincides with the period of maximum positivity observed in the potential measurements.

An increase in positivity also follows the administration of acidifying salts, such as ammonium chloride. The combination of ammonium chloride and acetazolamide maintained the increase in potential over a longer interval than either alone, which suggests a longer interval of intra- and extracellular acidosis.

A decrease in the potential gradient accompanied transient alkalosis with a relatively rapid recovery. Thus, the action of acetazolamide on the potential gradient between anterior aqueous and blood may be explained on the combination of alkalosis and acidosis. The pH of the sodium acetazolamide solution measured 9, and produced a momentary alkalosis followed by acidosis. The alkalosis decreased the electrical gradient momentarily and was immediately followed by carbonic anhydrase inhibition with resulting systemic acidosis.

It has been observed that systemic alkalosis blocks the action of acetazolamide upon aqueous secretion.[7] The infusion of sodium bicarbonate did not alter the potential changes produced by acetazolamide. However, a pressure decrease was obtained in the control eye following acetazolamide. Thus, under the conditions of these experiments, the reversal of acetazolamide effects by alkalosis was not verified either by tension or by potential measurements.

The change seen after the injection of fluoroacetate is similar to that of strophanthin in that the resting potential was reduced, with the initial phase of the acetazolamide cycle maintained. The continuation of the increase in positivity following carbonic anhydrase inhibition was not attributed to systemic acidosis since measurements have indicated that the decrease in plasma pH following acetazolamide was either blocked or delayed. One possible explanation for the prolongation of positivity may be the production of metabolic acidosis without systemic changes. This leads to the accumulation of acid radicals within the eye so that a diffusion current is maintained between blood and aqueous.

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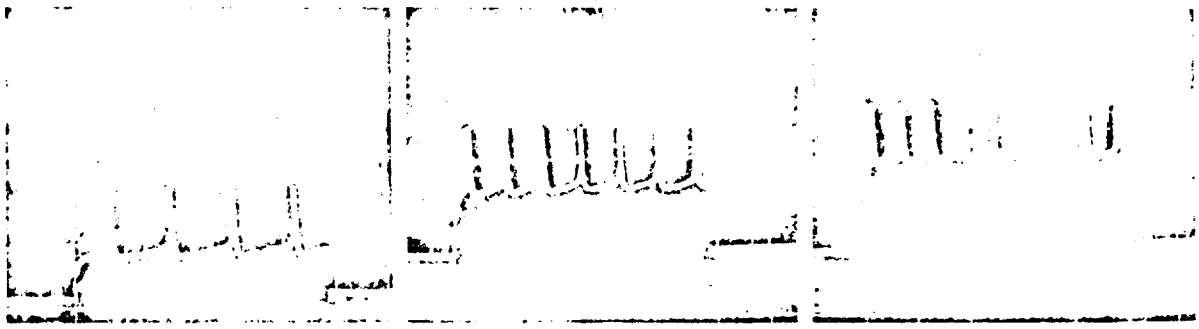
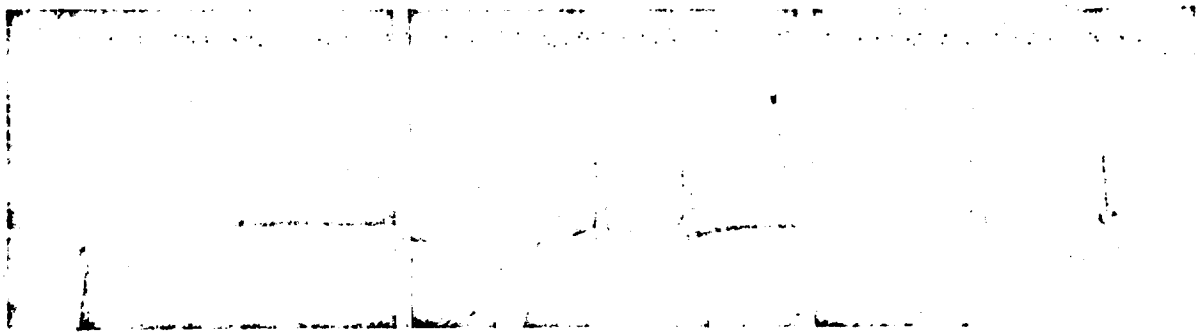
Transport of Ammonia by the Small Intestine of the Golden Hamster

HYPERAMMONAEMIA is thought to be a cause of hepatic coma. Ammonium which is contributed to the general circulation by the alimentary tract is derived from the degradation of protein and urea[1]. Ammonium chloride is absorbed readily from the small intestine *in vivo*, but a more precise characterization of ammonium transport can come only from *in vitro* investigations. Our report



a *b* *c*

Fig. 1. Muscle fibre response after positive intracellular stimulation. *a*, Stimulation current = $1{\cdot}0 \times 10^{-7}$ amp; *b*, stimulation current = 1×10^{-7} amp; *c*, stimulation current = $2{\cdot}0 \times 10^{-7}$ amp. Ordinate, 10 msec/division; abscissa, 50 mV/division.



a *b*

Fig. 2. Muscle fibre response after negative intracellular stimulation. *a*, Stimulation current = 6×10^{-7} amp; *b*, stimulation current = 1×10^{-6} amp; *c*, stimulation current = 1×10^{-6} amp. Ordinate, 10 msec/division; abscissa, 50 mV/division.

provides preliminary data on the movement of ammonium ions across the small intestinal mucosa of the golden hamster.

The everted sac of an isolated intestine is suitable for investigation of ion transport. Segments of duodenum, jejunum and ileum (4–8 cm) were prepared by the method of Wilson and Wiseman[2]. Particular care was given to cleansing both surfaces of the bowel so that secretions containing ammonium, debris and blood were removed. Sacs were incubated in Krebs–Ringer bicarbonate and 0·3 per cent glucose[3] for 1 h at 37° C in an atmosphere of 95 per cent oxygen and 5 per cent carbon dioxide or 95 per cent nitrogen and 5 per cent carbon dioxide. Identical solutions bathed both sides of the gut wall. Mucosal volumes were 35–50 ml. and serosal volumes were 0·9–1·8 ml. The initial serosal volume was measured with a calibrated syringe, and the final serosal volume was calculated by adding the change in weight of the sac after incubation to the volume originally introduced. Ammonia nitrogen was determined by a modification of the diffusion method of Seligson and Hirahara[4].

Control sacs, incubated in ammonium-free medium, liberated small amounts of ammonia nitrogen, which averaged 0·006 mmoles/l. in the mucosal fluid and 0·163 mmoles/l. in the serosal solution. The appearance of equivalent quantities in unincubated sacs suggested that this ammonium was extrinsic, not metabolic.

The simultaneous movement of ammonium, glucose, water, sodium and potassium was investigated at concentrations of ammonia nitrogen of 3·06–7·39 mmoles/l., a range physiologically suitable for the intestinal tract of man[5]. Ammonium transport was measured by three parameters: (*a*) percentage increase in serosal concentration (mmoles/l.) of ammonia nitrogen; (*b*) percentage increase in total amount (μmoles) of serosal ammonia nitrogen; and (*c*) μmoles/mg dry weight of ammonia nitrogen transferred from the mucosal to the serosal surface. Under aerobic conditions duodenal segments did not transport ammonium in either direction, while a small serosal accumulation occurred in the jejunum. Ileal sacs showed a significantly greater mucosal to serosal movement of ammonia nitrogen than did the other bowel segments. Differences in water, sodium, potassium and glucose transport were not significant. Results are summarized in Table 1.

The effect of various metabolic inhibitors on ammonium transport in the ileum was assessed (Table 2). Comparison with aerobic transference quotients revealed highly significant inhibition by anaerobiosis, 1×10^{-3} molar sodium cyanide and 1×10^{-3} molar and 1×10^{-3} molar 2,4-dinitrophenol ($P < 0·001$ by each inhibitor in all parameters of ammonia nitrogen transport). Despite these changes, the final serosal to mucosal ratio of ammonia nitrogen was greater than one in sixteen of seventeen sacs tested.

From the above data it is evident that although some mucosal to serosal movement of ammonium occurs in the jejunum, preferential transport of ammonium takes place in the ileum of the golden hamster. Active transport cannot be inferred, however, since we have not attempted to demonstrate ionic movement against an electrochemical gradient. The positive transference of ammonium even in the presence of minimal or negative water transport indicates that solvent drag is not the cause of the observed changes. Inhibition by cyanide and dinitrophenol points to an energy-dependent transport system, and so it is reasonable to suspect that aerobic metabolism may be essential for ammonium movement against a concentration gradient.

It is suggested that the movement of ammonium across the ileal membrane of the golden hamster may occur by a process other than that of simple diffusion.

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Table 1. COMPARATIVE TRANSPORT OF AMMONIUM, GLUCOSE, SODIUM, POTASSIUM AND WATER BY EVERTED SACS OF SMALL INTESTINE IN THE GOLDEN HAMSTER

	Jejunum	Ileum	*P*
No. of sacs	8	12	
Ammonia nitrogen transport (μmoles/mg/h)	−0·033–0·125 (0·045)	0·017–0·273 (0·131)	< 0·01
Percentage change (μmoles)	−15–62 (34·3)	33–174 (91·8)	< 0·01
(mmoles/l.)	−14·30 (8·4)	14–99 (59·5)	< 0·001
Water transport (ml/mg/h)	−1·3–12·8 (5·2)	0·2–15·5 (6·1)	> 0·6
Glucose transport (μmoles/mg/h)	0·103–0·508 (0·318)	0·272–0·861 (0·157)	> 0·1
Sodium transport (μmoles/mg/h)	−0·760–1·67 (0·512)	−0·140–1·73 (0·695)	> 0·6
Potassium transport (μmoles/mg/h)	−0·011–0·053 (0·018)	−0·054–0·060 (0·016)	> 0·9

Probability was determined by the *t* test.

Table 2. EFFECT OF INHIBITORS ON AMMONIUM TRANSPORT BY EVERTED SACS OF ILEUM IN THE GOLDEN HAMSTER

Inhibitor	No. of sacs	Ileal ammonia nitrogen transport (μmoles/mg/h)	Percentage change (μmoles)	Percentage change (mmoles/l.)
6 per cent nitrogen	6	−0·50–0·034 (−0·094)	−30–15 (−1·0)	3–29 (13·3)
Sodium cyanide 1×10^{-3}	3	−0·098–0·012 (−0·037)	−34–5 (−13·3)	−3–22 (10·3)
2,4-dinitrophenol 1×10^{-3}	3	−0·013–0·025 (0·002)	−4–17 (3·7)	13–23 (17·0)
1×10^{-4}	5	−0·030–0·050 (−0·003)	−14–13 (−3·0)	1–22 (9·2)

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MINERAL AND WATER CONTENT OF BONE[1,2]

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Depletion of the body stores of fixed base is the most serious complication of diabetic ketoacidosis, and losses of total body sodium of 25 to 50 per cent have been reported in diabetic acidosis in man (1–4). Recent work from several laboratories has indicated that from 30 to 45 per cent of the total body sodium exists out of solution in the mineral solids of the skeleton (5–7). It has also been demonstrated that this store of sodium is not fixed but may increase or decrease depending on whether the individual is subjected to excess sodium intake, uncompensated sodium loss or acidosis (8–10). The degree to which the bone sodium pool may be reduced seems to vary with the intensity and duration of the sodium loss and acidosis (7) and probably with the age of the animal (7, 8, 11). Therefore, experiments designed to examine effects of diabetes and of acidosis, separately and together, upon the size of this store of body sodium should be of value in designing rational electrolyte replacement therapy in diabetic acidosis.

In the experiments reported here the bone sodium content of rats with alloxan diabetes of both short and long duration was compared with normal controls of equal age. In addition, normal and diabetic rats were rendered acidotic by the intraperitoneal injection of ammonium chloride and the response of their bone sodium to this stimulus was examined. The results indicated that untreated alloxan diabetes, in the absence of acidosis, as indicated by normal blood pH's, had no effect on bone sodium stores, and that both normal and diabetic animals responded to acidosis with a comparable decrease of about 12 per cent in bone crystal sodium content.

[1] Supported by Public Health Service Grant A-525 and National Science Foundation Grant G-1307.

[2] Presented in part before the Twentieth International Congress of Physiology, Brussels, Belgium, August, 1956.

[3] Markle Scholar in Medical Science.

METHODS AND CALCULATIONS

All of these studies were carried out in male, albino rats of the Charles River strain, weighing 225 to 250 Gm., 9 to 10 weeks old at the initiation of the experiment. All of the animals were maintained, two to a cage, on an *ad libitum* diet of Purina Rat Checkers and tap water. All of the animals were fasted for 20 hours prior to sacrifice. The control animals for each group were obtained from the supplier at the same time as their experimental counterparts, and were maintained under identical conditions.

Diabetes was induced by the intravenous injection of alloxan monohydrate (40 mg. per Kg.) following a 24 hour fast. Criteria for the establishment of diabetes were two random blood sugar levels of over 300 mg. per cent one and two weeks after injection, together with a failure to gain weight in a normal fashion. Rats who became severely ill within 24 to 48 hours of alloxan injection were sacrificed and not used in this study, since we had previously found, at sacrifice and autopsy, that these animals had severe renal and hepatic damage, in addition to destruction of the beta cells of the pancreas, and abnormalities of these organs, especially the kidneys, might be expected to produce changes in the water and electrolyte content of the tissues, independent of the effects of diabetes. One group of diabetic rats was sacrificed three to four weeks after the injection of alloxan. Another group of diabetic rats was sacrificed at the same time following the induction of acidosis, together with a group of normal controls and nondiabetic acidotic animals. A third group of eight diabetic rats, together with six controls, was maintained for 23 weeks. During the twenty-third week two of the diabetic animals died, so the remaining six were sacrificed at that time, approximately six months after the establishment of diabetes. No insulin was administered to any of the diabetic animals.

Acidosis was induced by the intraperitoneal injection of normal saline, to which was added ammonium chloride (100 mM per L.). Eight ml. per 100 Gm. of body weight was administered four hours prior to sacrifice. It must be noted that ketoacidosis is almost impossible to induce in the alloxan-diabetic animal, since once diabetes has been established by this drug, loss of body fat occurs almost immediately unless insulin is administered, and within two to three weeks the fat depots have practically disappeared. Accordingly, aceto-acetic acid and other ketones in increased amounts do not appear in the plasma of these animals, presumably due to a lack of fatty acid

for metabolism, and intense, true ketoacidosis cannot therefore be produced. However, moderately severe hyperchloremic acidosis resulted from the procedure used here.

At sacrifice the rats were exsanguinated through the abdominal aorta under Nembutal® anaesthesia. A pH determination (using a glass electrode and a Model G Beckman pH meter) and a blood-sugar (by the Folin-Malmros method) were performed immediately on the whole blood. One fasted, alloxanized rat with a blood sugar of less than 200 mg. per cent was discarded and four rats given ammonium chloride who exhibited a pH of more than 7.32 were also excluded from the experiment. No analyses were performed on the bones of these animals. A semi-quantitative analysis for ketones [by the nitroprusside method (12)] was carried out on the plasma of each rat. Plasma water was determined by means of a micropyknometer, plasma chlorides by the method of Wilson and Ball (13), and plasma sodium by means of an internal, standard flame photometer (14), built by the authors. Because of the small amount of blood available, blood and plasma analyses were not carried out in duplicate.

Both femurs were removed for analysis. They were carefully scraped free of all adherent tissue, and the epiphyses removed with a small bone-cutter. The marrow was removed from the shaft by means of small wires. All possible precautions were taken to carry out these processes in a uniform, rapid manner and to avoid contamination with electrolytes or loss of water by evaporation.

Both femurs from each animal were placed in a tared weighing bottle and brought to constant weight at 102° C. The bones were then ground by hand in a mortar to a powder the consistency of fine talc. This powder was extracted three times with a mixture of equal parts of ethyl and petroleum ether to remove neutral fat. The marrow-free bones of young rats contain almost no fat (less than 1 per cent). However, the bones of the older animals contained significant amounts of fat (up to 5 per cent of wet weight). Therefore, all bones were fat-extracted. The methods used in the analysis of the bones for chloride and sodium have been described in detail elsewhere (9). Bone chloride and sodium analyses were all carried out in duplicate. Ashing was carried out on separate samples of 75 to 100 mg. The samples were ashed in platinum crucibles for 48 hours at 1,000° F.

Calculation of the crystal sodium, or that part of the sodium not contained in the extracellular fluid of the bone, was made on the assumption that all of the chloride pres-

TABLE I

Basic data

		Duration diabetes	Body wgt.	Blood sugar	pH	Blood ketones*
			Gm.	*mg. %*		
80–100 day old rats						
Normal (8)†	Mean		281	115	7.45	0
	S.D.		15.3	4.8	0.030	
Normal acidotic (7)	Mean		259	109	7.29	1 = 0, 1 = faint trace,
	S.D.		11.5	6.9	0.030	3 = trace, 2 = + to ++
	p‡		0.02	ns	0.001	
Diabetic (6)	Mean	3–4 weeks	234	218	7.43	5 = 0, 1 = faint trace
	S.D.		14.1	75.6	0.09	
	p		0.001	0.001	ns	
Diabetic acidotic (9)	Mean	3–4 weeks	218	269	7.29	5 = 0, 4 = faint trace
	S.D.		30.2	73.4	0.053	
	p		0.001	0.001	0.001	
Diabetic vs. diabetic acidotic	p		ns§	ns	0.01	
280 day old rats						
Normal (6)	Mean		484	106		
	S.D.		5.5	8.6		
Diabetic (6)	Mean	6 months	356	365		
	S.D.		12.6	65.0		
	p		0.001	0.001		

* Values of less than + represent less than 10 mg. per cent acetone; + to ++ represent 10 to 20 mg. per cent acetone (12).

† No. animals in group.

‡ p values are the significance of difference of means of normal and experimental animals. The 80 to 100 day rats are compared with the 80 to 100 day normals and the 280 day diabetic rats with the 280 day normals.

§ No significant difference ($p > 0.05$).

ent in bone is present in the extracellular and hydration shell water. Using the formulae of Hastings and Eichelberger (15), the amount of this water was calculated, and the sodium contained in it subtracted from the total sodium in the bone.[4] The remainder of the sodium was assumed to be present in association with the crystalline lattice of the bone. Accordingly, crystal sodium was calculated by dividing the concentration of total bone sodium (minus extracellular fluid sodium) in a Kg. of bone solids by the percentage of ash found per Kg. of solids, divided by 100. In the calculation of bone crystal calcium, the correction for calcium contained in the chloride space has been omitted, since it is always less than 1 mM.

Expression of calculated sodium and calcium values using this reference base has the advantage of eliminating fluctuations in total ions which may be due to changes in the volume or ion content of the chloride space of bone, and also presents the data as figures that are probably close to the actual concentrations of these ions in the bone mineral. It has the disadvantage that the chloride space as calculated may not be a true measure of this moiety of bone water.

Statistical analysis of the data was done using Fisher's t test for small samples.

RESULTS

Tables I through III present the results obtained. All experimental values were referred to the 80 to 100 day old normal controls, with the exception of the values for the 280 day old diabetic rats where control values were those found in the normal animals of the same age. In addition, statistical comparison of the data of diabetic versus diabetic acidotic animals was made.

In Table I it can be seen that diabetes caused a significant loss of weight. Short-term diabetic rats weighed 16.8 per cent less than normal controls, while after six months the diabetic animals weighed 26.5 per cent less than their nondiabetic littermates. In addition, acidosis caused loss of weight in both groups of animals, statistically significant in the normals. The acidotic animals had a marked diuresis following NH_4Cl, which probably accounts for the loss of weight. Blood sugars were markedly elevated in diabetes, and were higher in the rats who had had their disease for a longer period of time. Acidosis appeared to increase the blood sugar of diabetic rats, as compared with nonacidotic diabetic animals, perhaps due to the stress of this experimental procedure, but this difference was not statistically significant. Plasma pH was normal in the diabetic rats, and ammonium chloride did not result in a more marked drop in the diabetic animals. pH fell an average of 0.14 unit in the diabetic animals and 0.16 unit in the nondiabetic animals.

Results of plasma ketone analyses were of interest in that normal animals exhibited a much more marked degree of ketosis in response to acidosis than did diabetic animals, probably as a result of the previously mentioned fact that the presence of depot fat is a necessary precursor for the development of ketosis. This finding also suggests that lowered pH, such as occurred in the non-

TABLE II

Plasma water and electrolytes

		H_2O	Na	Cl
		Gm./L.	*mEq./L.*	
80–100 day old rats				
Normal (8)*	Mean	951	149	109
	S.D.	2.2	3.6	3.1
Normal acidotic (7)	Mean	952	144	121†
	S.D.	1.8	5.2	4.2
	p‡	ns§	ns	
Diabetic (6)	Mean	953	141	100
	S.D.	4.6	2.4	4.1
	p	ns	0.01	0.001
Diabetic acidotic (9)	Mean	955	150	116†
	S.D.	1.7	4.2	5.4
	p	0.01	ns	
Diabetic vs. diabetic acidotic	p	ns	0.001	ns
280 day old rats				
Normal (6)	Mean	945	148	103
	S.D.	3.5	3.5	1.3
Diabetic (6)	Mean	945	143	99
	S.D.	3.5	4.1	3.9
	p	ns	ns	0.05

* No. animals in group.
† Received NH_4Cl.
‡ p values are the significance of difference of means of normal and experimental animals. The 80 to 100 day rats are compared with the 80 to 100 day normals and the 280 day diabetic rats with the 280 day normals.
§ No significant difference ($p > 0.05$).

[4] The formula used for the calculation of the chloride space in bone is as follows:

$$CCS = \frac{Cl_s \times S}{Cl_p \div H_2O_p \times 0.96}$$

where

CCS = ml. "calculated chloride H_2O" per Kg. fat-free bone,
Cl_s = mM Cl per 100 Gm. fat-free bone,
S = Gm. solids per Kg. fat-free bone ÷ 100,
Cl_p = mM Cl per L. plasma,
H_2O_p = Gm. H_2O per L. plasma ÷ 1,000, and
0.96 = Donnan correction factor.

TABLE III

Basic bone data *

		H_2O	Ash	Cl	Na	Ca
		Gm./Kg. bone	*% solids*	*mM/100 Gm. solids*		
80–100 day old rats						
Normal (8)†	Mean	234	70.0 (7)	2.69	26.1	671
	S.D.	16.1	1.5	0.22	0.85	14.8
Normal acidotic (7)	Mean	243	71.1 (6)	2.99	23.2	643
	S.D.	10.4	1.0	0.23	0.41	7.6
	p‡	ns§	ns	0.01	0.001	0.01
Diabetic (6)	Mean	224	69.9	2.20	25.1	657
	S.D.	13.2	2.4	0.26	0.47	2.9
	p	ns	ns	0.02	ns	ns
Diabetic acidotic (9)	Mean	218	69.8 (7)	2.53	23.4	632
	S.D.	9.7	2.0	0.16	0.71	15.3
	p	0.05	ns	0.05	0.001	0.001
Diabetic vs. diabetic acidotic	p	ns	ns	ns	0.01	0.05
280 day old rats						
Normal (6)	Mean	192	70.9	2.63	26.8	
	S.D.	5.8	1.4	0.35	1.04	
Diabetic (6)	Mean	201	71.3	2.16	27.3	
	S.D.	11.5	2.1	0.22	0.88	
	p	ns	ns	0.02	ns	

* All data are expressed in terms of fat-free bone.
† No. animals in group.
‡ p values are the significance of difference of means of normal and experimental animals. The 80 to 100 day rats are compared with the 80 to 100 day normals and the 280 day diabetic rats with the 280 day normals.
§ No significant difference ($p > 0.05$).

diabetic animals in response to hyperchloremia, inhibited normal glucose utilization, resulting in a secondary ketoacidosis which might have been responsible for the slightly greater degree of pH change seen in the normal animals when compared to the diabetics.

Table II presents the average values found for plasma water and electrolytes in the different groups of animals. Plasma water was lower in the older animals, as has been described during aging (7, 11). Diabetes did not affect plasma hydration, but there was a slight increase in plasma water in the diabetic acidotic rats. Plasma sodium and chloride concentrations were lower in the diabetic animals than in the normals. In the younger rats both these differences were statistically significant, and plasma chloride was significantly lowered in the older diabetic animals. A rapid rate of urine formation, secondary to the osmotic diuresis of hyperglycemia, which prevented as complete reabsorption of these ions as normally occurs, is the probable explanation for these findings. The high plasma chloride values in the acidotic animals were the result of the large load of intraperitoneal ammonium chloride which they had received. The normal value for serum Na seen in the diabetic acidotic animals, as compared to the diabetics, may have been due to retention of Na from the NaCl solution in which the NH_4Cl was administered.

In Table III, it can be seen that the water and electrolyte content of the bones of diabetic animals did not differ significantly from that of the normal controls, with the exception of chloride. Although total bone water was significantly lower in the older animals, both control and diabetic, compared to the younger, as has been previously reported in association with aging (7, 11), the total bone sodium content was not lowered in diabetes either of short or long duration. In contrast, total bone sodium was significantly lower in both normal and diabetic animals rendered acidotic, the size of the decrease in bone sodium being similar in both groups of animals. Bone calcium also fell

TABLE IV

Calculated bone data *

		Calculated chloride space	Remaining water	Chloride space Na	Calculated "crystal" Na	Calculated "crystal" Ca
		Gm./Kg. wet bone		*Total mM/Kg. wet bone*	*mM/Kg. crystal*	
80–100 day old rats						
Normal (8)†	Mean	174	57	26.2	325 (7)	9,622 (7)
	S.D.	11.0	16.0	2.0	11.8	162
Normal acidotic (7)	Mean	171	70	25.6	280 (6)	8,994 (6)
	S.D.	12.8	9.4	1.5	7.6	54
	p‡	ns§	ns	ns	0.001	0.001
Diabetic (6)	Mean	148	76	21.1	322	9,414
	S.D.	7.2	10.0	0.8	8.6	108
	p	0.01	ns	0.001	ns	ns
Diabetic acidotic (9)	Mean	157	61	23.6	289 (7)	9,093 (7)
	S.D.	12.7	18.2	2.2	11.9	161
	p	0.02	ns	0.05	0.001	0.001
Diabetic vs. diabetic acidotic	p	ns	ns	ns	0.001	0.01
280 day old rats						
Normal (6)	Mean	182	10	26.7	331	
	S.D.	11.2	8.2	2.7	13.2	
Diabetic (6)	Mean	158	43	23.0	343	
	S.D.	12.3	18.3	2.7	7.7	
	p	0.01	0.01	ns	ns	

* All data are expressed in terms of fat-free bone.
† No. animals in group.
‡ p values are the significance of difference of means of normal and experimental animals. The 80 to 100 day rats are compared with the 80 to 100 day normals and the 280 day diabetic rats with the 280 day normals.
§ No significant difference ($p > 0.05$).

in both normal and diabetic acidotic animals. Bone chloride content was significantly lowered in both young and old diabetic animals, while acidosis raised the bone chloride content in both normals and diabetics, probably as a reflection of the abnormally high plasma concentrations of this electrolyte. This hypothesis is confirmed by the fact that acidosis did not significantly increase bone chloride spaces (Table IV).

DISCUSSION

While the total volume of bone water is amenable to direct measurement, the assumption that the chloride space is a true measure of the extracellular space of this tissue is open to considerable doubt. Histological studies and the investigations by Neuman, Toribara and Mulryan of synthetic hydroxyapatite (16) suggest that bone water may be divided into four moieties: *1*) "true" extracellular fluid (*i.e.*, plasma and interstitial fluid contained in the Haversian systems; *2*) intracellular water of the bone cells; *3*) the hydration shells of the mineral crystals; and *4*) water in the form of hydronium ions substituted for Ca^{++} or Na^{++} at the crystal surface. In previous publications the authors have assumed that the chloride space comprised only the "true" extracellular water. Forbes, Mizner and Lewis, on the other hand, have assumed that the chloride space includes the hydration shell water as well (11). Their theory is supported by two observations which suggest that our previous concept was in error. First, there appears to be exchange of ions between the bulk solution and the apatite crystal *in vitro* (17, 18), which implies that the hydration shell water must contain the same ions and be continuous with the bulk solution *in vitro*; and, by analogy, the extracellular fluid *in vivo*. Second, Neuman and associates' experiments with fresh bone indicate that the largest part of the water is bound, presumably

as an hydration shell (16). Since most of the bone water is included in the calculated chloride space, one must deduce that the hydration shell contains chloride.

The calculation of a chloride space in bone, then, presupposes that the concentration of chloride in the hydration shell is equal to that in the extracellular fluid. This may not be a valid assumption. It is currently believed that the surfaces of bone apatite crystals are charged (19); the concentration of ions in solution in the hydration shell water must therefore be at least in part influenced by this charge, and by the ability of a particular ion, depending on its size and charge, to exchange with the crystal surface. Neuman and Neuman have shown that phosphate appears to be restricted within the hydration shell water by the crystal charge (20), and this could also be true of chloride. If this is the case, the concentration of chloride in the hydration shell may be higher than in the extracellular fluid, as in tendon water where the concentration of chloride averages 10 to 20 mM higher than in extracellular fluid (21) due to binding by positively charged sites on the collagen molecules. If this is the case, one is overestimating the size of the bone chloride space and assigning to it more sodium than it actually may contain, thereby underestimating the concentration of sodium in the lattice. Furthermore, the concentration of sodium in the water of hydration may vary for the same reasons as in the case of chloride, introducing still another source of error.

Using the calculated chloride space as a measure of the sum of "true" extracellular fluid and water of hydration, the remaining water in young animals constitutes some 25 per cent of total bone water. Presuming that bone cells are hydrated to the same extent as muscle cells (765 Gm. H_2O per Kg.) (10, 22), then marrow-free cortical bone would contain some 8.5 per cent of cells. This figure seems high, but part of the water may be present as hydronium ions. On the other hand, in older rats, where nonchloride water constitutes only 6 per cent of the total bone water, cells would constitute only 1.3 per cent, which seems low, and there would be virtually no water present as hydronium ion.

It is also puzzling that the total water content of adult bone is inadequate to hydrate bone collagen to the same extent as collagen in other parts of the body. Pure collagen, as exemplied by tendon, has a water to dry solids ratio of 2:1 (21). In our normal 280 day old rats, water constituted 19.2 per cent of the total bone. Of the total solids (808 Gm.), ash weight constituted 70.9 per cent or 572 Gm. Dry collagen constituted almost 100 per cent of the remaining solids (23), or 236 Gm. If this collagen were hydrated to the same extent as tendon, it would, then, command the presence of some 472 Gm. of water, more than twice the total water content of adult bone found by us and other workers (11). Presuming that bone contains a considerable amount of crystalline water of hydration, as well as water in the Haversian systems, and a small amount of intracellular water, one is reduced to visualizing collagen in mature bone as virtually a "dry" substance. This same conclusion has been reached by Robinson (24), and similar findings in enamel have been reported by Deakins (25). One is tempted to speculate, in the light of this finding, that the dehydration of collagen in bone occurs during skeletal development by the substitution of calcium and phosphate ions directly into the collagen fiber, in exchange for hydronium or hydroxyl ions. In other words, the formation of the crystalline structure of bone may occur actually within the fibers of collagen by means of a chemical exchange between the water of this substance and the ions forming the bone crystal. The marked decline in total bone water from weanling to old rats (7, 10, 11) and the demonstration of calcification centers inside the collagen fibrils of embryonic bone (26) support this concept.

Despite these grave reservations as to the validity of the calculated chloride space in bone, it should be realized that some sodium in bone does exist that is not present as part of the mineral lattice, that the amount of this sodium may vary, and that the only possible method of estimating this variation at present is by the use of such a calculated chloride space. The quantitative accuracy of the data derived by this means is certainly open to criticism.

Table IV and Figures 1 and 2 present values calculated from the basic data for the water, sodium and calcium content of bone.

In the three groups of nondiabetic animals, the only significant changes found in either total bone water or calculated chloride spaces was a decrease

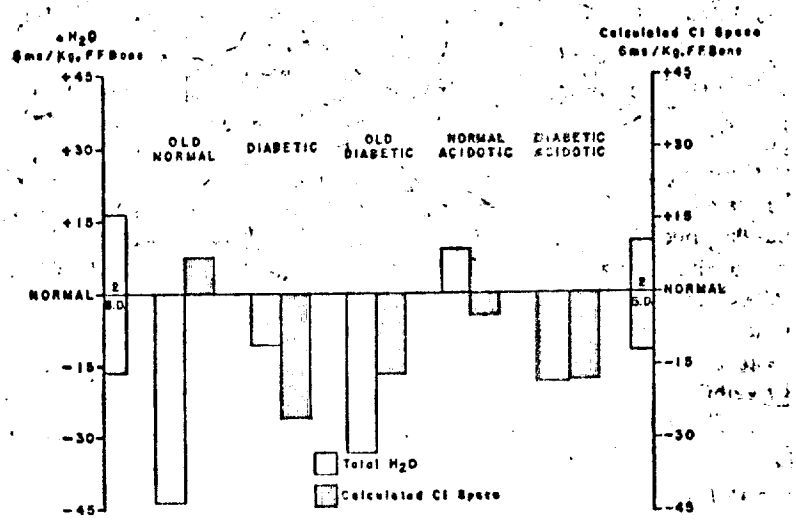

FIG. 1. CHANGES IN BONE WATER

The bars represent changes in total bone water and calculated Cl space in the old normals and the experimental groups, as compared with the values found in the normal 80 to 100 day control animals. One standard deviation above and below normal is shown on the scale for both total water and calculated Cl space.

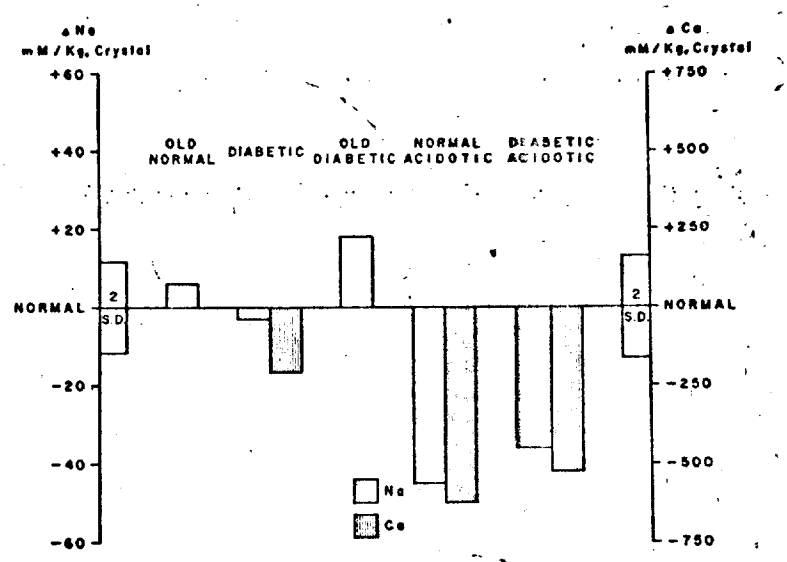

FIG. 2. CHANGES IN BONE CRYSTAL SODIUM AND CALCIUM

The bars represent changes in the concentration of calculated "crystal" Na^+ and Ca^{++} in the old normals and experimental groups, as compared with the values found in the normal 80 to 100 day control animals. One standard deviation above and below normal values is shown on the scale for both Na^+ and Ca^{++}.

in the total bone water of the older animals, consistent with previous reports (Table IV and Figure 1). Since the calculated chloride space did not decrease with age, this represented a significant decrease ($p < 0.01$) in the remaining fraction of the bone water, which fell from 25 per cent of the total bone water in the 80 to 100 day rats to 5 per cent in the older animals.

In the diabetic animals, total bone water was lowered significantly, compared to the normal 80 to 100 day controls, in the acidotic group. This lowering appeared to be a combination of the stress of diabetes and acidosis in these animals, since the values for total bone water in the nonacidotic animals did not differ significantly from the normal controls, nor were the values for the diabetic-acidotic rats significantly different from those for the nonacidotic diabetic animals. On the other hand, the calculated chloride space was significantly lower in all three groups of diabetic animals, compared with the normal 80 to 100 day controls. This change was also significant when the values for the older diabetic animals were compared with the values found in the older controls. The calculated chloride space in the 80 to 100 day old diabetics was 15 per cent less than in the 80 to 100 day controls, and 10 per cent less in the diabetic-acidotic animals, compared with the same controls. In the older animals, the calculated chloride space was 13 per cent less than in the control animals of the same age. Acidosis, as produced in these experiments, did not significantly alter the size of the calculated chloride space in either normal or diabetic animals. The mechanisms which underlie these apparent changes in the volume and distribution of bone water are as yet unknown. In the case of the reduced size of the calculated chloride spaces in the diabetic animals, one might speculate that there was a general reduction in total body extracellular fluid volume, as a result of osmotic diuresis, which was reflected in the calculated chloride space of bone. Since no measurements of total extracellular fluid were made in these animals, the validity of this concept cannot be determined.

Table IV and Figure 2 present calculated values for bone crystal sodium and calcium. It is apparent from these calculated values that bone crystal sodium concentrations were almost identical in the short-term diabetic animals with those found in the normal animals. There was, however, an increase in bone crystal sodium in the bones of the older animals, as has already been described by the authors and other investigators (7, 8, 11). This increase was actually greater in the old diabetic rats than in the normals, confirming the impression that uncontrolled diabetes of this type, despite causing a lowered serum sodium concentration, did not decrease the store of sodium in the bone crystal. It is of interest to note that age, rather than weight, is apparently the determining factor in bone crystal sodium concentration, since the diabetic rats weighed 18 to 20 per cent less than the normal controls.

In both normal and diabetic animals, however, acidosis caused a marked fall in the concentration of bone crystal sodium. In the normal animals bone crystal sodium fell from 325 to 280 mM in response to acidosis, a decrease of 13.8 per cent, while in the diabetics it dropped from 322 to 289, or 9.9 per cent. From these data, it would appear that there was little difference in the response of bone crystal sodium of the normal and the diabetic rats to the stimulus of acidosis.

The mobilization of bone crystal sodium observed in these rats in response to hyperchloremic acidosis is comparable to that observed by the authors in adult dogs, where in response to acute pure sodium deficiency some 11 per cent of bone crystal sodium was removed (9) and to the 11 per cent fall found by Levitt, Turner, Sweet and Pandiri in rats (27). Lesser degrees of bone sodium depletion were observed by Edelman, James, Baden and Moore in dietary sodium depletion of dogs (5.8 per cent) (28) and by Lobeck and Forbes (5.5 per cent) (29) in rats dying in acute acidosis 72 hours after the injection of alloxan monohydrate. It is of interest that in the latter experiments the degree of sodium depletion of bone was less than half that reported here, despite greater decreases in plasma pH and significantly decreased serum sodium concentrations. It is possible that adrenal stimulation accounted for this, since their animals were moribund at the time of sacrifice. Bergstrom and Wallace, using a dialysis technique identical with that used in the present series, but allowing their rats to equilibrate for 24 hours in the absence of exogenous sodium, were able to mobilize 31 per

cent of the "extra" sodium of bone (8). No bone waters are available in this series, and furthermore, since their values were expressed in terms of wet bone rather than crystal solids, it is difficult to compare them with our values.

Bergstrom and Wallace noted a fall in bone calcium in response to acidosis similar to that reported here, although this decrease was not statistically significant (8). In their animals, calcium fell from 8,000 to 7,800 mEq. per Kg. of wet bone, or 2.5 per cent. In the present series, bone calciums were comparable in the normal and diabetic animals, and acidosis caused a fall of 7 per cent in bone crystal calcium in the bones of the normal rats, and a drop of 3.4 per cent in the diabetic animals. These changes were both highly significant.

It would appear in these animals, that loss of bone sodium in response to acidosis was not accompanied by any major gain in water, and in fact there was a decreased amount of nonextracellular water in the diabetic animals. Hence no exchange of sodium for hydronium ion can be postulated. Nor, apparently, was sodium exchanged for calcium in the crystals, since bone crystal calcium also fell. An exchange of hydrogen ion for sodium and calcium cannot be postulated in the light of Neuman and Neuman's belief that hydrogen ion exchange at the crystal surface occurs only when hydrogen is present as hydronium or hydroxyl ion (19). It is probable, therefore, that actual dissolution of the crystalline matrix occurs in acidosis; some confirmation of this is found clinically in the demineralization of bone observed in conditions of chronic acidosis (30, 31). It is also probable that, in these animals, dissolution of organic matrix also occured, since there was virtually no change in the percentage of ash in the acidotic bones. If 7 per cent of the crystalline matrix had been lost, as suggested by the fall in calcium in the nondiabetic acidotic animals unaccompanied by a loss of organic solids, one would have expected to find a lowering of the ash to organic solids ratio in these bones. Furthermore, the ratio of calcium to the other mineral ions must have decreased, since a decrease in the concentration of this ion was observed in relation to both total bone solids and bone ash.

The syndrome of alloxan diabetes in the rat, while differing in many important respects from the spontaneous diabetes occurring in humans, does include hyperglycemia, glycosuria and polyuria, which might be expected to reduce stores of body sodium. However, from the data presented, it would appear that untreated alloxan diabetes in the presence of an adequate dietary intake of sodium exerted little or no effect upon body sodium stores, as exemplified by the storage of sodium in bone, despite the presence of a lowered serum sodium in these animals. It should also be noted that serum sodium was high in the diabetic-acidotic animals who lost considerable quantities of bone sodium. Apparently, then, the removal of sodium from bone is not completely dependent on extracellular fluid levels of this ion in the absence of acidosis. The concentration of extracellular fluid sodium may, however, play some part in the mobilization of bone sodium. We were able to mobilize some 70 mM of sodium in excess of that lost from the extracellular fluid, from dogs dialyzed against a low-sodium bath (9). In these animals plasma sodium fell 7 mM per L., while plasma pH remained normal. Much more sodium was mobilized from a second group of animals in whom plasma sodium fell 18 mM per L., accompanied by a fall in plasma pH (9). In the present series of rats, more sodium and calcium were mobilized in the normal acidotic animals who had a plasma sodium concentration of 144 mM than in the diabetic-acidotic animals with a plasma sodium of 150 mM per L. Plasma pH was comparable in the two groups. These findings suggest that either lowered extracellular fluid sodium in the presence of normal pH or acidosis in the presence of normal extracellular fluid sodium may cause a loss of bone sodium, but that maximal loss of bone sodium occurs when both of these factors are present.

SUMMARY

1. Untreated alloxan diabetes does not alter the total water or crystal sodium and calcium content of bone. It does diminish the size of the "calculated chloride space" of bone.

2. Acute hyperchloremic acidosis causes a fall in bone crystal sodium (10.2 per cent) and calcium (3.5 per cent) in diabetic rats which is comparable with the fall in bone crystal sodium (13.8 per cent) and calcium (7.0 per cent) obtained in normal rats.

3. A discussion of the present state of knowledge of bone water, and those factors limiting the accuracy of the "calculated chloride space" in bone, is presented.

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AMINO ACID EXTRACTION AND AMMONIA METABOLISM BY THE HUMAN KIDNEY DURING THE PROLONGED ADMINISTRATION OF AMMONIUM CHLORIDE *

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According to current concepts, renal ammonia synthesis may be attributed to the renal extraction and catabolism of certain plasma amino acids. In 1943, Van Slyke and associates demonstrated in the dog that the amide nitrogen of glutamine was removed from arterial plasma in quantities sufficient to account for approximately 60 per cent of the urinary ammonia excreted during metabolic acidosis (1). It was proposed that the remaining 40 per cent of urinary ammonia could be accounted for by the renal uptake of plasma α-amino nitrogen. In agreement with this hypothesis, many investigators have since shown that the administration of several amino acids other than glutamine is associated with an increased urinary ammonia excretion (2–4). Loading experiments of this type have indicated that—besides glutamine—glycine, alanine, asparagine, leucine, and histidine may serve as precursors of urine ammonia. The demonstration of appropriate enzyme systems within renal tissue capable of forming ammonia from these substrates has been offered as additional support for the thesis that several plasma amino acids may participate normally in the renal production of ammonia. Nevertheless, the specific amino acids that serve as renal ammonia precursors have not been identified clearly in conditions associated with normal acid-base balance and normal plasma amino acid concentrations. The present study was undertaken for two principal reasons: first, to characterize the typical patterns of uptake or release of individual amino acids by the normal human kidney, and second, to determine whether or not the adaptive increase of renal ammonia production during metabolic acidosis could be attributed to changes in the renal extraction of certain plasma amino acids.

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METHODS

Twelve renal clearance experiments were carried out on eleven healthy male volunteers between 21 and 38 years old. All studies were conducted with them in the recumbent position after an overnight fast. To facilitate the collection of timed urine specimens, urine flows of approximately 5 ml per minute were assured by the oral ingestion of 500 ml of tap water 1 hour before the test, followed immediately by a constant intravenous infusion of 5 per cent dextrose in water. After appropriate priming doses, an intravenous maintainance infusion of a solution of inulin and para-aminohippurate (PAH) was begun 45 minutes before the first clearance period via a Bowman constant-infusion pump. During the equilibration period, a no. 7 or 8 cardiac catheter was introduced into a renal vein through either the right brachial or femoral vein under fluoroscopic control. An indwelling Cournand arterial needle was placed in the left brachial artery. Three sequential clearance periods, each 20 to 40 minutes long, were obtained during all experiments. Simultaneous arterial and renal venous blood samples were drawn for analysis at the approximate mid point of each urine collection period. Urine was collected without the aid of bladder catheterization by voiding into a flask containing a small amount of mineral oil.

Six of the 12 experiments were performed during the presence of a mild metabolic acidosis induced by the daily oral ingestion of 6 to 8 g of nonenteric coated ammonium chloride for 6 to 9 days before the procedure. The first clearance period was obtained 8 to 12 hours after administration of the last dose of ammonium chloride. In one subject, G.J., experiments were performed both before and after the prolonged administration of ammonium chloride.

The free amino acid content of urine and arterial and renal venous plasma was determined by the ion exchange column chromatographic technique of Spackman, Stein,

and Moore (5) with a Beckman Spinco model 120 amino acid analyzer. With the exception of glutamine, an accuracy of ± 3 per cent was obtained regularly with this procedure when 0.1 to 0.3 μmole of an amino acid was placed on the column for analysis. The total quantity of most free amino acids placed on the columns fell within this range when plasma and urine samples were prepared for analysis by the following procedures.

Plasma. Protein precipitation was carried out immediately after collection by the addition of 50 ml of 1 per cent picric acid to 10 ml of plasma. After centrifugation, excess picrate was removed from the supernatant fluid by filtration of a 50-ml sample through a 2 × 2-cm column bed of Dowex 2 × 8 resin. The column bed was then washed with 15 ml of 0.02 N HCl, and the total eluate was lyophilized and reconstituted in 4.2 ml of citrate buffer at pH 2.2. These samples were stored at −20° C until analysis, when 2-ml samples were placed on the columns.

Since glutamine cannot be separated reliably from asparagine under the usual conditions of chromatography (6), arterial and renal venous plasma concentrations of glutamine were also measured by an enzymatic assay technique using a specific glutaminase obtained from *E. coli*, strain W. The enzyme was prepared according to Meister, Levintow, Greenfield, and Abendschein (7) and the glutamine assay was carried out in duplicate as described by Segal and Wyngaarden (8). Duplicate determinations agreed within ± 8 per cent and recoveries of glutamine added to plasma ranged between 94 and 106 per cent consistently. Plasma glutamine values obtained by enzymatic assay were between 20 and 30 per cent higher than the glutamine-asparagine concentrations measured by column chromatography.

Urine. The pH of 10-ml samples of urine was adjusted to between 11.5 and 12 by the drop-by-drop addition of 4 N NaOH. After 6 hours of vacuum desiccation, the urine samples were acidified to pH 2.0 to 2.2 with 6 N HCl and their volume readjusted to 10 ml by the addition of citrate buffer at pH 2.2. The samples were then stored at −20° C until analysis. The appropriate sample volume to be placed on the column for free amino acid analysis was calculated by the method of Stein (9).

Blood and urine ammonia was measured by a modification of the microdiffusion method of Brown, Duda, Korkes, and Handler (10). Inulin was determined by the resorcinol method of Roe as modified by Schreiner (11). PAH was measured by the technique of Selkurt (12). The pH of whole blood and urine was determined with a Cambridge model R pH-meter equipped with an enclosed glass electrode. Measurements were made at room temperautre and were corrected to 37° C with Rosenthal's factor (13). The total CO_2 content of arterial blood was determined by the method of Van Slyke and Neill (14). Assuming a pK of 6.11, the plasma CO_2 tension was calculated from the line chart of Van Slyke and Sendroy (15). Oxygen content and saturation of arterial blood were determined by the spectrophotometric method of Hickam and Frayser (16). The hematocrit of arterial blood was measured in duplicate by the method of Wintrobe (17).

Calculations. Glomerular filtration rate was measured by the clearance of inulin. True or total renal plasma flow was estimated from the clearance and extraction of PAH. Total renal blood flow was calculated from this value and the peripheral arterial hematocrit (18).

Estimates of the net uptake or release of each free amino acid across the renal circulation were obtained by multiplying the arteriorenal venous amino acid difference by total renal plasma flow. Because urinary amino acid excretion rates were not measured in all studies, these values were not used in the calculation of net renal uptake or release. Because of their small magnitude, however, their use in such calculations would not have affected materially the directional patterns of uptake or release that were obtained from the use of A-V differences alone. The total amino acid nitrogen values used in similar balance calculations reflect the summation of the nitrogen content of each amino acid measured by column chromatography.

Renal venous ammonia release was calculated from the A-V difference of ammonia and the total renal blood flow. The total bidirectional release of ammonia was obtained by summing the values for urine ammonia excretion and renal venous ammonia release.

RESULTS

Experiments in healthy human subjects without prior administration of ammonium chloride. For all clearance periods in these 6 experiments, glomerular filtration rate and total renal blood flow averaged 137 and 1,491 ml per minute, respectively. Extraction ratios for PAH remained steady throughout each procedure and averaged 88 ± 5 per cent. The plasma bicarbonate concentration averaged 25.5 ± 1.3 mmoles per L, plasma pCO_2 was 46 ± 3.9 mm Hg, and arterial pH, 7.35 ± .02 units. A modest decrease of arterial pH and elevation of arterial pCO_2 was observed in one subject, but could be attributed to the presence of mild pulmonary hypoventilation induced by sleep. Urine pH remained constant in each subject and averaged 5.98 ± .33 for the entire group.

Table I lists the individual values of each of the 6 patients for renal blood flow, urine pH, ammonia release into urine and renal venous blood, and the arterial and renal venous concentrations of ammonia and those amino acids whose net extraction or release appeared of definite magnitude. In addition, the average arterial plasma concentrations of all 19 free amino acids are summarized for the entire group in Table II, with the appropriate

TABLE I

*Net renal uptake or release of eight selected amino acids in healthy human subjects**

Subject	Total renal blood flow	Urine pH	$U_{NH_4}V$	Amino acids and ammonia								
				Ammonia†	Glutamine‡	Proline	Glycine	Valine	Glutamic acid	Serine	Alanine	Cystine
	ml/min		*μmoles/min*									
G.J.§	1,454	6.10	58.1									
Arterial plasma, μmoles/100 ml				3.9	66.1	15.4	16.3	19.1	6.6	7.2	24.3	8.0
Renal venous plasma, μmoles/100 ml				5.1	58.8	13.7	14.6	19.3	6.8	10.5	23.8	9.5
Net uptake (+) or net release (−), μmoles/minute				−17.1	+52.7	+12.3	+12.1	+ 0.5	− 0.7	−23.5	+ 3.4	+11.2
V.K.	1,237	6.32	28.1									
Arterial plasma, μmoles/100 ml				6.9	71.7	11.6	17.0	10.9	5.2	5.4	25.0	7.4
Renal venous plasma, μmoles/100 ml				9.7	63.6	11.5	18.6	11.7	7.6	11.4	30.0	10.2
Net uptake (+) or net release (−), μmoles/minute				−34.9	+50.5	+ 0.6	−10.0	− 5.1	−15.9	−23.3	−31.3	−17.9
H.S.	1,661	5.89	49.8									
Arterial plasma, μmoles/100 ml				6.8	68.7	12.9	20.6	17.7	13.8	8.9	22.2	9.0
Renal venous plasma, μmoles/100 ml				8.9	63.9	9.3	20.8	15.0	15.6	18.9	24.9	
Net uptake (+) or net release (−), μmoles/minute				−49.8	+40.2	+30.3	− 1.7	+22.3	−15.3	−84.5	−22.6	

* Only the amino acids that showed the most definite values of net uptake or release are included in this table.
† Ammonia concentrations are for whole blood rather than plasma.
‡ Glutamine concentrations are those determined by specific enzymatic assay.
§ Subject G.J. was re-examined after ammonium chloride administration. The results of that experiment are included in Table III.

TABLE I—(*Continued*)

Subject	Total renal blood flow	Urine pH	$U_{NH_4}V$	Amino acids and ammonia								
				Ammonia†	Glutamine‡	Proline	Glycine	Valine	Glutamic acid	Serine	Alanine	Cystine
	ml/min		*μmoles/min*									
N.S.	2,329	6.34	36.7									
Arterial plasma, μmoles/100 ml				6.8	71.0	16.8	15.8	16.2	13.1	10.0	20.0	9.8
Renal venous plasma, μmoles/100 ml				10.4	64.9	12.7	15.3	16.3	12.7	11.7	21.5	9.8
Net uptake (+) or net release (−), μmoles/minute				−84.2	+68.3	+11.3	+ 4.8	− 1.8	+ 3.9	−19.3	−17.8	0.0
J.D.	1,398	5,63	28.0									
Arterial plasma, μmoles/100 ml				4.0	59.8	23.0	16.6	20.0	17.4	7.4	29.6	11.9
Renal venous plasma, μmoles/100 ml				5.9	53.3	24.1	16.0	21.0	18.4	10.4	33.2	12.8
Net uptake (+) or net release (−), μmoles/minute				−27.2	+47.4	− 8.1	+ 4.2	− 7.0	− 9.4	−22.0	−26.7	− 6.6
M.W.	964	5.60	25.8									
Arterial plasma, μmoles/100 ml				4.1	52.3	15.2	15.7	13.7	15.0	7.2	24.2	6.3
Renal venous plasma, μmoles/100 ml				6.7	48.2	13.2	13.1	13.0	14.6	8.4	24.8	7.6
Net uptake (+) or net release (−), μmoles/minute				−25.0	+25.0	+12.4	+15.8	+ 4.5	+ 2.7	− 8.2	− 3.8	− 7.7

average values for either their net renal uptake or release. In these studies, urine ammonia excretion averaged 37.8 ± 25.6 μmoles per minute, renal venous ammonia release was 37.3 ± 24.6 μmoles per minute, total renal ammonia release, 77.4 ± 28 μmoles per minute, the arterial blood ammonia concentration, 5.2 ± 1.6 μmoles per 100 ml, and the venous blood ammonia level averaged 7.8 ± 2.2 μmoles per 100 ml.

Of those amino acids—glutamine, proline, and glycine—that exhibited the most definite figures of net uptake from plasma by the kidney (Tables I and II), only glutamine was extracted consistently by all 6 subjects. Net glutamine uptake averaged 47 ± 14 μmoles per minute for the entire group. Net renal extraction of proline occurred in 5 of 6 studies, and glycine exhibited net uptake in 4 of 6 experiments (Table I). Although valine also demonstrated net extraction during most experiments, the average net uptake of this amino acid was much less than that of either glutamine, proline, or glycine (Table II).

Serine, glutamic acid, alanine, cystine, and arginine comprised the group of amino acids that clearly exhibited average values for net release into renal venous plasma (Tables I and II). Of these, only serine was added to renal venous blood by all subjects (Table I), and usually in considerable quantity, averaging 30 ± 27 μmoles per minute. Net venous release of the other amino acids in this group was noted in at least 4 of 6 experiments, and in some instances (alanine and cystine), their addition to venous plasma was of respectable proportions (Tables I and II). Glutamic acid also exhibited net release in 4 of 6 studies, averaging 5.8 μmoles per minute for all subjects. Although leucine and tyrosine were usually added to renal venous plasma, the average values for their net release were smaller than those of either serine, glutamic acid, alanine, cystine, or arginine (Table II).

The arteriorenal venous differences of the remaining amino acids—taurine, threonine, methionine, isoleucine, phenylalanine, ornithine, lysine, and histidine—were so small or variable that no conclusions could be drawn as to the presence of either net uptake or net release (Table II).

Total ammonia nitrogen release by the kidneys during these experiments averaged 1.05 ± 0.36 mg per minute (Table V). Of this amount, 49.5 per cent (0.52 ± 0.34 mg per minute) was released into renal venous blood and the remaining 50.5 per cent, (0.53 ± 0.18 mg per minute) was excreted in urine. Despite the addition of more free amino acids to renal venous plasma than were extracted from arterial plasma, the net balance of total amino acid nitrogen across the renal circulation was positive (net uptake) in 5 of 6 subjects, averaging 0.53 ± 0.59 mg per minute for the entire group. From these data it is apparent that the total renal release of ammonia nitrogen cannot be accounted for solely by an analysis of the net renal extraction of total amino acid nitrogen from plasma. Similarly, the average net uptake of glycine and the amide nitrogen of glutamine (0.72 ± 0.22 mg per minute) could account for only 70 per cent of the total renal ammonia nitrogen release. The average net extraction of glycine and glutamine total nitrogen (1.39 ± 0.41 mg per minute), however, was more than sufficient (132 per cent) to provide the total amount of ammonia nitrogen released by the kidney. In fact, the renal extraction of glutamine total nitrogen (1.33 ± 0.41 mg per minute) was the major determinant of the modest positivity (net uptake) of total amino acid nitrogen extraction observed in these

TABLE II

*Net renal uptake or release of free amino acids in healthy human subjects**

Amino acids	Arterial plasma concentration		Net uptake (+) or net release (−)	
	Average	Range	Average	Range
	μmoles/100 ml		*μmoles/minute*	
Taurine	6.1	4.8– 8.7	+2.5	+15.2 to −5.7
Threonine	10.6	8.0–13.3	− 0.1	+ 9.0 to − 7.2
Serine	8.8	7.2–10.1	−30.1	− 8.2 to −84.5
Glutamine†	64.9	52.3–71.0	+47.4	+68.0 to +25.0
Proline	17.4	11.6–24.8	+ 9.8	+30.3 to − 8.1
Glutamic	12.4	5.2–17.4	− 5.8	+ 3.9 to −15.9
Glycine	18.4	15.7–24.5	+ 4.2	+15.8 to −10.0
Alanine	26.1	20.0–35.6	−16.5	+ 3.4 to −31.3
Valine	18.2	10.9–30.6	+ 2.2	+22.3 to − 7.0
Half-cystine	8.4	5.8–11.9	−10.8	0.0 to −17.9
Methionine	1.4	1.1– 2.5	+ 0.4	+ 5.0 to − 3.5
Isoleucine	4.3	1.7– 8.6	+ 2.0	+ 4.0 to − 0.8
Leucine	7.9	4.6–12.9	− 2.4	+ 4.5 to − 6.0
Tyrosine	3.3	2.0– 4.8	− 4.0	+ 0.4 to −11.1
Phenylalanine	3.6	2.8– 4.8	− 0.2	+ 3.4 to − 6.5
Ornithine	4.9	3.2– 6.9	− 0.8	+ 0.2 to − 1.8
Lysine	10.4	10.3–10.6	− 2.0	+ 1.3 to − 5.4
Histidine	6.8	6.1– 7.2	0.0	+ 4.0 to − 3.6
Arginine	6.4	4.4– 8.6	− 6.0	+ 1.8 to −16.1

* Net uptake or release was calculated by multiplying each arteriorenal venous amino acid difference by total renal plasma flow.

† Values listed for glutamine were obtained from enzymatic analysis rather than from column chromatography.

TABLE III

*Net renal uptake or release of eight selected amino acids after the prolonged administration of oral ammonium chloride**

Subject	Total renal blood flow	Urine pH	$U_{NH_4}V$	Amino acids and ammonia								
				Ammonia†	Glutamine‡	Proline	Glycine	Valine	Glutamic acid	Serine	Alanine	Cystine
	ml/min		*μmoles/min*									
G.J.§	1,591	5.38	104.0									
Arterial plasma, μmoles/100 ml				3.6	37.8	17.3	20.8	26.8	12.1	7.9	30.8	12.4
Renal venous plasma, μmoles/100 ml				6.1	29.6	13.3	19.2	22.0	10.4	13.7	33.7	12.4
Net uptake (+) or net release (−), μmoles/minute				−39.3	+ 68.4	+34.7	+12.7	+40.2	+14.2	−48.7	−24.2	0.0
W.A.	1,189	5.34	87.8									
Arterial plasma, μmoles/100 ml				4.7	31.7	10.1	12.8	17.4	9.2	4.7	22.6	11.9
Renal venous plasma, μmoles/100 ml				8.3	11.6	8.0	12.1	15.3	7.4	8.3	23.9	12.0
Net uptake (+) or net release (−), μmoles/minute				−42.7	+113.3	+11.8	+ 4.1	+11.8	+10.2	−20.0	− 7.1	− 1.8
W.W.	1,203	5.66	144.3									
Arterial plasma, μmoles/100 ml				5.6	43.4	10.5	13.5		6.3	6.1	13.5	
Renal venous plasma, μmoles/100 ml				9.4	33.1	9.5	13.4	12.6	4.9	8.9	17.1	6.3
Net uptake (+) or net release (−), μmoles/minute				−44.6	+ 69.1	+ 6.9	+ 0.7		+ 9.4	−19.0	−24.4	

* Only the 8 amino acids that showed the most definite values of net uptake or release are included.
† Ammonia concentrations are for whole blood rather than plasma.
‡ Glutamine concentrations are those determined by specific enzymatic assay.
§ Subject G.J. was also examined before ammonium chloride administration. The results of that study are included in Table I.

TABLE III—(*Continued*)

Subject	Total renal blood flow	Urine pH	$U_{NH_4}V$	Amino acids and ammonia								
				Ammonia†	Glutamine‡	Proline	Glycine	Valine	Glutamic acid	Serine	Alanine	Cystine
	ml/min		*μmoles/min*									
G.S.	1,336	5.41	118.0									
Arterial plasma, μmoles/100 ml				6.0	46.3	11.7	16.9	16.2	8.6	6.6	24.3	9.1
Renal venous plasma, μmoles/100 ml				10.9	16.3	11.2	16.6	16.5	7.3	10.1	27.0	10.6
Net uptake (+) or net release (−), μmoles/minute				−65.2	+203.8	+ 3.4	+ 1.6	− 2.5	+ 8.6	−23.5	−18.1	−10.1
F.H.	1,758	5.02	67.4									
Arterial plasma, μmoles/100 ml				3.3	39.1	20.8	14.7	19.6	9.7	5.5	25.1	8.9
Renal venous plasma, μmoles/100 ml				5.8	29.4	19.6	13.7	19.5	8.0	7.2	26.7	11.3
Net uptake (+) or net release (−), μmoles/minute				−43.4	+ 90.6	+11.6	+ 9.0	+ 1.1	+15.3	−16.4	−15.5	−22.1
E.C.	1,120	5.36	59.5									
Arterial plasma, μmoles/100 ml				2.9	43.5	17.8	15.7	15.1		3.9	22.9	6.8
Renal venous plasma, μmoles/100 ml				4.5	31.1	17.5	14.5	13.7	5.5	7.2	23.2	8.7
Net uptake (+) or net release (−), μmoles/minute				−18.4	+70.1	+ 2.0	+ 6.6	+ 7.9		−18.3	− 1.9	−11.0

subjects. If glutamine total nitrogen was excluded from this calculation, an average net addition of total amino acid nitrogen to renal venous blood occurred that averaged 0.80 ± .61 mg per minute.

It should be emphasized that the amino acid nitrogen balances listed above were obtained only across the renal circulation. They do not represent total balance calculations, since urinary amino acid excretion rates were not included. Although urine amino acid excretion was measured in two subjects, the values were normal and their inclusion in the calculations would not have appreciably altered the individual amino acid balances (net uptake or release) across the renal circulation.

Experiments in healthy human subjects during mild ammonium chloride acidosis. Values for glomerular filtration rate and total renal blood flow were within accepted normal limits during all six studies. Filtration rate averaged 125 ml per minute and total renal blood flow 1,366 ml per minute. PAH extraction ratios were almost identical to those of the nonammonium chloride subjects and remained constant throughout each individual study. Plasma bicarbonate concentrations averaged 22 ± 2 mmoles per L, arterial pCO_2, 39 ± 5 mm Hg, and arterial blood pH, 7.33 ± 0.05. Urine pH was stable throughout each experiment, averaging 5.36 ± 0.20 for the entire group.

The individual values of renal blood flow, urine pH, renal venous ammonia release, urine ammonia excretion, and the arterial and renal venous concentrations of ammonia and certain amino acids are listed in Table III for each subject. As before, the group averages for the arterial plasma concentration, urine excretion rate, and net uptake or release of all 19 amino acids are summarized in Table IV. As anticipated, the prolonged administration of ammonium chloride was associated with a higher average value for total renal ammonia release (139.1 ± 42 μmoles per minute) than was observed in those subjects who did not ingest the drug. In these subjects, the arterial blood ammonia averaged 4.4 ± 1.3 μmoles per 100 ml, the renal venous ammonia concentration, 7.5 ± 2.4 μmoles per 100 ml, and the urine ammonia excretion, 96.8 ± 31.9 μmoles per

TABLE IV

*Net renal uptake or release and urinary excretion of free amino acids in healthy human subjects after prolonged oral ingestion of ammonium chloride**

Amino acids	Arterial plasma conc.		Net uptake (+) or net release (−)		Urinary excretion†	
	Average	Range	Average	Range	Average	Range
	μmoles/100 ml		μmoles/minute		μmoles/minute	
Taurine	6.4	3.7–10.4	+ 4.2	+ 13.3 to − 2.9	1.63	0.98–2.49
Threonine	7.8	6.0–11.4	+ 0.1	+ 5.4 to − 5.2	0.13	0.10–0.15
Serine	5.8	3.9–7.9	− 24.3	− 16.4 to −48.6	0.22	0.16–0.31
Glutamine‡	40.3	31.7–46.3	+102.5	+203.8 to +70.1	0.37	0.26–0.73
Proline	14.7	10.0–20.8	+ 11.5	+ 33.6 to + 2.0	Trace	
Glutamic	9.2	6.3–12.1	+ 11.5	+ 15.3 to + 8.6	Trace	
Glycine	15.7	12.8–20.8	+ 5.8	+ 12.7 to + 0.7	0.63	0.27–1.19
Alanine	23.2	13.5–30.8	− 15.2	− 1.9 to −24.4	0.24	0.15–0.37
Valine	19.0	15.1–26.8	+ 11.7	+ 40.1 to − 2.5	0.09	0.03–0.14
Half-cystine	10.3	6.8–14.4	− 9.0	− 0.2 to −22.1	0.21	0.15–0.27
Methionine	1.3	1.0–1.6	− 0.1	+ 1.8 to − 0.9	Trace	
Isoleucine	3.6	2.8–5.1	+ 0.1	+ 1.0 to − 1.4	Trace	
Leucine	7.6	4.4–12.2	+ 0.2	+ 4.0 to − 2.9	0.04	Tr–0.08
Tyrosine	3.7	2.6–4.6	+ 0.2	+ 4.6 to − 3.1	0.11	0.06–0.13
Phenylalanine	3.7	3.1–5.1	0	+ 1.5 to − 1.0	0.08	0.03–0.11
Ornithine	5.2	4.1–6.1	+ 2.7	+ 7.3 to − 1.6	Trace	
Lysine	12.9	11.8–14.4	+ 4.6	+ 24.7 to − 7.3	0.29	0.07–0.64
Histidine	5.9	5.1–7.5	+ 2.5	+ 8.6 to − 4.2	0.55	0.34–1.23
Arginine	4.9	3.2–6.4	− 5.2	− 2.4 to −12.3	Trace	

* Net renal uptake and release were calculated from A-V differences multipled by the total renal plasma flow.

† Measurable amounts of 1-methyl and 3-methyl histidine, α-aminobutyric, phosphoethanolamine, and ethanolamine were present in urine and accounted for an average 20 per cent of total amino acid nitrogen excretion. They have not been listed because of the lack of corresponding plasma values.

‡ Plasma values were measured by enzymatic assay. Urine values were determined by chromatography and represent the sum total of glutamine and asparagine concentrations, most of it asparagine.

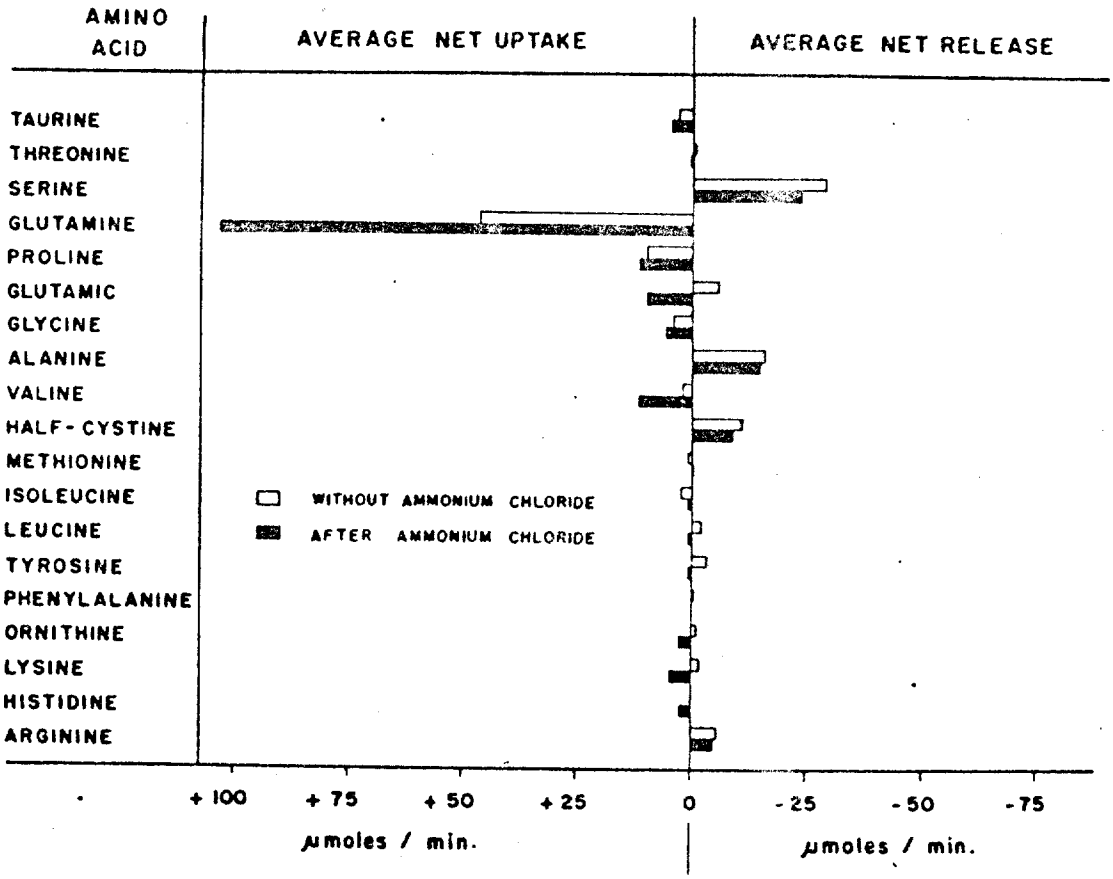

FIG. 1. CHANGES OF NET RENAL UPTAKE OR RELEASE OF AMINO ACIDS INDUCED BY AMMONIUM CHLORIDE ADMINISTRATION.

minute. Since the renal venous release of ammonia was essentially the same both before and after ammonium chloride administration (37.3 ± 24.6 and 42.3 ± 14.9 μmoles per minute, respectively) the elevated total ammonia release during mild acidosis appeared to be largely related to an increased urinary excretion of ammonia. This rise was highly significant statistically ($p < 0.01$), and could not be explained satisfactorily by the relatively small reduction of urine pH that occurred after ammonium chloride administration (Table III).

Values from both experimental groups for the average net uptake or release of each amino acid have been illustrated in Figure 1 for purposes of graphic comparison. Ammonium chloride administration was unassociated with a significant change in the plasma levels of the various amino acids (Tables III and IV), with the exception of glutamine. In general, plasma amino acid concentrations were similar to those of the nonammonium chloride group (Tables II and IV) and agreed closely with the values reported by Stein and Moore for peripheral venous plasma (6). During mild acidosis, however, the arterial plasma glutamine concentration averaged 40 ± 5 μmoles per 100 ml, a value significantly lower ($p < 0.01$) than the 65 ± 7.5 μmoles per 100 ml obtained in the nonammonium chloride group. A significant difference ($p < 0.05$) was also noted in the net renal extraction of glutamine from arterial plasma (Figure 1). During mild acidosis, glutamine extraction averaged 102 ± 53 μmoles per minute (Table IV), in contrast to 47 ± 14 μmoles per minute (Table II) in those subjects who did not receive ammonium chloride. As before, a similar percentage of the total renal release of ammonia could be accounted for by the renal extraction of glutamine amide nitrogen (74 per cent). As in the subjects who did not receive ammonium

TABLE V

Changes of net renal uptake or release of ammonia and amino acid nitrogen associated with the prolonged administration of ammonium chloride

Determination		Without ammonium chloride	After ammonium chloride
		mg/min	*mg/min*
Average renal release of ammonia nitrogen	Renal venous release	0.52	0.59
	Urine excretion	0.53	1.36
	Total renal release	1.05	1.95
Average net renal uptake of total amino acid nitrogen*		0.53	2.86
Average net renal uptake of glutamine nitrogen	Glutamine total nitrogen	1.32	2.88
	Glutamine amide nitrogen	0.66	1.44
Average net renal release of amino acid nitrogen (glutamine total nitrogen excluded)		0.79	0.02

* The total nitrogen content of glutamine was used in this caluclation.

chloride, however, the net uptake of glutamine amide nitrogen could account for all of the ammonia excreted in urine.

Although after ammonium chloride the net renal uptake of proline, glycine, and valine averaged 12 ± 4, 6 ± 5, and 12 ± 17 μmoles per minute, respectively, these somewhat higher extraction values were not significantly different from those of the nonacidotic subjects (Figure 1). In contrast, however, ammonium chloride administration was associated with a highly significant ($p < 0.01$) directional change in the net balance of glutamic acid across the renal circulation (Figure 1). An average net release (6 ± 9 μmoles per minute) of glutamic acid was observed in the nonammonium chloride group (Table II). During acidosis, however, glutamic acid was extracted (net uptake) consistently (12 ± 3 μmoles per minute) from arterial plasma by the kidney. Average figures for the net uptake or release of the remaining amino acids were essentially the same in both experimental groups (Figure 1).

The average net balances of total amino acid and ammonia nitrogen across the renal circulation from both experimental groups have been compared in Table V. After ammonium chloride, the average net uptake of total amino acid nitrogen (2.86 ± 1.38 mg per minute) was significantly higher ($p < 0.01$) than that observed in the absence of drug administration (0.53 ± 0.59 mg per minute), and was more than sufficient (147 per cent) to account for the total renal release of ammonia nitrogen (1.95 ± 0.60 mg per minute). As shown in Figure 1, higher extraction values of the total nitrogen content of glutamine and glutamic acid were largely responsible for this increased uptake of total amino acid nitrogen.

DISCUSSION

Previous measurements in nonacidotic dogs have suggested that arteriorenal venous differences of total free amino acid nitrogen are so small and variable that no conclusion may be formed as to the presence of either net renal uptake or release (19). The present observations demonstrate that such differences are also small across the human kidney (Table V). However, the consistent finding of values indicative of net renal extraction suggests that these small differences may well be significant. Regardless of the validity of small arteriorenal venous differences of total free amino acid nitrogen, the present results indicate clearly that several free amino acids exhibit definite patterns of either net renal uptake or release that are not apparent from determinations of total free amino acid nitrogen alone. Characteristically, the healthy human kidney extracts glutamine, proline, glycine, and valine from arterial plasma, whereas serine, glutamic acid, cystine, leucine, and arginine are usually added to renal venous blood. Glutamine is the only free amino acid extracted consistently by the kidney from arterial plasma, and serine is the only amino acid regularly released into renal venous blood.

The precise metabolic activities of the kidney that are responsible for either the net uptake or

release of certain amino acids are not identified by the present experiments. Nevertheless, quantitative differences between the net extraction or release of individual amino acids during conditions of normal acid-base balance and mild metabolic acidosis do permit certain statements to be made regarding the relationship of renal amino acid metabolism to ammonia production. It has been widely accepted for some time that the amide nitrogen of glutamine is the major precursor of urine ammonia. In addition, previous loading experiments have indicated that glycine, alanine, leucine, histidine, and asparagine may also serve as presursor materials for the renal production of ammonia (2–4). Since glutamine and glycine were the only members of this group that exhibited net extraction by the kidney, the present results can definitely implicate only two of these six amino acids as possible ammonia precursors at normal plasma amino acid levels. Net asparagine utilization, however, has not been excluded with certainty since the analytic technique used in this study prohibited its accurate measurement (6).

Under normal conditions of acid-base balance, the net renal uptake of glutamine amide nitrogen was sufficient to account for 63 per cent of the total amount of ammonia nitrogen released by the kidneys. The net extraction of glycine nitrogen was adequate to provide an additional 6 per cent of the total bidirectional release of ammonia. The net removal rates of other amino acids implicated as ammonia precursors were insufficient to account for the remaining total release of ammonia.

The remaining 30 per cent of the ammonia added to urine, or renal venous blood, or both, may be accounted for by consideration of the possible fates of the glutamic acid arising from the enzymatic deamidation of glutamine by glutaminase. This process yields one mole of both glutamic acid and ammonia from each mole of available glutamine. If the α-amino nitrogen of the glutamic acid produced by this reaction is not used in either ammonia synthesis or other metabolic reactions, a stoichiometric relationship between glutamine uptake and glutamic acid release should be found. That such is not the case is illustrated by the finding that only small amounts of free glutamic acid are actually released into either urine or renal venous blood. Although the present studies do not provide a definitive explanation of its fate, several possibilities must be considered. First, glutamic acid may contribute directly to renal ammonia production via its direct deamination to α-ketoglutarate and ammonia by glutamic dehydrogenase. Second, it may participate in various transamination reactions leading to the production of other amino acids which, in turn, are either released unchanged into renal venous blood, or deaminated directly to form ammonia and the appropriate keto-acid. A metabolic pathway of this type provides one possible explanation for the net venous release of such amino acids as alanine and serine. Third, it may be released into either blood or urine in a conjugated form undetected by the chromatographic technique used in this study. Fourth, it may be used directly in the synthesis of various peptides or proteins by kidney tissue. Although the results of these experiments are compatible with a proposal that both the α-amino and amide nitrogens of glutamine may serve as potential sources of renal ammonia, it is felt that complete utilization of glutamic acid for this purpose is unlikely for two principal reasons. First, the observed bidirectional release of ammonia is significantly less than would be expected if the α-amino and amide nitrogens of glutamine were both used completely for its synthesis. Second, previous balance studies during the administration of glutamic acid have suggested that this amino acid does not contribute significantly to renal ammonia production (2, 3). Nevertheless, the possibility does exist that at least a portion of the glutamic acid arising from glutamine deamidation is used in the formation of ammonia. It is obvious that further experiments are needed to clarify the exact role of glutamic acid in the renal synthesis of ammonia.

Although the possible transamination reactions of the glutamic acid derived from glutamine provide a potential source of the amino acids added to renal venous blood, another possibility should be mentioned. If current estimates of the normal protein content of glomerular filtrate are valid, the intratubular digestion of reabsorbed protein could also contribute to the observed patterns of net amino acid release. If so, however, only a small amount of reabsorbed protein must be degraded completely in view of the small quantity of nitrogen actually added to renal venous blood

as free amino acids. In addition, the dissimilarity between the amino acid composition of plasma proteins and the character of the free amino acids released into renal venous blood suggests that the degradation of reabsorbed protein by proteolytic enzyme systems is not solely responsible for their release.

The twofold adaptive rise of total renal ammonia production after ammonium chloride administration is in agreement with many previous observations (20, 21). Since renal venous ammonia release did not rise appreciably, this response was almost solely related to an increased urine excretion of ammonia. This observation raises the important question of whether or not the factors controlling renal venous ammonia release also participate in the adaptive response to ammonium chloride administration. Additional studies are urgently required to evaluate this possibility more fully.

After prolonged ammonium chloride administration, glutamine, valine, and glutamic acid were extracted by the kidney in increased amounts. Of these, only the increased net removal values of glutamine and glutamic acid were statistically significant, and only glutamine has been shown by previous loading experiments to be associated with an increased excretion of urine ammonia. Other amino acids such as glycine that have been suggested as renal ammonia precursors were not extracted in increased amounts. As under normal conditions of acid-base balance, most (74 per cent) of the total bidirectional release of ammonia could be explained by the increased extraction of glutamine amide nitrogen. Again, the data failed to account for approximately 25 per cent of the total ammonia released, unless the assumption is made that some, but not all, of the glutamic acid arising from glutamine deamidation is used in renal ammonia synthesis. The lower plasma glutamine concentration observed after ammonium chloride in association with an elevated renal extraction of glutamine has not been described previously. Its occurrence suggests, but does not prove, that the increased extraction of glutamine by the kidney is not counterbalanced by a compensatory rise in its availability to plasma.

Recently, the characteristic patterns of net renal uptake or release of free amino acids have been described in acidotic dogs by Shalhoub and associates (22). In most respects, their results appear to agree qualitatively with those observed during ammonium chloride administration to human subjects. They differ mainly in that mild metabolic acidosis in the human subject is associated with net renal extraction of glutamic acid rather than net release into renal venous plasma. The appearance of net glutamic acid uptake by the human kidney after ammonium chloride administration is difficult to relate to changes of renal ammonia metabolism unless, as discussed previously, one assumes either its direct deamination to ammonia and α-ketoglutarate, or its transamination to other amino acids that may then serve as direct precursors of renal ammonia nitrogen. That such may be the case is perhaps implied by the fact that glutamine and glutamic acid are the only amino acids whose renal extraction was significantly responsive to an induced change of acid-base balance.

Although these experiments provide information that must be explained by any complete and final description of the mechanisms of renal ammonia metabolism and excretion, it is apparent that the exact sources of urinary ammonia nitrogen cannot be ascertained by the simultaneous measurement of urinary amino acid excretion and arteriorenal venous amino acid differences. In this regard, it should be emphasized that net balance data reflect only the algebraic summation of the rates of total organ influx and efflux of a given substance. After ammonium chloride administration, it is conceivable that an amino acid might be extracted in amounts sufficient for ammonia synthesis without an apparent change of net extraction if its increased total influx is, for whatever reason, also accompanied by an appropriate change of total organ efflux. Nevertheless, if the factors responsible for the total efflux of amino acids into renal venous blood remain constant after ammonium chloride administration, a contribution of any amino acid to increased ammonia production should be reflected by either diminished net release or increased net uptake. In the present experiments, the only significant changes of this type were those exhibited by glutamine and glutamic acid.

SUMMARY

1. The net renal uptake or release of 19 free amino acids and the total bidirectional release of

ammonia (urinary excretion plus renal venous release) were measured in 6 healthy subjects under conditions of normal acid-base balance. Typically, serine, glutamic acid, alanine, cystine, and arginine were added to renal venous blood, whereas glutamine, proline, valine, and glycine usually exhibited net uptake. Only glutamine was extracted consistently by all subjects, and serine alone was added regularly to renal venous blood. The quantitative net release of glutamic acid was much less than the theoretical amount expected from the complete deamidation of extracted glutamine.

2. The net extraction of glutamine amide nitrogen accounted for only 63 per cent of the total bidirectional release of ammonia nitrogen. Other amino acids that have been implicated as renal ammonia precursors were not extracted in amounts sufficient to account for the remaining total ammonia release. If, however, the glutamic acid arising from glutamine deamidation is partially utilized for ammonia synthesis, then sufficient nitrogen is available from extracted amino acids to account for the observed total ammonia release.

3. Six similar experiments were performed after prolonged administration of ammonium chloride in an attempt to relate changes of renal amino acid extraction to the adaptive rise of renal ammonia production. With the exception of glutamine and glutamic acid, values for the net uptake or release of individual free amino acids resembled those of the subjects who did not receive ammonium chloride. Net glutamine uptake was significantly higher, and its amide nitrogen was extracted in amounts sufficient to account for 74 per cent of the increased total renal release of ammonia. The increased extraction of glutamic acid from plasma was small and insufficient to account for the remaining total release of ammonia. Again, however, total renal ammonia release could be satisfactorily explained by assuming that both nitrogens of extracted glutamine are potentially available to ammonia synthesis.

4. These data are compatible with the thesis that glutamine serves as the major precursor of renal ammonia under conditions of both normal acid-base balance and mild ammonium chloride acidosis, and that the glutamic acid arising from glutamine deamidation may also serve as a potential source of renal ammonia. They also imply that other amino acids do not contribute appreciably to renal ammonia production at normal plasma amino acid levels.

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Renal ammonia release during ammonium chloride acidosis[1]

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OWEN, EDWARD E., AND ROSCOE R. ROBINSON. *Renal ammonia release during ammonium chloride acidosis.* Am. J. Physiol. 208(1): 58–60. 1965.—The effect of prolonged oral ammonium chloride administration on the simultaneous release of ammonia into urine and renal venous blood was examined in the dog. Quantitatively, chronic ammonium chloride acidosis was accompanied by an almost identical average elevation of both urine ammonia excretion and renal venous ammonia release. Although not definitive, the results are entirely consistent with the prevailing concept that ammonia enters urine and renal venous blood via a pH-dependent process of non-ionic diffusion.

renal ammonia production ammonia release into urine and renal venous blood

THE APPEARANCE of an adaptive rise of urinary ammonia excretion has been well described following the chronic administration of strong acids or acidifying salts. In contrast, however, an increased renal venous release of ammonia has not yet been demonstrated in either man (3) or dog (2) during ammonium chloride acidosis. If always valid, this observation could be construed as evidence that the factors which control the entrance of ammonia into urine and renal venous blood are different, and that the renal venous addition of ammonia does not occur via a pH-dependent process of non-ionic diffusion as commonly supposed. For this reason, the present study was initiated to re-examine the effect of the prolonged administration of oral ammonium chloride on bidirectional renal ammonia release in experiments where each animal could serve as its own control.

METHODS

Twenty renal clearance experiments were performed on 10 healthy female dogs weighing between 14 and 24 kg. Each animal was studied twice, once before and once after the production of a moderate metabolic acidosis by the daily oral ingestion of 5–6 g ammonium chloride for 10 days. In each instance, a 5- to 6-day period elapsed between the first experiment and the initiation of ammonium chloride treatment. At that time, the animal was felt to have recovered from the initial or control experimental procedure.

Following pentobarbital anesthesia (25 mg/kg), an endotracheal tube bearing an inflatable cuff was inserted into the trachea and attached to a Harvard respirator. After suitable priming doses, a maintenance infusion of inulin and *p*-aminohippurate (PAH) in 5% mannitol was begun about 1 hr before the first clearance period via a constant-infusion pump. During the equilibration period a no. 7 cardiac catheter was introduced under fluoroscopic control into the left renal vein via the jugular vein. Using sterile technique, a small midline incision was made in the abdominal wall so that the tip of the catheter could be accurately positioned at a similar location in the left renal vein during each experiment. After equilibration had occurred, three or four sequential clearance periods of 20 min duration were obtained in each experiment. Simultaneous arterial and renal venous blood samples were drawn anaerobically at the approximate midpoint of each urine collection period. Bladder urine was collected under mineral oil via an indwelling Foley catheter.

Blood and urine ammonia concentrations were determined by a modification of the microdiffusion method of Brown, Duda, Korkes, and Handler (1). Inulin was determined by the resorcinol method of Roe, as modified by Schreiner (4), and PAH was measured by the technique of Selkurt (5). The pH and pCO_2 of arterial blood were determined polarographically in a constant-temperature water bath at 37 C, using a Severinghaus pCO_2 electrode and a glass capillary pH electrode. Urine pH was measured at 37 C using a Cambridge model R pH meter.

True or total renal plasma flow was estimated from PAH clearance and extraction of PAH. Total renal blood flow was calculated from this value and the arterial hematocrit. Renal venous ammonia release was calculated by

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TABLE 1. *Effect of prolonged ammonium chloride ingestion on total bidirectional release of ammonia by the kidney*

Dog No.		V	C_{IN}	E_{PAH}	TRPF	TRBF	B_{NH_4}	RV_{NH_4}	$A\text{-}RV_{NH_4}$	TRBF × $A\text{-}RV_{NH_4}$	$U_{NH_4}V$	Tot. Renal Ammonia Release	Urine pH	Art. Blood pH	Art. CO_2 Tension
		ml/min		%	ml/min		mM			μM/min			units		mm Hg
1	Control	2.5	51.2	73.4	176	296	.021	.056	.035	10.1	16.4	26.5	5.35	7.28	30.0
	NH_4Cl	2.0	51.2	53.2	375	650	.030	.076	.046	34.3	47.1	81.4	5.55	7.18	39.0
2	Control	2.3	30.0	81.3	96	177	.022	.077	.055	9.6	3.6	13.2	6.36	7.38	24.0
	NH_4Cl	3.5	55.8	62.9	235	440	.035	.084	.049	21.5	22.9	43.9	5.15	7.28	35.8
3	Control	2.5	48.1	66.0	170	264	.016	.051	.035	9.4	15.5	24.9	5.48	7.32	37.8
	NH_4Cl	3.1	41.7	54.0	356	588	.060	.130	.070	42.2	12.2	54.4	5.85	7.22	30.8
4	Control	4.0	48.5	58.7	276	427	.027	.046	.019	7.8	8.5	16.3	6.92	7.35	39.2
	NH_4Cl	4.2	60.5	61.5	293	414	.032	.076	.044	18.0	20.6	38.6	6.69	7.30	36.8
5	Control	1.8	59.2	75.9	180	295	.039	.082	.043	12.8	15.0	27.8	6.08	7.34	42.0
	NH_4Cl	4.2	44.4	51.4	294	498	.047	.114	.067	33.1	30.4	63.5	5.89	7.30	43.2
6	Control	1.8	73.0	74.2	318	570	.032	.104	.072	39.9	14.6	54.5	6.39	7.41	33.0
	NH_4Cl	2.8	108.7	69.7	415	878	.038	.111	.073	63.7	25.5	89.2	6.43	7.28	50.1
7	Control	2.9	51.3	64.7	269	467	.025	.069	.044	20.9	8.5	29.3	6.60	7.39	41.8
	NH_4Cl	2.7	48.5	59.9	179	367	.020	.080	.060	21.9	20.7	42.6	6.38	7.33	42.6
8	Control	2.3	65.6	73.1	268	446	.035	.066	.031	13.9	10.3	21.8	5.78	7.31	40.1
	NH_4Cl	2.3	42.8	47.5	274	477	.045	.105	.060	29.1	16.7	45.8	5.88	7.21	35.6
9	Control	3.8	56.1	62.3	162	237	.033	.067	.044	8.1	13.2	21.3	7.08	7.47	24.6
	NH_4Cl	4.0	64.8	74.9	266	395	.025	.054	.029	11.1	27.7	40.8	6.41	7.38	24.2
10	Control	3.5	69.6	76.8	223	465	.028	.065	.037	17.2	9.0	26.2	6.29	7.31	43.3
	NH_4Cl	3.2	62.0	58.7	264	433	.041	.122	.081	35.1	36.3	71.4	5.54	7.28	45.5
Mean control		2.7	54.3	70.6	214	364	.028	.068	.040	15.0	11.5	26.2	6.23	7.36	35.6
±1 SD		.8	16.3	7.3	68	127	.007	.016	.014	9.1	4.1	11.2	.57	.03	7.2
Mean NH_4Cl		3.2	58.0	59.3	295	514	.037	.095	.058	31.0	26.0	57.2	5.92	7.28	38.4
±1 SD		.8	19.6	8.5	70	147	.012	.023	.015	14.8	12.4	18.2	.43	.05	7.5
P		>.1	>.1	<0.02	<0.02	<0.02	<.1	<0.01	<0.05	<0.01	<0.01	<0.01	>.1	<0.01	>.1

V = urine flow; C_{IN} = inulin clearance; E_{PAH} = renal extraction of PAH; TRPF = total renal plasma flow; TRBF = total renal blood flow; B_{NH_4} = arterial blood ammonia concentration; RV_{NH_4} = renal venous blood ammonia concentration; $U_{NH_4}V$ = urine excretion of ammonia.

the arteriorenal venous difference of ammonia and the total renal blood flow. Total renal ammonia production was estimated by summation of the simultaneous values for renal venous ammonia release and urine ammonia excretion.

RESULTS

The average values from each experiment are listed in Table 1. After ammonium chloride administration, the pH of arterial blood fell from an average control value of 7.36 ± .03 to 7.28 ± .05 units, the pCO_2 of arterial blood stayed constant, and the urine pH decreased from 6.23 ± .57 to 5.92 ± .43 units (Table 1). On the average, the clearance of inulin was not altered during ammonium chloride acidosis. However, an appreciable rise of PAH clearance was observed in 7 of 10 dogs, and the extraction of PAH decreased moderately in 8 of 10 experiments. As a result, total renal blood flow increased significantly from an average control figure of 364 ± 127 ml/min to 514 ± 147 ml/min during acidosis (Table 1).

Predictably, the chronic oral administration of ammonium chloride was accompanied by an approximate twofold rise of total renal ammonia release (Table 1). Urine ammonia excretion, which averaged 11.5 ± 9.1 μmoles/min during the control experiments, rose to an average value of 26.0 ± 12.4 μmoles/min during the presence of metabolic acidosis (Table 1). This increase was highly significant statistically, and it could not be satisfactorily explained by the relatively small reduction in urine pH. In contrast to previous data (2, 3), ammonium chloride acidosis was also accompanied by a quantitative increase in the net addition of ammonia to renal venous blood. On the average, renal venous ammonia release rose from a control value of 15.0 ± 9.1 μmoles/min to 31.0 ± 14.8 μmoles/min after the appearance of metabolic acidosis (Table 1). Since the elevated renal venous release of ammonia was accompanied by an actual increase in the arteriovenous difference of ammonia, its appearance could not be solely ascribed to the associated rise in total renal blood flow (Table 1).

DISCUSSION

These data provide the first clear demonstration that the prolonged administration of oral ammonium chloride may be accompanied by an adaptive rise in the net addition of ammonia to renal venous blood. Although the results provide no information on the exact mechanism by which ammonia gains entrance into either urine or renal venous blood, they are perfectly consistent with the prevailing concept that ammonia is transported from renal tubular cells via a pH-dependent process of non ionic diffusion. Within the framework of this hypothesis

the relative magnitude of ammonia diffusion into either urine or renal venous blood must be dependent on such simultaneous variables as intracellular ammonia production, the pH of tubular fluid and peritubular blood, and the volume and velocity of flow (tubular fluid and peritubular blood) past the cellular site(s) of ammonia production and diffusion. During metabolic acidosis, a relatively greater reduction of urine pH as compared to arterial blood pH might be expected to favor the accumulation of a disproportionate amount of ammonia in urine. However, an accompanying rise of renal blood flow as seen in these experiments would tend to counterbalance this effect by facilitating an increased removal of ammonia in renal venous blood.

In contrast to other studies (2, 3) suggesting that the adaptive rise of renal ammonia release during metabolic acidosis is solely related to an increased urinary excretion of ammonia, the present experiments demonstrate that an almost identical elevation of both urinary ammonia excretion and renal venous ammonia release may occur under appropriate experimental conditions. Although definite statements cannot be made, the greater reduction of urine pH observed during earlier studies may have blunted the appearance of increased renal venous ammonia release by facilitating a disproportionate diffusion of ammonia into urine. In addition, however, the limited number of data in previous experiments and the lack of serial observations in the same subject may also have made the demonstration of increased renal venous ammonia release difficult.

Increased renal blood flow has not been described previously during ammonium chloride acidosis. Although an explanation for its occurrence is not apparent, the combination of increased PAH clearance and diminished PAH extraction does suggest that the increase in flow is a generalized phenomenon perfusing both functioning and nonfunctioning renal tissue.

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Relationship of pNH_3 of tubular cells to renal production of ammonia[1]

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PILKINGTON, LOU ANN, JOHN WELCH, AND ROBERT F. PITTS. *Relationship of pNH_3 of tubular cells to renal production of ammonia.* Am. J. Physiol. 208(6): 1100-1106. 1965.—Renal excretion of ammonia, addition of ammonia to renal venous blood, and total renal production of ammonia were measured in acidotic dogs before and during the intravenous infusion of ammonium chloride at rates of 0.5 and 1.0 mmole/min. Infusion of ammonium chloride increased urinary excretion of ammonia, reversed the direction of diffusion of ammonia between tubular cells and blood, i.e., induced the extraction of ammonia from blood, and reduced cellular production of ammonia. Production of ammonia was an inverse function of pNH_3 of tubular cells. The infusion of ammonium lactate N^{15} into one renal artery increased total ammonia excretion but decreased production of ammonia-N^{15} from normal blood-borne precursors. The intravenous infusion of ammonium chloride reduced extraction of glutamine from renal blood, yet increased the concentration of free glutamine in renal cortical cells. Our data suggest that an increase in pNH_3 of tubular cells results in competitive inhibition of the glutaminase-1 reaction. We further suggest that the pNH_3 of tubular cells is one factor governing renal production of ammonia; i.e., the higher the cellular pNH_3, the lower is the renal production of ammonia.

pNH_3 of cells ammonia production ammonia excretion amino acids ammonia-N^{15} excretion acidosis acid-base regulation glutamine ammonium chloride infusion diffusion of ammonia

THE FOLLOWING FACTS, describing the renal metabolism of ammonia, are well documented and generally accepted. Ammonia is formed within renal tubular cells from precursors extracted from arterial blood (13). The major precursor is glutamine; minor precursors include several of the circulating free amino acids (8, 11, 16, 22, 26, 29). Both the amide and amino nitrogens of glutamine contribute to ammonia formation, the former to about twice the extent of the latter (23). The ammonia, produced in tubular cells by the deamidation and deamination of these precursors, diffuses as the free base (NH_3) into acid urine, where it is trapped as relatively nondiffusible ammonium ion (NH_4^+), and, in lesser amounts, into peritubular blood, by which route it enters the general circulation (21). Tubular cells are freely permeable in both directions to the free base, relatively impermeable to ammonium ion (3, 27). Urinary excretion of ammonia increases markedly in chronic acidosis (2, 6, 20, 25) and plays a major role in combating and reversing the changes in body fluid composition which result from increased production of so-called fixed, nonvolatile acids.

Although the urinary excretion of ammonia is clearly a function of the hydrogen ion concentration of the urine (12, 14, 20, 21), the means by which intracellular production of ammonia is related to the acid-base requirements of the organism as a whole are not well understood. A variety of factors, summarized in a recent review (21), have been suggested as elements of the mechanism of control of ammonia production. One of these, namely the effect of cellular concentration of ammonia on cellular production of ammonia, is the subject of this investigation.

Studies in vitro have shown that ammonia is a competitive inhibitor of the glutaminase-1 reaction, namely the phosphate-activated, enzymatic hydrolysis of glutamine to form glutamic acid and ammonia (9). Glutamic acid, in the presence of oxidized nicotine adenine dinucleotide (NAD^+), is further degraded to α-ketoglutaric acid and ammonia by the enzyme, glutamic dehydrogenase. This latter reaction is reversible and, in the presence of an excess of ammonia and of the reduced nucleotide (NADH), results in the synthesis of glutamic acid (30). One would predict that an increase in production of ammonia, in excess of diffusion of ammonia into the urine and peritubular blood, would result in increased cellular concentration and hence check production of ammonia by both the glutaminase-1 and glutamic dehydrogenase reactions. Studies on the intact functioning kidney of the dog suggest that such inhibition may be one of several factors constituting the mechanism of

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control of production of ammonia. They further suggest that competitive inhibition of the glutaminase-1 reaction is a more significant element of control than is reversal of the glutamic dehydrogenase reaction.

METHODS

Experimental methods. Experiments on nine mongrel dogs of either sex, weighing from 20 to 25 kg, are reported in this paper. These animals were anesthetized with 20–30 mg/kg pentobarbital, administered intravenously and supplemented, as necessary, with additional small doses. In eight of the experiments, the animals were in a state of chronic metabolic acidosis, induced by feeding 10 g/day ammonium chloride mixed with the food for a period of 2–3 days. In these experiments, creatinine (Cr), for the measurement of glomerular filtration rate (GFR), and para-aminohippurate (PAH), for the measurement of renal plasma flow (RPF), were given as a prime and incorporated in an intravenous infusion, made two-thirds isotonic by the addition of sodium sulfate. In one experiment (*no. 4*) the dog was in a state of normal acid-base balance and the infusion was made two-thirds isotonic with mannitol. In the dogs in acidosis, sodium sulfate was administered to ensure the excretion of an acid urine; in the one dog in normal acid-base balance, mannitol was given to ensure the excretion of essentially neutral urine.

In *experiments 1–7* a radiopaque catheter was introduced into the right renal vein under fluoroscopic observation. A rapid determination of PAH extraction prior to starting the experiment and visual observation at autopsy confirmed its proper positioning. In these experiments urine was collected from the right ureter only and the data given in Tables 1 and 2 describe functions of the right kidney only.

In *experiments 6* and *7*, the left (control) kidney was exposed through a flank incision prior to starting the experiment and was rapidly removed and frozen in liquid nitrogen following the control period. The right (post-infusion) kidney was rapidly removed and frozen in liquid nitrogen at the termination of the experiment.

In *experiments 1–7*, the initial infusion, administered during the control period, contained no ammonium salt; the final infusion contained ammonium chloride in an amount such that its rate of administration was 0.5 or 1.0 mmole/min, as noted in the tables. All infusions were given at a rate of 5 ml/min.

In two experiments (*no. 8* and *9*) the left kidney was exposed through a flank incision and a curved no. 25 needle was introduced into the left renal artery close to its origin from the aorta. Either saline or saline containing isotopic ammonium lactate (96 atoms % N^{15}) was infused at a constant rate of 0.97 ml/min into the left renal artery. In these experiments urine was collected from both ureters separately.

Chemical methods and calculations. Creatinine was determined in plasma and urine by the method of Phillips (17). Para-aminohippurate was determined in plasma and urine by the method of Bratton and Marshall (4) and on whole blood as described by Pilkington et al. (19). Plasma, whole-blood, and tissue concentrations of free amino acids were determined by automatic column chromatography as described by Shalhoub et al. (26). Urine concentrations of total ammonia were determined by the aeration-nesslerization procedure of Van

TABLE 1. *Effects of increasing pNH_3 of renal cortical cells on excretion, extraction, and production of ammonia in acidotic dogs*

Exp. No.	Ammonium Chloride Infused	GFR	RPF	RBF	Arterial Plasma		Arterial Blood		Renal Venous Blood		Urine		Ammonia		
					HCO_3^-	pH	Ammonia	pNH_3	Ammonia	pNH_3	Flow	pH	Urine excretion	Renal blood add, subtract	Total production
	mmoles/min	*ml/min*			*μmoles/ml*		*μmoles/ml*	*mm Hg* $\times 10^6$	*μmoles/ml*	*mm Hg* $\times 10^6$	*ml/min*		*μmoles/min*		
1	0	55.0	232	379	15.7	7.29	0.035	16.8	0.082	38.4	0.95	5.15	54.5	+17.8	72.3
	0.5	55.5	194	318	15.5	7.28	0.197	92.3	0.119	54.5	1.06	5.39	91.3	−24.8	66.5
2	0	47.0	161	292	16.4	7.38	0.012	7.4	0.042	24.1	1.31	5.27	36.6	+8.8	45.4
	0.5	55.8	137	244	14.7	7.37	0.262	151.0	0.069	38.7	1.62	6.26	67.9	−47.0	20.9
3	0	51.9	174	272	16.5	7.35	0.031	17.1	0.070	37.6	1.50	5.40	41.1	+10.6	51.7
	1.0	56.2	163	248	15.5	7.35	0.360	198.0	0.160	85.0	1.69	6.32	72.8	−49.6	23.2
4	0*	41.6	120	195	19.1	7.40	0.048	29.5	0.109	65.5	1.40	7.03	11.2	+11.9	23.1
	1.0*	42.5	111	187	18.4	7.38	0.626	367.0	0.487	280.0	1.66	6.98	29.0	−26.0	3.0
5	0†	26.3	99.6	187	19.0	7.35	0.036	19.8	0.082	44.0	0.625	5.18	22.2	+8.60	30.8
	1.0†	19.3	68.0	136	16.9	7.33	0.832	436.0	0.428	219.0	1.00	6.37	58.5	−54.5	4.0
6	0	44.9	203	334	14.7	7.32	0.047	24.2	0.104	52.2	1.82	5.51	40.7	+19.1	59.8
	1.0	46.7	173	306	11.1	7.30	0.552	272.0	0.315	165.0	3.90	6.74	68.4	−63.4	35.0
7	0	54.6	232	437	13.5	7.30	0.048	23.5	0.098	46.9	2.18	5.43	54.8	+21.6	76.3
	1.0	49.4	158	303	10.5	7.25	0.758	338.0	0.488	214.0	4.10	6.43	141.0	−82.0	58.5

* Dog in a state of normal acid-base balance and infused with two-thirds isotonic mannitol rather than with sodium sulfate.
† Bilateral renal disease with scarring of kidneys.

Slyke and Cullen (28). Urine concentrations of ammonia-N^{15} were measured by the procedure described by Pitts et al. (23), using a Consolidated Nier isotope ratio mass spectrometer, model 21-201. Arterial and renal venous whole-blood concentrations of ammonia ($NH_3 + NH_4^+$) were measured by the vacuum-distillation method of Archibald (1); extractions were computed on the basis of whole-blood flows, either calculated or measured as described below.

In *experiments 1–5*, true renal plasma flows were calculated from plasma concentrations and arteriovenous extractions of PAH by the formula of Wolf (31), observing the precaution of rapid centrifugation of blood to minimize shift of PAH from cells to plasma in renal venous blood (18). Blood flows were calculated from plasma flows and hematocrit. In these experiments, free amino acids were measured in plasma. In *experiments 6* and *7*, true renal blood flows were calculated from whole-blood concentrations and arteriovenous extractions of PAH by the formula of Wolf (31). Such methods avoid the possibility of error due to shift of PAH from red cells to plasma. Renal plasma flows were calculated from whole-blood flows and hematocrit. In these experiments, free amino acids were measured in whole blood.

RESULTS

Table 1 summarizes seven experiments in which urinary excretion of ammonia, addition of ammonia to or its subtraction from renal blood, and cellular production of ammonia were measured. As Nash and Benedict (13) first observed, the kidney under normal circumstances adds ammonia to renal venous blood, i.e., the renal venous concentration exceeds the arterial concentration. However, if the arterial concentration of ammonia is significantly increased by the intravenous infusion of an ammonium salt, the kidney extracts ammonia from the blood and excretes it in the urine (5, 15). Denis et al. (5) have provided evidence that the free base (NH_3) is in diffusion equilibrium among the several fluid compartments of the renal cortex, namely proximal and distal tubular cells, proximal and distal tubular fluids, peritubular fluid, and peritubular capillary blood. They also have demonstrated that the concentration of free base, or expressed differently, the pNH_3 of renal venous blood, is a good approximation of the pNH_3 of cortical tubular cells.

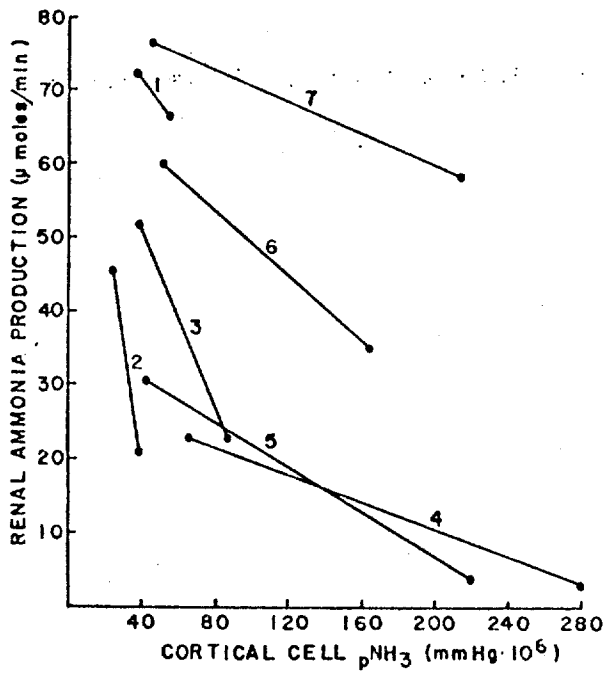

FIG. 1. Relation of renal production of ammonia to pNH_3 of cortical cells (renal venous pNH_3) in the acidotic dog. Numbers on lines correspond to experiment numbers in Table 1. Left point of each pair is the control value; right point is the value during infusion of ammonium chloride.

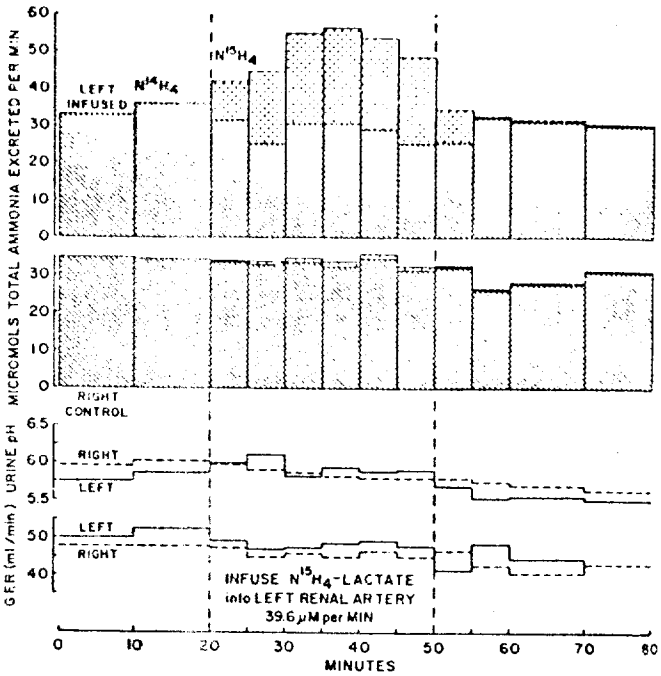

FIG. 2. Effect of infusion of ammonium lactate-N^{15} (96 atoms % N^{15}) at a rate of 39.6 μmoles/min into left renal artery on urinary excretion of ammonia by the two kidneys of an acidotic dog. Note that rate of excretion of ammonia-N^{14}, derived from normal blood precursors, decreases modestly on the left during infusion and excretion of the isotopic ammonia.

Table 1 shows that the renal venous concentration of ammonia ($NH_3 + NH_4^+$) exceeds the arterial value in the control period of each experiment. However, following the intravenous infusion of 0.5–1.0 mmole/min ammonium chloride to elevate the arterial concentration, ammonia is extracted from renal blood, i.e., both the renal venous concentration of ammonia (total) and the pNH_3 (calculated according to Jacquez (17)) are less than the corresponding arterial values. The quantity of ammonia extracted from the blood is added to the urine, increasing rate of excretion. However, of special significance in this study is the fact that the production of ammonia by renal tubular cells is in each instance reduced by elevation of the pNH_3 of the cells.

Figure 1 further summarizes the data of these

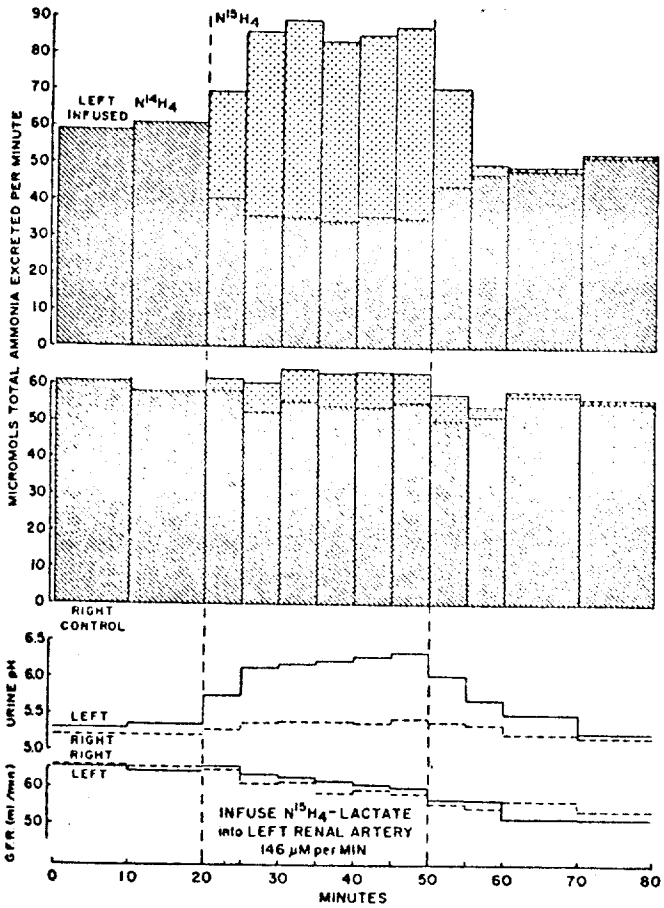

FIG. 3. Effect of infusion of ammonium lactate-N^{15} (96 atoms % N^{15}) at a rate of 146 μmoles/min into left renal artery on urinary excretion of ammonia by the two kidneys of an acidotic dog. Note that rate of excretion of ammonia-N^{14}, derived from normal blood precursors, decreases markedly on the left and modestly on the right during infusion and excretion of the isotopic ammonia.

experiments. Renal production of ammonia is plotted against cortical cell pNH_3, i.e., against pNH_3 of renal venous blood. For each experiment, the control datum and the datum derived during the infusion of ammonium chloride are connected by a straight line. The numbers beside the lines correspond to experiment numbers in Table 1. It is evident that an increase in cortical cell pNH_3 reduces rate of production of ammonia. In *experiments 1*, 2, and 3, a relatively small increase in cellular pNH_3 resulted in an appreciable reduction in rate of ammonia formation. However, Fig. 1 emphasizes the fact that cellular pNH_3 is by no means the sole or even the major factor controlling ammonia production. Thus at any given pNH_3, e.g., 120×10^{-6} mm Hg, production varies over a wide range.

Experiments 8 and 9, summarized in Figs. 2 and 3, constitute an independent confirmation of the fact that elevation of the pNH_3 of tubular cells decreases ammonia production. In these two experiments, isotopic ammonium lactate, highly labeled with N^{15} (96 atoms %), was infused into the left renal artery at rates of 39.6 and 146 μmoles/min, respectively. Rate of total ammonia excretion and atoms percent excess N^{15} in urinary ammonia were measured before, during, and after the infusion of the isotope into the left renal artery. The rates of excretion of ammonia-N^{14} and -N^{15} were calculated from these measurements.

It is evident that the isotopic nitrogen appeared promptly and in relatively high concentration in the ammonia excreted by the infused kidney, and in lesser amounts in the ammonia excreted by the control kidney.

Germane to our argument is the fact that, although total ammonia excretion increased, excretion of ammonia-N^{14}, formed from normal circulating precursors, decreased. This decrease is obviously much greater in the experiment summarized in Fig. 3 than in the one summarized in Fig. 2, due to the higher rate of infusion of isotopic ammonium lactate in the former. In fact, the excretion of ammonia-N^{14} by the opposite control kidney was reduced in *experiment 3*, due to the entry of sufficient isotopic ammonia into the general circulation to elevate the arterial pNH_3 and suppress ammonia production by the contralateral kidney.

Additional data obtained in *experiments 3*, 4, 6, and 7, described earlier, suggest that the suppression of ammonia production by elevation of cellular pNH_3 is the consequence of inhibition of the glutaminase-1 reaction. In these experiments, arterial and renal venous plasma (*exp. 3* and 4) or whole blood (*exp. 6* and 7) were chromatographed and free amino acids were measured. Concentrations and rates of extraction of six selected amino acids are presented in Table 2. Selection of these amino acids is based on the following facts, demonstrated by Shalhoub et al. (26) and Owen and Robinson (16). The kidneys of the acidotic dog and man extract glutamine and glycine from and add serine and alanine to renal blood more consistently and in greater amounts than other amino acids. Glutamic acid and aspartic acid are of interest because of their high concentrations in renal cortical tissue and their minor extraction from or addition to renal blood. Glutamic acid is of further interest because of its derivation from glutamine by the action of glutaminase.

It is evident from Table 2 that the intravenous infusion of ammonium chloride does not consistently alter the extraction of amino acids from or their addition to renal blood, with the exception of glutamine, the extraction of which is more or less markedly reduced. Values of net nitrogen extraction, given at the right of the table, refer only to the six amino acids listed. The arterial and renal venous concentrations of glutamine are given in terms of micromoles per milliliter, whereas extractions of glutamine are the product of renal plasma flow or blood flow and twice the arteriovenous micromolar concentration difference, to account for both the amide and amino nitrogens of the compound.

Table 3 demonstrates the interesting fact that the concentrations of all free amino acids of renal cortical tissue with the exception of glutamine decreased following the infusion of ammonium chloride. In contrast, the concentration of glutamine increased despite the fact that

TABLE 2. *Effects of increasing pNH_3 of renal cortical cells on extraction from blood of selected free amino acids by kidney of acidotic dog*

Exp. No.	Ammonium Chloride Infused	Aspartic Acid			Serine			Glutamine				
		Art.	R. ven.	Extr.	Art.	R. ven.	Extr.	Art.	R. ven.	Extr.		
		μmole/ml	μmole/ml	μmoles/min	μmole/ml	μmole/ml	μmoles/min	μmole/ml	μmole/ml	μmoles/min		
3	0	0.009	0.006	+0.5	0.198	0.259	−10.6	0.[illegible]5	0.406	+69.3		
	1.0	0.009	0.009	0.0	0.119	0.192	−11.9	0.794	0.631	+53.2		
4	0	0.005	0.016	−1.3	0.094	0.154	−7.2	0.431	0.320	+26.6		
	1.0	0.010	0.003	+0.8	0.067	0.102	−3.9	0.528	0.460	+15.1		
		Glutamic Acid			Glycine			Alanine			Net N Extr.	Ammonia Produced
		Art.	R. ven.	Extr.	Art.	R. ven.	Extr.	Art.	R. ven.	Extr.		
		μmole/ml	μmole/ml	μmoles/min	μmole/ml	μmole/ml	μmoles/min	μmole/ml	μmole/ml	μmoles/min	μmoles/min	μmoles/min
3		0.032	0.040	−1.4	0.202	0.170	+5.6	0.368	0.419	−19.3	+44.3	51.7
		0.028	0.043	−2.4	0.170	0.136	+5.5	0.261	0.401	−22.8	+21.6	23.2
4		0.037	0.040	−0.4	0.124	0.108	+1.9	0.132	0.163	−6.1	+13.5	23.0
		0.036	0.057	−2.3	0.114	0.113	+0.1	0.179	0.254	−8.3	+1.5	3.0
		Aspartic Acid			Serine			Glutamine				
		Art.	R. ven.	Extr.	Art.	R. ven.	Extr.	Art.	R. ven.	Extr.		
		μmole/ml	μmole/ml	μmoles/min	μmole/ml	μmole/ml	μmoles/min	μmole/ml	μmole/ml	μmoles/min		
6	0*	0.027	0.026	+0.3	0.173	0.195	−7.3	0.751	0.661	+66.8		
	1.0*	0.026	0.026	0.0	0.122	0.132	−3.1	0.651	0.595	+34.3		
7	0*	0.027	0.028	−0.4	0.275	0.282	−3.1	0.[illegible]5	0.600	+56.8		
	1.0*	0.021	0.024	−0.9	0.230	0.253	−7.0	0.723	0.669	+20.9		
		Glutamic Acid			Glycine			Alanine			Net N Extr.	Ammonia Produced
		Art.	R. ven.	Extr.	Art.	R. ven.	Extr.	Art.	R. ven.	Extr.		
		μmole/ml	μmole/ml	μmoles/min	μmole/ml	μmole/ml	μmoles/min	μmole/ml	μmole/ml	μmoles/min	μmoles/min	μmoles/min
6		0.056	0.065	−3.3	0.162	0.141	+7.6	0.252	0.273	−7.0	+56.5	59.8
		0.056	0.067	−3.4	0.133	0.109	+7.3	0.179	0.208	−15.0	+20.0	35.0
7		0.061	0.067	−2.6	0.178	0.156	+9.6	0.237	0.287	−21.8	+38.5	76.3
		0.064	0.067	−0.9	0.172	0.148	+7.3	0.245	0.286	−12.4	+7.0	58.5

* Amino acid concentrations of arterial and renal venous whole blood. Amino acid extractions calculated from whole blood analyses and measured renal whole blood flows.

the extraction of glutamine from arterial blood is reduced by increasing the pNH_3 of tubular cells (Table 2). Although we have no explanation for the decrease in concentration of all free amino acids of renal cortex other than glutamine, the fact that the concentration of glutamic acid decreased suggests that inhibition of the glutamic dehydrogenase reaction, if it occurs at all, is less significant than is inhibition of the glutaminase reaction.

DISCUSSION

Table 1 and Figs. 1–3 demonstrate clearly and in two independent ways that presentation of preformed ammonia to tubular cells reduces their rate of production of ammonia from blood-borne precursors. Since the extraction of glutamine from renal blood is reduced by elevation of arterial pNH_3, one might argue that ammonia suppresses the transport of glutamine into tubular cells, perhaps by altering the binding of glutamine by a membrane carrier in one manner or another. Reduced transport of glutamine into cells might thus be the ultimate cause of reduced utilization. However, the reabsorption of filtered glutamine from tubular urine is not significantly reduced, for excretion of glutamine was essentially zero both in control periods and during the infusion of ammonium chloride. If ammonia affects membrane transport of glutamine it must do so solely or predominantly at the peritubular border. The fact that the concentration of glutamine within renal cortical cells increases despite reduced extraction implies that the primary event is reduced metabolic utilization rather than reduced extraction. The additional fact that the glutamic acid content of tubular cells decreases further pinpoints a specific interference in the conversion of glutamine to glutamic acid and ammonia as the significant causal factor in reduced production of ammonia. We infer that an increase in pNH_3 of tubular cells suppresses the glutaminase reaction, most probably

TABLE 3. *Changes in concentration of selected free amino acids in renal cortex resulting from intravenous infusion of ammonium chloride*

Exp. No.	Condition	Aspartic Acid	Serine	Glutamine	Glutamic Acid	Glycine	Alanine
6	Control	1.917	0.391	0.426	5.440	0.584	0.847
	+ Ammonia	0.377	0.181	0.615	2.910	0.431	0.551
	Δ	−1.540	−0.210	+0.189	−2.530	−0.153	−0.296
7	Control	1.190	0.622	0.637	4.380	0.652	0.976
	+ Ammonia	0.630	0.439	0.868	3.060	0.561	0.982
	Δ	−0.560	−0.183	+0.231	−1.320	−0.091	+0.006

Values are expressed in μmoles/g.

competitive inhibition, resulting in accumulation of glutamine and diminished production of glutamic acid and ammonia. If the glutamic dehydrogenase reaction is suppressed, it is suppressed to a lesser extent than is the glutaminase-1 reaction, for the glutamic acid content of the kidney decreases.

We recognize the fact that the above arguments are not entirely conclusive. First, it is possible that, as a consequence of increased cellular pNH_3, synthesis of glutamine from glutamic acid is increased. This would result in a higher cellular concentration of glutamine and a lower cellular concentration of glutamic acid. We believe that the virtual absence of the glutamine synthetase system in the kidney of the dog (10, 24) negates this explanation of our results. The glutaminase-1 reaction may not be competitively inhibited by ammonia under the conditions of our experiments. Thus diffusion of free base into tubular cells may alter pH of the cells to such an extent that enzyme activity is reduced and ammonia production is suppressed. We have no answer to this argument; it may be correct.

A point of interest is the reduction in the hydrogen ion gradient between blood and urine which occurs when the arterial and renal venous pNH_3 are significantly increased. This obviously is a consequence of diffusion of free base from blood to urine and titration of the secreted hydrogen ions to form ammonium ions. It is probable that the mechanism which we propose operates in this context. Thus any reduction in secretion of hydrogen ions results in the formation of urine of higher pH, in a reduction in the diffusion gradient for free base (NH_3) from cells to urine, cellular accumulation of ammonia, and suppression of ammonia formation. We do not wish to imply that this is the only or even the major mechanism of control of ammonia production. Rather we believe it to be one of several elements of significance in the control of ammonia formation.

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Experimental Metabolic Acidosis: The Enzymatic Basis of Ammonia Production by the Dog Kidney *

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Ammonia excretion by the kidney has long been known to increase when a constant acid load is given to healthy subjects. This was clearly delineated by Sartorius, Roenmelt, and Pitts (1), who reported that when healthy humans were given a constant amount of ammonium chloride daily, there was a gradual increase in the excretion of ammonia, accompanied by an increased excretion of protons, and by an adaptation that resulted in the conservation of sodium and potassium ions.

These experiments were preceded by those of Van Slyke and his co-workers (2), who had demonstrated that glutamine was extracted from the renal arterial blood of acidotic dogs in amounts sufficient to account for about 60% of the NH_3 excreted by the kidneys—an observation recently confirmed and extended by the experiments of Pitts and his colleagues (3, 4). The infusion of a variety of amino acids into the acidotic dog has also been shown to increase the rate of excretion of ammonia (5–8), L-glutamine, L-asparagine, L- and D-alanine, and L-histidine being the most effective.

The nature of the metabolic pathways involved and the enzymes responsible for catalyzing the relevant reactions are not yet clearly understood. Davies and Yudkin were the first to demonstrate an adaptive increase in glutaminase activity in kidney slices from rats made chronically acidotic by the ingestion of HCl in their drinking water (9). Rector, Seldin, and Copenhaver observed a concordant relationship between glutaminase activity in homogenates of rat kidney and the increase in ammonia excretion during successive days of ammonium chloride administration (10). On the other hand, an increase in glutaminase activity occurred in both experimental acidosis and alkalosis in the guinea pig (11), and glutaminase activity did not appear to increase in the acidotic dog (12).

The present experiments were designed *1*) to investigate the influence of ammonium chloride induced acidosis on the activity of enzymes catalyzing ammonia metabolism and the production and excretion of hydrogen ions, and on representative enzymes of glycolysis and the citric acid cycle in the kidney and liver of the dog; and *2*) to attempt to relate quantitatively enzyme activity in the kidney to the ammonia production and hydrogen ion excretion by the kidney. The activity of most enzymes was studied quantitatively by ultramicrobiochemical techniques in the anatomical units of the nephron, individually dissected from frozen-dried 10-μ sections; others were studied in homogenates. Enzyme activity in the liver was studied in homogenates.

Methods

Selection of dogs for study. That there is a high incidence of interstitial nephritis in dogs is well known (13), and in our experience interstitial nephritis is common in kidneys obtained from unselected dogs. The five male mongrel dogs used in these experiments were selected because they had good renal function. After de

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hydration for 20 hours the urine specific gravity was 1.036 to 1.060, the urine osmolality 1,442 to 2,188 mOsm per kg. There were no significant abnormalities on microscopic examination of the urine sediment. The serum creatinine levels varied from 0.67 to 0.79 mg per 100 ml and the urea nitrogen levels from 7.5 to 22 mg per 100 ml. The weights of the dogs chosen ranged from 10.2 to 15.7 kg.

Design of the experiment. One dog was chosen at random as a control; the other four were designated the experimental animals. The dogs were kept in individual metabolic cages throughout the experiment. They were allowed water ad libitum and were fed a constant diet of Purina dog meal, horsemeat and gravy, and puppy kibbles mixed in the ratio 2:1:1. The dogs ate approximately 450 g of this mixture daily, containing about 97 g of protein, 157 g of carbohydrate, and 26 g of fat. The control dog was pair-fed the average food intake of the four experimental dogs on the preceding day.

Twenty-four-hour urine collections were made using toluene and thymol crystals as preservatives. Feces were removed promptly from the cages to prevent contamination of the urine. Immediate analyses were made of urine volume, pH, NH_3, and bicarbonate; portions of the remaining urine were frozen for subsequent chemical analysis. Preliminary experiments were done in which dog urine samples, collected in identical manner with toluene and thymol, were stored for 24 to 36 hours at room temperature. Under these conditions, the pH, NH_3, titratable acid, and bicarbonate concentrations did not change.

Each dog was studied during a 7-day control period. At the end of this period needle and wedge biopsies of the kidney and liver were taken transperitoneally under pentobarbital anesthesia. The dogs were allowed 1 week to recover from the operation and were then studied during a 7-day experimental period in which they received ammonium chloride, 112 mEq daily in gelatin capsules in three divided doses. The control dog did not receive ammonium chloride, but was pair-fed and studied in an identical manner. At the end of the experimental period needle and wedge biopsies of kidney and liver were again taken transperitoneally under identical conditions. The kidney tissue of all five dogs was normal on histologic examination.

Handling of the renal biopsy specimens. Part of each biopsy specimen was frozen immediately in liquid nitrogen and prepared for analysis of enzyme activity as described previously (14). The methods of identifying and dissecting out the individual anatomical units of the nephron, with the stained section as control, and of weighing the tissue fragments on quartz fiber balances have been published (14, 15). For each enzyme studied, save for glutaminase, the two kidney specimens from a single dog were analyzed in a single experiment on the same day.

TABLE I

*Optimal conditions for the assay of enzyme activities in the dog kidney**

Enzyme	Buffer	Substrate	Cofactor	Other	Incubation product	
					Volume	Measured
	M	*mM*			*μl*	
LDH	Tris, 0.2 pH 7.15	Na-pyruvate, 1.0	$NADH_2$, 1.25 mM	Nicotinamide, 25 mM	10	NAD†
MDH	Tris, 0.18 pH 8.6	Oxaloacetate, 1.1	$NADH_2$, 1.30 mM	Nicotinamide, 20 mM	10	NAD
GDH	Tris, 0.10 pH 7.4	2-Oxoglutarate, 2.5 $(NH_4)_2SO_4$, 75	$NADH_2$, 2.8 mM	Nicotinamide, 20 mM	2	NAD
AspAT	Tris, 0.10 pH 7.6	L-Aspartate, 48 2-Oxoglutarate, 10		$NADH_2$, 2.7 mM MDH Nicotinamide, 20 mM	5	NAD
AlAT	Tris, 0.15 pH 7.7	2-Oxoglutarate, 10 L-Alanine, 43	Pyridoxalphosphate, 0.17 mM	$NADH_2$, 2.1 mM LDH Nicotinamide, 20 mM	5	NAD
DAO	Tris, 0.07 pH 7.8	D-Alanine, 2.5	FAD, 7.8×10^{-6} M	$NADH_2$, 0.3 mM LDH	210	$NADH_2$‡
CS	Tris, 0.05 pH 8.7	Acetyl S-CoA, 0.7		K-malate, 6.0 mM NAD, 0.9 mM MDH	160	$NADH_2$
Glutaminase I	Tris, 0.04 pH 9.0	L-Glutamine, 48		Phosphate, 0.2 M	25	NH_3§
Glutaminase II	Tris, 0.04 pH 8.75	L-Glutamine, 48	Pyruvate, 0.1 M		25	NH_3
Glutaminase nonactivated	Tris, 0.04 pH 8.75	L-Glutamine, 48			25	NH_3

* LDH, MDH, and GDH = lactate, malate, and glutamate dehydrogenase; AspAT and AlAT = aspartate and alanine aminotransferase; DAO = D-alanine oxidase; CS = citrate synthase; FAD = flavin-adenine dinucleotide.
† Measured by fluorometry.
‡ Measured by spectrophotometry.
§ Measured by diffusion and titration.

A second part of each renal biopsy specimen, consisting of a wedge of cortex, was used for preparing tissue homogenate in ice cold distilled water using a Potter-Elvehjem all glass homogenizer. The liver biopsy specimen was handled similarly. A small portion (100 to 200 mg) was cut from each specimen and was used to determine dry weight by drying at 110° C until the weight was constant.

Measurement of enzyme activity. Full details of the methods used to measure lactate dehydrogenase activity (16), carbonic anhydrase activity (17), glutaminase I and II activities (18), and the activity of the other enzymes examined (19) have been published. The optimal conditions, determined for the dog kidney (19), are summarized in Table I. In previous publications we have expressed enzyme activity in moles of substrate metabolized per kilogram dry tissue per hour (MKH). To comply with the suggestions of the Commission on Enzymes of the International Union of Biochemistry, the results in this paper are expressed in micromoles per gram dry tissue per minute (1 MKH = 16.6 μmoles per g per minute).

Nomenclature of enzymes. The nomenclature for enzymes as suggested in the Report of the Commission on Enzymes (20) is used in this paper. The classification number is given in brackets after the systematic name, and the suggested common name is in parentheses together with the abbreviation: D-aminoacid: O_2 oxidoreductase [1.4.3.3] (D-aminoacid-oxidase, with D-alanine as substrate D-alanine-oxidase, DAO); citrate oxaloacetate-lyase [4.1.37] (citrate synthase, CS); L-lactate: NAD oxidoreductase [1.1.1.27] (lactate dehydrogenase, LDH); L-malate: NAD oxidoreductase [1.1.1.37] (malate dehydrogenase, MDH); L-glutamate: NAD oxidoreductase [1.4.1.2] (glutamate dehydrogenase, GDH); L-alanine: 2-oxoglutarate aminotransferase [2.6.1.2] (alanine aminotransferase, AlAT);[1] L-aspartate: 2-oxoglutarate aminotransferase [2.6.1.1] (aspartate aminotransferase, AspAT);[1] carbonate hydrolyase [4.2.1.1] (carbonic anhydrase, CA); and L-glutamine-aminohydrolyase [3.5.1.2] (glutaminase, Glu).

Statistical analysis. The activity of each enzyme was analyzed in each renal structure independently. The design model for the analysis of variance was: $Y_{ijk} = \mu + T_i + D_j + (T \times D_{ij}) + e_{k(ij)}$, where Y_{ijk} = observed response of the kth determination of the jth dog receiving i^{th} treatment; μ = general mean; T_i = treatment effect due to the ith treatment; D_j = effect attributable to dog j; $T \times D_{ij}$ = interaction effect of the ith treatment on the jth dog; $e_{k(ij)}$ = effect attributable to the kth determination made on dog j treated with treatment i. The mean square estimators in the analysis of variance are:

Source of variation	Degrees of freedom	Estimators of mean square
Between dogs	$d - 1$	$\sigma^2_e + \eta_0\sigma^2_{T \times D} + \eta_0 t\sigma^2_D$
Between treatments	$t - 1$	$\sigma^2_e + \eta_0\sigma^2_{T \times D} + \eta_0 d\sigma^2_T$
Treatment × dog interaction	$(t - 1)(d - 1)$	$\sigma^2_e + \eta_0\sigma^2_{T \times D}$
Determinations within treatment within dog	$\Sigma(n_k - 1)$	σ^2_e
Total	$N - 1$	

where

$$\eta_0 = 1/(dt - 1) \times (\Sigma\eta_{ij} - \Sigma\eta^2_{ij}/\Sigma\eta_{ij}).$$

The design is balanced in respect to dogs and treatments, but not necessarily in respect to the number of determinations made on each structure of each biopsy within each dog.

When the F ratio of treatment × dog interaction was significant at the 5% probability level, the interaction mean square was used as the error term for testing the significance of differences between treatments; when this interaction was not significant, a pooled error term was used.

Differences in treatments might be due to the effect of the ammonium chloride administration itself or to one or more of the effects of time and of the surgical trauma associated with the biopsy or to all of these. To estimate the effects of surgical trauma and time, independent of treatment, the single control dog was analyzed for differences between biopsies. It is desirable to use more dogs as controls, but the volume of the enzyme analyses involved precluded this.

Results

Metabolic studies

The dogs were in good health throughout the study, did not vomit, and did not lose weight during the period of ammonium chloride administration. The dogs had not been trained, and the urine

[1] The report did not include suggestions for abbreviations for enzymes. Although the abbreviations used hitherto for most enzymes are still meaningful, some abbreviations must be changed. Thus for two enzymes included in this study, new abbreviations are introduced in accordance with new systematic and common names: *a*) alanine-amino transferase, hitherto known as glutamate-pyruvate-transaminase (GPT), is now abbreviated AlAT, and *b*) aspartate-amino transferase, hitherto known as glutamate-oxaloacetate-transaminase (GOT), is now abbreviated AspAT.

volumes obtained, although complete, were not true 24-hour collections. Thus there was a large variation in the day-to-day urine volumes and in the daily excretion of the substances measured. Nevertheless the basic trends are clear as summarized in Figures 1 and 2, in which the average values for the four experimental dogs are plotted in solid lines, the values for the single control dog in broken lines. In the control period the urine pH and the daily excretion of titratable acid, NH_3, hydrogen ion, sodium, potassium, chloride, and phosphate were of similar magnitude in the experimental and control dogs. With ammonium chloride loading the urine pH fell promptly from an average of 6.85 to 5.89 on the first day and

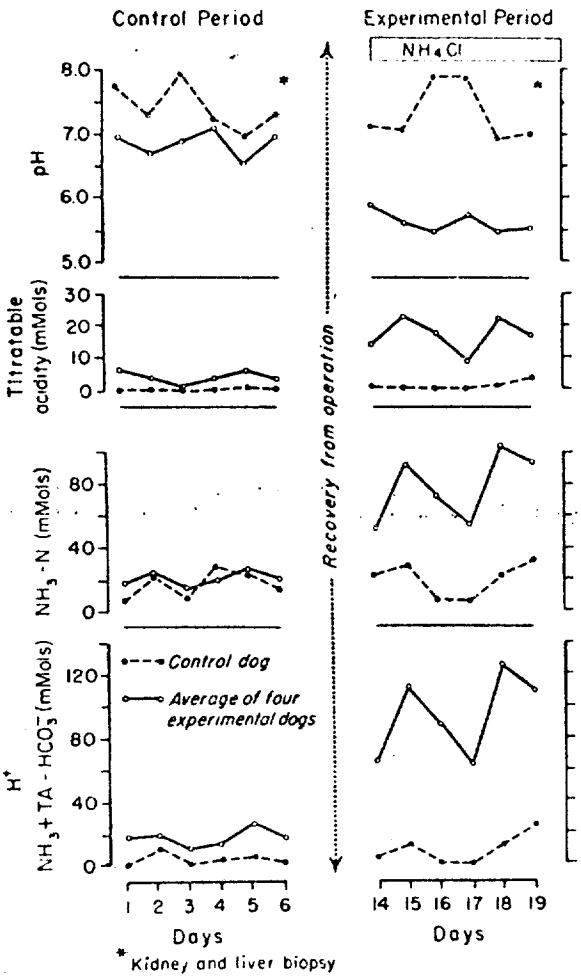

FIG. 1. URINE pH AND 24-HOUR TITRATABLE ACID, AMMONIA, AND TOTAL HYDROGEN ION EXCRETION BEFORE AND DURING THE DAILY ADMINISTRATION OF 112 mEq OF AMMONIUM CHLORIDE.

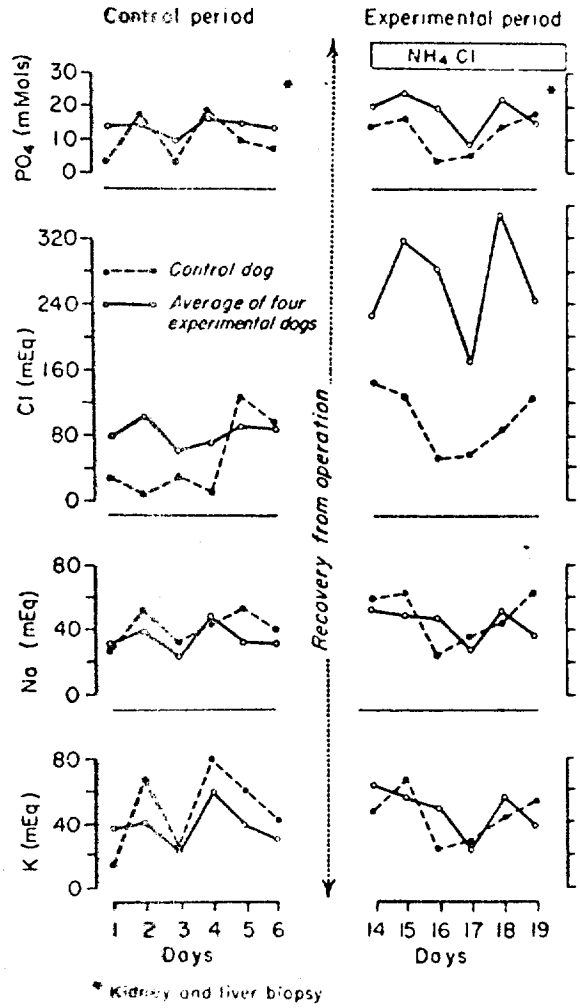

FIG. 2. TWENTY-FOUR-HOUR URINE CHLORIDE, INORGANIC PHOSPHATE, SODIUM, AND POTASSIUM EXCRETION BEFORE AND DURING THE DAILY ADMINISTRATION OF 112 mEq OF AMMONIUM CHLORIDE.

varied thereafter from 5.47 to 5.73. The excretion of titratable acid increased from an average of 3.6 nmoles to 17.4 nmoles per day, of ammonia from 20 to 78 nmoles per day, and of net H^+ excretion ($TA + NH_3 - HCO_3^-$) from 16 to 95.5 nmoles per day. There was a significant relationship ($r = -0.65$, $p < 0.001$) between pH and the logarithm of NH_3 excretion in the control period but no relationship ($r = -0.17$, $0.50 > p > 0.40$) in the experimental period. The excretion of sodium and potassium did not change significantly at the beginning of or throughout the period of ammonium chloride loading.

The plasma bicarbonate levels were not meas-

TABLE II

*The effect of ammonium chloride administration on enzyme activities in the nephron of the dog**

Structure	No. of dogs	Average no. of determinations per biopsy	Enzyme activity: Normal metabolic conditions: Mean	Normal metabolic conditions: Range	During NH_4Cl administration: Mean change	95% confidence limits of mean change
			μmoles substrate metabolized/g dry tissue/minute			
			Glutaminase†			
Cortical homogenate	4	3.6	18.6	16.0–21.5	−3.4	(−10.4 and +3.7)
			D-Alanine oxidase‡			
Cortical homogenate	4	2.0	8.5	8.0–9.0	−0.7	(−2.5 and 1.1)
			Citrate synthase‡			
Cortical homogenate	4	2.0	21.6	19.2–25.2	+0.8	(−4.0 and +5.5)
			Glutamate dehydrogenase§			
Glomeruli	3	4.8	13.6	9.7–15.8	+2.8	(−10.0 and +15.6)
Proximal convolutions	3	9.8	73.8	62.8–87.5	+6.4	(−37.8 and +50.6)
Distal convolutions	3	9.8	84.7	64.7–110	+11.6	(−323 and +346)
			Aspartate aminotransferase§			
Glomeruli	4	4.3	70.2	45.0–132	+53.7	(−27.9 and +135)
Proximal convolutions	4	7.6	199	158–235	+98.9	(−14.8 and +213)
Distal convolutions	4	6.5	372	243–530	+115	(−87.0 and +317)
Medullary rays	2	4.5	188	138–250	+100	(−15.4 and +215)
			Alanine aminotransferase§			
Glomeruli	3	4.1	12.2	4.8–21.6	−3.5	(−15.6 and +8.7)
Proximal convolutions	4	7.5	52.2	36.8–78.2	−2.1	(−19.4 and +15.2)
Distal convolutions	4	7.7	44.3	29.2–68.3	+1.9	(−31.1 and +34.9)
Medullary rays	2	3.8	67.8	39.8–98.3	+12.4	(−87.7 and +112)

* The first assay was made at the end of a 7-day control period, the second at the end of a 7-day period of ammonium chloride administration, 112 mEq daily.

† Glutaminase I activity was measured at pH 9.5 at 37° C. The conditions for this assay, which were taken from the literature, were in fact suboptimal. Later, optimal conditions for glutaminase were reinvestigated (18), and the full study was repeated on two dogs (see text and Table III).

‡ D-Alanine oxidase and citrate synthase activities were measured at 22° C.

§ GDH, AspAT, and AlAT activities were measured at 37° C.

ured because of an error in the laboratory. When the second biopsy was taken, the average arterial pH of the four experimental dogs was 7.25, and the average serum chloride concentration was 112 mEq per L.

Enzyme activity in the kidney

Glutaminase. Glutaminase I activity (Table II) was measured in cortical homogenates according to optimal conditions described by Richterich and Goldstein (21). It could not be measured in the anatomical units of the nephron, as the enzyme activity is destroyed by freeze-drying (18). The average activity was 18.6 μmoles per g per minute. With ammonium chloride loading it decreased slightly in all four dogs; a decrease of similar magnitude was also observed in the control dog.

Subsequently, further studies were made of the optimal conditions for the assay of glutaminase activity (18), and we found that the conditions under which enzyme activity had been assayed were not in fact optimal. When new optimal conditions had been established, dogs 1 and 3 were restudied under metabolic conditions identical to those of the original experiment. The other three dogs were studied only under normal metabolic conditions. The results are given in Table III. Glutaminase I activity in the cortex was found to be 200 μmoles per g per minute, i.e., 11 times higher than in the original experiment. It did not change significantly with ammonium chloride administration. Glutaminase II activity was low, 19.8 μmoles per g per minute; it increased slightly in both dogs. Nonactivated glutaminase activity was low, 8.5 μmoles per g per minute, and in

TABLE III

*Glutaminase I, glutaminase II, and nonactivated glutaminase activities in homogenates from canine kidney**

Enzyme	Structure	Normal metabolic conditions						During NH_4Cl administration	
		Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Average	Dog 1	Dog 3
Glutaminase I	Cortex	230	170	222	188	217	206	257	243
	Medulla					100		95.8	84.7
Glutaminase II	Cortex	19.7	19.7	22.2	17.3	20.7	19.8	28.5	32.0
	Medulla					9.3		17.7	17.7
Nonactivated glutaminase	Cortex	9.0	7.2	8.5	8.0	9.8	8.5	23.8	17.3
	Medulla					4.3		9.8	9.0

* The data are from second experiments in which the enzyme activities were assayed under the optimal conditions determined in our laboratory (18). The results are expressed as micromoles of ammonia formed per gram dry weight of tissue per minute at 37° C.

creased twofold in the kidney homogenates of both dogs.

D-*Alanine oxidase and citrate synthase.* DAO and CS activities were measured in cortical homogenates only (Table II). DAO activity could not be measured in frozen-dried sections because the oxygen tension cannot yet be kept high enough to saturate the enzyme during 1-hour incubation in ultramicro test tubes. An ultramicro method for citrate synthase activity has not yet been developed. In cortical homogenates the average DAO and CS activities were 8.5 and 21.7 μmoles per g per minute, respectively, and did not change with ammonium chloride administration.

Glutamate dehydrogenase activity was measured in the control dog and in three experimental dogs (Table II). GDH activity was relatively low in the glomeruli (mean, 13.6 μmoles per g

TABLE IV

*The effect of ammonium chloride administration on enzyme activities in the nephron of the dog**

Structure	No. of dogs	Average no. of determinations per biopsy	Enzyme activity: Normal metabolic conditions: Mean	Normal metabolic conditions: Range	During NH_4Cl administration: Mean change	95% confidence limits of mean change
			μmoles substrate metabolized/g dry tissue/minute			
			Carbonic anhydrase†			
Glomeruli	4	5.4	106,500	94,800–120,000	−5,400	(−62,900 and +52,200)
Proximal convolutions	4	17.7	365,000	296,700–455,000	+134,600	(−102,300 and +371,600)
Distal convolutions	4	14.5	354,700	189,500–513,300	−16,300	(−870,600 and +930,200)
Medullary rays	2	4.5	195,800	164,300–213,800	+81,500	(−122,300 and +285,300)
			Lactate dehydrogenase‡			
Glomeruli	2	4.8	694	645–743	−58	(−468 and +352)
Proximal convolutions	2	7.2	2,170	1,917–2,417	+88	(−881 and +1,060)
Distal convolutions	2	7.0	2,345	1,850–2,917	+156	(−1,548 and +1,859)
Medullary rays	2	5.0	1,782	1,650–1,917	+33	(−895 and +961)
			Malate dehydrogenase§			
Glomeruli	2	4.2	979	725–1,180	+23.2	(−121 and +470)
Proximal convolutions	2	7.2	3,154	2,800–3,550	+135	(−1,172 and +1,441)
Distal convolutions	2	7.5	4,600	4,500–4,683	+66.7	(−2,711 and +2,845)
Medullary rays	1	5.0	2,917		384	

* The first assay was made at the end of a 7-day control period, the second at the end of a 7-day period of ammonium chloride administration, 0.12 mEq daily.

† Carbonic anhydrase activity was measured at 0° C.

‡ Lactate dehydrogenase activity was measured at 37° C.

§ Malate dehydrogenase activity was measured at 25° C; the values given have been converted to activity at 37° C with the experimentally determined conversion factor, 2.30.

per minute), and there was no significant difference in activity in proximal and distal convolutions (mean, 73.8 and 84.7 μmoles per g per minute, respectively). There was no significant or consistent change in GDH activity with ammonium chloride loading.

Aspartate amino transferase activity (Table II). AspAT activity was lowest in the glomeruli (mean, 70.2 μmoles per g per minute) and was higher in the distal (mean, 372 μmoles per g per minute) than in the proximal convolutions (mean, 199 μmoles per g per minute). This difference was statistically significant ($F_{24,30} = 3.77$, $p < 0.001$). There was no significant difference in AspAT activity in the first and second biopsies of the control dog. With ammonium chloride loading AspAT activity increased significantly in glomeruli ($F_{1,20} = 7.79$, $p < 0.01$), proximal ($F_{1,50} = 24.85$, $p < 0.001$) and distal ($F_{1,47} = 8.49$, $p < 0.01$) convolutions, and medullary rays ($F_{1,15} = 15.17$, $p < 0.01$).

Alanine aminotransferase activity (Table II). The mean AlAT activity in the glomeruli was 12.2 μmoles per g per minute. The activity was slightly but not significantly higher in proximal (mean, 52.2 μmoles per g per minute) than in distal convolutions (mean, 44.3 μmoles per g per minute). There was no significant change in AlAT activity with ammonium chloride administration.

Carbonic anhydrase activity (Table IV). The mean CA activity in the glomeruli was 106,500 μmoles per g per minute at the end of the control period. The mean CA activity in the proximal and distal convolutions was 365,000 and 354,700 μmoles per g per minute, respectively. The activity was higher in the proximal convolutions of three dogs, in the distal convolutions of the other two. With ammonium chloride loading there was no change in CA activity in the glomeruli or distal convolutions; in the proximal convolutions the mean activity increased by 134,600 μmoles per g per minute. This increase appears to be significant ($F_{1,125} = 17.68$, $p < 0.001$) but is interpreted with caution because the variance of the control and treatment data was different.

Lactate dehydrogenase activity (Table IV). As reported previously (16) LDH activity was lowest in the glomeruli; it was slightly but not significantly higher in the distal than in the proximal convolutions. LDH activity was studied in only two experimental dogs and did not change with ammonium chloride loading.

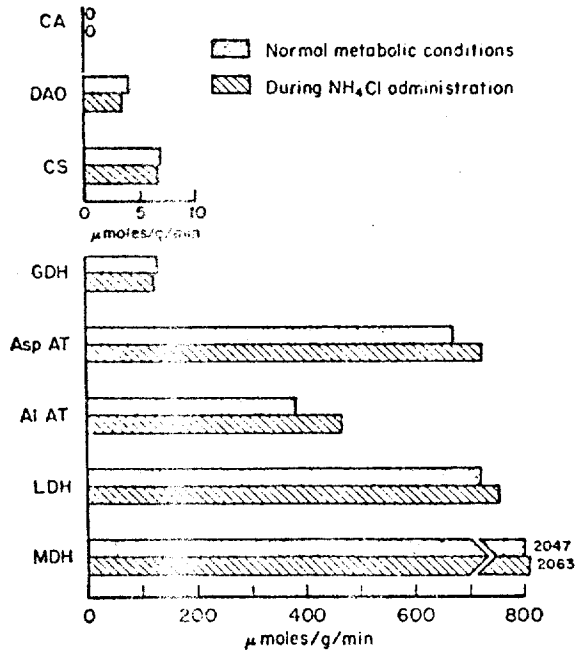

FIG. 3. ENZYME ACTIVITY IN HOMOGENATES OF BIOPSIES OF DOG LIVER BEFORE AND DURING THE DAILY ADMINISTRATION OF 112 mEq OF AMMONIUM CHLORIDE. The activity is expressed in micromoles of substrate split per gram dry weight of tissue per minute at 37° C. CA = carbonic anhydrase; DAO = D-alanine oxidase; CS = citrate synthase; GDH, LDH, and MDH = glutamate, lactate, and malate dehydrogenase; AspAT and AlAT = aspartate and alanine aminotransferase.

Malate dehydrogenase activity (Table IV). The mean MDH activity in glomeruli was 979 μmoles per g per minute. The MDH activity was significantly higher ($F_{2,25} = 4.49$, $p < 0.001$) in the distal (mean 4,600 μmoles per g per minute) than in the proximal convolutions (mean, 3,154 μmoles per g per minute). No change in MDH activity occurred with ammonium chloride loading.

Enzyme activity in the liver

Glutaminase activity was not studied in liver homogenates, as the optimal conditions for assay in the dog liver differed from those for the kidney and had not been clearly defined when the experiment was done. The results of the assays for the activity of the other eight enzymes studied are summarized in Figure 3. No carbonic anhydrase activity could be detected in the liver. Alanine

amino transferase activity increased slightly with ammonium chloride administration, but the difference was not significant ($F_{1,3} = 6.74$; $0.10 > p > 0.05$). Aspartate amino transferase activity also increased slightly ($F_{1,39} = 7.15$, $p < 0.02$), but in contrast with the findings in the kidney, the difference was very small and is probably not significant biologically. There was no change in activity of any of the other enzymes studied.

Discussion

The dogs studied in this experiment excreted an average of 20 mmoles of NH_3 per day (i.e., 13.9 μmoles per minute) under normal metabolic conditions; the excretion increased to 78 mmoles per day (i.e., 54.2 μmoles per minute) in metabolic acidosis. The amount of NH_3 added to the renal venous blood was not measured but was assumed to be 12 to 18 μmoles per minute in metabolic acidosis (2, 4). Thus, the total NH_3 production by the kidney in acidosis was about 70 μmoles per minute. In the discussion that follows we attempt to explain on a quantitative basis the interrelationships between enzyme activity and NH_3 production under both normal conditions and acidosis.

The role of glutaminase in ammonia production. In 1943 Van Slyke and his colleagues (2) showed that about 60% of the NH_3 excreted by the kidneys of acidotic dogs could be accounted for by extraction of glutamine from the renal arterial blood. This finding has recently been confirmed by Pitts and his co-workers in the dog (3, 4) and by Owen and Robinson in man (22). In the acidotic rat, in which glutamine extraction has not been determined, Davies and Yudkin (9) and Rector, Seldin, and Copenhaver (10) found that glutaminase activity increased *pari passu* with the increase in ammonia excretion. On the other hand Rector and Orloff (12) have demonstrated that glutaminase activity in the kidney of the acidotic dog was the same as that in the alkalotic animal. Although they did not study glutaminase activity in the normal dog, they concluded that phosphate- and pyruvate-activated glutaminase activity did not increase in metabolic acidosis in the dog. This conclusion was not completely justified, because it is not known whether glutaminase activity increases in metabolic alkalosis in the dog. In the guinea pig, the only animal so studied, glutaminase activity is increased in both alkalosis and acidosis (11). The fourfold increase in NH_3 excretion in metabolic acidosis in the present experiments in the dog was not accompanied by any change in phosphate- and pyruvate-activated glutaminase activity.

After the optimal conditions for the *in vitro* assay had been finally determined in our laboratory, glutaminase I activity was found to be considerably higher than that reported previously (18). The optimal values for glutaminase activity are used in making the calculations which follow and which attempt to correlate the measured excretion and production of NH_3 by the kidney with the measured glutaminase activity.

In vitro conditions differ considerably from *in vivo* intracellular conditions. Consequently, enzyme activity measured *in vitro* is unlikely to be the same as the *in vivo* activity. For no enzyme system are all the intracellular conditions known. In the case of glutaminase the concentration of substrate and cofactors in the kidney has been described. Thus, it is possible to attempt to calculate the activity *in vivo*. In making all such calculations we are fully aware that unknown factors could influence derived data on *in vivo* activity. The optimal glutamine concentration for the glutaminase I assay *in vitro* was 48 mM. This is much higher than the glutamine concentration of 1.38 μmoles per g wet weight in the dog kidney cortex in acidosis (3). As the water content of dog kidney cortex is about 77%, the glutamine concentration is about 1.79 mmoles per L. Glutaminase I activity, determined *in vitro* at the *in vivo* glutamine concentration, was about 7.5% of the activity measured with the optimal *in vitro* glutamine concentration (18). The inorganic phosphate concentration, optimal *in vitro* at 0.2 M, is probably about 0.01 to 0.02 M within the cell (23). The glutaminase I activity measured with 0.02 M phosphate was 36% of that measured with 0.2 M phosphate (18). Correcting for *in vivo* glutamine and phosphate concentrations would reduce the activity to 2.75% of the optimal *in vitro* activity. The pH optimum *in vitro* was 9.0, considerably higher than the cellular pH of about 7.1 (24), although the pH at the intracellular site of action of glutaminase is unknown. At this pH glutaminase activity *in vitro* was about 40% of the activity at pH 9.0 (18). Thus

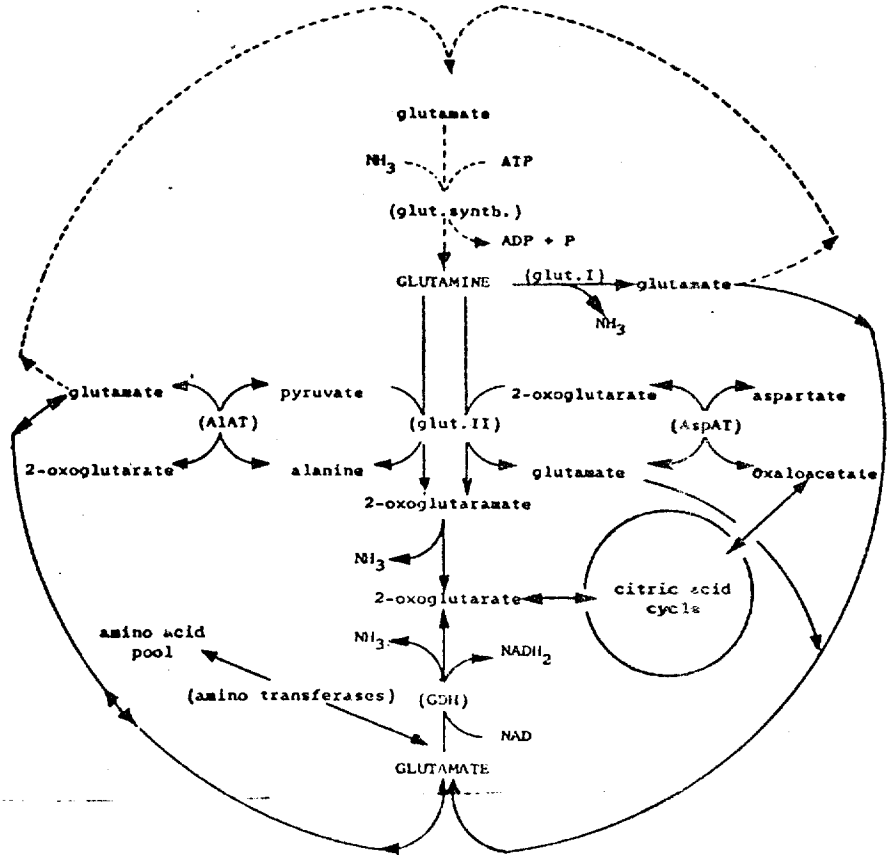

FIG. 4. INTERRELATIONSHIP OF THE REACTIONS INVOLVED IN AMMONIA METABOLISM IN THE KIDNEY. See text for detailed discussion. As there is doubt about the glutamine synthase activity in the dog kidney, the reactions involved are represented by a dotted line.

when we correct for glutamine and phosphate concentrations and for pH, the *in vivo* glutaminase activity is 1.1% of the measured *in vitro* activity of 205 μmoles per g per minute in the cortex and 90 μmoles per g per minute in the medulla. If these derivations are valid, the phosphate-activated glutaminase of the kidney cortex would split 39.2 μmoles of glutamine per minute, freeing an equimolar amount of NH_3;[2] the glutaminase I of medulla and papilla would split only 3.3 μmoles of glutamine per minute. The pyruvate-activated glutaminase of cortex, medulla, and papilla would split 4.4 μmoles per minute under *in vivo* conditions. Thus, the total glutaminase activity of the two dog kidneys would be 46.9 μmoles per minute. In the nonacidotic dogs this is 3.3 times the amount of NH_3 excreted; in the acidotic dogs it is about 87% of the amount excreted and about 66% of the amount produced.

If these derivations are valid, the glutaminase activity present in the normal dog kidney has the capacity to split from glutamine the increased amount of NH_3 produced in metabolic acidosis without an increase in enzyme activity.[3] This may explain the prompt rise in NH_3 excretion in acidotic dogs to maximal levels by the second day of ammonium chloride administration (Figure 1). Why, then, does enzyme adaptation occur in the

[2] Assuming that the two kidneys weigh 98 g (25); the dry weight of the kidneys was found to be 23%.

[3] Under the present experimental conditions the possibility has not been excluded that the increment in NH_3 production could be very much greater when larger ammonium chloride loads are given. On two occasions we attempted to give a larger load of ammonium chloride, but the dogs vomited. Thus it was not possible to determine whether a much greater increment in NH_3 production would be accompanied by an adaptive increase in glutaminase activity.

rat? In the nonacidotic rat the derived *in vivo* glutaminase activity of cortex, medulla, and papilla has the capacity to split from glutamine only the same amount of NH_3 as is excreted. Thus an increase in enzyme activity would be necessary to split the increased amount of NH_3 excreted in metabolic acidosis. In the acidotic rat NH_3 excretion increases slowly over a period of days (10), and this increase is accompanied by a parallel and progressive increase in glutaminase activity (10, 12).

The role of glutamate dehydrogenase in NH_3 production. Ammonia excretion in acidotic dogs increases when certain amino acids other than glutamine are infused, particularly L-asparagine, L- and D-alanine, L-histidine, L-leucine, and glycine (5–8). However, L-amino acid oxidases have been reported to be virtually absent from dog kidney (26). We have confirmed this observation and have found in preliminary studies that asparaginase activity is extremely low. D-Alanine oxidase activity has been reported in kidney tissues by Krebs (27), but we found that the activity was low (8.49 μmoles per g per minute) and did not change during acidosis. The contribution of amino acids other than glutamine to NH_3 production by the acidotic kidney is about 30 to 40% (2, 4). Since L-amino acid oxidases are apparently absent, the production of NH_3 is likely to occur as a result of reactions through the amino transferase-glutamate dehydrogenase system, which is also linked with the α-ketoacid-activated glutaminase (glutaminase II). These interrelationships are outlined in Figure 4.

Glutamine is desaminated to glutamate by glutaminase I. The glutamate formed can be desaminated by GDH. Glutamine is also desaminated and desamidated by glutaminase II via 2-oxoglutaramate. The cofactor is pyruvate, or 2-oxoglutarate or another 2-oxo acid. In Figure 4 two possibilities are considered: *1*) With pyruvate as cofactor, alanine, 2-oxoglutarate, and NH_3 are the reaction products; pyruvate can be regenerated in the ALAT reaction, giving rise to glutamate. *2*) With 2-oxoglutarate as cofactor, glutamate, 2-oxoglutarate, and NH_3 are the reaction products; 2-oxoglutarate can feed back into the glutaminase II reaction. Glutamate is the most abundant amino acid in kidney tissue, constituting on a molar basis 39 to 49% of the free amino acid content of the kidney cortex of the dog (3). Although glutamate is added to the venous blood of acidotic dogs, the amount added is small and is equivalent on a molar basis to only 4% of the glutamine extracted (3). This suggests that the glutamate generated in the several reactions outlined in Figure 4 is metabolized to 2-oxoglutarate and NH_3 in the GDH reaction.

The amount of glutamate contributed by the amino acid pool through other amino acid amino transferase reactions is unknown. The GDH activity *in vitro* was about 75 μmoles per g per minute in the kidney cortex. For technical reasons this was measured *in vitro* at pH 7.4 with 2.5 mM 2-oxoglutarate as the substrate. *In vivo* NH_3 is split from glutamate in the reaction, glutamate + NAD → 2-oxoglutarate + NADH + NH_3. Experimentally we determine that the rate of desamination of glutamate at pH 7.1 and at the *in vivo* concentration of 14.6 mM (3) was 2% of the rate of the reverse reaction measured under the *in vitro* optimal conditions. The derived *in vivo* GDH activity in the kidney cortex was 1.50 μmoles per g per minute or 25 μmoles per two kidney cortices per minute. Making similar assumptions the GDH of medulla and papilla would split only 4.4 μmoles of glutamate per minute, giving rise to a total of 29.4 μmoles of NH_3 per two kidneys per minute. This is equivalent to 55% of the total NH_3 excretion in the acidotic dogs, and to 42% of the NH_3 production, and agrees closely with the conclusions of Van Slyke and Pitts, whose experimental evidence suggests that about 30 to 40% of the NH_3 production by the acidotic dog kidney is contributed to by amino acids other than glutamine. Glutamate could also be the substrate for the synthesis of glutamine by glutamine synthase, but we found, as have others (12, 21), little or no glutamine synthase activity in the dog kidney.

Canessa-Fischer and colleagues (8) infused L-alanine into the left renal artery of acidotic dogs; when the amino acid was infused at a rate of 300 μmoles per minute, they observed an increment of NH_3 excreted by the two kidneys of about 30 μmoles per minute. Since L-alanine oxidase is virtually absent from the dog kidney, it is probable that the amino group of L-alanine is transferred to 2-oxoglutarate by ALAT. The derived *in vivo* ALAT activity is about 490 μmoles per two kidneys per minute—sufficient to transfer the entire load

of NH_3. The increment of NH_3 excreted would be derived from the desamination of glutamate by GDH (Figure 4). The limiting factor in determining the increment of NH_3 excreted is therefore likely to be GDH. The GDH activity was calculated to be 29.4 μmoles per two kidneys per minute, a figure agreeing closely with the observed increment in NH_3 excretion in the infusion experiments. Pertinent to this is the observation that for all amino acids infused by Canessa-Fischer and associates, the increment in NH_3 excretion did not exceed this value (8).

The derived data in the previous discussion must of course be interpreted with caution. That the assumptions underlying these derivations may be substantially correct is suggested by the fact that the derived data satisfactorily explain in a quantitative manner the following: *1*) the production of ammonia from glutamine and from glutamate, *2*) the different responses of the dog and the rat to experimental metabolic acidosis, and *3*) the increment in NH_3 excretion during infusion of amino acids other than glutamine.

The influence of metabolic acidosis on the other enzymes studied. The derived *in vivo* AspAT activity was about 2,000 μmoles per two kidneys per minute and increased in metabolic acidosis. The reason for this increase is unexplained. The derived *in vivo* AlAT activity was about 490 μmoles per two kidneys per minute and did not change significantly in metabolic acidosis. Glutaminase and GDH are related directly to NH_3 production, whereas AspAT and AlAT participate only indirectly. These aminotransferases must be considered in many aspects of amino acid metabolism, and their activity cannot be related quantitatively only to NH_3 production.

The activity of two enzymes of the citric acid cycle, citrate synthase and malate dehydrogenase, did not increase in metabolic acidosis; nor was there any increase in LDH activity. These findings do not exclude the possibility that the activity of other enzymes of the cycle or of glycolysis may have changed in metabolic acidosis.

The role of carbonic anhydrase in the kidney. Although measured for technical reasons at 0° C, CA activity was very high throughout the nephron. At 37° C the CA activity would probably be five times greater (28). CA activity of a similar order of magnitude has been found in the kidney of man (29) and rat (30), and in erythrocytes (17), choroid plexus (31), and the salt gland of the herring gull (32). In the dog liver, by contrast, any activity present was less than the sensitivity of the method could detect. High CA activity occurs in those tissues concerned with H^+ excretion, HCO_3^- transport, or both, but the activity measured *in vitro* is very much higher than expected. Nothing is known of intracellular regulation of CA activity, and no derivations for *in vivo* activity can be made.

Summary

1. Studies were designed to investigate quantitatively the enzymatic basis for ammonia production and hydrogen ion excretion in healthy dogs under normal metabolic conditions and during ammonium chloride-induced metabolic acidosis.

2. During the administration of 112 mEq of ammonium chloride daily for 7 days, the urine pH fell from 6.85 to 5.47. The excretion of titratable acid increased from 3.6 to 17.4 mmoles per day, of ammonia from 20 to 78 mmoles per day, and of net hydrogen ion excretion from 16 to 93.5 mmoles per day.

3. Enzyme activities were measured in kidney and liver biopsies taken at the end of a 7-day control metabolic study and at the end of a 7-day period of ammonium chloride administration. In the kidney there was no change in the activity of glutaminase I and II, glutamate dehydrogenase, alanine amino transferase, D-alanine oxidase, citrate synthase, carbonic anhydrase, lactate dehydrogenase, or malate dehydrogenase; aspartate amino transferase activity increased throughout the nephron in metabolic acidosis. In the liver there was no change in the activity of any of these enzymes, save for a slight increase in aspartate amino transferase activity.

4. From the *in vitro* data the total *in vivo* glutaminase I and II and glutamate dehydrogenase activities of the two dog kidneys were derived. It was calculated that in metabolic acidosis the glutaminases of the two kidneys would have split 46.9 μmoles of glutamine per minute and that the glutamate dehydrogenase would have split 29.4 μmoles of glutamate per minute, in each case giving rise to equimolar amounts of ammonia. The ammonia production of the dogs was 70 μmoles per

minute. Thus sufficient glutaminase I and II and glutamate dehydrogenase activities appeared to be present to split all the ammonia produced in metabolic acidosis. About 60% of the ammonia would have derived from glutamine, the remainder from the other amino acids through the amino acid amino transferases and desamination of glutamate.

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CV. THE EFFECT OF DESICCATED THYROID, IRRADIATED ERGOSTEROL AND AMMONIUM CHLORIDE ON THE EXCRETION OF CALCIUM IN RATS.

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Parhon [1912] observed a marked loss of calcium in rabbits which received lethal doses of thyroid substance by mouth. Aub *et al.* [1929] reported that patients with hyperthyroidism showed an excretion of calcium much greater than that observed on similar diets in hyperparathyroidism or after the administration of acids; he pointed out that the high calcium excretion of hyperthyroidism was unusual in that it occurred chiefly by the intestinal route. Parhon *et al.* [1932] found that demineralisation of the skeleton occurred in dogs during thyroid feeding and was not increased by simultaneous administration of parathyroid extracts. Tibbetts *et al.* [1932] announced that the high faecal excretion of calcium in clinical hyperthyroidism was not influenced by administration of irradiated ergosterol; Watchorn [1930], Pugsley [1932] and others have found however that irradiated ergosterol tends generally to decrease the faecal and increase the urinary excretion of calcium. The present paper reports a study of the effect of desiccated thyroid on the calcium excretion of rats and the influence of irradiated ergosterol[1] thereon, while some experiments on the effect of ammonium chloride acidosis are included for comparison.

Methods.

The experiments were similar in general plan and in the analytical methods used to those described by Pugsley [1932] in a study of the effects of parathyroid extracts and of irradiated ergosterol on the calcium and phosphorus metabolism of rats. Adult male and female rats of Wistar strain, weighing approximately 240 g., were used. The diet, which contained 0·056 % calcium, was given as a constant weighed ration daily, and was not increased during thyroid treatment in spite of the greater appetite of the animals: the weight of unconsumed food was also recorded. Metabolic rates were found by determination of oxygen consumption in a closed-circuit apparatus, similar to that described by Benedict and Macleod [1929], save that the fall of the oxygen reservoir-spirometer is traced on a rotating smoked drum, the tracing serving also as a record of activity. The metabolic rates are expressed as the percentage increase in oxygen consumption over the oxygen consumption of the individual animals determined in the pre-experimental period.

The desiccated thyroid was supplied by Messrs Ingram and Bell, and was found to contain 0·141 % calcium; it was thoroughly mixed with the food each day, so that each rat received 2 grains daily if, as was almost invariably the case, the entire ration was consumed. Irradiated ergosterol (Mead, Johnson & Co.)

[1] The authors are indebted to Mead, Johnson & Co. for the ergosterol used in these experiments.

was suitably diluted with olive oil and given from a pipette in daily doses of 0·1 ml. Ammonium chloride was given daily at the time of feeding by stomach tube, subcutaneous injection at the dosage required having proved highly toxic.

Results.

The effect of administering desiccated thyroid (2 grains daily) is shown in Fig. 1, which is a composite graph representing the average of six similarly

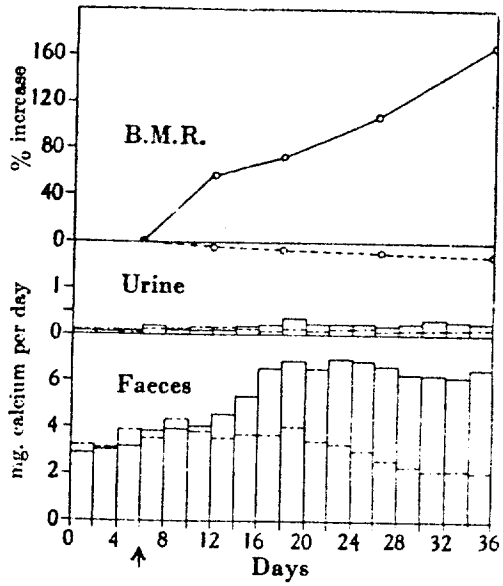

Fig. 1.

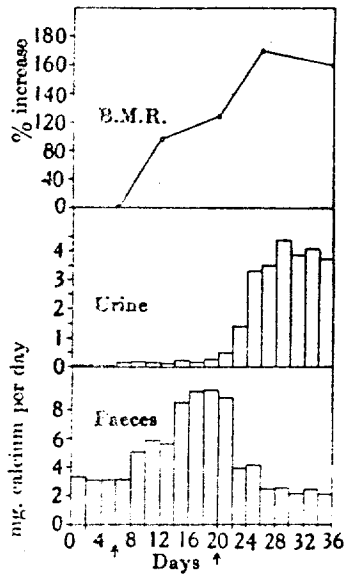

Fig. 2.

Fig. 1. Effect of administering 2 grains desiccated thyroid daily on calcium excretion (average of 6 rats); the dotted line shows the calcium excretion of controls.

Fig. 2. Effect of administering 20,000 units of irradiated ergosterol daily upon the increased calcium excretion induced by thyroid feeding (average of 6 rats). The first arrow indicates the commencement of thyroid treatment, the second the addition of irradiated ergosterol.

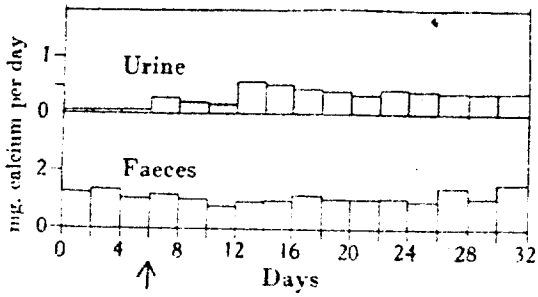

Fig. 3. Effect of administering 125 and (later) 250 [illegible] calcium excretion (average of 6 rats).

treated animals. The rats weighed approximately 250 g. at the beginning of the experiment and lost about 70 g. under thyroid treatment. Thyroid medication began on the sixth day, as indicated by the arrow. The dotted lines in the

figures represent the average behaviour of four untreated control rats, in a simultaneous study.

The administration of thyroid soon leads to a marked increase in the faecal excretion of calcium, which becomes approximately constant at the new level, while the basal metabolic rate (B.M.R.) increases progressively. There is also a decided increase in the urinary excretion of calcium (shown, for clearness, on a larger vertical scale) and the ratio between urinary calcium and faecal calcium, initially very small, does not appear to be greatly altered under thyroid treatment.

Fig. 2 represents another experiment on a series of six rats; thyroid treatment was begun as before on the sixth day, but from the twentieth day (second arrow) the animals also received 20,000 international units of irradiated ergosterol daily by mouth. This dosage has been found in similar experiments on normal rats [Pugsley, 1932] to produce a very marked rise in the urinary calcium excretion with a corresponding decrease in the faecal excretion and without any very pronounced effect on the total calcium balance or the general well-being of the animals. It is clear from the figure that in the present experiments the irradiated ergosterol has again largely diverted the calcium excretion from the faeces to the urine, the total excretion of this element (on practically constant intake) being decreased rather than increased, though still far above the levels observed before thyroid feeding.

Smaller doses of irradiated ergosterol have proved to be less effective. Table I summarises an experiment similar in plan to the last, carried out on another series of six rats. The usual effect of thyroid administration was

Table I. *Effect of administration of 1000 and 10,000 units of irradiated ergosterol daily on the increased calcium excretion induced by thyroid feeding.*

Average of four rats.

Treatment	Day	Urine-Ca mg. day	Faecal Ca mg. day	Ca balance mg. day	Body wt. g.	% increase in O_2 consumption
Control period	2	0·04	4·93	−2·33	224	—
	4	0·06	4·80	−2·76	229	—
	6	0·05	4·59	−2·71	230	0
2 g. desiccated thyroid daily	8	0·04	4·57	−2·63	228	—
	10	0·05	4·60	−1·51	—	—
	12	0·05	5·77	−1·13	221	—
	14	0·03	7·37	1·40	—	—
	16	0·11	9·25	−3·36	216	—
	18	0·15	10·50	−3·65	—	—
Added 1000 units irradiated ergosterol daily	20	0·11	11·50	−4·61	209	112
	22	0·37	11·50	−3·87	—	—
	24	0·50	12·10	−4·48	—	—
	26	0·57	11·47	−3·79	197	—
	28	0·71	13·67	−5·98	—	—
	30	0·48	12·05	−4·13	188	—
	32	0·74	13·00	−5·34	—	—
10,000 units daily: thyroid continued	34	0·70	13·80	6·10	180	100
	36	1·22	10·90	−3·72	—	
	38	1·52	9·17	−2·29	168	—
	40	1·47	9·32	−2·39	—	—
	42	2·47	9·10	−3·17	172	—
	44	2·85	5·58	0·03	163	—
	46	3·70	4·60	−0·10	—	—
	48	4·24	4·46	−0·30	149	156
	50	3·77	5·62	0·99	—	—
	52	5·00	3·30	−0·10	—	—
	54	6·72	2·60	−0·92	142	—

observed. The administration of 1000 units of irradiated ergosterol daily, from the twentieth day, may have slightly increased the urinary excretion of calcium but had no discernible effect on the faecal or the total excretions. On the thirty-fourth day the dosage was therefore increased to 10,000 units daily; the table shows that this led in the course of a few days to a definite shift of excretion from faeces to urine, with an unmistakable decline in the total calcium excretion, the negative calcium balance being greatly reduced.

Table II summarises another similar experiment on a series of six rats, in which the dosage selected was 2000 units of irradiated ergosterol daily, from the

Table II. *Effect of administration of 2000 units of irradiated ergosterol daily on the increased calcium excretion induced by thyroid feeding.*

Average of four rats.

Treatment	Day	Urine-Ca mg. day	Faecal Ca mg. day	Ca balance mg./day	Body wt. g.	% increase in O_2 consumption
Control period	2	0·04	4·36	+2·60	231	—
	4	0·05	4·40	+3·47	236	—
	6	0·03	3·77	+3·76	238	0
2 g. desiccated thyroid daily	8	0·06	3·30	+3·64	236	—
	10	0·05	3·00	+3·35	—	—
	12	0·04	4·57	+2·11	231	—
	14	0·02	7·20	−1·64	—	—
	16	0·05	7·30	−1·19	223	—
	18	0·07	8·87	−0·82	—	—
Added 2000 units irradiated ergosterol daily	20	0·02	10·25	−2·21	216	79
	22	0·11	10·12	−1·83	—	—
	24	0·13	9·12	−1·01	—	—
	26	0·22	10·30	−2·12	210	—
	28	0·47	10·48	−2·55	—	—
	30	0·54	10·50	−2·65	207	—
	32	0·48	10·51	−2·75	—	—
	34	0·54	10·77	−2·91	206	83
	36	0·67	10·00	−2·27	—	—
	38	0·38	10·00	−1·98	196	—
	40	0·66	10·00	−2·26	—	—
	42	0·24	10·00	−1·84	188	—
	44	0·40	10·90	−2·90	—	—
	46	0·48	10·80	−2·80	165	—
	48	0·45	11·20	−3·25	—	200

twentieth day. Such doses have been found to reduce the faecal excretion of calcium, in normal animals, very decidedly [Pugsley, 1932]; but in the present experiments, although the faecal excretion and negative balance are somewhat lower and the urinary excretion definitely higher than might have been expected in view of the earlier results, there is not convincing evidence that this dose was in any degree effective.

From a series of 12 rats receiving 2 grains of desiccated thyroid daily, blood was collected by the method previously described [Pugsley, 1932] for determination of the serum-calcium by the method of Clark and Collip [1925]. The values obtained fell within the normal range for rats in this colony, the average being 10·3 mg. per 100 ml. (minimum 10·1, maximum 10·4). This agrees with the general opinion [Thomson and Collip, 1932] that there is no simple relation between thyroid function and the level of the serum-calcium.

The effect of ammonium chloride on calcium excretion was studied in six rats, and the results are summarised in Fig. 3. 1 ml. of a 12·5 % solution of the salt was administered daily by stomach tube from the sixth day; as this had little effect, the dose was increased to 2 ml. from the fourteenth day onward.

A marked diuresis naturally resulted, and the excretion of calcium in the urine was considerably increased, the faecal excretion being scarcely affected. This observation confirms the view of Aub *et al.* [1929] that the thyroid hormone does not affect calcium metabolism merely by producing an acidosis.

Discussion.

The work of Aub *et al.* [1929] on the effect of the thyroid hormone on calcium excretion has, curiously enough, stimulated little experimental study. We have entirely confirmed his view that the thyroid hormone stimulates calcium excretion and promotes loss of calcium from the body, and that the channel of excretion is the intestine rather than the kidney (a matter in which this effect differs strikingly from the increased calcium excretion produced by parathyroid hormone, by ammonium chloride or by very large doses of irradiated ergosterol). It seems at present futile to speculate on the factors responsible for directing calcium excretion to one channel rather than another, but it should be emphasised that in our experiments with thyroid feeding the amount of calcium lost in the faeces was often considerably greater than the total calcium intake. Our work also supports the contention of Aub that the action of the thyroid hormone is not to produce acidosis or parathyroid stimulation, and moreover does not seem to be directly correlated with the increase in basal metabolic rate. In experiments not reported herein, we have found that the injection of parathyroid extract into rats receiving desiccated thyroid increases the total loss of calcium from the body. The failure of Tibbetts *et al.* [1932] to observe a reduction in faecal calcium excretion and negative calcium balance, as we did, after administration of vitamin D, may perhaps be ascribed to inadequate dosage; Taylor and Weld [1932] obtained results similar to ours in dogs.

Summary.

The administration of 2 grains of desiccated thyroid daily, by mouth, to adult rats, leads to a marked increase in calcium excretion and establishment of a negative calcium balance. The increase is observed in the faecal rather than in the urinary calcium. The serum-calcium is not elevated.

The administration of irradiated ergosterol, in doses of 10,000 international units or more daily by mouth, to rats receiving desiccated thyroid reduces the faecal and increases the urinary excretion of calcium, and partly or wholly corrects the negative calcium balance. Smaller doses are comparatively ineffective.

The administration of 250 mg. ammonium chloride daily, by mouth, to normal adult rats leads to a marked increase in the urinary excretion of calcium, with little influence upon the faecal excretion.

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MECHANISM OF THE KETOGENIC EFFECT OF AMMONIUM CHLORIDE

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When ammonium chloride is added to liver mince (1) or to rat liver slices (2), there is an increase in ketone body production. In the absence of other possibilities, it was earlier suggested (3, 4) that the ketone bodies arising on addition of ammonium chloride were coming from acetate formed during the "acetylation amination" of α-keto acids. According to this view, amination of α-keto acids is an oxidative process involving condensation with ammonia and pyruvate to yield carbon dioxide and the corresponding *N*-acetylamino acid, which, on hydrolysis, would then yield the corresponding α-amino acid and acetate, the latter condensing to form acetoacetate. However, it has not been possible to bring experimental evidence to bear on the formation of ketone bodies through such a mechanism.

More recently, a knowledge of the reductive amination of α-ketoglutaric acid to glutamic acid (5–7) has led to the concept of ammonia fixation by reductive amination, followed by transamination (8–10). In addition, experiments with liver homogenate by Krebs *et al.* (11), and unpublished experiments[1] from this laboratory, indicated that the primary locus of action of added ammonia, even under aerobic conditions, was in the reductive amination of α-ketoglutaric acid. Meanwhile, the experiments of Lehninger (12) had clearly shown that ketone body precursors (pyruvate, octanoate) would form acetoacetate in liver in the absence of oxalacetate, and that degradation via the Krebs citric acid cycle was the preferred pathway provided oxalacetate was available. These considerations suggested that the ketosis induced by ammonium chloride might be due fundamentally to the absence of oxalacetate, a condition brought about by the preferential removal of α-ketoglutarate, in the presence of added ammonia, to glutamic acid. The experimental data presented in this paper support such a mechanism for the ketosis induced by ammonium chloride.

* National Research Council Fellow in Zoology, 1949–50.

[1] Ackermann, W. W., and Potter, V. R., unpublished.

EXPERIMENTAL

General—The conventional Warburg apparatus was used. Well fed male rats,[2] 3 to 6 months old, weighing between 200 and 230 gm., were decapitated, bled, and the livers excised and chilled to 0° before further treatment. Liver slices were cut in the cold to a thickness of 0.5 mm. in an apparatus essentially that of Stadie and Riggs (13), and suspended in Medium A1 of Robinson (14), to which the appropriate substrates were added. Glucose was omitted. Oxygen was used in the gas phase; the temperature was 38°. One slice was used per flask. Final dry weights varied between 10 and 20 mg. Data from the liver slice experiments are expressed as Q values; *i.e.*, microliters per mg. of dry weight per hour. 1 μM of any substance is taken as equivalent to 22.4 μl.

Homogenates were prepared in the Potter and Elvehjem homogenizer (15) at concentrations of 10 to 20 per cent, in an ice-cold medium of the following composition: 5.75 gm. of KCl, 16 mg. of $KHCO_3$, 494 ml. of H_2O, pH adjusted to 7.6. The ice-cold homogenates were pipetted directly into the iced Warburg flasks containing all the necessary additions, and equilibrated for 10 minutes at 38° before oxygen uptake measurements were made.

The homogenate system employed was essentially the "oxalacetic acid oxidase" system of Potter *et al.* (16). The main constituents were not varied and had the following final concentrations: 0.067 M KCl (not including the KCl of the homogenate), 3.3×10^{-3} M $MgCl_2$, 6.7×10^{-3} M potassium phosphate buffer of pH 7.4, 1×10^{-3} M adenosinetriphosphate (K salt). The final pH was 7.2; the final volume was 3.0 ml. Other additions, such as cytochrome *c*, diphosphopyridine nucleotide (DPN), and nicotinamide, are indicated in connection with the pertinent data.

Pure adenosinetriphosphate and 80 per cent pure DPN were obtained from Dr. G. A. LePage. Cytochrome *c* was prepared according to the method of Keilin and Hartree (17), as described by Potter ((18) p. 211). The oxalacetic and α-ketoglutaric acids used were supplied through the courtesy of C. Heidelberger and W. W. Ackermann, respectively.

Analytical Methods

Pyruvic acid disappearance was followed by the direct method for total keto acids (19). Although α-ketoglutaric and oxalacetic acids react under these conditions, it was shown by chromatography (20) that the level of α-ketoglutaric acid did not exceed 0.2 μM per flask, while the level of oxalacetic acid was not detectable. Acetoacetic acid interferes to the extent of 10 per cent in the direct method, but the maximum amount present

[2] Holtzman-Rolfsmeyer Company, Madison, Wisconsin.

was always small enough to make corrections of little significance. *β-Hydroxybutyric acid* was determined by the method of Greenberg and Lester (21) after boiling to remove acetoacetic acid and after treating with copper lime (22) and then with $Ca(OH)_2$ alone to remove citric acid. *Acetoacetic acid* was determined by Lehninger's modification[3] of the method of Greenberg and Lester (21). Protein-free filtrates containing acetoacetic acid equivalent to 5 to 30 γ of acetone were brought to pH 5.0 in a volume of 1.5 ml. Molar acetate buffer (pH 5.0, 0.5 ml.) and 4 M aniline hydrochloride (2.0 ml.) were added, and the mixture was incubated in glass-stoppered test-tubes for 90 minutes at 30°. The acetone 2,4-dinitrophenylhydrazone was then formed (21) and extracted into 5.0 ml. of CCl_4 with prolonged (50 minutes) shaking. The CCl_4 layer was washed twice with H_2O and once with 0.5 N NaOH to remove any excess reagent and pyruvic acid 2,4-dinitrophenylhydrazone, and read at 420 mμ. β-Hydroxybutyric acid is completely inert in the procedure, and pyruvic acid does not interfere. When this method was tested with acetoacetic acid prepared according to Ljunggren (23), the results agreed with those obtained by the use of the aniline citrate method ((2), (18) p. 175) within ±3 per cent.

Glutamic acid was measured according to the manometric method of Gale (24). The glutamic decarboxylase of *Clostridium welchii* SR12 was a gift from Dr. P. P. Cohen. This enzyme has some activity against aspartic acid (10). In some experiments the specific glutamic decarboxylase of *Escherichia coli* (American Type Culture Collection strain 4157) was used. The enzyme was prepared according to Umbreit and Gunsalus (25) with slight modifications. At pH 5.0 and 38°, the enzyme had a Q_{CO_2} (glutamic acid) of 60 and was completely inactive against L-aspartic acid and DL-alanine. *Lactic acid* was measured according to the method of Barker and Summerson (22), as described by LePage ((18) p. 192), *citric acid* by the method of Natelson *et al.* (26), as employed by Potter *et al.* (16). Flask contents were deproteinized by addition of 0.3 ml. of 70 per cent perchloric acid. If glutamic acid was to be measured, 0.5 ml. of 2.00 N HCl was added; the pH was then adjusted to 5.0, and the precipitated protein was removed by centrifugation.

Results

Liver Slices—Four experiments with liver slices from fed rats were in close agreement and completely confirmed the ketogenic effect of ammonium chloride as described by Edson (2). In the presence of 20 μM of pyruvate, the Q_{AcAc} was 0.73 without added ammonium chloride. When

[3] We are indebted to Dr. A. L. Lehninger for giving us unpublished details, made available here through his permission.

ammonium chloride was added to a final concentration of 0.04 M, acetoacetate production was increased almost 4-fold (Q_{AcAc} = 2.67). The endogenous production of acetoacetate was low (Q_{AcAc} = 0.51) and rose to Q_{AcAc} = 0.78 on addition of ammonium chloride. With pyruvate as substrate, β-hydroxybutyric acid production was increased from $Q_{\beta\text{-OH}}$ = 0.18 without added NH_3 to 0.26 when ammonium chloride was added. No endogenous β-hydroxybutyric acid was found. Addition of ammonium

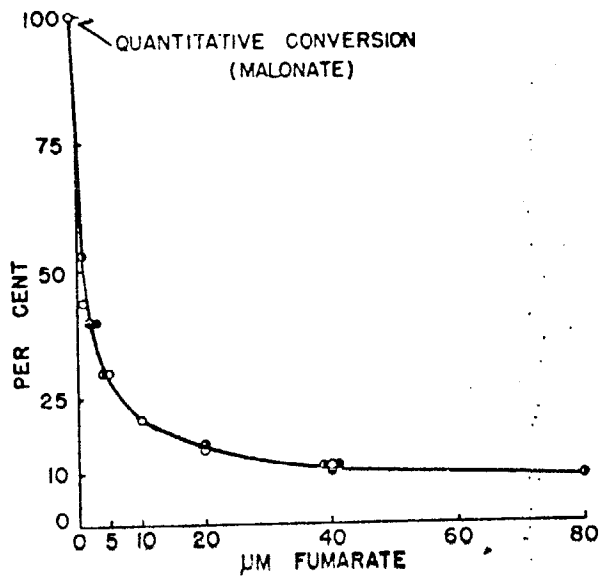

FIG. 1. Acetoacetate production from pyruvate as per cent of pyruvate disappearance. Conditions, per flask, 80 mg. of whole rat liver homogenate, 20 μM of pyruvate, and either 12 μM of malonate or fumarate in the quantities indicated. 30 minutes incubation. Data from different rats are represented by the respective symbols.

chloride lowered oxygen consumption and lactic acid production about 25 per cent; pyruvic acid disappearance was depressed about 10 per cent.

Liver Homogenates. Malonate Effect—Lehninger (12), using washed residues of rat liver homogenates, showed that pyruvic acid has two major degradative pathways open to it in the liver, one leading to acetoacetate, the other to citrate. If adequate levels of the dicarboxylic (C_4) acids of the Krebs citric acid cycle are present, pyruvate is *preferentially* removed via citrate and the citric acid cycle. If recycling of the C_4 acids is prevented by addition of malonate, conversion of pyruvate to acetoacetate is accelerated, since the pathway to citrate is no longer available. It will be shown that addition of ammonium chloride to the rat liver homogenate results in a similar increase in the production of acetoacetate from pyruvate.

We first established the properties of the whole homogenate with respect to the oxidation of pyruvic acid, both in the presence and in the absence of a functioning Krebs citric acid cycle. The data of Fig. 1, compiled from a number of experiments, confirm Lehninger's basic observation concerning the alternative pathways open to pyruvate. Without added intermediates of the Krebs citric acid cycle, 75 per cent of added pyruvate is converted to acetoacetate. That this is due simply to the dilution of the intermediates of the Krebs cycle is evident from the marked depression in acetoacetic acid production resulting from addition of fumaric acid. Similar results were obtained on addition of oxalacetate, malate, succinate, α-ketoglutarate, and citrate. Even with the highest levels of added fumaric acid, only 1 μM of β-hydroxybutyric acid was found, indicating that the decrease in the yield of acetoacetic acid in the presence of fumarate was not due to extensive formation of β-hydroxybutyrate. When malonate was added, pyruvate was converted to acetoacetate quantitatively. These experiments show that in the whole homogenate, in the presence of adequate amounts of the C_4 acids, the pathway of pyruvate to citrate has a high priority over the pathway to acetoacetate.

Ammonia Effect—The ketosis resulting from addition of ammonium chloride to the whole homogenate is shown in Fig. 2. In experiments in which the reaction was followed at shorter time intervals, it was established that the linear rate of conversion of pyruvate to acetoacetate was maintained down to levels of pyruvate approaching 0.1 μM per flask or less. A maximum ketogenic effect can be obtained with as little as 9 μM of added ammonium chloride. The ketosis induced by ammonia is easily prevented by addition of an equivalent quantity of fumarate. Glutamate acts like α-ketoglutarate, and not like ammonia; *i.e.*, addition of glutamate depresses acetoacetic acid production from pyruvate. Occasionally, in experiments with added ammonium chloride, and rarely with added malonate, conversion of pyruvate to acetoacetate was less than quantitative (*cf.* Fig. 3). The reason for this finding is not clear, but is believed to be due to correspondingly high endogenous levels of the Krebs cycle intermediates convertible to oxalacetate under the respective experimental conditions.

Non-Utilization of Acetoacetate—The quantitative estimation of the extent of the conversion of pyruvic acid to acetoacetic acid in these homogenate systems and the effects of various additions on this conversion depend on the fact that acetoacetate, once formed, is not further metabolized, except for the very small amounts reduced to β-hydroxybutyric

acid under certain conditions. That significant quantities of acetoacetic acid were not metabolized, even in the presence of added fumarate, was shown by the following experiment. Acetoacetate production from pyruvate was followed in a rat liver homogenate system to which ammonium chloride had been added. In parallel flasks, fumarate was tipped in from the side arm after 20 and 40 minutes of incubation. The reactions were stopped at 20 minute intervals for the next 60 minutes, and the contents of the flasks were analyzed for pyruvic, acetoacetic, and β-hydroxybutyric

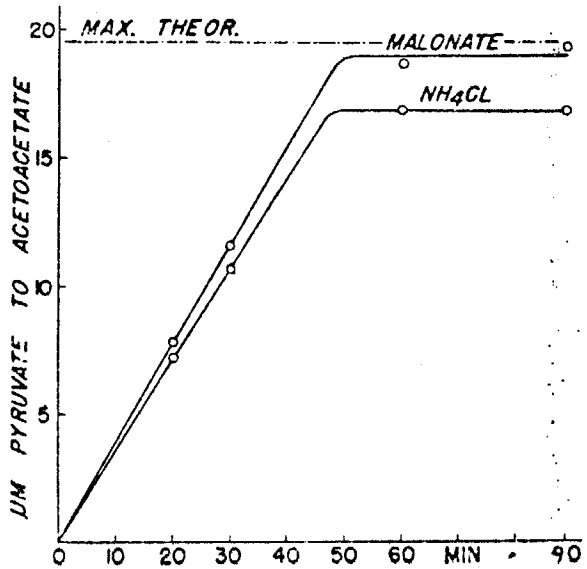

FIG. 2. Ketogenic effect of NH_4Cl. Conditions, per flask, 100 mg. of whole rat liver homogenate, 20 μM of pyruvate, and either 12 μM of malonate or 30 μM of NH_4Cl. The dash line corresponds to the maximum possible recovery of acetoacetic acid calculated on the basis of pyruvic acid disappearance.

acid. The data are shown in Fig. 3 and clearly indicate the abrupt cessation of acetoacetic acid production when fumarate was added to the system. The slight depression in the level of acetoacetic acid following addition of the fumaric acid was completely accounted for by reduction to β-hydroxybutyric acid, as demonstrated by analyses. This indicates that no condensation of acetoacetic acid with fumaric acid occurred in these liver homogenate systems. The conclusion that acetoacetic acid is not significantly metabolized by rat liver homogenates supports those of Lehninger (12) and of Buchanan *et al.* (27) to the effect that liver tissue does not utilize acetoacetic acid to any appreciable extent.

Mechanism of Ammonia Effect. Krebs, Eggleston, and Hems (11) demonstrated that the rat liver homogenate would synthesize glutamic

acid vigorously from certain precursors (citrate, α-ketoglutarate) in the presence of added ammonia. On the basis of this observation it seemed likely that the ketosis induced by ammonium chloride was due to the same general mechanism that caused the ketosis induced by malonate; *viz.*, the failure of the system to deliver adequate levels of oxalacetate for the Krebs condensation. It appeared that addition of ammonia resulted in an interruption of the Krebs citric acid cycle at the stage of α-ketoglutarate oxidation to succinate, and that in the presence of added

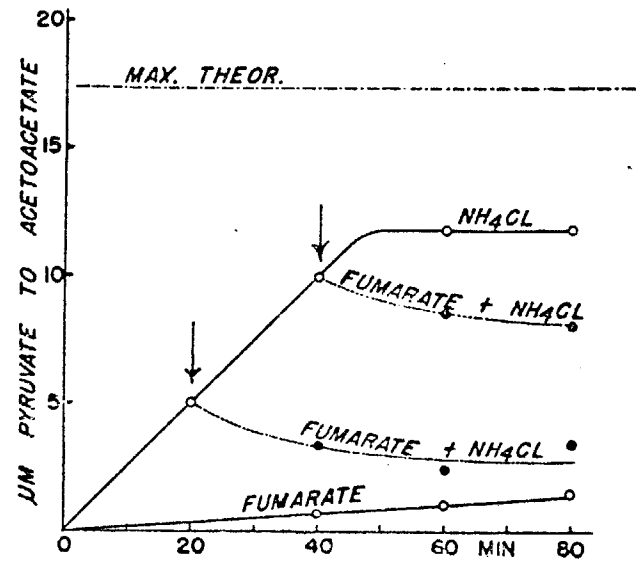

FIG. 3. Failure of rat liver homogenate to metabolize acetoacetate. Conditions, per flask, 80 mg. of whole rat liver homogenate, 20 μM of pyruvate. Initially, all flasks contained either 30 μM of NH_4Cl or 20 μM of fumarate. The arrows indicate addition from the side arm of 20 μM of fumarate to flasks already containing NH_4Cl. Dash line, same as in Fig. 2.

ammonia α-ketoglutarate was preferentially removed via reductive amination to glutamate. If α-ketoglutarate is preferentially converted to glutamate in the presence of added ammonia, then it could be expected that the catalytic effect of *small* amounts of added fumaric acid on pyruvate oxidation would soon be exhausted, as the recycling of the added fumaric acid was prevented. Failure of the ammonium chloride system to regenerate the C_4 acids of the Krebs cycle could in turn be expected to result in a switch in pyruvate oxidation to acetoacetate formation as the supply of added fumaric acid was used up. In the presence of ammonium chloride the homogenate system containing an excess of added pyruvate should therefore exhibit a *delay* in the onset of ketosis in proportion to the amount

of added fumaric acid. Furthermore, once initiated, the rate of the conversion of pyruvate to acetoacetate should be independent of the time of onset of the ketosis, and, finally, since recycling of the added fumarate is prevented, there should be a deficit in the final quantities of acetoacetic acid produced corresponding stoichiometrically to the amount of fumarate added. All of these expectations were repeatedly borne out by experiment. Typical data are shown in Fig. 4, *A* and 4, *B*, which present data for two different rats.

Recovery of Added Fumaric Acid—Net recoveries of glutamic acid from experiments on five different rats are shown in Table I. Addition of fumaric acid to the whole rat liver homogenate, in the presence of pyruvate and ammonium chloride, leads to a marked production of glutamic acid. No net increase in glutamic acid was observed if either ammonium chloride or fumaric acid was omitted from the system. If the theory advanced in this paper to explain the ketogenic effect of ammonium chloride is correct, the added fumaric acid should all be metabolized beyond the stage of oxalacetate at the time of onset of the ketosis, and should therefore be accountable as glutamic and citric acids at this time. In Fig. 5 are shown the results of analyses for these two acids for the experiment recorded in Fig. 4, *B*, in which the ketosis began after 30 minutes of incubation. The plot of the sum of citrate plus glutamate reveals that about 85 per cent of the added fumaric acid had already been metabolized past the level of oxalacetate after 40 minutes of incubation. The glutamic decarboxylase of *C. welchii* was used to measure production of glutamic acid. In a similar experiment in which the glutamic decarboxylase of *E. coli* was used for measurement of glutamic acid, the sum of citrate plus glutamate accounted for 60 per cent of added fumaric acid at 30 minutes of incubation, but a maximum recovery of only 75 per cent was reached on further incubation. The same early rapid production of citric acid was observed, followed by a fall in the citric acid and a rise in the glutamic acid concentration of the medium. These data support the hypothesis that, in the presence of added ammonium chloride, the onset of the ketosis is coincident with the reduction of the concentration of the C_4 acids to a point at which the condensation reaction can no longer occur.

Effect of CO_2—Formation of oxalacetate via fixation of CO_2 in the Wood-Werkman reaction (28) could be expected to result in a decrease in the conversion of pyruvate to acetoacetate. Data from an experiment designed to test this possibility are shown in Table II. Addition of ammonium chloride alone exerted a pronounced ketogenic effect. If 4 μM of fumarate were added in addition to ammonia, the deficit in the final recovery of acetoacetate corresponded exactly to 4 μM of pyruvate, indicating that, in the presence of added ammonia, fumarate and pyruvate

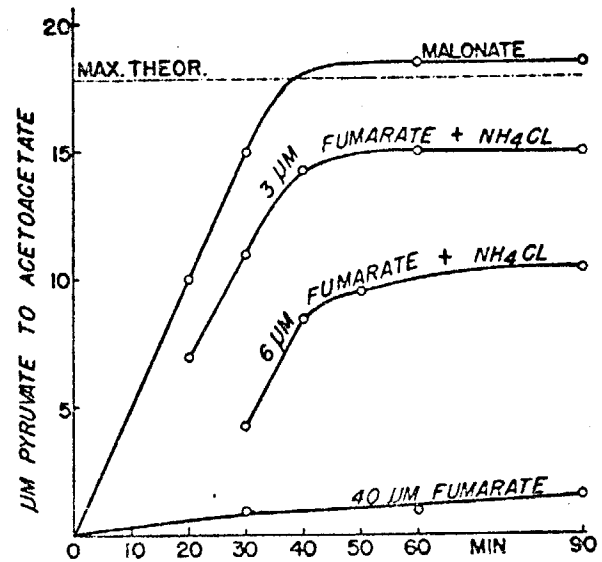

FIG. 4, *A*

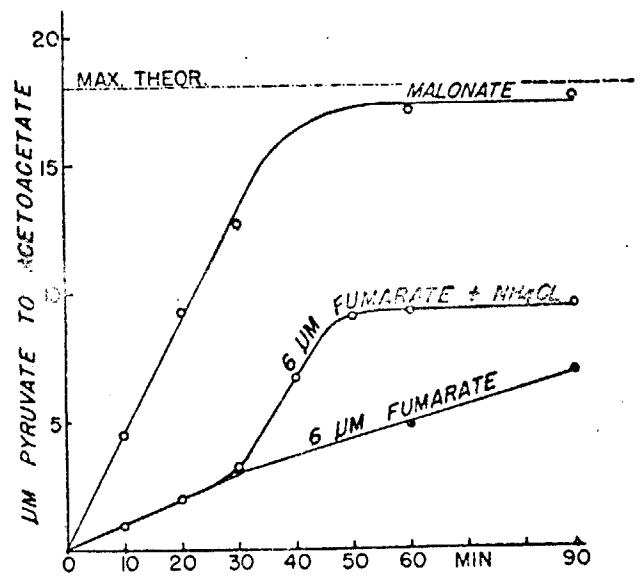

FIG. 4, *B*

FIG. 4. Delayed NH_4Cl ketosis in the presence of varying amounts of added fumaric acid. Conditions, per flask, 100 mg. of whole rat liver homogenate, 20 μM of pyruvate, and either 12 μM of malonate or 30 μM of NH_4Cl, with fumarate as indicated. Dash lines, same as in Fig. 2. *A* and *B* represent experiments on two different rats.

reacted stoichiometrically. Under conditions favoring fixation of CO_2, the deficit in the final production of acetoacetate was 2.2 μM of pyruvate, indicating that one-half of this amount, or 1.1 μM of pyruvate, had been

TABLE I

Production of Glutamic Acid by Rat Liver Homogenates

Pyruvate added	NH_4Cl added	Fumarate added	Glutamic acid recovered	Source of glutamic decarboxylase
μM	μM	μM	μM	
	30		None	*E. coli* 4157
20	30		"	" " 4157
30		10	"	" " 4157
30	30	10	5.7	" " 4157
20	30	6	4.3	" " 4157
20	30	6	4.8	" " 4157
20	30	6	5.5	*C. welchii* SR12
20	30	6	6.2	" " SR12

Conditions, 100 mg. of whole rat liver homogenate per flask in all cases except for the experiment reported in the last line, in which 150 mg. of liver were used.

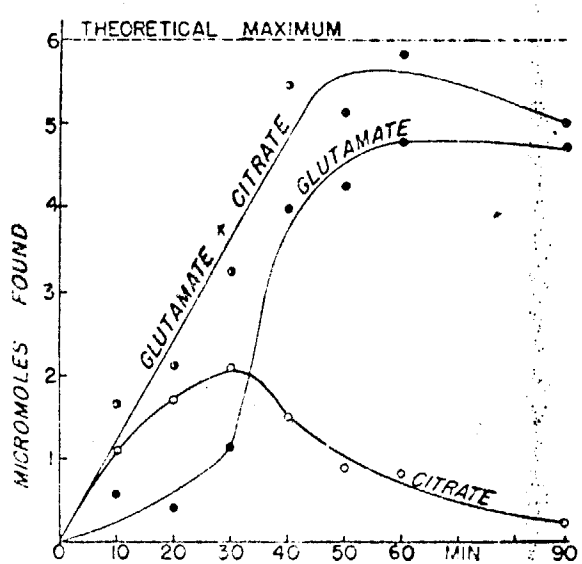

FIG. 5. Recovery of added fumaric acid (see the text)

fixed as oxalacetate. Assuming the dry weight as 30 per cent of the wet weight, the rate of fixation of CO_2 in terms of the production of oxalacetate was $Q_{oxalacetate}$ = 1.03. Stern (29) found no increase in the sum of succinate and α-ketoglutarate on addition of pyruvate to rat liver mince

treated with malonate. He emphasized that the failure of his method to detect fixation of CO_2 indicated only that the rate of the Wood-Werkman reaction in the mammalian liver was below $Q_{oxalacetate}$ = 1.0. Our experiments, in which conversion of pyruvate to acetoacetate was used as a more sensitive measure of the availability of oxalacetate, indicate that the rate of this reaction in rat liver is near the limit of detection in the experiments of Stern.

Effect of Cytochrome c and DPN—Addition of cytochrome *c* to a final concentration of 2.67×10^{-5} M had a slight stimulatory effect on the rate of O_2 consumption. It had no effect on the rate of pyruvic acid removal or acetoacetate production, either with added malonate or with added fumarate. However, as in the case of kidney (30), cytochrome *c* increased the maintenance of activity by 30 per cent, as indicated by the time to

TABLE II

Effect of CO_2 on NH_4Cl Ketosis

Additions	Gas	Pyruvate to acetoacetate	Deficit
		μM	μM
30 μM NH_4Cl	Air	16.6	
30 " " 45 μM $KHCO_3$	5% CO_2	14.4	2.2
30 " " 4 " fumarate	Air	12.6	4.0
4 " fumarate	"	3.8	12.8

Conditions, 80 mg. of whole rat liver and 20 μM of pyruvate per flask. Incubation time, 60 minutes. No alkali absorbers in flasks containing added bicarbonate-CO_2.

half the maximum rate of O_2 consumption. Addition of cytochrome *c* 2.67×10^{-5} M, DPN, 1200 γ, and nicotinamide, 30 μM, had little effect on the rate of conversion of pyruvate to acetoacetate in the malonate-treated system, although the rate of removal of pyruvate from the system was increased about 20 per cent. Analysis for lactic acid in the DPN-fortified system revealed that all the pyruvate disappearing from the system which could not be accounted for as acetoacetate was present as lactic acid. In the absence of added DPN, less than 0.5 μM of lactate was found.

Octanoate Oxidation—With 112 mg. of whole rat liver homogenate and 3 μM of added octanoic acid, and in the presence of 12 μM of malonate, we observed a vigorous O_2 uptake, and recovered acetoacetate in yields of 91 and 93 per cent of the theoretical 2 moles of acetoacetate per mole of octanoate utilized. With 18 and 80 mg. of tissue, the yields were 80 per cent. In the absence of malonate, and on addition of fumarate, we were able to depress the yield of acetoacetate down to, but not below, 1 mole of acetoacetate per mole of octanoate. Lehninger and Kennedy (31),

using washed residues from rat liver, obtained a much greater depression of octanoate conversion to acetoacetate in the malate-supplemented system (acetoacetate yield, only 13 per cent of the theoretical maximum). However, our experiments with octanoate oxidation confirm in general those of Lehninger (12) and of Lehninger and Kennedy (31), which indicate that availability of oxalacetate is the controlling mechanism in the disposition of ketone body precursors arising from oxidation of octanoic acid.

DISCUSSION

These data have certain implications regarding the control of metabolic pathways in the whole organism. In two separate instances, a substrate having two alternative metabolic pathways open to it was shown to proceed preferentially by one pathway when a second substrate was available and to proceed via another pathway when the second substrate was not available. In these experiments it was possible to arrange the conditions so that other potential reactions were minimized and the maximum shifts were obtained. It is recognized that the controlling mechanisms in the utilization of pyruvate in the liver of the intact animal will involve many controlling factors not operating in the reaction systems employed here. However, by limiting the reactions to two main alternatives, it became feasible to demonstrate the decisive rôle played by the delivery of the second substrate. The extent to which these mechanisms operate in the intact animal can only be shown by experiments designed to this end. Unpublished studies on rats injected with fluoroacetate have shown that the accumulation of citrate in liver *in vivo* can be greatly modified by altering the conditions of the experiment along lines suggested by the data reported above.

SUMMARY

The ketogenic effect of added ammonium chloride in the rat liver slice has been confirmed and the phenomenon has been extended to the rat liver homogenate, supplemented with adenosinetriphosphate, magnesium, potassium, and phosphate. Data are presented which indicate that the ketosis induced by ammonium chloride involves the following steps: (1) a primary vigorous reductive amination of α-ketoglutaric acid to form glutamic acid; (2) because of the interruption of the Krebs cycle at the stage of α-ketoglutarate oxidation to succinate, the system fails to supply levels of oxalacetate sufficient for the Krebs citric acid condensation; (3) with the preferential pathway to citric acid formation no longer available, the metabolism of ketone body precursors is shunted in the direction of acetoacetate formation.

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(Submitted for publication July 22, [illegible]; accepted September [illegible])

The response of urinary ammonia to strong acid loads has been repeatedly observed to follow a characteristic pattern (1–3). Initially, ammonia excretion increases to a slight extent, then slowly attains maximum intensity after three to four days, and is maintained at a rate which parallels the magnitude of the acid load. This enhanced rate of ammonia excretion has usually been ascribed to the accelerated transport of ammonia into the tubular urine as a result of a sharp fall in urine pH (4–8). Pitts, however, found that chronic acidosis resulted in the excretion of more ammonia at any given urine pH than did acute acidosis (5) and suggested that prolonged acidosis might stimulate certain adaptive changes in the ammonia-producing system. The subsequent demonstration by Davies and Yudkin (9) of increased activity of renal ammonia-producing enzymes in rats given acid loads strongly supports the thesis that the production of ammonia may also constitute a regulatory mechanism of ammonia excretion. Apparently, therefore, two processes control the excretion of ammonia: 1) The *production* of ammonia in the tubular cell by the action of various ammonia-producing enzymes on precursor amino acids; and 2) the *transport* of the ammonia thus produced from tubular cell into tubular urine.

The purpose of the present investigation was three-fold: 1) To determine the relationship between ammonia excretion and renal glutaminase activity during two circumstances: the early period of adaptation following the administration of an acid load, and the period of maximum ammonia excretion following the administration of different amounts of strong acid; 2) to determine if possible, the factor [illegible] for the adaptation of renal glutaminase; and 3) to determine the effect of urine pH upon the relationship between ammonia excretion and renal glutaminase activity.

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PROCEDURE

Male Sprague-Dawley rats, weighing 350 to 400 gm., were tube fed 10 ml. of standard electrolyte deficient diet twice daily. Each 10 ml. of diet contained 1.0 gm. of protein, 0.75 gm. of fat, 1.0 gm. of glucose, essential salts and soluble vitamins, but no sodium, potassium, or chloride. Preliminary unpublished observations in this laboratory have shown that variations in dietary intake result in altered excretion of acid by the kidneys. In order to obviate variations in acid excretion arising from differences in dietary intake, rats were tube fed constant amounts of diet. All rats were kept in an air-conditioned room maintained at a constant temperature to avoid any changes in the excretion of acid resulting from metabolic responses to temperature changes.

The first group of experiments was undertaken to determine the relationship of glutaminase adaptation to the rise in ammonia excretion during the early phase of a constant acid load. In order to avoid variations in NH_3 excretion resulting from different antecedent diets, 32 rats were [illegible] electrolyte deficient diet for five days. At the end of this period 4 of these rats were sacrificed and their kidneys analyzed for glutaminase to serve as controls. The remainder then received [illegible] of NH_4Cl [illegible] rats were sacrificed in groups of 4 at [illegible] hours, 6 days, and 14 days, and their kidneys analyzed for glutaminase activity. Daily twenty-four hour urine samples were collected and analyzed [illegible] acidity and ammonia throughout [illegible]

To determine the relationship between ammonia excretion [illegible] different acid [illegible]

METHODS

Daily twenty-four hour urine samples from each rat were collected under mineral oil using phenyl mercuric nitrate and toluene as preservatives. The ammonia in the urine was determined by a modification of the micro-diffusion method of Conway (10). The pH of the urine was measured with a Cambridge pH meter using external glass electrodes, and corrected to 37.5°C. by a factor of 0.01 pH unit per degree. The urine was titrated to corrected pH 7.4 with 0.1 N NaOH to determine the titratable acid.

At the end of each experimental period, the rats were anesthetized by an intraperitoneal injection of hexabarbital (Evipal® sodium) and arterial blood was collected anaerobically from the abdominal aorta. Muscle samples were removed from the hind legs. The kidneys were then removed and placed in chilled isotonic saline.

Maximum glutaminase activity was measured by adding 0.2 ml. of cold 5 per cent kidney homogenate (11) to a test tube containing 0.3 ml. of 0.25 M tris (hydroxymethyl) amino methane and 0.4 ml. of 0.6 M sodium phosphate (both solutions were adjusted to pH 8.0), 0.15 ml. of 0.12 M sodium cyanide, 0.25 ml. of 0.2 M glutamine and 0.2 ml. of water. The tubes were then incubated for 30 minutes at 38°C. The reaction was stopped by the addition of 0.15 ml. of 50 per cent perchloric acid. With each determination a blank was run in which the enzyme was replaced with water. The amount of ammonia produced was measured by nesslerization of an aliquot (12). Dry weights were determined by adding an aliquot of the 5 per cent homogenate to a weighed tube and drying for two or more days at 90°C. to a constant weight. The activity of glutaminase was expressed as micro moles of ammonia per 100 mgm. of dry kidney per hour.[3]

The muscle samples were weighed and dried; fat was removed with ether; electrolytes were extracted with 0.75 N nitric acid (13). The content of sodium and potassium in the serum and in the muscle extracts was determined on the flame photometer (14). Chloride was measured in the serum and extracts of muscle by the Hald modification of the open Carius method (15). The CO_2 content was determined on 0.2 ml. serum (15).

RESULTS

Following the start of the constant daily administration of 5 mM NH_4Cl (2½ mM every 12 hours) renal glutaminase activity remained unchanged for 24 hours, then rose in a stepwise manner, reaching maximum activity in approximately six days (Figure 1). The rise in ammonia excretion preceded the rise in glutaminase activity,

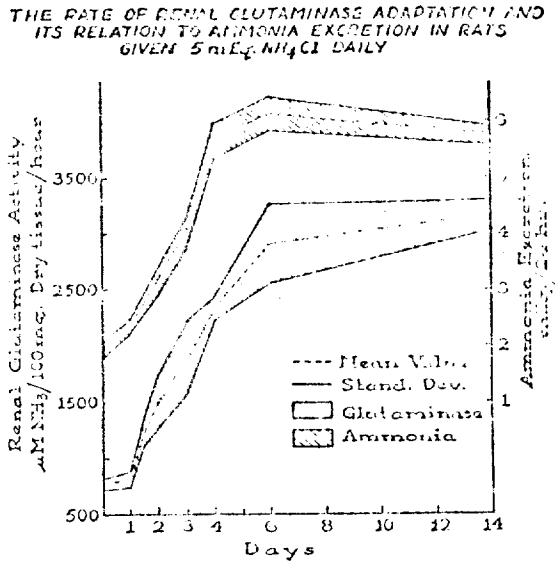

FIG. 1

showing a slight increase during the first 24 hours, but thereafter roughly paralleled the rise in renal glutaminase activity. The progressive rise in ammonia excretion during the early period of acid loading was, therefore, related to a similar rise in the activity of renal glutaminase.

In order to determine whether different loads of acid stimulated ammonia excretion by activating glutaminase, the ammonia excretion and glutaminase activity were compared in seven groups of rats, each group being maintained on a different acid load. Renal glutaminase activity was determined at the end of 14 days, when constant maximum ammonia excretion had been attained. Urine pH did not vary significantly among the rats receiving different loads of NH_4Cl. Under such circumstances, there was an excellent correlation between maximum ammonia excretion and the activity of renal glutaminase (Figure 2). During chronic acid loading the excretion of ammonia, therefore, appears to be more closely related to glutaminase activity than to urine pH.

The effect of increased urine pH upon the relationship between ammonia excretion and renal glutaminase activity is illustrated in Figure 3. The regression line was calculated from the [illegible] tained in studies on rats receiving varying [illegible] loads; the urine pH [illegible] among these groups. Rats whose urine pH was

[3] It has been demonstrated (Copenhaver, unpublished results) that the conditions outlined here are optimal for the determination of phosphate-activated glutaminase in water homogenates of rat kidney.

elevated, either by the administration of $NaHCO_3$ or the injection and ingestion of 9 mg. of Diamox® every six hours (16), excreted far less ammonia than could be predicted from the level of glutaminase activity. The disproportionately low ammonia excretion in these rats may possibly be linked with the increased urine pH, which may have repressed some passive diffusion of NH_3 out of the tubular cell. It appears, therefore, that in addition to renal enzyme activity, urine pH may play a contributory role in the regulation of ammonia excretion.

In an attempt to identify the factors responsible for the adaptation of renal glutaminase during acid loading, the changes in the electrolyte composition of plasma and muscle were determined; mean values with standard deviations are listed in Table I. The rise of glutaminase activity in rats receiving 6 mM NH_4Cl daily was accompanied by a decrease in muscle potassium from 51.4 mEq. per 100 gm. fat-free dry tissue (FFDT) to 47.3 mEq. per 100 gm. FFDT. The addition of 6 mM of KCl to the diet (Group III) had no significant effect upon the response of glutaminase to the acid load. However, the muscle potassium of rats given KCl + NH_4Cl (Group III) was not significantly different from the muscle potassium of rats given NH_4Cl alone (Group II) owing to the large standard deviation of the former which resulted from a marked divergence from the mean

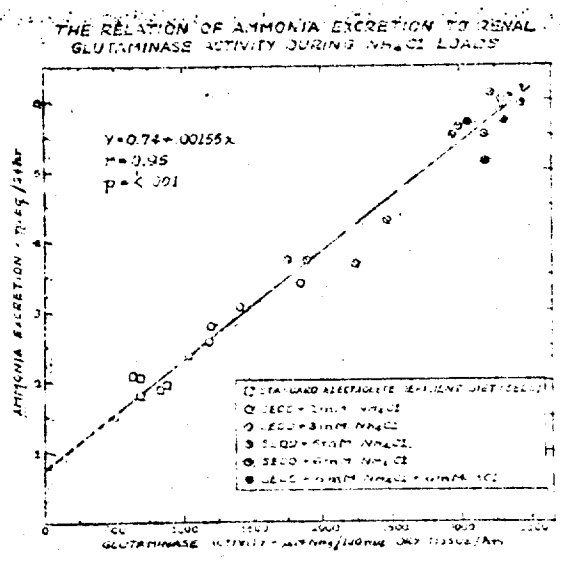

FIG. 2

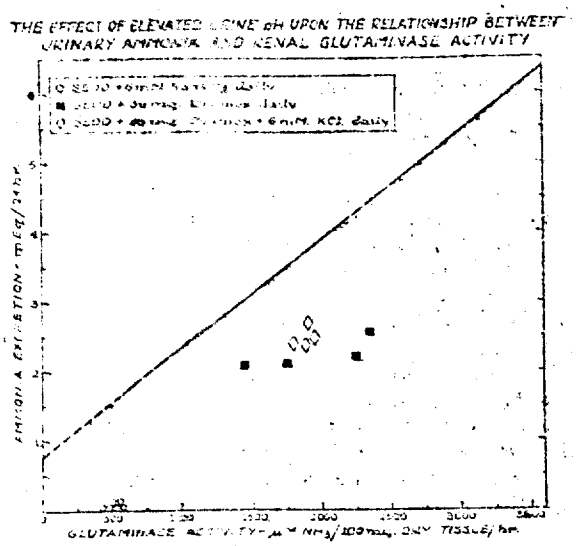

FIG. 3

in one rat. In other studies on rats given Diamox® reported elsewhere (16) correction of the potassium deficiency was accomplished by the administration of KCl without depressing glutaminase activity. It appears likely, therefore, that changes in tissue potassium, as reflected by the potassium content of muscle, are not responsible for the adaptation of renal glutaminase. Although the rise of glutaminase activity in rats receiving 6 mM NH_4Cl was accompanied by a decrease in the serum bicarbonate content from 23.9 mEq. per L. in controls to 17.8 mEq. per L., no significant change in bicarbonate content supervened in rats given 2 mM NH_4Cl daily (Group IV) despite a significant rise in glutaminase activity. Therefore, changes in serum bicarbonate content can not be causally related to the acceleration of renal glutaminase.

DISCUSSION

The excellent correlation between ammonia excretion and the activity of renal glutaminase during both the early period of adaptation in rats given constant acid loads and the period after steady ammonia excretion had been attained in rats given different acid loads strongly suggests that the rate at which ammonia is excreted is, at least partially, regulated by the activity of the ammonia-producing enzymes. The activation of the NH_3-producing enzymes by the administra-

TABLE I

THE RELATION OF SERUM AND MUSCLE ELECTROLYTES TO AMMONIA EXCRETION AND RENAL GLUTAMINASE ACTIVITY

Group	Treatment	Duration Days	PDM				MUSCLE			NH_3 excretions	GLUTAMINASE
			Na Eq./L	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	μM NH_3/100 mg. dry tissue/hr.
I (5 rats)	Standard electrolyte deficient diet (SEDD)	14	140.2 ±4.15	[illegible]	[illegible]	25.9 ±1.[illegible]	[illegible] ±.41	[illegible] ±1.2	1.94 ±.15	786 ±39
II (4 rats)	SEDD + 6mM. NH_4Cl daily	14	40.3 [illegible]	[illegible]	[illegible]	[illegible] ±3.3	3.13 [illegible]	[illegible]	[illegible] ±.26	3230 [illegible]
III (4 rats)	SEDD + 6mM. NH_4Cl + 6mM. KCl daily	4	143.7 ±[illegible].2	[illegible]	[illegible]	[illegible] ±0.7	[illegible] ±.[illegible]	[illegible] ±[illegible]	[illegible] ±.26	3131 ±1[illegible]
IV (5 rats)	SEDD + 2mM. NH_4Cl daily	14		[illegible] ±.[illegible]		[illegible] ±.[illegible]			[illegible].80 ±.37	1327 ±107
V (6 rats)	SEDD + 6mM. $NaHCO_3$ + 1mM. KCl daily	14		[illegible] ±.33		25.2 ±[illegible]			[illegible].04 ±.31	[illegible] ±42

tion of strong acids apparently augments ammonia excretion by accelerating the rate at which ammonia is formed within the tubular cell.

Since the administration of NH_4Cl produces a shift of potassium out of the cells and subsequent loss of potassium into the urine (1) and may produce a systemic acidosis, at least three changes in the internal environment might have been causally related to the activation of renal glutaminase: 1) Intracellular potassium deficiency; 2) extracellular acidosis; and 3) changes in intracellular acid-base equilibrium. The cellular content of potassium, as reflected by the potassium content of skeletal muscle, does not appear to be responsible for glutaminase adaptation, since the administration of supplemental potassium corrected the muscle deficit in three out of four rats in the present study and in all rats in a study reported elsewhere (16) (in which Diamond resulted in glutaminase adaptation), yet prevented neither the rise in ammonia excretion nor the activation of renal glutaminase. However, since it has been demonstrated (17) that during the development of potassium deficiency the cellular content of different tissues may vary widely, the possibility remains that the potassium content of the renal tubular cells may be diminished even when muscle potassium is normal. Such a possibility was not excluded by the present study, since kidneys were not analyzed for their potassium content. Although acidosis of the extracellular fluid resulting from large acid loads (6 mM NH_4Cl) might be implicated as the causative factor for the increased glutaminase activity, rats receiving smaller acid loads (2 mM NH_4Cl) had increased glutaminase activity in the absence of a systemic acidosis. Moreover, other experiments from this laboratory in animals receiving DOCA and the sodium-low potassium diets have demonstrated a similar rise in glutaminase activity in the presence of a severe alkalosis (18). Therefore, no constant relationship between the HCO_3^- content (and probably the pH as well) of the extracellular fluid and glutaminase activity can be established. Changes in intracellular acid-base equilibrium are more difficult to analyze, since there is no satisfactory method for measuring the [illegible] of the tubular cells. A fall in intracellular pH, resulting from an acid load, might be a factor in the activation of renal glutaminase. However, [illegible] experiments have shown the pH for optimum glutaminase activity to be approximately [illegible]. Nevertheless, Robinson (20) has recently demonstrated that the maximum rate of ammonia [illegible]

tion by kidney slices is attained at pH 5.5. This suggests either that the optimum pH *in vivo* differs radically from the optimum pH *in vitro*, or else that intracellular acidosis stimulates the formation of increased amounts of ammonia producing enzymes. While such *in vitro* data indicate that intracellular pH could modify enzymatic activity, the absence of any information of either the actual pH of the tubular cell or of the impact of pH on the integrated *in vivo* operation of the ammonia producing enzymes renders any inference from *in vitro* experiments to *in vivo* operation highly tenuous at best. The factors, therefore, which are responsible for the activation of renal glutaminase during ammonium chloride acidosis could not be identified. The present experiments indicate only that glutaminase adaptation is proportional to the magnitude of the acid load, but independent of the concentration of serum bicarbonate and the potassium content of cells (as reflected by muscle potassium).

Although the excellent correlation between ammonia excretion and glutaminase activity during NH_4Cl loading strongly suggests that the rate of ammonia excretion is, in part, regulated by the rate of *production* of ammonia within the tubular cell, the manner in which the ammonia thus produced is *transported* into the tubular urine is not precisely clear. Two theories have been formulated: 1) Free molecular ammonia (NH_3) may enter the tubular urine by a process of passive diffusion (4-8); 2) ammonium ions (NH_4^+) within the tubular cell may be transported into the urine by an active process of ion exchange for tubular Na^+ (16, 21). These theories are illustrated in the two model cells of Figure 4.

According to the theory of passive diffusion, the rate of ammonia transport is regulated by the H^+ concentration of the tubular urine, the H^+ promoting the conversion of NH_3 to NH_4^+, thereby establishing a concentration gradient favoring continued passive diffusion of NH_3 out of the tubular

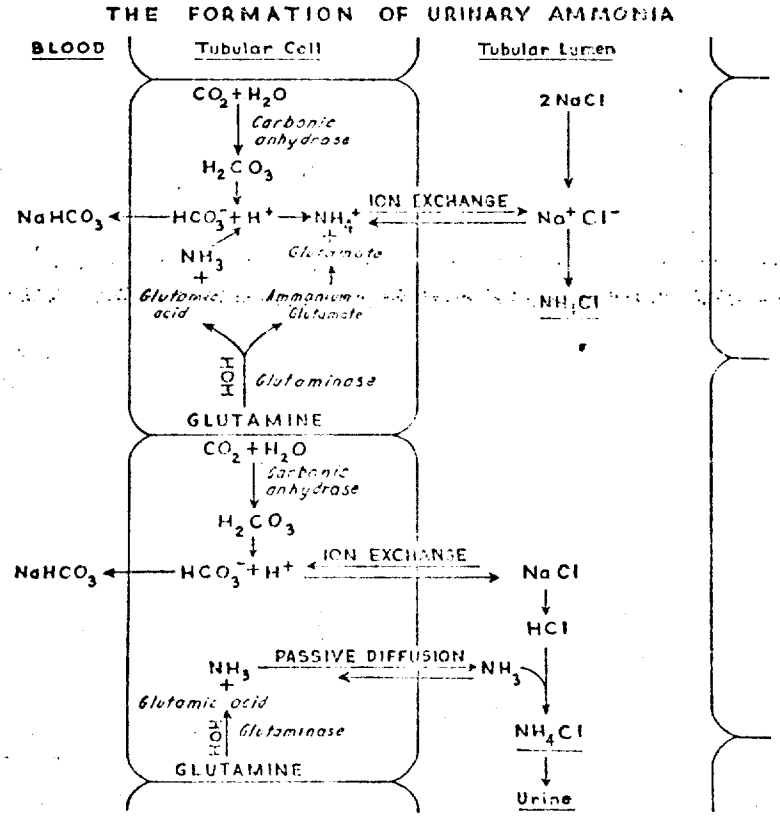

FIG. 4

cell. The data on which this theory is based have been assembled principally during acute experiments of less than 24 hours' duration (4–8). During such short intervals, major changes in the activity of the ammonia producing enzymes—judging from the data in Figure 1—do not occur, and ammonia excretion seems closely correlated with urine pH. However, during chronic acidosis, dogs excrete more ammonia at any given urine pH following an infusion of acid than do previously normal dogs subjected to the same acute acid load (5). Moreover, in the present study, with rats given varying amounts of NH_4Cl for a prolonged period of time, variations in ammonia excretion could not be attributed to any changes in urine pH. Since the rate of ammonia excretion in chronically acidotic rats varies in direct proportion to renal glutaminase activity, it is possible that accelerated formation of NH_3 within the cell may be sufficient to maintain a concentration gradient favorable for increased passive diffusion into the tubular lumen at levels of urine pH which, in acute acidosis (when the ammonia producing enzyme systems are not activated), are associated with much less ammonia excretion. The effect of urine pH on ammonia excretion can be evaluated properly, therefore, only when the rate of ammonia production can be estimated. In order to explore this relationship, ammonia excretion and glutaminase activity were determined in rats whose urine pH was elevated either by the administration of $NaHCO_3$ or Diamox® (Figure 3). These rats excreted less ammonia per unit enzyme than did rats with acid urines. The apparent discrepancy between ammonia excretion and glutaminase activity in these rats may be linked with the elevated urine pH, which could have repressed some passive diffusion of NH_3 out of the tubular cell. If passive diffusion is accepted as the primary means by which ammonia is transported into the urine it is necessary, therefore, to postulate that both the rate of ammonia formation within the cell and the pH of the tubular urine influence the concentration gradient of NH_3 across the cell membrane. This would mean, as Pitts suggested earlier (5), that the rate of ammonia excretion is pH dependent *at any given level of enzymatic activity.*

Since approximately 99 per cent of the ammonia present in the cell at physiologic pH is in the form of ammonium ion,[4] an alternative hypothesis explains the transport of NH_3 into the tubular urine on the basis of an active process of ion exchange, Na^+ of the tubular urine exchanging for NH_4^+ of the tubular cell. Although the accelerated excretion of ammonia under conditions unfavorable to passive diffusion (*i.e.*, increased urine pH) in animals receiving Diamox® (16) is highly suggestive that a mechanism of active ion exchange may be operative, sufficient data comparing urine pH and ammonia excretion with the level of enzymatic activity are not available to validate either the theory of passive diffusion or the theory of active ion exchange.

SUMMARY

1. The administration to rats of a constant NH_4Cl load resulted in a progressive, stepwise increase to a plateau in the activity of renal glutaminase, which was closely paralleled by a similar increase in ammonia excretion.

2. With the administration of different NH_4Cl loads, maximum ammonia excretion rose in direct proportion to increased glutaminase activity.

3. The adaptation of renal glutaminase was proportional to the administered acid load, but independent of the concentration of serum bicarbonate and the content of intracellular potassium.

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[4] The pK for the reaction $NH_4^+ \rightleftharpoons NH_3 + H^+$ is 9.4 (13). Therefore, in simple solution at pH 7.4 the fraction dissociated (α) can be estimated from the equation $7.4 = 9.4 + \log \alpha/1-\alpha$. Since α is 0.01, 99 per cent of the total ammonia within the cell exists as NH_4^+.

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PROFOUND ACIDOSIS RESULTING FROM EXCESSIVE AMMONIUM CHLORIDE IN PREVIOUSLY HEALTHY SUBJECTS*

A Study of Two Cases

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AMMONIUM chloride has been widely used as a therapeutic agent ever since its introduction by Keith, Barrier and Whelan[1] in 1925. Given intermittently for a few days at a time, it has a slight diuretic effect that is sometimes useful in the treatment of relatively mild degrees of fluid retention. More potent diuretics for oral use have recently become available,[2] but ammonium chloride is still in general use, not only as a diuretic agent but also as an acidifying agent that appears to potentiate the action of mercurials and may be used to prevent or treat mercurial-induced hypochloremic alkalosis. It is also a constituent of certain patent medicines sold directly to the public for weight reduction, and it is occasionally prescribed by physicians for that purpose.

Soon after ammonium chloride was introduced it became apparent that serious degrees of metabolic acidosis might result from its use. Over the past three decades a number of reports describing the syndrome of acidosis due to chronic administration of ammonium chloride have appeared.[3-15] Almost all the patients have been in late middle age or older, or have had renal disease. In view of the defect in acid excretion that characterizes renal disease, and considering the reported delays in acid-base adjustments in aged subjects,[16] it is to be expected that such patients would be susceptible to intoxication with ammonium chloride.

It is not generally appreciated, however, that ammonium chloride can be an exceedingly dangerous drug even in young and healthy subjects. It is the purpose of this report to call attention to that fact by describing 2 cases of nearly fatal acidosis produced by excessive ingestion of ammonium chloride in previously healthy young women. Several complications not previously reported in ammonium chloride poisoning are also described — namely, transient hyperglycemia, calcium-deficiency tetany and severe potassium depletion, with subsequent alkalosis. In addition, the clinical and biochemical observations made in these 2 patients provide some interesting insights into the compensatory responses of the normal subject to exogenous acidosis of extraordinary severity.

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CASE REPORTS

CASE 1. An 18-year-old photographer's model, who had always been in good health, was taken to the hospital by her parents because of hyperventilation and confusion of 1 day's duration. It was learned that for the past 6 months, in an effort to stay slim and photogenic, she had been taking up to 15 1-gm. enteric-coated ammonium chloride tablets daily for short periods. No ill-effects had been noted except on 1 occasion, 4 weeks earlier, when she became nauseated and weak, with transient hyperventilation.

During a period of 48 hours, ending 2 days before admission, she took 82 gm. of ammonium chloride. Thereafter, although she took no more medication, headache and nausea developed; she vomited once or twice and was noted to become progressively drowsy and confused.

On physical examination she was partially disoriented and was alternately stuporous and agitated, with deep respirations. She appeared to be moderately dehydrated. Examination was otherwise within normal limits except for hyperactive deep tendon reflexes.

The temperature was 99° F. by rectum, the pulse 88, and the respirations 36 to 40. The blood pressure was 120/80.

The hemoglobin was 15.4 gm. per 100 ml., and the white-cell count 27,400, with 72 per cent neutrophils. Urinary pH was 4.82 (pH meter), the specific gravity was 1.010, and there was a + test for protein and a ++ qualitative test for glucose. No acetone was present in urine or in serum, and the urinary sediment contained only 2 or 3 white cells per high-power field and an occasional granular cast. Analysis of venous blood drawn on admission (Table 1) revealed a serum sodium of 144 milliequiv., potassium of 5.2 milliequiv., carbon dioxide content of 2.4 millimols and chloride of 137 milliequiv. per liter. Whole-blood pH was 6.78 (determined in triplicate), and calculated carbon dioxide tension was 13.7 mm. of mercury. The blood sugar was 237 mg., and the urea nitrogen 37 mg. per 100 ml.

Soon after admission the patient lapsed into a comatose state. Aspiration of the stomach revealed nothing of note, and x-ray study of the abdomen revealed 2 or 3 round densities that presumably represented undigested pills. Treatment and the subsequent blood chemical changes are summarized in Table 1. Large amounts of sodium bicarbonate solution were administered intravenously. At the end of the 1st 90 minutes, when she had received 2000 ml. of 5 per cent dextrose solution containing a total of 264 milliequiv. of sodium bicarbonate, the serum carbon dioxide content had risen to 8.5 millimols per liter. She was given another 97 milliequiv. of sodium bicarbonate in the course of the next hour and a half, at the end of which the blood pH was found to be 7.32. At this point she began to respond, although hyperventilation was still marked. With further infusion of sodium bicarbonate, clinical recovery was rapid, and the respirations returned to normal during the

next few hours. There was a steady rise in serum carbon dioxide content, which reached normal levels in 20 hours and then became *higher* than normal thereafter. The serum potassium fell abruptly soon after treatment was begun, reaching a low of 2.1 milliequiv. per liter at 6 hours, and there were confirmatory electrocardiographic signs characteristic of hypokalemia. She was then given 60 milliequiv. of potassium chloride intravenously, and the serum potassium concentration rose slightly. The urinary pH remained acid despite the subsequent rise in blood carbon dioxide content to abnormal levels. The blood urea nitrogen had not changed on the morning of the 2d day, when the creatinine was 2.1 mg. per 100 ml. At discharge on the 3d hospital day the blood urea nitrogen and creatinine were 27 mg. and 1.4 mg. per 100 ml. respectively; the serum potassium was 3.2 milliequiv. per liter. Phenol-red excretion was 12 per cent in 15 minutes and 42 per cent in 2 hours. The white-cell count gradually fell from 27,400 on admission to 11,600 on discharge. Fasting and 2-hour postprandial blood sugars were normal.

TABLE 1. *Blood Chemical Values and Therapy Administered in Case 1.*

Elapsed Time	Serum Concentration					Whole Blood			Intravenous Therapy	
	Sodium	Potassium	Chloride	Carbon Dioxide Content	Creatinine	Blood Urea Nitrogen	pH*	Carbon Dioxide Tension	Sodium Bicarbonate	Potassium Chloride
	milliequiv./liter	*milliequiv./liter*	*milliequiv./liter*	*millimols/liter*	*mg./100 ml.*	*mg./100 ml.*		*mm. Hg.*	*milliequiv.*	*milliequiv.*
0 hr.	144	5.2	137	2.4		37	6.78	13.7		
1½ hr.	133	3.0	118	8.5					264	
3 hr.							7.32	16.9‡	97	
6 hr.	146	2.1	118	10.8					132	
12 hr.	151	2.7	119	19.6	2.1	37	7.44	28.4	92	28
20 hr.	150	3.5	113	25.2					106	32
36 hr.	147	2.6	109	32.5						
60 hr.	143	3.2	106	32.2	1.4	27				
19 days	141	3.7	97	37.0	0.9	8	7.36	64.3		—†
30 days	143	3.9	109	30.3	0.9	9				

*Venous blood.
†3 gm. daily by mouth on days 5-12.
‡Calculated from interpolated value for carbon dioxide.

CASE 2. A 29-year-old housewife, in the 8th month of pregnancy, was admitted to the hospital because of hyperventilation and progressive mental confusion of about 3 days' duration. Three months earlier she had been admitted to the obstetric service because of ankle edema. At that time the blood pressure was normal, and urinalysis was negative; the serum creatinine was 0.4 mg., and the blood nonprotein nitrogen 25 mg. per 100 ml. The maximum specific gravity of the urine was 1.027, and phenolsulfonphthalein excretion was normal. The serum carbon dioxide content at that time was 26 milliequiv. per liter. No specific cause for the edema could be found, and she was advised to restrict her salt intake and to take 6 gm. of ammonium chloride daily for 3 consecutive days of each week.

The history immediately before the current admission could not be accurately obtained, but it was known that she had been depressed and had been under psychiatric care intermittently for some time before this. Apparently she had appeared to be well until 3 days before entering the hospital. Subsequently, it was observed that she became increasingly tired and hyperpneic and that she vomited several times. She later admitted having taken a large but indeterminate amount of ammonium chloride, with suicidal intent.

On admission she was restless and somewhat confused. Respirations were 42 per minute and deep; other vital signs were normal. There was slight pitting edema of the ankles, with no evidence of dehydration. Deep tendon re-

TABLE 2. *Blood Chemical Values and Therapy Administered in Case 2.*

Elapsed Time	Serum Concentration						Whole Blood			Intravenous Therapy	
	Sodium	Potassium	Chloride	Carbon Dioxide Content	Calcium	Phosphorus	Nonprotein Nitrogen	pH*	Carbon Dioxide Tension	Sodium Bicarbonate	Potassium Chloride
	milliequiv./liter	*milliequiv./liter*	*milliequiv./liter*	*millimols/liter*	*milliequiv./liter*	*mg./100 ml.*	*mg./100 ml.*		*mm. Hg.*	*milliequiv.*	*milliequiv.*
0 hr.	140	3.4	125	3.0			54	7.12‡	8.7		
[illegible]			117	8.1				7.30	16.0	378†	36
[illegible]	141	2.5	113	14.4	3.8	1.5	54	7.40	23.0	131	
[illegible]	142	2.2	108	23.0			53			131	24
[illegible]	138	2.3	109	23.3			45				
[illegible]	140	3.5	107	25.4			27				
[illegible]	141	3.6			3.7	3.1					

[illegible] blood.
[illegible] blood.
[illegible]ding 200 milliequiv. of sodium lactate.

[illegible] discharge the patient took no more ammonium [illegible], had a normal diet and felt well. She was given [illegible] of potassium chloride daily for 7 days, and was seen [illegible] 16 days after discharge, when the white-cell count [illegible] 350 and the blood urea nitrogen and creatinine 8 [illegible] and 0.9 mg. per 100 ml. respectively. Routine urinalysis [illegible] a specific gravity of 1.025 and was entirely within [illegible] limits; phenolsulfonphthalein excretion was 22 per [illegible] 15 minutes and 47 per cent in 2 hours. Serum electrolytes revealed a compensated metabolic alkalosis, with [illegible] fully low serum potassium (3.7 milliequiv. per liter) and an elevated carbon dioxide tension. At a final visit 11 [illegible] later the serum potassium and carbon dioxide concen-

flexes were hyperactive. The uterus was at the level of the umbilicus, and the fetal heartbeat was audible.

On initial examination the patient was thought to be hysterical, and she was treated overnight with sedation and an intravenous infusion of dextrose and water. On the next morning she could not be roused, and hyperventilation was even more pronounced. The specific gravity of the urine was 1.001, with a pH of 5.0, a + test for protein, a negative test for sugar and a sediment containing 60 to 80 white cells, 0 to 2 red cells and a few granular casts per high-power field. The hemoglobin was 10.4 gm. per 100 ml., and the white-cell count 14,000, with 91 per cent neutrophils. The nonprotein nitrogen was 54 mg., and the blood sugar 77 mg. per 100 ml. As shown in Table 2, the serum sodium was 140 milliequiv., the potassium 3.4 milliequiv., the chloride 125 milliequiv., and the carbon dioxide content 3.0 millimols per liter. Arterial-blood pH was 7.12, and the calculated carbon dioxide tension was 8.7 mm. of mercury.

On the basis of these data, and with more historical information available, a diagnosis of ammonium chloride intoxication was made. Treatment was instituted with alkali given intravenously; the course is summarized in Table 2. Six hours later, after having received a total of 378 milliequiv. of sodium lactate and sodium bicarbonate, the patient was more responsive, although hyperventilation was still present. At this time the serum carbon dioxide content was 8.1 millimols per liter. The venous-blood pH was 7.30, and the calculated carbon dioxide tension was 16 mm. of mercury. Twenty-four hours after therapy had started she had received a total of 509 milliequiv. of alkali and 30 milliequiv. of potassium chloride. She was quite alert, and the respirations were normal. The serum carbon dioxide content was 14.4 millimols per liter, the venous-blood pH 7.40, and the calculated carbon dioxide tension 23 mm. of mercury. The serum potassium had fallen to 2.5 milliequiv. per liter, and the blood nonprotein nitrogen remained at 54 mg. per 100 ml.

During the next few days the patient was given more potassium and alkali by mouth and by vein, and she improved steadily. On the 5th day the carbon dioxide content was 25.4 millimols, and the serum potassium only 3.5 milliequiv. per liter, and the nonprotein nitrogen had returned to normal. On this day she went into labor spontaneously, and a normal infant was delivered without undue difficulty. One day post partum she began to complain of tingling and cramps in the extremities, and positive Trousseau and Chvostek signs were elicited. At this time the serum potassium was 3.6 milliequiv., and the calcium 3.7 milliequiv. per liter, and the phosphorus 3.1 mg. per 100 ml.

She was then given calcium lactate by mouth, and the symptoms of hypocalcemia disappeared within a few days. On the 8th post-partum day she was discharged, feeling well. At the time of discharge urinalysis was within normal limits, the blood creatinine was 0.5 mg. per 100 ml., and phenolsulfonphthalein excretion was 63 per cent in 2 hours. On subsequent observation this patient continued to have normal renal function by the usual clinical criteria, including urinalysis, and normal serum levels of potassium and calcium.

Discussion

Metabolic acidosis of the severity noted in these 2 patients is relatively uncommon, and has been reported mainly in diabetic ketosis[9,10] and advanced renal insufficiency.[11] The venous-blood pH of 6.78 observed in Case 1 is the lowest such value in our experience, and must surely be close to the limit of tolerable acidity. In both patients, virtually all extracellular bicarbonate had been exhausted (Table 3). At such extremely low levels of bicarbonate in the serum, respiratory compensation becomes critical. This is illustrated by the following consideration: given the observed levels of bicarbonate, it can be calculated that if there had been no respiratory compensation (that is, if the carbon dioxide tension had remained normal) the resulting blood pH would have been 6.32 in Case 1 and 6.45 in Case 2 (Table 3). Such degrees of acidosis would surely have been fatal.

It is of interest that the patient in whom respiratory compensation was most effective (Case 2) was in the eighth month of pregnancy. Normal pregnancy is characterized by mild hyperventilation,[12] some evidence suggesting that respiration may be stimulated by the action of progesterone.[13-15] This may have accounted for the extraordinarily low carbon dioxide tension of 8.7 mm. of mercury in this patient, and also for the fact that one day after institution of therapy, when extracellular pH had been restored to 7.40, she still had a carbon dioxide tension of only 23 mm. of mercury (Table 2). However, persistence of hyperventilation during recovery from severe metabolic acidosis has been described as a frequent phenomenon,[10] and it is of interest that the carbon dioxide tension also remained moderately low in Case 1 at the twelfth hour of treatment, when the whole-blood pH had returned fully to normal (Table 1).

Total Acid Load and Response to Treatment

Treatment consisted mainly of intravenous administration of large quantities of alkali to counteract the acidosis (Tables 1 and 2). Thus, in Case 1 the plasma carbon dioxide content was raised from 2.4 to 25.2 millimols per liter in a period of twenty hours by a total of 691 milliequiv. of sodium bicarbonate. A comparable result (carbon dioxide content raised from 3.0 to 23.0 millimols per liter) was achieved in Case 2 in a period of two days by 643 milliequiv. of alkali.

Table 3. *Acid-Base Measurements in Cases 1 and 2.*

	Observed Value		Calculated Value		
	Blood pH	Serum carbon dioxide content	Blood carbon dioxide tension	Serum carbonic acid	Serum sodium bicarbonate
		millimols/liter	*mm.-Hg.*	*millimols/liter*	*milliequiv./liter*
Actual observations:					
Case 1	6.78*	2.4	13.7	0.4	2.0
Case 2	7.12†	3.0	8.7	0.3	2.7
Assuming no respiratory compensation:					
Case 1	6.32‡	3.2‡	40.0§	1.2§	2.0
Case 2	6.45‡	3.9‡	40.0§	1.2§	2.7

*Venous blood
†Arterial blood
‡Calculated.
§Assumed.

Table 4 shows some pertinent data on the composition of the urine and renal function in Case 1. It is seen that during the first twenty-four hours the urine remained acid, indicating that little or none of the infused bicarbonate had been excreted. Only 76

milliequiv. of sodium was excreted during this time, indicating also that most of the large sodium load was retained. Complete collections of urine were not made in Case 2, but the pH of random specimens obtained during the first two days was between 5.0 and 6.0, suggesting that most or all of the infused alkali was also retained in this patient.

TABLE 4. *Composition of the Urine in Case 1*

Time	Total Urine Volume	pH	Ammonium	Chloride	Sodium	Potassium
	ml.					
On admission	(Random specimen)	4.8	68 milliequiv./liter	151 milliequiv./liter	39 milliequiv./liter	45 milliequiv./liter
1st day	1520	5.3	84 milliequiv./day	146 milliequiv./day	76 milliequiv./day	[illegible] milliequiv./day
2d day	940	5.9	81 milliequiv./day	103 milliequiv./day	2 milliequiv./day	17 milliequiv./day

With little or no bicarbonate in the urine, the total alkali required to restore blood carbon dioxide content approximately to normal can be taken as a rough estimate of the total acid load in the body, provided that no significant quantity of acid continues to be derived from delayed absorption and metabolism of ammonium chloride and that renal excretion of acid is not increased. These conditions seem applicable to Case 1 because forty-eight hours had elapsed between the last ingestion of drug and admission to the hospital. Gastric lavage did not reveal any unabsorbed pills, and x-ray study of the abdomen showed only a few shadows consistent with at most 2 or 3 undissolved tablets in the intestines. Furthermore, as shown in Table 4, only 84 milliequiv. of ammonium ion was excreted during the first hospital day. This quantity is only a little more than might be expected under normal circumstances. The titratable acidity of the urine was not measured, but since ammonium excretion usually constitutes half or more of total acid excretion, it is unlikely that renal excretion of acid played any large part in restoring the plasma bicarbonate level to normal.

As estimated from the bicarbonate administration, therefore, the retained acid load in Case 1 was roughly 15 milliequiv. per kilogram of body weight. Such a calculation cannot confidently be made in Case 2 because there were no urinalyses for ammonium ion or acid, and because less was known about the interval between final ingestion of drug and the onset of treatment. However, if the conditions are assumed to be similar in both cases, the estimated acid load would also be approximately the same.

A value of 15 milliequiv. per kilogram of body weight is remarkably close to the estimate of the maximum whole-body buffer capacity, which can be calculated from a study of the acute effects of administration of hydrochloric acid to dogs.[16] It should be noted that *acute* acidosis may not call into play the large potential buffer capacity of bone,[17] which in more chronic situations may become important. Since renal compensation would not be of great significance for the first day, this estimation of the rapidly available buffer capacity approximately defines the limits of tolerance for acute acid loads. On this basis, 40 to 50 gm. of ammonium chloride given over a short period would be expected to exhaust the available body buffers of an average-sized adult and thus to produce fatal or near fatal acidosis even in a previously healthy subject.

Potassium Depletion

Both patients had evidence of severe potassium depletion, apparently owing to the ingestion of ammonium chloride. Although there are no previous reports of potassium depletion resulting from ammonium chloride acidosis, it is not surprising that such a complication should develop. Kaliuresis of varying degree regularly follows administration of large doses of ammonium chloride, and, when sodium retention is stimulated, potassium may comprise the major part of the fixed cation in the urine.[18] Despite body depletion of potassium, serum concentration may be normal or even elevated if glycolysis, tissue breakdown or impairment of renal function is present.[19] This is believed to explain the paradoxical hyperkalemia frequently noted in potassium-depleted patients with severe diabetic or uremic acidosis. Presumably, these mechanisms were also operative in the present cases. The rapid fall of serum potassium during treatment was probably due mainly to the abrupt rise in blood pH, and in part also to increased glycogenesis. The later development of alkalosis in Case 1 may have been a direct consequence of the potassium depletion. On the other hand, it could have represented a delayed but excessive compensatory renal response to the initially severe acidosis. Analogous but much less marked "overshoots" of plasma bicarbonate were noted after the administration of ammonium chloride was stopped in other studies.[20,21]

Calcium Deficiency

Calcium depletion represents another electrolyte deficiency that, although not previously reported in ammonium chloride intoxication, might have been predicted from the known acidotic effects of this drug. Calciuria induced by ammonium chloride is

usually of slight degree.[21] However, when ingested in large quantities over a prolonged period, particularly by a patient late in pregnancy, ammonium chloride might well be expected to add significantly to the loss of calcium from the maternal blood. Thus, in Case 2 symptoms and signs of latent tetany developed on the sixth day after recovery from the acidosis. This coincided with the onset of lactation and with a progressive increase in serum concentration of potassium. As seen in Table 2, the serum calcium level on the day tetany appeared was 3.7 milliequiv. per liter, almost the same as it had been five days before. The initial absence of tetany can probably be ascribed in part to the acidosis, which tends to decrease the protein binding of calcium, and also to the neuromuscular-depressant effects of the very low serum potassium levels.[22] The subsequent rise in serum potassium probably combined with the correction of acidosis to evoke the manifestations of calcium deficiency, even though total serum calcium had not changed.

Two other interesting manifestations noted in Case 1 remain to be discussed — namely, leukocytosis and hyperglycemia. Despite the absence of fever and of any other known cause of *leukocytosis*, the white-cell count in this patient was 27,400 on admission, and it returned only gradually to normal over the next several days. The differential count showed the increase to be due to neutrophilic granulocytes, with a relative eosinopenia and lymphopenia. Case 2 also manifested a transient leukocytosis, but to a much lesser degree: the white-cell count was 14,000 on admission and fell to 5500 in four days. It is generally recognized that marked neutrophilic leukocytosis may accompany a variety of other acidotic states, such as diabetic ketosis and uremia. The mechanisms involved have never been clearly elucidated, although increased adrenocortical activity and dehydration and hypertonicity[23] have been implicated.

The initial discovery of *hyperglycemia* and *glucosuria* in Case 1 naturally prompted a consideration of diabetic ketosis as a cause of the acidotic state. However, the history of ammonium chloride ingestion, the absence of acetone from the serum and the normal serum level of "unmeasured anions" (as estimated by the difference between sodium and the sum of chloride and carbon dioxide content) argued strongly against this possibility. The carbohydrate defect proved to be transient, subsequent study of fasting and postprandial blood sugars yielding entirely normal results. This patient's father had diabetes. Thus, it is quite possible that the transient hyperglycemia represented nothing more specific than a temporary diabetic state evoked by acute stress in a patient with latent diabetes. It is interesting, however, to recall the fact that ammonium chloride acidosis can impair glucose utilization in intact dogs,[24] presumably by affecting hexokinase activity.[25] This raises the possibility that transient diabetic manifestations are occasionally due solely to profound acidosis.

Summary and Conclusions

Two previously healthy young women in whom profound metabolic acidosis followed the ingestion of large amounts of ammonium chloride are described. The potential dangers of ammonium chloride even with previously normal renal function are thus emphasized. Biochemical observations in these patients demonstrate some of the important features of the normal homeostatic response to acute acidosis of maximal severity. Transient impairment of renal function, potassium depletion, calcium deficiency and hyperglycemia are described as complications of this condition.

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Reports

Relation of Ammonia Excretion to Urine pH in the Guinea Pig

In 1856 Claude Bernard (*1*) observed that the urinary *p*H of carnivorous animals is markedly lower that that of herbivorous animals. Some years later Walter (*2*) described the excretion of ammonia in various animals that had been rendered acidotic and stressed the protective and base-saving features of this mechanism. Since then, numerous studies on the patterns of ammonia excretion in man, dog, and rat, especially during acidosis, have been published. On the other hand, very few studies on these patterns in the rabbit have been made and, as far as we are aware, none in the guinea pig. The current theories of ammonia excretion are, therefore, largely based on studies in species that normally excrete an acid urine.

In spite of much work in this field, three major questions await conclusive answers. What is the physiological stimulus for ammonia excretion? What is the relative importance of enzymatic and physicochemical factors in the mechanism of ammonia excretion? What is its physiological significance? During studies on ammonia excretion in the guinea pig, we have made observations that are surprising when they are considered in the light of present concepts of ammonia formation and excretion.

The ammonia output of guinea pigs (1 to 4 microequivalent/30 min kg) is low compared with that of rats or dogs and varies with age, sex, and dietary habits. In acute experiments (*3*) guinea pigs were rendered acidotic or alkalotic; and urine, collected under oil, was analyzed for *p*H (*4*) and ammonia content (*5, 6*). The urinary ammonia concentration was expressed as microequivalents per milliliter and the output as microequivalents per 30 minutes, per kilogram of body weight. Data from seven acute experiments are plotted on Fig. 1, using the urinary *p*H as abscissa and ammonia concentration or output as ordinate (*7*). The graph shows that both the concentration and the relative output of ammonia in the urine rise, not only during acidosis as in man, dog, and rat, but also during alkalosis. The same is true if the relative ammonia output for each animal per 30 minutes is plotted against the *p*H. The correlation between deviations of *p*H from the normal range and increased ammonia output is not strict but is statistically significant. A similar pattern has been observed in a few experiments with rabbits.

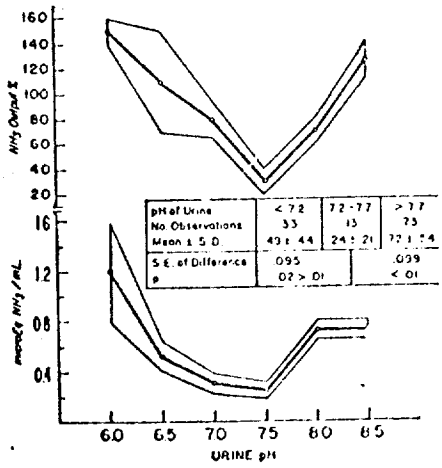

Fig. 1. The relation of ammonia excretion to urine *p*H in the guinea pig.

The increased ammonia concentration in alkaline urines cannot be explained as a function of urine flow, as could an increased output. It must, therefore, be assumed that there is increased ammonia production or transfer into alkaline, as well as acid, urine.

These observations suggest reconsideration of current hypotheses regarding the stimulus. Decreased plasma bicarbonate or *p*H or increased titratable acidity or decreased *p*H of the urine have been proposed as the proximate stimulus for the increase in ammonia output during acidosis. None of these, however, could cause the marked increase in ammonia output observed during alkalosis. Information now available does not indicate whether the same or different stimuli act during acidosis and alkalosis.

Ammonia excretion is currently considered to be the resultant of two independent mechanisms, one being enzymatic, the other being physicochemical in nature. Investigations during the past few years indicate that glutaminase activity in the kidneys is one of these factors (*8*). Transport of ammonia from tubular cells into urine, be it by passive diffusion (*9*) or active exchange for sodium (*10*), is held to be the second limiting factor for ammonia excretion. Our experiment demonstrate that ammonia excretion i. the guinea pig is nearly as efficient in th alkaline as in the acid range. It seem improbable, therefore, that physicochem ical transport is the limiting factor i ammonia excretion in the guinea pig. I it were, different mechanisms in the aci and alkaline ranges would have to b postulated.

At urine *p*H's of 8, 8.5, and 9 the frac tions of ammonia present in an un-ionize form as free ammonia are respectively 4 11, and 29 percent (*11*). Back diffusio of the ammonia during the flow of th urine from the distal tubules to the blad der should, therefore, also be taken int account in studies on ammonia excretio in the alkaline range. This aspect has ne yet been explored.

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4. Determinations of *p*H were done with a Cam bridge Research *p*H meter using the syring type glass electrode.
5. A modification of the microdiffusion metho of D. Seligson and H. Seligson [*J. Lab. Cli Med.* 38, 324 (1951)] was used. The workin range of this method in our hands was betwe 0.5 and 100 microequivalents of ammon nitrogen.
6. This investigation was supported by a resear grant, RG-3795, from the National Institut of Health, U.S. Public Health Service.
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METABOLISM OF AMMONIA
I. BIOCHEMICAL ASPECT OF AMMONIA INTOXICATION[1]

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ABSTRACT — Thirteen ammonium salts given by intraperitoneal injections to rats at the concentration of 10.6 mmoles of NH_4-N/kg of body weight (LD 99.9) were toxic. Nevertheless, the various times for the manifestation of comatose state obtained after the injection of ammonium sulfate, bicarbonate and phosphate were independant of blood pH variations and were related to the speeds with which ammonia crossed the blood-brain-barrier. These different dissociation speeds of ammonium ion into free ammonia at the level of cell membrane influenced greatly the protective action of L-arginine. This compound gave a full protection with ammonium sulfate and phosphate. A relative protection (70%) was obtained only when L-arginine was given 15 minutes prior to the injection of ammonium bicarbonate. L-arginine, L-citrulline, L-ornithine and L-ascorbic acid were the most effective substances against ammonia intoxication. The fact that labeled ^{14}C-L-arginine and ^{14}C-L-citrulline were not accumulated in brain after their intraperitoneal injections and that L arginine, L-citrulline and L-ornithine were ineffective against experimental ammonia intoxications with chicks pointed out the biochemical aspect of the protection. Biochemical mechanisms involved in ammonia intoxication and detoxication are postulated.

Accumulation of ammonia in the blood and body fluids in advanced hepatic diseases has stimulated the need for better understanding of the biochemical mechanisms of production, fixation and excretion of ammonia[13]. A great number of publications have appeared on the different aspects of this problem[11,12,19,24]. Even with the theoritical discussions of the possible biochemical aspects evolved in ammonia toxicity, it seems that the true mechanism whereby ammonia exerts its toxic effect on the neural cell is still unknown[12,18]. From this point of view, a study of biochemical aspect of ammonia toxicity becomes valuable and urgent. Experiments were made to evaluate the differencial toxicity of ammonium salts and the relation of ammonia toxicity with blood pH. The effects of various compounds against toxicity of these salts were also tested. Finally blood-brain-barrier and biochemical role of arginine in ammonia intoxication were investigated.

MATERIALS AND METHODS

White rats male Wistar weighing between 180 g to 220 g were used. In some experiments, chicks (Rhode Island X Sussex) weighing about 100 g were used. The animals were fed on a Purina laboratory chow diet and kept in individual cages. They received intraperitoneal injections of NH_4Cl or other ammonium compounds at a dose of 10.6 mmoles of NH_4-N/kg body weight in one ml of a freshly prepared solution. This dose represents a LD 99.9. The times for the initiation of comatose convulsions and for the death of animal were noted. The surviving animals were kept under observation during 24 hours after the injection. In the experiments on protection, the different compounds used were generally injected intraperitoneally 30 minutes prior to the injection of the lethal dose of ammonium salt.

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Every experiment was performed with at least 10 animals in each experimental group. In many instances larger numbers of rats, up to 30 per group, were employed. All ammonium salts were reagent grade. The amino acids, the α-ketoacids and all other substances to be injected were purchased from Nutritional Biochemicals Corporation, Cleveland. These substances were dissolved in water neutralized before being given in injection.

Blood pH were determined with an Astrup microequipment type AEME I on blood obtained by cardiac blood puncture on living animals. The effect of each ammonium salt on blood pH was measured at the beginning of convulsions. The ammonia was estimated by the method of Miller and Rice[14]. Ammonia levels were determined in blood which was obtained by cardiac blood puncture on living animals and in a sample of high supernatant of a brain quickly homogenized in ion free ammonia water. The effect of each ammonium salt on blood and brain ammonia levels was measured at the beginning of convulsions.

Adult white albino male mice 129J, weighing 20 to 30 g, were used for autoradiographic studies. ^{14}C-L-arginine (uniformly labeled) and ^{14}C-L citrulline (labeled on C 5 position) were injected intraperitoneally at a dose of 0.15 μ curies/g of body weight. The mice were killed by freezing in a mixture of acetone and dry ice at −78°C, and were treated according to the method of Berlinguet *et al.*[1].

RESULTS

The differential toxicity of ammonium salts. The toxicity of ammonium chloride at the concentration of 10.6 mmoles of NH_4-N/kg of body weight as related to the other ammonium salts at the same concentration is presented in Table I. The thirteen ammonium salts tested were toxic for rats under experiment. Times for convulsions and death were more or less prolongated. The beginning of convulsions after the injection of ammonium salts varied from 5 to 11 min and the death, from 7 to 13 min with the exceptions of the injection of ammonium oxalate and citrate where the animals died without any sign of convulsions.

TABLE I

The differential toxicity of ammonium salts

Salts injected *	Times in min for convulsions	Times in min for death	% of survival
NH_4Cl	5′ **	10′	0
$(NH_4)_2SO_4$	8′	13′	0
NH_4HCO_3	5′	8′	0
$(NH_4)_2HPO_4$	11′	13′	0
CH_3COONH_4	6′	8′	0
$(NH_4)_2HC_6H_5O_7$	—	8′	0
$(NH_4)_2C_2O_4, H_2O$	—	6′	0
NH_4NO_3	6′	10′	0
$NH_4H_2PO_4$	8′	9′	0
$NH_4C_3H_5O_2$	8′	10′	0
NH_4CHO_2	6′	8′	0
PO_4 buffer ***	8′	12′	0
$(NH_4)_2CO_4$	6′	8′	0

* All these salts were injected i.p. at the basic concentration of 10.6 mmoles of NH_4-N/kg of body wt.

** These values represent the mean from 10 rats. All animals weighed about 200 g.

*** Ammonium PO_4 buffer, pH 7.4.

TABLE II

Blood and brain ammonia levels related to blood pH

Rats injected * with	pH of solutions	Blood pH	NH_4 - N in blood (μg/ml)	NH_4 - N in brain (μg/g of wet tissue)	Time for convulsions (min)
$(NH_4)_2SO_4$	6.01	7.05 ± 0.01 **	78 ± 3 ***	141 ± 15 ***	8′
NH_4HCO_3	7.48	7.38 ± 0.02	87 ± 3	177 ± 11	5′
$(NH_4)_2HPO_4$	8.55	7.25 ± 0.01	127 ± 5	107 ± 16	11′
Normal	—	7.36 ± 0.01	0.95 ± 0.006	3 ± 0.04	—

* The rats were injected i.p. with the same concentration of NH_4 - N, i.e. 10.6 mmoles/kg.

** This result represents the mean value of 50 determinations, and SE are given. Blood pH was determined on blood obtained from cardiac blood puncture at the beginning of convulsions.

*** These results represent the mean from 10 rats, and SE are given. Ammonia concentration was determined at the beginning of convulsions. Blood obtained from cardiac blood puncture and samples of high supernatant of brain homogeneization were used.

Blood and brain ammonia levels related to blood pH after a lethal dose of ammonium salts. Among the ammonium salts tested, ammonium sulfate, bicarbonate and phosphate were chosen. This selection was based on the fact that their solutions had an acid, neutral and basic pH as seen in Table II. A drastic fall in blood pH (7.05) was noted at the moment of convulsions when ammonium sulfate was injected. Blood pH was neutral (7.38) after the injection of ammonium bicarbonate and acid (7.25) when ammonium phosphate was used.

At the same concentration of NH_4-N, ammonium bicarbonate, sulfate and phosphate initiated the convulsions after 5, 8 and 11 min respectively. These various times of onset of convulsion were independant of blood pH. The injection of ammonium phosphate demonstrated elevated ammonia blood level along with a decrease in ammonia brain concentration as compared to the two other salts. The ammonium bicarbonate injection caused an increase in ammonia brain level as compared to two other salts. This was accompanied by a decrease in ammonia blood concentration as compared to the injection of ammonium phosphate. In this case, time for the beginning of convulsions was very rapid. The action of ammonium sulfate on time of convulsions and on ammonia brain concentration was also intermediary.

Effect of arginine on ammonia toxicity. The most effective substance for the protection against the toxicity due to these ammonium salts was found to be L-arginine. When 6 mmoles of this substance/kg of body weight was administered at various times prior to the injection of lethal dose (LD 99.9) of one of the three ammonium salts, the results described in Figure 1 were obtained. The data indicate that 30% survival was obtained when L-arginine was administered at the beginning of convulsions (-10 min) which were caused by an intraperitoneal injection of ammonium phosphate. If the convulsions were due to a lethal dose of ammonium sulfate or bicarbonate, no protection was noted.

The two compounds being injected simultaneously (0 min), 60% and

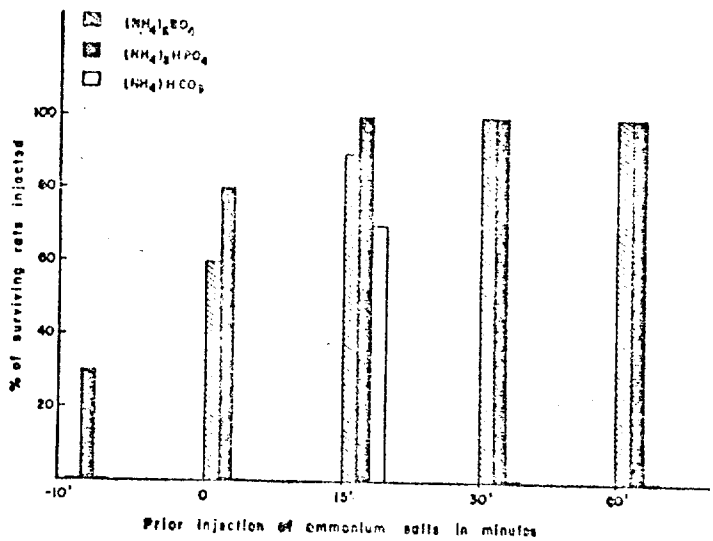

FIG. 1: Protective effect of 6 mmoles of L-arginine/kg of body weight administered intraperitoneally to male rats at different times prior to the intraperitoneal injection of a $LD_{99.9}$ (10.6 mmoles NH_4-N/kg) of ammonium salts. Each experiment was performed with a minimum of twenty rats. −10 min indicate that injection of arginine was given at beginning of convulsions; 0 min, at the same time than ammonium salts; 15 min prior to the injection of ammonium salts, etc.

80% survival were obtained with ammonium sulfate and phosphate respectively while no protective effect was observed in the case of ammonium bicarbonate. When arginine was given 15, 30 and 60 min prior to the administration of the lethal dose of ammonium sulfate and phosphate, a complete or almost complete protection was noted. When L-arginine was injected 15 min prior to the injection of ammonium bicarbonate, only 70% of the animals survived. No protective effect was observed at 30 and 60 min.

TABLE III

Effects on ammonia toxicity of different substances

Substances injected *	No. of rats	Times in min for convulsions	for death	% of surviving rats
L-arginine	20	—	—	100%
L-citrulline	20	—	—	100%
L-ornithine	20	—	34′	90%
L-arginine methyl ester **	20	10′	28′	90%
L-ascorbic acid	20	13′	20′	60%
L-canavanine	20	12′	13′	55%
L-homoarginine	20	11′	27′	40%
Oxaloacetic acid	20	6′	17′	30%
Reduced glutathione	20	17′	23′	30%
Acetyl-L-arginine	20	22′	26′	20%
L-methionine	10	7′	12′	10%
Arginine ethyl ester	10	7′	9′	0%

* 6 mmoles/kg of these substances were injected i.p. 30 min prior to the injection of a lethal dose of ammonium sulfate (LD 99.9).

** 3 mmoles/kg of this substance were injected i.p.

Effects on ammonia toxicity of different substances. The effectiveness of L-arginine was compared with that of several other substances and the results obtained against ammonia toxicity are summarized in Table III.

The following amino acids were given by intraperitoneal injection at the concentration of 6 mmoles/kg of body weight: L-glycine, L-methionine, L-lysine, L-alanine, L-glutamic acid, N-acetyl-L-glutamic acid, L-citrulline, L-homocitrulline, L-ornithine, L-arginine, L-aspartic acid, carbamyl-D-L-aspartic acid and N-acetyl-L-aspartic acid. Among these substances, L-arginine, L-citrulline and L-ornithine gave a complete protection and all the other compounds were ineffective against ammonia toxicity. The use of creatine and guanidine compounds such as creatine, creatinine, guanine, guanilyc acid, guanidoacetic acid, γ-guanidinobutyric acid showed no protective action.

In the course of these studies, arginine analogues and derivatives such L-arginine methyl ester, L-canavanine, L-homoarginine, N-acetyl-L-arginine, argininic acid, D-arginine and L-arginine ethyl ester were used. L-arginine methyl ester was almost equally effective as arginine while arginine ethyl ester gave no protection. A relative protection was noted with L-canavanine, L-homoarginine and N-acetyl-L-arginine.

With the exception of oxaloacetic acid where a slight protection (30%) was observed, all other compounds such as pyruvic, malic, fumaric, succinic, a-ketoglutaric acids were ineffective against this toxicity. Out of the compounds: ascorbic acid, reduced glutathione, L-glutamine, γ-aminobutyric acid, urea, orotic acid and uridine, only ascorbic acid gave 60% survival.

Localisation of the radioactivity. Two minutes after the intraperitoneal

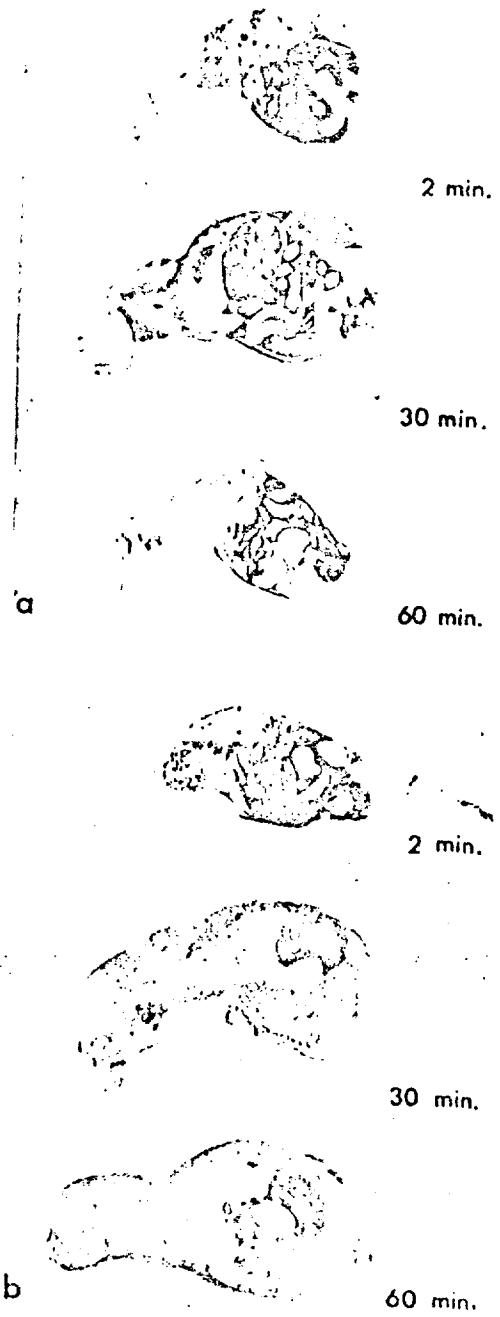

FIG. 2: Autoradiograms of thin sections of mice which had received intraperitoneal injections of labeled L-arginine (a) and of labeled L-citrulline (b). The animals were killed 2, 30 and 60 min after the injection of radioactive material. Dark spots represent the localised radioactivity.

injection of labeled ^{14}C-L-arginine, the intraperitoneal cavity contained all the radioactivity (Fig. 2). After 30 min, while most of the radioactivity was still in the intraperitoneal cavity, a fairly large concentration of radioactive material was localised in liver and in the region of the thyroid gland. There was also a faint trace of radioactive material distributed throughout the whole body. No radioactivity was observed in the brain. After 1 hour, the radioactivity was still concentrated in the peritoneal cavity particularly around mesentery, intestine and peritoneal walls.

When ^{14}C-L-citrulline labeled on carbon five was injected intraperitoneally, the distribution was somewhat similar to that of arginine except that much of the radioactivity was present in kidneys after 30 min. The same general pattern was observed in the distribution of the radioactive material in mice receiving an injection of non labeled arginine or citrulline (6 mmoles/kg) followed immediately by an injection of labeled arginine or labeled citrulline 30 min prior to the administration of a lethal dose of ammonium sulfate (Fig. 3).

Protection of chicks against ammonia toxicity. The most effective compounds that protected rats against experimental ammonia intoxication were tested in chicks and the results obtained are described in Table IV. The lethal dose of ammonium sulfate was experimentally determined. The injection of 15.8 mmoles of NH_4-N/kg of body weight killed all the animals. L-arginine, L-citrulline and L-ornithine were administered intraperitoneally 30 min prior to the injection of the ammonium salt at the concentration of 9 mmoles/kg body weight. No protective action was noted with the injection of these three amino acids. Even if the concentration of these compounds (18 mmoles/kg) was doubled, no change occured in the results.

DISCUSSION

When a lethal dose (LD 99.9) of ammonium chloride (10.6 mmoles of NH_4-N/kg) was given intraperitoneally in a single dose, a reaction be-

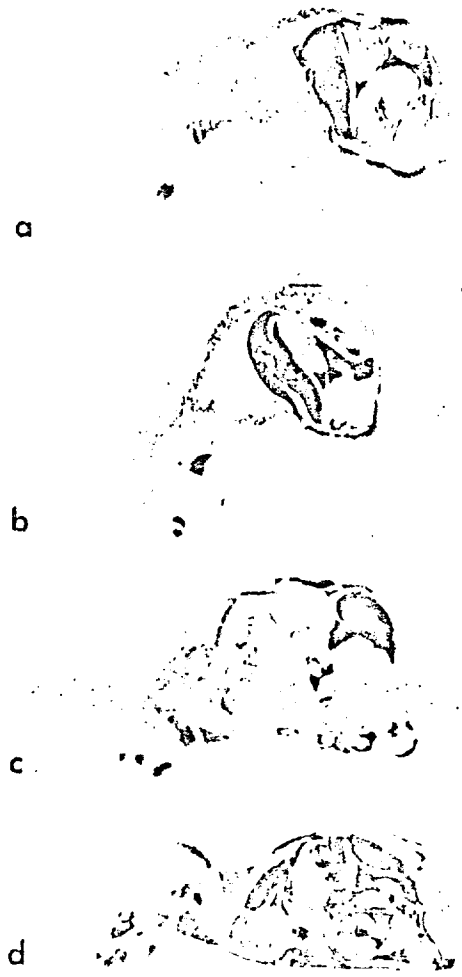

FIG. 3: Autoradiograms of mice which had received (a and b) an injection of non labeled arginine (6 mmoles/kg) followed immediately by an injection of labeled arginine. 30 min later, a lethal dose of ammonium sulfate (10.6 mmoles/kg was administered to mice (b)). The animals were killed 10 min after the injection of ammonium salt. In c and d, the mice received injection of citrulline instead of arginine.

TABLE IV

Effects on ammonia toxicity of different compounds injected to chicks

Substances injected *	No. of animals	Times in min for convulsions	for death	% of surviving chicks
L-arginine	20	4′	5′	0%
L-citrulline	20	5′	8′	0%
L-ornithine	20	4′	7′	0%

* 9 mmoles and 18 mmoles of these substances were injected i.p. 30 min prior to the injection of a lethal dose of ammonium sulfate (15.8 mmoles NH_4-N/kg).

gan within 8 min characterized by hyperventilation and violent convulsions. The rats remained in a comatose state for approximately 4 to 6 min moving convulsively to noise stimuli. During this time, death could occur at any time. At doses varying between LD_{50} to $LD_{99.9}$, ammonium chloride and ammonium acetate have been routinely used by several investigators to produce experimental ammonia intoxication[4,5,6,17]. The results of these different studies were based on the tacit assumption that all ammonium salts had similar toxicity. This assumption seems to be confirmed since all ammonium salts injected intraperitoneally at the same concentration as that of ammonium chloride initiated comatose convulsions and death in all the animals under experiment (Table II). The interval between injection and onset of coma varied by a factor of 2 when these salts were compared among themselves. This suggests a delay in the toxic action of some of them even if the final end was comatose convulsions and death of rats.

The toxicity of ammonium citrate and oxalate appeared to develop in a manner somewhat different from that of the other ammonium compounds. In this case according to Warren the death was related to hypocalcemic tetany induced by chelation of calcium by citrate and oxalate moieties[21].

The various times in the manifestation of comatose state after the injection of these ammonium salts can be explained by the different speeds with which ammonia enters from the blood into brain cells by passing across the cell membrane. Indeed cell membranes of neurons are formed by lipid layers permeated by aqueous pores which represent only a small part of the total surface area. Compounds highly soluble in lipids can penetrate by a larger surface than those which are water soluble and lipid-insoluble. The latter substances penetrate only through the aqueous pores. In accord with this general assumption, the ammonium ion (NH_4^+) was found to be highly soluble in water and the speed of its penetration into cell membranes was low. The free ammonia (NH_3) was very soluble into lipids and could penetrate cell membranes more rapidly[2,18]. This passage across the blood-brain-barrier was related by several workers[3,21,22] to blood pH although other investigators have found no such relationship[7,9,10].

Our results (Table II) have shown that the various times for the manifestation of comatose state obtained after the injection of three selected ammonium salts were related to the dissociation speed of ammonium ion into free ammonia at the level of cell membrane. This dissociation seemed to be influenced in some way by the

anionic moiety itself or by the ionic strength. In fact the speed with which ammonia derived from ammonium bicarbonate crossed the cell membrane to enter into brain cells was more elevated than that of ammonium phosphate. Consequently these various speeds were reflected by the ammonia brain and blood levels and also the interval before the onset. This relationship could not be explained by variations observed in blood pH since ammonium sulfate produced convulsions more rapidly than ammonium phosphate.

Even if the final end is comatose convulsions and death of rats, this delay resulting from the different dissociation speeds of ammonium salts has an important influence on the protective action of L-arginine. In this way, the percentage of surviving rats obtained by an injection of L-arginine prior to that of ammonium phosphate or sulfate confirmed the effectiveness of this compound against ammonia intoxication (Fig. 2). Nevertheless, L-arginine gives almost no protective effect against ammonium bicarbonate. The toxicity of this salt is due to the ammonium moiety and not to the anionic component[21]. These data seem to be of primordial importance when we consider the fundamental role played by bicarbonate ions in the organism and the possible relationship of this ion with ammonia during intoxication due to hepatic failure.

Besides L-arginine, a few other compounds have an antitoxic action against ammonia intoxication. From our results (Table III), the following interesting observations can be made. The three amino acids essential for the synthesis of urea, i.e. L-arginine, L-citrulline and L-ornithine gave the most effective protection against experimental hyperammonaemia. The results obtained with arginine methyl ester are probably due to the fact that this compound is hydrolysed by the tissue esterases to free arginine. To be efficient, arginine must have its chemical structure intact. Our results are in agreement with those of other workers[4,5,8,15,17].

The significant protection noted after an injection of ascorbic acid is important and interesting from a biochemical point of view. The protective mechanism of this compound is probably different from that of the three Krebs-Henseleit amino acids. This vitamin has been used in normal and cirrhotic patients and was effective against hyperammonaemia[23].

The intraperitoneal injection of L-arginine in living rats points out to the problem of transport and passage of this compound across the cell membrane; problem of primordial importance at the level of brain where the entry of almost all substances is dependent on the properties of the "blood-brain-barrier".

The use of autoradiographic technique shows that radioactive arginine and citrulline do not accumulate significantly in brain[20] (Fig. 3). It may be argued that all ^{14}C-arginine and ^{14}C-citrulline injected intraperitoneally is rapidly metabolized by liver or rapidly eliminated by kidneys. This seems to be confirmed since radioactive arginine is concentrated in liver and radioactive citrulline, in kidneys. Nevertheless, the autoradiographic studies obtained after 1 hour indicate clearly that a fairly large quantity of radioactive material is still concentrated around mesentery, intestine and peritoneal walls. In this line of reasoning, it is possible to conceive that arginine and citrulline have retarded the diffusion of ammonium sulfate from peritoneal fluid into blood by acting as a kind of barrier on peritoneal walls.

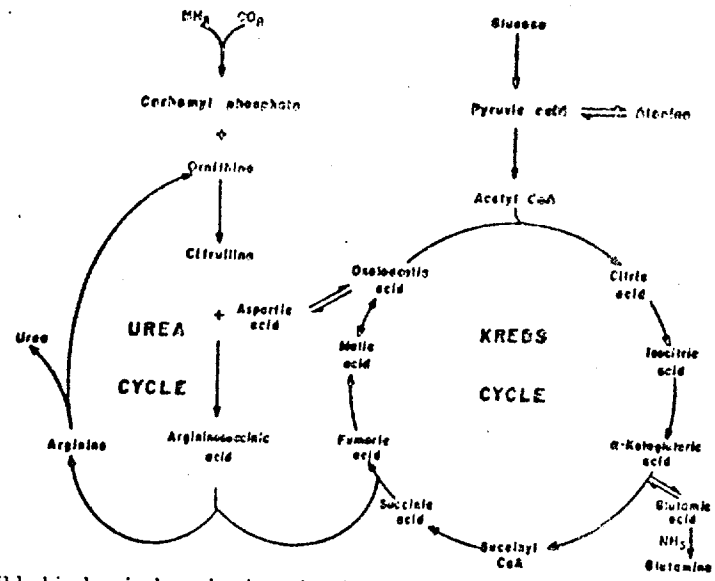

FIG. 4: Possible biochemical mechanisms involved in ammonia intoxication and detoxication by protection.

Such a protective mechanism at the blood-brain-barrier or at the level of peritoneal walls must be viewed with caution since the three amino acids arginine, citrulline and ornithine, effective against ammonia intoxication in rats, become completely ineffective when they are injected to chicks 30 min prior to the injection of a lethal dose of ammonium sulfate (Table IV). An important difference between these animals and rats is that in the former ammonia is eliminated as uric acid and no urea cycle enzymes have been found in the livers of chicks[10]. Thus L-arginine and L-citrulline act against ammonia intoxication by a biochemical mechanism involved in liver.

With these results, it would be now possible to postulate the biochemical mechanisms which could involved in ammonia intoxication and detoxication (Fig. 4). The normal biochemical mechanism to prevent the toxic accumulation of ammonia in brain would be the synthesis of glutamine. After a massive injection of ammonium salts, this protective mechanism would be exhausted and to prevent ammonia intoxication, there would be reamination of pyruvic, a-ketoglutaric and oxaloacetic acids producing a diminution in Krebs cycle substrates, a diminution in respiration and in energy production with concomitant convulsions. The injection of arginine would stimulate directly or indirectly the activities of the urea cycle enzymes in liver causing a decrease in ammonia level in blood and also in brain. Experimental verifications of these biochemical mechanisms are now in progress in our laboratory.

ABRÉGÉ — L'injection intrapéritonéale de treize sels d'ammonium à la concentration de 10.6 mmoles de NH_4-N par kg de poids corporel s'est révélée toxique pour tous les rats selon nos conditions expérimentales. Les variations de temps que nous observons dans l'apparition des convulsions après l'injection de sulfate, de bicarbonate et de phosphate d'ammonium ne sont pas dues aux variations du pH sanguin mais sont plutôt reliées aux vitesses de pénétration de l'ammoniaque à travers la barrière hématoéphalique. Ces différentes vitesses de dissociation de l'ion ammonium en ammoniaque libre conditionnent l'efficacité de l'arginine comme substance protectrice. L'injection de cette substance protège les rats qui ont

reçu le phosphate et le sulfate d'ammonium mais elle ne donne qu'une protection relative (70%) avec le bicarbonate d'ammonium. Chez le rat, les substances les plus efficaces contre une telle intoxication sont l'arginine, la citrulline, l'ornithine et l'acide ascorbique. L'arginine et la citrulline radioactives injectées intrapéritonéalement ne s'accumulent pas au niveau du cerveau. La protection manifestée par ces substances chez des poulets intoxiqués est nulle. Ces faits soulignent l'importance des mécanismes biochimiques lors de l'intoxication à l'ammoniaque et lors de la protection.

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No.41 (10):333-337

From the First Medicinal Clinic of the Latvian University in Riga (Director: Prof. M. Sihle)

Experiments on the Influence of Several Gas and Vapor Forming Media on the Human Organism

by Dr. K. Rudsit, Assistant at the Clinic
Foreword by Prof. M. Sihle

Gas and vapor forming media have long been in use for therapeutic purposes, and in fact, chiefly for the purpose of influencing certain diseased conditions in the respiratory tract. Little is known of a possible effectiveness of vapor and gas forming media in relation to other hollow organs of the human organism. In the last war years - during my clinical activities in Odessa -- I began to examine the influence of several gas-forming substances on the digestive tract. However, the revolution soon set in, and the observations were interrupted for quite a long time. In recent years, here in Riga, I have resumed these observations, and with the help of my assistant Dr. Rudsit, have developed a certain organization in the examinations.

Of the body cavities susceptible to influence by the said media, we dealt with the stomach, the intestine, the bladder, and the renal pelvis. We did not undertake examinations of the vagina. Our purpose lay in a tenting to obtain an approximate overview of both the direct and the reflex-induced modes of reaction of the said hollow organs. Further, we wished to determine whether any of the media possess disinfecting, deodorizing, or detoxicating capabilities, whether there might appear future reflex effects on the blood-cell formula or on the entire organism, and to determine whether and which of the media have, in a specific concentration, a hyper- or hypoaesthetic, an inflammatory or inflammation-diminishing effect, etc.

On the basis of all these observations it was then to be considered and examined whether a therapeutical significance could be attributed to any of the media employed, all the more because Dr. Pernou of Riga believes to have obtained good results from formalin and iodine, the clinical effectiveness of which he asked us to verify. We are not nearly so optimistic as said doctor. Although we have in certain cases received the impression that one or another of the media could in fact have a certain therapeutic value, it is nonetheless clear to us that one cannot be too cautious in drawing therapeutic conclusions, and that, therefore, concerning this aspect of the inquiries, it will only be possible after a sufficiently lengthy observation period to report anything essential.

A. On the influence of gas forming media on gastric secretion (with a contribution to the question of paradoxic gastric secretion)

In recent years, there has appeared a whole series of articles which regard as insufficient the methods of gastric juice analysis which have hitherto been used for diagnostic purposes, and therefore propose new methods. As is known, the diagnostic difficulties are founded to a large extent in the fact that the composition of the gastric juice which is siphoned out is dependent upon many factors which are not easily controllable quantitatively and qualitatively, such as the secretion and the motility, the backflow of duodenal juice, as well as the amount and composition of the test meal. These last two factors are the only ones that we can vary to our liking within certain limits, by, for example, applying either Ewald-Boas's procedure, or the Boas dry meal, or the fractionated siphonage method of Ehrmann. Each one of these methods has its advantages. The Ewald test meal corresponds for the most part to the usual concept of the meal. With the Boas dry meal, it is believed that one can obtain an almost undiluted gastric juice. With the method of fractionated siphonage, we can obtain in general a better total overview of the entire secretion process, since the hydrochloric acid is not bound after the introduction of a protein-free fluid, as is the case in the alcohol process of Ehrmann. However, each of the processes also has certain disadvantages. In the first (Ewald) the hydrochloric acid is partially bound, partially diluted. The meal acts as a poor digestive stimulus. The second method (the Boas dry meal) does not guarantee that the gastric juice remains undiluted (admixture of saliva); the hydrochloric acid is bound, as in the Ewald method. Besides that, we come across instances where the gastric juice is so thin that there is virtually nothing to be siphoned out. In the method of fractionated siphonage after a liquid test meal (Talma, Fuld, Ehrmann, etc.), the fact of the dilution of the gastric juice remains an unavoidable source of error. In the Ehrmann method we often see a strong duodenal backflow (Woenckhaus[1]), by which the stomach hydrochloric acid is bound.

Our present task is not to check off all the hitherto existing methods of gastric juice analysis and to criticize them. We should just like to point out that so far there exists no method in which the gastric secretagogue factor does not bind with the hydrochloric acid, or does not dilute it. In our examinations of secretion in a certain number of patients we have therefore, upon the instigation of Dr. Fähle, refrained from using solid and liquid stimuli, and have begun to test how gaseous and vaporous substances influence the secretory action of the stomach. Gases have been introduced in certain cases, admittedly as diagnostic aids for specific stomach diseases, e.g. for the purpose of expanding the stomach; it

appears, however, that it has not hitherto been attempted to employ gas as a secretagogue medium. In order to achieve something positive in this direction, we must first of all ascertain whether gases are at all capable of functioning in a secretagogue manner on the gastric mucosa.

We undertook our experiments on 88 individuals, employing gasified formalin, tetraiodine, ammonium causticum, oil of menthol, oil of mustard, allylsulphide and oxygen, whereby it became evident that all these substances, with the exception of oxygen, really could promote a secretion of gastric juice. All the control experiments with oxygen revealed that this substance is not capable of any stimulation of secretion. From the same experiments we also came to another conclusion, namely, that in and of itself the mechanical influence of the flatulence of the stomach caused by the introduction of an indifferent gas does not evoke any secretion. The control experiments were of course not carried out on the same days on which the insufflations took place. Of all the above-named substances, formalin was the one we selected for our further investigations, because it was also useful to us for other purposes. We used 2-3 drops of medicinal 40% formalin, gasified it by heating it in a small piston, and insufflated the gas which formed therein by means of a stomach and duodenal probe, with the aid of a small rubber bulb and a size 150 cm^3 syringe. The amount of the insufflated gas volume usually equalled 100 to 150 cm^3. Since under the conditions of our experiment a portion of the gas created in the piston remained in the piston or the probe as a condensational residue, we estimated to have introduced only about one drop of medicinal formalin solution into the stomach. One source of error of this method which must be taken into consideration is the fact that after insufflation belching occasionally occured, whereby of course a portion of the gas was emptied from the stomach, and the amount of the secretory stimulus being examined was reduced. Cases were even observed where the belching began immediately after the introduction of just the smallest amounts of gas, so that the gas method proved to be entirely unsuitable for these cases. Experiments with gasified formalin were carried out on a total of 50 people, and in 20 of these cases, a control experiment using Ehrmann's method, or the Ewald test meal, were made on the following day. The experiments were carried out with a slender drop probe, and the juice was drained out with a syringe every ten minutes. Most of the siphonage was done with the patient lying down, as this position, according to Schoendube and Kalk[12], impedes the regurgitation of the contents of the duodenum to a certain degree. For the present, on the basis of our experiments, we are able to conclude the following:

1. The formalin gas promotes the secretion of stomach hydrochloric acid.

2. The peak of the HCl secretion curve after insufflation was on the average somewhat higher than that obtained with the Ewald meal; in some cases however, the formalin secretion curve far surpassed the Ewald and alcohol curves.

3. The maximum of acidity was reached in ten minutes after insufflation in 4 cases, in 20 minutes in 8 cases, 30 minutes in 13 cases, 40 minutes in 9 cases, 50 minutes in one case, and in 90 minutes in several others. The maximum is therefore attained sooner than it generally is by using either the method of the Ewald meal or the alcohol potion.

4. The secretion and acidity curves are not consistently parallel to one another. That is to say, that in some cases we obtained the most plentiful gastric juice ten minutes after insufflation, but the presence of any hydrochloric acid could not be established. The color of the secretion betrayed no traces of bile. The regurgitation of the pancreatic juice occurs, according to Iwanow, simultaneously with the appearance of bile. Other authors as well (Sallinger[2]) recognize the duodenal backflow in the bilious color of the extract. We believe that such cases can be so interpreted, that in these instances, the maximum of secretion had already been reached before the secretion of hydrochloric acid had even begun. Certainly the possibility of a dissociated regurgitation of the duodenal juice and the bile (Iwanow[3]) cannot be entirely dismissed. However, it is noteworthy that the secretion produced an acid reaction, so that it could not have originated from the duodenum alone. Secondly, in all the cited instances, we did not observe any backflow of bile during the entire observation period; these cases were therefore not inclined to regurgitation. Finally, the large quantity of the secretion (80 cm^3 in one case) seems to speak for gastric secretion.

5. The examination of the pepsin values yielded no corresponding results, so that we can agree entirely with those authors who maintain that the fractionated examination of pepsin is of no essential significance. (Manasse[4])

It has often been emphasized that, particularly in the cases of clinical function tests, a single standard procedure should be used, in which the direction of the experiments and the conditions are always the same. Our gas method is naturally not to be interpreted as an ideal one; it does seem to us, however, that with improvement, the technique of this procedure might have certain advantages, and especially in those cases where it is a question of obtaining a pure gastric juice without binding or diluting of the hydrochloric acid. We now wish to cite thoses cases in which the gas method seems to find its justification.

It is generally known that there are cases where the gastric juice of a fasting stomach plainly reveals a high level of HCl, whereas after a meal, it yields a low one. These cases are often overlooked, or explained by the supposition that the HCl was either diluted or bound by the meal. Where this does not seem to be the case, we can assume, according to Lawlow's views, that the high HCl-values of the fasting stomach are to be interpreted as the result of a psychic stimulation of secretion, and the low values after the meal as the consequence of a psychic check. The possibility of a neutralization of the gastric juice by the duodenal juice also comes into question. When we examined such cases with the gas method we usually

found, instead of a decrease in acidity such as occurs after the Ewald or Ehrmann meals, a definite increase in the HCl values. In such cases, the decrease in acidity after the Ewald or Ehrmann meals can be viewed as a consequence of thinning or binding. Psychic influences may also contribute. When we sought to pursue our question further, we came across three cases where a decrease in acidity took place after the test insufflation of the gas. However, upon repetition of the same procedure, a normal reaction occurred. We believe that these cases may be explained by the fact that the first time we observed a strong psychic secretion in a fasting stomach, which was followed after insufflation by a weak chemical secretion. In all these cases, we were dealing with individuals who are easily stimulated psychically. The first was a businessman who had had to interrupt pressing business because of his illness and had therefore suffered losses; the second was a traumatic neurotic, and the third suffered from psychogenic diarrhea.

Finally, we also observed cases where the high HCl values in the fasting stomach fell to zero after insufflation of gas, and this reaction persisted even upon repetition of the experiment.

B. Paradoxic gastric secretion

It seemed doubtful to us whether in the just cited cases it could have been merely a question of psychic influence upon secretion. Indeed, it is even maintained by some (Vandorfy[5]) "that the acidity values of the contents of the fasting stomach are never higher than the maximal values which appear after a suitable digestive stimulus is introduced", and that "if one obtains a superacid content in the probe of a fasting stomach", then one may well conclude that one can also obtain superacid values after the introduction of a suitable digestive stimulus. The same conclusions can be drawn from the material of Michelson[11]. We do not agree these views insofar as we admit the existence of cases where the secretion, owing to the strong psychic predisposition of the patient, can be reduced even after a suitable (in Vandorfy's sense) test meal. However, we could also fully agree with Vandorfy if we considered each digestive stimulus unsuitable, that is connected with the examination of a patient who is strongly susceptible to psychic influence. On the basis of all these considerations, we were inclined to view the decrease in secretion in the above-mentioned cases as the direct response of the gastric mucosa itself to the stimulus, which under other circumstances acted in a secretagogue manner. Here I should like to point out several works which can to a certain extent support our assumption. It is namely known (Kalk and Siebert[13]) that in some cases the stomach can react to the intake of atropine with an intensification of secretion. Sallinger[2], for his part, found that there are cases of a sinking in the hydrochloric acid values, for the explanation of which he could find no clues (p.341), and he attempts to explain this occurrence as the blunting of the acid through the activity of the stomach itself (p.343).

It remains unclear whether Sallinger considers the so-called gastric water secretion -- the alkali secretion of the stomach according to Vandorfy -- an acid-blunting factor, or whether he has thought of the possibility which we have just expressed. Now, it could be objected that it would be perhaps too bold to consider this last possibility before a more objective support for such a view is produced. The clues, on the basis of which we would be justified in assuming the possibility of the above-named paradoxic gastric activity, were, however, soon to be found.

Here I should like to draw attention to my work ("On leucocytic blood changes under the influence of physicochemical processes"), although it does not directly touch upon the questions which are being dealt with here. In this article I showed that the number of leucocytes in the peripheral blood can be influenced by the intravenous injection of chemical substances. These changes continue for some two minutes after the injection, after which they disappear. Among other things it was found that the injection of $CaCl_2$ caused an increase in the number of leucocytes. In the same article it is also mentioned that, on the other hand, in some pathological cases, the $CaCl_2$ injection evoked a state of leukopenia. When we tested the $CaCl_2$ phenomenon parallel with our gastric secretion experiments, we consistently found a leucocytosis, with the exception of exactly those cases in which we had ascertained the above-mentioned paradoxic gastric secretion reaction to gases. We find it difficult to believe that this was a question of coincidence in all these cases. We had to examine whether a regularity can be found in this coincidence. We were able finally to predetermine the paradoxic reaction of the stomach with the help of the corresponding leukopenic $CaCl_2$ phenomenon. It was proven besides that in the case of a normal $CaCl_2$ reaction (leucocytosis), different types of gastric secretion can indeed be observed, but never a paradoxic one in the manner induced by the gas stimulus. On the other hand, a reversal of the $CaCl_2$ effect combined only either with an achylically or with a paradoxically reacting stomach. Of great importance finally is the circumstance that besides the reversal of the $CaCl_2$ reaction, we were also able to find, in the case of paradoxic gastric secretion, some other phenomena of a paradoxic nature peculiar to these cases. In consideration of this coincidence, we should like to draw the conclusion that, in the cases referred to, we are dealing with a pathologically altered reaction or constitution, which is capable under appropriate conditions of releasing a whole chain of paradoxic reactions.

Paradoxic gastric secretion may therefore be interpreted as a necessary consequence of certain pathological facts or tendencies. At present I should prefer not say anything more definite about the abovementioned remaining paradoxic reactions, since a special article will be devoted to this question.

In the literature to which I have access I could find, with the exception of the aforementioned three unexplained cases of Sallinger, no reference to the existence of a description of paradoxic gastric secretion. There is now no

doubt about the occurrence of such an abnormal secretory gastric function, and we may designate it terminologically as "paradoxochylia" or "parachylia".

Parachylia is a rather rare phenomenon. In 20 cases, during which we were occupied with this question, we found, among circa 1500 patients, only 8 parachylics.

Physiologically (theoretically) we divide gastric secretion into the resting stage and the functioning stage. We observed the first in a fasting state, the second at the time of digestion. If we wished to determine all the theoretically possible conditions a priori, we could put together the following table.

		1	2	
		Nüchtern	Nach dem Sekretions-Reiz	Termina
Physiologisch	1.	—	+	Normaciditas
	2.	—	+	Ansubaciditas
	3.	+	++	Hyperaciditas
Pathologisch	4.	+	—	Parachylia resp. Paraciditas
	5.	+	+	Isochylia (??)

1 = fasting stomach 2 = after the secretion stimulus
3 = physiological 4 = pathological

These types seem to exhaust all mathematically conceivable possibilities. (We have not included heterochylia, as it is only to be viewed as a variation of a type.) The first three types are all generally known, and their equivalents are used daily. The fourth possibility is the one which I term parachylia, or respectively par aciditas. The fifth mathematically conceivable case, which may be designated as isochylia, should be understood as a case in which the secretion is established at a relatively high level, and cannot be forced from this level by any secretagogue exogenous stimulus. That would be the case of a ventricle-automatism, when for certain as yet undetermined pathological reasons, the stomach has lost the capability of reaction to exogenous stimuli. At present we cannot yet establish to what extent and how often such an isochylia occurs. We have only expressed these thoughts because of those cases in which the HCl values in the gastric juice exhibited almost no vacillations over the course of an hour. Unfortunately, a more extensive observation of these cases could not be carried out; we were, however, able to establish that both times a reverse $CaCl_2$ reaction, or a tendency toward parachylia, was revealed. The other question which concerns us is whether we can draw any practical therapeutical conclusions from the diagnosis of parachylia. The number of observed parachylics is much too small to make a decisive conclusion possible, but we can note certain peculiar characteristics, i.e., there were cases which

could be interpreted therapeutically as subacidic, but which tolerated no stomachics ingested before the meal. Two cases could be interpreted therapeutically as hyperacidic. In these cases, the subjective complaints disappeared after doses of stomachics. In our opinion, the stomachics (tincture of cinchona, tincture of bitters) functioned in both cases in a secretion-decreasing manner. Where the parachylic reaction took place in low figures, the stomachics produced an anacid condition, but where the parachylic reaction produced high figures, we were only able to effect a reduction in the excess with the stomachics.

In relation to parachylia, we should yet like to observe the following: if we interpret gastric secretion as a reflex action, then we must assume, in the case of parachylia, the existence of an increased vagal tone in a fasting state. The vagal tone would therefore have to be so highly increased before the test stimulus is administered, that the attempt to achieve a further increase would have to result in overstimulation. As a consequence of this overstimulation, a weakening of the vagus could be expected, and as the manifestation of that -- a decrease in secretion. But this explanation cannot thoroughly satisfy us, for the following reasons:

1. It remains unanswered why secretion (and respectively the vagal tone) is elevated in a fasting state.

2. The connection between parachylia and the reverse $CaCl_2$ effect on the leucocyte count is not explained, for the $CaCl_2$ phenomenon, as we have explained in the above-cited work, can by no means be interpreted as the consequence of a change in the vagal tone, or in the tonus of the sympathetic nervous system. In the same way, the connection between parachylia and other paradoxic phenomena, which are still to be discussed, remains unexplained.

We do not intend to question that the autonomic nervous system participates in all life processes. But as for what concerns the question with which we are dealing, it seems to us necessary to find an explanation which renders all the parallel appearing symptoms comprehensible, without operating with vague and abstract concepts ("tonus", "stimulus", etc). After all our deliberations, we reached the conviction that in certain pathological conditions, parachylia must inevitably appear, when certain definite alterations in the chemistry of the stomach wall are present. On the basis of clinical observations, other authors have also reached the conviction that in some cases the anomaly of the secretion of gastric juice is only understandable when we admit primary alterations in the chemistry of the secretory cells themselves (Strumpel[7]). Some authors, who do not take chemical factors into account, speak of "regional circumstances", of the asthenia of secretion (Boenheim[8]).

The gastric gland cell can be viewed as a colloidal particle, which functions in a particular milieu. The nature and direction of this function is dependent upon the environment. There is no doubt that the function of the gastric gland is inhibited by certain chemical substances, and stimulated by others. Depending upon the circumstances, at one time the effect of the inhibiting

substances is stronger, at others, that of the stimulating substances. Whether these substances have their origin in the activity of the autonomic nervous system, as Otto Löwe and Asher have shown, or whether they originate in the course of the metabolic process, as do carnosine and carnitine (Krimberg[9]), is not essential to our further discussion. When therefore a change takes place in the composition of the environment concerned, this can lead to a reversal of the effect of a stimulus. Whether the chemical reversibility of many reactions lies at the basis of a paradoxic gastric activity remains unclear, although it cannot be doubted that even during a parachylic gastric secretion reversible chemical reactions take place in the stomach wall. Reversibility is, of course -- as physiological chemistry teaches us, and Knoop[10] stresses in his recent essays -- indispensible to life. Since however we come across a chain of other reverse (paradoxic) reactions in cases of parachylia, it would be plausible to wonder whether a common reason, a common cause for all paradoxic phenomena might not be found. Fluid substances, in which, from the standpoint of physical chemistry, the ions are especially important, are common to the stomach, the blood, and all other organs. In the light of physical chemistry, there would seem to come into question in this respect, besides reversibility, the possibility as well of a reverse colloidal reaction in Hoffmeister's sense. If we accept the possibility of a reversal in Hoffmeister's sense, then we can arrive at the following consideration: the colloid (the secretory cell) responds to a secretagogue stimulus in the case of parachylia with a reduction in function, due to a concentration of H-ions which has exceeded the isoelectric point of this colloid. This interpretation, which seems acceptable to us, will doubtless not be acknowledged by many, because physical chemistry declares that no acid reactions can take place in the blood of a living organism. Even though we do not wish by any means to question the experimentation methods of physical chemistry, even if we assume that the discovered H-ion values agree exactly with reality, we must nonetheless at present stress that the values of H-ion concentration found employing the most exact methods are only valid for fluid *in vitro*, not *in vivo* in the organism.

Summary

A) 1. The insufflation of certain above-mentioned gases into the stomach promotes gastric secretion.

2. The gastric secretion curve after insufflation is steeper and shorter in comparison with the secretion curves obtained by the methods of Ehrmann and Ewald.

3. The gas method possesses certain advantages over the other methods in relation to binding and thinning of the hydrochloric acid in the stomach.

4. The gas method is diagnostically useful with regard to paradoxic gastric secretion.

B) 1. Paradoxic gastric secretion may be designated terminologically as parachylia or par aciditas.

2. Parachylia can be viewed as the manifestation of a constitution pathologically altered in the chemical sense.

3. Parachylia might possibly be explained in a purely physicochemical light.

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TOXICITY IN THE RAT OF D-AMINO ACIDS, ISONICOTINOYL HYDRAZIDE, AND AMMONIUM CHLORIDE

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Naples Section -- Meeting of March 15 1954

The therapeutic antitubercular action of isonicotinoyl hydrazide is indeed limited by a series of toxic phenomena, the most evident manifestations of which develop in the ganglion of the central nervous system. (1)

Of maximum importance is, therefore, the study of the relation between the toxicity and the chemical structure of both the hydrazide and the products of demolition in the animal organism which derive from it.

The numerous and now irrefutable series of experiments of Cedrangolo et al. (2) indicate that one of the principal factors of toxicity of hydrazide is the ammonia which is enzymatically freed (3) from the molecule of INI.*

In fact, the convulsive symptomology following the administration of hydrazide is in all ways analogous to that following the administration of ammonium salts (4.5), and like glutammic acid is capable, in suitable doses, of neutralizing the toxicity of NH_4Cl (6), and thus is also in a position to neutralize the toxic phenomena due to the administration of hydrazide (5,7,8). In both cases the glutammic acid would bind with the ammonium cation into an undissociable compound: glutamine (5,7); the enzyme responsible for such syntheses is the glutamminose present chiefly in the kidney and the central nervous system.

The present research tends to confirm the very important role played by NH_3 in the toxic activity of hydrazide. Towards this end, since Kamin and Handler (10) had caused death in a dog in consequence of intravenous injections of D-L amino acids, it seemed to me interesting to compare the toxic effects resulting from hydrazide and ammonium chloride with those of the amino acids; besides this, I dosed the NH_3 present in the central nervous system of rats intoxicated with hydrazide and NH_4Cl.

Description of experiments and results. -- In masculine albino rats of our breeding, and in white mice, I injected D-alanine and L-alanine intramuscularly in increasing doses until death occurred. To incur death, doses of 6-6.5 g/kg of body weight were necessary; such high quantities are evidently

* Porcellati and Preziosi have described how hydrazide is split into isonicotinoyl acid and ammonia in various organ pulps (for every µmol of INI which disappears there are extraformed 2 µmol of NH_3). The tissues in which this enzymatic fission occurs principally are: the brain, the kidney, the liver, the muscles,

necessary because the velocity of the deaminative fission is such as to provoke a formation of NH_3 which cannot be entirely neutralized by the mechanism of the ureogenesis.

I was able to note a greater toxicity for the D-alanine than for the L-alanine: this fact is to be placed in relation to the noted greater activity of the D-amino acid oxidases (11) in comparison to the L-, for which the lethal concentration of NH_3 is more rapidly attained. We hope somehow to communicate later results concerning the toxicity of the D-amino acids, as well as the notable technical difficulties encountered because of their poor solubility.

Next there came the examination of the toxicity of hydrazide and ammonium chloride controlled once again by me: in masculine albino rats, fasting for 24 hours, 450 mg of NH_4Cl or 300 mg of hydrazide (solution distilled in H_2O) were injected endomuscularly. After 20-25' of the injection in the first case, and after 45-50' in the second case, convulsions of the kind already described (5) began.*

The greatest amount of time needed for the unchaining of the convulsions in the case of hydrazide is certain to be placed in relation to its enzymatic fission, by which the ammonia which is released increases progressively in the tissues until it attains the lethal concentration.

When NH_3 was dosed into the rats thus treated, they died a few minutes before the convulsions.* The central nervous system (encephalus) was removed and weighed, and then cut up into small pieces and then pounded in a mortar until reduced to a fine pulp. A double quantity of phosphate powder (acc. Sorensen) was added, at a pH of 7.4, and then tricloractic acid at 10% in a quantity equal to that of the entire suspension. This was then centrifuged at a speed of 3000 turns per 10', and in an aliquot of the remains NH_3 was determined in the micro-Kjeldahl.

The experiment reported in the table represents one example from among numerous parallel examples, which yielded concordant results.

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the heart; wishing to make a comparison between the various tissues, this is the result which I measured: for every 100 disappearances of INI in the liver, there were 217, 133, 97, and 35 respectively for the brain, the kidney, the muscle and the heart. Besides this, the authors cited also observed that the lung, the spleen and the testicles do not split INI to an appreciable degree.

* Such doses should be considered for experiments conducted with various concentrations of the two substances (hydrazide and NH_4Cl), explained previously by Cedrangolo et al. (5) and later also by me as the minimum capacities which provoked death in a high percentage of rats (90-100%) within a few hours.

* In order to be sure of being found in the experimental conditions, we injected other control rats with the same doses, in which we observed the insurgence of convulsions, a condition which was always verified after 5' by the death of the rats on which the experiments were conducted.

TABLE -- Concentrations of NH_3 in the encephalus of rats intoxicated with ammonium chloride and hydrazide.

	µg di NH_3 per g	A. Differenza in µg di NH_3 rispetto al controllo
1. Ratto controllo	190	—
2. Ratto iniettato con 225 mg di NH_4Cl pro Kg (dose sub-letale)	220	+ 30
3. Ratto iniettato con 450 mg di NH_4Cl pro Kg (dose letale)	260	+ 70
4. Ratto iniettato con 300 mg di INI pro Kg (dose letale)	262	+ 72

1.= control rat
2.= rat injected with 225 mg of NH_4Cl per kg (sub-lethal dose)
3.= rat injected with 450 mg of NH_4Cl per kg (lethal dose)
4.= rat injected with 300 mg of INI per kg (lethal dose)
A.= Difference in µg NH_3 compared to control rat

From such results we can note immediately that the quantity of NH_3 in the central nervous system of the rats treated with lethal doses of NH_4Cl and that in the central nervous system of rats treated with hydrazide is substantially equal. This fact demonstrates once again that the instigator of the toxic phenomena, at least of those in the ganglion of the central nervous system, is in effect NH_3.

The lethal dose of hydrazide (300 mg/kg), which allowed a fission of 100% of the $-NH-NH_2$ group, might provide at most a quantity of NH_3 equal to 75 mg/kg. Now, 75 mg of ammonia correspond stechiometrically to 225 mg of ammonium chloride, which dose is certainly not lethal (see table); upon examination of the central nervous system there were found, for such a dose, only 30µg/g of extraformation of NH_3.

To obtain the lethal effect -- resulting, as has been seen, from the presence of about 70 µg/g of NH_3 in the central nervous system -- about 450 mg of ammonium chloride per kg of body weight are needed (see table), corresponding stechiometrically to 150 mg of NH_3.

Such an apparent disagreement is easily explained by the fact that while ammonium chloride distributes unifromly in all tissues, hydrazide splits more in the central nervous system and a few other tissues (3), and succeeds in provoking convulsions and death exactly because it permits the accumulation of that concentration of NH_3 in the encephalus which we have seen to be responsible for the aforesaid phenomena.

Conclusions. -- From the above quoted results of my research, the very important role exercised by ammonia in the toxic phenomena of hydrazide is once again demonstrated:

a) by the parallelism between the toxic phenomena of the D-amino acids, ammonium chloride and hydrazide in the rat;

b) by the direct dosage of ammonia into the central nervous system after the administration of lethal doses of ammonium chloride and hydrazide, which yielded the result of perfect identity of the concentration of NH_3 in the central nervous system of the above-mentioned animal.

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SODIUM AND WATER DEPLETION AND AMMONIA FORMATION BY THE KIDNEYS *

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Gamble and associates' work on the mechanism of action of acidifying salts (1, 2) originated the impression that the increase in renal excretion of ammonia in response to acidosis of several days' duration might be related to the loss of sodium and water induced by the first doses of the drug (3). This impression has never been adequately substantiated or disproven over the last three decades. In 1952 Peters (4) thus summarized his views on this matter: "The precise stimulus for the secretion of ammonia has not yet been identified. . . . The impression is given that it is substituted for sodium only after a certain amount of this base has been sacrificed and continues to be secreted until the normal supply of sodium in the body has been restored."

Pitts (5) admitted the same possibility while postulating that an increased secretion of adrenal cortical hormones, stimulated by sodium depletion, was the mechanism whereby ammonia excretion increased when ammonium chloride was given to rats.

The degree of acidosis does not seem to be the main regulating influence (6) because in Gamble's experiments ammonia excretion continues to increase even after the acidosis has reached its maximum, and minimal values of ammonia excretion occur while extracellular acidosis is being reversed. Many years ago Briggs (7, 8) expressed the opinion that renal excretion of ammonia had nothing to do with regulation of acid-base balance of the "milieu interieur," and that it served the only purpose of avoiding the harmful action of an excessively acid urine on the walls of the urinary tract. His views were based on experiments in which sodium sulfate solution was infused into dogs with subsequent increase in the excretion of ammonia in a urine of low pH, although there was no sodium or water depletion. Comparable findings had been reported by Hendrix and Sanders (9), and were recently demonstrated by Schwartz, Jenson and Rehman (10).

However, all those experiments in which an increase in ammonia excretion could be demonstrated in the absence of sodium and water depletion were of short duration (minutes to hours). It is very likely that this immediate increase in ammonia excretion results from the passage of preformed ammonia from within the tubular cells into the tubular lumen (11). The slow and stepwise increase in ammonia excretion over the period of several days, such as was seen in Gamble's experiments and subsequently confirmed by many others, seems to result from an intensified activity of ammonia-forming enzymes of the tubular cells (12), and might well be controlled by factors different from those which determine the release of preformed ammonia.

The following experiments were planned to investigate whether the depletion of sodium and water which follows the ingestion of acidifying salts is an indispensable factor in determining the coincident increase in urinary ammonia excretion.

Our results demonstrate that even in the absence of depletion of the subjects' own reserves of sodium and water, acidosis induced by ammonium chloride ingestion is accompanied by a stepwise increase in urinary ammonia, comparable to that seen in the experiments of Gamble, Blackfan and Hamilton (2) and of Sartorius, Roemmelt and Pitts (13). Although the increase in ammonia excretion does contribute to the economy of fixed cation, it is not the state of water and sodium depletion which constitutes the stimulating agent for the increase in activity of ammonia-forming enzymes of the renal tubular cells.

* This paper is based on a thesis accepted as part of the requirement for the Professorship of Medicine at the University of Bahia, Bahia, Brasil. The work was aided by a grant from the Conselho Nacional de Pesquisas (National Research Council), Brasil.

TABLE I

Effect of ammonium chloride on electrolyte metabolism

Day	Body weight*	Urine							Plasma*	Serum*		
		Vol.	pH	Ammonia	Titr. acid	Cl^-	Na^+	K^+	Total CO_2	Cl^-	Na^+	K^+
	Kg.	*ml./day*		*mEq./day*	*mEq./day*	*mEq./day*	*mEq./day*	*mEq./day*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*
1	56.00	1,620	7.10	24.29	8.75	328.2	392.8	64.8	25.4	102	140	4.1
2	57.80	2,920	7.03	39.71	16.64	663.1	695.0	77.7				
3	57.65	3,660	6.84	44.90	16.84	675.6	702.7	71.7				
4†	57.25	3,280	6.52	48.80	27.22		705.2	75.4	26.1	104	142	4.5
5	57.35	4,100	5.10	102.32	56.58	917.9	820.0	96.4	24.0	109	142	4.5
6	56.65	3,480	5.01	120.56	60.20	741.9	631.6	91.0	22.5	111	148	4.2
7	56.35	3,210	5.12	133.08	57.14	803.3	680.5	86.7	18.6	117		4.2
8	56.45								22.5		143	4.2

* Values in these columns were obtained at the beginning of each experimental day.
† 10 Gm. of ammonium chloride was administered orally on each day starting on the fourth day.

MATERIAL AND METHODS

Five healthy adults, aged 22 to 29 years, were maintained on a constant and adequate diet for a period of seven days, during which they ingested 2.5 L. of a solution containing 9 Gm. per L. of sodium chloride and 2.3 Gm. per L. of sodium bicarbonate. In addition, the subjects drank a constant amount of distilled water, which was always more than 1 L. per day.

The first three days of each experiment were taken as controls. From the fourth through the seventh day of each experiment, while still on the same diet and drinking the same amount of the above described solution, the subjects were given orally 10 Gm. of ammonium chloride daily, in four equally divided doses.

The urine was carefully collected under toluene for 24 hour periods, and on each specimen the following determinations were done: *1)* volume per 24 hours, expressed in milliliters; *2)* pH, measured with a "Cambridge" pH meter, Research Model; *3)* titratable acidity, by Henderson and Palmer's method, as described by Consolazio, Johnson and Marek (14); *4)* ammonia, by Conway's method (15); *5)* chlorides, by Van Slyke and Hiller's method, as described by Hawk, Bernard and Summerson (16); *6)* sodium, measured with a "Baird" flame photometer, using an internal standard of lithium sulfate; and *7)* potassium, same as for sodium. Values referring to concentration of ammonium, titratable acidity, chloride, sodium and potassium are expressed in mEq. per day.

Blood samples were taken at the beginning of Days 1, 4, 5, 6 and 7, and by the end of the seventh metabolic day. All specimens were taken from the veins of the metacarpal region after immersing the forearm in water at 46° C. for 20 minutes. One aliquot of blood was handled under anaerobic conditions and mixed with heparin. On the plasma separated from that blood, total carbon dioxide content was measured in a manometric Van Slyke apparatus. The rest of the blood was allowed to clot in contact with air in another test tube. On the serum separated therefrom, chloride, sodium and potassium concentrations were measured. The methods were the same as for the respective urine determinations.

TABLE II

Effect of ammonium chloride on electrolyte metabolism

Day	Body weight*	Urine							Plasma*	Serum*		
		Vol.	pH	Ammonia	Titr. acid	Cl^-	Na^+	K^+	Total CO_2	Cl^-	Na^+	K^+
	Kg.	*ml./day*		*mEq./day*	*mEq./day*	*mEq./day*	*mEq./day*	*mEq./day*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*
1	72.25	1,480	6.84	10.75	10.51	470.8	509.1	88.8	24.6	114	146	4.2
2	73.85	2,367	7.10	39.09	9.23	562.9	601.2	60.6				
3	74.00	3,460	6.93	16.03	18.03	746.2	751.5	65.0				
4†	73.50	4,100	6.64	56.09	30.34	936.0	910.2	70.5	28.5		150	4.5
5	72.30	2,550	5.25	62.76	43.09	780.3	772.6	72.7		115	147	4.9
6	72.30	3,150	4.98	105.80	45.51	827.5	715.0	85.0	19.8	112	146	4.6
7	71.95	3,360	5.05	126.64	49.06	791.3	756.0	90.7	18.4	112	146	4.5
8	71.90								19.2	113	145	4.4

* Values in these columns were obtained at the beginning of each experimental day.
† 10 Gm. of ammonium chloride was administered orally on each day starting on the fourth day.

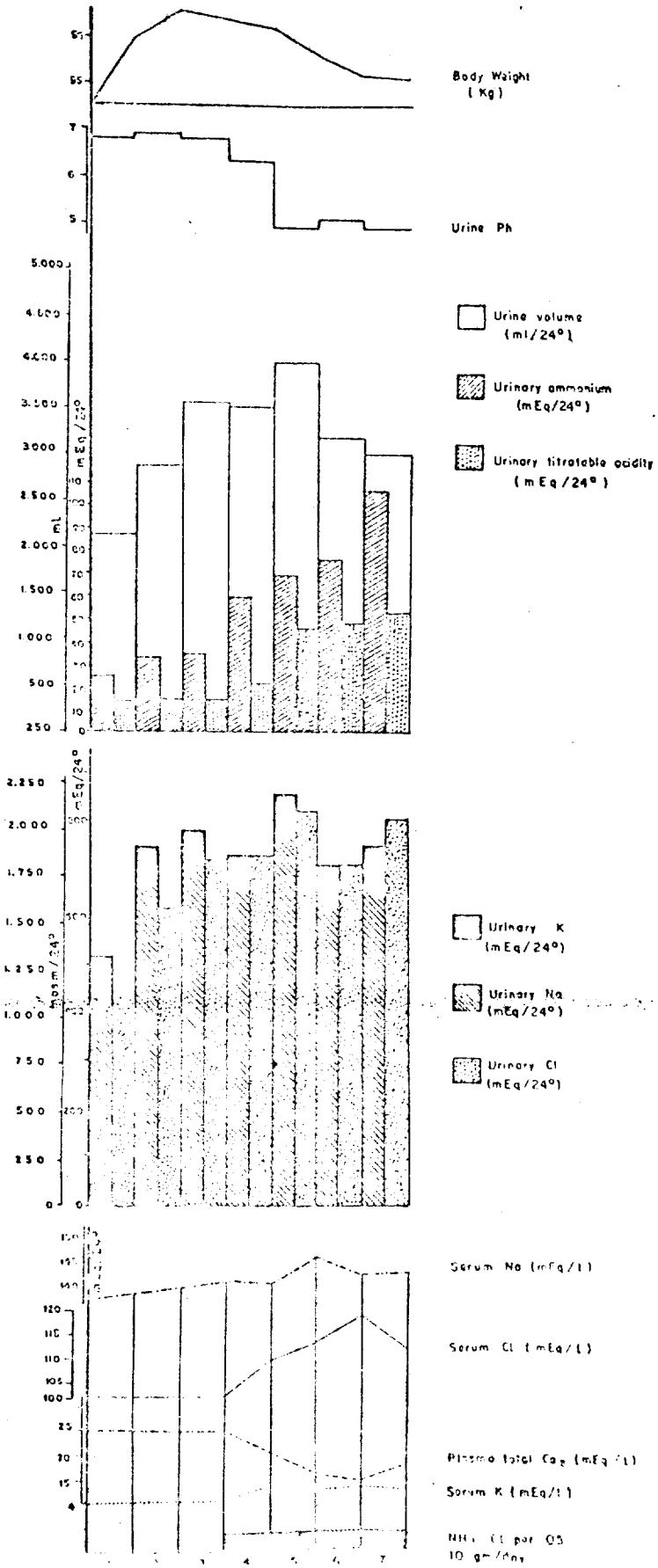

FIG. 1. EFFECT OF AMMONIUM CHLORIDE ON ELECTROLYTE BALANCE

During the first three days of the study, body water and sodium content were expanded by repeated ingestion of a solution containing sodium chloride and sodium bicarbonate. The ingestion of ammonium chloride starting on the fourth experimental day resulted in loss of body weight which, however, did not fall beyond control levels. In spite of the absence of depletion of the body's own stores of sodium and water, the progressive stepwise increase in urinary ammonia was observed.

RESULTS

The results of typical experiments are summarized in Figure 1 and in Tables I and II. None of the subjects complained of any untoward reactions to the experimental regimen. Throughout the experiments, they were capable of carrying on their routine activities in medical wards and laboratories.

Figure 1, for one of the typical experiments, shows how the body weight increased during the control days as a result of the ingestion of sodium chloride and bicarbonate solution. Ingestion of ammonium chloride starting on the fourth experimental day, with ensuing acidosis, caused a decrease in body weight which, however, did not reach precontrol levels. The subject did not sacrifice his own reserves of sodium and water. Ammonia excretion, which had been approximately constant during the control period, increased progressively in a stepwise fashion to levels comparable to those reported by others (2, 13) under conditions of progressive sodium and water depletion.

Results comparable to those described above were also found in two other experiments, one of which is shown in Table I. Two other subjects were also submitted to the same experimental regimen, but their body weights fell to less than precontrol levels (see Table II) during administration of ammonium chloride. They, however, showed an increase in urinary ammonia excretion comparable to that found in the three subjects who had obviously not been depleted of their own reserves of sodium and water.

Control levels of urinary pH were high, and those of titratable acidity were low in all experiments consequent to the ingestion of sodium bicarbonate. As would be expected, urinary pH decreased and the urinary excretion of chloride and titratable acid increased in all experiments while ammonium chloride was being administered.

The ingestion of ammonium chloride resulted in a marked increase in sodium and in total solutes excretion over and above control levels which were already high. However, we did not find a progressive decrease in the urinary excretion of sodium over several days, such as Gamble and coworkers (2) and Sartorius and associates (13) described in subjects who were being depleted of sodium and water. Nor was the increase in potassium excretion following ammonium chloride administration of such magnitude as that found in Sartorius' experiments.

The total carbon dioxide content of the arterial plasma increased during the control days, due to the ingestion of sodium bicarbonate, and decreased when ammonium chloride was administered. By the fourth day of acidosis, the carbon dioxide content of the plasma was increasing while ammonium chloride was still being given. Serum chloride values were high in all experiments during the control periods and increased further when ammonium chloride was administered. Serum sodium concentration increased during the control days and showed a slight and irregular decrease while the subjects were on ammonium chloride. No regular pattern could be made out of the variations in serum potassium concentration.

DISCUSSION

Gamble and associates demonstrated that the excretion of fixed cation is maximal on the first day of administration of an acidifying salt and decreases progressively on the following days, while ammonia excretion increases (2). The initial loss of fixed cation is associated with high urinary volume and loss of weight. After discontinuing the acidifying salt, a positive sodium balance occurs, and in spite of elevation of urinary pH, urinary ammonia excretion continues at high levels. This was thought to last until the body regained its stores of fixed cations and water.

Our results demonstrate the same characteristic stepwise increase in ammonia excretion consequent to the ingestion of an acidifying salt over several days, even when the depletion of sodium and water was avoided.

Various types of experiments, some of which were published long ago, have shown that urinary ammonia excretion can increase in the absence of sodium and water depletion. This appears, for instance, when normal human subjects breathe gas mixtures containing carbon dioxide in a concentration of 5 to 6 per cent (17), as well as after infusion of sodium salts of nonreabsorbable anions (8-10). In all those experiments urinary pH was suddenly brought to very low levels.

Several lines of evidence suggest that renal

excretion of ammonia can be modified according to two entirely different patterns. Sudden changes in urinary pH are promptly accompanied by the *release* of larger or smaller amounts of ammonia, which very likely were previously formed and present inside the tubular cells (11). On the other hand, conditions prevailing inside the tubular cells seem to determine a faster or slower rate of *formation* of ammonia (12). Thus, a drop in urinary ammonia was found to occur simultaneously with an increase in urinary pH caused by carbonic anhydrase inhibitors (18), while the prolonged use of carbonic anhydrase inhibitors, with ensuing acidosis, was found to cause increase in urinary ammonia excretion (19). This difference in patterns of change in urinary ammonia excretion is further illustrated by data reported by Sartorius (13), who measured the immediate change in ammonia excretion after administration of ammonium chloride to normal humans and found an increase of several fold within the first two hours. This rate was obviously not maintained throughout the several days during which ammonium chloride was given. It is likely that this large initial increase was due to transfer of preformed ammonia from within the tubular cells to the tubular lumen, as a result of sudden acidification of the urine. The progressive, but much slower, increase of several days' duration would result from the intensified action of ammonia-forming enzymes under the stimulus of acidosis.

These two patterns of change in urinary ammonia excretion seem to be influenced by factors of a different order. Increased ammonia excretion in the absence of sodium and water depletion had been found during experiments of a few hours' duration. This did not rule out the possibility that the same factor of sodium and water depletion might be the stimulus for the stepwise progressive increase in ammonia excretion consequent to the administration of acidifying salts for several days.

In our experiments ammonium chloride was given to normal human subjects preloaded with sodium chloride and bicarbonate. Sodium bicarbonate was added in order to avoid the acidifying effect of large amounts of isotonic sodium chloride solution given prior to the administration of ammonium chloride. Administration of ammonium chloride from the fourth to the seventh experimental days resulted in loss of some of the surplus of water and fixed cation, but in three out of the five experiments, body weight did not reach precontrol levels during the experimental period. These subjects excreted the excess chloride ingested as ammonium chloride along with the fixed cation and the water accumulated during the control days, and did not have to sacrifice their own reserves of sodium and water. The progressive decrease in sodium excretion, which in the experiments of Gamble and of Sartorius indicated the need for the body to save fixed cation, was not present in our experiments. In spite of that, the daily increase in ammonia excretion proceeded at a rate comparable to that described by Gamble (2), and subsequently confirmed by many others (13, 20, 21), under conditions in which water and sodium depletion did take place. No significant difference in the pattern of ammonia excretion could be detected amongst our own subjects, between those whose body weight remained above precontrol levels throughout the experiment and those who lost some of their own reserves of sodium and water.

The factors that control urinary excretion of ammonia have not yet been fully described. It was already pointed out that in Gamble's experiments the increase in ammonia excretion and the extracellular acidosis failed to follow parallel courses. This can also be seen in the data herein reported, and points to the existence of other factors which determine the progressive and stepwise increase in urinary ammonia excretion. The latter part of Gamble's experiments (2) indicates that urinary ammonia excretion can still be high in long-term experiments in spite of elevation of urinary pH. The increase in excretion of potassium following ammonium chloride administration in our experiments was irregular and small, much less than in Sartorius's experiments. This indicates that potassium depletion in itself should not be considered a prerequisite for the increase in ammonia excretion in response to acidifying salts.

As shown by Davies and Yudkin (22), adaptation of ammonia-forming enzymes of the renal tubular cells to a low pH proceeds at a slow rate. Schwartz, Jenson and Rehman (10, 23) suggested that the progressive loss of cation during the first days of administration of the acidifying salt

would aggravate the difference between the excretion of fixed anions and the fixed cations available for excretion. This would stimulate ammonia formation in a stepwise manner.

CONCLUSIONS

1. Electrolyte and acid-base studies were made on five normal subjects to determine if cation depletion is essential for progressive stepwise increase in urinary ammonia excretion secondary to administration of acidifying salts.

2. Subjects were maintained on constant diet and fluid intake and given sufficient sodium chloride and sodium bicarbonate orally to expand the extracellular fluid by approximately 1.5 to 2 L.

3. When extracellular fluid volume was increased, 10 Gm. of ammonium chloride was administered daily for four days. This resulted in a progressive excretion of ammonia although body weight measurements indicated that the extracellular fluid volume was still excessive.

4. These results demonstrate that depletion of sodium is not a prerequisite for a sustained increase in urinary ammonia excretion.

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Preparation and Some Properties of a Phosphate-activated Glutaminase from Kidneys*

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Glutamine participates in such diverse processes as urinary ammonia formation, transamination reactions, and biosynthesis of glucosamine, proteins, and purines (*cf.* (1) for pertinent references). The amide group of glutamine can be liberated as ammonia by two types of enzymes. One catalyzes the hydrolysis of glutamine to glutamic acid and ammonia and is dependent upon the presence of phosphate ion (or arsenate ion) (2, 3). The other catalyzes the transamination of glutamine with keto acids with the formation of α-ketoglutaramic acid, which is subsequently deamidated by an ω-amidase to give ammonia and α-ketoglutaric acid (4). The transamination and deamidation enzymes have been separated and their properties described (5, 6). Progress in the study of the phosphate-activated glutaminase has been slow because of the difficulties encountered in extracting and stabilizing this enzyme, which has only recently been obtained in a soluble form (7). A 40- to 80-fold purification of the enzyme has been reported (8). In the present study, some properties of the soluble enzyme prepared from dog kidney cortex are reported, and a relatively simple procedure is described by which 100- to 400-fold purification can be achieved.

EXPERIMENTAL

Materials and Methods

Source of Enzyme—Dog kidneys were frozen immediately after removal and stored. Extraction of the enzyme was greatly enhanced by prolonged storage in the frozen state. Kidneys routinely were kept frozen for at least a month before use.

Analytical Methods—Ammonia determinations were made by a diffusion procedure. Aliquots of the deproteinized samples were added to 20-ml. serum bottles which contained 1 ml. of saturated potassium carbonate. The bottles were stoppered with rubber serum bottle stoppers in which were inserted ground glass rods dipped into 1 N sulfuric acid. Diffusion of ammonia was allowed to take place over a period of 1 hour while the bottles were rotated on a large wheel. After diffusion was complete, the ammonia that was trapped as ammonium sulfate on the glass rods was washed into optically matched colorimeter tubes. 2 ml. of diluted Nessler's reagent (Folin) were added, the volume was brought to 10 ml., and readings were made in a Weston colorimeter with the 445 mμ filter. Quantitative recoveries of added ammonia were achieved by this method.

Protein concentrations were estimated usually by the method of Warburg and Christian (9), according to the formula of Kalckar (10). The results were found to be in excellent agreement with those obtained by the procedure of Lowry *et al.* (11).

Glutaminase Assay—Routine enzyme incubations proceeded for 15 minutes at 37° in a Dubnoff metabolic shaking incubator at a final volume of 2 ml. in 20 ml. beakers in 0.1 M phosphate buffer, pH 8.1. In many of the experiments to be described these conditions were varied as required. The reaction was stopped and the protein precipitated with 2 ml. of 25 per cent trichloroacetic acid. After centrifugation, aliquots of the supernatant fluid were assayed for ammonia content. Suitable enzyme and substrate blanks were subtracted.

Unit of Enzymatic Activity—The unit of activity was defined as the amount of enzyme which catalyzed the liberation of 1 μg. of ammonia in 15 minutes.

Fractionation Procedure—Frozen dog kidneys were allowed to thaw at room temperature until they could be dissected easily. All subsequent operations were carried out with cold solutions and in a refrigerated centrifuge. Only the cortex was used for enzyme preparation. The cortices were homogenized in a Waring Blendor for 4 minutes with 4 volumes of 0.1 M phosphate buffer, pH 8.1. The first 2 minutes were at reduced but gradually increasing speeds, and the final 2 minutes were at full speed. The homogenate then was centrifuged in the No. 30 head of the Spinco model L ultracentrifuge for 30 minutes at 30,000 r.p.m. (80,000 × *g*, average), and the sediment was discarded. The supernatant solution usually had approximately the same activity per unit volume as the original homogenate. The supernatant fluid was made 0.5 M with respect to sodium sulfate by the addition of the solid salt with stirring. After standing for 15 minutes, the material was centrifuged for 30 minutes at 30,000 r.p.m. in the No. 30 rotor of the Spinco ultracentrifuge. The precipitate was discarded, and the concentration of sodium sulfate in the clear supernatant fluid was increased to 1.0 M. After 15 minutes, the precipitate containing the enzyme was removed by centrifugation as above and taken up in 0.1 M phosphate buffer, pH 8.1. The supernatant fluid was discarded.

Salt fractionation was repeated several times, each time with precipitation of the enzyme within a slightly narrower range of salt concentrations. The final precipitate was collected between 0.55 and 0.65 M sodium sulfate. Preparations of highest specific activity were usually obtained after 5 successive salt fractionations. The ratio of optical density at 280 to 260 mμ approached

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TABLE I

Fractionation of dog kidney glutaminase

A representative fractionation of the phosphate-activated glutaminase from dog kidney cortex is presented. Original homogenate consisted of a 1:4 ratio of homogenate of cortices in 0.1 M phosphate buffer, pH 8.1; supernatant fluid A, was the supernatant obtained from centrifuging 30,000 r.p.m. (average 80,000 × *g*) in the No. 30 head of the Spinco model L ultracentrifuge. P-1, 1.0 M represents the first sodium sulfate fractionation, with enzyme precipitated between 0.5 and 1.0 M sodium sulfate; P-2, 0.8 M, the second sodium sulfate fractionation, with enzyme precipitated between 0.5 and 0.8 M sodium sulfate concentrations; P-3, 0.7 M, the third fractionation, with enzyme precipitated between 0.5 and 0.7 M sodium sulfate; P-4, 0.65 M, the fourth fractionation, with enzyme precipitated between 0.5 and 0.65 M sodium sulfate; and P-5, 0.65 M, the fifth fractionation, with enzyme precipitated between 0.55 and 0.65 M sodium sulfate concentrations. The optical density ratio at 280 to 260 mμ approached 1.6 by P-3, 0.7 M.

Preparation	Specific activity	Purification	Total units recovered
	μg.NH_3/mg.		
Homogenate	5.7	1	181,400
Supernatant fluid A	14.7	2.6	134,800
P-1, 1.0 M	134	23.4	114,000
P-2, 0.8 M	220	38.3	66,000
P-3, 0.7 M	385	67.2	37,400
P-4, 0.65 M	707	123	29,200
P-5, 0.65 M	1,590	277	7,100

1.6 after the third fractionation and changed very little subsequently, despite significant increases in specific activity. A 400-fold purification over the starting material was obtained in the best preparations. The most highly purified fractions lost 75 per cent of the activity within a week. Less highly purified preparations retained activity for considerably longer periods of time. Details of a typical fractionation are shown in Table I.

Other conventional methods of enzyme fractionation were unsatisfactory because of inactivation or low yield.

RESULTS

pH Activity Curve—Fig. 1 shows enzymatic activity as a function of pH. Optimal activity was obtained in the range from pH 7.9 to 8.1, a value in accord with other reports for renal glutaminase (12). Crude human placental glutaminase showed a pH optimum between 8 and 10 (13). The activity decreased rapidly and irreversibly at pH values of 7.0 or below. This is in sharp contrast to the bacterial glutaminase of *Clostridium welchii* which possesses optimal activity at pH 5 (14).

Effect of Temperature—A study of enzymatic activity as a function of temperature showed maximal activity at 40°, under the conditions of assay. The energy of activation as determined by the Arrhenius equation for the 30–40° range was found to be 8250 calories per mole. The heat of inactivation, as determined by measurement of residual activity after heating a solution of the enzyme for 5 minutes at different temperatures, was estimated to be approximately 37,500 calories per mole.

Effect of Enzyme Concentration—The activity observed was linear with regard to enzyme concentration over a 10-fold range.

Time Course of Reaction—The reaction was linear with time up to about 60 minutes. Quantitative determination of glutamine can be readily accomplished by these preparations when an excess of enzyme is used.

Effect of Substrate Concentration—The initial reaction rates for the deamidation of glutamine were measured at different substrate concentrations. From these data, the Michaelis-Menten constant for the reaction has been determined to be 5×10^{-3} moles per l. from a Lineweaver-Burk (15) plot.

Substrate Specificity—The purified enzyme deamidated glutamine, but not asparagine, nor a variety of amides, nor several glutamine analogues (Table II). α-Methylglutamine and isoglutamine were decomposed at a slower rate than glutamine itself. The lowered activity when glutamine and isoglutamine

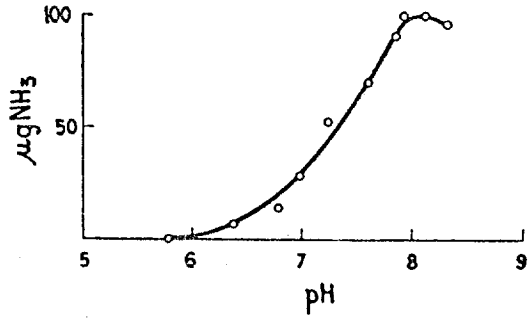


FIG. 1. Effect of pH on glutaminase activity. 0.1 M phosphate buffer at specified pH.

TABLE II

Action of glutaminase on various substrates

Compounds were tested at 0.025 M concentration in 0.1 M phosphate buffer, pH 8.1, under the conditions described in the text under "Glutaminase Assay." Incubation mixtures were analyzed for ammonia in the case of the amides, hydrazine for γ-L-glutamylhydrazide, or glutamic acid for L-glutamic-γ-methyl ester, γ-L-glutamylmethylamide, and γ-DL-glutamylbutylamide.

Substrate	Activity
L-Glutamine	100
L-Isoglutamine + L-glutamine*	88
L-Isoglutamine†	34
α-Methyl-DL-glutamine†	21
γ-L-Glutamylhydrazide‡	5
L-Glutamic-γ-methyl ester†	0
L-Asparagine	0
α-Methyl-DL-asparagine†	0
γ-L-Glutamylmethylamide§	0
γ-DL-Glutamylbutylamide†§	0
Nicotinamide	0
Acetamide	0
Adenine	0

* 0.025 M concentration of both substances.

† Samples of L-isoglutamine were kindly supplied by Dr. Alton Meister of Tufts University and Dr. Fredrick Carpenter of the University of California. α-Methyl-DL-glutamine, γ-L-glutamylhydrazide, L-glutamic-γ-methyl ester, α-methyl-DL-asparagine, and γ-DL-glutamylbutylamide were a gift of Dr. Karl Pfister of Merck and Company, Inc. γ-L-Glutamylmethylamide was kindly supplied by Dr. F. C. Meyer of Monsanto Chemical Company.

‡ Hydrazine measured by Lutwack's method (16).

§ Glutamic acid estimated by a chromatographic procedure.

were mixed in isomolar amounts suggests that the latter compound may be a competitive substrate. The γ-methyl ester of L-glutamic acid was not acted upon; it inhibited to the extent of 64 per cent when present in isomolar amounts with glutamine. γ-L-Glutamylmethylamide, although not acted upon itself, consistently enhanced activity on glutamine by approximately 20 per cent when present in isomolar amounts with glutamine. γ-DL-Glutamylbutylamide did not show this enhancement of activity. A slow liberation of hydrazine from γ-glutamylhydrazide occurred. When hydroxylamine was present during the action of the enzyme on glutamine, hydroxamate formation could not be detected by the method of Lipmann and Tuttle (17), nor was it possible to demonstrate a transfer function for the enzyme in several other test systems.

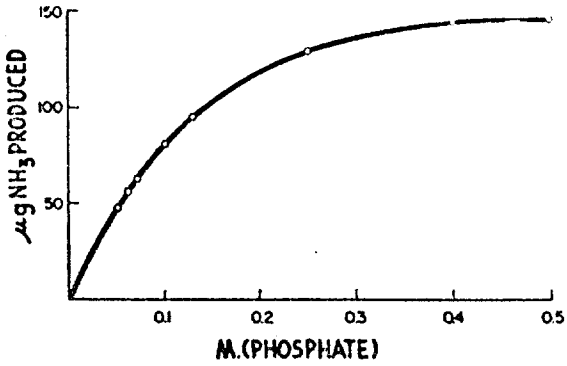


FIG. 2. Effect of phosphate on activity of dog kidney glutaminase. The glutamine concentration was 0.025 M, and the pH of all samples was 8.1. Each sample contained 0.6 mg. of protein, 0.025 M glutamine, and the indicated amount of phosphate at pH 8.1 (all pH readings made with glass electrode at 0.1 M phosphate concentration).

Effect of Anions—Glutaminase has been reported to be activated by phosphate, arsenate, and sulfate ions, with phosphate reported as the most effective (2). These results have been confirmed. Arsenate and sulfate were observed to activate in the presence of suboptimal phosphate concentrations, but inhibited in the presence of phosphate at optimal concentration. Sodium chloride, bromide, and cyanide, which have been found to activate bacterial glutaminase (14), inhibited the kidney enzyme in the presence of phosphate.

The effect of phosphate on glutaminase activity is shown in Fig. 2. The findings are in agreement with previous reports in which the optimal molar ratio of phosphate to glutamine was estimated to be approximately 17. A plot of values obtained from Lineweaver-Burk plots of results of experiments performed at different phosphate concentrations (Fig. 3) indicates that the affinity of enzyme for substrate is increased with increasing concentrations of phosphate up to approximately 0.2 M.

Effect of Inhibitors—The action of a number of inhibitors has been studied in an attempt to determine the characteristics of the active site of glutaminase. Three types of inhibition of the enzyme have been observed; sulfhydryl, competitive with glutamine, and competitive with phosphate.

The sulfhydryl reagents, mercuric chloride, *p*-chloromercuribenzoate, iodoacetamide, *N*-ethylmaleimide, and *p*-benzoquinone were found to be potent inhibitors of glutaminase. Inhibition by mercuric chloride, *p*-chloromercuribenzoate, and quinone was prevented by reduced glutathione or cysteine, whereas the effects of *N*-ethylmaleimide and iodoacetamide were not. Quinone had been previously reported to inhibit glutaminase (18), al-

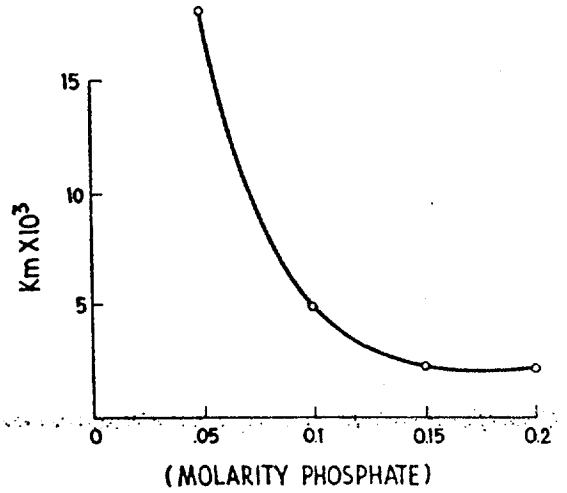


FIG. 3. Effect of phosphate concentration on Michaelis constants

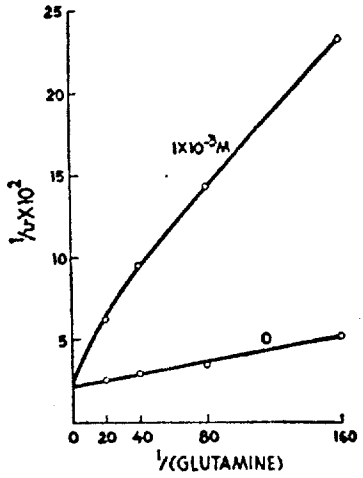


FIG. 4. Lineweaver-Burk plot of N-ethylmaleimide inhibition as a function of glutamine concentration. The phosphate buffer concentration was 0.1 M at pH 8.1.

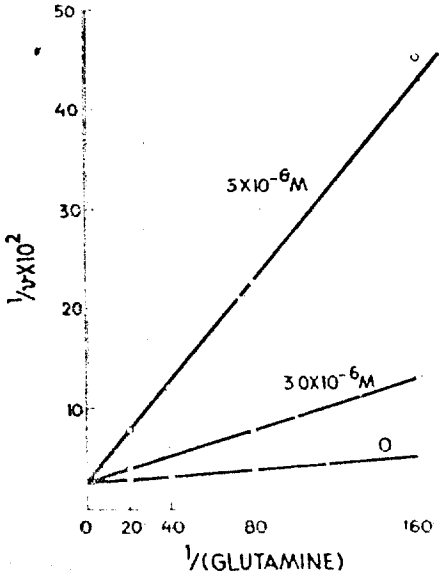


FIG. 5. Lineweaver-Burk plot of *p*-chloromercuribenzoate inhibition as a function of glutamine concentration. The phosphate buffer concentration was 0.1 M at pH 8.1.

though no evidence for its action as a sulfhydryl inhibitor was presented. A Lineweaver-Burk plot (Fig. 4) of inhibition by *N*-ethylmaleimide yielded a curve which showed inhibition to be more nearly competitive with glutamine than noncompetitive, whereas the results with *p*-chloromercuribenzoate gave Lineweaver-Burk plots typical of competitive inhibition (Fig. 5). Inhibition by *p*-chloromercuribenzoate was shown to be independent of phosphate concentration. From these data it seems probable that a sulfhydryl group of the enzyme is a site of attachment for the substrate.

Ammonia Inhibition—Ammonia was found to inhibit glutaminase in a manner competitive with glutamine (Fig. 6).

Phthalein Inhibition—Glutaminase has been reported to be inhibited by a number of phthalein dyes. Bromocresol green (3,3′,5,5′-tetrabromo-*m*-cresol-sulfonphthalein) and bromosulfalein (disodium phenoltetrabromophthalein-disulfonate) were reported to inhibit the phosphate-activated glutaminase from *C. welchii* in a manner competitive with glutamine (14). These results were not confirmed with the preparations from dog kidney. Bromocresol green was found to be a noncompetitive inhibitor with respect to glutamine (Fig. 7), but when studied as a function of phosphate concentration, the Lineweaver-Burk plots were characteristic of competitive inhibition (Fig. 8). Similar data were obtained for bromosulfalein and flavianic acid (2,4-dinitro-1-naphthol-7-sulfonic acid). The relative effectiveness of these inhibitors can be seen in Table III. The differences in the type of inhibition produced by the phthaleins in the bacterial and mammalian kidney glutaminase may be a function of the pH of incubation or may be a result of fundamental differences in the enzymes obtained from these two sources.

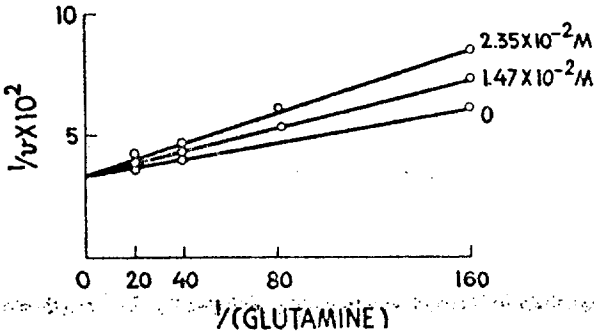


FIG. 6. Lineweaver-Burk plot of ammonia inhibition as a function of glutamine concentration. Reactions were run in 0.1 M phosphate buffer at pH 8.1.

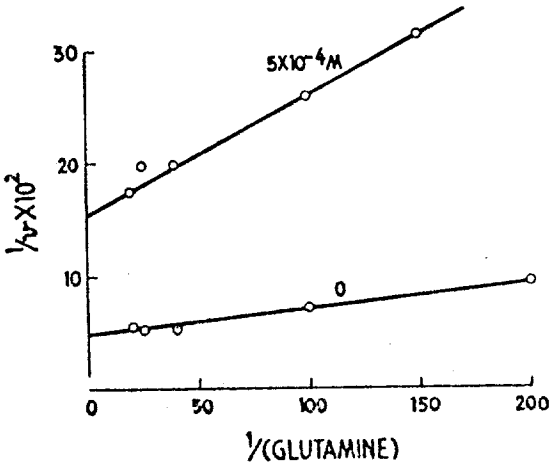


FIG. 7. Lineweaver-Burk plot of bromocresol green (tetrabromo-*m*-cresol-sulfonphthalein) inhibition as a function of glutamine concentration in 0.1 M phosphate buffer, pH 8.1.

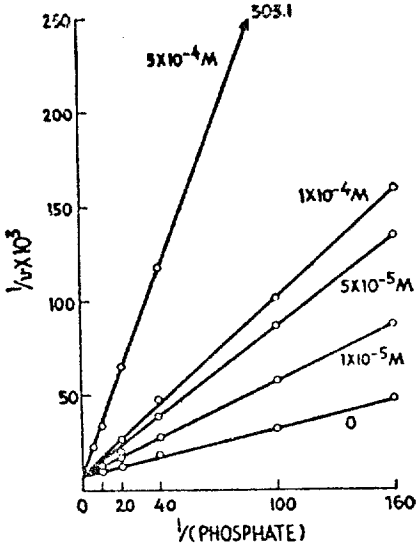


FIG. 8. Lineweaver-Burk plot of bromocresol green inhibition as a function of phosphate concentration. Glutamine was present at 0.025 M and phosphate buffer, at desired concentration at pH of 8.1.

TABLE III

Inhibitors of dog kidney glutaminase

Enzymatic activity was determined as described in the text in the presence of inhibitors at the specified concentrations.

Inhibitor added	Molar concentration of inhibitor	Inhibition
		%
Bromocresol green	1×10^{-3}	100
	5×10^{-4}	74
Bromosulfalein	1×10^{-3}	100
	5×10^{-5}	87
	1×10^{-5}	4
	1×10^{-6}	0
Bromocresol purple	1×10^{-3}	82
Flavianic acid	1×10^{-3}	79
	5×10^{-4}	68
	2.5×10^{-4}	45
Phenolphthalein	2×10^{-3}	0
p-Chloromercuribenzoate	1×10^{-3}	100
	5×10^{-4}	82
Mercuric chloride	1×10^{-3}	100
	5×10^{-4}	89
p-Benzoquinone	1×10^{-3}	92
N-ethylmaleimide	1×10^{-3}	100
	1×10^{-4}	50

Glutamic Acid Inhibition—Inhibition by glutamic acid was found to be noncompetitive with glutamine (Fig. 9) but com-

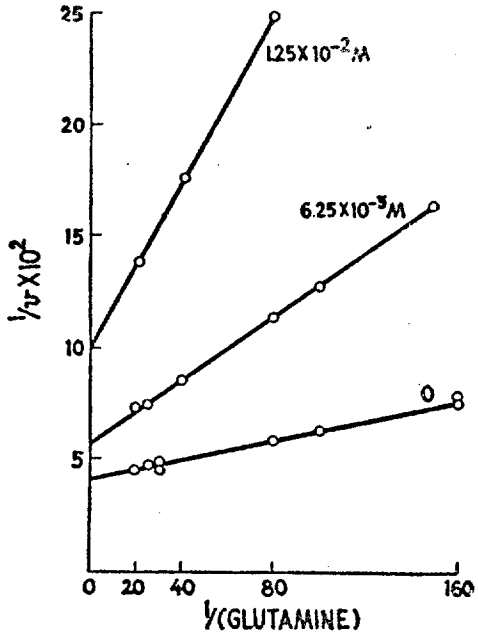


Fig. 9. Lineweaver-Burk plot of glutamic acid inhibition as a function of glutamine concentration. The phosphate buffer concentration was 0.1 M at pH 8.1.

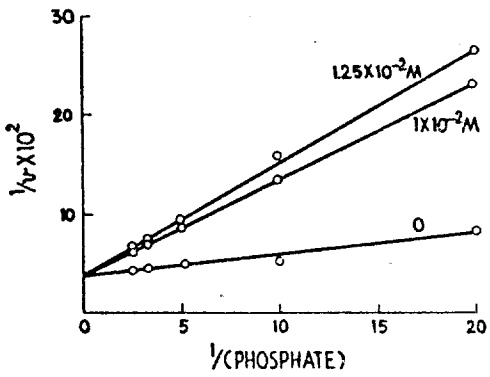


Fig. 10. Lineweaver-Burk plot of glutamic acid inhibition as a function of phosphate concentration. Glutamine was present at 0.025 M concentration. The pH of each sample, at a dilution of 0.1 M, was 8.1.

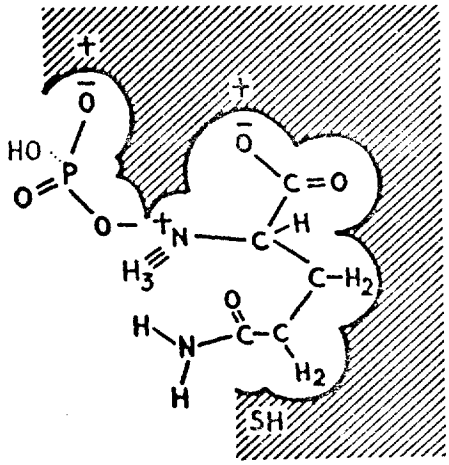


Fig. 11. Two-dimensional projection of scale models (Catalin) showing postulated attachment of glutamine to active sites of glutaminase (see the text).

petitive with phosphate (Fig. 10). Glutaric, α-ketoglutaric, γ-aminobutyric, and α-amino-ε-hydroxycaproic acids were not inhibitory in concentrations comparable to those found to be effective in the case of glutamic acid. It seems, therefore, that the α-amino and γ-carbonyl groups are necessary for inhibition.

DISCUSSION

The data presented provide a tentative explanation of the mechanism of glutaminase action. Glutaminase may be postulated to have a sulfhydryl and two cationic sites. One of the cationic sites may serve as a point of attachment for a phosphate ion, with the substrate attaching to the other cationic site through the α-carboxylate group and to the second negative charge of the phosphate group through a positively charged α-amino group. Such a mechanism would presume that glutamine could attach most readily to an active site already containing phosphate (or other suitable divalent anion). Hydrolysis of the amide would be expected to take place in the vicinity of the sulfhydryl group. A sketch made from the two-dimensional projections of scale models (Catalin models) illustrates this concept (Fig. 11).

The increasing affinity of substrate for enzyme with increasing levels of phosphate (Fig. 2) suggests that phosphate plays an important role in the attachment of substrate to enzyme. The high levels of phosphate ion required for maximal activity indicate that the affinity of phosphate for enzyme is relatively low as would be expected if an ionic bond with a cationic site on the enzyme protein were involved. Ammonia, an inhibitor competitive with glutamine, might be expected to compete with the α-amino group of the substrate for attachment to the phosphate. Although phosphate is known to enhance the rate of nonenzymatic deamidation of glutamine, sufficient differences exist between the enzymatic and nonenzymatic reactions to preclude a similar function for the anion in the two processes (3). It is not known whether or not the high levels of phosphate required in the enzymatic studies can occur at intracellular sites of glutaminase action, or whether phosphate is substituting in the system *in vitro* for some naturally occurring, more effective activator.

Superposition of the two-dimensional projections of models of flavianic acid and bromosulfalein, two potent inhibitors of glutaminase, on the projection of the postulated active site revealed a good fit of the inhibitor molecules to the proposed site (Figs. 12 and 13). In bromosulfalein, the distance between two negatively charged oxygen atoms on the sulfonic acid groups, and in

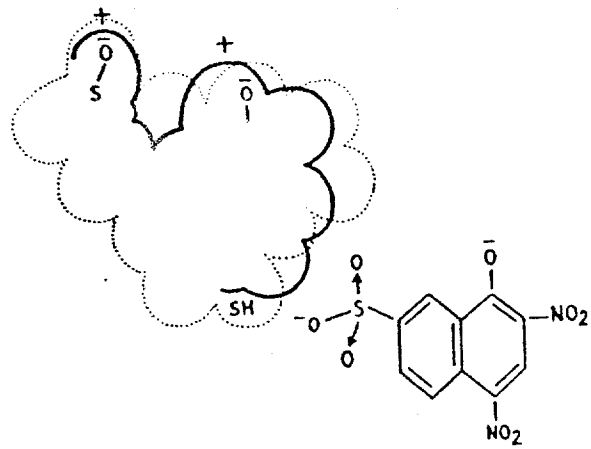


Fig. 12. Superposition of two-dimensional projection of flavianic acid model on the projection of the postulated active site.

flavianic acid, the distance between negatively charged oxygen atoms of sulfonic acid and phenolate groups approximate the distance between the cationic sites opposing the negative phosphate and carboxylate oxygen atoms of the phosphate-glutamine complex shown in Fig. 11. The finding that inhibitions by these substances were competitive with phosphate and not with glutamine is compatible with the hypothesis that the active site for attachment and subsequent hydrolysis of glutamine actually includes phosphate. Substances which compete with phosphate for attachment to the enzyme would inhibit enzyme activity, but their action could not be reversed by glutamine. Substances competitive with glutamine would then have to possess structures with affinity for the enzyme-phosphate complex. In a model of isoglutamine, a competitive substrate, the α-amino, γ-carboxyl, and α-amide groups can be made to lie in positions similar to those shown for the α-amino, α-carboxyl, and γ-amide groups, respectively, of glutamine.

Inhibition by *p*-chloromercuribenzoate seems to be competitive with glutamine which indicates that a sulfhydryl group is a point of attachment of substrate with enzyme. The participation of a sulfhydryl group in enzymatic deamidation might be envisioned by consideration of the possible interaction of two of three resonance hybrids of the amide structure with a sulfhydryl group (see Reaction 1). Structure A undoubtedly would be the most stable form and would give the greatest contribution. Although B has a greater separation of charge than C, it would probably be favored over C because of the greater number of covalent bonds and because the octet rule is violated by structure C. It might be expected that a significant proportion of the —SH groups on the enzyme would be in the S^- form at pH 8.1, the optimal pH for glutaminase activity (19). The reactions of B and C with the S^- groups of the enzyme could then be formulated as shown in Reactions 2 and 3.

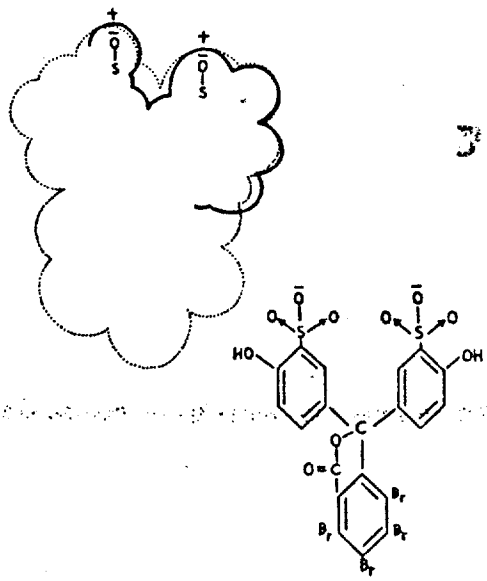
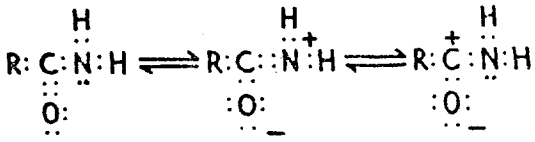
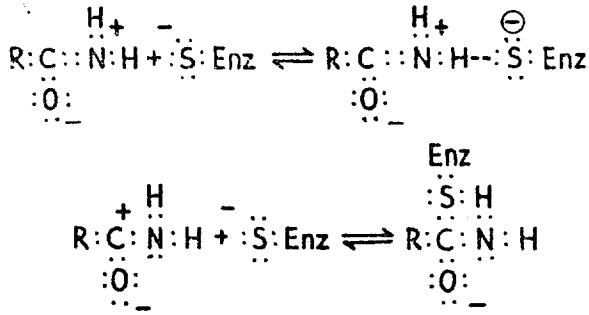


Fig. 13. Superposition of two-dimensional projection of bromosulfalein model on the projection of the postulated active site. The three rings are not coplanar.



A B C

Reaction 1



Reactions 2 and 3

Both intermediates suggested in Reactions 2 and 3 above could undergo spontaneous hydrolysis at alkaline pH to give ammonia, the carboxylate ion, and Enzyme-S^-. The addition compound formed in Reaction 3 is similar to that formed in the interaction of sulfhydryl groups with aldehydes and ketones whereas an ionic interaction is postulated in Reaction 2. There is no basis for favoring either mechanism at the present time. Neither of the above mechanisms requires the formation of high energy bonds. A thiol ester has been postulated as an intermediary step in the amidase action of papain (20).

SUMMARY

1. A procedure has been described for the purification of a phosphate-activated glutaminase from dog kidneys, which results in purifications from 100- to 400-fold.

2. α-Methyl-DL-glutamine and α-isoglutamine were deamidated by the purified enzyme at much slower rates than was glutamine. Other amides and glutamine analogues were not acted upon.

3. Inhibition by *p*-chloromercuribenzoate, mercuric ion, iodoacetate, and quinone was prevented or partially reversed by glutathione or cysteine, but not by glutamine. Irreversible inhibition was observed with the *N*-ethylmaleimide.

4. Inhibition by ammonia was competitive with glutamine, whereas inhibition by glutamic acid, bromosulfalein, bromocresol green, or flavianic acid was found to be noncompetitive with glutamine but competitive with respect to phosphate.

5. A tentative mechanism of glutaminase action has been proposed.

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39(4): 550-563, 1927

CHRONIC ACIDOSIS IN RABBITS AND IN DOGS

WITH RELATION TO KIDNEY PATHOLOGIC CHANGE *

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Change in the ability of the kidney to eliminate acids has been recognized as one of the fairly constant symptoms of renal disease. Palmer and Henderson [1] report an abnormally high acidity of the urine caused by a decrease of urinary ammonia in patients with nephritis usually of the glomerular type. The same investigators,[2] also Sellards,[3] Hösslin,[4] and Peabody,[5] find that the amount of alkali which must be ingested before the urine becomes alkaline is increased in nephritis. Sellards reports that this is particularly true in interstitial renal injury. Austin and Cullen [6] review the literature and draw the conclusion that nephritis gives rise to a disturbance of the acid base equilibrium, and that it may lower both the alkali reserve and the plasma p_H.

These investigators and many others, while recognizing the constant appearance of a disturbed acid excretion, have supposed that this was a consequence of the renal damage. Fischer,[7] on the other hand, believes that acid elimination may play an etiologic rôle in nephropathologic change. He supports his theory by experiments in which he injects acid intravenously in rabbits and finds albumin and casts in the specimens of urine in a few hours. Other work of this nature has been reported as far back as 1878, when Kobert [8] found that 5 per cent phosphoric acid given to dogs and rabbits either by stomach tube or by intravenous injections resulted in albumin, casts and even blood in the urine.

* [illegible] Medical School.

1 [illegible] 16: [illegible]

2 [illegible] 12: [illegible]

3 [illegible] 25: 141 [illegible]

4 Hösslin, [illegible] Sauregrad des Urins [illegible] Albuminurie, Diurese und [illegible] Deutsches Arch. f. klin. Med. 105: 147 [illegible]

5 Peabody, F. W. [illegible] Chronic Nephritis, Arch. Int. Med. 16: [illegible]

6 Austin, J. H., and Cullen, G. E. [illegible] Health and Disease, Medicine 4: 275 (Aug.) 1925.

7 Fischer, Martin H.: Edema and Nephritis, New York, John Wiley & [illegible]

8 Kobert, [illegible] Beitrag zur Phosphosaurewirkung, Schmidt's Jahrb. 179: [illegible]

Ewing [9] obtained albumin and hyaline casts in fifty-two hours in a rabbit that had received 2.16 Gm. hydrochloric acid subcutaneously. Microscopically, he found granular and hydropic degeneration of the renal tubule cells. MacNider [10] conducted a series of four hour experiments in which dogs received two doses of two-tenths normal hydrochloric acid, 5 cc. per kilogram. Albumin and casts appeared in the urine. Microscopically, the kidneys showed injury to the convoluted tubules, advanced cloudy swelling, granules in the cytoplasm and irregularly staining nuclei. Goto [11] observed that acid (from 0.5 per cent hydrochloric acid to 1 cc. per kilogram) administered by mouth to two dogs produced an "acute nephritis."

Acid has been used in these experiments in relatively large quantities over a short period of time, and the effect has been similar to that produced by other more toxic chemicals. Acute tubular injury has resulted.

PLAN OF EXPERIMENTS

The experiments described in this paper were planned to determine whether a mild chronic acidosis, or a lowering of the plasma carbon dioxide capacity, extending over a long period of time, would result in kidney damage.

The acidosis was produced by administering by stomach tube one-tenth normal hydrochloric acid or one of the hydrochloric acid forming substances, ammonium chloride or calcium chloride, to rabbits and dogs. The dosage of acid-forming substances was so regulated that the carbon dioxide capacity of the plasma became and remained slightly below normal over periods of months. It was difficult to regulate this dosage for rabbits. They often contracted a severe acidosis. Specimens of urine were examined at regular intervals for albumin, the p_H was determined and the sediment was examined microscopically.

ACIDOSIS IN RABBITS, MATERIAL AND METHODS

Fifteen young animals weighing approximately 2 Kg. were used. Six of these were kept as controls. All animals were maintained under the same living conditions, on a diet of oats and various greens and water as desired. For spontaneous ear infection with B. necrophorus, which made its appearance in a few animals, these animals were treated by pouring ether into the ear every other day until the infection disappeared completely.

9 Ewing, James: Acidosis and Associated Conditions, Arch. Int. Med. 2: 330 (Nov.) 1908.

10 MacNider, W. deB.: Studies Concerning the Influence of a Disturbance in the Acid Base Equilibrium of the Blood on Renal Function and Pathology: Study I. The Effect of Acid and Alkali on Solutions on Renal Function and Pathology in Normal Dogs, J. Metabolic Research 3: 511 (April) 1923.

11 Goto, Kingo: A Study of Acidosis, Blood Urea and Plasma Chlorides in Uranium Nephritis in the Dog, and of the Protective Action of Sodium Bicarbonate, J. Exper. Med. 25: 693 (May) 1917.

Weekly examinations of the urine were made on urine freshly obtained by pressure on the bladder. In order to make certain that the bladder should contain a sufficient volume of urine, the animals were put into rat cages for three or four hours previous to taking the specimen of urine. They were sufficiently crowded and uncomfortable in these small cages so that they did not urinate during that period. The p_H of the urine was determined colorimetrically with the LaMotte standards on all specimens of urine acid to litmus. Albumin was determined by heat and acetic acid. Acid was also added to urine which was cold, as it was found that frequently a precipitate formed under these conditions. Albumin as reported refers to the density of the cloud in the heated specimen after that in the cold urine has been subtracted. It is reported as a faint trace, trace, one plus, two plus or three plus. Microscopic examination of the sediment obtained by centrifugalization was made promptly.

Blood for plasma carbon dioxide capacity was drawn at weekly or bi-weekly intervals as the condition of the animal warranted. The specimen was collected by inducing hyperemia in the ear with ether, cutting the marginal vein and allowing the blood to fall into an oxylated tube. After gently mixing with a rod, oil was poured on the surface, and the blood was centrifugalized. This is not an accurate way of collecting blood for gas analysis. The method was checked, however, by drawing simultaneous specimens from the heart. The difference of about 3 per cent by volume was of no practical importance in these experiments. A rabbit's plasma carbon dioxide capacity has normal limits as wide as 45-70 per cent by volume. The method of analyzing the blood was that described by Van Slyke.[12] The analyses of the last six months were made on the constant volume Van Slyke machine.[13]

Nine of the rabbits received tenth normal hydrochloric acid, calcium chloride or ammonium chloride by stomach tube over periods of time which varied from ten days to one year. The time and dosage, together with urine, blood and post-mortem observations, are given in tables 1 and 2.

The use of tenth normal hydrochloric acid as a means of lowering the reserve alkali of the blood needs no explanation. Ammonium chloride and calcium chloride increase the chloride anions of the blood, because in the case of ammonium chloride, the ammonia is probably converted into neutral urea, while in the case of calcium chloride, a large portion at least of the calcium is eliminated in the stool, while the chlorine ion is absorbed. Either substance, therefore, increases the hydrochloric acid metabolism of the body. Haldane[14] has reported the production of acidosis in himself both after the ingestion of calcium chloride and ammonium chloride. Gambler, Ross, and Tisdall[15] and Gamble and Ross[16] administered calcium chloride, ammonium chloride, and hydrochloric acid in the treatment of tetany and proved that calcium chloride

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and ammonium chloride as well as hydrochloric acid lowered the plasma bicarbonate. Hydrochloric acid and presumably the other substance also lowered the p_H of the plasma. They found that only about 40 per cent of the calcium chloride ingested acts as hydrochloric acid, while probably all of the ammonium chloride ingested exerts an acid effect. One gram of ammonium chloride, which contains approximately 0.66 Gm. chlorine, would be equivalent to 180 cc. tenth normal hydrochloric acid. One gram of calcium chloride, if all the calcium were eliminated via the stool, and all the chlorine absorbed, would be equivalent to approximately the same, 180 cc. tenth normal hydrochloric acid.

Because of the possibility that ammonium chloride administered over a long period of time might introduce extraneous effects caused by ammonia and not by acid, three of the rabbits received tenth normal hydrochloride acid over long intervals of time during the experiments. There was no difference in the results. Calcium chloride was given to the rabbits during a large part of the experimental period. Tenth normal hydrochloric acid was finally stopped because such large volumes of fluid were necessary. The ammonium chloride and calcium chloride were given in 2 per cent solution.

The animals, with the exceptions noted in the tables, were killed, and autopsy was performed immediately. Sections were fixed in Zenker's solution and were stained with hematoxylin and eosin stain or by Giemsa's method.

EXPERIMENTAL OBSERVATIONS ON RABBITS

The individual variations in dosage and in the response of the animal to the acid make it impracticable to average the figures and present the data in a single table. The animals will be described as a single group during the first few days. They will then be divided into two groups: animals that never had a severe acidosis, that is a plasma carbon dioxide combining power below 30 per cent by volume, and the animals that developed a severe acidosis. Tables 1 and 2 contain the data for these two groups.

Previous to giving the acid, the specimens of urine of the nine animals used in the experiment were alkaline, there were no casts in the sediment, and eight of the nine had no albumin. One showed a faint trace. The plasma carbon dioxide capacity varied between the limits of 46 per cent by volume and 70 per cent by volume. These observations were made over a control period of one month.

The administration of from 5 to 7 Gm. of ammonium chloride to four animals over a period of three days to one week resulted in the appearance of albumin in the urine, which was a faint trace in one animal but a one plus amount in the others. The specimens of urine became acid; the p_H dropped as low as 5.4. Casts had appeared in the urine of one animal. After 7 Gm. of calcium chloride given over the period of a week to one animal, the albumin test gave a plus two reaction. The administration of from 100 to 350 cc. of tenth normal hydrochloric acid, given to four rabbits over a similar period of time, produced an acid urine, and in all but one case albumin varying from a faint trace to one plus. In all these animals the plasma carbon dioxide capacity was unchanged.

At the end of the first few days, without changing the reserve alkali of the body, the animals had been forced to excrete an acid urine and in all but one case varying amounts of albumin, while one rabbit had showed casts. It soon became evident, however, that the rabbit that does not produce ammonia to neutralize the acid eliminated in the course of

TABLE 1.—*Severe Acidosis*

Rabbit	Duration of Experimental Period	Dosage of Drugs Administered	Urine Observations: Average p_H	Urine Observations: Average Albumin	Urine Observations: Casts	Carbon Dioxide per Cent by Volume: Average	Carbon Dioxide per Cent by Volume: Lowest	Hours Post-mortem Cause of Death	Microscopic Kidney Observations
4	11 days	16.2 Gm. NH_4Cl	6.4	+	Hyaline, granular	39	12	0 Killed	Epithelium of convoluted tubules, swollen, vacuolated, completely filling lumen; nuclei showed karyolysis; slight spontaneous nephritis
7	18 days	18.6 Gm. NH_4Cl, 8.0 Gm. $NaHCO_3$	4.9	++	Hyaline, granular	37 61*	20	0 Acidosis	No swelling of tubular epithelium; nuclei filled with many deep purple punctate dots of chromatin; numerous casts in tubules; no spontaneous nephritis
11	36 days	26.8 Gm. NH_4Cl	5.2	+	All types terminally	45	15	Biopsy	Tubular epithelium swollen, vacuolated; both pyknosis and karyolysis of nuclei; slight spontaneous nephritis
	3 weeks	.....	7.0	++	All types terminally	15	..	0 Killed	Tubular epithelium in places showed regeneration; many epithelial cells with no cytoplasm, and pyknotic nuclei; pyknosis much more extensive; numerous casts in tubules
14	[illegible]	[illegible]	[illegible]	+	Granular, occasionally all types	50	11	Biopsy†	Some cellular débris and casts in tubules; slight spontaneous nephritis; essentially normal
	7.5 wk	[illegible]	[illegible]		All types	47	14	0 Killed	Slight swelling of convoluted tubular epithelium; some nuclei of epithelial cells pyknotic; slight spontaneous nephritis
[illegible]	[illegible]	[illegible]	[illegible]		All types	[illegible]	[illegible]	Biopsy	Tubular epithelium swollen, vacuolated; many nuclei pyknotic; no spontaneous nephritis
	[illegible] weeks							[illegible] Killed	Condition practically unaltered, slightly improved

* Per cent by volume of carbon dioxide at time of death.
† No acidosis at time this kidney was removed.

metabolism rapidly acquires a severe acidosis. Two of the animals were killed by the acidosis, one in eleven days and one in eighteen days, before this fact was fully appreciated. Even with careful watching subsequent animals were thrown into periods of severe acidosis from which they recovered.

The group of nine animals, therefore, is divided into two groups, one group consisting of rabbits 15, 16, 17 and 18, that at no time had a plasma carbon dioxide capacity below 30 per cent by volume. Animals 4, 6, 7, 11 and 14 had severe acidosis one or more times.

Table 1 summarizes the procedures and results in the group that had severe acidosis. Grossly and microscopically, the pathologic observations were uniform. The kidneys, grossly, were moderately swollen and pale. When spontaneous nephritis, so characteristic of rabbits, was present, it was recognized by small diffuse pits on the surface. On section, the proportion of cortex to medulla was normal. Extreme paleness was the outstanding feature.

Microscopically, with Giemsa's stain, the epithelium of the convoluted tubules was undergoing acute degeneration. The cells were so swollen as to fill the lumen completely; the cytoplasm was granular and vacuolated, the nuclei were usually deep violet, shrunken and irregular in contour, although rabbits 4 and 11, under treatment a short time, showed swollen, pale nuclei also. There were casts in the collecting tubules and a finely granular precipitate in some of the glomerular spaces. The glomeruli were normal. The observations were the same irrespective of the duration of the experiment. There was no intertubular proliferation of cells. Although the kidneys of these animals had been subjected to prolonged irritation, which was sufficient to cause the constant appearance of albumin and casts in the urine, no indication of a chronic response on the part of the tissues was obtained. The picture was that of a mild acute injury to the tubular epithelium and was essentially that of cloudy swelling.

Animals 4 and 7 are interesting in that they both received approximately the same amount of ammonium chloride and both were killed because they were in a moribund condition caused by acidosis. Rabbit 7, fig. 2, however, had received 8 Gm. of sodium bicarbonate during a period of two days previous to his death. His plasma carbon dioxide had returned to normal. With the exception of the great number of casts in the tubules and a slight degree of pyknosis of the nuclei, his kidneys were not microscopically abnormal, while those of rabbit 4, fig. 1, showed the tubular degeneration described in the foregoing. The nuclei were swollen and pale, or pyknotic.

Rabbit 11 was operated on in the stage of severe acidosis, and a portion of the right kidney was removed. This showed microscopically the characteristic picture of tubular degeneration. The kidney became infected postoperatively. Leukocytes and bacteria appeared in the urine, and the animal was killed seven days after the operation. In addition to the local area of infection in the right kidney, an early thrombus was found microscopically on the mitral valve. Besides the tubular degeneration in the kidneys, there often were adhesions between the glomerular

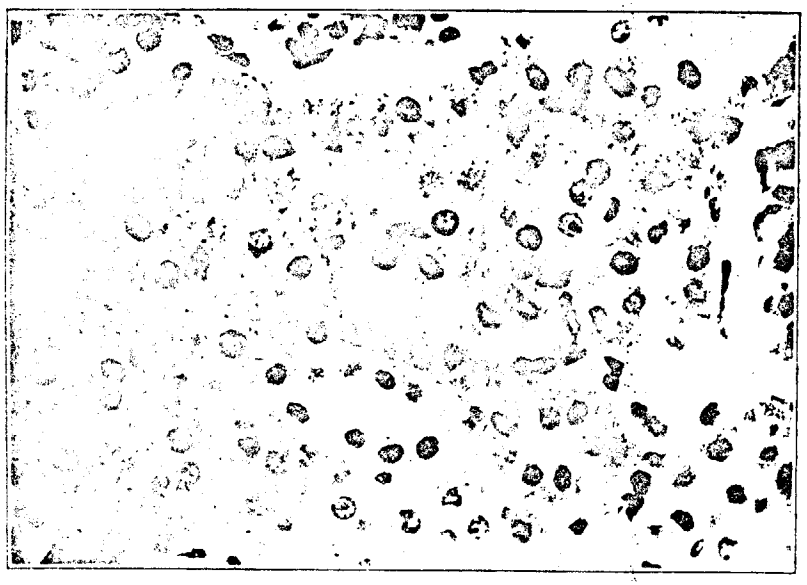


Fig. 1.—Convoluted tubules from the kidney of rabbit 4. The animal had received 16.2 Gm. of ammonium chloride over a period of two days. The plasma carbon dioxide had fallen to 12 per cent by volume. The combination of swollen cytoplasm with nuclei which are either swollen and pale or pyknotic is characteristic of early acute acidosis.

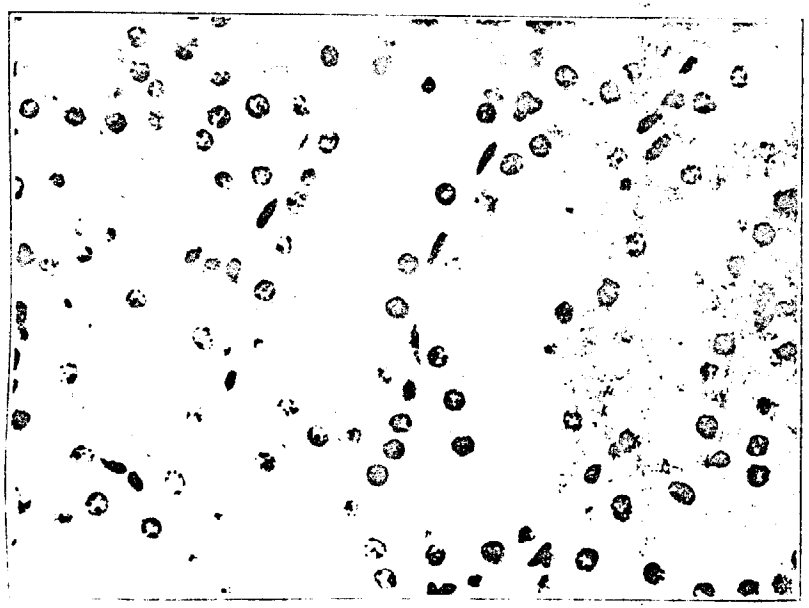


Fig. 2.—Convoluted tubules from the kidney of rabbit 7. The animal had received 6 Gm. of ammonium chloride over a period of seventeen days. The plasma carbon dioxide capacity was less than 20 per cent by volume two days before death, but due to the ingestion of sodium bicarbonate the plasma carbon [illegible] death. The tubules are

tuft and the capsule. In view of the negative observations in the glomeruli of the other animals, this must be attributed to the superimposed infection.

The left kidney was removed from rabbit 14 one month after the animal's plasma carbon dioxide had fallen to 10.7 per cent by volume. At this time the urine contained all types of casts in great numbers and a three plus albumin reaction. The blood urea nitrogen was 28 mg. per hundred cubic centimeters of blood. By way of treatment the animal was given sodium chloride, 4 Gm. daily by stomach tube, for two days. At the time of operation, one month later, the results of all tests were normal, except that there was a trace of albumin in the urine. Macroscopically and microscopically, the kidney removed for biopsy was normal with the exception of a few casts in some of the collecting tubules.

TABLE 2.—*Mild Acidosis*

Rabbit	Duration of Experimental Period	Dosage of Drugs Administered	Urine Observations: Average pH	Urine Observations: Average Albumin	Urine Observations: Casts	Carbon Dioxide per Cent by Volume: Average	Carbon Dioxide per Cent by Volume: Lowest	Hours Postmortem Cause of Death	Microscopic Kidney Observations
17	24 days	650 cc. N/10 HCl	5.6	+	Occasional hyaline and granular	59	59	<17 Pneumonia	Postmortem swelling of tubular epithelium; serous precipitate in capsular space; spontaneous nephritis
18	2 mo.	1,850 cc. N/10 HCl 6 Gm. NH_4Cl	6.0	+	Occasional hyaline and granular	61	58	<3	A few pyknotic nuclei in tubular epithelium; spontaneous nephritis
16	2½ mo.	3,125 cc. N/10 HCl	6.0	Trace	Hyaline, terminally all types	56	34	0 Killed	Serous and cell débris in tubule; many pyknotic nuclei; slight spontaneous nephritis
15*	13 days	13 Gm. $CaCl_2$	...	-	0	57	55	.....	Same as 16; also convoluted tubular epithelium swollen; no spontaneous nephritis
	7 mo.	5,570 cc. N/10 HCl, 12 Gm. NH_4Cl	5.5	-	All types in moderate numbers	56	30	<5	

* There was an interval of six weeks between the two experimental periods.

During the following seven and a half weeks the animal received 22 Gm. of the ammonium chloride. In the middle of this period a severe acidosis developed. The plasma carbon dioxide capacity was reduced to 13.7 per cent by volume. The animal recovered from this, however, and at the time it was killed, the plasma carbon dioxide capacity was 25 per cent by volume; the urine showed a plus one albumin reaction and a few of all types of casts. Grossly, the kidney was somewhat pale but otherwise normal and weighed just under 9 Gm. Microscopically, the changes already described were found but to a less marked degree. Only occasional pyknotic epithelial nuclei were found, and the cytoplasm was less granular, swollen and vacuolated. There were a few scars of spontaneous nephritis.

Rabbit 6 was operated on and his right kidney removed at a time when his plasma carbon dioxide capacity was 41 per cent by volume. The urine had a p_H of 5.1 albumin + + +, and showed hyaline and granular casts. There was severe bleeding at the time of the operation; the animal failed to gain weight afterward. He was finally killed three weeks later because of a stitch abscess which persisted and extended in spite of treatment. The abscess had involved the peritoneal wall at the site of the incision, and the intestines in the vicinity were matted together by dense adhesions. In spite of this secondary infection, the condition

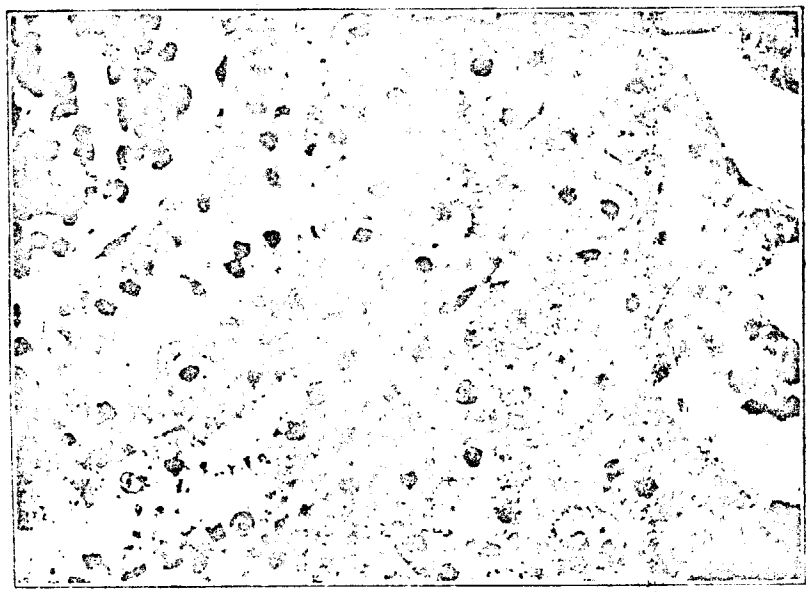


Fig. 3.—Convoluted tubules from the kidney of rabbit 6, removed at operation one year after the onset of acidosis. The plasma contained 41 per cent by volume of carbon dioxide. The marked pyknosis of the nuclei, with swelling and granulation of the cytoplasm is characteristic of protracted acidosis.

of the remaining left kidney, fig. 3, was somewhat better than of that removed at biopsy. The epithelium was not so swollen, and the pyknosis of the nuclei had in part disappeared. The kidney removed at biopsy showed the changes already described.

Of the four animals, table 2, that at no time contracted a severe acidosis, three died unexpectedly and autopsy was not performed immediately. Rabbit 16, which was killed, therefore furnishes the best example of this group. The same changes in convoluted tubules but to a less marked extent, were found microscopically.

The six control animals lived in the laboratory for from one month to sixteen months. All but one were killed and autopsy immediately performed. The animals that were kept ten months and longer occasionally showed a trace of albumin and, in some instances, a plus albumin reaction in the urine. This was most often associated with the spontaneous development of an acid urine. Three of the animals had hyaline and finely granular casts at times. The albumin and casts persisted in the case of no. 9 who at autopsy showed extremely extensive spontaneous nephritis. All had at least a few pits in the surface of their kidneys which microscopically were due to localized areas where tubules were collapsed, accompanied by round cell infiltration and connective tissue proliferation.

GENERAL BODILY REACTION OF RABBITS

The weight curve went down whenever the animals acquired a severe acidosis or a moderate acidosis extending over a long period of time. At autopsy the body fat was much decreased.

Pathologic changes elsewhere than in the kidneys were conspicuously absent. The aorta was examined particularly with reference to the development of arterial lesions, but only the small plaques which represent spontaneous degeneration of the media were found.

Rabbit 6 had progressive softening of his teeth during the last two months of his life, so that when he died the lower front teeth were practically gone. Dr. P. R. Howe had two of the teeth analyzed for calcium and found them deficient. The bones of the skull were so thin that they could readily be cut with an ordinary scissors. After twenty-four hours in Zenker's solution, they could be sectioned with a microtome. The long bones did not show any gross thinning, but the ribs were extremely soft. Loss of calcium and of fat in experimental acidosis has already been reported by Goto.[17] The epithelium of the lungs of this animal, no. 6, was vacuolated as though undergoing hydropic degeneration.

When an animal has severe acidosis, his respirations become slow and deep. His head is retracted. He sits quietly unless disturbed, and when he attempts to walk, he falls toward one side. Later, he lies on one side and exhibits spasmodic twitching of the extremities.

CHRONIC ACIDOSIS IN DOGS

Seven female mongrel dogs were used for the experiment. They ran together on the roof of the building during the day and occupied separate pens at night. Their diet consisted of Spratt's Dog Biscuit and meat. They all had the mange and were treated with sulphur ointment. It persisted in only one, no. 3.

17. Goto, Kingo: Mineral Metabolism in Experimental Acidosis, J. Biol. Chem. 36:255 (Nov.) 1918.

Experimentally the dogs were treated in the same manner as the rabbits, except that the plasma carbon dioxide determinations were made much less frequently because the onset of acidosis in the dogs was more gradual.

EXPERIMENTAL RESULTS

In table 3 are summarized the experimental procedures in the five animals who were given ammonium chloride, calcium chloride and tenth normal hydrochloric acid. About two weeks after the onset of treatment, the specimens of urine showed a faint trace of albumin. It was

TABLE 3.—*Acidosis in Dogs*

Dog	Wt., Kg.	Duration of Experimental Period	Dosage of Drugs Administered	Urine Observations: Average p_H	Urine Observations: Average Albumin	Urine Observations: Casts	Carbon Dioxide per Cent by Volume: Average	Carbon Dioxide per Cent by Volume: Lowest	Hours Post-mortem Cause of Death	Microscopic Kidney Observations
1	14	4 mo.	56 Gm. NH_4Cl, 498 Gm. $CaCl_2$	6.0 Terminally 4.9	+	Hyaline, granular, in moderate numbers	30	21	0 Killed	Convoluted tubular epithelium vacuolated; nuclei showed karyolysis and occasional pyknosis; casts in collecting tubules; spontaneous nephritis
2	17	5½ mo.	26,500 cc. N/10 HCl, 124 Gm. NH_4Cl, 519 Gm. $CaCl_2$	5.9	Trace	Hyaline casts in about one-half the examinations	45	31	..	Animal showed leukocytes and bacteria in the urine and the experiment was stopped
3	15	5 mo.	35,475 cc. N/10 HCl, 196.5 Gm. NH_4Cl, 171.5 Gm. $CaCl_2$	5.9	Trace	A few hyaline	40	27	..	Urine free from albumin and casts six weeks after stopping acid
4	17	3½ mo.	27,875 cc. N/10 HCl, 222.5 Gm. NH_4Cl, 277.0 Gm. $CaCl_2$	6.0	Trace	Occasional hyaline and granular rare cellular	42	32	..	Urine clear of casts in two weeks, of albumin in four weeks after treatment
[illegible]	[illegible]	[illegible] weeks	395 Gm. $CaCl_2$	5.9	Trace	Occasional hyaline	..	..	..	Urine returned to normal in two weeks

not until acidosis was well established, about 35 per cent by volume carbon dioxide, that casts appeared constantly and then only in small numbers. With the exception of dog 1, which was pressed to a point where it became necessary to kill her, the albumin test was never more than plus one. The urine p_H did not vary between wider limits than 5.7 to 6.1 during the whole course of the experiment. Two dogs were starved for seven days but still maintained the same urine p_H even when given 4 Gm. calcium chloride by stomach tube.

Terminally, when dog 1 had a plasma carbon dioxide capacity of 21 per cent by volume, her urine p_H fell to 4.9; she had a two plus albumin

reaction and all types of casts in considerable numbers. She was killed because she had lost weight and vomited every thing that she ate or drank.

The right kidney weighed 70 Gm., the left 75 Gm. This is above normal weight for a 14 Kg. dog.[18] The capsule stripped readily, although the surface of the kidney was irregularly pitted. It presented the same picture as that of the spontaneous nephritis kidney in a rabbit. On section abnormalities were not noted grossly, with the exception of the infrequent fine white striations radiating in from the capsule and caused by the spontaneous lesions. All the other organs were also normal in appearance.

Microscopically, the convoluted tubules of the kidney were slightly swollen and showed extensive vacuolization suggesting hydropic degeneration. The nuclei were irregular in shape, usually larger than normal and pale. There were casts in the collecting tubules. There was no intertubular cellular proliferation. The picture as before was similar to that of cloudy swelling.

In addition to this change, which may be attributed to the acidosis, there were localized areas of spontaneous nephritis. These consisted in proliferation of the epithelium of the capsule with hyalinization and obliteration of the capsular space. This usually involved two or three contiguous glomeruli. The adjacent tubules were collapsed. An occasional small arteriole showed hyaline change. Goodpasture[18] described similar changes. MacNider[19] also describes spontaneous lesions in the dog's kidney. One animal that was killed as a control had smaller lesions of a less marked character in the glomerular tufts resembling those described by MacNider.

Of the other four animals, experimentation was stopped on no. 2 because she developed an infection of the urinary tract. The calcium chloride was stopped on the other dogs when the results from no. 1 were found to resemble those obtained on rabbits. The specimens of urine became free from casts and albumin in from two to six weeks.

In summary, therefore, the effect of an acidosis on dogs' kidneys was similar to that on rabbits' kidneys. An acute degeneration of the epithelium of the convoluted tubules resembling cloudy swelling resulted, which did not produce any of the reaction normally associated with a chronic process. The main difference between the dogs and the rabbits was the extreme difficulty in forcing the dogs to form a more than normally acid urine.

18. Goodpasture, E. W.: Anatomical Study of Senescence in Dogs with Especial Reference to the Relation of Cellular Changes of Age to Tumors, J. M. Research **38**:127 (May) 1918.

19. MacNider, W. deB.: A Pathological Study of the Naturally Acquired Chronic Nephropathy of the Dog, J. M. Research **34**:177 (May) 1916.

COMMENT

Rabbits have been used extensively for work in experimental nephritis. Often much encouragement has been derived from the appearance in the urine of large quantities of albumin and numerous casts. Both are readily obtained in rabbits if the animal is forced to excrete an acid urine for a few days. Even when the irritation is carried over as long a period of time as a year, and the acidosis is forced to a point of almost lethal severity, with a resulting three plus albumin reaction and all types of casts, the injury proves microscopically to be only an acute tubular one resembling cloudy swelling. No permanent or chronic injury to the kidney is effected, and repair may take place rapidly and completely when the acidosis is stopped.

In carniverous dogs the same transitory type of injury to the tubular epithelium accompanies an acidosis. The animal protects himself against the development of an acid urine efficiently, however, and the albumin and casts are slower to appear and small in quantity until a drop in the p_H of the urine occurs.

The experimental production of nephritis with uranium nitrate is accompanied by acidosis. The fact as reported by MacNider [20] and Goto [21] that the toxicity of uranium nitrate is diminished by sodium carbonate may well depend on the neutralization of acids which are causing destruction of tubular epithelium. The same condition probably accounts for the diminution of albumin and casts in certain human patients with nephritis following alkali therapy as reported by Hösslin [4] and others.

It is also interesting, in view of the use of calcium and ammonium chloride to relieve tetany and edema, that such large quantities may be given to animals over long periods of time, and that such a severe acidosis may be incurred apparently without any permanent injury.

Our results emphasize the importance that an acid urine may have in producing the clinical picture of an injured kidney. Post and Thomas [illegible] that in a large majority of patients with orthostatic albuminuria, neutralization or alkalinization of the urine by administering alkaline salts or by regulating the diet prevents the development of the albumin and casts. We are at present trying to confirm this and to determine urine [illegible] in patients with orthostatic albuminuria.

Another circumstance under which albumin and casts occur in the urine of a person who does not have nephritis is following severe muscular exercise. At this time the urinary acidity is increased. Bornstein

[illegible] MacNider, W. [illegible] The Inhibition of the Toxicity of Uranium Nitrate [illegible] Med. **23**:171 (Feb. 1916).

[illegible] Post, W. [illegible] and Thomas, W. A.: Orthostatic Albuminuria, J. A. M. A. **80**:[illegible] (Feb. 3) 1923.

and Lippmann [22] report that giving sodium bicarbonate by mouth lessens or does away with the albumin and casts from this cause.

SUMMARY

Calcium chloride and ammonium chloride both increase the acid metabolism when injected into the stomach of an animal.

Rabbits were given varying doses of tenth normal hydrochloric acid and ammonium chloride over periods of time extending from eleven days to one year. The plasma carbon dioxide capacity was lowered, and the animals excreted an acid urine. Casts and albumin were obtained constantly throughout the experimental period. At autopsy, however, the only demonstrable pathologic change in the kidney was acute degeneration of the convoluted tubules, similar to cloudy swelling, but also accompanied by marked pyknosis of the nuclei in cases of longer duration. When acid-forming substance was stopped, the kidney promptly returned to normal.

The results with dogs were comparable except that a greater dose of chloride had to be given in order to produce an acidosis, and that there were less albumin and casts.

The possible causal relation of acidosis to orthostatic albuminuria following strenous exercise is discussed.

I wish to thank Miss Lewis of the Peter Bent Brigham Hospital for the Bun determination on rabbit 14. This was the only rabbit who showed any retention of blood urea nitrogen.

22. Bornstein, A., and Lippmann, A.: Weitere Beiträge zur nicht Nephritischen Albuminurie, Ztschr. f. klin. Med. **86**:345, 1918.

Pharmazie 21(5):307-309 (1966).

Influence of Ammonium Chloride on the Human Body

by K. H. Seidel and H. Krabisch

Already in 1921 Haldane (16) produced acidosis in himself through administration of high doses of NH_4Cl. In 1954 Fazekas (4,5) reported that he could verify, in addition to the already known damages, lipemia and histological damage to the brain in connection with ammonia poisoning in humans as well as in experimental animals. In a later work he was able to experimentally confirm this observation again (6). He declared already at that time the supposition that the lipemia present with ammonia poisoning is a symptom of the poisoning and plays an important role in the cause of death. Moreover he found in the work already cited above (6), just as in one published in 1937 (7), an ascent of the blood sugar and of the serum phosphorus, as well as a fall in the serum calcium. Likewise Fazekas obtained at that time from the poisoning with NH_4OH, a strong reduction of the alkaline reserve of the blood serum and a shift of the serum-pH toward the acid side as a symptom of a serious, uncompensated acidosis. Gaizler (14, 15) was able to demonstrate acetonuria in children and adults who had been poisoned for a long time. In later works Fazekas bestowed upon himself the ammonium chloride therapy and investigated the effect on the neuro-endocrine system. He was able to verify that a treatment with ammonium chloride over a long period of time could cure a chronic headache (13). Disturbances of the estrus cycles with frigidity as well as sterility could also be favorably influenced (8-11).

Fazekas showed test animals with which, through ammonia, ammonium salts, phosphate, hydrochloric acid and other simple compounds, he caused an acidosis and thereby observed an enlargement of the adrenals and the ovaries and was able to experimentally demonstrate an improvement of the ovary functions (8). He was also able to observe that the "target organs" of the endocrine system were influenced; for example, enlargement of the uterus in rabbits (9-11) and elimination of the sterility of goats and rabbits.

The question to be discussed now is whether both of the established observations, the acidosis and the endocrine effect, which we were able to clinically and experimentally confirm (18-22), are to be brought into agreement with a hypothesis on the mechanism of effect of the ammonium chloride.

We must here distinguish two questions.
1. How does the acidosis come about?
2. How does the ammonium chloride effect the "target organs" and also the endocrine organs?

Haldane (16) explained the occurrence of the acidosis produced by intake of NH_4Cl in the following way:

$$NH_4Cl \rightleftharpoons NH_4^+ + Cl^-$$
$$NH_4^+ \rightleftharpoons NH_3 + H^+$$
$$H^+ + Cl^- \rightleftharpoons HCl$$

Only some fish and the crabs are in the position to eliminate ammonia through the gills. The mammals must seek other methods since ammonia acts as a cell poison even in low concentrations. The gross reaction is as follows:

$$HCO_3^- + NH_4^+ + NH_3 + O = C\begin{matrix}NH_2\\NH_2\end{matrix} + 2\,H_2O$$

$\Delta G^0 = 3.54$ kcal/Mol = $\Delta G_{physiol.}$ ~14 kcal/Mol (18).

ΔG^0 indicates the change of the free enthalpy of the system relative to the standard condition, at the concentration of 1 Mol/l dissolved in pure solvent

$\Delta G_{physiol.}$ in contrast, relative to the concentration present under physiological conditions, thus ΔG is dependent on concentration.

The spent energy of approximately 14 kcal/Mol made the participation of energy-rich intermediates necessary, as they are known in the urea cycle.

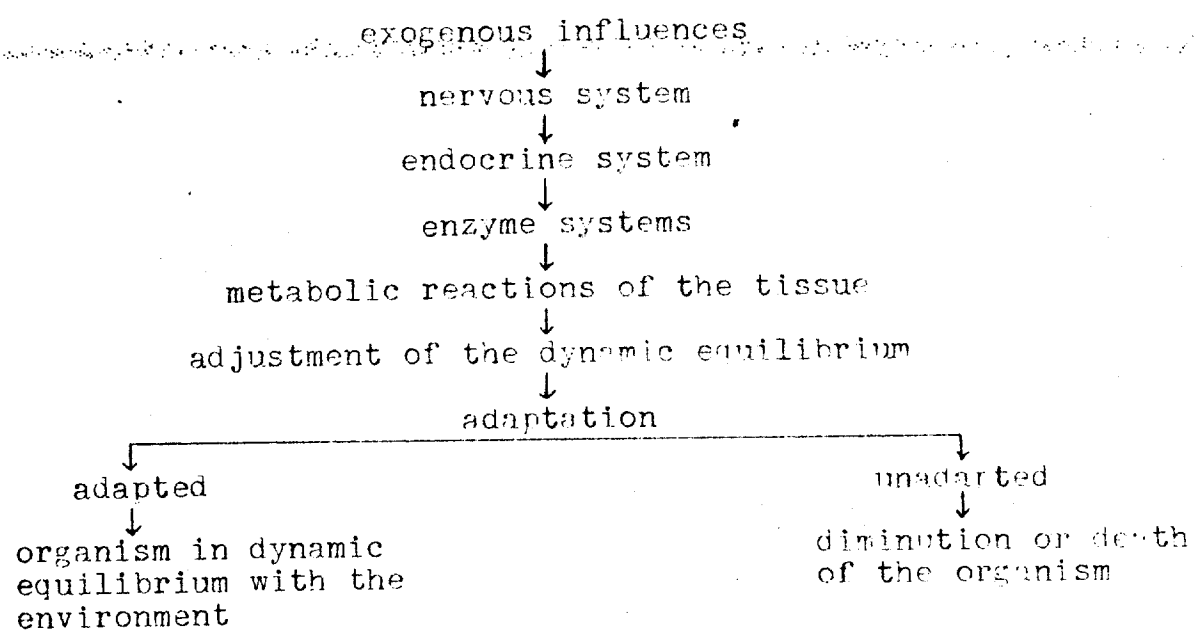
The structural formula of the urea cycle was shown by Karlson (18).

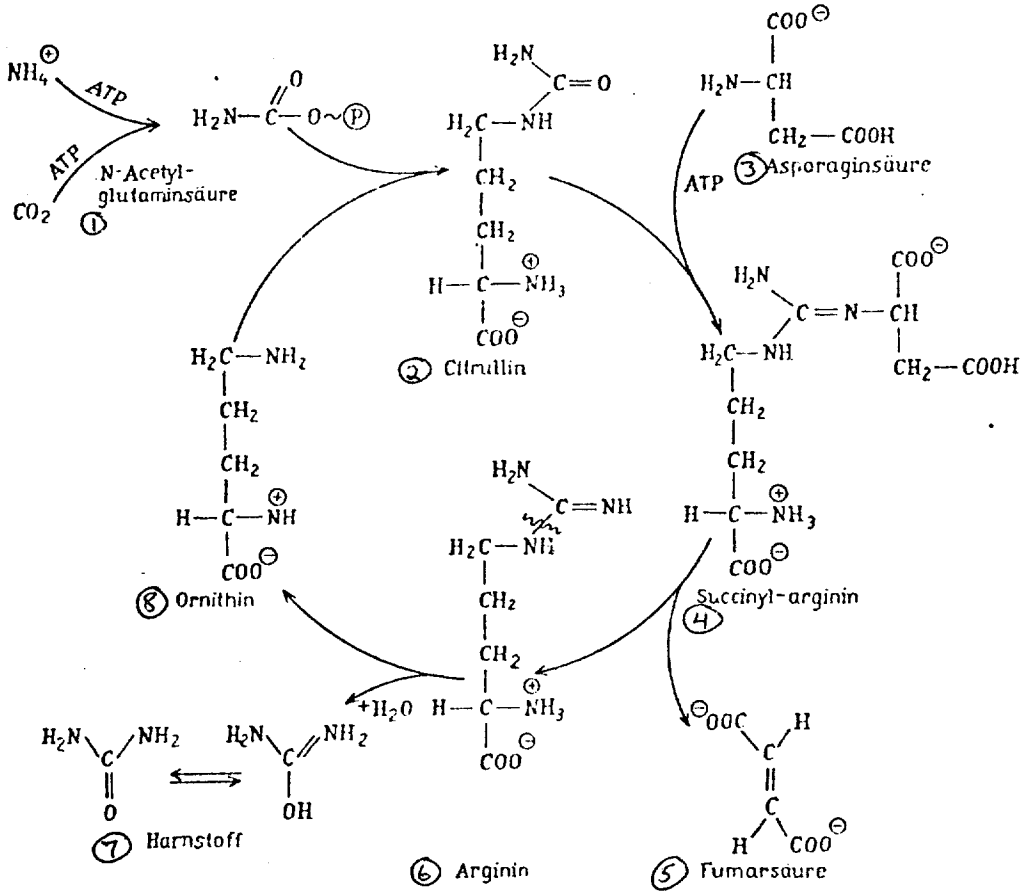
We see that carbamylphosphate is an important intermediate and that in the end effect 2 moles of NH_3 (from glutamic acid and from aspartic acid) combine with 1 mole of CO_2 to form urea and that thereby 3 moles of ATP is consumed. The carbonic acid in the liver of mammals needs, previous to activation, a cofactor, N-acetylglutamic acid. This binds, with consumption of 1 mole of ATP, with CO_2 to form the "active carbonic acid." With that comes a dominant role of glutamic acid (1). This agrees in general with the opinion of Schoen and Sudhof (24), who explained the metabolic acidosis with a withdrawal of α-ketoglutaric acid from the citric acid cycle. The α-ketoglutaric acid, catalyzed through pyridoxaminphosphate, could indeed take up NH_3 and transfer it to glutamic acid. It is conceivable that through the massive withdrawal of α-ketoglutarate, which results from a sudden supply of a large quantity of NH_3, a temporary disturbance of the Krebs cycle results. Such a metabolic disturbance would be allowed, by a therapy, to exist for a short time at most. It allows itself thus to proceed only by a reversible displacement, therefore no attendant phenomenon of such a medication appears. As we already described (22), we were able to verify such a reversible displacement.

Loeschke and Sugioka (22) postulated the appearance of acidosis through the diverse distribution of NH_3 and HCl in the organism. Similar to Winterstein and Gokhan (27), they based their hypothesis on the basis of the easy transfer of NH_3 through the cell membranes, while H^+ and Cl^- remained predominately in the extra-cellular phase. By a mathematical derivation, which corresponded to the actual degree of acidosis occurring, the hypothesis for the distribution was arrived at by Loeschke and Sugioka through registration of the blood- as well as serum-pH. They showed moreover, that,

in contrast to Winterstein and Gokhan, either no or increased respiration occurred such that it could no be due to abnormal elimination of CO_2.

Since NH_3 still exhibits a considerable cell toxicity, the organism will strive to eliminate it as quickly as possible. Although this rapid elimination is, according to the distribution hypothesis of Loeschke and Sugioka, not necessary for the production of acidosis with NH_4Cl, in our opinion it is physiologically involved and takes place, as we were able to show (20). Our investigations also showed, through the significantly increased Na elimination, that the organism attempts to neutralize to some extent the free hydrochloric acid with Na-ions and to thus eliminate it (partly combined also with available K^+ and Ca^{2+}). Through the intense burden of the urea cycle the course of the metabolism was mobilized, and also the energy contributing enzyme system. This explains the fact that we always found high NH_4^+ excretion, as expected (20). This stimulation of the energy-yielding enzyme system also gives an explanation for the improvement of the general health of the ammonium chloride-treated patients. In earlier works we investigated the influence of ammonium chloride on the different endocrine systems. We were able to prove that with ammonium chloride-therapy, the estrogen (20), the 17-ketosteroids and corticosteroids (21) as well as the catecholamine (22) could be normalized. It is difficult to provide clarity to the course of the mechanism of the effect and especially on the location of the activity. The pituitary must have been affected since it otherwise would not be obvious that such various places of hormone formation were influenced by an exogenous factor. Dirscherl (31) spoke of a close connection between the nervous system, especially its autonomic portions, and the hormones as well as of a doubtlessly existing relationship between the pituitary, especially its anterior lobes, and the midbrain and its autonomic center. Hane (14) gave a scheme for the incorporation of the hormones into the entire system of regulation of the organism. According to this scheme the exogenous influences





KEY:

1. N-acetyl-glutamic acid
2. Citrulline
3. Aspartic acid
4. Succinyl-arginate
5. Fumaric acid
6. Arginine
7. Urea
8. Ornithine

stand in a direct line with their effect on the nervous system and further on the endocrine system, the enzyme system and the metabolic reactions. Thereby results the adjustment of the dynamic equilibrium and the adaptation. The outer arrows also indicate a direct, exogenous influence on the dynamic equilibrium. Such a shifting of the dynamic equilibrium must result in a feedback of the metabolic reactions such that an additional arrow in the oppoite direction corresponds to a reversible reaction at that place.

This interpretation of the effect of ammonium chloride was supported by our findings, which show a significant influence of the catecholamine (22), as well as the similar observations of Fazekas, which were able to attain an improvement of chronic headaches. Since the catecholamine belongs to the class of neurohormones of the sympathetic nerve fibers and the "NNM" (2), the correlations are relatively clear, particularly since Bersin defined the "NNM" as a system of highly specialized, neuro-secretory cells lacking appendices.

SUMMARY

By means of a hypothesis of the mechanism of the effect of ammonium chloride, it was attempted to make the numerous experimental findings and clinical observations of the author and other writers agree.

It was determined that this is in best accord when one assumes that the ammonium chloride affects the energy metabolism. The assumption of such central activity explains most simply also the experimentally demonstrable influence of various endocrine glands with regard to the relation between the autonomic nervous system and the endocrine system.

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UTILISATION OF AMMONIUM NITROGEN BY THE ADULT HEN

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SYNOPSIS

Colostomised hens were given one of 3 diets: A, basal diet containing 10·8 per cent crude protein; B, diet A supplemented with the equivalent of 1·8 per cent crude protein as diammonium phosphate; C, diet A supplemented with the equivalent of 1·7 per cent crude protein as diammonium citrate. The mean percentages of dietary nitrogen absorbed were 82·9, 85·2 and 85·2 for the three diets, respectively, the percentages of the nitrogen of diammonium phosphate and diammonium citrate absorbed being 97·8 and 99·0, respectively. Retention of nitrogen was variable but was lower with diet A than with diet C but higher with diet A than with diet B.

INTRODUCTION

There is some evidence that the fowl may be able to utilise ammonium nitrogen (Featherstone, Bird and Harper, 1962; Young, Griffith, Desai and Scott, 1965; Chavez, Thomas and Reid, 1966). However, several authors have failed to show utilisation by poultry of nitrogen in this form, particularly in the case of the chick (Ackerson, Ham and Musselil, 1940; Jones and Combs, 1953; Moran, Summers and Pepper, 1967; Akintunde, Davis and Sykes, 1968; Blair and Waring, 1969).

In view of the conflicting evidence a nitrogen balance experiment was conducted in which adult colostomised hens were given diets supplemented with diammonium phosphate and diammonium citrate to test whether these forms of nitrogen could be utilised.

MATERIALS AND METHODS

Plan and diets. There were 3 diets:

A: basal diet containing 10·8 per cent crude protein.
B: basal diet (A) supplemented with the equivalent of 1·8 per cent crude protein as diammonium phosphate, and
C: basal diet (A) supplemented with the equivalent of 1·7 per cent crude protein as diammonium citrate.

The basal diet contained (parts by weight) 47·60 ground wheat, 40·00 ground maize, 5·00 white fish meal, 6·00 ground limestone, 1·00 dicalcium phosphate, 0·25 salt (NaCl) and vitamins and trace minerals at the required levels estimated by the Agricultural Research Council (1963).

During a preliminary period of 3 d and an experimental period of 4 d each bird received a daily ration of 70 g of air-dry diet to maintain a reasonably constant dry matter intake. Uneaten food was weighed back carefully so that accurate records of food consumption were obtained.

Birds and management. Four colostomised birds were used, diets A and B being given each to 2 birds in the first experimental period and diets A and C being given to 2 birds in the second. The birds were housed in individual cages. Water was provided *ad libitum*. During the trial ambient temperature was maintained at 15·5° C and the birds were allowed 16 h of artificial light daily.

Faeces were collected in polythene bags attached to the birds and urine was collected on metal trays placed beneath the cages. Collections were made thrice daily and the collected material was stored at −20° C.

Techniques. At the end of each experimental period the samples of faeces and urine obtained from each bird were thawed, bulked separately and mixed thoroughly. Samples of diets, faeces and urine were then analysed for nitrogen content by the standard Kjeldahl procedure.

RESULTS AND DISCUSSION

The experimental results are set out in Table 1.

The mean percentages and standard errors of dietary nitrogen absorbed were

TABLE 1

Nitrogen intakes and outputs of hens given diets with and without supplements of ammonium nitrogen

Bird no.	Bird weight (kg)	Diet	Food consumed (g)	N intake (g)	Faecal N (g)	Urinary N (g)	Percentage N absorbed	Percentage N retained
1	1·82	A	260·7	4·50	0·75	2·08	83·3	37·1
2	4·18	A	272·4	4·71	0·81	2·54	82·8	28·9
3	3·07	A	251·1	4·34	0·76	2·93	82·5	15·0
4	3·18	A	208·9	3·61	0·61	2·18	83·1	22·7
1	1·89	B	263·3	5·31	0·75	3·03	85·9	28·8
2	4·28	B	236·7	4·77	0·74	3·39	84·5	13·4
3	3·01	C	247·1	4·94	0·73	3·47	85·2	15·0
4	3·12	C	227·4	4·55	0·68	2·61	85·1	27·7

82·9 ± 0·18, 85·2 ± 0·70 and 85·2 ± 0·05 for diets A, B and C, respectively. The percentages of the nitrogen of diammonium phosphate and diammonium citrate absorbed were 97·8 and 99·0, respectively. From these results it appears that ammonium nitrogen in the above forms is almost completely absorbed by the adult hen.

Values for total nitrogen retentions were somewhat low and were variable. Probable reasons for these results were the maturity and hence low-retentive state of the birds and poor egg production, only one bird laying consistently throughout the trial. Mean retention by birds on the basal diet was lower than by birds fed diet C

and higher than birds fed diet B, the respective percentage values being 21·3 (C) and 18·9 (A), and 21·1 (B) and 33·0 (A).

Further studies are planned using rapidly growing young birds to obtain more accurate estimates of the utilisation of ammonium nitrogen by the fowl.

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Ammonium Chloride as a Urinary Acidifier in the Dog

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Summary and Conclusions

In 5 dogs, the normal urine pH was approximately 6.6. Ammonium chloride at a level of about 200 mg./kg./day given in 3 divided doses was found adequate to maintain normal urine pH below 6 at an average value of about 5.5 and to at least lower it from an alkaline to an acid range in the presence of acute bacterial cystitis. Systemic acidosis was mild at this dosage. In the treatment of bacterial cystitis, attention should be given to control of urine pH both for its direct influence on the infecting organism, and for its influence on the activity of some antibacterial agents.

CANINE CYSTITIS caused by infection may be acute, but in many instances it is chronic and vigorous treatment is required to eliminate it. Many recurrences of infectious cystitis, where no secondary etiologic factor is apparent, probably result from inadequate treatment initially. Lack of attention to the influence of urine pH on the effectiveness of some antibacterial agents and directly on the infectious agent may contribute to the inadequacy of treatment.

It has been demonstrated that streptomycin, neomycin, kanamycin, penicillin, and erythromycin have increased antibacterial activity at alkaline pH; that tetracyclines, cycloserine, and novobiocin are more active in acid pH; that the activity of sulfonamides and chloramphenicol is greater against some organisms at alkaline pH and against others at acid pH; and that nitrofurantoin is unaffected by pH.[1] In view of the influence of urine pH on the effectiveness of chemotherapy of the urinary tract, and since infectious cystitis is often accompanied by alkaline urine, evaluation of a simple means of reliably producing acid urine was undertaken. The purpose was to determine a dose and dose schedule for ammonium chloride which would maintain urine pH below 6 in a normal dog and would acidify the urine when urine pH is abnormally high as commonly occurs with bacterial cystitis.

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Materials and Methods

The experimental subjects were adult male mongrel dogs weighing 38 to 57 lb. None were considered old as judged by the condition of their teeth and by their general physical appearance. All except one, which developed distemper, were clinically healthy. The diet consisted of dry dog food* in all experiments except one in which canned dog food was used.** Water was available at all times.

Ammonium chloride was given orally as 15- and 7½-gr. enteric-coated tablets and 5-gr. uncoated tablets. Since uncoated tablets often produce gastric irritation, enteric-coated tablets were used for the main portion of each dose and uncoated tablets to supplement the main dose. Administration was accomplished by giving one-third of the total calculated daily dose at 6:00 a.m., one-third at 1:00 p.m. and one-third at 8:00 p.m. Samples of urine and blood were collected between 9:00 and 10:00 a.m. daily unless otherwise indicated.

The anaerobic pH of urine and blood was deter-

*Canine Checkers, Ralston Purina Co., St. Louis, Mo.
**Hill's "Special Pack" 16% Protein, Hill Packing Co., Topeka, Kan.

TABLE 1—Effects of Ammonium Chloride on Urine and Blood of 4 Dogs

Period and measurement	Dog No. and daily dose of NH_4Cl			
	1*(25 mg./kg.)	2(46 mg./kg.)	3(91 mg./kg.)	4(170 mg./kg.)
One day before NH_4Cl administration (single value)				
Packed cell volume	35.0	38.0	47.0	44.0
Plasma pH	7.33	7.37	7.36	7.37
Plasma bicarbonate (mM/l)	20.3	20.2	19.3	22.8
Plasma pCO_2 (mm. Hg)	40.1	36.1	34.0	42.2
Urine pH	.....	6.02	6.23	7.22
During NH_4Cl administration (7-day av.)				
Packed cell volume	34.0	34.0	44.0	42.0
Plasma pH	7.39	7.38	7.37	7.34
Plasma bicarbonate (mM/l)	22.8	21.4	22.2	21.5
Plasma pCO_2 (mm. Hg)	37.8	36.4	39.1	39.8
Urine pH	6.02	6.32	5.69	5.60
Urine sp. gr.	1.044	1.045	1.035	1.013
One day after last NH_4Cl administration (single value)				
Packed cell volume	35.0	30.0	40.0	43.0
Plasma pH	7.38	7.35	7.40	7.42
Plasma bicarbonate (mM/l)	22.2	22.3	23.8	24.5
Plasma pCO_2 (mm. Hg)	38.0	41.6	38.7	38.1
Urine pH	6.07	6.46	6.25	6.71
Urine sp. gr.	1.034	1.050	1.050	1.056

*Dog 1 developed distemper and was administered ammonium choride for only 4 days. On the 5th day postadministration samples were drawn, and he was eliminated from the experiment.

mined at 40 C. by a Beckman expanded scale pH meter with a constant temperature block.* Urine specific gravity was measured with a urinometer or refractometer. Plasma CO_2 was measured manometrically.[7] Packed red cell volume (PCV) was determined by the microhematocrit method. Urine and blood samples which could not be measured immediately were stored in an ice-water bath for less than 3 hours. Storage up to 6 hours at 0 C. does not significantly alter the pH or CO_2 content of these fluids.[4] The pCO_2 (plasma CO_2 tension in mm. Hg) and plasma bicarbonate were calculated from the plasma pH and CO_2. The measured blood pH was corrected for the suspension effect of erythrocytes and the difference between body temperature of the dog, taken as 39 C. and temperature at which pH was measured.[6] The pK' (apparent ionization constant) of plasma carbonic acid was assumed to be 6.08 or 6.09 depending on plasma pH.[8]

Results

Normal Urine pH.—From 5 dogs fed dry food for at least 15 days, urine was collected anaerobically by catheterizing each dog and withdrawing urine via the catheter into an oiled 10-ml. syringe. From 15 separate urine samples the mean pH was 6.63, with a standard deviation of 0.71. Since urine pH can be affected by diet, measurements were made on 3 urine samples from a dog on canned dog food. The average pH was 6.87.

The Dose of Ammonium Chloride.—Prior to administration of ammonium chloride to 4 dogs used in this phase of the study, blood samples were taken for measurement of pH, PCV, and plasma CO_2 content. Venous blood was withdrawn anaerobically from the unoccluded cephalic vein. After normal blood values had been measured for 2 days, ammonium chloride administration was initiated according to the following dose schedule:

Dog	Dose	
	mg./kg./day	gr./lb./day
1	25.5	0.178
2	45.6	0.319
3	91.0	0.637
4	170.0	1.19

The dogs, except 1, were maintained on ammonium chloride for 7 days. Dog 1 developed distemper and ammonium chloride treatment was stopped after 4 days. Beginning 24 hours after the initial ammonium chloride administration and continuing until a day after the last dose, blood samples were measured for pH, PCV, and plasma CO_2 content. Urine pH and specific gravity were determined each day (Table 1). Results given for the period following cessation of ammonium chloride are single values, not averages. For evaluation of the effect of a change in diet, 1 dog was fed canned food and given ammonium chloride, 220 mg./kg./day. Urine pH was measured for 3 days and averaged 5.56.

Variation in Urine pH. Because urine pH may vary considerably during a 24-hour period, urine pH was measured during a

*Available from Beckman Instruments, Inc., Fullerton, Calif.

period of ammonium chloride administration. For this purpose 1 dog was given 220 mg./kg./day of ammonium chloride for 5 days prior to and during the period of measurement. Urine pH and specific gravity were measured every 2 hours for 24 hours. Average urine pH was 5.48 and ranged from 5.22 to 5.90. The specific gravity averaged 1.017 and ranged from 1.003 to 1.042.

Urine Acidification in the Presence of Infectious Cystitis.—Primary cystitis was initiated by depositing 10 ml. of a 0.1% alcoholic solution of salicylic acid in the urinary bladder of a dog. On the following day, 5 ml. of a 24-hour veal broth culture of *Proteus mirabilis** was deposited in the urinary bladder. This organism's proteolytic and urea-splitting activities produce marked alkalinity of the urine. The urine pH rose from 6.76 on the day of inoculation to 7.66 on the following day and averaged 7.98 during the next 2 days. With increased pH, the urine developed an ammoniacal odor, became brownish red, and sometimes contained blood clots. At this point, ammonium chloride treatment was initiated at a dose of 197 mg./kg./day. By the 2nd day of treatment, urine pH had dropped to 6.71 and during the next 2 days averaged 6.57. As the pH remained below 7, the color of the urine changed from brownish red to pale amber and became clear. At this point, ammonium chloride was discontinued. After withdrawal of ammonium chloride, the urine pH rose to 7.68 and 8.10 at 42 and 67 hours postwithdrawal, respectively. The urine again became brownish red and contained blood clots. During this experiment clinical signs of cystitis accompanied the urine changes.

Discussion

In this study, the normal urine pH of the dog was higher than values usually given. This difference was probably due in part to the nature of the diet and the care taken to maintain anaerobic conditions during the process of sample collection and pH determination.

The objective in arriving at a dose of ammonium chloride for acidifying the urine was to find a dose which produced a minimal urine pH without producing a marked systemic acidosis. In the dog, a dose of about 200 mg./kg./day appeared to meet this requirement. Lower doses resulted in higher urine pH, and since this dose produced a mild acidosis, a higher dose would increase the degree of acidosis and hence the stress on the dog.

Changing the diet from dry to canned food did not result in a significant alteration in the normal urine pH nor in urine pH during ammonium chloride administration. The dose of ammonium chloride to acidify bovine urine has been found to be about 630 mg./kg./day.[3] In an adult man, a daily dose of about 100 mg./kg./day has been found to be adequate.[8] The ash content of the prepared dog foods may have been related to the relatively high dose for the dog as compared with that for man. Due to the similarity in composition of dry dog foods the results obtained here probably are applicable to most normal feeding situations.

In evaluating antibiotics enhanced by an acid medium, a pH of 5.5 was used.[1] In this study, without antibiotic therapy, ammonium chloride, at a dose of about 200 mg./kg./day, reduced urine pH in the presence of a bacterial cystitis from about 8.0 to 6.6. In the presence of an appropriate antibiotic, it would be reasonable to expect a reduction in the number of bacteria in the bladder and a further reduction in urine pH to about 5.5. Evaluation of this point was beyond the scope of this work. Change of urine pH in itself did not appear to constitute effective treatment of infectious cystitis as indicated by the rapid return of urine pH to an alkaline level and reappearance of other signs of cystitis upon withdrawal of ammonium chloride. Although this observation was made in only 1 dog, it was consistent with earlier findings that simple acidification of the urine had little if any curative effect.[7]

*Obtained from Dr. John A. Newman, Department of Veterinary Bacteriology and Public Health, University of Minnesota, St. Paul.

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Aujeszky's Disease in the Dog and Cat

Aujeszky's disease in dogs has been reported infrequently, mainly from European countries. The clinical syndrome in domestic carnivores is characterized by rapidly fatal illness with intense pruritus commonly localized around the face or mouth. In a recent report the neuropathologic and histologic lesions in 4 dogs and 1 cat were described. Four of these 5 affected animals came from farms on which pigs or cattle were affected with Aujeszky's disease several days to several weeks previously.

The pattern of lesion and virus distribution was found to be similar to that observed in experimentally infected ruminants. In the affected animals there was a cutaneous area of nonspecific inflammation associated with characteristic viral lesions in ganglia of the related spinal or cranial nerves. Lesions within the brain or cord were most severe in the neurones receiving afferent fibers from the affected ganglia.

Diagnosis was confirmed when Aujeszky's virus was isolated in tissue culture and the characteristic pruritus syndrome reproduced in rabbits. In the affected cat, virus was isolated from the wall of the pharynx but not from nasal or oral secretions.—*C. Dow and J. B. McFerran, Vet. Rec., 75, (Oct. 26, 1963): 1099.*

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OBSERVATIONS ON THE ETIOLOGY OF THE TOXEMIAS OF PREGNANCY.

II. Production of Acute Exacerbation of Toxemia by Sodium Salts in Pregnant Women with Hypoproteinemia.*

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The designation "toxemia of pregnancy" has included at one time or another almost all the non-septic, non-mechanical ills of the pregnant state. At the present time certain of these conditions, such as

* Aided by a grant from the William W. Wellington Memorial Research Fund, the Harvard Medical School.

anemias[7] and the polyneuritis[8] of pregnancy, are regarded as due to nutritional deficiency. It has also become apparent that the diagnosis "toxemia of pregnancy" has in the past included such conditions as essential hypertension, acute and chronic glomerulonephritis, pyelonephritis, and rarely malignant nephrosclerosis. In fact, probably five-sixths of patients said to have "toxemia" of pregnancy are suffering from some independent and frequently pre-existing disease in which the pregnancy is merely an incident. Nevertheless there remains a group of patients who have been apparently well prior to pregnancy and who exhibit no evident abnormalities after the puerperium, but who do develop relatively acute arterial hypertension in the last trimester of pregnancy, and who do not exhibit the clinical or laboratory findings of glomerulonephritis, pyelonephritis, or malignant nephrosclerosis. These patients have been shown to have markedly diminished plasma proteins invariably associated with water retention.[9,10] The condition has usually been designated "low reserve kidney," "pre-eclampsia," or "eclampsia," depending on the severity of the condition.[5] Lastly there are patients with pre-existing arterial or renal disease in whom the development of increasing hypertension or albuminuria is associated with hypoproteinemia and water retention.

The studies to be reported here indicate that hypoproteinemia is the primary factor permitting significant water retention in pregnancy and that such retention may be accompanied by considerable rises in blood pressure, increasing albuminuria and the appearance of such pre-eclamptic manifestations as severe headache, visual disturbances, and vertigo. It is known that the administration of sodium salts to normal individuals may produce slight increments of water retention, whereas in individuals with lowered plasma proteins marked retention may occur in inverse proportion to the plasma protein level.[4] There is evidence that the bicarbonate is more effective than the chloride of sodium in inducing water retention.[4] The mechanism by which sodium salts produce water retention is a complicated one. For a thorough discussion of this problem Peters' "Body Water"[3] may be consulted.

Methods. Women in the last trimester of pregnancy were admitted to the Thorndike Ward of the Boston City Hospital, but *not* put to bed. Each patient received the usual ward diet. Fluids were allowed *ad libitum*. The patients were not encouraged either to increase or decrease their fluid intake. Each patient was weighed upon arising in the morning. About 2 hours after breakfast, the patient having been in a chair for at least 20 minutes, the blood pressure was measured. The daily fluid intake and output were recorded. On the morning after admission to the hospital and at intervals thereafter 20 cc. of blood were withdrawn without stasis from an antecubital vein for analysis. The patients were recumbent for ½ hour prior to this procedure. The 24-hour urine was analyzed daily for albumin by the quantitative method of Tsuchiya. The total plasma protein and the plasma albumin were determined by a micromodification of Howe's method.[2] In the tables, the "initial" blood pressure represents the *average* of the first

3 days' readings. The change in blood pressure recorded represents the difference between this average and the *average* of the three readings obtained on the mornings of the last 2 days of the test period and that of the morning after the test period. The "initial" weight represents the reading on the day the test period began. The change in weight represents the difference between this weight and that recorded on the first morning after the test period.

TABLE 1.—RESPONSE TO THE ADMINISTRATION OF SODIUM SALTS FOR 7 DAYS IN 8 PREGNANT WOMEN WITH PLASMA COLLOID OSMOTIC PRESSURES ABOVE 220 MM. H_2O.

Case No.	Weight gain.	Blood pressure change.		Highest blood pressure prior to admission.		Average "initial" blood pressure.		Change in albuminuria.	Average daily fluid exchange during test period.		Calculated plasma oncotic pressure.
	%	mm. Hg.		mm. Hg.		mm. Hg.					
		Diast.	Syst.	Syst.	Diast.	Syst.	Diast.		Intake cc.	Output cc.	
1	0	-6	+19	154	7[illegible]	125	74	0	2507	2057	[illegible]
2	[illegible]	[illegible]	0	147	[illegible]	138	85	0	2452	1639	255
3	1.8	-9	-2	171	[illegible]	130	77	0	1410	1514	[illegible]
4	1.2	-1	-13	154	98	124	87	0	3030	3026	248
5	1.4	-1	-11	146	[illegible]	139	86	0	2391	1993	[illegible]
6	1.6	-1	18	224	11[illegible]	148	100	0	3214	1875	257
7	1.8	-2	-2	184	1[illegible]8	139	91	0	2468	2063	[illegible]
8	1.8	-1	0	15[illegible]	[illegible]	125	85	0	2700	1470	252
Av.	1.4	-1	-3	169	[illegible]	138	85	0	2535	1963	244

Case 6 received sodium bicarbonate.

Observations. I. Eight women known to have had some degree of arterial hypertension either before or early in pregnancy were studied in the last trimester of pregnancy. The osmotic pressure of the plasma proteins, calculated by the method of Wells, Youmans and Miller, varied from 230 to 260 mm. of water (Table 1). None of these women had significant albuminuria. In practically every instance the initial average blood pressures recorded in the test period were lower than those recorded prior to admission to the hospital. Such hospitalization without bed rest should result in a fall in blood pressure is not for discussion here.

On the third morning of their hospital stay 7 of these women commenced receiving 16 gm. of sodium chloride (= 6.3 gm. of Na) daily, and one (Case 6) commenced taking 23 gm. of sodium bicarbonate (= 6.3 gm. of Na) daily for a minimum period of one week. Gains of weight up to 1.8% of the initial body weight were observed (Table 1). None developed visible edema. No significant changes in diastolic blood pressure occurred (Table 1). In no instance did albuminuria increase nor did cylindruria or hematuria appear. No subjective symptoms occurred. Observations in two typical cases are charted (Charts I and II).

It thus appears that the daily administration of 6.3 gm. of Na (either in the form of 16 gm. of sodium chloride or 23 gm. of sodium bicarbonate) results in small increments of weight, but is without

effect on women with arterial hypertension in the last trimester of pregnancy who have essentially normal plasma protein values.

TABLE 2.—RESPONSE TO THE ADMINISTRATION OF SODIUM SALTS FOR 7 DAYS IN 10 PREGNANT WOMEN WITH PLASMA COLLOID OSMOTIC PRESSURES BELOW 220 MM. H_2O.

Case No.	Weight gain.	Blood pressure change.		Highest blood pressure prior to admission.		Average "initial" blood pressure.		Change in albuminuria.	Average daily fluid exchange during test period.		Calculated plasma oncotic pressure.
	%	mm. Hg.		mm. Hg.		mm. Hg.					
		Diast.	Syst.	Syst.	Diast.	Syst.	Diast.		Intake cc.	Output cc.	mm. H_2O.
11	2.5	-1	-10	125	85	123	78	0	2618	2652	172 [illegible]
	3.4	+6	+15	135	86	125	89	0	2734	2623	217 [illegible]
12	3.3	+16	+14	182	108	153	79	0	2661	2080	203
13	3.6	+5	+3	184	106	147	81	0	4160	3820	212
14	4.7	+27	+31	152	84	131	82	+	2301	2585	192
15	4.8	+24	+30	154	100	139	93	0	3808	2320	210
16	5.5	+33	+11	172	100	132	80	+	6266	7253	193*
17	5.7	+12	+16	150	120	153	100	+	1920	1593	182*
18	5.9	+18	+7	165	90	128	73	0	2668	1332	180
19	7.2	+1	+7	156	100	144	92	+	2600	2047	212
20	7.7	+6	+17	144	100	135	98	+	4185	2270	120*
Av.	4.9	+11	+14	156	98	134	86		3329	2524	192

* The test period was limited to 4 days or less in Cases No. 17, 20 because of the development of pre-eclamptic symptoms.

The test period in Case 15 was limited to 5 days because of severe headache.

Cases 11, 14, 18, 19 received sodium bicarbonate; Cases 11, 14 had 6 rest periods. Chart IX (Case 12) had arterial hypertension prior to the pregnancy. Case 13 had chronic nephritis prior to the pregnancy.

TABLE 3.—RESPONSE TO THE FEEDING OF A HIGH PROTEIN DIET TO 6 PREGNANT WOMEN WITH PLASMA COLLOID OSMOTIC PRESSURES BELOW 220 MM. H_2O.

Case No.	Weight loss.	Blood pressure change.		Highest blood pressure prior to admission.		Average "initial" blood pressure.		Change in albuminuria.	Average daily fluid exchange during test period.		Calculated plasma oncotic pressure.
	%	mm. Hg.		mm. Hg.		mm. Hg.					
		Diast.	Syst.	Syst.	Diast.	Syst.	Diast.		Intake cc.	Output cc.	mm. H_2O.
31	2.9	-2	-9	160	108	148	90	0	2562	1935	206
32	3.4	-5	+19	150	102	128	85	0	2371	1250	205
33	3.4	+3	-3	170	110	143	87	0	3902	2735	218
34	3.7	-9	-9	168	104	143	95	0	2331	2281	229
35	3.8	-5	-8	160	120	136	100	0	2540	2544	210
36	4.1	-6	-8	166	100	157	96	0	2880	2985	204
Av.	3.5	-4	-8	162	107	142	92	0	2614	2288	210

II. Ten women were studied in the last trimester of pregnancy. All of them had some degree of arterial hypertension and 8 were known to have had normal blood pressures before or early in pregnancy. One patient had definite evidence of having essential hypertension and another chronic glomerulo- or pyelonephritis prior to pregnancy. The osmotic pressure of the plasma proteins, calculated as above, varied from 120 to 217 mm. of water. Five

patients showed albuminuria of from 0.3 to 2.8 gm. per liter. None had hematuria. On the third morning of their hospital stay 6 commenced receiving 16 gm. of sodium chloride (= 6.3 gm. of Na)

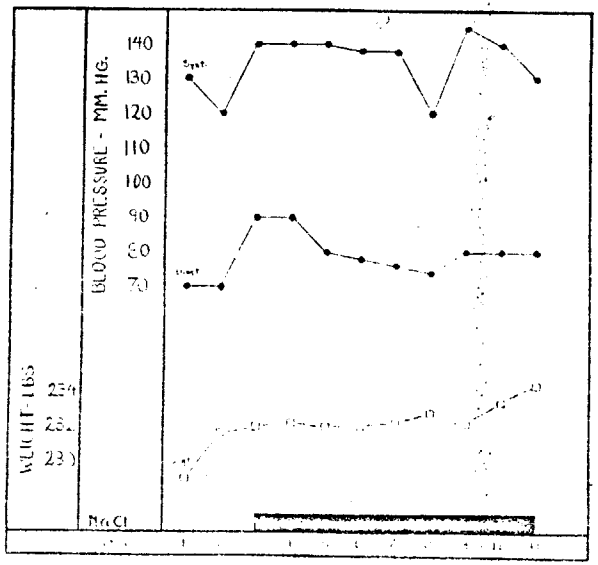

CHART I.—Weight and blood pressure changes in Case 3. Plasma oncotic pressure 250 mm. H_2O. Sodium chloride 16 gm. daily (solid block).

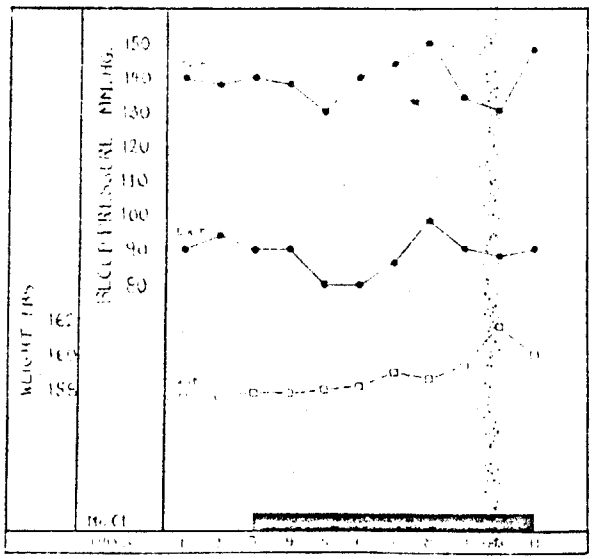

CHART II.—Case 7. Plasma oncotic pressure 230 mm. H_2O. Sodium chloride 16 gm. daily (solid block).

and 4 commenced taking 23 gm. of sodium bicarbonate (= 6.3 gm. of Na) daily. Gains in weight of from 2.5 to 7.7% of the initial weight occurred, associated in each instance with the appearance of

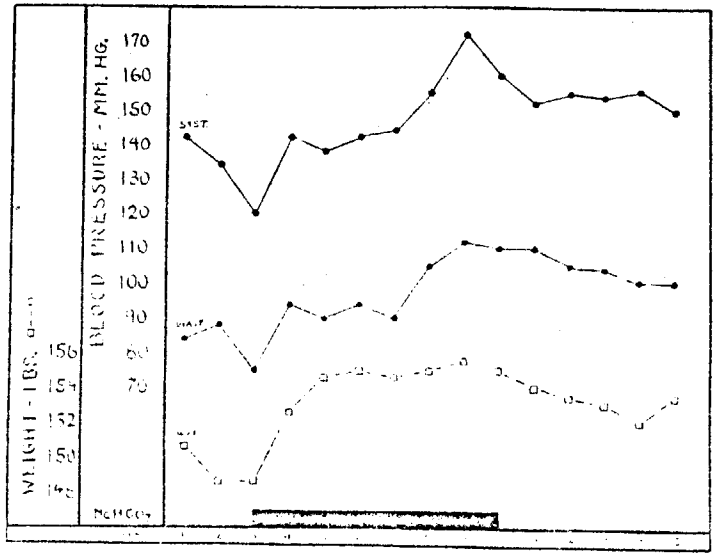

CHART III.—Case 14. Plasma oncotic pressure 192 mm. H_2O. Sodium bicarbonate 23 gm. daily (solid block).

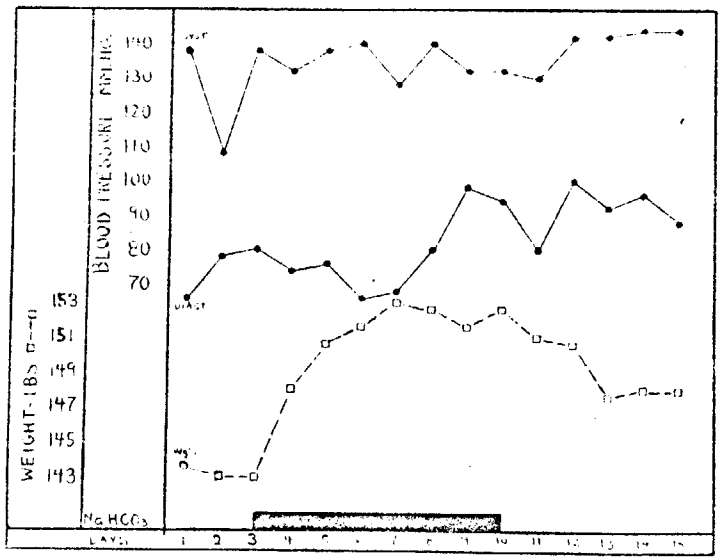

CHART IV.—Case 18. Plasma oncotic pressure 180 mm. H_2O. Sodium bicarbonate 23 gm. daily (solid block).

visible edema involving the extremities and frequently the [illegible]. Nine of the 10 women showed an elevation of arterial blood pressure, the diastolic being more consistently affected (Table 2) (Charts III to IX). Five showed significant increases in albuminuria so that the albumin varied from 1 to 5 gm. per liter. Cylindruria occurred in 2 patients and microscopic hematuria in one. In 2 patients the development of severe headache, visual disturbances, and vertigo necessitated the omission of sodium after 3 days (Charts VI and VII). A third patient was so uncomfortable from severe headache and edema that therapy was omitted on the fourth day (Chart VIII), and in a fourth, severe headache necessitated the omission of sodium after 5 days (Chart V).

It is thus apparent that the daily administration of 6.5 gm. of sodium (either in the form of 16 gm. of sodium chloride or 23 gm. of sodium bicarbonate) results in significant gains of weight together with the appearance of visible edema in women with hypoproteinemia during the last trimester of pregnancy. In these women significant rises of arterial blood pressure occurred. In 5 albuminuria increased, and in 3 symptoms of pre-eclampsia appeared necessitating immediate cessation of the sodium administration.

One patient, Case 11 (Chart IX), had 3 test periods. During the first period, when her calculated oncotic pressure was 172 mm. of water, she received 16 gm. of sodium chloride daily for 9 days. Her weight increased slowly by 2.5%. The blood pressure was unchanged. During the next 6 days she received 23 gm. of sodium bicarbonate daily. Her weight increased slowly by an additional 1.5%. No change in blood pressure occurred. At the end of this period her plasma proteins had risen so that the calculated oncotic pressure was 207 mm. of water. One month later she was readmitted to the hospital with plasma proteins giving a calculated oncotic pressure of 217 mm. of water. At this time she was given 23 gm. of sodium bicarbonate daily. Her weight increased by 3.4% in 2 days, edema appeared, and her blood pressure rose from an average of 125 systolic and 80 diastolic to 140 systolic and 95 diastolic (Chart IX). However, during the continued administration of sodium her weight declined to its original level, edema disappeared, and concomitant with this her blood pressure fell to an average of 126 systolic and 74 diastolic.

This phenomenon of spontaneous diuresis and loss of edema during the continuous administration of sodium salts has been observed in patients with hypoproteinemia due to malnutrition or nephrosis and in animals subjected to plasmapheresis as well as those with nutritional hypoproteinemia.[illegible] No satisfactory explanation for this has been suggested.

III. Five patients of Group II were given ammonium chloride daily. One (Case 16) received 16 gm. daily 4 days after the omission of sodium chloride (Chart VI) for a period of 4 days during which

time she lost 3.5% of her body weight and her blood pressure fell from an average of 146 systolic and 103 diastolic to 125 systolic and 70 diastolic.

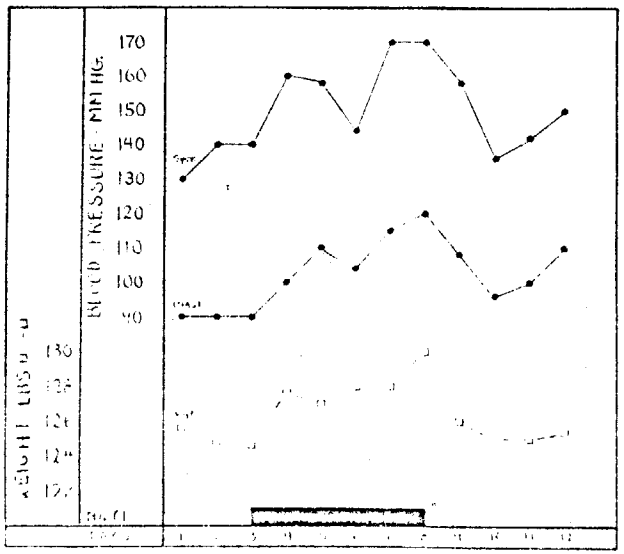

CHART V.—Case 15. Plasma oncotic pressure 210 mm. H_2O. Sodium chloride 16 gm. daily (solid block).

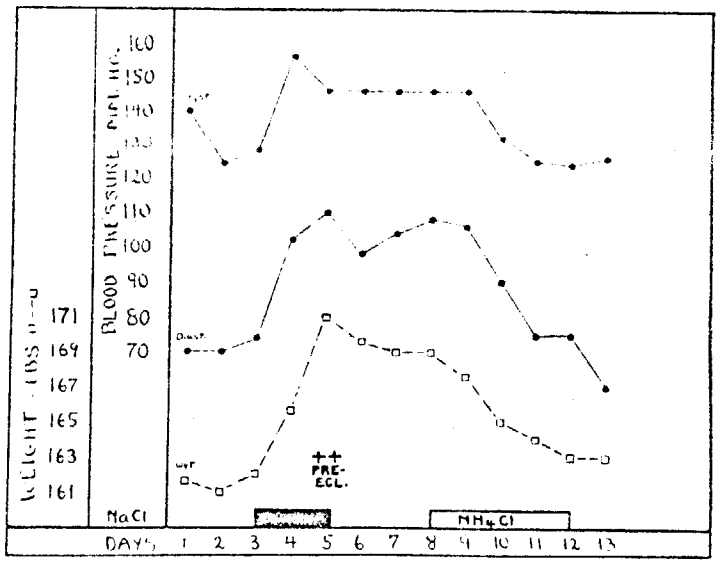

CHART VI.—Case 16. Plasma oncotic pressure 193 mm. H_2O. Sodium chloride 16 gm. daily for 2½ days (solid block). Ammonium chloride 16 gm. daily for 4 days (open block).

The second patient (Case 17) received 16 gm. of ammonium chloride daily for 6 days, 3 days after admission to the hospital

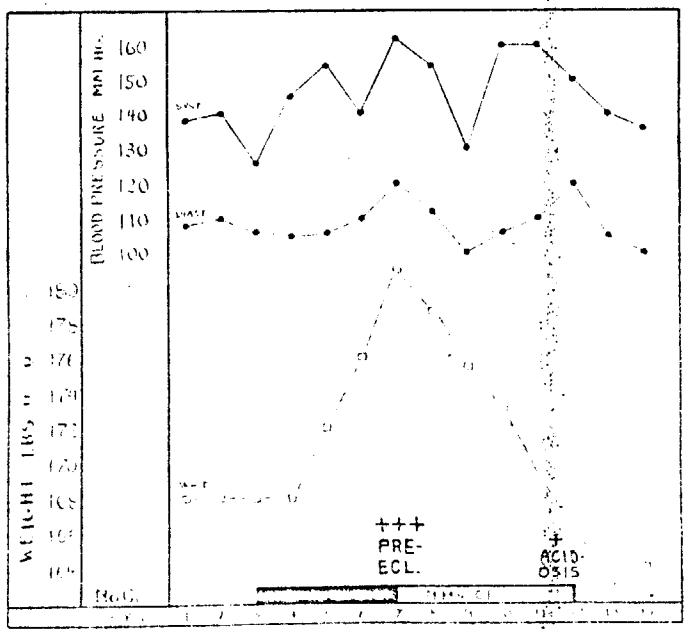

Chart VII. Case 20. Plasma oncotic pressure 120 mm. H.O. Sodium chloride 16 gm. daily for 4 days (solid blocks). Ammonium chloride 8 gm. daily for 7 days (open blocks).

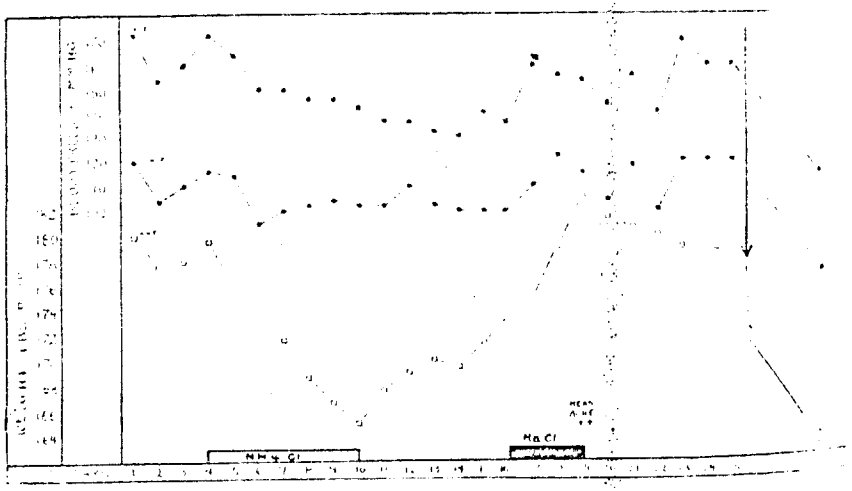

Chart VIII. Case 17. Plasma oncotic pressure 182 mm. H.O. Ammonium chloride 16 gm. daily for 6 days (open blocks). Sodium chloride 16 gm. daily 3 days (solid blocks).

(Chart VIII). Her weight dropped by 8.1% in 6 days and her blood pressure fell from an average of 166 systolic and 110 diastolic to 144 systolic and 102 diastolic.

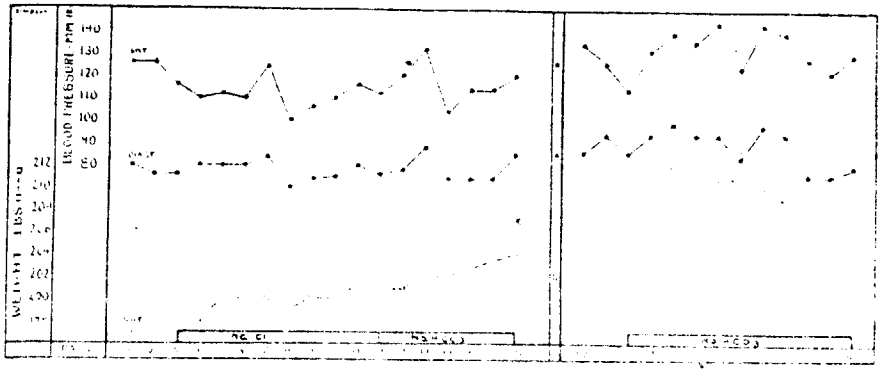

Chart IX. Case 11. Plasma oncotic pressure 172 to 217 mm. H.O. Sodium chloride 16 gm. daily for 6 days. Sodium bicarbonate 24 gm. daily for 6 and 10 days in second and third periods.

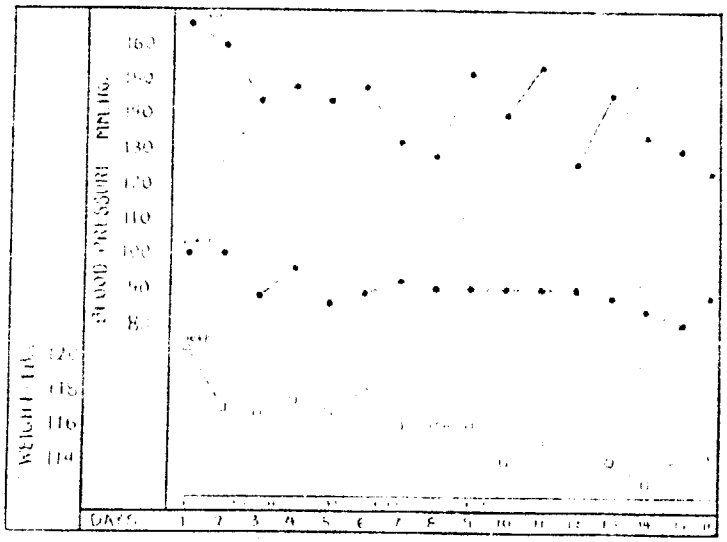

Chart X. Case 36. Plasma oncotic pressure 201 mm. H.O. 200 gm. protein diet for 16 days.

The third patient (Case 20) received 8 gm. of ammonium chloride daily immediately after pre-eclamptic symptoms had been produced by sodium chloride (Chart VII). Acidosis occurred after 4 days with hyperpnea, dyspnea, dehydration, and nausea. Her weight fell by 9.9% and her blood pressure decreased from an average of 152 systolic and 112 diastolic to 142 systolic and 108 diastolic.

Two other patients (Cases 13 and 14) were unable to tolerate ammonium chloride due to the occurrence of nausea, vomiting, and acidosis within 48 hours of its first administration.

Four patients, in addition to the 24 referred to above, with normal blood pressures in the last trimester of pregnancy, received 16 gm. of ammonium chloride daily for 1 week. Weight losses of 4.6, 5.0, 5.7, and 5.8% occurred. There were no consistent blood pressure changes.

IV. It is remotely possible that the 10 women with hypoproteinemia would have developed the changes recorded even if sodium had not been administered. Accordingly, 6 other women, whose condition was comparable to the 10 of Group II, with calculated plasma oncotic pressures varying from 206 to 220 mm. of water, were placed on a daily diet consisting of 260 gm. of protein, 100 gm. of carbohydrate, and 50 gm. of fat immediately upon admission to the hospital. No sodium other than that contained in the food or taken as seasoning on the food was administered. It is apparent from Table 3 that no significant change occurred in blood pressure or albuminuria. Rather than gains in weight, weight losses of from 2.9 to 4.1% occurred in 1 week's time. In no case did symptoms appear to become worse. A typical case has been charted (Chart X).

Discussion. It is generally conceded that only women who gain weight excessively during pregnancy develop the signs and symptoms of true "toxemia."[5] Previously[6b] evidence was presented which suggested that this excessive gain in weight was the result of water retention with either occult or gross edema. Further it was indicated that this water retention resulted not from hormonal influences or renal disease, but was the simple effect of lowered oncotic pressure of the plasma proteins. It was also shown that concomitant with a loss of retained water following the administration of a high protein diet the signs and symptoms of "toxemia" abated. In the present study, it has been shown that water retention may be induced in pregnant women, as in non-pregnant subjects, by the administration of sodium salts, and that the degree of retention varies inversely with the oncotic pressure of the plasma proteins. The difference in behavior of women with low oncotic pressures and those with normal pressures is quantitative rather than qualitative insofar as water retention is concerned. It has further been shown that pregnant women with low plasma proteins, who exhibited rapid and marked water retention following the administration of sodium salts, concomitantly developed rises in arterial blood pressure, and in 5 instances increased albuminuria. In 3 of the 10 cases studied symptoms and signs of "pre-eclampsia" appeared. With our present state of knowledge concerning the nature of arterial hypertension it would be foolhardy to speculate on the mechanism involved in producing the hypertension in the pregnant women studied. The fact remains that rapid gains in weight were accompanied by rises in arterial blood pressure.

In previous papers,[6a,b] it was indicated that lowered plasma proteins could result from a variety of factors, such as poor protein intake, protein loss, and malabsorption, and possibly failure of protein manufacture—all in the presence of the fetal demand for protein building materials. The importance of preventing the operation of the first factor by supplying an adequate amount of dietary protein to all pregnant women has been pointed out.

Summary and Conclusions. 1. Sodium given to 10 pregnant women with hypertension and hypoproteinemia resulted in significant weight gains, the occurrence of visible edema, and rises in the arterial blood pressure in each instance. In 5 women albuminuria increased and in 3 symptoms of pre-eclampsia appeared.

2. An identical amount of sodium given to 8 pregnant women with hypertension but no hypoproteinemia resulted in small increments of weight but was without other effect.

3. Ammonium chloride given to 3, and a high protein diet to 6, pregnant women with hypertension and hypoproteinemia resulted in significant weight losses and declines in arterial blood pressure.

4. The evidence presented suggests that the manifestations of "toxemia" of pregnancy in the patients studied resulted from water retention conditional upon hypoproteinemia.

5. The administration of sodium is dangerous in pregnant women with hypoproteinemia. The bicarbonate is fully as dangerous as the chloride. Its indiscriminate use in pregnancy is contraindicated.

The writer is indebted to Dr. F. H. L. Taylor and Miss Margaret Adams for carrying out the chemical determinations reported in this paper.

This work was made possible through the coöperation of the visiting surgeons and house staff of the Obstetrical Service of the Boston City Hospital, to whom the writer acknowledges his indebtedness.

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12: 417-424, 1929

A Note on the Hyperpnoic Action of Ammonium Chloride in Cats.

By

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Working with the hyperglycaemic action of ammonium chloride Morita[1] came to notice the absence of the marked acceleration of breathing when the salt was administered to cats, the splanchnic nerves of which had been interferred with bilaterally.

I have now undertaken to develop this question put forward by him by applying the salt to cats, whose splanchnic nerves on one side were divided and the suprarenal gland on the other side was removed. And the repetition of the experiments quoted in his communication was tried in the first place with results much similar to his. Further some unilaterally splanchnectomized cats with extirpating the suprarenal body have been also experimented on. All the data will be summarized in the following table, a protocol from the series of experiments on cats, whose splanchnic nerves on one side and the gland on the other had been interferred with.

Adult cats of good nutrition were employed. They were fed with a mixed diet (rice (mainly), fish, potato, radish and other vegetables cooked together with a little table salt and soy) for a few weeks at least before the experiment or operation. The sex was at random.

Ammonium chloride (Merck) was made in 4 mol solution warmed to about 38° and injected hypodermically in the hind limbs. Since a smaller dose of ammonium chloride than 0.7 grm. per kilo of body weight was often found insufficient to bring about a quickening of respiration and general excitation in normal cats, it was given in a dose of 0.7 grm. per kilo through-

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1) Sachi. Morita, Tohoku J. Exp. Med., 1922, 3, 410.

TABLE.

(0.7 gm NH_4Cl per kilo of the body weight.)

	Number of cases	Date of splanchnicotomy	Date of suprarenalectomy	Date	Body weight and sex	Respiration: Frequency (per minute) Before injection	Respiration: Frequency (per minute) Maximum after injection	Respiration: Time till hyperpnoe	Respiration: Duration of hyperpnoe	Suger in urine %	Body temperature: Before injection	Body temperature: Minimum after injection
Normal cats	1			15. XII. '25	3.1 ♂	19	200	25′	25′	—	38.2	36.5
	2			15. I. '26	2.8 ♂	25	160	35′	1h30′	0.61	39.3	35.6
	3			15. I.	2.6 ♀	26	180	5′	1h45′	0.80	39.4	—
	4			20. I.	3.0 ♂	26	42	—	—	—	38.7	36.3
	5			20. I.	3.3 ♀	27	94	—	—	—	38.7	35.6
	6			25. V.	1.7 ♂	24	26	—	—	—	38.9	36.5
	7			1. VI.	2.5 ♂	22	260	40′	55′	0.10	39.3	34.0
	8			8. VI.	2.4 ♂	26	116	50′	30′	0.15	38.1	—
	9			6. VII.	3.0 ♀	32	143	30′	2h20′	—	38.4	35.8
	10			20. VII.	3.0 ♀	26	180	45′	20′	0.28	38.6	—
	11			27. VII.	3.0 ♀	36	320	40′	3h0′	0.30	39.5	36.3
	12			4. VIII.	2.7 ♀	30	56	—	—	0.10	39.1	38.8
	13			11. VIII.	3.5 ♂	26	210	10′	3h30′	0.55	39.3	37.3
	14			28. II. '27	2.1 ♂	28	76	—	—	0.30	38.6	35.2
	15			7. III.	1.8 ♀	20	160	5′	1h15′	0.20	38.4	—
	16			14. III.	3.3 ♂	24	152	5′	25′	0.60	38.0	35.8
	17			23. III.	1.9 ♀	22	36	—	—	—	38.2	35.8
Bilaterally	1	16. II. '26	23. II.	9. III.	2.1 ♀	19	20	—	—	0.010	38.1	36.0
				13. III.	2.0	14	18	—	—	—	38.0	36.8
				27. III.	2.4	18	100	55′	15′	—	38.3	34.3
				12. IV.	1.8	13	16	—	—	—	38.6	36.6
				19. IV.	1.9	21	70	—	—	0.013	38.1	34.3
	2	10. III.	19. III.	27. III.	2.2 ♀	20	20	—	—	—	39.0	38.5
Unilaterally splanchnicotomized and contralateral suprarenalectomized cats				12. IV.	2.2	22	26	—	—	—	38.3	36.8
				19. IV.	2.1	20	48	—	—	—	38.0	34.8
				27. IV.	1.9	24	34	—	—	—	38.1	—
	3	10. III.	14. IV.	27. IV.	1.8 ♂	23	46	—	—	—	38.7	—
				4. V.	1.8	20	36	—	—	—	39.1	38.6
				11. V.	1.7	19	41	—	—	—	38.9	—
	4	19. III.	21. IV.	4. V.	3.1 ♂	20	38	—	—	0.18	38.7	37.2
				11. V.	2.5	18	26	—	—	0.02	38.0	37.3
	5	28. IV.	5. V.	18. V.	3.4 ♂	21	110	35′	10′	0.11	38.0	32.0
	6	5. V.	12. V.	18. V.	2.4	22	84	—	—	—	38.2	33.6
				25. V.	1.9	20	20	—	—	—	38.6	38.2
				1. VI.	1.5	15	16	—	—	—	37.0	37.0
	7	19. V.	2. VI.	8. VI.	2.5 ♀	18	72	—	—	—	38.4	35.5
	8	9. VI.	23. VI.	6. VII.	2.0 ♀	19	132	5′	35′	—	38.8	33.7
				13. VII.	1.8	19	120	5′	20′	—	38.4	—
	9	30. VI.	7. VII.	13. VII.	2.6 ♂	15	120	2h0′	20′	0.025	38.2	37.3
				20. VII.	2.2	16	24	—	—	0.025	37.3	36.4
Bilaterally splanchnicotomized cats	1	21. VII. '26		27. VII.	1.9 ♀	14	40	—	—	—	37.9	37.5
	2	28. VII.		4. VIII.	2.1 ♀	26	30	—	—	—	38.9	38.5
				11. VIII.	1.7	16	22	—	—	—	38.0	36.5
	3	15. IX.		28. X.	3.4 ♂	24	84	—	—	0.016	38.0	33.0
				4. XI.	3.3	24	36	—	—	0.011	38.2	35.6
				12. XI.	2.5	24	24	—	—	0.005	38.0	36.5
	4	15. IX.		28. X.	2.6 ♂	24	28	—	—	0.010	38.0	36.6
				4. XI.	2.5	20	22	—	—	0.012	37.3	36.2
	5	29. IX.		29. X.	3.1 ♂	28	40	—	—	—	38.1	37.5
	6	22. IX.		29. X.	2.8 ♀	27	32	—	—	0.010	38.1	37.5
				6. XI.	2.9	28	60	—	—	0.030	38.4	34.2
				12. XI.	2.9	28	68	—	—	0.240	38.3	33.5
	7	6. X.		30. X.	3.1 ♂	24	108	40′	15′	0.014	38.5	36.5
	8	29. IX.		30. X.	1.7 ♀	18	24	—	—	—	37.2	33.5
	9	6. X.		6. XI.	2.6 ♀	24	88	—	—	0.020	38.1	33.5
				11. XI.	2.7	28	144	40′	1h0′	0.050	38.8	33.8
				18. XI.	2.5	28	56	—	—	0.032	38.8	37.6
	10	13. X.		11. XI.	3.1 ♂	24	79	—	—	0.080	38.1	35.2
				18. XI.	2.9	28	76	—	—	0.042	38.9	35.8

	Number of cases	Date of splanchnicotomy	Date of suprarenalectomy	Date	Body weight and sex	Respiration: Frequency (per minute) Before injection	Respiration: Frequency (per minute) Maximum after injection	Respiration: Time till hyperpnoe	Respiration: Duration of hyperpnoe	Suger in urine %	Body temperature: Before injection	Body temperature: Minimum after injection
Unilaterally splanchnicotomized rats	1	22. XII. '26		8. I. '27	2.7 ♀	28	120	15′	1h0′	0.075	39.1	36.0
				14. I.	2.5	28	44	—	—	—	39.1	38.2
				24. I.	2.1	36	52	—	—	0.250	37.0	31.7
	2	12. I. '27		31. I.	3.0 ♂	20	200	50′	1h0′	0.086	37.5	33.5
	3	15. I.		31. I.	4.2 ♂	24	60	—	—	0.080	37.8	36.2
	4	22. I.		3. II.	3.0 ♂	20	40	—	—	0.050	37.9	35.8
	5	26. I.		9. II.	2.1 ♀	24	124	1h30′	50′	0.486	38.3	35.3
				18. II.	2.1	20	120	35′	1h0′	0.079	38.1	34.0
				24. II.	1.8	20	104	15′	1h20′	0.120	37.9	32.0
	6	29. I.		18. II.	2.7 ♂	24	28	—	—	—	37.7	33.0
	7	16. II.		28. II.	3.5 ♂	26	92	—	—	0.110	37.8	32.0
	8	23. II.		7. III.	3.1 ♂	18	24	—	—	0.050	38.0	37.3
	9	26. II.		10. III.	2.3 ♀	20	28	40′	20′	0.220	38.0	—
	10	2. III.		14. III.	2.0 ♀	20	124	2h50′	2h50′	0.630	38.1	34.5
				17. III.	1.7	24	60	—	—	—	38.0	35.8
	11	5. III.		17. III.	2.5 ♀	24	88	—	—	0.280	38.0	35.3
				23. III.	2.3	24	48	—	—	0.130	38.0	—

EXAMPLE.

Cat. ♀

10. III. 1926. r. splanchnectomy.
19. „ „ l. suprarenalectomy.
27. „ „ Experiment. Body weight 2.2 kilo.

Date	Time	Room temperature	Body temperature	Respiration		Urine					Remarks
				Rate (per min.)	Type	Volume	Special weight	Reaction	Protein	Sugar	
27. III.	9:30 a.m.	9	37.3	20	normal						
	10:30	11		18	"						
	11:30	13		19	"						
	12:30 p.m.	14		18	"						
	1:30	15	39.0	18	"						
	1:45	Injected 7.3 c.c. 4 mol NH_4Cl (0.7 grm. per kilo).									
	1:55	16		17	normal						
	2:10	16		19	"						
	2:30	16		20	"						
	2:45	17		20	"						Screamed. Salivation.
	3:0	16		16	"						
	3:30	16		18	"						
	4:0	15	38.5	19	"						
	4:30	15		18	"						
	5:0	15		18	"						
	5:30	14		17	"						
	6:0	14		16	"						
29. III.	10:0 a.m.	14		17	"	45	1025	weakly acid	+	no trace	Urination.
											Good appetite.

out all the series of experiments. The dose was proved sufficient as a rule to manifest its action.

Suprarenalectomy and splanchnicotomy were carried out extraperitoneally by the lumbar route some weeks prior to the experiment.

Sugar in urine was measured by Bertrand.

Results.

1. Experiments on normal cats.

Soon after the hypodermical injection of the salt solution the cat screamed, behaved itself as if it would bite somewhere in the region where the injection was made, and rolled over violently. The pupils widened ad maximum, and the nictating membranes drew back. Before long the animal became quiet, while salivation, vomiting, defaecation and micturition took place for about half an hour after the injection.

In almost all instances the breathing became very rapid immediately after the injection. The maximum in the rate was noted as 100–150 a minute or more which occurred within about half an hour after the injection. Inspiratory movements of the wings of the nose and convulsion-like breathing were also often observed. In a few cases a great acceleration in the breathing did not occur, but it was of convulsion-like type. The hyperpnoic interval lasted from half an hour to three hours or longer.

After the exciting period had passed away, the animal lay down calmly on its side and fell asleep. For a few days after the experiment the animal looked weak and had no good appetite. The body temperature descended invariably during the poisoning. Sugar and protein were found almost always in the urine.

2. Experiments on cats, whose splanchnic nerves on one side and suprarenal body on the other were interferred with.

For this set of experiments nine cats were prepared by unilateral splanchnicotomy and contralateral suprarenalectomy, and with each cat some experiments were carried out as a rule.

Ammonium chloride in a dose of 0.7 grm. per kilo was capable of producing in this sort of cat screaming, salivation, vomiting, defaecation and micturition as in normal animals, but they seemed of a somewhat minor degree. In some cases the hyperpnoea was remarkable, but its duration was distinctly shorter than in the normal individuals. An acceleration such as exceeding 150 a minute was never noticed in this set of experiments. And in the remaining cases, that is in the majority, the respiration, both in rate and type, remained absolutely or nearly unaltered notwithstanding the manifestation of the other symptoms above related. In one and the same animal the hyperpnoea occurred occassionally, while on other days no alteration took place in breathing; why it was so, I am not able to make any suggestion. The body temperature also decreased; sugar was only seldom discovered in the urine and only in a very small quantity. The recovery of the animals from the poisoning was seemingly somewhat sooner in comparison with the normal ones.

3. Experiments on bilaterally splanchnicotomized cats.

The number of cats employed for this set of experiments was ten, and the half of them was utilized two or three times. Of the poisoning symptoms, their severity and the velocity of recovery from poisoning it was difficult to distinguish this set of experiments from that quoted in the above paragraphs. The breathing became rapid on the injection of ammonium chloride in rare cases. Even when the acceleration set in, it lasted only

for a while. Namely in a great number of instances the respiration was not materially influenced by administration of the salt. Of the frequency of non-occurrence of rapid breathing on the injection of NH_4Cl this set of experiments is seemingly a little superior to that of cats which were unilaterally suprarenalectomized instead of cutting the splanchnic nerves, but it may not be justifiable to state this definitely because of the small number experiments. Of the body temperature and sugar in the urine similar results to those in the above section must be repeated here.

4. *Experiments on unilaterally splanchnicotomized cats.*

The intensity of the excitements following the administration of the salt in the same quantity was in the unilaterally splanchnicotomized cats rather similar to the normal individuals. In relation to the respiratory movements the results of this set of experiments were intermediate, that is the acceleration occurred more frequently as in the bilaterally splanchnicotomized ones or the unilaterally splanchnicotomized and contralaterally suprarenalectomized, but not so commonly as in the normal cats. In about one third of the cases the rapid breathing as over 100 a minute was observed, and it continued one hour or longer. Even when no rapid rate was counted, the breathing was usually laboured. Glycosuria and albuminuria were also detected in almost all cases. The body temperature descended.

Now by again epitomizing the data given in the above table the following small table is prepared. The classification of the intensity of acceleration in three degrees, strong, intermediate and weak or none, is certainly artificial; there are naturally no boundaries between the degrees.

Acceleration of the frequency of respiration

Kinds of cat	Total number of cases	Degree		
		Strong	Intermediate	Weak or none
Normal cats	17	11 (65%)	4 (23%)	2 (12%)
Unilaterally splanchnicotomized and contralaterally suprarenalectomized cats	23	5 (22%)	6 (26%)	12 (52%)
Bilaterally splanchnicotomized cats	19	2 (10%)	7 (37%)	10 (53%)
Unilaterally splanchnicotomized cats	17	6 (35%)	7 (41%)	4 (23%)

Summary.

As is readily seen by examining the above small table, the results with the intact cats and the bilaterally splanchnicotomized fell into the same line

with those of Morita; that is, the normal cats respond against the subcutaneous injection of ammonium chloride with an increase in the rate of breathing, remarkable or moderate; a definite acceleration was unperceived in a few individuals only. On the other hand, the double splanchnicotomy was capable of abolishing this reaction to a great extent, namely in over half of the cases an unmistakable increase in the respiration rhythm failed to occur.

The events caused by the salt in the cats, whose suprarenal body on one side was removed and the splanchnic nerves on the other divided, did not differ very materially from those with the bilaterally splanchnicotomized, but plainly differed from those with the unilaterally splanchnicotomized but not suprarenalectomized ones.

In relation to the hyperpnoic action of ammonium chloride the suprarenalectomy appears, thus, to have a similar bearing as to the splanchnicotomy, though it may be somewhat inferior.

I practised great care not to interfere with the splanchnic fibres which do not destinate in the suprarenal gland, but it is a matter of great difficulty to speak with confidence of the integrity of all or nearly all of such fibres. We are acquainted with the facts, which indicate that the removal of the suprarenal gland may run a risk of damaging the splanchnic nerves in an unexpected manner. The reports, for example, that the change in the blood pressure curve caused by sensory stimulation or stimulation of the peripheral end of the splanchnic nerve disappears after the extirpation of both the suprarenal glands, were annihilated by subsequent researches. The failure of the change was really caused by injury of the splanchnic nerves and not by the removal of the glands themselves.

On perusal of the literature it may be justifiable to assume that the rôle of the suprarenal gland in the hyperpnoic action of ammonium chloride cannot be explained by the oversecretion of epinephrine. It is open to further investigation.

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The Epinephrine Load in the Suprarenal Glands of Rabbits after Administration of Ammonium Chloride and Magnesium Sulphate.

By

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That salts such as ammonium chloride and magnesium sulphate are capable of evoking hyperglycaemia in animals, when properly dosaged, is generally accepted.[1]

Special attention was paid in respect to the dosage by some writers, such as Láng and Rigó. For example, 0.01-0.1 grm. $MgSO_4$ per kilo causes an elevation in the blood sugar content, while a smaller amount such as 0.005 grm. and less brings about, on the contrary, a decrease.

It has been also well established that the double splanchnictomy largely or almost entirely diminishes the magnitude of the hyperglycaemia causable by ammonium chloride while that by magnesium salts does not undergo so large an interference by those nerve cut-

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1) Hirsch, Hoppe-Seyler's Ztschr., 1915, 94, 227 ($MgSO_4$: rabbits); Naito, Tohoku J. Exp. Med., 1920, 1, 131 (NH_4Cl, $MgSO_4$: rabbits (old references there); Morita, Ibid., 1922, 3, 363 (NH_4Cl, $MgSO_4$: cats); Satake, Nippon Igakukwai Zassi, 1922, 6, 3; Tohoku J. Exp. Med., 1926, 8, 26 (NH_4Cl: rabbits); Pollak, Ergeb. inn. Med. u. Kinderh., 1923, 23, 391 ($MgSO_4$: rabbits); Masamune, Tokyo Izi Sinsi, 1925, 135 (Ammonia salts: rabbits); Karger, Kl. W., 1925, 4. year 1165 ($MgCl_2$: rabbits); Heianzan, Biochem. Ztschr., 1925, 165, 57 ($MgCl_2$: rabbits, no hyperglycaemia by 0.04 g $MgCl_2$ per kilo. Previous reports with negative outcome are quoted in Naito.), Fukuda and Itahasi, Chiba Igakukwai Zassi, 1926, 4, 197-216 (NH_4Cl: rabbits); Masamune, Fukuoka Ikadaigaku Zassi, 1927, 20, 511 (Ammonia salts: rabbits); Idem, Ibid., 1460 (Magnesia salts: rabbits); Yosimura, Nagasaki Igakukwai Zassi, 1927, 5, 803 ($MgCl_2$: rabbits); Suekawa, Mitt. med. Akad. Kioto, 1929, 3, part A, 225 ($MgSO_4$: rabbits); Láng and Rigó, Arch. f. exp. Path. u. Pharm., 1929, 139, 1 ($MgCl_2$, $MgSO_4$: rabbits); Fahrig and Wacker, Beitr. z. path. Anat. u. allg. Path., 1930, 83, 126 ($MgSO_4$: rabbits); Ohara, Mitt. med. Akad. Kioto, 1933, 9, 391 & 979 (NH_4Cl: rabbits); Miyoshi, Ibid., 1935, 13, 837 (NH_4Cl: rabbits).

tings (Naito, Morita, Satake, Masamune, Fukuda, Suekawa, Ohara, Miyoshi) the latter removed one gland and denervated another; this was done also by Satake. The double removal of the suprarenal glands also interferes with the intensity of hyperglycaemia causable by ammonium chloride (Satake).

By thus reviewing the existence of some definite bearings of the splanchnic nerves and the suprarenal glands upon the blood sugar increase by these salts, it seemed to me worth while investigating the epinephrine content of the suprarenal glands of animals poisoned with such salts.

In fact Morita already noted of his observation in cats that the double suprarenalectomy wholly abolishes the paradoxical pupil dilatation, observable in the ammonium chloride and magnesium sulphate poisoning. Of ammonium chloride, Miyoshi recently reported similar findings yielded in rabbits.

Further Morita undertook with Watanabe some experiments,[2] quite the same as the present. At that time they estimated the chromaffine substance by means of the Negrin and Brücke method. As they were not able to carry out a sufficient number of experiments, they reported only preliminarily: Ammonium chloride could not diminish the chromaffine substance in the denervated gland. In the unilaterally splanchnicotomized rabbit, which was poisoned with magnesium sulphate, the denervated gland was coloured more intensively than the non-denervated fellow in some cases, but not in others.

As their announcement seems to me inconclusive, I have attacked the same line of investigation, Suto-Kojima's colorimetric method[3] for determining the epinephrine in the tissue extract being used instead of staining the organ in toto by bichromate.

Methods:

Adult rabbits, mostly male individuals, were used.

The splanchnic nerves were unilaterally sectioned per laparotomiam or extraperitoneally; usually 14–38 days, in some cases 66 days were allowed to elapse from the denervation until the poisoning experiments.

Ammonium chloride and magnesium sulphate (both Merck) were

2) Watanabe and Morita, J. of Biophysics, 1927, 2 XLIX (Proc. in 1926).
3) Kojima, Tohoku J. of Exp. Med., 1928, 10, 281.

prepared to respectively 4 mol and 2 mol solution and injected subcutaneously in doses of 10 c.c. and 5 c.c. respectively. They are 2.14 grms. NH_4Cl and 2.46 grms. $MgSO_4+7H_2O$ per kilo of body weight. Before injecting, the solution was warmed up to the body temperature.

The suprarenals were examined for epinephrine by removing them in the ammonia experiments after death of poisoning, and in the magnesia experiments after death or after killing in the stadium of fully developing poisoning, or in the recovery stadium.

Results:

At first the results with ammonium chloride will be cited, the data concerning the epinephrine load being given in Table I.

Soon after the injection the rabbit hobbled about with crying, with the leg where the salt solution was injected being held up. The respiration accelerated then, the body appeared rigid, laying on its side or belly. Next cramps occurred at first only lightly, but they became stronger and stronger, with some seconds interval between the attacks. The pupils dilated widely. Convulsion attacks then step by step replaced by developing paralysis; the body temperature fell remarkably and the respiration diminished in the rate. In some cases the animal urinated. All the animals experimented on, died eventually. One side splanchnicotomy had no bearing upon the intensities of the poisoning symptoms.

9 normal rabbits, which were poisoned with ammonium chloride, died 24–57 minutes, in one case 135 minutes, after the injection. R glands weighed mean 0.146 grm. (0.083–0.279 grm.), L mean 0.16 grm. (0.078–0.306 grm.), and their total epinephrine content were R 0.084 mgrm. (0.058–0.111 mgrm.), L 0.086 mgrm. (0.052–0.129 mgrm.) and that per grm. of gland R 0.62 mgrm. (0.39–0.85 mgrm.), L 0.58 mgrm. (0.40–0.74 mgrm.) and that per kilo of body weight R 0.046 mgrm. (0.036–0.071 mgrm.), L 0.046 mgrm. (0.033–0.071 mgrm.).

These figures coincide well with those obtained from the normal rabbits, such as those reported from this laboratory. By saying this, it must be concluded that we have not been able to find any definite alteration of the epinephrine load in the suprarenal glands of the rabbits which died of the ammonium chloride poisoning.

This inference is also to be supported by the experiments on the unilaterally splanchnicotomized rabbits. In seven rabbits the right side splanchnic nerves were previously cut. 18 to 66 days later ammonium chloride was injected, and in seven other rabbits the opposite

TABLE I.

Epinephrine content in suprarenal glands of rabbits in NH_4Cl poisoning.

No.	Sex	Date of experiment	Body weight (kilos)	Dose of 4 mol NH_4Cl solution (c.c.)	Duration after injection to death (min.)	Weight of suprarenal gland (gram) r.	Weight of suprarenal gland (gram) l.	Total epinephrine content r.	Total epinephrine content l.	Epinephrine content (mcrm) per gram of gland r.	Epinephrine content (mcrm) per gram of gland l.	Epinephrine content (mcrm) per kilo of body weight r.	Epinephrine content (mcrm) per kilo of body weight l.	Days between splanchnicotomy and experiment	General symptoms
A. Normal rabbits.															
A. 1	♂	31. V. 1935	1.29	12.9	46	0.123	0.124	0.063	0.091	0.75	0.74	0.071	0.071		Very severe
2	♂	11. VI. "	2.10	21.0	42	0.145	0.158	0.093	0.090	0.61	0.57	0.044	0.043		"
3	♂	14. VI. "	1.85	18.6	46	0.107	0.116	0.091	0.095	0.85	0.74	0.049	0.046		"
4	♂	15. VI. "	1.55	15.5	21	0.090	0.099	0.058	0.055	0.64	0.55	0.037	0.035		"
5	♂	15. VI. "	1.15	11.5	21	0.043	0.078	0.038	0.052	0.70	0.66	0.040	0.036		"
6	♀	11. IX. "	2.11	21.1	51	0.128	0.161	0.088	0.100	0.69	0.62	0.041	0.047		Severe
7	♀	11. IX. "	1.62	16.2	57	0.119	0.118	0.058	0.053	0.53	0.45	0.036	0.033		"
8	♂	13. VI. 1936	2.13	21.3	42	0.253	0.270	0.108	0.120	0.42	0.46	0.061	0.061		"
9	♂	13. VI. "	2.50	25.0	135	0.279	0.306	0.111	0.123	0.39	0.40	0.044	0.049		Moderate
		Mean	1.85			0.146	0.160	0.084	0.086	0.62	0.58	0.046	0.046		
B. Unilaterally splanchnicotomized rabbits.															
B. 1	♂	7. VI. 1935	1.88	18.8	80	0.166	0.171	0.084	0.076	0.51	0.45	0.045	0.041	21	Moderate
2	♂	14. VI. "	1.68	16.8	141	0.062	0.086	0.042	0.040	0.51	0.47	0.025	0.024	18	"
3	♀	5. VII. "	1.75	17.5	23	0.146	0.165	0.090	0.108	0.62	0.65	0.051	0.061	38	Very severe
4	♂	5. VII. "	2.00	20.0	80	0.175	0.121	0.093	0.092	0.81	0.61	0.046	0.041	31	Severe
5	♂	5. VII. "	1.60	16.0	38	0.095	0.096	0.072	0.070	0.76	0.73	0.045	0.044	31	Very severe
6	♀	12. VII. "	1.61	16.3	66	0.081	0.091	0.070	0.063	0.86	0.70	0.043	0.038	31	Moderate
7	♂	12. VII. "	1.62	16.2	26	0.111	0.157	0.052	0.049	0.36	0.31	0.032	0.030	66	Very severe
		Mean	1.74			0.119	0.125	0.072	0.069	0.63	0.55	0.041	0.040		
C. 8	♂	26. VII. 1935	1.97	19.7	45	0.163	0.136	0.063	0.069	0.61	0.51	0.032	0.030	88	Very severe
9	♂	6. IX. "	1.95	19.5	97	0.125	0.112	0.070	0.061	0.56	0.55	0.035	0.035	66	Moderate
10	♀	29. VIII. 1936	1.80	18.0	32	0.094	0.060	0.069	0.069	0.73	0.71	0.039	0.039	36	Very severe
11	♂	12. IX. "	1.88	18.8	90	0.161	0.157	0.070	0.076	0.43	0.49	0.037	0.042	36	Moderate
12	♂	12. IX. "	1.76	17.6	110	0.147	0.169	0.085	0.099	0.58	0.53	0.049	0.050	50	"
13	♀	18. IX. "	2.08	20.8	65	0.155	0.133	0.071	0.066	0.46	0.49	0.034	0.032	15	"
14	♀	18. IX. "	2.40	24.0	75	0.183	0.206	0.117	0.123	0.64	0.60	0.049	0.050	21	"
		Mean	1.97			0.139	0.163	0.078	0.078	0.57	0.51	0.039	0.039		

The italicized figures refer to the values obtained from the denervated glands.

side splanchnicotomy was carried out. 15–50 days later the salt injection followed.

Comparison of the figures from the unilaterally splanchnicotomized rabbits with those in the normal rabbits, and the figures from the glands, intactly innervated and those denervated, makes it difficult to find any definite differences here. There are detectable some differences, but there are no definite tendencies in general. And that the sectioning of the splanchnic nerves effects no alteration in the epinephrine load in the suprarenal capsule in the rabbit poisoned with the salt, checks well with the fact that the ammonium chloride poisoning does not alter the epinephrine load of the gland intactly innervated, as above shown.

Next the data from the rabbits intoxicated by magnesium sulphate will be quoted, the epinephrine load being given in Table II.

Immediately after the injection there were some rabbits which hobbled about, but after a while they became also quiet. In some cases the respiration became quiet transitorily. With developing paralysis the animal lay down weakly, the body temperature fell gradually but in a large extent. The respiration became shallow and less frequent. After several hours they gradually recovered. Unilateral sectioning the splanchnic nerves seemed not to alter the severity of the poisoning symptoms.

The figures in the animals poisoned with magnesium sulphate, with intact innervation on both sides, are somewhat smaller in comparison with those for the animals poisoned with ammonium chloride, but if reckoned on the weight of the gland, the epinephrine content is wellnigh the same as that of the latter. Three animals (A 1, 2 & 3) were killed after setting in of the recovery, four animals (A 4, 7, 9 & 10) during full development of the poisoning symptoms and three (A 5, 6 & 8) died on poisoning. There are not detectable any definite differences between them.

The data in 7 rabbits, splanchnicotomized on the right side and 7 other rabbits, denervated on the opposite side, yield no evidence for elucidating any definite influence of the splanchnicotomy upon the epinephrine load of the suprarenal gland under the poisoning. In these animals, there was also detectable no influence of in what period of poisoning the suprarenals were examined.

Thus these data lead the present writer to think of non-influence

4) Z. Kanowoka, Tohoku J. of Exp. Med., 1934, 24, 575 and 576.

TABLE II.

Epinephrine content in suprarenal glands of rabbits in magnesium sulphate poisoning.

No.	Sex	Date of experiment	Body weight kilo	Dose of 2 mol $MgSO_4 \cdot 7H_2O$ solution (c.c.)	Duration after injection to death or slaughter (min.)	Weight of suprarenal gland (grm.) r.	Weight of suprarenal gland (grm.) l.	Epinephrine content (mgrm.) Total epinephrine content r.	Total epinephrine content l.	per gram of gland r.	per gram of gland l.	per kilo of body weight r.	per kilo of body weight l.	Days between splanchnicotomy and examination	General symptom
		A. Normal rabbits.													
A. 1	♂	13. VI. 1936	1.52	7.6	280*	0.058	0.070	0.036	0.043	0.60	0.63	0.023	0.026		Moderate, killed.
2	♀	15. VI. „	1.80	9.0	335*	0.118	0.112	0.064	0.069	0.54	0.48	0.036	0.038		„ „
3	♂	15. VI. „	1.18	7.4	270*	0.109	0.135	0.043	0.046	0.39	0.34	0.029	0.031		„ „
4	♀	10. VII. „	1.58	8.0	90§	0.058	0.061	0.050	0.048	0.86	0.75	0.031	0.030		„ „
5	♂	10. VII. „	1.59	8.0	93	0.078	0.081	0.058	0.055	0.75	0.66	0.036	0.033		Severe, death.
6	♂	11. VII. „	2.05	10.0	75	0.069	0.070	0.055	0.058	0.78	0.83	0.026	0.028		Moderate, death.
7	♀	11. VII. „	2.35	12.0	100§	0.079	0.078	0.066	0.056	0.78	0.72	0.028	0.023		„ , killed.
8	♂	11. IX. „	2.20	11.0	25	0.164	0.184	0.070	0.077	0.44	0.42	0.032	0.038		Very severe, death.
9	♀	19. IX. „	2.34	11.7	30§	0.235	0.262	0.106	0.112	0.92	0.85	0.039	0.042		Severe, killed.
10	♀	19. IX. „	1.56	7.8	30§	0.067	0.078	0.061	0.066	0.45	0.42	0.045	0.048		„ , killed.
		Mean	1.84			0.096	0.117	0.061	0.063	0.65	0.61	0.032	0.034		
		B. Unilaterally splanchnicotomized rabbits.													
B. 1	♀	22. IX. 1936	1.70	8.5	55§	*0.091*	0.091	*0.073*	0.075	*0.80*	0.80	*0.043*	0.044	14	Severe, killed.
2	♀	29. IX. „	1.98	6.5	46§	*0.095*	0.110	*0.072*	0.069	*0.76*	0.62	*0.036*	0.034	22	Moderate, killed.
3	♀	29. IX. „	1.82	6.0	71§	*0.112*	0.123	*0.075*	0.078	*0.67*	0.63	*0.042*	0.043	22	„ „
4	♂	2. X. „	1.68	6.0	200*	*0.075*	0.080	*0.052*	0.051	*0.69*	0.63	*0.031*	0.032	25	Mild, killed.
5	♀	13. X. „	1.90	7.0	60§	*0.198*	0.213	*0.076*	0.090	*0.38*	0.42	*0.040*	0.043	22	Moderate, killed.
6	♀	13. X. „	1.85	9.0	300*	*0.150*	0.139	*0.105*	0.094	*0.70*	0.68	*0.057*	0.051	22	„ „
7	♀	8. XI. „	1.72	8.5	90§	*0.157*	0.176	*0.118*	0.114	*0.75*	0.67	*0.058*	0.061	26	Severe, killed.
		Mean	1.81			*0.127*	0.1411	*0.081*	0.082	*0.68*	0.61	*0.045*	0.045		
C. 8	♀	22. IX. 1936	2.08	10.4	22	0.099	*0.133*	0.075	*0.085*	0.76	*0.61*	0.036	*0.041*	26	Very severe, killed.
9	♀	20. X. „	1.80	8.0	60§	0.120	*0.101*	0.070	*0.066*	0.58	*0.63*	0.039	*0.037*	22	Severe, killed.
10	♂	25. X. „	1.55	6.0	120§	0.099	*0.121*	0.082	*0.091*	0.83	*0.71*	0.053	*0.059*	14	Very mild, killed.
11	♀	30. X. „	1.62	8.8	305*	0.179	*0.183*	0.064	*0.082*	0.36	*0.45*	0.039	*0.041*	14	Very severe, killed.
12	♀	6. XI. „	1.52	7.0	205*	0.079	*0.078*	0.070	*0.070*	0.89	*0.92*	0.046	*0.048*	17	Mild, killed.
13	♀	6. XI. „	1.56	6.0	115§	0.127	*0.147*	0.082	*0.083*	0.64	*0.56*	0.053	*0.053*	17	Moderate, killed
14	♂	6. XI. „	1.50	6.0	95§	0.110	*0.125*	0.063	*0.065*	0.57	*0.52*	0.042	*0.043*	17	Very severe, killed.
		Mean	1.63			0.116	*0.128*	0.072	*0.077*	0.66	*0.63*	0.044	*0.046*		

The italicized figures refer to the values obtained from the denervated ganlds; * The animal was killed after setting in of recovery; § it was killed during full development of poisoning symptoms.

of magnesium sulphate poisoning, too, upon the epinephrine load of rabbits.

Summary.

The epinephrine content of the suprarenal glands in rabbits was measured by means of the Sato-Kojima method when poisoned with ammonium chloride or magnesium sulphate. The subcutaneous dose was respectively 2.14 grm. NH_4Cl and 2.46 grm. $MgSO_4 + 7H_2O$ per kilo of body weight.

All the rabbits died by this dose of ammonium chloride and some died by that of magnesium sulphate, but some was able to recover. Of the latter some were killed in the full intoxication period and some after recovery.

The present writer failed to see any alteration in the epinephrine load in these rabbits poisoned in various periods of poisoning, above related. The unilateral sectioning of the splanchnic nerves yielded no noticeable modification in respect to the epinephrine-load under the salt poisoning.

CLXIX. THE INFLUENCE OF VARIATIONS IN SYSTEMIC ACID-BASE BALANCE UPON CARBOHYDRATE TOLERANCE IN NORMAL SUBJECTS.

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THE relationship of changes in acid-base balance of the blood to carbohydrate storage has attracted the interest of several workers. Elias [1913], working with dogs, showed that the administration of HCl by mouth was followed by hyperglycaemia and glycosuria. Underhill [1916] showed that administration of acid tended to raise the blood-sugar, whereas alkalis reduced it. Hendrix and McAmis [1924] found that the alkalosis produced in dogs by the injection of hydrazine sulphate was associated with hypoglycaemia. Peters and Geyelin [1917], studying adrenaline hyperglycaemia in three human diabetics and two normal subjects, suggested that the hyperglycaemia and glycosuria were the results of an intermediate acidosis. Haldane [1924] reports "an upset of carbohydrate metabolism like that of diabetes" during the alkalosis induced by over-breathing or alkali ingestion. This observer also states that during ammonium chloride acidosis the ingestion of 100 g. of glucose produced hyperglycaemia and glycosuria.

The subject is one of considerable interest owing to the association of severe acidosis with human diabetes. It seems possible that the acidosis of diabetes, which is incidentally the severest acidosis met with in human beings except for some cases moribund from uraemia, may have a contributory effect upon the changes in carbohydrate metabolism encountered in this disease. Unfortunately most of the experimental work up to the present suffers from the obvious drawback of having been performed on laboratory animals and not on the human subject.

The object of the present investigation was to study carbohydrate tolerance in young healthy male adults during experimental acidosis of a degree comparable to that encountered in severe untreated diabetes. Such an acidosis can readily be induced by the oral ingestion of massive doses of ammonium chloride [Haldane, 1921]. Similar observations were performed on the same subjects during alkalosis induced by the ingestion of large amounts of sodium bicarbonate.

Method. Diet was kept constant throughout, except as regards water and salts. Metabolic changes occurring during acidosis and alkalosis were followed by estimations of the CO_2-combining power of the blood-plasma and of the total daily excretion of acid, chloride and ammonia in the urine. In each case there was a preliminary control period on normal diet, followed by a period of

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Table I. *Urine analyses.*

Day	Volume cc.	Total daily output: Acid cc. N/10 NaOH	Total daily output: Ammonia cc. N/10 NaOH	Total daily output: Chlorides g NaCl	CO_2 plasma vols. %	Ammonium chloride or sodium bicarbonate ingested	Remarks
Subject 1:							
1	1325	239	599	11·6	—	Nil	— —
2	1530	201	563	8·75	—	Nil	— —
3	1220	220	488	4·9	63	Nil	Control glucose tolerance test
4	1740	400	974	16·2	—	15 g. NH_4Cl	Symptoms: headache, insomnia; continuous nausea, no actual vomiting. No dyspnoea. Only 17 g. on account of nausea. Better next day
5	2600	520	1625	27·0	—	20 g. NH_4Cl	
6	1960	643	2399	24·5	—	17 g. NH_4Cl	
7	1000	376	1972	9·3	33	Nil	Acidosis glucose tolerance test
8	945	208	431	6·9	—	Nil	— —
9	760	207	403	4·1	—	Nil	— —
10	1090	76	283	5·7	—	20 g. $NaHCO_3$	Symptoms: marked thirst, otherwise nil
11	2030	41	81	2·4	—	40 g. $NaHCO_3$	
12	2560	Nil	51	3·9	—	70 g. $NaHCO_3$	
13	—	—	—	—	68	—	Alkalosis glucose tolerance test
Subject 2:							
1	—	—	—	—	52	Nil	Control glucose tolerance test
2	1020	379	388	9·4	—	Nil	— —
3	1130	289	378	13·6	—	Nil	— —
4	1025	291	394	10·5	—	Nil	— —
5	2320	483	724	18·7	—	15 g. NH_4Cl	Salivation; slight nausea
6	2420	600	1142	29·6	—	20 g. NH_4Cl	Nausea more marked; watery diarrhoea
7	2030	665	1697	26·1	—	20 g. NH_4Cl	Headache; severe nausea; vomited at 3 a.m.
8	—	—	—	—	32	Nil	Acidosis glucose tolerance test
9	870	390	313	9·2	—	Nil	— —
10	1390	234	289	14·2	—	Nil	— —
11	1680	275	262	14·4	—	Nil	— —
12	2050	156	172	10·9	—	20 g. $NaHCO_3$	— —
13	2510	80	90	14·4	—	20 g. $NaHCO_3$	Symptoms nil
14	2690	81	83	14·8	—	20 g. $NaHCO_3$	— —
15	—	—	—	—	57	Nil	Alkalosis glucose tolerance test
Subject 3:							
1	—	—	—	—	66	Nil	Control glucose tolerance test
2	1130	429	760	15	—	Nil	— —
3	1205	414	733	14	—	Nil	— —
4	1330	463	734	16	—	20 g. NH_4Cl	Symptoms nil except for increased salivation
5	2080	649	1440	30	—	20 g. NH_4Cl	
6	2490	936	1972	36	—	20 g. NH_4Cl	
7	2170	1068	2930	35	—	20 g. NH_4Cl	
8	2470	929	3359	39	38	25 g. NH_4Cl	Acidosis glucose tolerance test
9	1385	526	1233	7	—	Nil	— —
10	1600	307	678	8	—	20 g. $NaHCO_3$	Symptoms nil
11	2310	129	286	13	—	25 g. $NaHCO_3$	Symptoms nil
12	2360	Nil	Nil	9	—	30 g. $NaHCO_3$	Symptoms: marked tingling in hands and feet
13	—	—	—	—	68	—	Alkalosis glucose tolerance test

acidosis and finally a period of alkalosis, diet being kept constant throughout. Glucose tolerance was estimated during each period by following the changes in venous blood-sugar after the oral ingestion of 100 g. of B.D.H., A.R. standard dextrose dissolved in 250 cc. of water. Specimens of blood were withdrawn at 15-minute intervals during the first hour of the test, and then at 30-minute intervals. Urine specimens were examined for reducing substances at half-hour intervals.

Urine analysis. A. Quantitative.

(1) Total acidity by Folin's method.

(2) Ammonia by Malfatti's formaldehyde titration method. This includes amino-acids.

(3) Chlorides by Volhard's method.

B. Qualitative.

(1) Protein by salicylsulphonic acid.
(2) Sugar by Benedict's qualitative method.
(3) Ketone bodies by Rothera's method.

Blood-sugar was determined in specimens of venous blood by Folin and Wu's colorimetric method. The figures obtained include non-glucose reducing substances [Herbert and Groen, 1929].

Induction of acidosis. This was accomplished by the ingestion of ammonium chloride in 2 % solution. The total amounts taken were 52 g., 55 g. and 105 g. in 3 days.

Induction of alkalosis. Sodium bicarbonate suspended in water was ingested in amounts as shown in Table I. The mixture was stirred up and immediately swallowed. Thus in spite of low solubility 5 or 10 g. could be taken in a small volume of water.

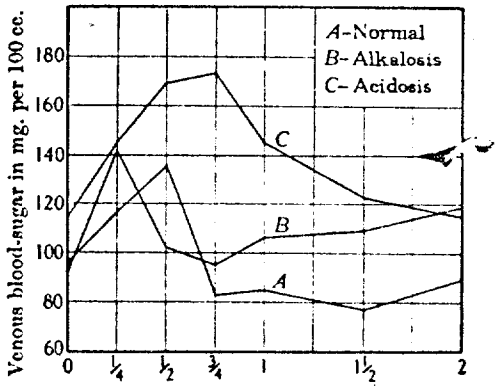

Fig. 1. Blood-sugar curves in subject 1.

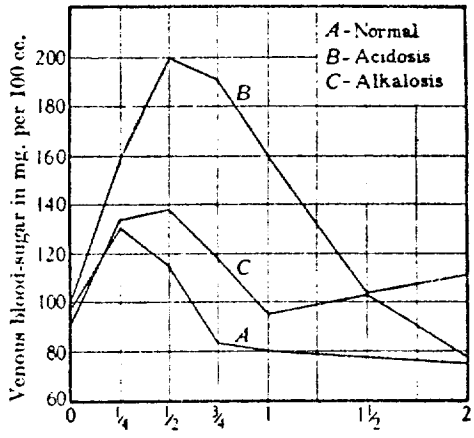

Fig. 2. Blood-sugar curves in subject 2.

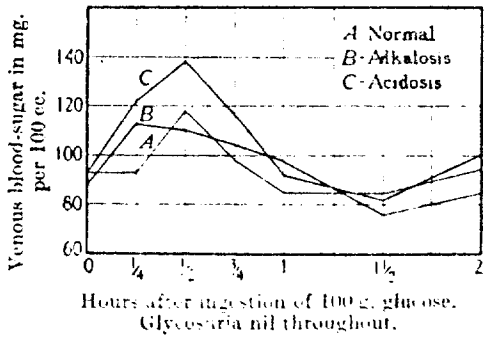

Fig. 3. Blood-sugar curves in subject 3.

Determination of CO_2-combining power of plasma. In each case this determination was performed on the first specimen of blood (fasting) taken for the glucose tolerance test. About 5 cc. of venous blood were rapidly withdrawn

under oil without stasis, oxalated and rapidly centrifuged under oil. The plasma was sucked off and equilibrated with the subject's own alveolar air. The CO_2 content of 1 cc. of equilibrated plasma was then determined in the Van Slyke apparatus, duplicate determinations being performed in each case. Results, after appropriate corrections for temperature and pressure, are shown in Table I.

Discussion and conclusions.

It was found possible by the ingestion of large amounts of ammonium chloride to reduce the alkali reserve of the blood-plasma to a level comparable with that found in diabetic coma and to raise the urinary excretion of acid by 100–200 % and of ammonia by 300 %. The acidosis so produced caused a definite failure in glucose tolerance, shown by hyperglycaemia and slow return of the blood-sugar to fasting level following glucose ingestion, but without any trace of glycosuria. These points are strikingly shown in subjects 1 and 2, but are less marked though still apparent in subject 3 for whom the curves for venous blood tend to be of a flat type. The absence of glycosuria is surprising in view of the fact that in two of the subjects the blood-sugar level exceeded what is usually regarded as the normal renal threshold.

The ingestion of sodium bicarbonate in large doses, sufficient to reduce the urinary excretion of ammonia and acid to zero, was not followed by marked increase in the CO_2-combining power of the plasma. In subject 1 the last dose of alkali (10 g.) was taken $2\frac{1}{2}$ hours before the determination of plasma-CO_2. These observations were carefully performed on three occasions with the same result. Palmer and Van Slyke [1917] found it necessary to raise the plasma-bicarbonate to 75–80 volumes % by alkali ingestion before the sum of urinary ammonia and titratable acid fell to zero. It appears, however, that the CO_2-combining power of the plasma may sometimes be a misleading guide to the extent of the total upset in acid-base balance following alkali ingestion.

Ingestion of alkali caused very little variation in the blood-sugar response to glucose ingestion. There was a tendency to a sharper rise in the initial phase, but thereafter the curves were little different from normal.

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The Effect of Diet on the Composition of the Kidney

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In the rat (and other animals) unilateral nephrectomy is followed by compensatory hypertrophy of the remaining kidney (Smith, 1951; Goss, 1964). The mechanism of this is unknown but it may be a response to one kidney having to carry out the duties normally shared between two. This hypothesis is supported by the observation that high protein diets (which presumably increase urea excretion) produce a similar hypertrophy (Osborn, Mendel, Park & Winternitz, 1927; Konishi, 1962; Halliburton & Thomson, 1965). The present experiments were undertaken to determine whether any degree of kidney hypertrophy could be produced by increasing the excretory load of an otherwise normal animal. The hypertrophy resulting from unilateral nephrectomy was characterized, 4 days after the operation, by a 30% increase in the weight of the remaining kidney, a 17% increase in its cell number (as estimated from total DNA content) and a 35% increase in RNA per cell. If normal unoperated rats were maintained for the same period on a diet supplemented with 10% of urea, their urinary output of urea was trebled but this was not accompanied by any increase in kidney weight or cell number and the increase in RNA per cell was only 11%. It is therefore unlikely that the remaining kidney is stimulated to hypertrophy after unilateral nephrectomy solely because it has to excrete twice as much urea as before.

An alternative possibility is that compensatory hypertrophy is a response to inadequate performance of other kidney functions, such as electrolyte excretion and maintenance of acid–base balance. This was investigated by determining the effect on normal animals of supplementing the diet with various salts. Addition of ammonium chloride (3%) for 6 days increased kidney weight by 25% and RNA per cell by 18%. Equivalent amounts of ammonium citrate or sodium chloride did not produce these effects, so the action of ammonium chloride would seem to be related to its acidotic effect rather than to the contribution it makes to either nitrogen excretion or electrolyte balance.

In summary, it seems unlikely that the excretory function of the kidney is by itself responsible for compensatory renal hypertrophy.

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Effect of Convulsion Inducing Agents on the Acetylcholine Content and on the Electrical Activity of the Brain

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THE VALIDITY of the hypothesis that an accumulation of acetylcholine is the universal factor initiating convulsions has been questioned on the basis of experimental evidence, namely: the effects of various convulsion-inducing agents on the acetylcholine metabolism imply that an accumulation of acetylcholine does not always precede convulsions (1). Electroshock convulsions are not preceded by an accumulation of acetylcholine in the brain (2). Furthermore, atropine inhibits the appearance of convulsions induced by acetylcholine and potentiates the effect of some agents in inducing convulsions (3).

In the experiments here described the acetylcholine content of the brain has been ascertained during the preconvulsive and convulsive phases induced by various agents in order to gain insight in the role of acetylcholine in the initiation of convulsions. An attempt was also made to correlate quantitative changes in the acetylcholine content of the brain and changes in the electrical activity of the brain.

METHOD

The experimental animals used were male and female albino rats of the Wistar strain. Rats of 95-105 gm body weight were used for acetylcholine content studies; and rats of 200-250 gm body weight were used for electroencephalographic studies. All animals received a mixed diet consisting of dog pellets (G.L.F. Cooperative), milk, bread and vegetables ad libitum.

Acetylcholine Content Estimation. The total acetylcholine content of the brain was ascertained by the following procedure (4,5). Immediately after separation of the head from the body the brain was removed, weighed and immersed in 2 ml of frog's Ringer solution containing physostigmine salicylate in a concentration of 0.4 mg/100 ml, at pH 7.2, 0°C. The brain was rapidly homogenized by means of a Potter glass apparatus and the homogenate was boiled for 2 min. It was diluted to 10 cc by frog's Ringer solution (5). After centrifugation at 3,000 rpm the supernatant fluid was decanted and cooled. The acetylcholine content of the supernatant fluid was assayed biologically on the sensitized rectus abdominis muscle of the frog using a previously described method (4, 5).

Changes in the acetylcholine content of the brain were considered significant if the values obtained in animals receiving the various convulsion-inducing agents differed from the values obtained in the control brains by more than twice the square root of the sum of the squares of the standard error of mean of the controls and the standard error of mean of the experiments ($2\sqrt{S.E.^2 \text{ (control)} + S.E.^2 \text{ (experiments)}}$).

Electroencephalography (6). Under light ether anesthesia of a few minutes' duration the trachea was incannulated. Positive pressure artificial respiration at the rate of 60 cpm was instituted by an air-driven respirator similar to the one described by Pierce (7). The power for operating the interruptor valve of the respirator was derived from compressed air, acting through the agency of a reciprocating air motor similar to those used in automobile windshield wipers. Air alone was passed to the animal. The speed of the motor, and hence the number of respirations per minute, was controlled by a knurled screw. The amount of air passing to the animal was regulated by a needle valve. About 30 min after the animals had recovered from general anesthesia they received by intraperitoneal administration about 3 U/kg body weight of a curare preparation (Intocostrin, Squibb, 8) to prevent muscle potential artifacts in the electroencephalogram. Occasionally, when muscle action potentials started to appear in the EEG (9), Intocostrin was reinjected intraperitoneally in amounts of 1 U/kg body weight. Since the rat readily suffers from both respiratory deficiencies and accumulation of fluid in the trachea if artificial respiration is delayed (10), the artificial respiration was applied prior to curarization.

After a lapse of about 30 min the animal was prepared for measurements of electrical brain potentials. It was placed in a large wire gauze shield and needle electrodes were pressed into the skull over the two occipitals and the right frontal cortex with the point embedded in the bone. The reference electrode was a needle inserted into the neck. The electrodes were led into a conventional resistance capacity coupled 3-channel Grass ink-writing electroencephalograph. Simultaneously electrocardiograms were recorded on one of the channels using an electrode inserted above the apex of the heart and in the left leg.

The paper speed through the inkwriter was 6 cm/sec. The frequency of brain waves was counted as previously described (6) and the predominant frequency was of the range reported by Bergen (11). The frequency and voltage of the brain waves of the control rats were maintained unaltered for several hours.

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Chemical Agents Used. Physostigmine salicylate, pentamethylene tetrazol and ammonium chloride were administered in a single intraperitoneal injection in concentrations indicated in the description of the results.

RESULTS

Acetylcholine Content Estimation. The acetylcholine content of the brain of control rats averaged 3.70 μg/1 gm brain tissue. This value was in agreement with the acetylcholine content of brain reported by Elliott and Henderson (12).

TABLE 1. EFFECTS OF CONVULSION-INDUCING AGENTS ON THE ACETYLCHOLINE CONTENTS OF THE BRAIN*

Substance	Conc., mg/100-gm rat	Time elapsed between administration of the substances and sacrificing of the rat, min.†				
		1	2	5	10	15
Pentamethylene tetrazol	2	138 ±1.2	143 ±1.1	156 ±1.5		
	4	139 ±0.9	144 ±1.4		150 ±0.8	
	12		137 ±1.2	148 ±1.6	121 ±0.6‡	110 ±1.0‡
	20		120 ±0.4‡	108 ±0.7‡	102 ±0.9‡	
Ammonium chloride	1		72 ±0.9	76 ±1.0		
	5			75 ±0.8		
	10		70 ±0.7	78 ±0.3		
	20		80 ±0.9	94 ±0.7		
	40		97 ±0.6‡	102 ±0.5‡		
Physostigmine salicylate	0.01			110 ±0.6	119 ±0.4	
	0.02			134 ±1.0	136 ±0.9	
	0.1			143 ±0.8	167 ±1.1	273 ±2.1‡
	0.2				258 ±2.4‡	
	0.5			350 ±2.7‡		

* Acetylcholine content of control brains averaged 3.70 ± 0.13 μg/1 gm of brain tissue (average of 60 expers).

† Acetylcholine content of the brain expressed as percentage of control values followed by the standard error of the mean. Each value represents the average of 10 separate experiments.

‡ Animals killed from a few seconds to several minutes after the beginning of visible convulsions.

Pentamethylene tetrazol was administered in amounts from 2–20 mg/100 gm body weight in a single intraperitoneal injection. Visible convulsions were induced by an injection of 12 mg in 10 min, and by an injection of 20 mg in less than 2 min. The acetylcholine content increased during the preconvulsive phase, it reached a peak of about 60% at the initiation of the convulsions and decreased gradually during convulsions (table 1).

Physostigmine salicylate was administered in a single intraperitoneal injection in amounts from 0.01–0.5 mg/100 gm body weight. Convulsions were induced by an injection of 0.1 mg in 15 min, by 0.2 mg in 10 min and by 0.5 mg in less than 5 min. The acetylcholine content of the brain increased during the preconvulsive phase. The increase was gradual depending on the concentrations of physostigmine salicylate administered and on the time lapsed between injection and killing of the animal. Convulsions occurred when the acetylcholine content exceeded 2.5 times the values found in control brains (table 1).

Ammonium chloride was administered in a single intraperitoneal injection in amounts from 1–40 mg/100 gm body weight. Convulsions occurred in less than 2 min in rats receiving 40 mg/100 gm and did not occur in rats receiving from 1–20 mg/100 gm. During the preconvulsive phase the acetylcholine content of the brain decreased on the average by 30%. The initiation of convulsions coincided with the return of the acetylcholine content to values approximating the one obtained in control rats (table 1). The acetylcholine content did not exceed the control values in animals undergoing convulsions of few minutes' duration.

Electroencephalography. *Physostigmine salicylate* administered in a single intraperitoneal injection in amounts of 0.1 mg/100 gm body weight induced a gradual increase of the voltage of brain waves and occasional spikes; within 15 min the pattern gradually changed to the 'convulsion equivalent' consisting of high-voltage fast activities (fig. 1). The convulsion coincided with an acetylcholine content of the brain exceeding 2.5 times normal values (table 1). Physostigmine concentrations below 0.1 mg/100 gm and within 15 min only the 'preconvulsive' changes in the electrical activity of the brain and increase of 10% of the acetylcholine content of the brain coincided already with a

ant increase of the voltage. When physostig-mine salicylate was administered in multiple, small doses, the total amount required to induce convulsions exceeded the above quoted values for a single convulsive dose. Several animals died during the preconvulsive phase.

In the absence of an increase of acetylcholine content of the brain the electrical changes occurring during the preconvulsive phase differed from the changes found after administration of physostigmine salicylate.

Ammonium chloride given in an intraperitoneal injection in concentrations from 1 20 mg 100 gm body weight induced paroxysmal runs of low frequency waves. With ammonium chloride in concentrations of 40 mg 100 gm the following changes in the electroencephalogram were observed during the preconvulsive phase: occasional bursts of low frequency waves becoming progressively abnormal and interspersed by an occasional 3 5 cps wave. Within min the 'convulsion equivalent' appeared suddenly, consisting of a continuous run of high-voltage fast activities (fig. 2). Death often occurred during the preconvulsive phase.

When *pentamethylene tetrazol* was given in a single intraperitoneal injection in a concentration of 10 mg/100 gm body weight the following changes in the electroencephalogram were observed during the preconvulsive phase: increased voltage of the brain waves, occasional spiking, paroxysmal runs of low frequency high voltage waves (10 20 cps), followed by progressively more abnormal bursts interspersed at times with spikes. The voltage of the low frequency waves decreased and occasional bursts consisting of 1 or 2 spikes followed by a large wave (3 5 cps) appeared. About 10 min the 'convulsion equivalent' appeared consisting of high-voltage fast activities (6; see fig. 3). Pentamethylene tetrazol in lower concentrations induced some or all the changes in the electrical activity of the brain described as occurring during the 'preconvulsive phase.'

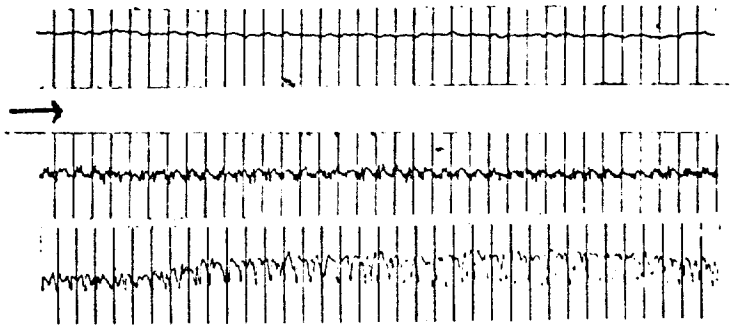

FIG. 1. Effect of physostigmine salicylate on electrical activity of rat brain. *First row:* record taken before injection of physostigmine salicylate (0.1 mg/100 gm), *arrow* indicates the time of injection of the substance. *Second row:* increase of voltage of brain waves. *Third row:* 'Convulsion equivalent' consisting of high-voltage fast activities appeared about 15 min after injection of physostigmine salicylate.

DISCUSSION

On the basis of the available experimental evidence the following inferences may be drawn: *a*) convulsions do not occur as long as the acetylcholine content of the brain is below normal limits (2; see table 1); and *b*) an additional factor is requisite. This factor is probably an excess of acetylcholine in convulsions induced by physostigmine salicylate and is not acetylcholine in convulsions induced by ammonium chloride and electroshock.

The nature of the electrical activity of the brain during the preconvulsive phase induced by pentamethylene tetrazol suggests the presence of both, an increased acetylcholine content of the brain (table 1) and an additional, not yet identified, factor. Pentamethylene tetrazol induces the increase of the acetylcholine content of the brain by increasing the synthesis of acetylcholine (1).

An increase of the acetylcholine content of the brain up to 150% coincided with an increase of the voltage of the brain waves and with occasional spiking (physostigmine salicylate, pentamethylene tetrazol; (see table 1 and figs. 1, 3; and other agents, 13). In the absence of an increase of acetylcholine content electrical changes of a different nature occurred in the electroencephalogram during the preconvulsive phase (ammonium chloride-injected rats).

Convulsions coincided with similar high-

voltage fast activities regardless of the nature of the agent inducing convulsions (figs. 1–3) and regardless of whether or not the acetylcholine content was within normal limits or exceeded it. The increase of the acetylcholine content necessary to initiate convulsions was unusually high (physostigmine salicylate-injected rats). Physostigmine salicylate induced the increase of the acetylcholine content through inhibition of the activity of acetylcholine esterase without significantly modifying the activity of choline acetylase (1, 5, 14).

During convulsions the acetylcholine content of the brain decreased. Convulsions were suspended when the acetylcholine content of the brain decreased significantly below normal limits (2). The decrease of the acetylcholine content was due to inhibition of the synthesis of acetylcholine by metabolites released during convulsions. Ammonium ion represents one of these metabolites since the intracerebral concentration of ammonium ion increases during convulsions to a degree (15, 10) sufficient to induce an inhibition of the activity of cho-

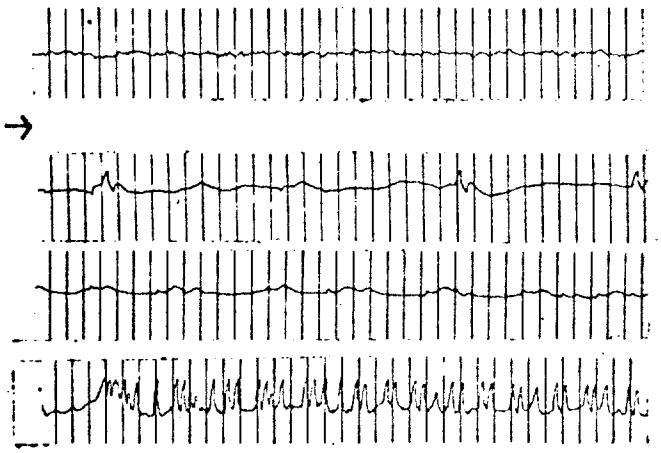

FIG. 2. Effect of ammonium chloride on the electrical activity of rat brain. *First row:* record taken before injection of ammonium chloride, *arrow* indicates the time of injection of 40 mg/100 gm body weight of ammonium chloride. *Second row:* occasional bursts of low-frequency waves. *Third row:* continuous run of low-frequency waves. *Fourth row:* 'convulsion equivalent' consisting of high-voltage fast activities.

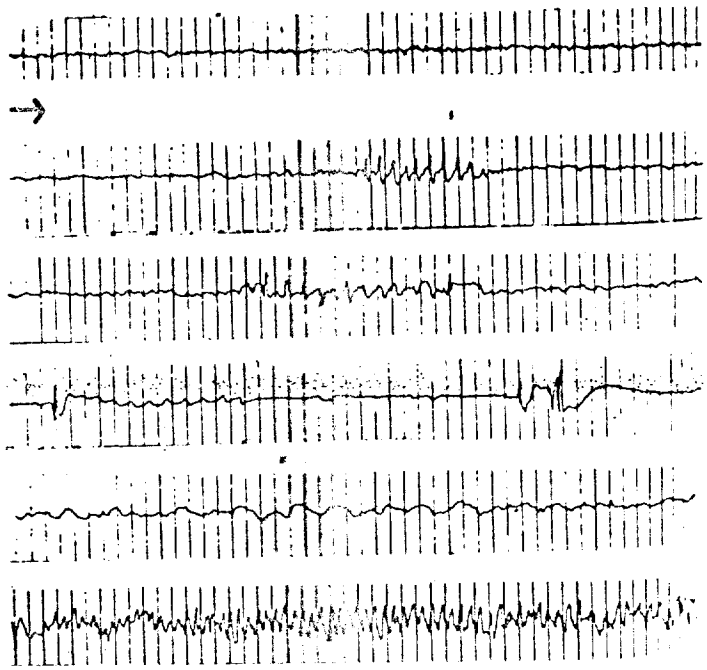

FIG. 3. Effect of pentamethylene tetrazol on electrical activity of rat brain. *First row:* record taken before administration of pentamethylene tetrazol, *arrow* indicates the injection of 12 mg/100 gm body weight of pentamethylene tetrazol. *Second row:* first paroxysmal run of low-frequency high-voltage waves. *Third row:* later paroxysmal run of low-frequency high-voltage waves. *Fourth row:* continuous run of low-frequency low-voltage waves. *Fifth row:* burst of spikes followed by a 3–5 cps wave. *Sixth row:* 'convulsion equivalent' consisting of high-voltage fast activities occurring within 10 min after injection.

acetylase exceeding 50% (17). Ammonium ion in physiological concentrations does not modify the activity of acetylcholine esterase (18).

SUMMARY

During the preconvulsive phase the acetylcholine content of the brain increased from 10 to 150% after administration of physostigmine salicylate and to about 60% after administration of pentamethylene tetrazol. The acetylcholine content decreased about 30% after administration of ammonium chloride. Convulsions were initiated with ammonium chloride only when the acetylcholine content of the brain previously returned within normal limits. During convulsions the acetylcholine content gradually decreased. The nature of changes of the electrical activity of the brain during the preconvulsive phase depends on the convulsion-inducing agent and its mechanism of action. The convulsion equivalent in the electroencephalogram is similar regardless of the nature of the convulsion-inducing agent. It is concluded that convulsions do not occur with brain acetylcholine content below normal limits. The factor initiating convulsions may or may not be acetylcholine. During convulsions the acetylcholine content of the brain decreases, probably due to inhibition of the synthesis of acetylcholine by a metabolite, e.g. ammonium ion. Convulsions are suspended when the acetylcholine content of the brain decreases significantly below normal limits.

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Systemic Lupus Erythematosus and Latent Renal Tubular Dysfunction

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Renal tubular acidosis (1), an impairment of renal acid excretion out of proportion to reduction of glomerular filtration rate, has been described in a variety of clinical settings. Its characteristic clinical manifestations are hyperchloremic acidosis, an inability to excrete a highly acid urine, and impaired renal concentrating ability. It may be associated, in the primary form, with renal calculi, nephrocalcinosis, osteomalacia, and potassium depletion (2). Subclinical cases—either asymptomatic or lacking the laboratory findings of the overt cases of renal tubular acidosis—have been uncovered by an acid-loading test designed to detect acid excretion inappropriate to the induced metabolic acidosis (3, 4).

Several recent reports have suggested that some forms of renal tubular dysfunction, including renal tubular acidosis, may occur in certain rheumatic disorders such as Sjögren's syndrome (5, 6) as well as in hypergammaglobulinemic states of diverse etiology (7-11). Since systemic lupus erythematosus (SLE) may overlap with other diseases such as Sjögren's syndrome (12), a study was undertaken to investigate whether subclinical impairment in renal acid excretion might exist in patients with SLE. The purpose of the present communication is to describe the results of this study.

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Materials and Methods

Twelve patients with SLE, nine women and three men whose mean age was 42 years, were studied. The diagnosis of SLE was established on the basis of a clinical picture strongly suggesting lupus erythematosus according to criteria employed by Harvey and his associates (13).

The pertinent data are summarized in Table 1. Twenty-one apparently healthy subjects, members of the medical staff and employees (10 men, 11 women) whose mean age (38 ± 2.4) did not differ significantly from that of the patient group (42.0 ± 1.2) ($P > 0.20$), served as controls.

All patients were free of circulatory failure and arterial hypertension at the time of study. All were eating well. Since none were azotemic, no patient was under dietary restriction of protein intake. The only restriction of sodium intake was for Patients 9 and 11, who were on mild (500 mg daily) sodium restriction. None had a history of polydipsia. The mean 24-hr urinary excretion of solutes, determined in four patients (9, 10, 11, and 12), was 745 mOsm/1.73 m². This was statistically not significantly different ($P < 0.60$) from the mean value of 821 mOsm/1.73 m² observed in 17 of the control subjects. In no patient was there evidence of metabolic acidosis or an abnormal serum concentration of sodium, potassium, chloride, carbon dioxide, calcium, or phosphorus. All patients showed some evidence of renal involvement, mainly limited to minimal to moderate proteinuria (Table 2), microscopic hematuria, and cylindruria. A clean voided specimen of morning urine from each prospective subject had been cultured for bacteria; all persons whose bacterial count exceeded

TABLE 1. Clinical Data: Patients with Systemic Lupus Erythematosus (SLE)

Patient Number	Age	Sex	LE Preparation	ANA*	Serum Gamma Globulin†	Duration of Disease‡	Duration of Medication	
							Steroid§	Aspirin
	yr				g/100 ml	yr	yr	yr
1	42	F	Negative	++	1.59	2	1	–
2	18	F	Positive	++	1.41	5	2	–
3	64	F	Positive	+++	2.40	6	2	–
4	37	F	Positive	+++	1.06	4	4	2
5	54	F	Positive	++	3.80	14	10	–
6	51	F	Negative	+++	1.86	6	6	–
7	60	F	Negative	Not done	1.57	10	–	–
8	46	M	Positive	+++	1.51	4	1.5	1.5
9	46	F	Negative	++	1.65	0.25	0.2	–
10	19	F	Positive	Not done	1.28	13	4	–
11	23	M	Negative	+++	2.02	0.1	0.1	–
12	44	M	Positive	Not done	0.38	12	12	–

* ANA = antinuclear antibody test.
† Normal mean value derived from a separate group of 100 normal subjects (Mean, 1.08 g/100 ml; SE, 0.02.)
‡ Duration of disease dates from the onset of clinical signs or symptoms of SLE.
§ The dosage of steroid was 10 to 80 mg a day for all patients except Patient 4, who was given 40 mg a day, Patients 9 and 11, who were on 60 mg daily for the indicated period in years.
The dosage of aspirin was in excess of 10 gr a day.

10^5/ml at the end of a 24-hr culture period were excluded from the study. (When a kidney biopsy was performed, the needle was cultured; no organism grew in any of these preparations.)

The ability of the kidney to excrete acid was tested by the short-term method of Wrong and Davies (4): ammonium chloride powder in gelatin capsules (a total of 0.1 g/kg body weight) was ingested over a 1-hr period. Beginning 100 min before administration of the ammonium chloride load and for 6 hr thereafter, the urine was analyzed hourly, within 15 min of voiding, for each of the following values: pH, determined with a glass electrode (Beckman pH meter) at room temperature; titratable acid (TA), by titrating urine to pH 7.4 with N/50 sodium hydroxide; ammonium, measured by the microdiffusion method of Conway (14). The excretion rates per minute of TA and ammonium were calculated from the total amount in the urine voided between the beginning of the second and the end of the sixth hour after the ingestion of ammonium chloride. An index of glomerular filtration rate was determined by the endogenous 24-hr creatinine clearance. The urine and the plasma creatinine were assayed by measuring alkaline picrate-reactive chromogen with the Technicon Autoanalyzer. Rates of endogenous creatinine clearance, TA excretion, and ammonium excretion were corrected to a body surface of 1.73 m².

Before the ingestion of ammonium chloride, with the subject in a fasting state, peripheral venous blood was drawn and analyzed by standard clinical laboratory methods for serum sodium, potassium, chloride, carbon dioxide content, calcium, phosphorus, and uric acid. Serum protein was analyzed by paper electrophoresis (15). Before the ingestion of ammonium chloride and 2 to 4 hr thereafter, usually at times coinciding with minimal urine pH values, arterialized capillary blood was collected anaerobically and analyzed for pH with a Radiometer at 37 C.

Urine osmolality was determined by means of a Fiske osmometer in a morning urine specimen after 14 to 15 hr of no fluid intake.

The mean values of data from the SLE group were compared with those of the control group by Student's *t* test.

Renal biopsy was performed with a modified Vim-Silverman needle by the technique of Kark and Muehrcke (16) with modification (17). The kidney tissue was fixed in Helly's solution.

RESULTS

The urinary pH, titratable acid (TA), and ammonium excretion rates and the blood hydrogen ion concentration before and after ammonium chloride loading in patients and controls are summarized in Table 2.

TABLE 2. Results of Renal Function Tests in Patients with Systemic Lupus Erythematosus and in Normal Subjects

Patient Number	Creatinine Clearance	Maximum Urine Osmolality	Urine Protein	Blood [H^+] Before Ammonium Chloride	Blood [H^+] After Ammonium Chloride	Minimum Urine pH	Titratable Acid*		Ammonium*	
	ml/min	*mOsm/kg*	*mg/24 hr*	*mμEq/liter*	*mμEq/liter*		*μEq/min*	*μEq/min/100 ml C_{cr}*	*μEq/min*	*μEq/min/100 ml C_{cr}*
1	99.9	990	500	40.74	51.29	4.90	33.14	33.17	33.41	33.44
2	69.6	760	4,000	38.02	47.87	5.00	43.45	62.86	36.39	52.38
3	117.0	740	300	43.66	45.71	5.50	10.63	9.09	22.87	19.55
4	85.4	680	2,000	42.65	44.67	5.20	19.61	22.96	41.05	48.07
5	70.6	480	40	40.74	43.66	5.52	19.29	27.32	36.05	51.06
6	104.7	645	40	38.94	39.82	5.20	27.83	26.58	40.47	38.66
7	101.3	600	10	38.94	40.74	5.10	34.19	33.75	30.89	30.49
8	103.0	899	200	37.16	38.02	5.20	44.89	43.58	34.00	33.00
9	105.8	612	40	42.66	48.98	5.10	24.68	23.33	28.70	27.13
10	155.0	566	60	38.02	42.66	5.20	35.42	22.85	43.46	28.03
11	95.3	745	200	44.67	43.66	5.10	32.10	33.68	33.26	34.90
12	90.7	560	•200	35.49	41.69	5.00	40.81	45.50	37.45	41.29
Systemic lupus erythematosus										
Mean	99.7	691.3	630	40.14	44.06	5.17	30.50	32.08	34.83	36.49
SE	6.2	39.8	330	0.79	1.08	0.05	3.03	3.97	1.65	2.90
Normal subjects										
Mean	110.0	958.0	28	39.65	42.00	4.81	51.70	46.94	50.76	46.75
SE	5.1	29.0	3	0.55	0.68	0.03	3.45	2.42	3.23	2.56

* C_{cr} = creatinine clearance.

In controls, the blood hydrogen ion concentration rose significantly after loading (42 mμEq/liter ($P < 0.02$). Within 2 to hr after loading the urine pH decreased to a minimum, and the excretion rates of TA and ammonium reached peak values (Figure 1). As shown in Figure 2, the rate of ammonium excretion was an inverse function of urinary pH.

In the patients with SLE, the group mean values for blood hydrogen ion concentration, for the urinary pH, and for urinary TA and ammonium excretion rates before ammonium chloride loading did not differ significantly from those of the control group (Table 2) ($P > 0.10$). After the ingestion of ammonium chloride, the blood hydrogen ion concentration rose to 11.1 mμEq/liter ($P < 0.01$), but this value was not significantly higher than the value for the mean of the control group ($P > 0.10$). The group mean urinary flow rate (1.5 ml/min (SD, 0.6)) was the same as for the control group ($P > 0.80$).

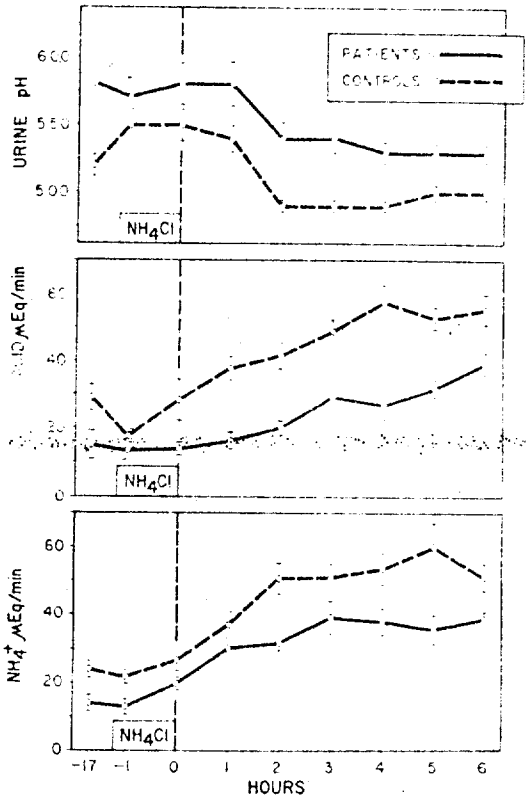

FIGURE 1. Renal response to acute loading of ammonium chloride. Time zero was set at completion of ingestion of a total dose of ammonium chloride (0.1 g/kg). The mean values of the respective groups are connected by solid or broken lines. Two standard errors of the mean are indicated by the vertical bars.

The renal response of patients with SLE to the ammonium chloride loading (Figure 1) was qualitatively similar but quantitatively different from that of the controls. The SLE group mean value for minimal urine pH (5.2 units) was significantly higher than that of the control group ($P < 0.001$). The SLE group mean rates of urinary TA and ammonium excretion were significantly lower than those of the normal subjects ($P < 0.001$ in each instance).

In the patients, as in the controls, the ammonium excretion rate proved to be an inverse function of urinary pH. Because of the decreased rates of TA and ammonium excretion, the total quantities of these components excreted were decreased. Since practically no bicarbonate is excreted at the range of pH of urine specimens (less than 6.0 units), the sum of urinary TA and ammonium is essentially the rate of hydrogen ion excreted in the urine (total hydrogen ion excreted = TA + ammonium − bicarbonate excreted in urine).

The renal acidification of urine and the renal excretion of hydrogen ions relative to the blood hydrogen ion concentration after ammonium chloride loading are shown in Figure 3.

The group means for rate of excretion of TA and ammonium per 100 ml of creatine clearance in SLE patients were 32.1 ± 0.4 and 36.4 ± 2.9 μEq/min, respectively—significantly less than those of the control group ($P < 0.005$ and 0.02, respectively).

The group mean of urine osmolality after fluid restriction in the SLE group (691 mOsm/kg), an estimate of renal ability to concentrate urine, was significantly

less than that of the control group (958 mOsm/kg) ($P < 0.001$).

RENAL BIOPSY

Sufficient kidney tissue for histologic examination was obtained by needle biopsy from six patients with SLE. The general architecture was well preserved in all specimens. Significant glomerular abnormalities consisted of focal endothelial proliferation consistent with active glomerulitis as described by Pollak, Pirani, and Schwartz (18) in two patients. The interstitial tissue showed focal fibrosis with some cellular

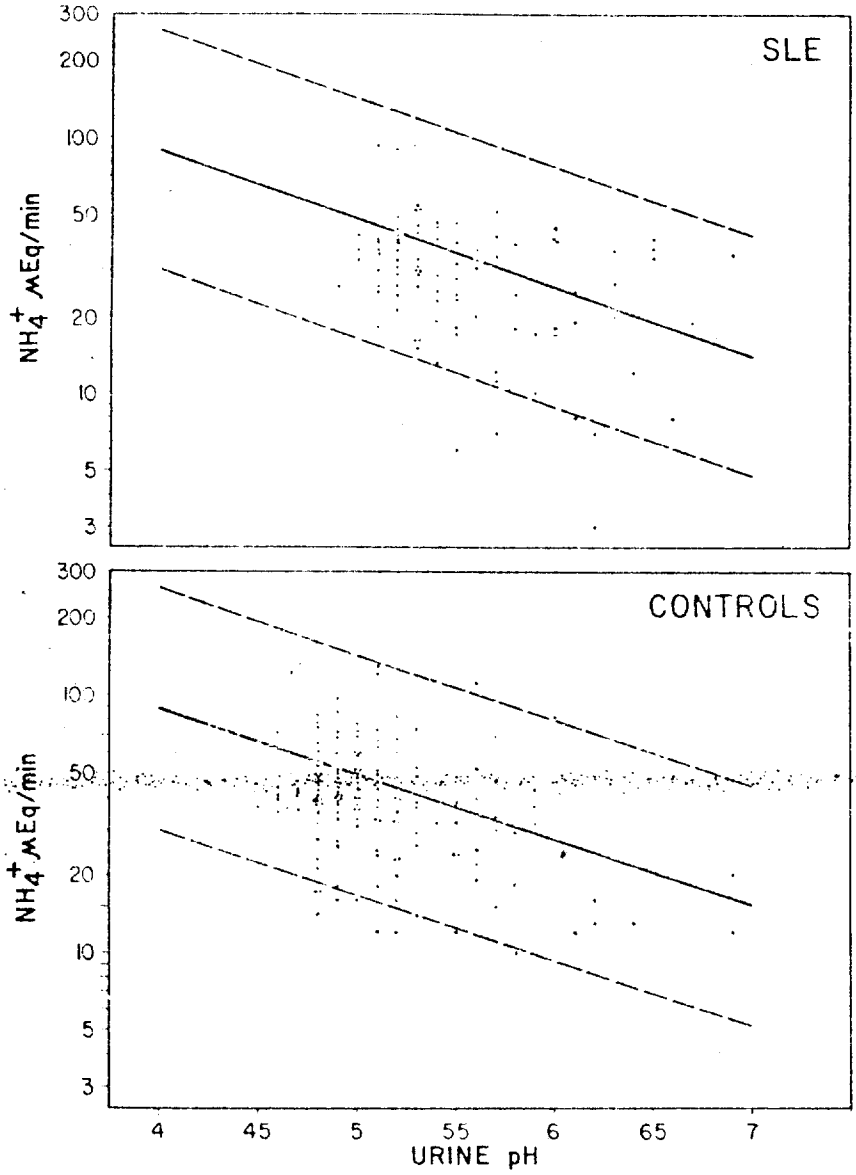

FIGURE 2. Relationship between renal excretion of ammonium and urinary pH. The regression line was derived from data from all the control subjects. Two standard errors of estimate are shown by broken lines (*below*). A similar relationship for patients with systemic lupus erythematosus (*SLE*) is shown with the range of observation in the control subjects (the regression line with 2 SE of estimate).

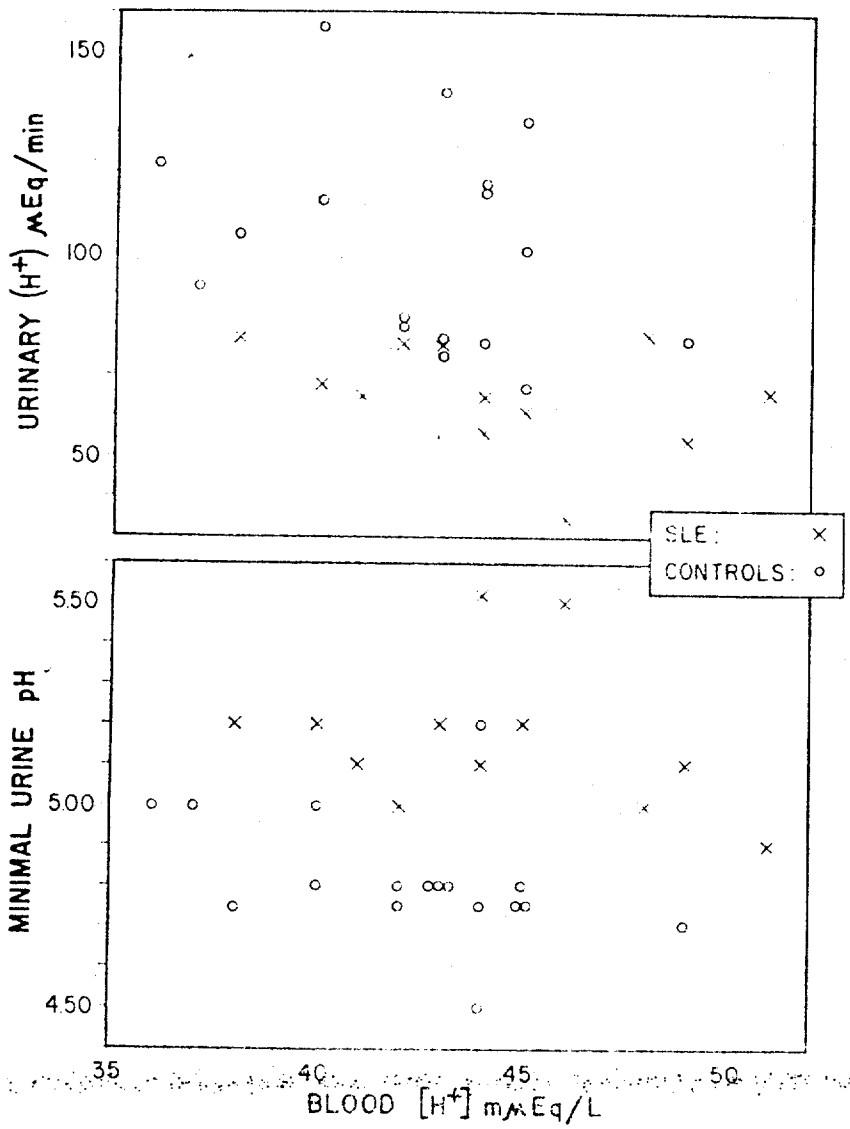

FIGURE 3. Relationship of the minimal urinary pH (*below*) and the net hydrogen ion excretion (*above*) with blood hydrogen ion concentration after ammonium chloride loading.

elements. Tubular abnormalities included atrophy, dilatation, and slight to moderate degrees of thickening of the basement membrane, as noted with periodic acid-Schiff stain (Figure 4). These findings are nonspecific and similar to those observed by others in renal tubular acidosis (3, 19, 20). No changes consistent with arteritis or necrosis of blood vessels were noted.

DISCUSSION

Renal excretion of acid is accomplished by reabsorption of bicarbonate, acidification of buffers in the urine (measured as TA) as a result of transfer of hydrogen ion by the tubular cells into the tubular fluid against a concentration gradient, and excretion of ammonium. The rate of excretion of titratable acid is determined by

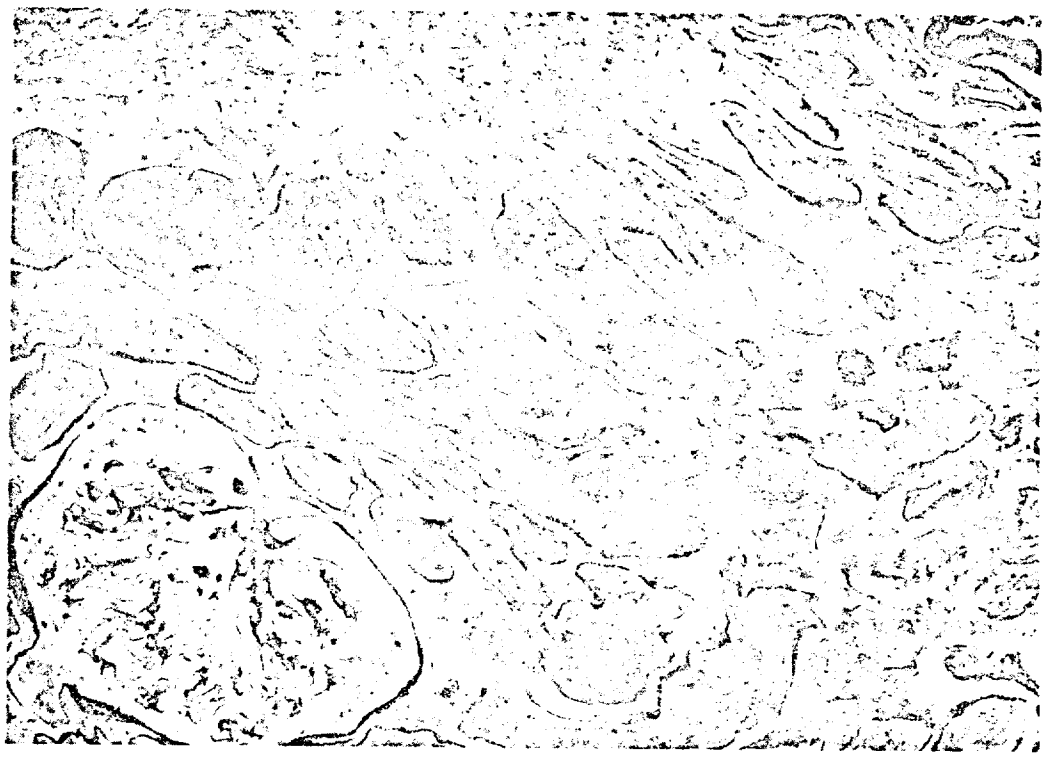

FIGURE 4. Renal biopsy specimen from Patient 3 showing interstitial fibrosis and irregular thickening of the basement membrane of the renal tubules. Glomerular structure is well preserved. (Periodic acid-Schiff stain, ×130.)

the acidity of the urine, by the pK and amount of buffers excreted in the urine (21), and by the rate of urine flow (22). The ammonium excretion rate is largely a function of the glomerular filtration rate (1) and is usually inversely related to the urine pH (23). The calculated regression line in our controls ($Y = 2.991 - 0.255X$) * was in fair agreement with those reported in similar acute loading studies by Wrong and Davies (4) ($Y = 2.89 - 0.259X$) and by Clarke, Evans, MacIntyre, and Milne (24).

Impaired renal acid excretion may represent an aspect of general renal failure (uremic acidosis). In patients with uremic acidosis (25) renal ability to acidify urine is maintained during increases in the degree of metabolic acidosis. The impairment of acid excretion parallels the decrease of the glomerular filtration rate. Although at a certain phase hyperchloremic acidosis may develop, the associated urinary pH tends to be low (26). Diminished acid excretion may be found as an isolated tubular defect (synonyms: renal tubular acidosis, renal hyperchloremic acidosis, nephrocalcinosis infantum, renal acidosis resulting from tubular insufficiency without glomerular insufficiency, and other terms) (27). The essential feature is generally believed to be insufficient acidification of the urine relative to the degree of metabolic acidosis. Consequently, in renal tubular acidosis the rate of urinary TA excretion is decreased and the rate of ammonium excretion is low, although when considered in relation to the pH of the urine it may be normal (1).

In the present study, the patients with SLE responded to the challenge of an am-

* Y = logarithm of the rate of ammonium excretion in μEq/min; X = pH unit of urine.

monium chloride load by an increase of blood hydrogen ion concentration comparable to that of the control subjects, but their renal response was less effective (Figure 3). Their rates of net hydrogen ion excretion were less than normal, apparently because the excretion rates of ammonium and TA were decreased. An inverse relationship between the rate of ammonium excretion and urine pH, similar to that in controls, was seen in the patients with SLE, and practically all ammonium excretion rates in patients were within the 95% confidence limits of the control group for the corresponding urinary pH. This suggests that the ammonium excretion mechanism patients with SLE is normal and that decreased (absolute) rate of ammonium excretion is related to the higher urinary pH. The inability to adequately lower the urine pH is believed to be the most frequent fundamental defect in renal tubular acidosis.

Other factors known to affect renal acid excretion or concentrating ability, such as age (28), deficiency of potassium (1) or of sodium (29), hypercalcemia (30), osmotic diuresis (31), dietary restriction of protein or sodium (32), polydipsia (33), and acute tubular necrosis (3), can be ruled out by data in the present study. The rate of urine flow in the patients did not differ from that in the control subjects and was not of a magnitude reported to reduce TA excretion (22). The mean age of the patient group did not differ significantly from that of the control group. A careful review of drug history in our patients revealed no apparent relationship between the nature, the dose, and the duration of medications and the observed renal function. The two patients (3 and 5) who showed the greatest acidification defect had been on the lowest dose of prednisone (5 and 10 mg daily, respectively) and took no other drugs. That the decreased acid excretion is independent of glomerular filtration rate was indicated by the significantly diminished patient group mean of the rate of TA and ammonium excretion per unit of creatinine clearance. Thus, the diminished capacity for excreting acid and for concentrating urine under standard stresses must be ascribed to primary tubular defects of undetermined mechanism.

With respect to tubular function, the difference between the patients with SLE observed here and those with classic renal tubular acidosis may be a quantitative one, as was reflected in the respective urinary pH values: The defect in the patients with SLE was so mild that metabolic acidosis did not develop when the acid load was limited to that derived from the usual dietary and endogenous sources, but a defect in acid excretion became apparent when the patients were challenged acutely with an extra load of acid derived from the metabolism of ammonium chloride. This finding suggests that such a subclinical impairment of acid excretory mechanism may be not infrequent in SLE while glomerular function rate is preserved and histological lesions are minimal. That this may not be limited to SLE but may also occur in other rheumatic disorders is suggested by results recently noted (34) in patients with Sjögren's syndrome. A form of renal tubular acidosis without overt metabolic acidosis was reported by Wrong and Davies (1), to which they applied the term "incomplete form of renal tubular acidosis." Unlike the patients in the present study, the subjects reported by Wrong and Davies had renal calcinosis, and their ammonium excretion in response to acid loading was above normal. The exact mechanism responsible for this subclinical tubular dysfunction is not known. However, the reported cases of acid excretion defect in the hypergammaglobulinemic states (7–11) suggest that some relationship to an immunological abnormality may exist.

In our series, 8 of 12 patients had abnormally elevated serum gamma globulin concentration (> 1.48 g/100 ml = control mean

+ 2 SD). Two of those (Patients 3 and 5) showed the greatest acidification defects (urine pH, 5.50 and 5.52) and the highest serum gamma globulin concentrations (2.1 and 3.8 g/100 ml) of the group. Further study, such as an analysis of serum gamma globulin by immunochemical methods as reported recently by Wilson, Williams, and Tobian (11), may help to define the relationship of serum gamma globulin to renal tubular function. At present there is no convincing evidence that renal tubular dysfunction is the result of serum protein abnormalities.

Summary

Renal response to acute loading of ammonium chloride and to fluid restriction was evaluated in 12 patients with systemic lupus erythematosus and compared to that in 21 normal subjects of similar age and glomerular function. Although the patients showed no clinical features of renal tubular dysfunction, they demonstrated a subclinical defect of renal acidification and an impairment of renal concentrating ability (latent renal tubular acidosis).

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PHARMACOLOGY

THE EFFECT OF VARIOUS ANESTHETICS ON THE DEVELOPMENT OF TOXIC PULMONARY EDEMA CAUSED BY AMMONIUM CHLORIDE

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Information about the pathogenesis of toxic pulmonary edema, produced by various substances (nitrites, phosgene, adrenalin, ammonium chloride, silver nitrate and others), is scant. The majority of investigators believed that the reason for the development of toxic edema was purely local injury to the vessels, changing the permeability of their walls with subsequent passage of liquid into the alveolar spaces.

The significance of neural mechanisms in the development of pulmonary edema has been shown in the works of national [1-3] and foreign [4-7] authors. In particular, the possibility of preventing the toxic pulmonary edema caused by adrenalin and ammonium chloride has been shown in the experiments of I. A. Serebrovsky. It remained unclear whether only ether has such an effect and whether this action of ether anesthesia is dependent on the inhibition of certain portions of the nervous system or on some side effects.

In order to clarify these questions, we carried out experiments on a model of toxic pulmonary edema while using anesthetics differing in their pharmacological and chemical characteristics.

The experiments were carried out on white rats. The first series of experiments was carried out on 32 animals. When 0.75 ml per 100 g weight of a 6% solution of ammonium chloride was administered intraperitoneally, as a rule by the 6-7th minute after the injection the rats developed an attack of acute tetaniform spasms. At the end of the attack, which usually lasted about 1-1 1/2 minutes, in all cases the animals were in a markedly depressed state, breathing infrequently, lacking corneal and pain reflexes. Later, breathing became less frequent, rales began. In a number of cases, the secretion of froth from the upper respiratory tract was observed. At the 30-40th minute the animals died.

At autopsy, the typical picture of pulmonary edema was observed even with macroscopic observation. A considerable amount of frothy liquid was found in the bronchial spaces. The lungs were solid, poorly aerated; on squeezing, depressions remained on their surface which did not disappear for a long time. In section, the pulmonary tissue was reddish-yellow in color, with hemorrhagic areas; a large amount of frothy reddish liquid ran off from the surface of the cut. The ratio of the weight of the lungs to the body weight of the animal was usually over 1%, reaching 2-2.2% in some cases, i. e., it was considerably greater than normal (0.7-1%).

Microscopic investigation showed that the alveolar spaces were filled with a proteinaceous liquid, in places with an admixture of erythrocytes. In some areas the erythrocytes filled the alveoli completely. In a number of cases a sharply demarcated perivascular and peribronchial edema of the connective tissue was found. The vessels were found to be plethoric. In the other organs (liver, kidneys, brain, spleen), plethora of the vessels was observed; in the liver and brain, in addition, slight perivascular edema was found.

In the second series of experiments, ammonium chloride was administered in the same doses during anesthesia produced by the subcutaneous injection of a 12% solution of urethane, using 1 ml per 100 g of weight. Fifteen animals were beheaded after 1-7 hours, 4 the next day. In 17 cases out of 19, pulmonary edema was not found in the course of either macroscopic or microscopic analysis. The weight coefficients of the lungs varied between 0.6-0.82. Only in two cases were separate hemorrhages and the appearance of edematous liquid in the alveoli observed; the coefficient reached 0.95-0.98.

In the third series of experiments, carried out on 20 animals, ammonium chloride was administered during ether anesthesia. Anesthesia was maintained for 1-2 hours, after which the rats were beheaded. In 17 cases pulmonary edema did not develop; the weight coefficients remained within normal limits. The ammonium chloride had a toxic effect on 3 rats and they died in 1-1 1/2 hours (from pulmonary edema); the weight coefficients were 1.1-1.45.

In the fourth series of experiments, carried out on 12 animals anesthetized with chloroform, the administration of ammonium chloride produced pulmonary edema in only one rat.

In the fifth series, 18 animals were anesthetized by the subcutaneous administration of a 5% solution of hexenal, using 0.3 ml per 100 g of the rat's weight. When anesthesia was achieved, ammonium chloride was injected. In 1 1/2 hours the animals were killed. Pulmonary edema did not develop in 14 rats.

In the sixth series, anesthesia was produced by the injection of 0.5-0.75 ml per 100 g of the rat's weight of a 6% solution of chloral hydrate. Out of 15 experiments, pulmonary edema was observed to develop in only 3 (the weight coefficients were 1.1-2.1; in the remaining cases they remained within normal limits).

In the next series of experiments, set up with 54 animals, the effect of anesthesia produced by the subcutaneous injection of a 1% solution fo chloralose using 0.5, 0.7 and 1-1.5 ml per 100 g weight with subsequent administration of ammonium chloride was studied.

In 10 experiments with a dose of 0.5 ml, which produced superficial anesthesia, pulmonary edema developed in all the animals. Administration of chloralose in a dose of 0.7 ml per 100 g weight prevented development of edema in 7 cases out of 25. When fatal doses of chloralose were administered (1-1.5 ml per 100 g weight) the animals died 1-1 1/2 hours after the experiment began, while in 9 cases out of 19 pulmonary edema did not develop.

The results of our experiments are shown in the illustration.

Microscopic investigation also indicates the absence of pulmonary edema in the cases when the weight coefficients were within normal limits.

In the supplementary group of control experiments, in which the animals were killed by the injection or inhalation of a lethal dose of anesthetic (hexenal, urethane, ether, chloroform, chloralose), pulmonary edema was only found in individual cases of death caused by inhalation of ether and chloroform; in all the rest of the experiments, neither macro- nor microscopic pulmonary edema was found. The weight coefficients corresponded to normal.

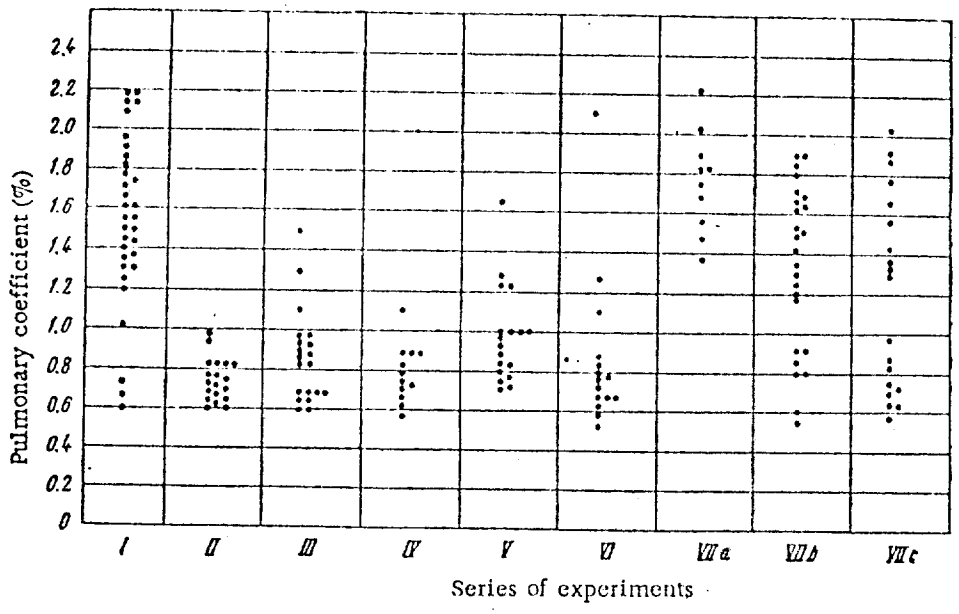
Summarizing the data we obtained, it can be observed that in the majority of cases preliminary anesthetization of the animal prevented the development of the toxic pulmonary edema caused by the administration of ammonium chloride. However, in a number of cases (experiments with chloral hydrate, ether, hexenal) pulmonary edema still developed after the administration of ammonium chloride, a fact basically connected, apparently, with the insufficient depth of the anesthesia.

The experiments using chloralose as an anesthetic should be put in a special group. The results of this series varied. But here also certain rules can be found; with light anesthesia, pulmonary edema developed in all the experiments, with medium anesthesia it was absent in 7 cases out of 25 and in deep anesthesia it was absent in 9 out of 19.

At the time our experiments were finished, Ya. A. Lazaris communicated* the data obtained in his laboratory on the possibility of preventing toxic pulmonary edema produced by ammonium chloride, by means of

*Enlarged quorum of the executive committee of the All-Union Society of Pathophysiologists (Moscow, 1953).

various anesthetics with the exception of chloralose. The author expressed the opinion that the indicated effect is dependent on the interruption during anesthesia of pathological reflex arcs which arise during the action of ammonium chloride. The author considers the brain stem area to be the level of these reflexes by which he explains the failure of chloralose, "a typical cortical" anesthetic by Pick's classification, as a preventative.



Effect of various anesthetic substances on the development of toxic pulmonary edema produced by ammonium chloride. I) Control group; II) urethane anesthesia; III) ether anesthesia; IV) chloroform anesthesia; V) hexenal anesthesia; VI) chloral hydrate anesthesia; VII) chloralose anesthesia; a) dose of 0.4 ml of a 1% solution per 100 g of weight; b) 0.7 ml; c) 1-1.5 ml.

In spite of the coincidences in the basic results of our experiments with the data of Ya. A. Lazaris and M. A. Serebrovskaya, we do not consider it possible to agree fully with the opinion of the indicated authors.

The results of the experiments with chloral hydrate which are characterized, in common with chloralose, by primarily cortical action do not fall within the framework of their concept. In experiments with various anesthetics (including hexenal and chloralose) a certain relationship can be observed between depth of anesthesia and its prevention of pulmonary edema, without regard to its pharmacological characteristics. Ya. A. Lazaris and I. A. Serebrovskaya did not take into account also the possible pathogenic significance of the direct action of ammonium chloride on the central nervous system and the sharp rise in its excitability.

The results of the experiments which were carried out permit denial of the importance of direct injury of the vessel walls by ammonium chloride in the pathogenesis of pulmonary edema. The prevention of pulmonary edema by the action of anesthetics also, apparently, is not determined by their direct effect on the permeability of the vessel membranes.

The speculation regarding the levels of the "pathological reflexes", based on Pick's representations regarding the localization of the action of anesthetics, cannot be admitted to be sufficiently well founded. The pathogenesis of toxic pulmonary edema (especially of its neurogenic components) requires further deeper study.

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THE EFFECTS OF ACIDS AND ALKALIS ON AMMONIA EXCRETION IN THE URINE

by
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Despite the great number of works on ammonia excretion in the urine[1], in connection with the reaction to the alimentation and the influence of the added bases or acids, some details have not been clarified, and the last investigations of Goebel and Tislowitz, with their assertions that ammonia causes an acidification of the organism, introduce a certain confusion into our considerations. Moreover, we believe that much can still be said even about the best known facts, especially if the question is considered from a personal standpoint, and if some personal experience is contributed to the consideration of the matter.

Therefore, we undertook to deal once again with the question of ammonia excretion in the urine, taking into consideration among other things the content of NH_3 in the blood, examining the possible representation of NH_3 by other cations, and studying the influence of the nitrogen content of the food on the excretion of NH_3. Besides this, the dissociation of the acids and its infkuence on water excretion was calculated, and the influence of the neutral salts (e.g. $(NH_4)_2SO_4$, NH_4-OOC-CH_3, etc.) on NH_3 secretion was compared with that of the corresponding acids.

The experiments were carried out on a dog, which was fed a mixed diet which remained unchanged for the duration of the experiments. The urine was examined daily, and after a constant composition was achieved, the appropriate acid or salt was administered. In the urine were evaluated: the Na, K, Mg, Ca, NH_3, the specific gravity, the pH, the titratable acidity, and that titratable with methyl red. Of the figures thus obtained, the most important results are quoted in the following.

The first period includes those experiments which were carried out using a mixed, therefore albuminous, diet. Mineral acids or their NH_4-salts were administered, and therewith the quantity of urine excreted was generally found to be reduced. So we see that after HCl, the quantity of urine decreases from 620 to 575 ccm, after H_2SO_4 from 600 to 400 ccm. The hydrochloric acid was administered in a quantity of 2.02 g, the sulphuric

[1] Benedict, R.St. and T.P. Nash: J. of biol. Chem. 69, 381 (1926); S. Bliss: Ibid. 78, 67.87; Fr. H. Willy and L.I. Wiley: Ibid. 83

acid in a quantity of 2.72 ccm. The simultaneously executed blood analysis (according to Wagner-Parnas) revealed no increase in the ammonia value, and this reaction of the blood NH_3 was observed during the entire course of the experiments. The NH_3 values hover between .0001 and .00018%; only after the administration of phosphoric acid and ammonium phosphate do we see an increase to .00026 and .00022%. The lowest values, .00007 and .00008%, were observed after alkalis and organic acids and their NH_3-salts. Ammonia, given in 4.13 ccm of an ammonia solution of 27%, effects no rise, as well as no compounds. The introduction of alkalis, such as NaOH, MgO and $Ca(OH)_2$, causes an increase in the urine volume, e.g. from 525 to 570 ccm after 2.06 g of $Ca(OH)_2$ or 1.12 g of MgO. NH_3 as such appears to exercise no influence upon the diuresis.

The excretion of cations, such as Na, K, Mg and Ca remained virtually unchanged throughout the entire period. Thus the Na values vary between .740 and 1.34; the highest were observed after NH_4Cl (3 g), after HCl we obtained 1.14, after NH_4Cl 1.34. The lowest values were obtained with organic acids (.725), with lactic NH_4, although with sulphuric acid similar low values were observed. It could be believed that the introduction of mineral acids causes a sinking in the Na values, but this phenomenon is not at all regular. In the same way, an increase after alkalis is barely indicated and cannot always be established. This should only be stressed because a similar and regular increase after acids and decrease after alkalis occurs in the case of calcium salt and magnesium salt, where it is explained by the increased solubility effected by acids and reduction in solubility effected by alkalis. Thus, after HCl and H_2SO_4, Ca and Mg rose from .0121 and .038 to .020 and .048 respectively, while after NaOH the Ca value falls from .0144 to .0105 and the Mg value from .0395 to .0181. We see the same sort of decreases after H_3PO_4 and $(NH_4)_3PO_4$, which can probably likewise be traced back to a reduced solubility.

The acidity of the urine and the two acid salts which can be determined by titration naturally undergo significant and clear changes. Thus the pH sinks from 6.4 to 6.2 after HCl, after NH_4Cl from 6.6 to 6.0, after H_2SO_4 from 6.9 to 6.6, after $(NH_4)_2SO_4$ from 6.5 to 6.2, and so forth. On the other hand, H_3PO_4 and its ammonium salt cause a smaller decrease, in both cases from 6.6 to 6.4. This is still more than the decrease which we observed after the administration of organic acids.

The proportion of acids titratable with phenolphthalein and methyl orange also rises upon administration of mineral acids: from 0.3 to 0.7 after HCl, 0.4 to 0.6 after H_2SO_4, and from 0.3 to 1.0 after H_3PO_4. We see a similar occurrence with the NH_4 salts of the corresponding acids, whereby phosphoric acid and its salt effect especially large differences.[2]

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[2]The values obtained after titration with phenolphthalein were divided by those titratable with methyl orange. The acids and bases were consistently exposed at a standard of 1/18.

Correspondingly, we see the NH_3 values in the urine rise after HCl from 3.6 to 6.38. In the second group of experiments it rises from 3.57 to 9.53, and for NH_4Cl from 3.05 to 5.33 and from 4.04 to 7.50 We can see that, despite the fact that with the salt the NH_4-ion is introduced, the ammonia excretion in the urine rises after acids. H_2SO_4 effects an increase from 3.15 to 8.9 and from 4.2 to 9.27; its ammonium salt from 3.45 in the morning to 7.68 and from 3.62 to 8.95. In contrast to that, phosphoric acid and its salt create a rather modest increase, from 2.3 to 3.0, and for $(NH_4)_2PO_4$ from 3.8 to 4.5. The figures quoted here indicate the ratio of NH_3 to N multiplied by 100, and prove, that the NH_3 does not nearly correspond to the N in the urine, which, moreover, after acids reveals a slight decrease along with a reduced diuresis.

Therefore, after mineral acids and their NH_3-salts we observe: 1. an increase in the pH and the titratable acids; 2. an increase in the NH_3 values in the urine, absolute as well as in relation to the total N content; 3. the NH_3 values in the blood undergo no changes, with the exception of those found after phosphoric acid and ammonium phosphate, and which show an increase. The other cations remain unaffected.

The second period includes the experiments with organic acids, of which acetic acid, lactic aicd, tartaric acid and their NH_4-salts were administered.

In contrast to the decrease observed in the case of mineral acids, the water excretion reveals an increase, which also continues after the NH_4-salts. Thus in the case of acetic acid, the quantity of urine rises from 540 to 580 ccm, after lactic acid from 500 to 520 ccm, and after tartaric acid from 550 to 600 ccm per day.

The NH_3 values in the blood are quite low (as mentioned above), e.g. 0.00013, 0.000093, even 0.00008 g to the hundred. We were unable to establish any influence on the excretion of Na or K. Though we occasionally noted a somewhat irregular decrease in K-excretion, especially after mineral acids, in these experiments the K-excretion remained unaffected. The same may be said of Na-excretion. As mentioned, the Ca values in the urine were decreased after phosphoric acid and phosphate, and increased after acids, which we explained by the solubility of the Ca salts; in these experiments, a noteworthy increase could only be seen after tartaric acid and tartaric NH_4, i.e. from .0197 to .0334 for Ca and from .0337 to .0475 for Mg. Nearly the same increased secretion was noted after tartaric NH_4.

The acidification of the urine, determined through titration, is not to be seen after acetic acid, sooner after NH_4-acetate (from .401 to .69), after lactic acid from 0.33 to 0.46, while after tartaric acid and tartaric NH_4 it is very significant: in the same manner, the NH_4 values in the urine are increased only after tartaric acid, while they remain unaffected by the other organic acids.

5.1 g of tartaric acid,	acidity	from	.421	to	.875
NH_4-salt	"	"	.303	"	.588
3.33 g acetic acid,	"	"	.468	"	.408
5.0 g lactic acid,	"	"	.354	"	.462

The NH_4 of the urine, expressed in relation to the N, sank after acetic acid and NH_4-salt. We purposely present the figures, in order to emphasize this phenomenon:

3.33 g acetic acid,	NH_3	of	the	urine	from	4.0	to	2.46
$NH_4OOC-CH_3$,	"	"	"	"	"	3.85	"	3.53
5.0 g lactic acid,	"	"	"	"	"	3.73	"	3.83
lactic NH_4,	"	"	"	"	"	4.04	"	4.30
5.1 g tartaric acid,	"	"	"	"	"	4.17	"	8.46
tartaric NH_4,	"	"	"	"	"	4.23	"	7.08

The observed increase of the NH_3 values in the urine can thus be compared with that which was noted after mineral acids and their NH_4-salts. It should be stressed, that at the same time, the NH_3 values in the blood hovered between .00012 and .00015, and can therefore be considered normal and unaffected.[3]

The <u>third</u> period includes the experiments made with the administration of alkalis and ammonia. After NH_3 and $(NH_4)_2CO_3$ we find a decrease of the titratable acids from 0.53 to 0.45, from 0.43 to 0.37, from 0.32 to 0.24, and the NH_3 figures in the urine sank correspondingly from 3.98 to 3.88, from 3.48 to 3.87, from 1.83 to 1.32, and from 2.84 to 2.37. These figures indicate that NH_3 has no sort of acidifying effects (Goebel and Tislovitz), and on the basis of repeated experiments, can be aligned with the alkalis. Thus after NaOH, the titratable acidity and the urine NH_3 are affected in a similar manner. If in general one finds a stronger reaction after solid bases, it may be due to the fact that NH_3 is in general a weaker base, and one can expect the same difference that we found in the case of mineral acids compared with the weakly dissociated organic acids. The figures are:

2.22 g NaOH,	titr.	acidity	from	.366	to	.102,	NH_3	4.9	to	2.0
1.12 g MgO,	"	"	"	.275	"	.196,	"	3.4	"	2.6
2.06 g $Ca(OH)_2$,	"	"	"	.77	"	.181,	"	3.8	"	3.4
3.17 g $(NH_4)_2CO_3$,	"	"	"	.435	"	.37,	"	3.9	"	3.8
4.13 ccm NH_3,	"	"	"	.325	"	.24,	"	1.83	"	1.3

From this we see, that the stronger the base, the greater the influence upon acid and NH_3 excretion. The other cations remained unaffected, outside of the self-evident increase of calcium salt by the alkalis.

Also worthy of mention, however, is the fact that the magnesium salts did not undergo an increase, but rather a decrease in excretion in the urine. In the same manner, we occasionally see a decrease in the calcium salts after NH_3 and an increase after NaOH. The pH rises from 6.5 to 6.8-7.4.

The <u>fourth</u> period was devoted to studying the influence of a diet poor in nitrogen on NH_3 excretion when acids are added. It is important to test whether the deficiency in nitrogen affects the other cations upon acidification. This new diet led initially to an increase in the diuresis, which rose from 550 to 700 ccm, then sank again, but always rose upon acidification, to some

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[3] The ammonium salts of the organic acids were obtained through neutralization of the acids with ammonia, using a litmus as indicator.

660 ccm. After 3 g of NH_4Cl, the pH falls from 6.3 to 5.6, after $(NH_4)_2SO_4$ from 6.4 to 5.8. The titratable acidity rises correspondingly. The NH_3 values in the urine rise from .235 to .583 per day, which, in view of the decreased N-excretion, increases the quantity of NH_3 relative to N even more. After ammonium sulphate, the increase from .208 to .583 is almost the same. The blood NH_3 values remained unaltered, and hovered around .000088%. The reaction of the K-salts, which rose after NH_4Cl from .391 to 1.06 and after $(NH_4)_2SO_4$ from .316 to 1.22, must be especially emphasized. The Mg values also reveal an increase, from .022 to .0412 and from .0198 to .0346. Less clear is the rise in the Ca-salts, from .0125 to .024 and from .0107 to .0178. This Ca-excretion can certainly be traced back to the increase of soluble Ca-salts, and the K-salts reveal no such rise after $CaCl_2$. The Na-salts always reveal a decrease after acidification, never an increase, e.g. from .131 to .098 or from .48 to .32. Only after NH_4-sulphate does an increase from .55 to .82 take place.

If one pictures the ratio of $NH_3/N \times 100$, the influence of the acidification is even clearer, e.g. after $CaCl_2$ from 10.4 to 13.5, after NH_4Cl from 11.4 to 19.4, after NH_4-sulphate from 12.8 to 20.0. One can see from the growing figure of the NH_4/N quotients, that the carbohydrate-rich and nitrogen-poor diet leads to an ever-rising acidification.

We used the fifth period to study the influence of the chlorine-poor diet on the acidification. As earlier, the food was mixed and administered without salt. Initially, the water excretion was increased, then became normal, from 630 to 575 ccm. The titratable acids, previously involved in an increase, now decreased, as did the pH (from 6.2 to 6.0), all of which indicated acidification. The ratio of NH_3/N during the course of the week was as follows: 8.2, 8.4, 8.6, 8.3, 8.6, 8.8, thus likewise indicating a constant acidification. After the administration of 2 g of NaCl, the NH_3/N fell to 8.1, the daily NH_3 excretion fell from 0.51 to 0.45, the K values, which were hovering around 1.4, fell to 0.95, and the Ca values fell from .0178 to .0161. The Mg-salts underwent a slight increase, from .0217 to .0235.

Thus the Cl-poor diet was accompanied by an acidification similar to that which we find with a nitrogen-poor diet, and the NaCl reacted like an alkali, which fact we had already previously observed and emphasized. Just as the change from a nitrogen-poor to a nitrogen-rich diet is accompanied by an alkalization -- probably through the NH_2 groups -- so sodium chloride seems to effect an alkalization with its Na-ion.[4]

In the sixth and last period we attempted to examine the influence of thirst on the acidification of the urine. It is known that a scanty urine is always more acid than a plentiful one, and that an increased diuresis causes an alkalisation of the urine. This was now once again confirmed.

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[4] Moraczewski, W.v. and St. Grzycki: Naunyn-Schmeideberg's Arch. 160, 703 (1931).

The quantity of urine went down to 250 ccm per day, and revealed a lowering of the pH from 6.6 to 6.4. The NaOh titratable acids however rose constantly from 33.8 to 64.8 per day. The daily NH_3-excretion rose from .153 past .288 to .318. In relation to N this is an increase from 2.33 past 3.46 to 4.82. The K-salts amounted on the average to 2.45, the Na-salts to .515, the Ca .0217, Mg .0191.

Now, when the diuresis rose to 900 ccm due to the abundant administration of water, the pH fell from 6.9 - 6.8, the titratable acids from 64 to 54, and the NH_3 values, due to the draining of the organism, showed an increase to .43 pro die, which increase however gave way on the next day to a decrease, .306. The ratio to N, which on the first day amounted to 6.4, fell now to 4.1, which still indicates a tendency toward acidulation, though no true acidulation. Nevertheless, in pursuit of the NH_3 values in the urine, we see a decrease in them, while in the case of thirst, an increase is noted. On the other hand, in the case of an abundant diuresis, the K-salts reveal a slight increase, from 2.45 to 2.55, and the Ca-salts from .0127 to .0356. The Mg-salts and Na undergo a decrease, Mg from .0191 to .0101, Na from 0.5 to 0.278. Contrary to our expectations, the Na/Cl ratio also was halved, falling from 8 to 4. The alkalization of the urine after much water intake is therefore not caused by the plentiful rinsing out of alkalis, and does not yield the NH_4 decrease which we otherwise find in alkalization of the urine. It seems as though only the K and Ca-salts react to water intake, while the Na and Mg-salts, on the other hand, are retained.

Summary

1. The introduction of mineral acids effects an increase in the ammonia values in the urine.

2. The introduction of alkalis and ammonia causes a decrease in the ammonia values.

3. Organic acids such as acetic acid or lactic acid cause no increase in the ammonia values.

4. Tartaric acid and tartaric NH_4 function like a mineral acid.

5. The introduction of acids and alkalis does not influence the NH_3 values in the blood.

6. In the cases of protein-poor and carbohydrate-rich diets, the K and Ca values as well as the NH_3 values in the urine are increased upon addition of acids.

7. A chlorine-poor diet functions as an acidification, and the introduction of sodium chloride as an alkalization.

8. A waterfree diet also leads to acidification, an abundant admixture of water to alkalization.

9. After phosphoric acid and ammonium phosphate, the NH_3 values in the blood are discovered to be increased.

The Differential Toxicity of Ammonium Salts

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The relationship between liver disease and ammonia toxicity has been known since the discovery of meat intoxication in Eck fistula dogs (1–4). The similarity between this syndrome and clinical hepatic coma stimulated the study of ammonia toxicity in humans. This proceeded from oral administration (5–10) to intravenous infusion (11, 12) and finally to the development of an "ammonia tolerance test" (13, 14). Ammonia toxicity studies have been performed in animals by the intravenous (15–24), oral, subcutaneous, and intraperitoneal routes (25–29).

Many different ammonium compounds were used in the above studies with the tacit assumption that their toxicities were similar. In order to test that assumption, intravenous LD50's were determined in mice under controlled conditions.

MATERIALS AND METHODS

Intravenous LD50's were determined in young Swiss albino mice, weighing 18 to 22 Gm. (average, 19.7 Gm.). One ml. of isotonic saline containing the ammonium compounds (reagent grade) in varying concentrations was injected into a tail vein during an average time of 1.9 seconds. The lethal effect was determined in 10 animals for each of three to five concentration levels. The mice were observed continually until all signs of acute toxicity disappeared and then were examined during the course of several days for the appearance of any latent toxicity.

Blood pH was determined with a Beckman Model GS meter, equipped with a 290-80 blood electrode, on heart blood obtained from live, unanesthetized animals. The effect of each ammonium salt on the blood pH was measured within one minute of its intravenous administration.

The LD50's and approximate 95 per cent confidence limits were obtained by the Carver method (30).

RESULTS

When the intravenous LD50's of ammonium chloride, citrate, acetate, bicarbonate, carbonate, and hydroxide were determined, differences in their toxicities were noted. On studying the relationship of the LD50's to the pH's of the compounds in isotonic saline and their effect on the blood pH (normal, 7.46 ± 0.02), it was observed that the toxicity of the compounds increased with a rise in pH, as shown in Figure 1. A notable exception was ammonium citrate which, although it had the lowest pH, displayed the highest toxicity. The data pertaining to this seemingly aberrant effect are not represented in Figure 1 but will be presented below, as the toxicity of ammonium citrate does not appear to be due to the ammonium moiety.

When the ammonium salts, with the exception of citrate, were administered in a single rapid intravenous dose (between the minimal and maximal lethal doses), a reaction began in 2 to 5 seconds, characterized by hyperventilation and clonic convusions. This was followed by either a fatal tonic extensor convulsion or the gradual onset of coma over the course of three to five minutes. The mice remained in a comatose state for approximately 30 to 45 minutes, showing no response to touch or light, but moving convulsively to sound stimuli. During this time a tonic convulsion and death, without cardiac arrest, could occur at any time, but those mice which survived made a rapid and complete recovery.

There was no discernible effect from the injection of 1 or even 2 ml. of isotonic saline. The injection of the maximum lethal dose of ammonium chloride over increasingly prolonged time periods of one-half, one, two, four, and six minutes revealed only a slight diminution of toxicity at four to six minutes. Since the toxicity of the ammonium salts appeared to vary with the pH, the effects of $Na_2CO_3/NaHCO_3$ and Na_2HPO_4/NaH_2PO_4 buffers with concentrations and pH's approximating the maximum lethal doses of ammonium chloride and ammonium hydroxide were studied. There was no evidence of toxicity. In

[1] Laboratory of Tropical Diseases.

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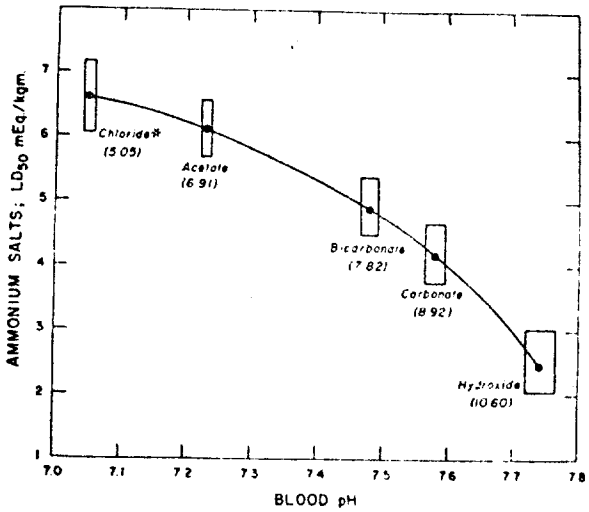

FIG. 1. LD50's OF AMMONIUM SALTS AND THE RESULTANT pH OF BLOOD ONE MINUTE AFTER INJECTION

The rectangle surrounding the points determined denotes, on the ordinate, the approximate 95 per cent confidence limits, and on the abscissa, the standard error of the mean pH—average for 10 animals.

* pH of the solution administered.

order to determine the effect of the anionic component of the ammonium salts, sodium salts of each compound were injected in a concentration equivalent to the maximum lethal dose, and again no toxicity was noted. Attempts to achieve a prior alkalosis, either metabolic or respiratory, were negated by the overpowering effect of the intravenously administered ammonium compounds.

Since the physical state in which ammonia exists might influence its toxicity, it was of interest to calculate the relative amounts of ammonium ion and ammonia gas at the pH's of the LD50 concentrations. In order to do this the following formula was derived from the mass action law:

$$\frac{X}{(Y - X)(H^+)} = 1.8 \times 10^9,$$ [2]

where X equals NH_4^+ and Y equals total ammonia. The data calculated from the above formula, summarized in Table I, reveal that as the blood pH rises, the amount of ammonia gas relative to ammonium ion increases markedly.

TABLE I

The effect of blood pH on the ratio of ammonia gas to ammonium ion

Ammonium salt	LD_{50} concentration mEq./L.*	Blood pH	NH_3 mEq./L.	NH_4^+ mEq./L.	Ratio NH_3/NH_4^+
Chloride	135.0	7.05	0.84	134.16	0.0063
Acetate	124.6	7.23	1.16	123.44	0.0094
Bicarbonate	100.9	7.48	1.67	99.23	0.0168
Carbonate	89.3	7.58	1.85	87.45	0.0212
Hydroxide	50.5	7.74	1.50	49.00	0.0306

* One ml. administered intravenously to each animal.

TABLE II

The toxicity of ammonium citrate

Solution administered	LD_{50} concentration of citrate salts mEq./Kg.	Solution pH
Ammonium citrate	1.58 (1.52–1.63)*	4.86
Ammonium citrate + calcium gluconate	8.40 (8.03–8.79)	4.63
Sodium citrate	1.94 (1.86–2.02)	4.90†

* Approximate 95 per cent confidence limits.
† pH adjusted to 4.90 with HCl.

When ammonium citrate was administered, toxicity appeared to develop in a manner somewhat different from that of the other ammonium compounds. The animals immediately began to leap about exhibiting tetanic movements of all limbs, followed by the sudden onset of apnea and death, with the heart still beating. They never developed the clonic-tonic convulsions typical of ammonia toxicity, and either died in a few minutes or recovered rapidly, with no subsequent development of coma. In order to test whether this effect was due to the citrate moiety and the induction of hypocalcemia, enough calcium in the form of calcium gluconate was added to ammonium citrate, on an equivalent weight basis to bind the citrate completely. As is shown in Table II, the toxicity was reduced below that of ammonium chloride. The toxicity of sodium citrate at a similar pH was approximately the same as that of ammonium citrate. Calcium gluconate had no effect on ammonium chloride toxicity.

DISCUSSION

The material presented in Table I, illustrating an increased ratio of ammonia gas to ammonium ion with a rise in blood pH, may explain the differential toxicity of the ammonium salts studied

[2] This figure was derived from the dissociation constant of NH_4OH

Preliminary data gathered in this laboratory (31) revealed that the greater part of the ammonia administered in a single injection passed from the blood in less than three minutes. In spite of this fall in ammonia level, our mice did not become comatose for 3 to 4 minutes and they remained in coma for at least 30 minutes. This may indicate a rapid and early passage of ammonia into the brain. Therefore, the effect of pH on the blood-brain-barrier may be related to either the greater permeability of free than ionized ammonia, or to an effect of pH directly on the barrier itself.

The pH of the medium in which ammonium salts are dissolved has been shown to influence the ability of ammonia to cross cell boundaries in both red blood cells (32) and other tissues (33). This appears to be due to the amount of ammonia gas versus ammonium ion in that the unionized ammonia has a much greater ability to penetrate cells. Jacobs and Stewart (33) have shown that the ammonium salts of weak acids, such as acetate, bicarbonate, and benzoate, penetrate all cells with great ease, while ammonium salts of strong acids, chloride, nitrate, and sulfate apparently do so no more readily than the corresponding potassium and sodium salts. The latter salts have been shown to cross the blood-brain-barrier at an extremely slow rate (34), requiring a much greater time to equilibrate with the brain than with other tissues. In all probability, ammonia gas can penetrate this barrier as rapidly as other gases, and as readily as it passes other tissue barriers.

A direct effect of pH changes on the blood-brain-barrier has been reported by Broman and Lindberg-Broman (35) who found that extreme changes in pH increase the permeability of the blood-brain-barrier to trypan blue. Fisher, Troast, Waterhouse, and Shannon (36) have shown that the amount of a given sulfa drug that crosses the barrier is dependent on the pK of the compound; the higher the pK the greater the passage. The question resolves itself, therefore, as to whether more ammonia crosses the barrier at high pH's because of the increased ammonia gas to ammonium ion ratio, or whether there is a direct effect on the blood-brain-barrier itself, or both. Animal studies now in progress may provide an answer.

Other investigators have studied the toxicity of different ammonium salts. As early as 1893, Marfori (15) noted that ammonium carbonate was much more toxic than either ammonium lactate or tartrate during slow intravenous infusion into rabbits and dogs. Roberts, Thompson, Poppell, and Vawnce (23) have recently speculated that an elevation of blood pH could enhance the toxicity of ammonia; nevertheless, several investigators have found no such relationship. Following the administration of ammonium compounds to dogs, Karr and Hendricks (20) felt that the effect of pH on ammonia within physiological limits was negligible, and although Iwahashi (29) noted differences in toxicity of the compounds in mice, he could find no consistent pattern. Eiseman, Fowler, White, and Clark (24) administered ammonium salts intravenously to dogs and concluded that there was no relationship between pH and ammonia toxicity. Since

TABLE III

Tabulation of the data of Eiseman and co-workers (24) on the infusion of ammonium compounds into dogs

Ammonium salt	Concentration and rate of infusion	Total time infused *hours*	Highest blood ammonia level *μg. NH_3/ml.*	Blood ammonia during coma *μg. NH_3/ml.*	Total NH_3 infused *Gm.*	Blood pH at coma	Remarks
Chloride	5% 2 ml./min. 2 hrs. 1 ml./min. 2 hrs.	4	175	90	5.73	6.9	Convulsion at 4 hrs.
Hydroxide and HCl, pH 8.8	1% 1 ml./min.	5	40	35	1.46	7.4	
Hydroxide	1% 1 ml./min.	4.5	50	20	1.31	7.6	
Citrate	4% 2 ml./min.	3	30	20	2.14	7.2	Convulsion, 2 hrs.; tremors, 40 min.

their data are particularly pertinent to this discussion, they are summarized in Table III. The concentrations of the drugs administered, as well as the rates and times of infusion and the blood ammonia levels at onset of coma, were not uniform. The symptomatology evinced by the dog given ammonium citrate did differ from those given the other salts in that there were tremors at an early stage and a convulsion about midway in the course of the infusion. Their data not only suggest a relationship between blood pH and ammonia toxicity, but also reveal the excessive toxicity of ammonium citrate.

Since ammonium citrate is being used with increasing frequency in both animal and human studies (12, 14, 24), it is important to note that in a single intravenous lethal dose in mice the citrate moiety is more toxic than the ammonium moiety. The mice die in tetany due to hypocalcemia rather than in a manner consistent with ammonia toxicity. Therefore, the use of ammonium citrate in human experimentation, particularly when administered via the intravenous route, might be contraindicated.

In relation to clinical hepatic coma and its therapy, Gyorgyi and Kleinschmidt (37) in 1927 found that dogs with Eck fistulas in meat intoxication were alkalotic. Vanamee, Poppell, Glicksman, Randall, and Roberts (38) showed, and it was confirmed by Robin, Whaley, Crump, and Travis (39), that the majority of patients in hepatic coma develop a respiratory alkalosis due to hyperventilation with blood pH's as high as 7.70. This alkalosis may effect significantly the toxicity of a given amount of ammonia in the blood and may be one of the factors accounting for the poor relationship between blood ammonia levels (arterial and venous) and clinical hepatic coma.

Gyorgyi and Kleinschmidt (37) noted further that the oral administration of hydrochloric acid brought about a rapid improvement even in severely intoxicated animals. The administration of acidifying agents or carbon dioxide to patients in hepatic coma may therefore prove to be beneficial.

SUMMARY

1. Intravenous LD50's of several ammonium salts were obtained in mice.

2. The ammonia toxicity of the chloride, acetate, bicarbonate, carbonate, and hydroxide salts increased in relation to their effect in raising blood pH.

3. This change appeared to be related either to the effect of pH on the NH_3/NH_4^+ ratio and the ability of ammonia gas to cross the blood-brain-barrier, or to a direct effect of pH on the barrier.

4. The toxicity of ammonium citrate, under the above conditions, does not appear to be related to ammonium toxicity, but to hypocalcemic tetany induced by chelation of calcium by the citrate moiety.

5. Evidence was cited from the literature supporting these results obtained in mice by similar experiments in rabbits and dogs.

6. Therapeutic implications are discussed in the light of the recent findings that respiratory alkalosis is a frequent accompaniment of the hepatic coma syndrome.

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Hypoxia and ammonia toxicity

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WARREN, KENNETH S AND STEVEN SCHENKER. *Hypoxia and ammonia toxicity*. Am. J. Physiol. 199 (6): 1105–1108. 1960.—Ammonia toxicity was strikingly potentiated by short periods of hypoxia. The toxicity (LD_{50}) of ammonium chloride administered intravenously to mice after 2 minutes in 15% oxygen was the same as that in 21% oxygen. However, its toxicity began to increase when they were exposed to 13% oxygen, and was markedly enhanced by each 2% decrement in oxygen concentration. The effect of a maximal lethal dose in 7% oxygen could be completely reversed in a matter of seconds by transferring the mice to 21% oxygen. Ninety-nine per cent oxygen did not alter the LD_{50}, but significantly prolonged life. Hypoxia had a relatively slight effect on Metrazol toxicity. Blood pH was unchanged by the short periods of hypoxia prior to ammonia injection. Furthermore, measurements of brain ammonia concentrations following a standard dose of ammonium chloride at 21, 11 and 7% oxygen revealed that hypoxia had no effect on ammonia uptake or detoxication by the brain. It appeared therefore, that hypoxia directly potentiated the toxic effect of ammonia on the brain.

RECENT STUDIES of ammonia toxicity in the mouse have shown that changes in blood pH may effect the passage of ammonia into the tissues (1, 2). In an attempt to produce a respiratory alkalosis via hyperventilation, mice were placed in moderately low oxygen concentrations just prior to and during the intravenous administration of ammonium chloride. As the toxicity of ammonia was so strikingly enhanced by this procedure that it could not be explained by blood pH changes alone this phenomenon was investigated further.

MATERIALS AND METHODS

The LD_{50}'s of intravenously administered ammonium compounds and pentylenetetrazol (Metrazol) were determined in young Swiss albino mice weighing 18–22 gm. One milliliter of isotonic saline containing the compounds in various concentrations (figs. 1 and 2) was injected into a tail vein during an average time of 1.9 seconds. The lethal effect was determined in 5–20 animals for each of three to six concentrations. The surviving mice were observed until all signs of acute or latent toxicity disappeared.

In order to determine the LD_{50} at different oxygen concentrations mice were placed singly into small wooden boxes with transparent removable tops. The oxygen mixture was introduced at the head end of the box and intravenous injections were made into the mouse's tail protruding from the opposite end. The oxygen atmosphere in the box was checked constantly with a Beckman D oxygen analyzer. For oxygen concentrations below 21% the instrument used had a scale of 0–21% and an accuracy within 0.5%.

For blood pH determinations the mice were kept in a large polyethylene bag into which the oxygen-nitrogen mixtures were passed. Heart blood was drawn from the unanesthetized animals at the end of 2 minutes. The pH of the blood was measured under strictly anaerobic conditions within 1 minute on a Beckman GS pH meter utilizing a 290-80 blood electrode maintained at a constant room temperature of 23°C.

Brain ammonia concentration was determined by the method of Nathan and Warren (3) on the whole brain of mice frozen alive in liquid nitrogen.

RESULTS

Acute toxicity of ammonium chloride in room air (21% oxygen). When ammonium chloride was administered in a single rapid intravenous dose (between the minimal and maximal lethal doses), a reaction, characterized by hyperventilation and clonic movements began in 2–5 seconds. This was occasionally followed by a fatal tonic extensor convulsion, but the usual sequel was the gradual onset of coma over the course of 3–5 minutes. The mice remained in coma for approximately 30–45 minutes, showing no response to touch or light, but moving convulsively to sound stimuli. Death would usually be preceded by a tonic convulsion but the mice which survived made a rapid and complete recovery.

There was no discernible effect following the injection of 1 or even 2 ml of isotonic sodium chloride. When the maximum lethal dose of ammonium chloride was injected during periods of ½, 1, 2, 4 and 6 minutes there was only a slight diminution of toxicity at 4–6 minutes.

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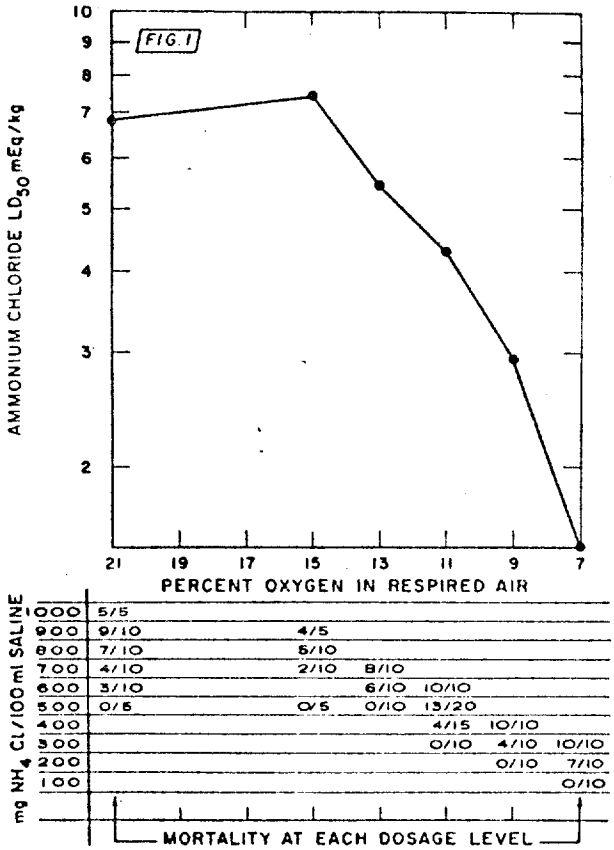

FIG. 1. Effect of lowering the respired O_2 concentration on the LD_{50} of ammonium chloride in mice.

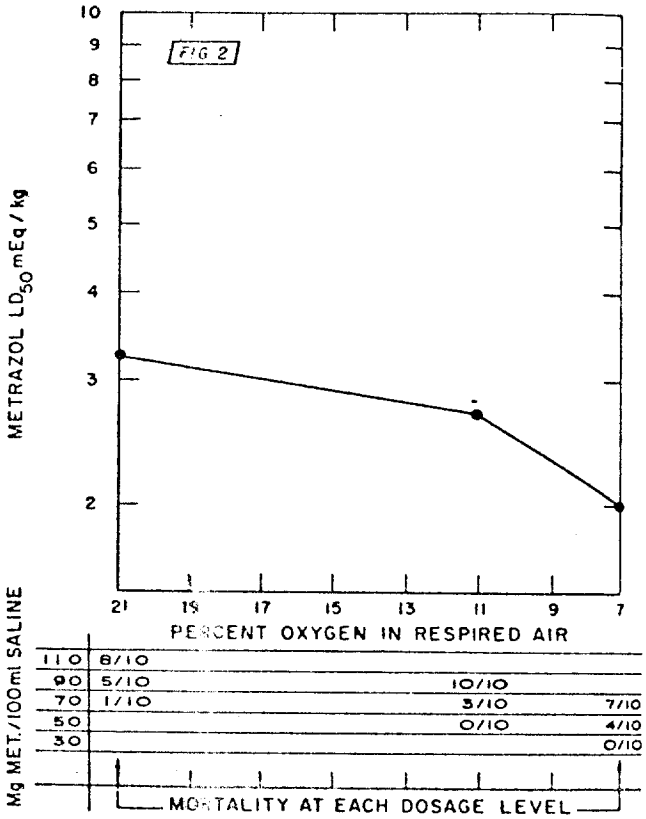

FIG. 2. Effect of lowering the respired O_2 concentration on the LD_{50} of Metrazol in mice.

A dose just below the minimal lethal dose administered once each day for 8 days showed no cumulatively toxic effect.

Decreased atmospheric oxygen concentration and ammonia toxicity. The LD_{50} of ammonium chloride to mice in 21% oxygen was previously determined (1), and was confirmed in our initial experiments (fig. 1). Mice were then placed in 15, 13, 11, 9 or 7% oxygen. Two minutes later ammonium chloride was injected into a tail vein and the mice were maintained in the lowered oxygen concentrations until death or recovery ensued.

There was no significant change in ammonia toxicity when mice were placed in 15% oxygen (fig. 1). However, there was a marked increase in ammonia toxicity as the respired oxygen concentration fell in 2% decrements from 13 to 7%. Five per cent oxygen was itself lethal within 3-6 minutes whereas mice remained alert and well in 7% oxygen for at least 30 minutes. At 7% oxygen ammonium chloride was 4.4 times more toxic than in 21% oxygen.

When the mice were placed in an atmosphere containing a mixture of 8% carbon dioxide and 10% oxygen in nitrogen the toxicity of ammonia was somewhat less than that following 10% oxygen without added carbon dioxide (LD_{50} 4.94 mEq/kg cf. fig. 1).

Between 21% oxygen and 13% oxygen most deaths occurred from 2 to 20 minutes following the injection of ammonium chloride, but at 9 and 7% oxygen the mice died in 15-30 seconds. At 11% oxygen ammonia was lethal from within 15 seconds to 20 minutes.

Increased atmospheric oxygen concentration and ammonia toxicity. When mice were exposed to 50, 75, 95 and 99% oxygen, ammonia toxicity was similar to that in 21% oxygen. The LD_{50} in 99% oxygen (7.48 mEq/kg) was not essentially different from that in room air or in 15% oxygen (cf. fig. 1). When 5% carbon dioxide and 94% oxygen was administered the toxicity of ammonium chloride was also unchanged.

Although the LD_{50} in 99% oxygen was the same as that at 21% oxygen, the mice lived longer. They died within 7-93 minutes, and many mice remained alive longer than an hour. Control mice kept in 99% oxygen for 2 hours showed no toxic effects.

Effect of rapid alterations in atmospheric oxygen concentration on ammonia toxicity. The intravenous administration of 1 ml of isotonic sodium chloride containing 400 mg (100 ml of ammonium chloride is not lethal to mice in 21% oxygen, but causes 100% mortality when they are in 7% oxygen (fig. 1). This dose was injected into mice after they had been in 7% oxygen for 2 minutes (as in determining the LD_{50}). If these mice were removed to 21% oxygen immediately following the injection none died. If they were taken out 10 seconds after injection 50% died, and by 20 seconds there was 100% mortality. If the time interval in 7% oxygen prior to an injection of this same dose of ammonium chloride was reduced to

1 minute, or the injection was made simultaneously with the change to 7% oxygen, the mice all died within 60 seconds.

Ammonium chloride (400 mg/ml) was then injected while the mice were in 21% oxygen, and they were subsequently transferred to 7% oxygen. When this was done at 1 minute there was a 90% mortality occurring within 60 seconds. When transfer into 7% oxygen was effected 2 minutes following injection the mortality fell to 20%.

Essentially the same effects occurred when 1 ml of 600 mg of ammonium chloride per 100 ml of saline (fig. 1) was injected while mice were in 11% oxygen. That dose was 100% lethal as compared to 20% mortality in 21% oxygen, and the alterations in its toxicity with changes in oxygen concentration were equally as rapid.

Decreased atmospheric oxygen concentration and Metrazol toxicity. The toxicity of another analeptic compound Metrazol was not strikingly different when administered under the same conditions to mice maintained at 21%, 11% and 7% oxygen (fig. 2). Metrazol was 1.6 times more toxic in 7% oxygen than in 21% oxygen, as compared to a difference of 4.4 times in the case of ammonium chloride.

Decreased atmospheric oxygen concentration and blood pH. The pH of the heart blood of ten mice in 21 per cent oxygen averaged 7.51 (table 1). When similar groups of mice were placed in 11% and 7% oxygen for 2 minutes, and heart blood was withdrawn while they remained at those oxygen concentrations, the blood pH remained unchanged.

Ammonium chloride causes an acute acidosis when given to mice under the conditions of these experiments (1). To determine whether the observed toxic effects at the various oxygen concentrations might be modified by blood pH changes the effect of ammonium carbonate which causes acute alkalosis was studied. Ammonium carbonate injected into mice in 11% oxygen was 1.76 times more toxic than when they were in 21% oxygen, whereas ammonium chloride under the same conditions was 1.59 times more toxic.

Decreased atmospheric oxygen concentration and brain ammonia concentration. A uniform dose of ammonium chloride (1.77 mEq/kg—the LD_{50} in 7% oxygen) was injected while mice were in 21% oxygen and 2 minutes after they were placed in 11 and 7% oxygen. Twenty seconds following the injection the mice were dropped into liquid nitrogen (they were all alive at that point) and the brain ammonia concentrations were measured.

The base-line brain ammonia concentrations were higher in the mice placed in the lower O_2 concentrations (table 2). When these values were subtracted from the concentrations found following intravenous injection of the standard dose of ammonium chloride noted above, the rises in brain ammonia concentrations were essentially similar at all the oxygen concentrations studied (table 2).

TABLE 1. *Blood* pH of Mice at 21% Oxygen and After 2 Minutes in 11% and 7% Oxygen*

$\%O_2$	pH
21	7.51±0.025†
11	7.50±0.022†
7	7.51±0.030†

* Heart blood. † Mean of 10 observations ±S.E.

TABLE 2. *Brain Ammonia Concentrations in Mice Breathing 21%, 11% and 7% Oxygen Before and 20 Seconds After an I.V. Injection of Ammonium Chloride*

$\%O_2$	Av.* Brain Ammonia† Conc.		Diff., μg/gm
	μg/gm	After 195 μg/ NH_4 N‡, μg/gm	
21	4.68±0.53	14.61±0.54	9.93±0.71
11	6.76±0.55	19.42±1.02	12.67±1.28
7	7.39±0.40	17.34±0.58	9.96±0.70

* Mean of 9-12 mouse brains ±S.E. † LD_{50} dose in 7% O_2. ‡ Ammonia nitrogen.

This dose of ammonium chloride, which was the LD_{50} dose in 7% oxygen, raised the average brain ammonia nitrogen concentration as much as 12.67 mg/gm (table 2). This change was considerably smaller than that following the larger LD_{50} dose in 21% oxygen, in which the average brain ammonia nitrogen concentration rose 35.30 mg/gm.

DISCUSSION

Hypoxia increased ammonia toxicity in the mouse to a marked degree. The question arose as to whether this was a unique property of ammonia or was related purely to its analeptic properties. However, when dosages which had no discernible effect in 21% oxygen were administered at lower oxygen concentrations they initiated convulsions followed by death. Moreover, the increase in toxicity of another analeptic compound (Metrazol) tested under similar conditions was relatively slight when compared to that of ammonia. Therefore it appears that hypoxia did not merely increase the mortality of convulsions, but that it acted synergistically with ammonia in increasing the frequency of onset of severe seizures.

Another unique aspect of this relation between hypoxia and ammonia toxicity was the speed of its onset and the great rapidity with which it could be altered. The extreme toxicity of ammonia in 7% oxygen could be completely reversed in a matter of seconds by transferring the mice to 21% oxygen.

In addition, hypoxia appeared to be almost unique in its ability to increase the acute toxicity of ammonia in the mouse. An alteration in blood pH can also do this, by changing the concentration of the freely permeable form of ammonia, but not to as great a degree (2). The authors have attempted to potentiate ammonia toxicity in the mouse by other means with no success. These included pretreatment with drugs implicated in the genesis of hepatic coma (which has been attributed to ammonia toxicity (4)) such as morphine, barbiturates, methionine, iproniazid, acetazolamide and chlorothiazide; with metabolic inhibitors such as methionine

sulfoximine and thiosemicarbazide; with hepatotoxins such as carbon tetrachloride; and with other drugs which might elucidate the relationship between hypoxia and ammonia toxicity such as cyanide, insulin and pyruvate (unpublished observations).

Three main factors might have been responsible for the potentiation of ammonia toxicity by falling oxygen concentrations: *1*) enhanced permeability of the blood brain barrier due to: *a*) a change in blood pH which increases the freely permeable form of ammonia, or *b*) a direct effect of anoxia on the permeability of the barrier; *2*) decreased detoxication of ammonia due to the effect of anoxia on cerebral and liver enzymes; *3*) an effect of anoxia on the brain, directly potentiating ammonia toxicity.

In order to determine whether more ammonia entered or accumulated in the brain (*factors 1* and *2*), or whether a given concentration of ammonia became more toxic as hypoxia increased (*factor 3*) the brain ammonia concentration was measured before and after the administration of a standard dose of ammonia. *Factors 1* and *2* did not seem to be tenable since the changes in brain ammonia concentrations at the different oxygen concentrations were essentially similar. Although there was a difference in the total ammonia present in the brain following the standard dose of ammonium chloride, this was because of a rise in the base-line ammonia concentrations due to anoxia, an effect which had been noted previously by Richter and Dawson (5). However, the change in total brain ammonia concentration after this dose, which was the LD_{50} in 7% oxygen, was never more than 50% of that following an injection of the larger LD_{50} dose in 21% oxygen. It appeared therefore, that anoxia directly potentiated the toxic effect of ammonia.

Several *in vivo* and *in vitro* effects of hypoxia and ammonia on brain metabolism have been previously recorded. In studying the *in vivo* effect of hypoxia on the dog, Gurdjian, Stone and Webster (6) found no change in any of the parameters measured until the oxygen concentration in the respired air was reduced to between 13 and 11%. At this stage the cerebral lactic acid concentration began to rise, increasing as the oxygen concentration diminished. There were no changes in phosphocreatine or inorganic phosphorus until 7% oxygen was reached. (It might be noted that ammonia toxicity in our mice began to increase at 13% O_2 and that hypoxia itself did not become lethal until the O_2 concentration in the respired air fell below 7%.)

Clark and Eiseman's (7) *in vivo* study on the effect of ammonia infusion in dogs revealed a marked accumulation of cerebral pyruvate. One explanation for this occurrence might be found in the *in vitro* studies of Muntz and Hurwitz (8) who described a direct stimulation of cerebral glycolytic pathways by ammonia. In addition, Weil-Malherbe (9) has shown a pronounced stimulation of glycolysis by ammonia in guinea pig brain slices. The concentrations in his studies were comparable to those found in the brains of mice (2.8×10^{-3} M) following an injection of the LD_{50} dose of ammonia (2) and in dog brain (2.8–4.3×10^{-3} M) at death due to prolonged ammonia infusions (10).

Bessman and Bessman (11) proposed an hypothesis of cerebral ammonia toxicity based on a depression of respiration due to a decrease in alpha-ketoglutarate. Although Clark and Eiseman (7) found a fall in brain alpha-ketoglutarate in their severely toxic dogs on ammonia infusion, Weil-Malherbe (9) in the above mentioned *in vitro* studies has shown a mild stimulation of respiration by concentrations of ammonia that would have been lethal *in vivo*. Concentrations as high as 0.1 M are needed to depress respiration *in vitro* (12).

Therefore both hypoxia and ammonia appear to have effects on the accumulation of lactic or pyruvic acid either through a stimulation of their production or an inhibition of their utilization, thus providing some biochemical evidence supporting the synergistic effect of hypoxia on ammonia toxicity.

Finally, it should be mentioned that the clinical syndrome of hepatic coma has been related to ammonia toxicity (4), that hypoxemia is a frequent concomitant of severe liver disease (13–15), and that the neurological syndrome that most closely resembles hepatic coma is chronic pulmonary insufficiency (16).

ADDENDUM

Two papers relating to the above results have recently come to the authors' attention. In the course of studies on water pollution the effect of dissolved oxygen concentration on the toxicity of unionized ammonia to minnows (17) and rainbow trout (18) was determined. These results in fish and our results in mice are essentially similar.

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Watanabe, A., and T. Yuasa: EFFECTS OF ACETYLCHOLINE ON THE LETHAL DOSE OF TOXIC COMPOUNDS IN RATS. J. Physiol. Soc. Japan, Vol. 24, pp. 521-524, 1962. Department of Physiology, Nihon University School of Dentistry.

Acetylcholine (ACH) was first identified by Dale /1/ as a chemical transmitter in the terminal of parasympathetic nerve, and it was later confirmed that it played the same role in the ganglion of sympathetic nerve and the terminal of motor nerve. ACH or its enzyme has also been discovered in the spinal marrow, brain stem, cerebral cortex, etc. as well as in nerves and muscles. Based on these facts, Nachmansohn /3/ reached an assumption that ACH not only takes part in synaptic transmission, but also plays an important role in the mechanism of nervous and muscular stimulation and the transmission mechanism of stimuli. The triggering activity of ACH in the central nervous system has been studied extensively. The concentration at which ACH triggers spasms from the cortex or brain stem is extremely high (over 2 mol). Yamada /5/ inserted a fine glass tube in skeletal muscle fiber and injected solutions of various toxic compounds through the tube. In his experiment, ACH produced no contraction of the muscle, and he assumed that ACH played no part in generation or transmission of stimuli. He also observed that all but isobaric KCl caused contracture at the site of injection, but not only an ACH solution failed to produce contracture but its presence in the contracture-causing substances (including H_2O) in a small quantity suppressed their stimulatory activity. Thus, he assumed that the physiological significance of AcH, widely distributed in the excitation system, resides in its protective action toward the system. Kikuchi /2/ ascribed such protective action to its capacity to prevent protein coagulation, substantiating this view with an experimental fact that the thermal coagulation of egg white was inhibited by the presence of a small amount of ACH. If this assumption is valid, ACH should be able to increase the lethal dose of toxic compounds in laboratory animals, and protect the animals from death due to poisoning. The authors conducted the following experiment in order to clarify this problem.

Rats weighing approximately 100 g were fixed by legs, and the solutions of test compounds were injected intraperitoneally at 0.5 cc at 5 - 10 min intervals in order to determine the lethal dose. The lethal dose of a NaCl solution was also determined by the same procedure. ACH at 10^{-3} - 10^{-9} was added to the convulsants, the lethal dose of which was already known, and the lethal doses before and after the addition of ACH were compared.

A. LETHAL DOSE OF NaCl (15%)

The data in Table 1 were obtained when 1 cc of 15% NaCl solution was injected intraperitoneally at 5-min intervals until death occurred. The animals used were rats weighing an average of 109 g, and the total amount of solution that caused death of the animals was 11.3 cc. The animals died in 55 min. The lethal dose was calculated as 15.54 g. When 1 cc of NaCl solution containing ACH at 5 x 10^{-5}% (15%) was injected at 5 min intervals, the animals, weighing an average of 112 g, died at a total quantity of 15.3 cc in 1 hr. 17 min, the lethal dose being 20.65 g/kg.

TABLE 1

A NaCl 15%, 1 cc 5′ 每注射

例数 a	致死时间 b	注入液量 c	体重 d	致死量 e g/kg
1	47′	10cc	113g	13.27
2	58′	12cc	99g	18.18
3	60′	12cc	115g	17.48
平均 f	55′	11.3cc	109g	15.51

B NaCl 15%, Ach 5×10^{-5}, 1 cc 5′ 每

例数 a	致死时间 b	注入液量 c	体重 d	致死量 e g/kg
1	59′	12cc	117g	15.37
2	81′	16cc	110g	21.82
3	91′	18cc	109g	24.77
平均 f	77′	15.3cc	112g	20.65

Keys: A, 15% NaCl, 1 cc, at 5-min intervals; B, 15% NaCl with ACH at 5 X 10^{-5}, 1 cc, at 5-min intervals; a, case No.; b, time up to death; c, amount of solution injected; d, weight; e, lethal dose; f, average

B. LETHAL DOSE OF SODIUM GLUTAMATE

The data shown in Table 2 were obtained when sodium glutamate (2 mol) was injected intraperitoneally. As shown in Table 2, the time up to death was 54.6 min, and the total amount of injection up to death, 5.67 cc. Since the average body weight of the animals was 112.6 g, the lethal dose should be 16.46 g/kg. When 2 mol of sodium glutamate with ACH at 5 x 10^{-7} was administered, the animals died at 8.3 cc of total amount of injection in 1 hr 16 min. The lethal dose in this case was 25.45 g/kg. The ACH mixed in the solution increased the lethal dose to a significant degree, to 1.5 times that of the solution without ACH.

TABLE 2

A [illegible]

a	b	c	d	e
1	[illegible]	[illegible]	107g	17.172
2	[illegible]	[illegible]	110g	15.85[illegible]
3	[illegible]	[illegible]	11[illegible]g	15.062
平均 f	[illegible]	[illegible]	112[illegible]g	16.46

B Glutamin 每 No 2 [illegible] Ach

a	b	c	d	e
1	[illegible]	[illegible]	1[illegible]	21.[illegible]
2	[illegible]	[illegible]	1[illegible]g	25.28
3	[illegible]	[illegible]	1[illegible]g	22.2[illegible]
平均 f	[illegible]	[illegible]	110.6g	25.4[illegible]

Keys: A, sodium glutamate, 2 mol; B, sodium glutamate, 2 mol, with ACH at 5 X 10^{-7}; a, case No.; b, time to death; c, amount of solution injected; d, body weight; e, lethal dose; f, average.

C. LETHAL DOSE OF H_2O AND AMMONIA

The data shown in Table 3 were obtained when H_2O was injected to

rats weighing an average of 110.67 g. The animals died in 2 hr 59 min at an average total dose of 36.33 cc, the lethal dose being 301.22 g/kg. With ACH added at 5 X 10^{-6}, death occurred in 3 hr 55 min at 47.67 cc of total amount of injection. The animals in this experiment weighed an average of 104 g. The lethal dose was 458.3 g/kg. When ACH was added at 5 X 10^{-7}, animals weighing an average of 95 g died in 4 hr 19 min at 52.33 cc of total injected solution. The lethal dose in this case was 550.9 g/kg.

TABLE 3

A 0.5 cc 5′ 每注入

例 a	致死時間 b	注入液量 c	体重 d	致死量 e g/kg
1	214′	43cc	89g	483.14
2	207′	42cc	123g	341.46
3	118′	24cc	120g	200.00
平均 f	180′	36.33cc	110.67g	301.22

B H_2O 5 × 10^{-6} Ach, 0.5 cc 5′ 每注入

例 a	致死時間 b	注入液量 c	体重 d	致死量 e g/kg
1	200′	41cc	93g	440.85
2	287′	58cc	94g	617.02
3	219′	44cc	125g	352.00
平均 f	235′	47.67cc	104g	458.3

C H_2O 5 × 10^{-7} Ach, 0.5 cc 5′ 每注入

例 a	致死時間 b	注入液量 c	体重 d	致死量 e g/kg
1	227′	46cc	103g	446.60
2	279′	56cc	96g	583.33
3	272′	55cc	86g	639.53
平均 f	259′	52.33cc	95g	550.9

TABLE 4

A Ammonia 5%, 0.5 cc 5′ 每注入

a	b	c	d	e g/kg
1	16′	2cc	105g	0.953
2	20′	2cc	113g	0.884
3	20′	3cc	120g	1.25
4	20′	2cc	118g	0.846
平均 f	22.25′	2.25cc	114g	0.989

B Ammonia 5%, 5 × 10^{-6} Ach

a	b	c	d	e g/kg
1	40′	4cc	119g	1.681
2	63′	6.5cc	106g	3.066
3	52′	5.5cc	110g	2.5
平均 f	51.67′	5.33cc	111.67g	2.388

C Ammonia 5%, 5 × 10 Ach

例 a	致死時間 b	注入液量 c	体重 d	致死量 e g/kg
1	40′	4cc	123g	1.626
2	31′	3.5cc	100g	1.750
3	49′	5cc	108g	2.314
平均 f	40′	4.17cc	110.33g	1.890

Keys: A, 0.5 cc at 5-min intervals;
B, H_2O + ACH at 5 x 10^{-6}
C, H_2O + ACH at 5 X 10^{-7},
0.5 cc at 5-min intervals

A, 5% ammonia, 0.5 cc at 5-min intervals;
B, 5% ammonia + ACH at 5 x 10^{-6};
C, 5% ammonia + ACH at 5 x 10^{-7}.

a, case No.; b, time up to death; c, amount of solution injected; d, body weight; e, lethal dose; f, average.

Table 4 shows the toxicity of 5% ammonia. When 0.5 cc of the solution was administered to rats weighing an average of 114 g at 5-min intervals, the animals died at 2.25 cc of total injection in 21.25 min. The lethal dose was 0.989 g/kg. In the presence of ACH at 5 X 10^{-6}, it required 5.33 cc of the solution and 52.07 min for rats weighing an average of 111.67 g to die, the lethal dose being 2.388 g/kg, 2.4 times larger than that without ACH. In the presence of ACH at 5 X 10^{-7}, the lethal dose was 1.890 g/kg, 1.9 times larger than that of the solution without ACH.

D. LETHAL DOSE OF PICROTOXIN

Table 5 shows the results of an experiment in which 0.5 cc of picrotoxin solution (0.05%) was injected at 5 min intervals. The total amount of solution injected up to death was 2.67 cc. The rats, weighing an average of 111.3 g, died in 22.07 min, and the lethal dose was 0.01048 g/kg. When ACH was added at 5 x 10^{-8}, the rats, weighing an average of 101.66 g, died with 3.83 cc of the solution in 37.33 min. The lethal dose was 0.0188 g/kg, 1.8 times that of the solution without ACH.

TABLE 5

A Picrotoxin 0.05%, 0.5 cc 5′ 间

例 a	致死时间 b	注入量 c	体重 d	致死量 e kg/g
1	18′	2cc	107g	0.0934
2	29′	3cc	120g	0.125
3	18′	2cc	107g	0.0934
平均 f	22.07′	2.67cc	111.3g	0.01048

B Picrotoxin 0.05%, 5 × 10⁻⁸ Ach

例 a	致死时间 b	注入量 c	体重 d	致死量 e g/kg
1	36	3.5cc	106g	0.01650
2	37′	3.5cc	95g	0.01842
3	11′	4cc	1[illegible]4g	0.02162
平均 f	37.33′	3.83cc	101.66g	0.0188

Keys: A, 0.05% picrotoxin, 0.5 cc, at 5-min intervals.
B, 0.05% picrotoxin + 5 X 10^{-8} ACH
a, case no.; b, time to death; c, amount of injection; d, body weight; e, lethal dose; f, average

E. LETHAL DOSE OF NICOTINE

A 0.5% nicotine solution was injected in the same manner. Table 6 shows that the animals, weighing an average of 101.5 g, died when 1.5 cc of the solution was injected. The time to death was 28 min, and the average lethal dose, 0.0739 g/kg. When ACH was added at 5 X 10^{-6}, it took 2 cc of the solution and 36.6 min for the rats, weighing an average of 127.66 g, to die. The lethal dose was 0.0783 g/kg, approximately 1.1 time that of the solution without ACH.

TABLE 6

A Nicotin 0.5%, 0.5 cc 5′

例 a	致死时间 b	注入量 c	体重 d	致死量 e g/kg
1	23′	1.5cc	105g	0.0714
2	33′	1.5cc	[illegible]8g	0.0764
平均 f	28′	1.5cc	101.5g	0.0739

B Nicotin 0.5%, 5 × 10⁻⁶ Ach.

例 a	致死时间 b	注入量 c	体重 d	致死量 e g/kg
1	40′	2cc	128g	0.0781
2	37′	2cc	126g	0.0793
3	33′	2cc	12[illegible]g	0.0775
平均 f	36.6′	2cc	127.66g	0.0783

Keys: A, 0.5% nicotine, 0.5 cc, at 5-min intervals
B, 0.5% nicotine + ACH at 5 x 10^{-6}
a, case number; b, time to death; c, amount of injection; d, body weight; e, lethal dose; f, average

F. LETHAL DOSE OF CARDIAZOL

The data in Table 7 were obtained when a 5% cardiazol solution was injected to rats weighing an average of 90.75 g. Death occurred in 13 min at 0.75 cc of total amount injected, the lethal dose being 0.413 g/kg. When ACH was added at 5 X 10^{-4}, the total amount of solution causing

death was 2.25 cc. The rats weighing an average of 75.5 g died in 41 min. The lethal dose was considerably larger than that without ACH, 1.490 g/kg. The lethal dose of the ACH-containing solution is 3.6 times larger than that of the solution without ACH.

TABLE 7

A Cardiazol 5%, 0.5 cc 5′ 毎

数a 例	致死時b間	注入液c量	体d重	致死量 e g/kg
1	19′	1cc	111g	0.45
2	7′	0.5cc	70.5g	0.37
平f均	13′	0.75cc	90.75g	0.413

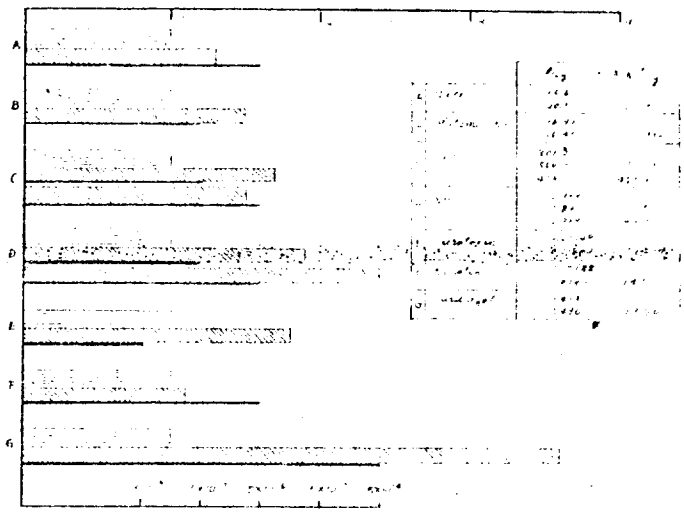
B Cardiazol 5% 5×10^{-4} Ach

数a 例	致死時b間	注入液c量	体d重	致死量 e g/kg
1	31′	2cc	77g	1.3
2	51′	2.5cc	74g	1.69
平f均	41′	2.25cc	75.5g	1.490

Keys: A. 5% Cardiazol, 0.5 cc, at 5-min intervals
B. 5% Cardiazol + ACH at 5×10^{-4}
a, case number; b, time to death; c, amount of injection; d, body weight; e, lethal dose; f, average.

DISCUSSION AND CONCLUSION

The experimental results are summarized in the following chart and Table. ACH increased the lethal dose by 1.3 times in Experiment A



(15% NaCl solution), 1.5 times in Experiment B (sodium glutamate, 2 mol), 1.9 - 2.4 times in Experiment C (H_2O), 1.8 times in Experiment D (5% ammonia), 1.1 times in Experiment F (0.5% nicotine) and 3.6 times in Experiment G (cardiazol, 5%). Thus, ACH at low concentrations, ranging from 10^{-4} to 10^{-7}, elevated the lethal dose to varying degrees. In the calculation of lethal dose, the time to death was not taken into consideration for the following reason. The animals died within a range of 13 to 58 min in all experiments except for those which received H_2O but survived for 179 min. It was deemed unlikely that recovering process took place during such short periods of time. In any event, the experiment confirmed that the presence of small amounts of ACH considerably increased

the lethal dose of the toxic compounds other than nicotine.

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DIAMMONIUM CITRATE AND DIAMMONIUM PHOSPHATE AS SOURCES OF DIETARY NITROGEN FOR GROWING-FINISHING SWINE[1]

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MANY scientists are concerned about the possibility of a protein shortage in the near future. Since the pig will be placed in direct competition with man for many of the proteins presently used in feeds, it has become increasingly important to determine if other sources of nitrogen can serve as a replacement for some of the non-essential amino acid nitrogen in natural proteins.

The exact relationship between "non-essential" and "essential" amino acid nitrogen of a given diet is not fully understood. It is assumed that if the diet does not supply an adequate quantity of nitrogen for the "non-essential" amino acids, they must be synthesized from nitrogen supplied by the "essential" amino acids that are present. Thus, provisions in the diet for adequate quantities of non-protein nitrogen (NPN) for "non-essential" amino acid synthesis should allow the use of minimal quantities of the "essential" amino acids in diet formulation.

Most, if not all, of the research concerning NPN utilization by swine has involved urea. While Liu *et al.* (1955) showed that limited amounts of ^{15}N-labeled urea nitrogen were incorporated into the proteins of various body tissues, growth studies with swine fed urea have not been promising (Hanson and Ferrin, 1955; Hays *et al.*, 1957; Kornegay *et al.*, 1964, 1965).

Since urea is a natural excretory product, some other nitrogen source may be more effective in supplying a portion of the nitrogen requirement of swine. Two experiments were therefore conducted in which a portion of the dietary nitrogen supplied by soybean meal in diets for growing swine was replaced by an equimolar mixture of diammonium citrate (DAC) and diammonium phosphate (DAP).

Methods and Materials

In Experiment 1, 60 Yorkshire x Hampshire pigs averaging 27.3 kg were randomly assigned by weight within sex and littermate groups to five replicated dietary treatments. Each replication consisted of three gilts and three barrows. Treatments were randomly assigned to each replication. The study lasted 81 days and was conducted during the winter months.

All pigs were housed in wooden sheds open to concrete pens. The pens were cleaned daily and the sheds were bedded with straw. The pigs were fed from self feeders and given free access to water. Supplemental light was provided by overhead yard lights during the night hours.

The composition of the experimental diets is shown in table 1. All diets contained approximately 16% crude protein (N x 6.25) until the pigs weighed approximately 50.5 kilograms. At this time, all diets were adjusted to 14% crude protein and fed for the remainder of the experimental period. Diet A was a standard corn-soybean meal diet used as a positive control. In diets B, C and D, respectively, 5, 10 and 20% of the dietary nitrogen was replaced by an equimolar mixture of DAC and DAP. Cornstarch was added to make the diets isocaloric. Diet E was the same as Diet D except DL-methionine, L-lysine and DL-trytophan were added to approximate the level of these amino acids found in Diet A.

The pigs were weighed and feed intake was determined at 2-week intervals throughout the experimental period. Approximately 6 ml of blood was withdrawn with heparinized Becton-Dickinson vacutainers from the brachial viens of two gilts and two barrows selected at random from each treatment within each replication on day 58 of the experimental period. The samples were frozen and analyzed at a later date. Blood nitrogen (BN) was determined by the micro-Kjeldahl method as outlined in A.O.A.C. (1960) and blood urea nitrogen (BUN) was determined by the method described by Conway (1957).

All pigs were probed for backfat at approximately 95.5 kg body weight. The barrows were slaughtered and carcass length and ham and loin weights were obtained. Since

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TABLE 1. COMPOSITION OF EXPERIMENTAL DIETS

Treatments	Experiment 1					Experiment 2		
	A	B	C	D	E	F	G	H
NPN, %	0	5	10	20	20+AA[a]	0	20	20+AA[a]
Crude protein (N x 6.25), %	16.00	16.00	16.00	16.00	16.30	12.80	16.00	16.30
Calcium, %	0.65	0.65	0.65	0.65	0.65	0.65	0.65	0.65
Phosphorus, %	0.65	0.65	0.65	0.65	0.65	0.65	0.65	0.65
Ingredients								
Ground yellow corn	77.74	77.74	77.74	77.74	77.74	77.74	77.74	77.74
Soybean meal (50%)	18.40	16.80	15.20	12.00	12.00	12.00	12.00	12.00
Diammonium phosphate	....	0.39	0.77	1.54	1.54	....	1.54	1.54
Diammonium citrate	....	0.52	1.04	2.07	2.07	....	2.07	2.07
Dicalcium phosphate (18.5% P)	1.82	1.45	1.05	0.30	0.30	2.04	0.30	0.30
Ground limestone	0.44	0.68	0.95	1.44	1.44	0.34	1.44	1.44
Trace mineral[b]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Salt (I)	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin antibiotic premix[c]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
DL-methionine (100%)	....	....	....	....	0.05	....	....	0.05
L-lysine (20%)	....	....	....	....	0.99	....	....	0.99
DL-tryptophan (100%)	....	....	....	....	0.05	....	....	0.05
Cornstarch	....	0.82	1.65	3.31	2.22	6.28	3.31	2.22

[a] 0.05% methionine, 0.02% lysine; 0.05% tryptophan.
[b] Composition (%): Mn, 10.0; Fe; 10.0; Cu, 1.0; Co, 0.10; I, 0.30; Zn, 10.0 Calcium Carbonate Company, Quincy, Ill.
[c] Composition per kg diet: vit. A, 3168 IU; vit. D_3, 475 IU; riboflavin, 2.11 mg; pantothenic acid, 7.90 mg; niacin, 21.1 mg; choline chloride, 132 mg; vit. B_{12}, 13 µg; chlortetracycline, 44 mg.

the DAP also served as a source of phosphorus, the left hind foot of each pig was collected at the time of slaughter to determine bone length (BL), width (BW), thickness (BT), and breaking strength (BKS) of the fourth metatarsal. The length of the diaphysis of the fourth metatarsal was measured to determine BL. The smaller diameter of the wide and narrow side of the diaphysis, respectively, was measured to determnie BW and BT. The BKS of the bone was determined according to the method described by Libal *et al.* (1969). Percent ash of the moisture-free, fat-free fourth metatarsal bone was determined according to the methods outlined in A.O.A.C. (1960).

Experiment 2 was conducted to determine the comparative nitrogen retention and blood characteristics of pigs fed NPN. Two Yorkshire x Hampshire barrows selected from each of three litters were utilized. Based upon average daily feed consumption within each pair of littermates, the pigs were divided into two groups (high and low daily feed intake). The amount of feed given each pig was based upon the lowest individual feed consumption within each group adjusted to metabolic body size ($W^{0.75}_{kg}$ Kleiber, 1947). The experiment was divided into three periods. Each period consisted of 8 days, 3 days for the pigs to adjust to their respective diets and 5 days for the collection period. Three experimental diets were randomly assigned and rotated within each group in a 3 x 3 latin square design.

The composition of the diets is shown in table 1. Diet F (low protein) was considered a negative control containing 12.8% crude protein (N x 6.25) from corn and soybean meal. Diets G and H were identical to Diets D and E of Experiment 1.

The pigs were kept in adjustable circular metabolism crates and the temperature of the experimental unit was maintained at approximateliy 24 centigrade. Collection procedures were similar to those described by Luce *et al.* (1966).

Nitrogen determination of the feed, feces and urine was in accordance with the Kjeldahl method as outlined in A.O.A.C. (1960). Calcium level of the urine was determined colorimetrically with a Beckman spectrophotometer by the Ferro-Ham Method (1957). Calcium level of the feed and feces was determined by titration following the method described by Hunt (1963). Phosphorus levels were determined colorimetrically as outlined by Sumner (1944).

Approximately 6 ml of blood was withdrawn from each pig on the last day of each experimental period. The method of collecting the blood and the analyses for blood urea nitrogen and percent nitrogen were as outlined in Experiment 1.

The data were analyzed statistically by

analysis of variance methods as outlined by Steel and Torrie (1960).

Results

A summary of the gain and feed conversion data for Experiment 1 is shown in table 2. Average daily gain decreased as increments of NPN were doubled. The addition of 0.20% L-lysine, 0.05% DL-methionine and 0.05% DL-tryptophan to the 20% NPN did increase average daily gain and feed intake in amounts that approached significance when compared to the 20% NPN diet without amino acids. Gains for the pigs fed Diets A, B and C were not significantly different; however, they were significantly ($P<.005$) greater than for the pigs fed Diets D and E. As might be expected, barrows gained significantly faster ($P<.025$) than gilts.

Gain per unit of feed was greater ($P<.005$) for the pigs fed Diets A, B and C compared to Diets D and E. There was no effect of added amino acids (Diet E) upon gain per unit of feed compared to Diet D (20% NPN diet).

A summary of the blood, bone and carcass data for Experiment 1 is also shown in table 2. While the percent total nitrogen in blood was unaffected by dietary treatment, there was a significant ($P<.05$) treatment effect on BUN levels. BUN levels decreased as the increments of NPN were doubled in the diet. The values for the pigs fed Diets A, B and C were significantly ($P<.01$) higher than for pigs fed Diets D and E. The addition of the amino acids to the 20% NPN diet resulted in a further decrease in BUN. Barrows had significantly ($P<.025$) higher BUN levels than the gilts.

A trend toward higher BKS for the pigs fed DAC and DAP was noted. BKS values were greater ($P<.10$) for the pigs fed Diets B and E, respectively, compared to the pigs fed Diets A and D. Bone Length, width,

TABLE 2. GAIN, FEED CONVERSION, AND CERTAIN BLOOD AND BONE CHARACTERISTICS OF GROWING-FINISHING SWINE FED NPN[a]

	Experiment 1				
Treatments	A	B	C	D	E
NPN, %	0	5	10	20	20+AA[b]
Criterion					
Avg daily gain, kg					
1–35 days	0.78	0.76	0.73	0.65	0.73
36–81 days	0.90	0.88	0.87	0.78	0.80
1–81 days	0.85	0.83	0.81	0.72	0.77[c]
Barrows	0.88	0.86	0.83	0.76	0.81[d]
Gilts	0.81	0.80	0.78	0.68	0.73[d]
Avg daily feed intake, kg	2.92	2.77	2.73	2.57	2.70[e]
Gain/feed	0.29	0.30	0.30	0.28	0.28[f]
Blood urea nitrogen (mg/100 ml)	19.57	17.58	16.35	15.73	13.73[g]
Barrows	20.30	20.60	16.57	16.89	15.07[d]
Gilts	18.84	14.55	16.13	14.58	12.40[d]
Blood nitrogen, %	2.93	3.02	3.06	[illegible]	3.04
Bone length, cm	5.68	5.66	5.66	5.79	5.76
Bone width, cm	1.53	1.54	1.56	1.53	1.54
Bone thickness, cm	1.24	1.26	1.29	1.25	1.29
Bone breaking strength, kg	161.30	189.10	174.70	[illegible]	194.80[h]
Bone ash, %	58.43	[illegible]	[illegible]	[illegible]	[illegible]
Backfat thickness, cm	3.80	3.40	[illegible]	[illegible]	[illegible]
Barrows	4.10	3.50	3.60	[illegible]	[illegible]
Gilts	3.40	[illegible]	[illegible]	[illegible]	[illegible]
Carcass length, cm					
Barrows	76.40	[illegible]	75.50	[illegible]	[illegible]
Ham and loin, %					
Barrows	36.40	37.00	[illegible]	[illegible]	[illegible]

[a] Two replicates, 6 pigs/pen; initial wt. [illegible] kg; final wt. [illegible] kilograms.
[b] Supplemented with 0.05% methionine, [illegible] lysine, 0.05% tryptophan.
[c] Treatment effect sig. $P<.05$. Difference between A, B, C [illegible] D, E sig. $P<.005$.
[d] Sex effect sig. $P<.025$.
[e] Treatment effect sig. $P<.10$. Difference between A, B, C [illegible] D, E sig. $P<.025$.
[f] Treatment effect sig. $P<.05$. Difference between A, B, C [illegible] D, E sig. $P<.05$. [illegible]
[g] Treatment effect sig. $P<.05$. Difference between A, B, C [illegible] D vs. E sig. $P<.10$.
[h] Difference between A vs. B sig. $P<.1$. Difference between D vs. E sig. $P<.10$.
[i] Difference between A vs. B sig. $P<.1$.

TABLE 3. NITROGEN, CALCIUM AND PHOSPHORUS RETENTION OF GROWING SWINE FED NPN

	Experiment 2								
Treatment[a]	F			G			H		
NPN, %	0			20			20% + AA[b]		
Crude protein (N x 6.25), %	12.80			16.00			16.30		
Calcium, %	0.65			0.65			0.65		
Phosphorus, %	0.65			0.65			0.65		
Criterion	N	Ca	P	N	Ca	P	N	Ca	P
Intake, g	24.06	8.52	7.84	29.94	7.92	7.17	33.70	8.83	7.19
Fecal, g	5.42	3.50	3.87	5.80	3.87	3.89	6.88	4.63	4.61
Urinary, g	9.87	0.19	1.22	16.02	0.27	1.08	16.23	0.36	1.24
Retained, g	8.77	4.83	2.75	8.12	3.78	2.20	10.59	3.84	1.34
Intake retained, %	36.45	56.63	35.04	27.10	47.70	30.68	31.42[c]	43.49[d]	18.56[e]
G retained/kg body weight	0.28	0.16	0.09	0.26	0.12	0.07	0.29	0.11[d]	0.04[f]

[a] Each experimental diet was rotated within 2 groups of three pigs each.
[b] Supplemented with 0.05% methionine; 0.20% lysine; 0.05% tryptophan.
[c] Treatment effect sig. P<.05; Difference between F vs. G, H sig. P<.025.
[d] Treatment effect sig. P<.10; Difference between F vs. G, H sig. P<.05.
[e] Treatment effect sig. P<.10; Difference between F vs. G, H and G vs. H sig. P<.10.
[f] Treatment effect sig. P<.05; Difference between F vs. G, H sig. P<.05; Difference between G vs. H sig. P<.10.

thickness, and percent ash were not affected by dietary treatment.

Carcass length and percent ham and loin were unaffected by the level of NPN in the diet. There was a trend toward reduced backfat in pigs fed NPN. Backfat thickness was greater (P<.025) for barrows than for gilts.

A summary of the nitrogen, phosphorus and calcium retention for Experiment 2 is shown in table 3. Pigs fed Diet F retained a greater (P<.025) percent of ingested nitrogen than pigs fed Diets G and H; however, nitrogen retention (expressed as grams retained per kg body weight) was not affected by dietary treatment. Percent ingested phosphorus retained was (P<.10) greater for the pigs fed Diet F than for those receiving Diets G and H. In addition, pigs fed Diet G retained more phosphorus (P<.10) than the pigs fed Diet H. A decrease in percent calcium (P<.05) retained was observed for pigs fed Diets G and H compared to those fed Diet F.

A summary of the blood data for Experiment 2 is shown in table 4. BUN levels were significantly (P<.005) lower for the pigs fed Diet F than for the pigs fed Diets G and H. Percent nitrogen of the blood was unaffected by dietary treatment.

TABLE 4. CERTAIN BLOOD CHARACTERISTICS OF GROWING SWINE FED NPN

	Experiment 2		
Treatment[a]	F	G	H
NPN, %	0	20	20% + AA[b]
Crude protein, %	12.80	16.00	16.30
Criterion			
Blood urea nitrogen (mg/100 ml)	16.28	23.18	21.25
Blood nitrogen, %	2.73	2.83	2.74

[a] Each experimental diet was rotated within 2 groups of three pigs each.
[b] Supplemented with 0.45% methionine; 0.20% lysine; 0.05% tryptophan.
[c] Overall treatment effect sig. P<.01; Difference between F vs. G, H sig. P<.005.

Discussion

The replacement of 0, 5, 10 and 20% of the dietary nitrogen by an equimolar mixture of DAC and DAP in a diet for growing swine resulted in decreased average daily gain and feed intake. The greatest decrease was noted when 20% NPN was added. This depression was similar throughout the experimental period. Similar results have been reported by Hays *et al.* (1957) when urea was used as the source of NPN, but Kornegay *et al.* (1965) found that 2.5% urea included to replace an equivalent amount of soybean meal nitrogen in a 17% crude protein corn-soybean meal diet did not significantly affect average daily gain and feed intake. The gain depression noted with the 20% NPN diet was not as great when 0.20% L-lysine, 0.05% DL-methionine and 0.05% DL-tryptophan were added.

Blood urea nitrogen decreased as the increments of NPN were doubled in the diets. A further decrease was noted when amino acids were added to the 20% NPN diet (table 2). This is in direct conflict with the results found in Experiment 2 (table 4). Hays *et al.* (1957) reported that serum blood urea nitrogen values were higher for pigs fed urea.

However, Kornegay *et al.* (1965) reported that serum blood urea was higher for pigs fed urea during the initial part of the growing-finishing period, but urea nitrogen values for these pigs were not different from those pigs fed no urea toward the end of the finishing period. Percent nitrogen of whole blood was unaffected by dietary treatment.

The tendency for pigs fed the control diet to have thicker backfat and a lower percent of ham and loin than the pigs fed NPN may be due to the reduced feed intake for the pigs fed NPN. Crampton, Ashton and Lloyd (1954) reported that restriction of feed intake during the finishing period reduced backfat thickness and increased percent ham and loin.

Nitrogen retention, expressed as grams of nitrogen retained per kg body weight, tended to be higher for the pigs fed Diet F (low protein) and Diet H (low protein plus NPN and amino acids) compared with Diet G. Rose *et al.* (1949) demonstrated that rats can utilize some of the nitrogen furnished by NPN if adequate amounts of the essential amino acids are present. The addition of NPN to Diet F (low protein) resulted in a 62% increase in urinary nitrogen.

Figure 1 shows nitrogen, phosphorus, and calcium retention expressed in grams retained per kg body weight. Calcium and phosphorus retention followed the same pattern noted for the nitrogen except when lysine, methionine, and tryptophan were added to the low protein plus NPN diet. The addition of the amino acids (Diet H) resulted in an apparent increase in nitrogen retained but less calcium and phosphorus were retained when compared with Diet G. The latter observation is unexplained at this time but was a consistent finding for every animal exposed to these dietary treatments.

BUN levels were significantly lower in pigs fed Diet F (low protein) compared with pigs fed Diet G (low protein plus NPN). This conflicts with the results reported in Experiment 1, but in Experiment 2 the total nitrogen level was greater in the NPN supplemented diets; whereas, in Experiment 1 all diets were isonitrogenous. Also the pigs were heavier when blood samples were collected in Experiment 1 and had a longer time to

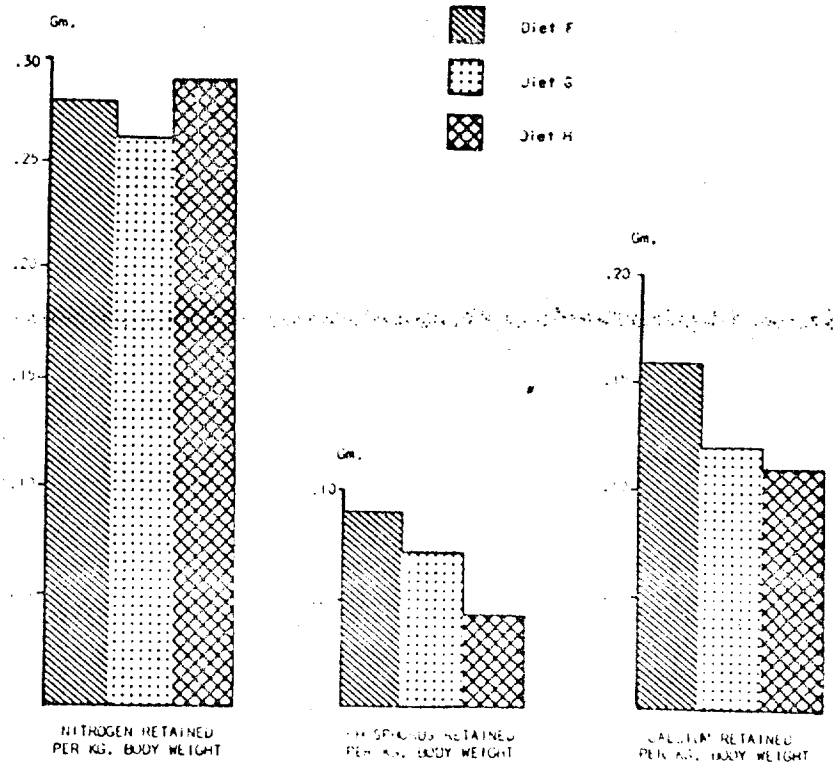

Figure 1. Nitrogen, Phosphorus, and Calcium Retention for Growing Swine fed NPN. (Low Protein, Diet F; Low Protein plus NPN, Diet G; Low Protein plus NPN and Amino Acids, Diet H).

adjust to the NPN diets. As in Experiment 1, total nitrogen of whole blood was not significantly affected by dietary treatment.

Summary

Two experiments were conducted with 66 pigs to determine the effect of replacing 0, 5, 10 or 20% of the protein in a diet for growing swine with non-protein nitrogen (NPN) from an equimolar mixture of diammonium citrate and diammonium phosphate. Criteria of response were rate of gain, gain/feed, nitrogen retention and certain blood and carcass characteristics. Since the DAP was also considered as a source of phosphorus, its effects on certain bone characteristics and the retention of calcium and phosphorus were determined.

Average daily gain, feed intake and blood urea nitrogen values decreased as the increments of supplemented NPN were doubled in the diets. However, the decrease in average daily gain was significant only for the pigs fed the 20% NPN diet compared to the control.

Results from the metabolism study showed that the addition of NPN to the diet reduced nitrogen retained per unit of body weight. This effect was reversed by the addition of 0.05% DL-methionine, 0.20% L-lysine and 0.05% DL-tryptophan to the diet. An unexplained decline in phosphorus retention was noted when the supplemental amino acids were employed.

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PN 10001

Activation of mammalian glycerate dehydrogenase by inorganic salts

The activation or inhibition of various dehydrogenases by inorganic salts has been reported by several groups of investigators. JOYCE AND GRISOLIA[1] observed reactivation of malate dehydrogenase (EC 1.1.1.37) by Tris Cl^- and by ammonium sulfate. BURTON AND WILSON[2] studied the effect of NaCl or fumarate on this enzyme. They concluded that the observed increase in the equilibrium constant was due to the effect of increased ionic strength on the activity coefficients of the reactants. THEORELL *et al.*[3] have demonstrated either activation or inhibition of liver alcohol dehydrogenase (EC 1.1.1.1) by Cl^-, depending on the concentrations of substrate and coenzyme used. The activity of glucose-6-phosphate dehydrogenase (EC 1.1.1.29) was stimulated as much as 6-fold by low concentrations of various anions, while inhibition resulted at higher concentrations[4]. In experiments with crystalline glyoxylate reductase (EC 1.1.1.26) from tobacco leaves, anion activation of hydroxypyruvate reduction has been reported[5]. HOLZER AND HOLLDORF[6] have demonstrated the activation of hydroxypyruvate reductase from spinach leaves by various anions, in the order $Br^- \gg NO_3^- \gg SO_4^{2-} \geq Cl^- \geq I^- \gg F^-$. The effect of various salts on the activity of mammalian glycerate dehydrogenase (D-glycerate: NAD^+ oxidoreductase, EC 1.1.1.29) is reported in this paper. The enzyme catalyzes the reaction:

$$\text{D-glycerate} + NAD^+ \text{ (or } NADP^+) \rightleftharpoons \text{hydroxypyruvate} + H^+ + NADH \text{ (or } NADPH)$$

Since Tris–Cl^- buffers were routinely used in our earlier studies, the effect of Cl^- was not observed until a study of pH optima in various buffers was initiated.

The method described by DICKENS AND WILLIAMSON[10] was used to prepare lithium hydroxypyruvate. NADH was purchased from Pabst Laboratories. The reagent-grade inorganic salts were commercial products. Beef liver glycerate dehydrogenase, free of lactate dehydrogenase (EC 1.1.1.27), was prepared as previously reported[8].

The 2.25 ml incubation medium contained: 0.250 μmole NADH; 1.43 μmoles lithium hydroxypyruvate (omitted from the control); 120 μmoles imidazole–acetate buffer (pH 6.3); 125 μmoles inorganic salt, added as indicated in the figures; and enzyme solution (0.059 mg of protein) added last. As shown in Fig. 1, the reaction rate was linear for at least 5 min. The values plotted are averages of two or more experiments with each salt.

Under the conditions specified the optimal chloride concentration was found to be from 0.056 *M* to 0.078 *M*. The final concentration of the salts used in this study was 0.056 *M* unless stated otherwise. In the first experiment the effect of various anions was determined (Fig. 1). These data indicating the following relationship: $Cl^- \geq Br^- \geq I^- \geq NO_3^- \geq F^-$. Results similar to those observed with NaF were obtained upon the addition of sodium or ammonium sulfate (0.028 *M*) or potassium or sodium phosphate buffer (pH 6.3) (0.042 *M* $H_2PO_4^-$, 0.014 *M* HPO_4^{2-}). Various chloride salts were tested at the chloride concentration found to be optimal with sodium as the cation (Fig. 2). Although lower activities were observed with lithium and ammonium chloride, the differences were not as great as those observed with the various anions. The possibility remains that the lower activities which the cations may have been due to impurities in the commercial salts used

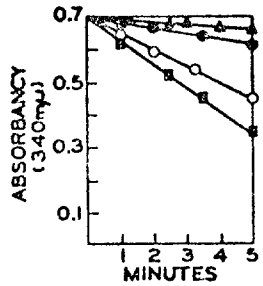

Fig. 1. Anion activation. Additions: ▲, none; ●, NaF; ○, $NaNO_3$; ■, NaCl, NaBr, or NaI.

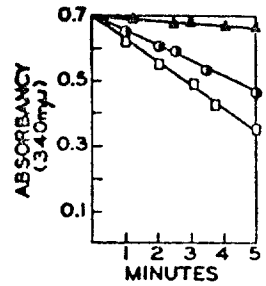

Fig. 2. Various chloride salts as activators. Additions: ▲, none; ●, LiCL or NH_4Cl; □, NaCl or KCL.

The mechanism of the ion activation of glycerate dehydrogenase is unknown. The activation of enzymes by cations and anions has been studied classically through the application of kinetic analysis[11-14]. Further studies of mammalian glycerate dehydrogenase are in progress in order to determine the effect of ions on the kinetic constants.

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On the ammonia content in the blood of the dog after the administration of urea, ammonium and acidifying salts.

By
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With 8 illustrations in text.
(Received on March 13,1943)

I.

A remark made in medical circles to the effect that after urea therapy of patients conditions of agitation as well as pulse slow-down can occur, led us to the question whether these symptoms as well as the diuresis caused by urea can be traced back to a decrease of the ammonia content in the tissue and in the blood. Tests were therfore performed once more to determine whether an increase of the ammonia content can be achieved by flooding the organism with large doses of urea. Besides the splitting of the urea, the inhibition of the synthesis of the ammonia produced by metabolism seemed conceivable.

Urea as diureticum has not been known very long in therapy. It was recommended by KLEMPERER[1] from the I. Clinic of Charité (under LEYDEN) on December 19,1895 to the Association of Charité Physicians. KLEMPERER noticed the diuretic action of urea accidentally when he prescribed it in a case of nephrolithiasis, believing that it could dissolve uric acid concretions. Urea is a type of the so-called "uric" (harnfähig) substances which, when accumulated in the blood, stimulate the secretion of the entire kidney epithelium. Therefore an artificial supply of urea increases the corresponding stimulus and shall be defined as a physiological diureticum". GREMELS[2] reached the conclusion on the strength of tests with heart-lung-kidney preparations according to STARLING that the diuretic urea effect is to be ascribed to the osmotic inhibition of water back-diffusion in the HENLE loop.

The formation of ammonia from urea has been repeatedly discussed earlier. Thus EFFLAGER[3] believed to be able to counteract the acid intoxication of rabbits with subcutaneously administered urea, although this could not be achieved with oral administration[3]. ADDIS and BARNETT[5] found in the same test animal an increase of the ammonia in the blood when they injected urea intravenously into the colon after laparotomy. Two intravenous tests were performed with 15 and 5 g per kg in each case; both animals died (after 1½ and 2 hours). After the larger dose 9 mg% of blood ammonia was found, and 2½ mg% was found after the smaller dose; the intitial values, as for the control test, were constant between 0,1 and 0,3 mg%. Subsequently, 3 g of urea was introduced into the intestines of three animals weighing approximately 1 kg; one of them showed no modification whatsoever in its blood value, nor any symptoms. A second animal died during respiratory malfunction after less than 1 hour with 2,7 mg% ammonia in the blood. The third animal likewise (died) with 4,5 mg% ammonia after 3/4 of an hour. It is remarkable, when evaluating these tests, that the steepest rise of the ammonia occurred considerably later after intravenous administration that after enteral administration.

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201: 520-528, 1943

Findings concerning the ammonia content of the urine after urea doses can be disregarded from the viewpoint of the question raised by us because the kidney is important for ammonia formation. Under these circumstances, and especially in view of the small number of reported tests, the powerful doses used for these tests and the neverthless consistent results, it seemed desirable to investigate the problem once more. The dog was selected as test animal.

II. Methodology.

I used the method of PARNAS and KLISICKI[7] for the determination of the ammonia content in the blood; with said method, 2 ccm of the blood made non-coagulant is added to 2 ccm borate powder (pH =9,2) in a small distilling flask, is overdistilled by water vapour in vacuum, into a concentration which contains 2 drops n 10 hydrochloric acid. The blood ammonia is found quantitatively as ammonium chloride in the distillate. It is mixed with NESSLER's reagent and is colorimetrically determined after 15 minutes. I made every effort to perform the investigation as fast as possible after taking the blood smaple, insofar as this was technically feasible.

III. Urea.

A sound dog (A), weighing 13,5 kg received in a fasting condition, after a blood sample was previously taken, 0,5 g urea per kg of body weight in 100 ccm water, through the esophagus probe. This application method comes closest to the clinically customary therapeutic use of urea, i.e. 30 g for a patient weighing 60 kg, per os. Illustration 1 shows a characteristic test result: 1 curve each for the untreated animal and for the same animal treated with urea are recorded. Both show a certain daily fluctuation insofar as the ammonia content decreases from 8 a.m. to 12 noon-1 p.m.; it rises again until 5 p.m. The previously obtained daily ammonia curves of the untreated animal were accordingly unchanged after urea therapy.

Since I assumed that I could have overlooked an early effect on the ammonia level during the first blood taking - 1 hour after administration of the urea, I took blood samples more frequently in the course of a second test, i.e. every 5, 15, 30, 60 and 120 minutes after the urea administration. Illustration 2 shows the result. No substantial effect was determinable during this test either.

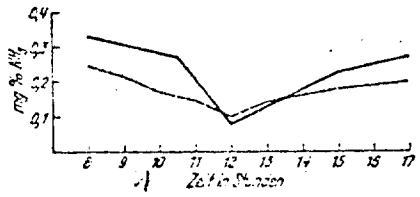

Illustration 1. Test 1: --- ammonia curve of the untreated animal.
Test 2: ____ ammonia curve of the animal treated with 0,5 g urea immediately after blood was taken.

1) Time in hours.

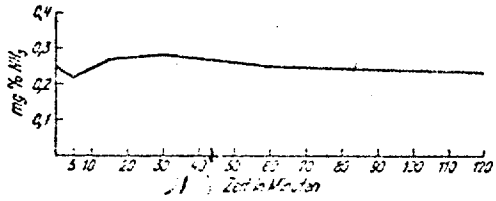

Illustration 2. Test 3: Ammonia curve during the first two hours after application of 0,5 g urea per kg.

1) Time in minutes.

The diuresis was very good during these tests and 3 consecutive urea tests. No specific excitation conditions were observed. The numerical findings for two additional tests with the same negative result are shown on the following Table:

Table 1. Dog A. Ammonia in the blood after 0,5 g urea per kg, orally applied following the taking of the first blood sample (8 a.m. and 8.30 a.m.).

Test 4.				Test 5.			
1 Zeit	mg% NH_3	Zeit	mg% NH_3	Zeit	mg% NH_3	Zeit	mg% NH_3
8h	0,21	12h	0,10	8h 30m	0,18	12h 30m	0,11
9	0,10	13	0,10	9 30	0,14	15	0,11
10	0,16	15	0,11	11	0,13	17	0,15
11	0,05	17	0,18				

1) Time.

Another dog (B) with a body weight of 24,5 kg, the fasting values of which were repeatedly examined and found to be between 0,13 and 0,16 mg% ammonia, showed a suddenly increased ammonia content without treatment, i.e. between 0,38 and 0,4 mg% ammonia, after the dog had refused to eat for unknown reasons during 4 days. 2 days before the animal died after having fasted for 4 days, the following values resulted for the ammonia content in the blood, without any treatment:

Table 2. Dog B. Ammonia in the blood without pretreatment.

1 Zeit	mg% NH_3	Zeit	mg% NH_3
8h 30m	0,39	12h 30m	0,22
9 30	0,40	15	0,24
11	0,28	17	0,31

1) Time.

PARNAS and KLISIECKI[8] indicated values for the dog between 0,01 and 0,3 mg%, with the remark, that "especially strong individual fluctuations" occur thereby. Alice ROHDE[10] determined in the "vividiffusate" from the blood of two live dogs, after equilibrium diffusion had occurred, according to the earlier method of FOLIN, an ammonia-nitrogen content of 0,18 and 0,30 mg%. Accordingly, my values coincide well with the results of the best earlier researchers.

IV. Ammonium salts

For the purpose of a comparison with urea I used the same test animal (A) to investigate the action of ammonium chloride. I gave the animal doses 1 to 4 g ammonium chloride, i.e. 0,08 to 0,3 g per kg. Still higher doses could not be applied because vomiting occurred. The analyses were limited here and during the subsequent tests to the first 2 hours after the application. I regularily obtained a rather strong increase of the ammonia level within the first ½hour; a decline resulted after 30 minutes which was once more followed by an increase, without repeated application of ammonium chloride. Illustration 3 shows the process after 1,2,3 and 4 g of ammonium chloride.

This remarkable curve tracing was regularily evident, although the decline after the first increase with a dose of 3 g ammonium chloride was negligible.

I was unable to determine a diuretic effect after ammonium chloride. On the other hand, the dog now was now under the effect of quite obvious excitatation conditions. He whined and ran restlessly up and down. Salivation occurred, and occasionally vomiting as well. Therefore--as known in principle--excitation conditions possibly could be triggered in the central nervous system by raising the ammonia level in the blood.

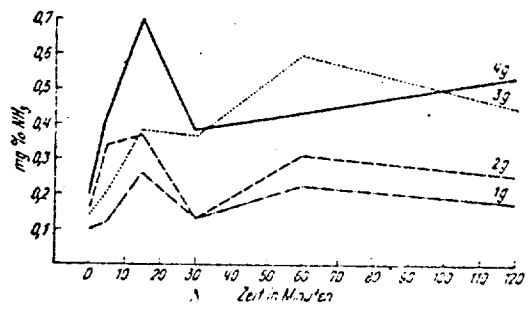

Illustration 3. Tests 6 to 9: Ammonia curves after 0,08, 0,15, 0,22 and 0,30 g NH_4Cl (= 1,4 to 5,6 millimol) per kg.

1) Time in minutes.

To clarify whether an artificially induced acidosis could likewise be involved in connection with the results obtained with ammonium chloride, I undertook on the one hand tests with an acidifying ammonium salt, namely with ammonium carbonate, and on the other hand, with primary potassium and sodium phosphate.

I again gave the same test animal 3,5 g ammonium carbonate dissolved in 100 ccm water through the esophagus probe. Illustration 4 shows the result. (The dotted curve represents the simultaneously determined hemoglobin values). I obtained, exactly as with ammonium chloride, an increase during the first 15 minutes; but this increase was not followed by any decline and renewed rise; instead, a gradual decline occurred, approximating the initial value within 2 hours.

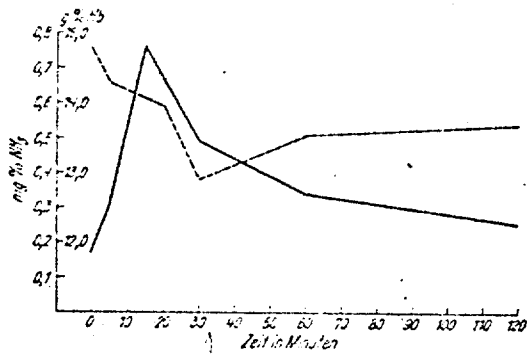

Illustration 4. Test 10: _____ NH_3-curve after 0,26 g (=6,8 millimol) ammonium carbonate per kg.
- - - hemoglobin curve.

1) Time in minutes.

The following values resulted with a parallel test, likewise using 3,5 g ammonium carbonate:

Table 3. Dog A; Test 11: 0,26 g ammonium carbonate per kg, orally. Ammonia in the blood:

1 Zeit	2 mg % NH_3	1 Zeit	mg % NH_3
2 Vorher	0,15	5 Nach 30 Min.	0,46
3 Nach 5 Min.	0,24	6 " 60 "	0,30
4 " 15 "	0,56	7 " 120 "	0,21

1) Time
2) Before
3) After 5 min.
4) " 15 "
5) After 30 "
6) " 60 "
7) " 120 "

V. Alkali salts.

I applied 4,5 g primary potassium phosphate (Ill. 5) for purposes of comparison. This curve first shows a conspicuous, very strong increase after 15 minutes, which proves that the ammonia level can be modified without ammonia application, merely by supplying acid. The above leads to the conclusion that the effect during the ammonium chloride tests is presumably not the result of the ammonia supply only, but is controlled by a more complex mechanism.

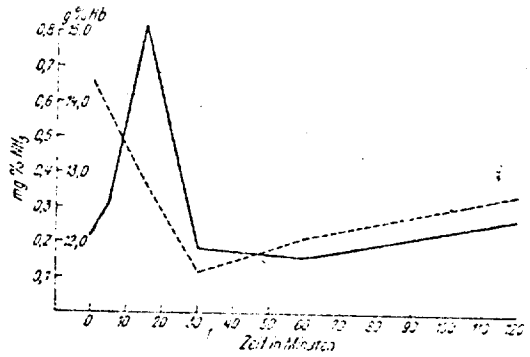

Illustration 5, Test 12: NH_3-curve after 0,33 g (= 2,45 millimol) primary potassium phosphate per kg;
1) Time in minutes. - - - hemoglobin curve.

As in case of the ammonium salts, a considerable decline occurs after 15 minutes which is followed during the second hour after the test start by a well-defined, even if minor, second increase--similar to (the findings) with acidifying ammonium chloride. The conclusion can presumably be reached from the above that at least this second increase--which we could not determine in case of neutral ammonium carbonate--is connected with the acidoctic component.

A test with 4,5 g. primary sodium phosphate took a fundamentally similar course (Ill.6).

To clarify how far the water shift parallels the modification of the ammonia level, I determined in the course of a few tests the hemoglobin content of the blood simultaneously with its ammonia content. The corresponding hemoglobin curves are represented in Ill. [illegible] by dotted lines. They indicate that no parallelism exists, since the course of said curves is initially quite different--while the ammonia curve course is similar in principle. The question [illegible] be raised whether this is valid for (curve) progress during the second hour (see, however, Ill. 4, p. of original text [illegible]).

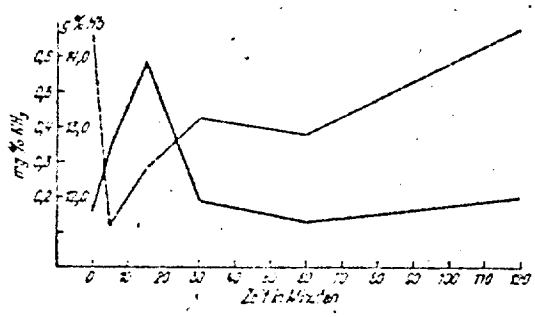

Illustration 6; Test 13: _____ NH -curve, after 0,[illegible] g [illegible] 2.13 millimol) of primary sodium phosphate per kg; - - - hemoglobin curve.

1) Time in minutes.

Furthermore, it seemed desirable to investigate the effect of an urea concentration in the tissue on the ammonia curve after acidifying salts, because the speed of the ammonia increase in the blood indicated that during acidification ammonia is transferred from the tissues into the blood. It likewise seemed conceivable that urea in the tissues could cause an increase of the ammonia content. To investigate this problem, I gave the test animal during 2 days 10g urea each in 100 ccm water. The diuresis lasted for [illegible] days. Following pretreatment with urea, I repeated the test shown in Ill.5, i.e. I administered 0,[illegible] g primary potassium phosphate in 100 ccm water. Ill. 7 shows the result.

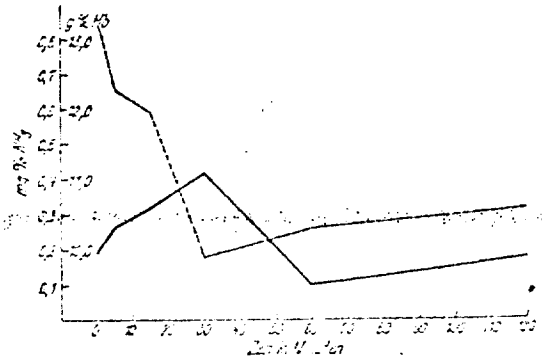

Illustration 7 : Test [illegible]: _____ [illegible] g [illegible] with [illegible] Test: [illegible].

1) Time in minutes.

In principle, the course of the hemoglobin curve [illegible] to its course during the earlier test with potassium [illegible]. However, it is evident from the ammonia curve [illegible] occurs later, namely only after 30 minutes, [illegible] customary elevation. The decline [illegible] another rising likewise occurs in the course [illegible] This test indicates that the saturation of the [illegible] does not result in an ammonia concentration, [illegible] rather leads to a reduction of the same. [illegible] the finding that no rise of the ammonia level [illegible] urea.

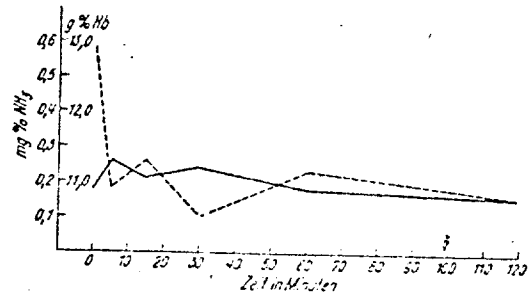

Illustration 8; Test 15: _____ NH_3-curve after 0,30 g (= 5,06 millimol) per kg; - - - hemoglobin curve.

1) Time in minutes.

Since I was able to obtain a speedy rising of the blood ammonia with alkali salts on the one hand, and also with ammonium salts on the other hand, I subsequently performed a control test with 4 g sodium chloride in 100 ccm water. Ill.8 shows the result; the hemoglobin curve is once more represented by a dotted line in said illustration. Exactly as in case of urea, sodium chloride has no effect on the ammonia level.

VI. Discussion.

My findings exclude the possibility that the diuretic effect of the urea could be a consequence of the intermediate ammonia formation. (The question might not yet be definitely settled whether part of the excessive urea supply can be chemically attacked in the kidney).

However, it is subject to discussion whether the occurrence of excitation conditions after urea [illegible] connected with the formation of ammonia. I [illegible] ammonia [illegible] excitation in the dog, as observable [illegible] but ammonium salts caused excitation in the dog as well, [illegible] in this context that [illegible] rabbits which showed ammonium content in the [illegible] as well, at least in the form of spastic respiration; [illegible] in an animal which had received similar treatment [illegible] ammonia increase. The reactions of man to this [illegible] neither merely carnivores nor vegetarians—must be reserved for future clarification.

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Failure of Antihistaminic Drug "Phenergan" to Protect Against Acute Pulmonary Edema. (17351)

CHARLES A. WINTER.

From the Merck Institute for Therapeutic Research, Rahway, N. J.

Halpern and his co-workers have developed the thesis that antihistaminic drugs owe much of their therapeutic effectiveness to a primary influence upon cellular permeability, aside from their property of competitive inhibition of histamine. This concept is based in part on the observation that N-(β-dimethylamino-α-methylethyl)-phenothiazine (3277 R.P. or Phenergan) protects guinea pigs and rabbits from pulmonary edema produced by an overdose of epinephrine[1,2] or by certain poison gases, such as chloropicrin.[3]

On the other hand, Stone and Loew[4] failed to obtain protection by pyranisamine (Neo-Antergan) in rabbits with epinephrine-induced pulmonary edema. The same authors[5] also could not confirm the observation that Phenergan protected rabbits. In one series (8 animals) they obtained a reduced mortality but no reduction in pulmonary edema, as judged by lung weight. It seemed of interest to determine whether Halpern's results could be confirmed in our laboratory, and also whether Phenergan would affect pulmonary edema produced by administration of ammonium salts, as described by Koenig and Koenig.[6] If it could be shown that pulmonary edema produced by two such widely different means could be alleviated by an antihistaminic drug, support would be added to Halpern's view that such drugs may primarily affect cellular permeability.

Methods. The rats used in this study weighed about 200 g. Both Carworth and Holtzmann rats were employed, and as no difference was noted in results obtained with the two strains, they have been combined in the summary. The guinea pigs varied from 300 to 500 g in weight. Pulmonary edema was produced as follows: epinephrine chloride was injected in the forelimb vein of guinea pigs, 0.6 mg per kg (the dose used by Reuse[2]); ammonium chloride was administered intraperitoneally, the dose being 400 mg per kg for rats, and 600 mg per kg for guinea pigs, as described by Koenig and Koenig.[6]

Phenergan was chosen as the antihistaminic drug, because Halpern has stated that its effect on cellular permeability was greater than any others he used. We administered Phenergan subcutaneously, except to a few of the rats, where the intraperitoneal route was employed. The dose was the same as that used by Halpern and by Reuse: namely, 20 mg per kg, except in 2 groups of rats where the dose was doubled. Thirty to 60 minutes (usually the latter) were allowed to elapse between injection of Phenergan and of epinephrine or ammonium chloride. The lot of Phenergan used was manufactured in France, and obtained from Société des Usines Chimiques Rhône-Poulenc, Paris.

Results. The syndrome of acute experimental pulmonary edema in rats and guinea pigs has been adequately described by Koenig and Koenig.[6] The signs and gross findings at autopsy described by them were seen in the majority of our animals.

The results are summarized in Table I. It is evident that Phenergan, even in the relatively large doses used, did not protect the animals from pulmonary edema, as judged by lung weight, nor did the antihistaminic drug reduce the mortality, nor prolong the survival

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1 Halpern, B. N., Hamburger, J., and Cruchaud, S., *Acta allerg.*, 1948, **1**, 97; Halpern, B. N., and Cruchaud, S., *Compt. rend. soc. biol.*, 1947, **141**, 1028; Vermeil, G., Halpern, B. N., and Cruchaud, S., *Compt. rend. soc. biol.*, 1949, **143**, 77.

2 Reuse, J., *Compt. rend. soc. biol.*, 1948, **142**, 638.

3 Halpern, B. N., and Cruchaud, S., *Compt. rend. Acad. d. sc.*, 1947, **225**, 1194.

4 Stone, C. A., and Loew, E. R., *Proc. Soc. Exp. Biol.*, 1949, **8**, 335.

5 Stone, C. A., and Loew, E. R., Proc. Soc. Exp. Biol. and Med., 1949, **71**, 122.

6 Koenig, H., and Koenig, R., Proc. Soc. Exp. Biol. and Med., 1949, **70**, 375.

TABLE I.
Lack of Protective Effect of Phenergan Against Acute Pulmonary Edema Produced by Epinephrine or Ammonium Chloride.

Exp. No.	Treatment	Avg body wt, g	Dose of phenergan, mg/kg	Mortality ratio*	Median survival time,† min.	Avg lung wt,‡ g/kg body wt
		Rats				
I	NH_4Cl	199	—	8/15	35	11.6
II	" + phenergan	220	20	4/10	20	18.2
III	" + "	206	40	8/10	24	14.6
IV	Phenergan	196	40	0/10	—	6.2
V	No drug	212	—	0/4	—	7.7
		Guinea pigs				
VI	NH_4Cl	461	—	8/10	8	10.4
VII	" + phenergan	408	20	12/12	8	8.6
VIII	Epinephrine	436	—	6/7	7	19.7
IX	Epinephrine + phenergan	423	20	8/8	7	20.3
X	No drug	450	—	0/5	—	6.8

* Mortality ratio = number dead/number used.
† Excluding those surviving indefinitely.
‡ Avg lung wt in experimental groups include only those animals which succumbed after injection. Control animals were sacrificed under pentobarbital anesthesia.

time of the animals which died. The difference in lung weight between Groups VI and VII is not significant ($P > 0.05$), but Group VII differs significantly from control Group X ($P = 0.02$), as does Group VI ($P < 0.01$). In all the other cases, the differences between experimental and control groups are obvious from inspection of the table.

Discussion. The lung weights of the guinea pigs succumbing to epinephrine were greater than in those receiving a lethal dose of ammonium chloride. It may therefore be assumed that pulmonary edema was not maximal in the latter group, and may not have been the sole cause of death. Stone and Loew[5] have also expressed the view that pulmonary edema may not have been the sole cause of death in their epinephrine treated rabbits. The overall mortality rate in all our animals receiving edema-producing drug without Phenergan was 22/32 or 69%, while in those receiving Phenergan in addition to the toxic agent it was 32/40 or 80%. Our results are in agreement with the statement of Stone and Loew,[5] that Phenergan did not protect the animals against pulmonary edema, although we did not observe the lower mortality rate which they reported in their animals.

Koenig and Koenig[6] believe that the pulmonary edema produced by ammonium chloride is a specific action of the ammonium moiety, and is not related to the acidosis produced by NH_4Cl. Other ammonium salts also induced pulmonary edema, while acidosis produced by other means did not. However, they did not publish data indicating the degree of acidosis produced by the various agents they used, and it is therefore impossible to judge the validity of their conclusions.

The divergence between our results and those of Reuse[3] is not easy to explain. He used guinea pigs about twice as large as those herein reported. He also used epinephrine ascorbate, while the epinephrine we used was Adrenalin chloride (Parke, Davis), a glandular extract containing an unknown amount of nor-epinephrine. Halpern[1] stated that Phenergan did not prevent the blood pressure and cardiac effects of epinephrine, but Reuse[7] found that Phenergan in large doses inhibited both the pressor effect and the apnea produced by small doses of epinephrine. This effect, however, lasted only a few minutes, and probably does not explain the partial protection which he observed. Even in Reuse's experiments Phenergan did not offer complete protection against epinephrine-induced pulmonary edema as the

7 Reuse, J., [illegible] 11, 1949.

mortality ratio was 5/14 in the Phenergan-treated animals, compared to 11/14 in those receiving 0.55 - 0.6 mg per kg of epinephrine without Phenergan. In the group to which 1.2 - 1.5 mg per kg of epinephrine was administered, 8 of 11 Phenergan-treated animals died.

Our results, as well as those of Stone and Loew, do not lend support to Halpern's hypothesis that Phenergan opposes the formation of pulmonary edema by virtue of the property of inhibiting cellular permeability.

Summary. The antihistaminic drug, Phenergan (3277 R.P.), in doses of 20 or 40 mg per kg, failed to protect rats or guinea pigs against the pulmonary edema induced by injection of ammonium chloride, and also offered no protection against epinephrine-induced pulmonary edema in guinea pigs.

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AMMONIUM CHLORIDE ACIDOSIS AND THE REACTION THEORY OF RESPIRATION CONTROL

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Two objections have been primarily raised against the theory according to which hematogenous respiratory control is exercized by the cH of the blood and not by a specific reaction of CO_2 (Winterstein, 1, 2): 1) the effect of an injection of bicarbonate through which the blood becomes more alkaline, yet pulmonary ventilation increases, at least at the outset; 2) contrary behavior in NH_4Cl acidosis, where particularly the investigations of Nielsen (3) have shown that the pH value of the blood decreases extraordinarily while respiration rises much less than during the extended inhalation of CO_2, although the latter takes place with a much lesser depression of the pH.

The first objection has been refuted a long time ago: after Jacobs (4) had shown that alkaline $H_2CO_3 \cdot NaHCO_3$ solution, due to the more rapid diffusion of the undissociated CO_2, induces a sour taste and shifts the reaction in animal and vegetable cells toward the acid side (a behavior demonstrated by Winterstein (5) on a lifeless model), Gesell and Hertzman (6) furnished direct proof of the fact that the injection of $NaHCO_3$ in the blood shifts the reaction of the blood toward the alkaline side, but that of cerebrospinal fluid (Csf) toward the acid side. Since the reaction of the centers certainly parallels that of the liquors, these findings, recently confirmed by the authors, satisfy the conditions of the theory.

Jacobs experimented not only with $H_2CO_3/NaHCO_3$, but also with NH_4OH/Nh_4Cl solutions and found that eggs of sea urchins submerged in the two solutions, both with a pH of 7.2, showed an acid reaction in the first case and an alkaline reaction in the second. It therefore appeared entirely possible that just as the injection of an alkaline solution of $NaHCO_3$ can cause a shift in the Csf reaction toward the acid side, NH_4Cl acidosis proceeds with a shift of the reaction of the liquor to the alkaline side, which would explain the weakening of the hyperpnea required by the reaction theory.

Concerning the effect of chloride acidosis on the liquor, the literature yielded only a single statement in Gellhorn & Regnier (7), according to which J. Thurzo & S. Katzenelbogen, in a paper not available to the authors in the original (Magy. orv. arch. 1934, 35, 256) state that the rapid decline of the alkali reserve in the blood is followed by a very slow decline in the liquor, an observation which, according to the authors cited, would indicate the difficult passage of H ions through the bloodbrain barrier.

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Experimental Method

To produce NH_4Cl acidosis, in the four experiments performed on humans for two days, 6 times daily in four-hour intervals, a total of 10-26 g NH_4Cl powder was given in wafers. Dogs received the same wrapped in meat, three times daily, a total of 300-500 g per day and kg. In the experiments with intravenous administration, 50-300 mg/kg were given in a 1% solution, either in individual doses in intervals of 15-20 minutes, or in a slow, continuous infusion. In a few cases, signs of nausea were observed without any other side effects.

The pH values were determined with a Marconi tube voltmeter; only the millivolt scale was used and not the direct pH reading. The Marconi glass electrode was surrounded by a special glass jacket which reduced the volume of the liquid to be measured to 4 cc. Measurements in flowing blood thus became possible. In the experiments on humans and in the first series of dog tests, the pH determination was performed at room temperature. Since the authors were interested not in absolute values but in changes taking place under different conditions, they felt that the effect of temperature could be neglected. It was found that differences between two different blood values and two different liquor values are indeed unaffected by temperature, but not the difference between the pH value of blood and of the liquor. While, as determined by Michaelis (8), the pH of blood at the temperature of the body is lower by approx. 0.2 than at 18-20°, the pH of the liquor varies with this difference in temperature only by approximately 0.1, similarly to a simple bicarbonate solution, for which, according to Hasselbalch (9), the difference of $pH^{18}-pH^{38}= 0.12$. Accordingly, the difference in pH between blood and Csf is lower at body temperature than at room temperature. In the remaining experiments, all determinations were thus performed at 38 degrees. Older data concerning the pH of the liquor regard it most often to be more alkaline than blood. This is undoubtedly due to the loss of CO_2 in the measurement. Under standard conditions the pH of the liquor is always substantially less than that of blood.

The pH determination of blood, performed in these investigations through intravenous injection of NH_4Cl 15-60 minutes after the completion of the latter, was conducted mainly in venous blood: arterial blood was used only in the liver elimination experiments. Liquor was obtained with humans through lumbal and with dogs through suboccipital punction. With the exception of one dog which remained very quiet and coul be punctioned without narcosis, narcosis of short duration through the injection (i.v.) of Exipan was used in order to prevent brain damage during the extraction of the liquor. In more substantial operations, Chloralose (110 mg/kg, i.v. in a 1-2% solution) or Nembutal Abbott (the contents of a 100 mg capsule dissolved in 4 cc of Ringer's solution; 1 cc of the solution for 700 g body weight = approx. 36 mg/kg, i.v.), for cats, urethane i.p. in a 50% solution (approx. 1 g/kg). In the experiment with aortic denervation, Nembutal was used as the base narcotic and then, after the larynx intubation, Protoxyd (N_2O) with oxygen under pressure.

Respiratory volumes were measured with the aid of special masks placed over the snouts of the dogs; the masks were sealed with inflatable rubber cushions lining the masks. The dogs readily adapted to the wearing of the masks periodically and usually fell into a kind of semi-sleep with regular breathing when stroked. Directly in front of the snout the mask connected with a valve, the intake and outlet openings of which lead to the meter.

The removal of chemoreceptors was in general restricted to those of the carotid sinus. The glomera aortica appear to play a minor role with respect to the effect on the pulmonary ventilation in dogs. Lobelin and sodium cyanate proved to be without effect even if only sinus receptors were eliminated, in doses twice those which were effective previously. A dog with its aortic arches denerved behaved with respect to NH_4Cl in the same manner as when only the carotid sinuses were denerved. Denerving was performed mainly by the Leriche method of removing the adventitia, which also removed the in- and out-going nerves. A 4% cocaine solution was injected under the adventitia which lifted them from the media so that they could be removed with a pair of fine scissors. Simultaneously, shock effects produced by chemoreceptor irritation were eliminated in this manner. In aortic denervation not only the adventitia of the arch, but also of the truncus anonymus and the initial part of the left subclavia were also removed, i.e. from all of the regions in which, according to de Castro (10), glomera are present. The authors owe gratitude to Dr. Zeki Turker for the performance of this operation; after 11 months, the dog is still in perfect health.

In previous experiments with liver elimination, already briefly reported (11), a U-shaped cannula, with its arch directed toward the head, was bound into the central stump of vena jugularis of one side, which has been cut. Next, a ligature was placed under the liver inlet and the vena portae, but not tightened. The blood of the animal was now rendered non-coagulating by the slow i.v. injection of Liquaemin Roche (1 cc liquaemin, diluted 10 times with Ringer's solution = 500 units/kg). The two ligatures are now tightened, another cannula rapidly bound into the peripheral stump of the vein and connected with a hose with the free leg of the jugular cannula. The blood of the portal vein now flows directly into the jugular vein, bypassing the liver. Since the arteria hepatica in the liver port is also tied off, no blood flows into the liver. A section of the latter does not cause bleeding.

Experimental Results

Table 1 presents the results of four experiments performed on patients of the neurological clinic with the adminstration of NH_4Cl; they were three cases of schizophrenia and a myoclonic suffering from violent cramps. In the case of the latter and with one of the schizophrenics, where the pH value of the blood showed a pronounced shift toward the acid side, such a shift was also found in the liquor. In the other two cases, however, in the presence of merely a weak hypohydration of the blood, the reaction of the Csf was strongly displaced toward the alkaline side.

The results may not be entirely reliable, as the examination of the samples had to take place several hours after sampling.

Much better agreement is exhibited by the experiments on dogs. As shown by Table II, a strong shift of the pH of the blood toward the acid and that of the Csf toward the alkaline side is found without exception.

TABLE I

NH_4Cl Effect per os on Humans

Fall von (1)	pH des Blutes (2)		pH der Csf (3)		pH-Differenz (4)	
	vorher (5)	2-3 St. nach der letzten Dosis (6)	vorher (5)	nachher (7)	im Blut (8)	im Liquor (9)
Schizophrenia	7,51	7,35	7,22	7,08	— 0,16	— 0,14
Myoklonia	7,52	7,40	7,27	7,15	— 0,12	— 0,12
Schizophrenia	7,61	7,59	7,09	7,19	— 0,02	+ 0,10
Schizophrenia	7,53	7,48	7,13	7,29	— 0,05	+ 0,16

Key: 1. Case of ...; 2. pH of the blood; 3. pH of Csf; 4. Difference in pH; 5. before; 6. 2-3 hours after the last dose; 7. after; 8. in the blood; 9. in liquor.

While the reaction of the blood returns rapidly to normal after the administration of NH_4Cl is discontinued, or possibly even swings to the alkaline side, the Csf retains its alkaline reaction, as observed also in other experiments, in some cases for several days or even weeks.

Heretofore, NH_4Cl acidosis was always produced by per os administration. The authors investigated the results of the intravenous introduction of the substance. As shown in Table III, the same behavior is obtained, if no excessive amounts are infused on one occasion (see No. 7). In the great majority of cases, particularly in experiments with slow continuous infusion (No. 8, 9, and 10), the reaction of the blood again shifts in a direction opposite to that of Csf: blood becomes acidic, the liquor alkaline.

Respiratory Experiments

The authors proceeded to comparatively examine the effects of ammonium chloride on the cH of blood and Csf with the effects on pulmonary ventilation. In order to accomplish this under conditions as normal as possible, the method of feeding NH_4Cl was again employed. Table IV presents the effect on two normal dogs. As is seen, the very substantial shift of the pH of the blood to the acid side is accompanied by a very slight hyperventilation, exactly as described by Nielsen (3) for humans. There should be no doubt that the respiration enhancing effect of the cH increase in the blood is opposed by the respiration decreasing effect of the decline of cH in the Csf.

TABLE II

NH_4Cl Effect per os on Humans

1 Nr.	2 Vers. Bedingungen	3 pH des Blutes	4 pH der Csf	pH-Differenz 5 im Blut 6	pH-Differenz 5 im Liquor 7
1	normal 8	7,49	7,08		
	3 Tage NH_4Cl	7,32	7,41	— 0,17	0,33
	2 Tage später 9	—	7,08		
	3 Tage NH_4Cl	7,29	7,57	— 0,20	0,49
	12 Tage später	7,61	7,06		
2	normal	7,48	7,23		
	3 Tage NH_4Cl	7,38	7,38	— 0,10	0,15
	21 Tage später	7,58	7,38		
3	normal	7,51	7,26		
	3 Tage NH_4Cl	7,38	7,35	— 0,13	0,09
	4 Tage später	7,54	7,32		
	14 Tage später	7,56	7,27		
4	normal	7,46	7,03		
	3 Tage NH_4Cl	7,38	7,47	— 0,08	0,44
	6 Tage NH_4Cl	7,38	7,42		
	28 Tage später	7,50	7,24		

Key: 1. No.; 2. Experimental conditions; 3. pH of blood; 4. pH of Csf; 5. pH difference; 6. in blood; 7. in liquor; 8. days; 9. later.

TABLE IV

NH_4Cl Effect on pH and Respiration in Normal Dog

No.	1 Datum	2 Versuchs-bedingungen	3 Blut-pH	Csf-pH	4 Min. Atemvo-lumen ccm
1	29.XI	normal 5	7,35	7,14	6500
	4.XII	3 Tage NH_4Cl	7,23 (— 0,12)	7,19 (+ 0,05)	7250 (+ 750)
	14.XII	normal	7,34	7,14	
2	9.I	normal	7,36	7,26	4300
	18.I	6 Tage NH_4Cl	7,12 (— 0,24)	7,34 (0,08)	4450 (150)
	24.I	normal	7,37	7,26	3950

Key: 1. Date; 2. Experimental conditions; 3. Blood pH; 4. Respiratory volume/min.; 5. Days.

TABLE III

NH_4Cl Effect on Dogs, Intravenous

No.	1 Versuchsbedingungen	2 Blut-pH	Csf-pH	pH-Differenz 3 im Blut 4	im Liquor 5
1	normal	7,47	7,18		
6	15 Min. nach NH_4Cl	7,47	7,28	0	0,10
2	normal	7,58	7,37		
	15 Min. nach NH_4Cl	7,39	7,38	— 0,19	0,01
7	4 Stunden später	7,53	7,21		
3	normal	7,51	7,38		
8	30 Min. nach NH_4Cl	7,48	7,44	— 0,03	0,06
4	normal	7,53	7,36		
6	15 Min. nach NH_4Cl	7,46	7,36	— 0,07	0
5	normal	7,56	7,27		
	15 Min. nach NH_4Cl	7,31	7,31	— 0,25	0,04
9	3½ Stunden später	7,53	7,21		
6	normal	7,53	7,29		
	15 Min. nach NH_4Cl	7,46	7,34	— 0,07	0,05
	3 Stunden später	7,56	7,21		
7	normal	7,48	7,28		
	30 Min. nach NH_4Cl	6,94	7,07	— 0,54	— 0,21
8	normal	7,36	7,05		
	65′ nach NH_4Cl-Inf.	7,27	7,13	— 0,09	0,08
9	normal	7,40	7,11		
	40 Min. nach NH_4Cl	7,31	7,20	— 0,09	0,09
10	normal	7,44	7,22		
	90 Min. nach NH_4Cl	7,36	7,29	— 0,08	0,07

Key: 1. Experimental conditions; 2. pH of blood; 3. pH difference; 4. in blood; 5. in liquor; 6. 15 min. after NH_4Cl; 7. 4 hours later; 8. 30 min. after NH_4Cl; 9. 3 1/2 hours after NH_4Cl;

In experiments 1, 2, 5 and 6, 300 mg and in exp. 3, 4 150 mg/kg NH_4Cl were given. The doses were injected in a 1% solution, in several portions, in 15-20 min. intervals. In exp. 7, the total dose of 300 mg/kg was injected at once. In exp. 8, 9, 10, 30 mg/kg was infused slowly, continuously.

TABLE V

NH_4Cl Effect on Respiration in Dogs Without Sinus Chemoreceptors

No.	1 Datum	Versuchs-2 bedingungen	3 Blut-pH	Csf-pH	Min. Atemvol. ccm 4
1	27.II	normal 5			2800
	1.III	3 Tage NH_4Cl			2050 (— 750)
	11.III	normal			2700
	14.III	3 Tage NH_4Cl			2300 (— 400)
	20.III	normal			3100
2	6.VIII	normal			6100
	11.VIII	4 Tage NH_4Cl	7,21	7,32	3600 (— 1500)
	29.VIII	normal	7,36 (+ 0,15)	7,21 (— 0,11)	5200

Key: 1. Date; 2. Experimental conditions; 3. Blood ph: 4. Respiratory volume/min.; 5. Days.

It is not likely that the two factors opposing each other would have the same point of attack. Since it is now known that hematogenic cH variations affect respiratory centers in two different ways, directly and by reflection through chemoreceptors (for literature see Winterstein, 2), the authors investigated the behavior of animals in NH_4Cl acidosis after removal of carotid sinus chemoreceptors. Table V displays the behavior of two of these animals. In the case of the first, blood and liquor reactions were not examined, in the second only after the feeding of NH_4Cl and return to normal, so that the reverse of the usual phenomena, i.e. a rise of pH in the blood and a decrease in the Csf, were observed.

TABLE VI

NH_4Cl Effect on Respiration Before and After Elimination of Sinus Receptors

Datum 1	Versuchsbedingungen 2	Minuten-Atemvolumen ccm 3
3.III	normal 4	2800
5.III	2 Tage NH_4Cl	3100 (+ 300)
6.III	normal	2900
11.III	normal	2900
14.III	3 Tage NH_4Cl 5	3200 (+ 300)
24.III	Beide Sinus denerviert	
8.IV	normal	2800
10.IV	2 Tage NH_4Cl	2500 (— 300)
11.IV	1 Tage NH_4Cl	2650
14.IV	normal	3000

Key: 1. Date; 2. Experimental conditions; 3. Respiration volume/min.; 4. Days; 5. Both sinuses denerved.

TABLE VII

NH_4Cl Effect on the pH of Blood and of Csf and on Respiration Volume Before and After Elimination of Sinur Receptors and Denerving of the Aorta

1 Datum	Versuchs- 2 bedingungen	3 Blut-pH	Csf-pH	Min. Atemvol. 4 ccm
		Tier normal 5		
4.X	normal 6	7,36	7,28	5750
9.X	5 Tage NH_4Cl	7,28 (— 0,08)	7,34 (+ 0,06)	6000 (+ 250)
24.X	normal	7,42	7,34	6000
31.X	6 Tage NH_4Cl	7,24 (— 0,18)	7,37 (+ 0,03)	6250 (+ 250)
5.XI	Entfernung des linken und Denervierung des rechten Sinus Caroticus 7			
14.XI	normal	7,32	7,12	4500
22.XI	5 Tage NH_4Cl	7,22 (— 0,10)	7,24 (+ 0,12)	3650 (— 850)
5.XII	normal	7,31	7,14	4500
18.XII	normal	—	—	6000
24.XII	7 Tage NH_4Cl	7,26 (— 0,05)	7,31 (+ 0,17)	4600 (— 1400)
4.I	normal			5000
11.II		Denervierung des Aortenbogens 8		
18.II	normal	7,60 (?)	7,34	4500
19.II	normal 6	7,40	—	—
21.II	3 Tage NH_4Cl	7,36 (— 0,04)	7,37 (+ 0,03)	3850 (— 650)
28.II	normal	7,41	7,19	4800

Key: 1. Date; 2. Experimental conditions; 3. pH of blood; 4. Respiratory volume/min.; 5. Normal animal; 6. Days; 7. Elimination of the left and denervation of the right carotid sinus; 8. Denervation of the aortic arch.

In Table VI, the effect of NH_4Cl on the respiratory volume was investigated on the same animal, before and after elimination of the sinus chemoreceptors.

Table VII presents the results of an experiment in which the reaction of the blood, the Csf and the respiratory volume were investigated on the same dog initially under normal conditions, then after elimination of both carotid sinus and, finally, after denervation of the aorta.

The experiments indicate uniformly that following the elimination of the chemoreceptors, the respiration-stimulating hematogenic effect of ammonimum chloride acidosis disappears and only the respiration decreasing effect ("liquorogenic") hematogenic effect appears. The elimination of the aortic glomera by denerving the aortic arch, truncus anonymus and the initial part of the left art. subclavia, as performed at the conclusion of the last experiment, did not fundamentally change the result.

Discussion of Experimental Results

In the authors' opinion, the experiments reported above permit important conclusions in three different areas:

1. The nature of Ammonium Chloride Acidosis

J. B. S. Haldane (12), who was the first to describe NH_4Cl acidosis, felt that it had to be explained by the assumption that the ammonia arriving in the liver is converted into urea, thus settling hydrochloric acid free. This explanation has been generally accepted. Proof apparently has been attempted only by Bourdillion (13), who, on the basis of self-experimentation, stated that following the per os administration of NH_4Cl the increase in serum urea corresponds to the assumption that the retained nitrogen is entirely converted into urea. The finding that the same acidosis can be achieved by circumventing the portal vein circuit through direct intravenous infusion -- an observation made also by other authors (see Mackler & al. (14) -- renders this explanation highly improbable, since it is known from experience with Eck's fistula that the mere elimination of the portal vein circulation greatly depresses the formation of urea. The authors have shown in an experimental series, already briefly reported (11), that even after completely chunting the liver out of circulation, by tieing off both the portal vein and the hepatic artery and direct diversion of the portal vein blood into the jugular vein, acidosis occurs in the same manner, following intravenous infustion of NH_4Cl.

The following, as yet unpublished Table VIII presents a number of such experiments. Many animals tolerate the operation for a short period only. The impression is created that the nausea frequently observed after NH_4Cl infusion (which seldom leads to actual vomiting in narcosis) is more readily triggered after the liver has been eliminated. In Experiments 1 and 2, pH measurement was continuous; the glass electrode was inserted in the blood stream of a carotis. In the rest of the experiments, due to inadequate accuracy of the 0 point of the apparatus, pH was determined in samples of arterial blood. Experiments 1-4 were performed on cats and 4-5 on dogs.

The physico-chemical explanation of NH_4Cl acidosis must therefore be attempted on another basis, obviously in analogy to the previously mentioned (S.-) experiments of Jacobs (4) and observations with bicarbonate infusion (S.-). The acid reaction of a NH_4Cl solution follows from the dissociation reaction $NH_4^+ \quad NH_3 + H^+$. In the concentration of 1% used by the authors, the cH of the solution with a pH between 6 and 7 is much too low for an explanation of the observed shifts of the blood toward the acid side. The latter is possible only if it is assumed that the hydrolytic alkalizing effect of NH_3 is eliminated or restricted. This occurs not by the formation of urea as heretofore assumed, but simply because the electrically neutral ammonia passes readily and rapidly through lipoid membranes, such as the blood liquor barrier, while H ions are unable to do so, or at least not at the same rate. On the other side of the membrane, therefore, the actually observed alkaline reaction shift takes place in the liquor. However, even this is not sufficient to explain the acidification of blood, as shown by a simple consideration: let us assume that the 50 mg/kg NH_4Cl injected in many of the authors' experiments is uniformly distributed over all of the tissues; then they would contain approx. n 1000 NH_4Cl. An injection of HCl corresponding to this concentration in order of magnitude leads, as demonstrated particularly by the experiments of Banus & al. (15), to a hardly measurable reaction shift of blood, while, as seen above, values of pH 0.1-0.2 can be obtained with NH_4Cl. How is this difference to be explained?

TABLE VIII

NH_4Cl Acidosis Following the Elimination of the Liver

Nr	Versuchsbedingungen 1	pH des Blutes 2
1	Leber ausgeschaltet 3	7,14
	11 Min. nach 10 ccm NH_4Cl (1 %)	7,05 (— 0,09)
	8 Min. später 4	7,14
2	normal 5	7,08
6	16 Min. nach Injection von insgesamt 7 ccm NH_4Cl	6,95 (— 0,13)
	Leber ausgeschaltet 3	
	47 Minuten später 5	6,92
6	14 Min. später nach Inj. von insgesamt 7 ccm NH_4Cl	6,79 (— 0,13)
	27 Minuten später 5	6,88
3	Leber ausgeschaltet 3	7,27
6	3 Min. nach Inj. von 5 ccm NH_4Cl	6,98 (— 0,29)
4	Leber ausgeschaltet 3	7,08
7	Im Verlaufe von 90 Min. 70 ccm NH_4Cl, 5 Min. später	6,96 (— 0,08)
	19 Min. später 5	7,01
5	Leber ausgeschaltet, künstliche Atmung 8	7,11
9	Im Verlaufe von 34 Min. 65 ccm NH_4Cl, 4 Min. später	6,95 (— 0,16)
	26 Minuten später	7,05

Key: 1. Experimental conditions; 2. pH of blood; 3. Liver eliminated; 4. after; 5. later; 6. min. after injection of a total of; 7. During a period of 90 minutes 70 cc NH_4Cl, 5 min. later; 8. Liver eliminated, artificial respiration; 9. During a period of 34 min. 65 cc NH_4Cl, 4 min. later and 26 min. later.

Evidently by the fact that with other acids the body immediately introduces measures to control the reaction and to return it to normal through increased respiration and the resulting CO_2 washing; in NH_4Cl acidosis this is not possible, at least not to the required extent. The result is, as determined by Nielsen (3), that the increased respiration during NH_4Cl acidosis does not correspond to a decrease in pH, but conversely, the inadequacy (due to Csf alkalinity) of respiration enhancement is producing the strong decline in pH or at least leaves it unchanged. NH_4Cl acidosis is a result of liquor or center alkalosis.

2. The problem of the BloodBrain Barrier

The effect of NH_4Cl on respiration is the counterpart to the effect of bicarbonate. In the latter case respiration is enhanced in spite of the shift of the reaction of the blood toward the alkaline side, because cH rises in the liquor; in the NH_4Cl effect, respiration is not increased correspondingly, even somewhat weakened after the elimination of the chemoreceptors, because the cH of the liquor declines. The relationship between the reaction of the Csf and the activity of the respiratory centers is again undeniable. Leusen investigated the matter recently. Leusen (16) devised a method of direct rinsing of brain ventricles in which a cannula is introduced in a side ventricle and a second cannula suboccipitally in the 4th ventricle. The liquid to be

examined with respect to its effect enters through the first cannula and exits through the second. Leusen (17) then observed that decreases or increases of the pH of the liquid are coupled with an increase or decrease of the respiratory volume. The change in the pH of the liquid was effected by varying the CO_2 content. If, however, the same changes in pH were obtained with CO_2-free solutions, they remained without effect. The author concluded that a specific effect of carbonic acid must be present and that changes in pH have no direct influence on respiratory centers. This is evidently a misconclusion, due to the neglection of pereability conditions, the great importance of which had been emphasized by Winterstein (5) a long ago. If the changes in pH between 6.9 and 7.8 would have arrived at the cells of the respiratory centers in the absence of carbonic acid, the variations in CO_2 tension necessarily produced in the cells would have been such that, regardless of whether the reaction or the CO_2 theory is correct, substantial changes in the respiratory volume would have necessarily occurred. The fact that such changes did not occur can only indicate that the pH changes never reached the cells of the respiratory centers. In the case of CO_2-containing solutions, on the other hand, due to the great diffusivity of carbonic acid, the changes affected the cells. This leads us to the important question of the manner in which the composition of blood and liquor affects the centers and thus to the much discussed problem of the blood-brain barrier.

Here there are two opposing views. According to one, the classical representative of which is Lina Stern (18), all substances must pass through the liquor to penetrate the centers and exercise their influence. The bloodliquor barrier thus becomes simply the bloodbrain barrier. According to the other view (see esp. Spatz (19), Friedemann (20), distinction must be made between the bloodliquor barrier and a separate bloodbrain barrier, and substances can pass into the central nervous system without entering the Csf. However, the authors feel that here again two different problems are present. The first problem: to what extent does the Csf intervene between the blood vessels and the brain substance? The brain ventricles are in open communication with the subarachnoidal spaces and the latter continue in the form of Virchow-Robin spaces, surrounding the blood vessels, into the brain substance. According to the concept of Clara (21), these liquor-filled spaces grow gradually narrower with the decrease in the diameter of the vessels and cannot be detected in the case of blood capillaries. In this sense it could be claimed correctly that substances can enter the central nervous system without passing through the Csf. This, however, certainly is no proof of the existence of a bloodbrain barrier separate from the bloodliquor barrier. The latter constitutes an entirely different problem. It appears that the only evidence for the statement that substances may penetrate the brain although they cannot pass the bloodliquor barrier are staining experiments which, due to the many factors affecting stainability, do not represent decisive proof. Basic pigments, which cannot be detected in the liquor following their introduction in the blood stream, may, as assumed by Stern (22) become fixated in the nervous tissue. In fact, in accordance with this assumption, they would disappear after a brief period in time, even if injected directly in the liquor.

The experiments of the authors support in a decisive manner, or so they feel, the identity of the bloodbrain and bloodliquor barriers. It has been shown that with the elimination of the reflector effect of the chemoreceptors, the effect of the blood cH on the respiratory centers is apparently completely removed and that an effect corresponding to the cH of the liquor appears. This obviously would not be possible if a bloodbrain barrier with a hydrogen ion

permeability different from that of the bloodliquor barrier would actually exist. This ionic action either proceeds in accordance with the older view, by way of the Csf itself, or it passes through a bloodbrain barrier having physicochemical properties identical with those of the bloodliquor barrier, at least with respect to hydrogen ions. In any case, the nature of the liquor and its variations may be interpreted as a measure and characteristic of changes occurring in the centers and, in the absence of a better nomenclature, the variations in center activity caused in this manner are designated liquorogenic.

3. Ammonium Chloride Acidosis and the Reaction Theory

The effect of NH_4Cl evidently attains its greatest importance in connection with the theory of the chemical control of respiration. It has been shown that the hematogenic respiratory effects originating in the blood are divided into two clearly defined phenomenal complexes which can be separated experimentally: I) the effects which transfer the characteristics of the blood (specifically the blood cH) through reflection by means of the chemoreceptors. Since these effects originate in the glomera, they are designated glomerogenic (Winterstein (2). The latter must be divided into two types: a) endoglomerogenic, where metabolic processes determine the cH within the glomus as a function of oxygen pressure and b) hematoglomerogenic, where cH variations occurring primarily in the blood (e.g. through varying CO_2 contents or changes in the concentration of acids introduced from the outside, thus affecting reflex impulses originating inside.

Class II of hematogenic effects are hemato-liquorogenic, where the character of the blood directly affects the composition of the liquid surrounding the cells of the respiratory centers, by way of the bloodliquor or bloodbrain barrier. Variations of this liquid, which, as may be assumed, are indicated by the character of the Csf, generally parallel those of the blood, but not necessarily so. They may differ quantitatively, or as shown by the examples of the bicarbonate and NH_4Cl effects, may proceed in the opposite direction.

The latter statement represents findings of inestimable consequences. Initially, it destroys all foundations of the so-called "multiple factor theory" of Gray (23). Based on voluminous statistical material, Gray attempted to prove that of the three factors responsible for respiratory regulation, oxygen pressure, CO_2 pressure and cH, none is capable of providing an adequate explanation by itself so that a cooperation of all three of the interacting but individually effective factors must be assumed; this interaction can be represente by a series of mathematical equations*.

* Similarly, in order to explain accomodation to altitudes, Gray is forced to assume "changes in the excitability of respiratory centers". It has been emphasized repeatedly that such an assumption explains nothing (Winterstein (2)). According to Gray (p. 72), only an increment in effect caused by a definite increase in stimulation should be designated as sensitivity or irritability; the "irritability threshold" is as different from this as a "horse chestnut" from a "chestnut horse"... Even if such a distinction would be justifiable, no one should assume the right to arbitrarily define a concept used in biology for 200 years. In fact, the distinction is entirely trivial from a concept-analytical standpoint, since it is only necessary to define the "irritability threshold" as the increment required to obtained a certain effect with respect to 0 or an arbitrary sub-threshold value, in order to channel one concept into the other. The lack of value of the concept as a principle of explanation is not altered in any way by the definition.

The prerequisite of Gray's theory, as admitted by the author, is the assumption of a constant connection between changes occurring in arterial blood and in the cells of the brain. This requirement, as has been shown, is not satisfied. Even in cases where -- e.g. during inhalation of CO_2 -- such a proportionality may be assumed, the factor of proportionality for the glomus and for the Csf may be entirely different from that of the blood. Thus, according to Gray, the reaction theory would qualitatively correct but not quantitatively, because associated with an increase in the CO_2 pressure is a rise in respiration much greater than the related and separately calculated rise in cH. An algebraic summation of the two separately acting factors must therefore be present. In fact, however, practically nothing is known of the summation of the reflectory (glomerogenic) and the hemato-liquorogenic cH effects. In addition, the cH increment in blood corresponding to the increase in the CO_2 pressure of the respiration air will be entirely different from that of the Csf, the buffering capacity of which is disproportionally lower. The apparent additive effect of cH and CO_2 may therefore very well be a simple summation of glomerogenic and liquorogenic cH increases.

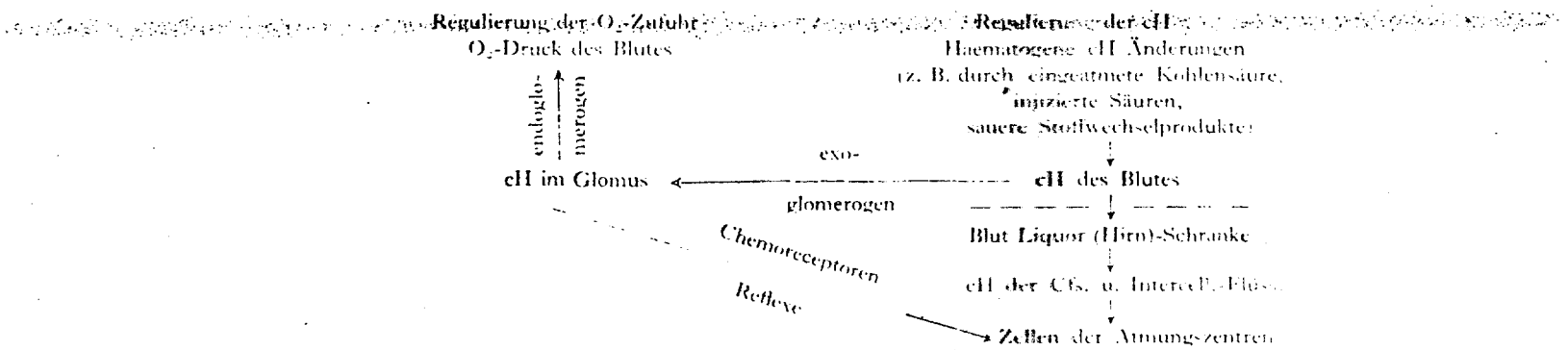
The finding that the behavior of respiratory centers depends not only on the nature of the blood but also on that of liquids located on the other side of the bloodbrain barrier, perhaps also offers a means to explain certain phenomena which heretofore defied understanding. These include, e. g. , the curious delayed effects of the inhalation of gas mixtures of different composition, such as described by Gollwitzer-Meier & al. They observed (24) a lasting effect of the excitation of respiratory centers following the inhalation of CO_2, in spite of a decline of cH and CO_2 tension below the initial value; this phenomenon was also observed after the elimination of the chemoreceptors. It is obvious that a decline of the cH of blood is not necessarily coupled with a similar effect in the Csf. While the preceding case represents a purely positive after-effect of excitation, Gollwitzer-Meier (25) observed a reduction in the respiratory volume as an after-effect of O_2-deficient respiration, which represented a negative or positive after-effect, depending on whether the experiments were performed on normal organisms (humans, dogs) or after the removal of the chemoreceptors, because the phenomenon was preceded in the first case by an increase and in the second by a decrease in respiration. A negative after-effect in a normal animal would be readily understandable, as admitted by the author, from the standpoint of the reaction theory, because with the cessation of the glomerogenic reflectory respiratory irritation, the blood cH reduced by the preceding increase in respiration must lead to a decrease in breathing. The same, however, is not immediately valid for experiments with chemereceptors eliminated, because here even initially not an increase bu a decrease in respiration takes place, which should lead to an increase in the blood cH. In fact, however, as shown by the experiments of Winterstein (26), in most cases a decrease in cH results here again, due to the enhanced reduction of oxyhemoglobin. In addition, according to this author, the decrease in pulmonary ventilation under these conditions may be caused by the inadequacy of oxidation processes in the respiratory centers, the acticity of which is insufficient for the generation of normal respiratory impulses. Acceptance of this hypothesis here again explains a positive after-effect, because the lack of O_2 in the Csf cannot be eliminated as rapidly as in the blood.

Finally, in a third series of experiments, Gollwitzer-Meier (27) detected an increase in respiration during the inhalation of pure oxygen as an after-effect of a decrease in respiration observed in normal animals and humans. Following the elimination of the chemoreceptors, enhanced respiration occurred during the inhalation of O_2 itself and remained as an after-effect. Here again, the effect observed is readily understandable in normal organisms, because the reduction in breathing must lead to an increase in blood cH through CO_2 accumulation. The effect must become detectable as soon as the weakening of the endoglomerogenic respiratory stimulation due to the oxygen ceases. The increase of the respiratory volume of animals without their chemoreceptors during increased O_2 pressure could perhaps represent a counterpoint to the reduction in breathing observed during a lack of oxygen. If both are caused by a direct dependence of center metabolism on oxygen pressure (the proper function of the glomus apparatus being the prevention of changes in oxygen pressure), the prevalence of an after-effect again becomes understandable, when blood returns to normal state.

Let us point out two facts from the standpoint of the reaction theory: the phenomenon of a barrier for respiratory effects originating in the blood represents novel proof of the fact that effects of cH and not of CO_2 but by H ions is supported convincingly by tge detection of carbonic anhydrase in the glomus (Klingenberg & Mahnert (28)). Carbonic anhydrase appears always when it is necessary to convert carbon dioxide into dissociated carbonic acid and to rapidly provide acid valences.

The reaction theory itself requires modifications based on the new findings; they are represented in the following diagram:

Reaction Theory of Respiratory Control



Key: 1. Regulation of O_2 supply O_2 pressure of blood; 2. Regulation of cH Hematogenic cH changes (e.g. through inhaled CO_2, injected acids, etc., acidic metabolic products), 3. Endoglomeragenic, 4. exo-glomerogenic 5. cH of blood; 6. bloodliquor (brain) barrier; 7. cH of Csf & intercellular liquid; 8. cells of respiratory centers; 9. chemoreceptor reflex.

Pulmonary ventilation is the chemical regulator of oxygen supply and of the tissue reaction (cH). Control of the O_2 supply, according to the discoveries of Heymans & al. (for literature see Winterstein (2), takes place by reflection through special peripheral organs, the glomera, especially the glomera carotica, which are characterized by a special sensitivity to all fluctuations of O_2 pressure and which therefore are able to maintain the oxygen atmosphere of the respiratory centers at a constant level within broad limits. The reflectory excitation of chemoreceptors occurs, as shown mainly by the investigations of Euler & al. (29), endoglomerogenically by variations of cH by metabolic processes depending on O_2 pressure. Hematogenic cH variations occurring primarily in the blood itself (e.g., through CO_2 inhalation, artificial supply of acids, acidic products of metabolism) are effective in two ways: first, through reflexes by way of chemoreceptors through changes in the cH of the glomus from the outside, exoglomerogenically or hematoglomerogencially, and second, through cH variations, which appear in the Csf and in the intercellular liquid surrounding the respiratory centers, having passed through the bloodliquor or bloodbrain barrier; they are designated hemato-liquorogenic. Fluctuations in pulmonary ventilation correspond to the algebraic sum of all glomerogenic and liquorogenic cH variations.

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I. INTRODUCTION

One of eight workers employed at a "dip brazing" zinc depositing factory in Wakamatsu-shi, Fukuoka-ken felt painful irritation in the throat while working amidst white ammonium chloride (NH_4Cl) fumes from the surface of molten zinc in a zinc bath. The symptom became aggravated after he returned home, and, following dyspnea, he died that night [1]. The exact cause of death could not be determined, but the symptoms could be related to a number of possibilities such as zinc fume fever [2-7], zinc chloride ($ZnCl_2$) or ammonium chloride (NH_4Cl) poisoning [7,9,10], etc., and the author determined to pursue the case for the clarification of the exact cause of death. There are a large number of clinical and experimental reports on zinc fume fever and toxicosis due to inhalation of $ZnCl_2$, but the literatures on the effects of NH_4Cl are limited in number. In his publication, Thompson [11] included a brief description of intoxication, characterized by collapse and severe dyspnea, caused by the inhalation of high concentration ammonium chloride fumes in a zinc electric smelting plant. Since NH_4Cl produces a volatile chloride by heating a metal oxide, it is generally used for the cleaning of metal surfaces in soldering, or the melting of zinc, platinum, nickel, etc., but Hunter [7] stated that ammonium chloride could be used for the control of fumes in a zinc production plant. In medicine, ammonium chloride is used as expectorant or diuretics [12], and in the field of industrial hygienics, its oral administration, along with low calcium diet, is prescribed for the treatment of chronic lead poisoning in order to eliminate the lead accumulated in the body [6,7]. As stated above, NH_4Cl has served many useful purposes in the form of solution, but it is assumed to exert negative effects on human respiratory system in the form of fume, although its toxicity has not yet been fully investigated. While studying the cause of death in the above case of toxicosis, the author administered a NH_4Cl solution to mice for prolonged periods of time, and observed the general symptoms, period of survival, and histopathological changes. Subsequently, he conducted NH_4Cl and $ZnCl_2$ mist inhalation tests with rabbits, and examined the general symptoms, period of survival, changes in peripheral blood and pathological image of the animals. This study provided new knowledge on the toxicity of ammonium chloride, which will be discussed below.

II. PROLONGED HYPODERMIC ADMINISTRATION OF AMMONIUM CHLORIDE (NH_4Cl) TO MICE

The concentrations at which NH_4Cl is to be administered to experimental animals were determined in a preliminary experiment. The mice were divided into two groups, the low concentration and the high concentration groups, and the toxicity of NH_4Cl was investigated by observing the changes following each injection.

SECTION 1. EXPERIMENTAL PROCEDURE

1. EXPERIMENTAL ANIMALS

Healthy, mature male and female D.D. mice weighing 20 to 30 g were used. They were fed corns, dried sea slugs and greens.

2. MODE OF ADMINISTRATION OF NH_4Cl SOLUTIONS

NH_4Cl, a special grade material of Wako Junyaku Co., was diluted with distilled water to 1 to 10% concentrations. The solutions were injected hypodermically in the thoracispinal region. The amount of each injection ranged from 0.2 to 0.5 cc. Luetin's syringe was used to inject the specific amounts of solution to the highest possible accuracy.

3. AMOUNT AND PERIOD OF ADMINISTRATION OF NH_4Cl SOLUTIONS

For the experiment involving prolonged administration of NH_4Cl solutions, the animals were divided into group A (low concentration group) and group B (high concentration group). Group A received 0.3 cc of a 1% NH_4Cl solution for the first 59 days, 0.4 cc of a 2% solution for subsequent 32 days, and 0.2 cc of 5% solution until death, as shown in Table 1. The total dose of NH_4Cl was 351.8 mg, the value being the average of 10, as shown in Table 1. Group B received 0.2 cc of a 3% solution for 32 days, 0.1 cc of a 10% solution until they died, as shown in Table 2. The total dose of NH_4Cl for group B was 234.5 mg, the value being the average of 8.

TABLE 1. HYPODERMIC ADMINISTRATION OF NH_4Cl TO GROUP A MICE

a 性別	b 個体No.	c 注射総量 mg	d 投与期間(日) 1% 0.3cc	1% 0.4cc	5% 0.2cc	e 生存期間(日)
f 雄	No. 1	281	59	26	—	85
	No. 2	293	59	29	—	88
	No. 6	281	39	26	—	85
	No. 7	415	59	32	11	102
	h 平均	317.5	—	—	—	90
g 雌	No. 3	305	59	32	—	91
	No. 4	293	59	29	—	88
	No. 5	415	59	32	11	102
	No. 8	415	59	32	11	102
	No. 9	405	59	32	10	101
	No. 10	415	59	32	11	102
	h 平均	374.5	—	—		97.7
総 i 平均		351.8	—	—	—	94.0

TABLE 2. HYPODERMIC ADMINISTRATION OF NH_4Cl TO GROUP B MICE

a 性別	b 個体No.	c 注射総量 mg	d 投与期間(日) 3% 0.2cc	10% 0.1cc	e 生存期間(日)
f 雄	No. 1	(48)	8	—	(8)
	No. 2	202	32	1	33
	No. 3	202	32	1	33
	No. 4	242	32	5	37
	No. 5	202	32	1	33
	平均	212(179.2)	—	—	34(28.8)
g 雌	No. 6	242	32	5	37
	No. 7	242	32	5	37
	No. 8	302	32	11	43
	No. 9	242	32	5	37
	No. 10	(42)	7	—	(7)
	平均	257(214)	—	—	38.5(32.2)
総 i 平均		234.5(196.6)	—	—	36.3(30.05)

Keys: a, sex; b, rabbit No.; c, total dose; d, period of administration (days); e, period of survival (day); f, male; g, female; i, total, h, total.

4. OBJECT OF EXPERIMENT

General symptoms and changes in body weight were observed until the animals died. The animals were subjected to patholohistological examination after death. Tissue specimens of major organs were fixed

with a 10% formalin solution and paraffin-embedded in order to prepare thin sections which were subsequently stained with a hematoxylin-eosin mixture.

SECTION 2. EXPERIMENTAL RESULTS

1. EFFECTS OF SINGLE LARGE-DOSE ADMINISTRATION

In order to determine the doses at which NH_4Cl should be administered to the animals in the experiment, high concentration NH_4Cl solutions were injected hypodermically. With 0.5 cc of a 10% solution and 0.25 cc of a 5% solution, the animals died within 10 minutes, with dyspnea and spasms. While 0.5 cc of a 2.5% solution caused death of one out of 6 animals, 0.25 cc of the solution merely suppressed motor activity and accelerated respiration in some of the animals. No death occurred with 0.5 cc of 1 and 2% solutions.

2. EFFECTS OF PROLONGED HYPODERMIC ADMINISTRATION

2.1 GENERAL SYMPTOMS, CHANGES IN BODY WEIGHT, AND NUMBER OF DAYS OF SURVIVAL

(1) Group A (low concentration NH_4Cl solutions). As shown in Figures 1 and 2, the body weight continued to drop for the first 2 weeks, but returned to the initial level in 7 weeks. Although some of the animals died, as shown in Table 1, neither abnormal symptom nor severe loss of weight before death was noted. Five out of 6 female mice became pregnant during the experiment. With regard to the period of survival and total dose, female mice exhibited slightly greater resistance than male mice. The time up to death became shorter with increasing dose of

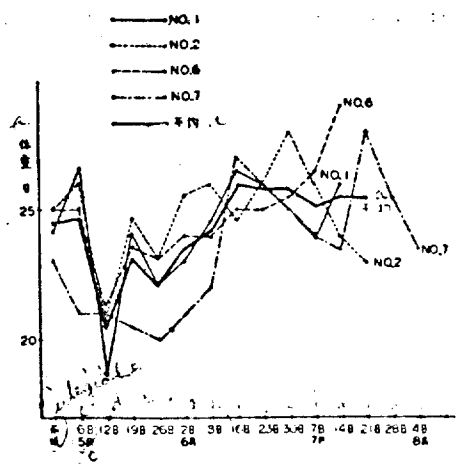


Figure 1. Changes in Body Weight following prolonged Hypodermic Administration of NH_4Cl (Group A, Male)

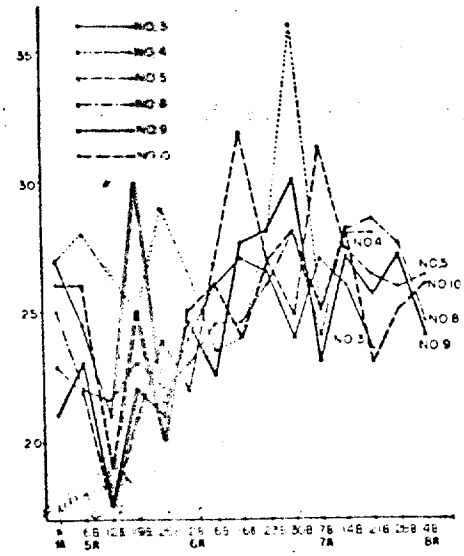


Figure 2. Changes in Body weight following prolonged hypodermic Administration of NH_4Cl (Group B, Female)

Keys: a, average; b, body weight; c, May 6, d, May 12; e, May 19; f, May 26; g, June 2, i, June 16; j, June 23; k, June 30; l, July 7; m, July 14; n, July 21; o, July 28; p, Aug. 4.

NH_4Cl. As shown in Table 1, the average period of survival was 90 days for male and 97.7 days for female, and 97.7 days for male and female inclusive.

(2) Group B (high concentration NH_4Cl solutions). The body weight continued to drop for a while but began to show a slightly upward trend as shown in Figures 3 and 4. However, some of the animals, mostly male mice, died before the initial level could be attained. With the exception of two mice which died in an early stage of the experiment, the average period of survival was 34 days for male and 38.5 days for female. As in Group A, the female mice showed stronger resistance. As compared to the 351.8 mg of group A, the lethal dose of group B was considerably lower, 234.5 mg. This experiment confirmed that a single large-dose administration of NH_4Cl exerts serious effects on laboratory animals.

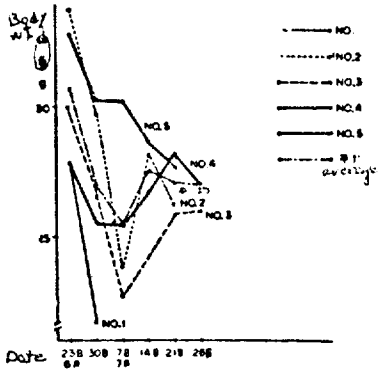


Figure 3. Changes in Body Weight following Prolonged Hypodermic Administration of NH_4Cl (Group G, male)

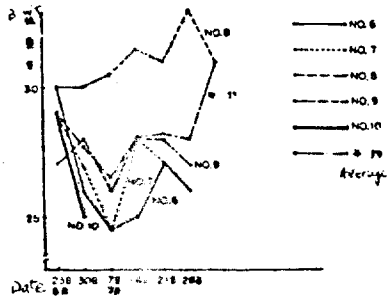


Figure 4. Changes in Body Weight following Prolonged Hypodermic Administration ofNH_4Cl (Group B, female)

2. PATHOLOHISTOLOGICAL FINDINGS

MOUSE GROUP A-NO. 2. MALE

HEART: Slightly congested. LUNGS: Localized hemorrhagic, exudative pneumonia. Apparently healthy portions were also congestive, with slightly edematous alveolar walls. Wandering cells were notably increased. The inflammatory region was marked with perivascular and peribronchial infiltration of small round cells, with black foreign particles and well developed reticular cells deposited extensively. The bronchial epithelia appeared swollen and proliferative, some, exfoliating. Healthy portions also revealed nodular aggregation of round cells and perivascular and peribronchial infiltration of small round cells.
LIVER: The central veins were congestive. The cord of liver cells showed slight distortion, with nuclear condensation. Star cells indicated absorption of dark brown pigments, appearing enlarged. The sinusoids were slightly enlarged, with foreign particles in the lumina. KIDNEYS: The cellular components of the glomeruli indicated a slight increase, and the uriniferous tubules in the cortex, slight swelling. Localized infiltrations of small round cells were noted in the interstitial connective tissue, particularly in the perivascular region. No marked change was noted in the uriniferous tubules and interstice of the

medullary substance. SPLEEN: Both lymphatic follicles and the sinuses of the spleen contained masses of hemosiderin particles. ADRENALS: Normal. PANCREAS: Normal. TESTES: The normal structure of germinal epithelia of the tubules was distorted.

MOUSE GROUP A-NO. 5, FEMALE.

HEART: Normal. LUNGS: Slightly congested. The alveolar walls exhibited edematous thickening. The epithelial cells of bronchia and bronchioli were swollen and proliferative, with foreign particles and phagocytes ingesting the particles. LIVER: The central veins were congestive. There were architectural distortion of the cord of liver cells and pronounced turbidity and swelling of liver cells. Star cells grew in size and number, ingesting black foreign particles. Glisson's capsule was marked with pronounced infiltration of small round cells and masses of black foreign particles and hemosiderin deposit. Small round cells were also contained in hepatic lobules, but not to any notable degree. (Cf. photograph 6) KIDNEYS: The glomeruli appeared normal. The convoluted tubules in the cortex exhibited slight epithelial swelling, with severe localized infiltrations of small round cells in some areas. The medullary substance remained normal (cf. photograph 11). SPLEEN: The connective tissue and reticulo-endothelial system of the lymphatic follicles proliferated causing morphological distortion. Phagocytes were also present. The sinuses of the spleen indicated splanchnostaxis, and proliferation of reticulo-endothelial system and connective tissue. Hyaline degeneration was also present in some areas. Giant spleen cells showed an increase in number (cf. photograph 14). ADRENALS: Vacuolar degeneration was noted over the fascicular and reticular zones, and black foreign particles appeared in the boundary between the reticular zone and the medullary substance. PANCREAS: Normal. OVARIES: Normal.

MOUSE GROUP A-NO. 7, FEMALE.

HEART: Normal. LUNGS: Localized hemorrhagic bronchopneumonia, with pronounced infiltration of small round cells and swelling of bronchial epithelia in the lesion. An increase in reticular cells was also noted. Small round cells infiltrated around blood vessels and bronchia of apparently healthy regions, and wandering cells grew in number in the alveolar walls (cf. photograph 1). LIVER: The normal architecture of the cord of liver cells was distorted, with marked irregularity in the size of liver cells. Relatively large legions of hemorrhagic coagulation necrosis were noted in hepatic lobules. In some areas of lobules, nuclear condensation, morphological distortion, and turbidity of interstitial cells were eminent. Star cells grew in size and number, and Glisson's capsule displayed severe infiltration of small round cells and accumulations of black foreign particles and hemosiderin deposits. The cellular components of the sinusoids showed an increase (cf. photograph 7). KIDNEYS: Generally congested. The glomeruli and Bowman's capsule were joined by adhesion, and the epithelia of the connecting main tubules were degenerated and exfoliated, with localized infiltration of small round cells in that region. Small round cells also appeared in the convoluted uriniferous tubules, the interstice and perivascular connective tissue of the cortex. No abnormal

change was noted in the medullary substance (cf. photograph 12). SPLEEN: The lymphatic follicles were atrophic and irregular in size. The sinuses of the spleen were distended with blood, showing well-developed reticulo-endothelial system. Black foreign particles and hemosiderin deposit were slightly increased. The splenic pulp appeared without abnormality. PANCREAS, OVARIES: Normal.

MOUSE GROUP A-NO. 8, FEMALE.

HEART: Normal. LUNGS: Slightly congested. There was a small amount of black foreign particles, but no major abnormality was noted. LIVER: The cord of liver cells appeared distorted, and the nuclei were irregular in shape and size. Some liver cells appeared turbid. The central veins were severely congested, with sporadic hemorrhagic lesions. In the lobules, the liver cells indicated small localized coagulation necrosis and atrophy, surrounded by small round cells. Star cells increased in number and enlarged, exhibiting an image of having ingested black foreign particles and hemosiderin. Glisson's capsule was marked with pronounced infiltration of small round cells, with black particles and hemosiderin deposited to an intermediate degree. KIDNEYS: The glomeruli varied in size to some degree, but no marked change was noted in the cortical or medullary tubules. Interstitial infiltration of small round cells was present but slight. SPLEEN: The lymphatic follicles and the sinuses of the spleen exhibited proliferation of reticulo-endothelial system and connective tissue. Giant spleen cells showed a notable increase in the sinuses of the spleen. Hemosiderin and black particles were widely distributed. No marked change was present in the splenic pulp (cf. photograph 15). PANCREAS AND OVARIES: Normal.

MOUSE GROUP A-NO. 10. FEMALE.

HEART: Slight interstitial infiltration of round cells was noted. LUNGS: Slightly congested. The alveolar walls were edematous. The alveoli and their walls were filled with blood corpuscles and wandering cells, occasionally revealing cells with hydropic degeneration. The epithelia of the bronchial system indicated proliferation, partly exfoliating in the lumina. Pronounced infiltration of small round cells was noted around the blood vessels and bronchial system, and nodular masses of small cells were dispersed in the lung fields. There were accumulations of black foreign particles.(cf. photograph 2). LIVER: The cord of liver cells was obliterated. Localized coagulation necrosis was observed in lobules, with localized infiltration of small round cells and hemosiderin deposit. Generally, the nuclei of liver cells varied in size, some being giant nuclei. Star cells grew in size and number, exhibiting a clear image of having ingested black particles and hemosiderin. Glisson's capsule indicated infiltration of small round cells and accumulation of hemosiderin (cf. photograph 8). KIDNEYS: No abnormal change was noted in the glomeruli or tubules in the cortex or medullary substance. Slight infiltration of small round cells was present in the interstices. SPLEEN: Proliferation of reticulo-endothelial system and connective tissue was observed in the lymphatic follicles. The splenic pulp indicated slight increase and fibroid degeneration. There were increased number of of giant cells. No abnormal change appeared in the adrenals, spleen, or ovaries.

MOUSE GROUP B-NO. 3, MALE.

HEART: Normal. LUNGS: Relatively severe hemorrhagic, exudative bronchopneumonia. The infiltration of small round cells around the blood vessels and bronchial system was highly advanced, and an overall edematous image was noted. The epithelia of the bronchial system were swollen and some, exfoliated. The accumulation of black foreign particles was relatively conspicuous. LIVER: The cord of liver cells exhibited distortion, with nuclear irregularity. Vacuolar degeneration was also present. Lobules were marked with diffuse infiltrations of small round cells, with growing star cells, which had absorbed black particles and hemosiderin, reaching the size of a liver cell and aggregating in masses in some areas. Glisson's capsule also revealed pronounced infiltration of small round cells (cf. photograph 5). KIDNEYS: Generally congested. The glomeruli became enlarged, over-extending in Bowman's capsule. The uriniferous tubules in the cortex exhibited a tendency of swelling. The medullary substance was normal. SPLEEN: Slight fibroid degeneration appeared in the sinuses of the spleen and lymphatic follicles. A slight increase of spleen cells was observed. TESTES: The germinal epithelia exhibited distortion, with a decrease in spermatozoa. No marked change was noted in interstitial Leydig's cells.

MOUSE GROUP B-NO. 6, FEMALE.

HEART: Normal. LUNGS: Hemorrhagic, hydropic, and exudative bronchopneumonia. Pronounced infiltration of small round cells was noted around the vascular and bronchial systems. The swelling, proliferation, and exfoliation of bronchial epithelia was also notable. The alveoli contained cells with hydropic degeneration and reticular cells, and their walls revealed many wandering cells and black foreign particles. There were sporadic distribusion of dead small round cells. LIVER: The cord of liver cells appeared obliterated. There were many star cells which had grown in size due to active ingestion of black particles and hemosiderin. Slight infiltration of small round cells was present in Glisson's capsule. The accumulation of hemosiderin was particularly notable in Glisson's capsule, forming round masses. KIDNEYS: The glomeruli appeared normal, but slight epithelial destruction was noted in the uriniferous tubules in the cortical region. Interstitial infiltration of small round cells was particularly severe in perivascular connective tissue. SPLEEN: The lymphatic follicles were atrophic. In the sinuses of the spleen, the accumulation of hemosiderin and the number of giant cells showed slight increases. ADRENALS: Slight, diffuse infiltration of small round cells occurred over the fascicular and reticular zones of the cortex. Aggregations of black foreign particles and hemosiderin were present in the reticular zone. PANCREAS: Normal. OVARIES: Normal.

MOUSE GROUP B-NO. 7, FEMALE.

HEART: Normal. LUNGS: Hemorrhagic, hydropic bronchial Pneumonia. Pronounced infiltration of small round cells was noted around the vascular and bronchial systems. Alveolar walls indicated hydropic degeneration, and the alveoli were obstructed by blood corpuscles, wandering cells,

and cells exhibiting hydropic degeneration. Bronchial epithelia showed swelling, proliferation and exfoliation in some portions. LIVER: Pronounced congestion of the central veins. The cord of liver cells exhibited distortion, with nuclear irregularity. Liver lobules were marked with the atrophy of liver cells and localized necrosis, accompanied by the infiltration of small round cells. There was pronounced proliferation of star cells. Absorption of hemosiderin and black foreign particles was clearly noted. Glisson's capsule also indicated infiltration of small round cells and accumulation of hemosiderin and black particles. KIDNEYS: The glomeruli were normal. The epithelial cells of the uriniferous tubules in the cortical region were destroyed and gelatinous substances were present in the lumina. There was infiltration of small round cells in the perivascular connective tissue. SPLEEN: The lymphatic follicles were irregular and contained hemosiderin. No marked change was noted in the splenic pulp. ADRENALS: Pronounced congestion was noted in the reticular zone of the cortex and the medullary substance. OVARIES: Normal.

MOUSE GROUP B-NO. 8, FEMALE.

HEART: Normal. LUNGS: Normal. LIVER: Liver cells exhibited nuclear irregularity, occasionally nuclear destruction. Star cells grew in size and number, showing active absorption of black particles and hemosiderin, and occasionally aggregating in the size of a liver cell. Glisson's capsule indicated infiltration of small round cells and accumulations of hemosiderin and black foreign particles. KIDNEYS: The glomeruli varied in size to a marked degree. The uriniferous tubules remained without change. Interstitial infiltration of small round cells was present. ADRENALS: Slight cortical congestion and medullary infiltration of small round cells were observed. The fascicular zone exhibited hydropic degeneration, with swollen cells. (See photographs 17 and 18).

MOUSE GROUP B-NO. 9, FEMALE.

HEART: Normal. LUNGS: Hemorrhagic and exudative bronchopneumonia. Pronounced accumulation of black hemosiderin particles was noted. The alveoli and their walls contained numerous reticular cells, wandering cells, and phagocytes of foreign particles. Perivascular and peribronchial infiltration of small round cells was present. LIVER: The congestion of the central veins was extremely severe. The cord of liver cells appeared obliterated, and hepatic lobules indicated localized infiltrations of small round cells. Star cells grew in size and number, having ingested black hemosiderin particles, occasionally aggregating in the size of a liver cell. Glisson's capsule also showed hemosiderin deposits. KIDNEYS: Notable congestion was present, but the glomeruli and uriniferous tubules remained without change. SPLEEN: The lymphatic follicles exhibited irregularity. The accumulation of hemosiderin was slight but present in the follicles and sinuses of the spleen.

III. AMMONIUM CHLORIDE AND ZINC CHLORIDE SOLUTION MIST INHALATION EXPERIMENT

In order to investigate the effects of NH_4Cl on the respiratory

tract, changes caused by the inhalation of $ZnCl_2$ mist were also studied for a comparative purpose. Since ammonium chloride is highly deliquescent, and cannot be inhaled in the form of powder, both NH_4Cl and $ZnCl_2$ were administered in the form of mist.

SECTION 1. EXPERIMENTAL PROCEDURE

1. EXPERIMENTAL ANIMALS

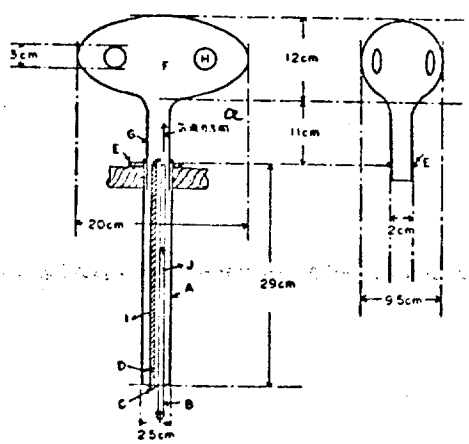
Male, mature white rabbits weighing approximately 2 kg were raised for a given period, and only those in good health were employed. They were fed 300 g of bean curd refuse and greens. Five (Nos. 1 - 5) were subjected to NH_4Cl mist and 4 (Nos. 6 - 9), to ZnCl mist.

2. EXPERIMENTAL MATERIALS

NH_4Cl and $ZnCl_2$, special grade materials of Wako Junyaku Co., were diluted with distilled water to prepare 10, 20, and 30% NH_4Cl solutions and a 10% $ZnCl_2$ solution, which were administered to the animals in the form of mist by means of the following equipment.

3. EQUIPMENT AND METHOD OF ADMINISTRATION

A special equipment, shown in Figure 5, was designed for the mist formation of the NH_4Cl and $ZnCl_2$ solutions, and placed in the center of the inhalation chamber. The inhalation chamber, of low cylindrical shape,



A: Liquid tank duct (calibrated to 70 cc)
B: Joint to the air duct
C: Liquid tank cleaning hole (with a rubber plug)
D: Small hole
E: Mount
F: Cap with windows
G: Can be detached from the tank
H: Window
I: Capillary
J: Air duct

Key: a, direction of air flow

Figure 5. Schematic Diagram of the Sprayer

could house 8 rabbits, fixed radially at equal intervals. A schematic diagram of the sprayer appears in Figure 5. A flow of air is introduced with an air pump under specific pressure. The pressure is kept at a specific level at the height of the wind pressure indicator by controlling the faucet. The amount of air was measured with a flow meter. Mist was produced by the principle of a mist sprayer with the equipment shown in Figure 6. The air sent by the pump is pushed stright upward from the pointed glass head of the sprayer, and the negative pressure thereby caused transforms the solution into mist. At this time, large droplets

condense on the wall of the cover and are returned to the liquid duct, thus only small particles emerge into the inhalation chamber through the circular side windows. The interior of the chamber is so designed that the moisture due to the mist, large droplets and dews be prevented and the entire solution be consumed by the rabbits in the form of mist. During the experiment, the interior of the chamber was filled with dense white-fume-like mist until the animals were no longer visible.

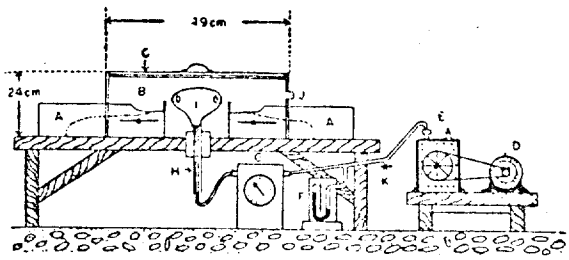


Figure 6. Mist Inhalation Device

A: Device for fixing animals
B: Inhalation chamber (124,556 l)
C: Transparent canopy
D: Motor
E: Air pump
F: Wind pressure indicator
G: Flow meter
H: Calibrated liquid duct of the sprayer
I: Cap with windows of the sprayer
J: Window
K: Faucet for controlling the amount of air.

4. AMOUNT OF ADMINISTRATION, INHALATION TIME, AMOUNT OF AIR FLOW, AND PERIOD OF EXPERIMENT

The single, daily dose of the solutions at various concentrations was 50 cc. The animals were subjected to the experiment once daily before feeding. Efforts were made to maintain a specific period of inhalation throughout the experiment and to have the entire solution inhaled by the animals. As shown in Tables 3 and 4, the average period of time required for the complete inhalation of the solution was 60, 96.2, and 98.6 minutes for the 10, 20, and 30% NH_4Cl solutions, respectively, and 72.7 minutes for the 10% $ZnCl_2$ solution. In terms of the amount of air, an average of 820.02, 147.57, 1501.34, and 1205.81 liters were required for the 10, 20, and 30% NH_4Cl solutions and the 10% $ZnCl_2$ solution, respectively. The NH_4Cl mist group were subjected to the test at a concentration of 10% for 25 days, 20% for 20 days, and 30% for 7 days. The period of experiment was 56 days. The experiment with the 10% $ZnCl_2$ solution required only 11 days. The total inhalation times of for the NH_4Cl and $ZnCl_2$ are shown in Table 4.

TABLE 4. NUMBER OF DAYS, INHALATION TIME AND AMOUNT OF AIR IN AVERAGE OF THE INHALATION EXPERIMENT USING RABBITS

項目 / 吸入濃度 c 及び種類 b	吸入時間(分)	1日平均吸入時間 d (分)	総風量 (l) e	1日平均風量 (l)	総時間(分)	1日平均吸入時間 h (分)	総風量 (l)	1日平均風量 (l)
10% $ZnCl_2$	800	72.72	13263.94	1205.81	—	—	—	—
10% NH_4Cl	1500	60.00	20500.73	820.02	—	—	—	—
20% NH_4Cl	1923	96.15	29451.58	1472.57	3423	76.06	49952.31	1110.05
30% NH_4Cl	690	98.60	10509.42	1501.34	4113	79.09	60461.73	1162.72

Keys: a, concentration; b, solution; c, ihhalation time (min); d, average daily inhalation time (min); e, total amount of wind (l); f, average daily amount of wind; g, total time (min); h, daily inhalation time in average (min); i, total amount of wind; j, daily amount of wind

TABLE 3. NUMBER OF DAYS, INHALATION TIME, AND AMOUNT OF WIND OF THE INHALATION EXPERIMENT USING RABBITS (56-day experiment)
(No inhalation test was performed for two days after the completion of each of the 10% and 20% NH_4Cl mist experiments.)

10% $ZnCl_2$			10% NH_4Cl			20% NH_4Cl			30% NH_4Cl		
a 吸入日数(日)	b 吸入時間(分)	c 風量 (ℓ)	a 吸入日数(日)	b 吸入時間(分)	c 風量 (ℓ)	a 吸入日数(日)	b 吸入時間(分)	c 風量 (ℓ)	a 吸入日数(日)	b 吸入時間(分)	c 風量 (ℓ)
1	45	696.05	1	60	511.25	26	105	1416.93	46	120	1981.41
2	60	1470.19	2	60	761.90	27	60	1058.00	47	65	1024.95
3	55	907.60	3	60	685.45	28	60	935.08	48	125	1173.16
4	55	905.03	4	60	781.45	29	85	1204.05	49	110	1179.13
5	60	959.02	5	60	838.15	30	63	1110.00	50	95	1640.50
6	90	1 50.47	6	60	773.90	31	80	1416.95	51	75	1675.27
7	110	1828.49	7	60	754.32	32	80	1313.29	52	100	1835.00
8	90	1415.00	8	60	806.73	33	85	1416.05	d 累計	690	10509.42
9	80	1030.36	9	60	921.93	34	90	1400.75	e 平均	98.60	1501.34
10	70	1155.41	10	60	822.77	35	75	1276.98			
11	85	946.32	11	60	800.03	36	85	1308.24			
d 累計	800	13263.94	12	60	821.35	37	105	1710.98			
e 平均	72.72	1205.81	13	60	847.95	38	115	1822.02			
			14	60	914.57	39	165	2796.72			
			15	60	759.01	40	95	1651.14			
			16	60	767.13	41	135	1785.43			
			17	60	877.29	42	95	1463.18			
			18	60	908.88	43	90	1491.27			
			19	60	890.93	44	90	1551.82			
			20	60	877.90	45	85	1522.70			
			21	60	850.95	d 累計	1923	29451.58			
			22	60	854.01	e 平均	96.15	1472.57			
			23	60	944.82						
			24	60	851.91						
			25	60	786.15						
			d 累計	1500	20500.73						
			e 平均	60	820.02						

Keys: a, number of days; b, inhalation time (min); c, amount of wind; d, total; e, average

5. OBJECT OF OBSERVATION

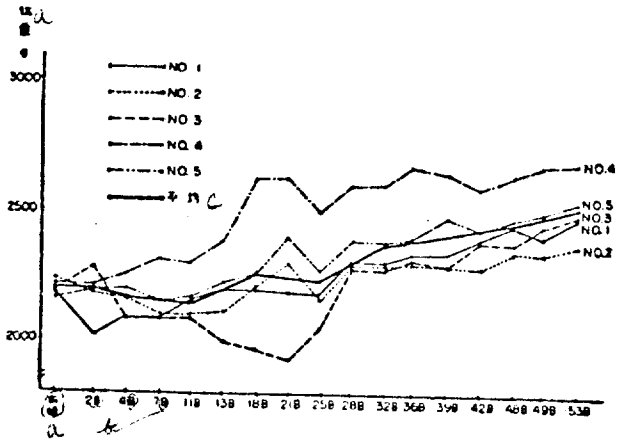
Changes in body weight, general condition, and peripheral blood were observed. Upon death or completion of the experiment, the animals were killed by inducing air embolism and subjected to autopsy. The patholohistological specimens of the lungs and other major internal organs were prepared by the method described in Part I. The peripheral blood was studied by drawing samples from the auricular vein before and after inhalation of the mist. The blood corpuscle melangeurs (C.95551, B.77033, C.95282, C.95287, C.95582), leucocyte melangeur (C.932926, B.30938, C.96427, B.27778, B.36490) and Thoma's counting cell (K.3075) approved by the Japan Hamatological Society were used.

SECTION 2. EXPERIMENTAL RESULTS

1. CHANGES IN GENERAL CONDITION AND BODY WEIGHT

(1) NH_4Cl MIST GROUP

Some of the rabbits in the inhalation chamber became hyperactive for a while after the start of the experiment, but soon became calm. None indicated abnormal symptoms such as dyspnea. This was observed in the animals of the 10, 20, and 30% solution groups without exception. After each inhelation test, the animals were willing to eat, showing no sign of conjuctival injection, wheezing or dyspnea. As shown in Figure 7, and Tables 5 and 6, some indicated loss of weight for the first few days during a total of 55 days with 52 tests, but the initial level was soon recovered and some even indicated further gain. None of the 5 died nor developed abnormal symptoms or lack of appetite.



Keys: a, initial value; b, days; c, average

Figure 7. Changes in Body Weight following the Inhalation of NH_4Cl Mist (Male Rabbits)

TABLE 5. CHANGES IN BODY WEIGHT FOLLOWING INHALATION OF NH_4Cl MIST (MALE RABBITS)

吸入日数 a	No. 1	No. 2	No. 3	No. 4	No. 5
前 初 b	2195 g	2240 g	2200 g	2210 g	2260 g
2(日)	2020	2190	2280	2210	2210
4 〃	2090	2160	2090	2250	2250
7 〃	2090	2100	2090	2310	2150
11 〃	2160	2100	2090	2300	2170
13 〃	2200	2100	2000	2390	2230
18 〃	2200	2210	1970	2630	2260
21 〃	2190	2300	1930	2630	2440
25 〃	2180	2170	2050	2500	2270
28 〃	2310	2300	2280	2610	2580
32 〃	2310	2300	2280	2610	2580
36 〃	2350	2310	2320	2690	2410
39 〃	2350	2300	2300	2660	2490
42 〃	2400	2290	2390	2600	2440
46 〃	2460	2350	2390	2650	2480
49 〃	2420	2350	2460	2690	2530
53 〃	2490	2380	2490	2700	2550

Keys: a, number of days; b, initial value; c, days

TABLE 6. CHANGES IN THE AVERAGE VALUES OF BODY WEIGHT, ERYTHROCYTE AND LEUKOCYTE COUNTS FOLLOWING INHALATION OF NH_4Cl MIST

吸入日数 a	体重(g) b	赤血球×10⁴ c	白血球 d
前 値 e	2200	545	6400
4 日 目 f	2158	501	5800
11 〃	2164	510	7200
18 〃	2254	570	8400
25 〃	2234	590	7900
32 〃	2378	591	6800
48 〃	2466	573	7200
55 〃	2522	509	10700

Keys: a, number of days; b, body weight; c, erythrocyte count; d, leukocyte count; e, initial value; f, days

(2) $ZnCl_2$ MIST GROUP

Some of the rabbits indicated a hyperactive tendency in the beginning of the experiment, and the tendency was more notable than that of the previous group. After the experiment, the animals exhibited weakness and hypomotility. Rabbits No. 8 and No. 6 which died during the experiment indicated cyanotic symptoms such as bluish purple lips and eyeballs and watery cornea when removed from the chamber. Tachypnea was indicated by most of the animals, but No. 8 and No. 6 developed dyspnea marked with alar collapse, severe cyanosis and wheezing shortly before death. However, none indicated bleeding from the conjuctiva, lips, or nasal membrane, signs of inflammation or ulceration, or depilation. As stated above, No. 8 and No. 6 died with severe dyspnea on the 2nd and 5th days, respectively, of the experiment. A slight drop in body weight was noted, but death occurred before severe loss of weight. The surviving two indicated no marked change after the 11 days of the experiment, except for somewhat wheezy respiratory sound.

Thus, the changes in general condition, such as respiratory disturbance, and body weight in the 10% $ZnCl_2$ mist group were more notable than those of the 10, 20, and 30% NH_4Cl mist group.

2. CHANGES IN PERIPHERAL BLOOD

2.1 ERYTHROCYTE COUNT

(1) NH_4Cl MIST GROUP

As shown in Figure 8, and Tables 6 and 7, the erythrocyte count dropped slightly for the first two weeks of the 10% solution experiment, but returned to normal subsequently. Even with the 20% solution, changes were slight. With the 30% solution, slight decrease began to show in one week. However, in the 30% solution experiment, changes were not notable even before or after the experiment, as shown in Table 8.

(2) $ZnCl_2$ MIST GROUP

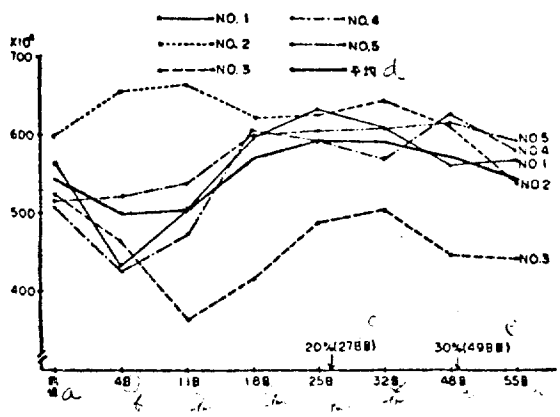


Figure 8. Changes in Erythrocyte Count following Inhalation of the mist of NH_4Cl solutions.

Keys: a, initial value; b, days; c, 27th day; d, average; e, 49th day.

TABLE 7. CHANGES IN ERYTHROCYTE COUNTS FOLLOWING INHALATION OF NH_4Cl SOLUTION MIST

吸入日数 a	No. 1 ×10⁶	No. 2 ×10⁶	No. 3 ×10⁶	No. 4 ×10⁶	No. 5 ×10⁶
前 値 b	563	609	526	507	520
4日目 c	432	658	466	428	523
11 〃	504	664	364	479	541
18 〃	599	623	420	607	603
25 〃	632	629	489	595	606
32 〃	612	647	506	579	610
48 〃	563	613	448	627	615
55 〃	567	560	443	580	590

Keys: a, number of days; b, initial value; c, days

TABLE 8. DIFFERENCE IN ERYTHROCYTE COUNT BEFORE AND AFTER THE INHALATION OF THE MIST OF A 30% NH_4Cl SOLUTION

家兎 No. a	吸入前値 b ×10⁶	100分吸入後値 c	差 d ×10⁶
No. 1	567	556 ×10⁶	−11
No. 2	565	612	+47
No. 3	443	420	−23
No. 4	585	552	−33
No. 5	589	528	−61
平均 e	546.8	533.6	−16.2

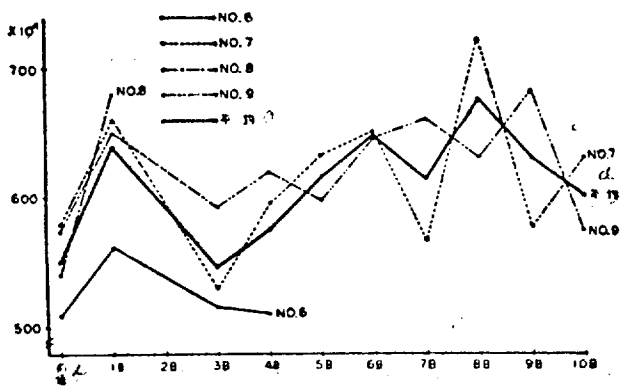
Keys: a, rabbit No.; b, initial value; c. value after 100 minutes of inhalation; d, difference; e, average.

As shown in Figure 9, and Tables 9 and 10, a slight variation was noted. Examining the changes shown by the 4 cases including two which died, over a period of 11 days, slight increases were noted before and after the first inhalation experiment, and the changes became more notable in 24 hours. However, the normal condition was recovered in 72 hours, the 4th day, and the values subsequently indicated an upward trend.

2.2 LEUKOCYTE COUNT

(1) NH_4Cl MIST GROUP

As shown in Figure 10 and Table 11, slight variation was noted upon inhalation of the mist of the 10 and 20% NH_4Cl solutions, but the changes were not significant. An elevation in leukocyte count from the initial 7200 to 10700/mm^3 in average was noted one week after the inhalation of the 30% solution, clearly indicating hyperleukocytosis. However, as compared to the minimal change before and after the inhalation of the 10% solution mist, shown in Table 12, the elevated leukocyte count showed a downward trend before and after the 30% solution mist experiment, from the initial 9720 to 7400/mm^3 in average.



a, average
b, initial value
c, day(or days)
d, average

Figure 9. CHANGES IN ERYTHROCYTE COUNT FOLLOWING THE INHALATION OF THE MIST OF A $ZnCl_2$ SOLUTION

TABLE 9. CHANGES IN ERYTHROCYTE COUNT FOLLOWING A 10% $ZnCl_2$ SOLUTION MIST INHALATION EXPERIMENT

家兎 No.	1日目	2日目	4日目	5日目	6日目	7日目	8日目	9日目	10日目	11日目
No. 6	50900	56200	51600	51100	死亡	—	—	—	—	—
No. 7	57900	66200	53000	59600	63400	65300	56700	72500	57800	63000
No. 8	54100	68100	死亡	—	—	—	—	—	—	—
No. 9	57400	65100	59200	62000	59800	64700	66100	63100	68400	57500
平均	55100	63900	54600	57600	61600	65000	61400	67800	63100	60250

Keys: a, rabbit No.; b, days; c, died; d, average

TABLE 10. DIFFERENCES IN ERYCHROCYTE COUNT BETWEEN BEFORE AND AFTER THE INHALATION OF THE MIST OF A 10% $ZnCl_2$ SOLUTION

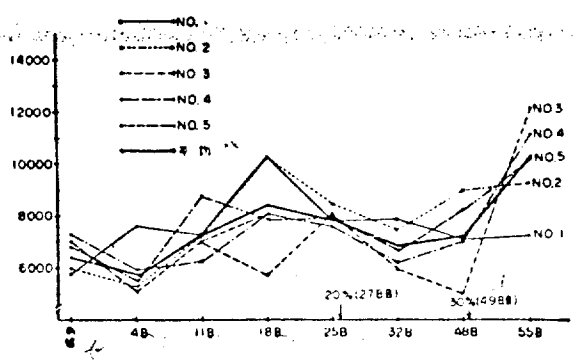
家兎No. a	1 日 g			2 日 g			7 日 g		
	吸入前値 c	吸入後値 d	差 e	吸入前値 c	吸入後値 d	差 e	吸入前値 c	吸入後値 d	差 e
No. 6	50900	52700	+1800	56200	51900	−4300	死 f	—	—
No. 7	57900	59300	+1400	66200	67200	+1000	65300	64100	−1200
No. 8	54100	61600	+7500	68100	66900	−1200	死 f	—	—
No. 9	57400	63000	+5600	65100	67100	+2000	647[illegible]	63200	−1500
平均 b	55100	59200	+4100	63900	63300	1600	65[illegible]	63700	−1350

Keys: a, rabbit No.; b, average; c, value before inhalation; d, value after inhalation; e, difference; f, died; g, day (or days)

(2) $ZnCl_2$ GROUP (AT A 10% CONCENTRATION)

As shown in Figure 11, and Tables 13 and 14, no significant change was noted before and after the first inhalation test, but an increase from the initial 9000 to 14500/mm^3 in average was noted 24 hours later, before the 2nd inhalation test. Subsequently, values of approximately 15000/mm^3 were maintained for 10 days, after which clear hyper-leukocytosis was indicated. Rabbit No. 8 which died after the third day of inhalation showed an abrupt increase, to 25700/mm^3 after the third test. No specific pattern was shown by the changes in leukocyte count before or after each mist inhalation test, as shown in Table 14.

As stated above, hypderleukocytosis was indicated in 24 hours by the peripheral blood of those subjected to the mist of 10% Zn solution, but the mist of the 10 and 20% NH_4Cl solutions caused no such abnormality and a hyperleukocytosis was present only in the experiment with the mist of the 30% NH_4Cl solution. This experiment also revealed that the mist of NH_4Cl solution exhibited lower toxicity than the mist of a ZnOH solution.



Keys: a, average
b, initial value
c, 27th day
d, 49th day
e, days

Figure 10. Changes in Leukocyte Count following Inhalation of the Mist of NH_4Cl Solutions,

TABLE 11. CHANGES IN LEUKOCYTE COUNT FOLLOWING INHALATION OF THE MIST OF NH_4Cl SOLUTIONS

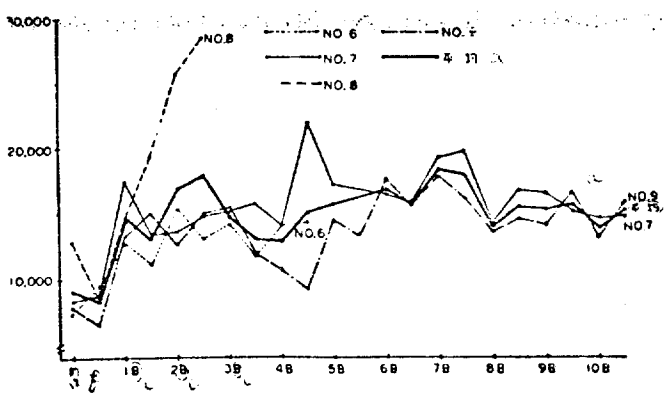
吸入日数 a	No. 1	No. 2	No. 3	No. 4	No. 5
前 値 b	5600	6000	6600	7200	6800
4日目 c	7600	5200	5000	5800	5200
11 〃	7200	7200	7000	6200	8600
18 〃	10200	10200	5600	8000	7800
25 〃	7800	8400	8000	7600	7800
32 〃	7800	7400	6000	6200	6600
48 〃	7000	9000	5000	7200	8200
55 〃	11200	9200	12000	11000	10200

Keys: a, number of days; b, initial value; c, days

TABLE 12. DIFFERENCES IN LEUKOCYTE COUNT BEFORE AND AFTER THE 10% and 30% NH_4Cl SOLUTION MIST INHALATION EXPERIMENTS

家兎No. a	NH_4Cl 10%溶液 f 吸入前値 b	吸入後値 60分 c	差 d	NH_4Cl 30%溶液 g 吸入前値 b	吸入後値 100分 e	差 d
No. 1	—	—	—	10300	7000	−3300
No. 2	—	—	—	9800	8000	−1800
No. 3	—	—	—	8000	6200	−1800
No. 4	7200	6800	−400	10100	7600	−2500
No. 5	6800	6800	0	10400	8200	−2200
平 均 h	7000	6800	−200	9720	7400	−2320

Keys: a, Rabbit No.; b, initial value; c, value after inhalation (60 min); d, difference; e, value after inhalation (100 min); f, 10% solution; g, 30% solution; h, average



Keys: a, average; b, initial value; c, day (or days)

Figure 11. Changes in Leukocyte Count following the Inhalation of the mist of a 10% $ZnCl_2$ Solution (values before and after inhalation test)

TABLE 13. CHANGES IN LEUKOCYTE COUNT FOLLOWING INHALATION OF A 10% $ZnCl_2$ SOLUTION MIST (VALUES BEFORE INHALATION TEST)

家兔No.	9月18日	19日	20日	21日	22日	23日	24日	25日	26日	28日	29日
No. 6	7300	12800	15300	14200	14100	死亡	—	—	—	—	—
No. 7	8100	17400	13500	15000	14000	17200	16600	19300	14400	16900	14900
No. 8	12800	14600	25700	死亡	—	—	—	—	—	—	—
No. 9	7800	13300	12600	15400	10800	14400	17500	17900	13700	14200	13200
平均	9000	14500	16800	14800	12900	15800	17100	18600	14100	15550	14050

Keys: a, rabbit No.; b, September 18; c, 9/19; d, 9/20; e, 9/21; f, 9/22; g, 9/23; h, 9/24; i, 9/25; j, 9/26; k, 9/28; l, 9/29; m, died; n, average

TABLE 14. CHANGES IN LEUKOCYTE COUNT BEFORE AND AFTER THE INHALATION OF THE MIST OF A 10% $ZnCl_2$ SOLUTION

家兔No.	9月18日			19日			20日			21日		
	吸入前值	吸入後值	差	吸入前值	吸入後值	差	吸入前值	吸入後值	差	吸入前值	吸入後值	差
No. 6	7300	9400	+2100	12800	11100	-1700	15300	13000	-2300	14200	11700	-2500
No. 7	8100	8500	+400	17400	13200	-4200	13500	14900	+1400	15000	15700	+700
No. 8	12800	8600	-4200	14600	19400	+4800	25700	28600	+2900	死亡	—	—
No. 9	7800	6400	-1400	13300	15000	+1700	12600	15000	+2400	15400	11800	-3600
平均	9000	8200	-775	14500	13000	+600	16800	17900	+1100	14800	13100	-1800

家兔No.	22日			23日			24日			25日		
	吸入前值	吸入後值	差	吸入前值	吸入後值	差	吸入前值	吸入後值	差	吸入前值	吸入後值	差
No. 6	14100	14400	+300	死亡	—	—	—	—	—	—	—	—
No. 7	14000	21900	+7900	17200	13400	-3800	16600	15700	-900	19300	20000	+700
No. 8	—	—	—	—	—	—	—	—	—	—	—	—
No. 9	10800	9100	-1700	14400	13100	-1300	17500	15400	-2100	17900	16300	-1600
平均	12900	15100	+6500	15800	13300	-2550	17100	15600	-1500	18600	18200	-450

家兔No.	26日			28日			29日		
	吸入前值	吸入後值	差	吸入前值	吸入後值	差	吸入前值	吸入後值	差
No. 6	—	—	—	—	—	—	—	—	—
No. 7	14400	17000	+2600	16900	15400	-1500	14900	15100	+1800
No. 8	—	—	—	—	—	—	—	—	—
No. 9	13700	14700	+1000	14200	16800	+2600	13200	14800	+2600
平均	14100	15900	+1800	15550	16100	+550	14050	14950	+2200

Keys: a, rabbit No.; b, September 18; c, 9/19; d, 9/20; e, 9/21 f, 9/22; g, 9/23; h, 9/24; i, 9/25; j, 9/26; k, 9/28; l, 9/29; m, average; n, value before inhalation; o, value after inhalation; p, difference.

3. PATHOLOGICAL FINDINGS

3.1 NH_4Cl MIST GROUP

(1) ANATOMICAL FINDINGS: All of the animals indicated sporadic localized hemorrhagic lesions in the lungs, but no gross change was noted in any other organ.

(2) PATHOLOGICAL FINDINGS:

RABBIT No. 1

HEART: Normal. LUNGS: Generally congestive and edematous. Incomplete expansion of alveoli was noted, with intermediate degree of atelectasis. Alveolar walls were edematous with an increased number of wandering cells. In the bronchial system, epithelial cells were swollen, particularly in the bronchia and respiratory bronchioli where exudates were found in the lumina. In the bronchia, small round cells infiltrated in the layer of epithelial cells and surrounding connective tissue. TRACHEA: The pavement epithelial cells appeared hypertrophic, but no cellular infiltration and ulceration was noted (cf. photograph 3). LIVER: Generally congested to a slight degree, with enlarged sinusoids. Star cells and Glisson's capsule appeared normal. KIDNEYS: Slightly congested. The glomeruli indicated an increase of cellular components and adhesion to Bowman's capsule in some areas, showing irregularity in shape and size. The uriniferous tubules in the cortical region showed swelling. The tubules in the medullary substance were normal. No interstitial change was noted. SPLEEN: The lymphatic follicles appeared atrophic. A slight increase in the reticulo-endothelial system in the follicles was noted. The sinuses of the spleen exhibited a slightly hemorrhagic image. PANCREAS: Normal. ADRENALS: The reticular zone indicated distortion and infiltration of small round cells. The medullary cells were swollen due to hydropic degeneration. TESTES: Normal. LYMPHATIC GLAND IN THE HILA OF THE LUNGS: The reticulo-endothelial system indicated a slight development. CONJUCTIVA: Normal.

RABBIT No. 2

HEART: Normal. LUNGS: Slightly incomplete expansion of alevioli was noted, but no histological change was noted in the alveoli or their walls. The peribronchial or perivascular regions remained without change. The lymphatic follicles showed no abnormal development. TRACHEA: Epithelial cells remained intact, and no cellular infiltration was present in the submucous coat. LIVER: Slightly congested. Glisson's capsule appeared normal. The sinusoids showed slight enlargement. KIDNEYS: Glomaruli appeared normal. The epithelia of the convoluted tubules in the cortical region were slightly turbid and swollen. Colloidous cylinders appeared in the lumina of the medullary tubules. There were localized infiltrations of small round cells in the perivascular connective tissue of the cortex (cf. photograph 13). ADRENALS: Normal. LYMPHATIC GLAND IN THE HILA OF THE LUNGS: Normal. CONJUCTIVE: Normal.

RABBIT NO. 3

HEART: Normal. LUNGS: Incomplete alveolar expansion and swelling of alveolar walls were noted. Reticular cells in the alveoli and their walls were swollen, but indicated minimal ingestion of foreign particles. Wandering cells increased to a slight degree. The epithelia of the bronchia, bronchioli, and respiratory bronchia were slightly swollen. The lymphatic follicles of the bronchia indicated some degree of development. TRACHEA: Normal. LIVER: Slightly congested. The sinusoids were enlarged. Extremely slight vacuolar degeneration was shown by hepatic lobules. Small round cells infiltrated slightly in Glisson's capsule (cf. photograph 9). KIDNEYS: Congestion was generally severe. Glomeruli were distended with blood, and the convoluted uriniferous tubules of the cortex were turbid and swollen to a slight degree. The interstices remained normal. SPLEEN: Slight fibroid degeneration took place on lymphatic follicles. The sinuses of the spleen remained normal. PANCREAS, ADRENALS: Normal. TESTES: Normal. LYMPHATIC GLAND OF THE HILA OF THE LUNGS: Normal.

RABBIT NO. 4

HEART: Normal. LUNGS: Congested, with a tendency of atlectasis due to incomplete expansion of alveoli. Alveolar walls appeared slightly edematous, with increased wandering cells. There was perivascular infiltration of small round cells in the bronchial system, development of lymphatic follicles. Bronchial epithelia showed swelling, particularly in the bronchioli and respiratory bronchia where epithelia became exfoliated and exudates accumulated in the lumina (cf. photograph 4). TRACHEA: Normal. LIVER: The central veins were congested, but liver cells remained intact. Small round cells infiltrated inside Glisson's capsule in hepatic lobules. Star cells appeared normal. (Photograph 10). SPLEEN: The lymphatic follicles indicated distortion. Thesinuses of the spleen indicated the so-called splenic hemorrhage. ADRENALS: Localized infiltrations of small round cells were present in the fascicular zone of the cortex. The reticular zone and medullary substance indicated signs of congestion. PANCREAS: Normal. LYMPHATIC GLAND OF THE HILA OF THE LUNGS, TESTES, CONJUCTIVA: Normal.

RABBIT NO. 5

HEART: Normal. LUNGS: Alveolar walls remained without change. Bronchial epithelia showed slight swelling. Lymphatic follicles were well developed. There were reticular cells in alveolar walls and the alveoli. TRACHA: Normal. LIVER: Slightly congested. In hepatic lobules, localized infiltration of small round cells was noted to a slight degree. Glomeruli appeared normal. The uriniferous tubules in the cortical region showed slight swelling. No interstitial change was noted. SPLEEN: There was pronounced proliferation of reticulo-endothelial system and connective tissue in the lymphatic follicles. The sinuses of the spleen revealed slightly increased blood components, hemosiderin deposit and components of the connective tissue. The splenic pulp was slightly enlarged (photograph 16). ADRENALS: Normal. PANCREAS: Normal. LYMPHATIC GLAND OF THE HILA OF THE LUNGS: Normal. TESTES: The germinal epithelia of the seminiferous tubules remained intact.

(2) $ZnCl_2$ MIST GROUP

Rabbit No. 8, which died during the experiment, exhibited a hemorrhagic image over the entire pulmonary lobes, atelectasis and lack of elasticity. Exudative pneumonia was indicated. The thoracic gland was swollen, and the adrenals and testes, atrophied. No marked change was noted in the liver, Kidneys, or spleen. Rabbit No. 6 showed hemorrhagic lesions in the lower lobe of both lungs. The upper middle lobe showed hemorrhagic lesions and pulmonary emphysyma, with fresh pin point hemorrhagic spots. Other organs were normal. Rabbits No. 7 and No. 9, which were sacrificed upon completion of the experiment, showed hemorrhagic spots in the lungs, with localized pneumonia. Other organs remained normal.

IV. DISCUSSION

A NH_4Cl solution exhibits a slightly acidic tendency due to the evaporation of NH_3, particularly after prolonged boiling [9,13]. As stated in the introduction, the toxicity of hypodermically administered NH_4Cl has rarely been discussed in literatures. According to the author's experiment, the mice died when 0.25 cc of a 5% NH_4Cl solution was administered hypodermically. Thus, the total lethal dose for mouse is assumed to be approximately 600 mg/g of body weight. In prolonged hypodermic administration, NH_3 solutions of relatively low concentrations, from 1% to 3%, were given to the animals of Group A. In this case, the total dose amounted to an average of 351.8 mg, more than the 234.5 mg of Group B to which solutions of higher concentrations were given. It was noted that, despite the larger total dose, the low concentration group survived longer, for an average of 94.6 days against 38.5 days of the high concentration group. At low concentrations, the animals showed no drop in body weight, and the values at the time of death were almost the same as those at the start of the treatment, whereas at high concentrations, loss of weight continued until death. Two out of 10 of the high concentration group died within one week. These results may be attributed to the fact that a NH_4Cl solution exhibits weak acidity upon hydrolysis. As Goodman and Gilman [12] stated, NH_4Cl probably induces acidosis once it is introduced in the body. Up to a certain amount, the NH_3 ion becomes urea, and the Cl ion is substituted with CO_2, and, even though the buffer system may exhibit slight disturbance, the original state is soon be recovered. In a larger amount, however, the substitution of Cl ion does not proceed sufficiently, as a result of which excessive amount of Cl ions are retained in the body fluid and acidosis is induced. If the amount is adequate, NH_4Cl serves as an acidifying diuretics and stimulates the excretion of lead from various organs in chronic lead toxicosis. The optimum daily dose as a diuretics is considered to be within a range of 8 - 12 g for oral administration. The patholohistological changes in the mice subjected to prolonged administration of NH_4Cl in this experiment cannot be evaluated on a comparative basis since no literature has yet provided such data. Generally, the heart showed mild congestion but was otherwise normal, except for the infiltration of small round cells in the muscular layer of the heart in one of the low concentration group. At high concentrations, the lungs exhibited an image of hemorrhagic, exudative, exfoliating bronchopneumonia. Small round cells were deposited in thick layers concentrically around the blood vessels and bronchial

system. The lymphatic follicles were well developed, and the alveolar walls thickened, appearing edematous with increased wandering cells, black foreign particles and hemosiderin deposit. The epithelial cells of the bronchial system showed emphysematous thickening and some vacuoles were filled with exudates, wandering cells, and ruins of epithelial cells. At low concentrations, the image of pneumonia was milder and localized, and all changes were generally less severe. The major changes comprised hydropic hypertrophy of alveolar walls, increase of wandering cells, and infiltration and nodular aggregation of small round cells. At high concentrations, sudden inflammation occurred in the lungs, causing death in some instances, whereas, at low concentrations, the inflammation took a chronic course.

In the liver, the congestion of the central veins, distortion of the cord of liver cells, irregularity of liver cells in size, nuclear condensation, swelling or destruction of liver cells, and lobular hemorrhage and infiltration of small round cells were the histological findings for the low concentration group. The sinusoids were enlarged, and star cells grew in size after having ingested hemosiderin-like pigments and black foreign particles, reaching the size of a liver cell in some instances and exhibiting nodular aggregation. Glisson's capsule also indicated infiltration of small round cells and deposit of hemosiderin, which aggregated in liver cells. The severity of these changes was extremely high. On the other hand, the administration of high concentration solutions caused necrobiosis and vacuolar degeneration of liver cells, but the degree of damage was relatively lower, without well-defined localized necrotic legion. Changes in star cells, and accumulation of hemosiderin and infiltration of small round cells in hepatic lobules and Glisson's capsule were also present with the severity comparable to that observed in the low concentration group. In general, these changes were the major histopathological findings of the low concentration group whereas they were secondary after the changes in the lungs for the high concentration group.

The animals which received low concentration NH_4Cl solutions developed renal damages of higher severity than those which received high concentration solutions. At low concentrations, the glomeruli became irregular in size and exhibited an atrophic tendency, accompanied by an increase in cellular components, and adhesion to Bowman's capsule. At high concentrations, these changes could not be seen. The turbidity and swelling of epithelial cells of the convoluted uriniferous tubules in the cortical region, epithelial destruction, and localized infiltration of small round cells were shown by both groups.

The development of lymphatic follicles and the reticulo-endothelial system in the sinuses of the spleen and connective tissue exhibited more advanced image in the case of low concentration group. Both groups showed considerable amounts of lymphatic follicles and hemosiderin particles in the sinuses of the spleen, and a notable increase of spleen cells containing giant nucleus. The atrophy of lymphatic follicles was severe in the high concentration group.

None of the low concentration group showed any change in the adrenals. Of the high concentration group, hemosiderin deposit was noted in the fascicular and reticular zones of the cortex in one case, the swelling and hydropic degeneration of the cells and nuclear destruction in the cortex, and medullary infiltration of small round cells, in another case. These histological changes in the suprarenal glands of the animals treated with high concentration NH_4Cl solutions are manifestations characteristic to acute intoxication, and can be attributed to a number of chemical and

physiological reactions following the acidosis: the Cl ion becomes excessive following acidosis, the adrenals are closely related to Cl ion metabolism, and the adrenals react under stress as in adaptation syndrome. In the sexual organs, epithelial distortion in the seminiferous tubules of the testis was indicated in one case, but there was no change in the ovaries. The pancreas remained normal.

As stated above, in chronic intoxication of mice due to prolonged hypodermic administration of NH_4Cl solutions, the changes were concentrated mainly in the liver, with parenchymal degeneration, but the administration of high concentration solutions for relatively short periods resulted circulatory disturbance, particularly acute bronchopneumonia as the primary manifestation, and parenchymal degeneration of the liver and other organs.

In regard to the NH_4Cl mist inhalation experiment, it is difficult to produce fumes of NH_4Cl because of its deliquescent property. The compound becomes colorless vapor only when heated to temperatures above 450°C. However, the fume and vapor become a mixture of NH_2 and HCl, and inhalation of such mixture does not permit observation of the effect of NH_4Cl fume only /9,12/. Thus, the author prepared a special equipment for producing aqueous solution mists of NH_4Cl to be used for this experiment.

There is hardly any literature discussing the harmful effect of NH_4Cl fumes. As stated in the introduction, Hunter /11/ stated that NH_4Cl is often spread over a zinc filter in order to prevent the formation of zinc fume. However, in the dip brazing deposit plant where the author experienced the clinical case introduced at the beginning of this paper, NH_4Cl was applied primarily for the elimination of the scums containing zinc oxide on the surface of molten zinc in the furnace. Immediately after NH_4Cl was spread, grayish blue fumes filled the work area. The author experienced no irritation after standing admist the fumes for a short time. Thompson mentioned that fumes occurred when NH_4Cl was added to molten zinc, and described that, when the fumes reached a certain amount, it became highly irritating, causing a series of symptoms including collapse, severe dyspnea, cyanosis and weak pulse, and fatal pulmonary edema. The fumes thus produced are considered to contain the fume of volatile metal chloride, ZnCl in the above case, as well as NH_4Cl fume, since the molten zinc in the furnace is approximately 450 - 470°C /3/. Thus, in the discussion of the effect of such fumes on the respiratory system, NH_4Cl and $ZnCl_2$ must receive equal amount of attention. The toxicity of $ZnCl_2$ and the respiratory disturbance due to the inhalation of the toxic fume of $ZnCl_2$ has been studied by Evans /10/ in relation to the mass intoxication incident due to a gas generator in Conadino Tunnel. In this incident, 10 out of 70 victims died, following intra-thoracic discomfort, dryness of the throat, coughing, invasion of naso-laryngeal and respiratory mucuous membranes. Hunter /7/ encountered cases of intoxication due to smogs in a harbor on Malta Island, with the similar symptoms. In his book /9/, Holland described the characteristics of $ZnCl_2$ toxicosis as follows, based on 40 clinical cases. This compound is a dehydrant, coagulates and hardens tissues, and possesses corrosive action. Its corrosive action is particularly strong on the gastrointestinal tract when taken orally, causing metallic taste, burning sensation in the oral and pleural cavities and the stomach, excessive salivation, vomitting, hematemesis, tenesmus, hemafecia, and persistent diarrhea. The victim normally dies in several hours after collapse and coma. In the event that the victim survives, neurological

sequela, aphasia, and stricture of the esophagus and gastric pylorus persist permanently. Callender and Gentzkow [14] and Webster [15] also published reports on a mass $ZnCl_2$ dietary poisoning. Tanaka [16] and Noiri [17] confirmed strong corrosive action and toxicity of the compound in their experiments using rabbits to which the compound was administered hypodermically and intraperitoneally, respectively. According to Noiri, the pathohistological changes caused by the compound included the adhesion of peritoneal organs, perforation of intestinal tubes, fatty degeneration, infiltration of small round cells and swelling of the liver, pronounced irregularity in the size of glomeruli and epithelial destruction of the uriniferous tubules in the cortex of the kidneys, and increased trabeculae of spleen.

The author conducted an experiment in which the mist of 10, 20, and 30% NH_4Cl solutions and 10% $ZnCl_2$ solution were administered to rabbits through inhalation, and the toxicity was examined. Changes in body weight occurred in all 5 rabbits during the period of 55-day NH_4Cl mist inhalation experiment, but no change in general condition was noted. After the experiment, the animals appeared healthy with normal appetite. On the other hand, inhalation of a 10% $ZnCl_2$ solution mist caused cyanotic changes in the eyeballs, palpebral mucuous membrane and lips in 2 out of 4 cases after 2 to 4 times of inhalation, although the period of experiment and amount of air introduced were only 1/5 of those in the NH_4Cl mist experiment. The animals also developed wheezing and dyspnea, and died after 3 to 5 administrations of the mist. The remaining 2 showed slight wheezing after 11 administrations. In peripheral blood, NH_4Cl mist caused no change in erythrocyte count at 10, 20, and 30% concentrations, but hyperleukocytosis was noted after 7 inhalation tests at 30%, with leukocyte count rising from the initial 7200 to 10700/mm^3 in average, which indicates that the mist of NH_4Cl is potentially inflammatory at high concentrations. On the other hand, the 10% $ZnCl_2$ solution mist produced an upward trend in erythrocyte count after the first test, then a downward trend, and an upward trend again from the fourth day. The reason for this pattern of change is not clear. In leukocyte count, an increase from the initial 9000 to 14500/mm^3 in average was noted after the first administration, and the values remained at that level for 11 days. A sudden rise was shown by the animals which died during the experiment. Hyperleukocytosis is reported to be consequential to zinc fume fever, but it is undetermined whether the hyperleukocytosis following the present $ZnCl_2$ mist experiment can be ascribed to the same cause. From the pneumonic changes noted in the lungs of the rabbit which died during the experiment, the author assumes that it was caused by the corrosive and inflammatory action of $ZnCl_2$, rather than the action that caused the similar symptom in zinc fume fever.

Histopathological examination revealed localized hemorrhagic lesions in the lungs of those subjected to the mist of NH_4Cl solutions, but no abnormal condition in the trachea and lungs. In this group, the heart remained without change. The lungs indicated incomplete expansion of the alveoli and enlarged alveolar walls with increased wandering cells. The bronchial system revealed epithelial swelling, and the lumina contained epithelial ruins and exudates. There was perivascular and peribronchial infiltration of wandering cells and small round cells, and reticular cells exhibited development. The connective tissue was generally edematous, and accumulations of hemosiderin were clearly seen in 1 out of 5 cases. Thus, an image of catarrhal inflammation of the

bronchia was observed. However, the trachea exhibited no inflammatory change, except for slight swelling of epithelial cells shown by one case. The lymphatic gland in the hila of the lungs was normal, and no inflammatory change was present in the conjuctiva. The respiratory tract or the mucuous membranes which come in contact with the mist at the time of inhalation developed no abnormality during the period of this experiment. Thus, the changes were extremely slight as compared to those following a large-dose hypodermic administration of the compound to mice.

The liver showed slight congestion of the central veins and enlargement of sinusoids. Vacuolar degeneration of liver cells were noted in one case, and infiltration of small round cells in hepatic lobules and Glisson's capsule, in three cases. These changes were also milder than those due to large-dose hypodermic administration in mice.

With regard to the kidneys, localized glomerular irregularity and adhesion to Bowman's capsule was present. Epithelial cells of the convoluted uriniferous tubules were turbid and swollen. One case indicated growth of renal casts and interstitial infiltration of small round cells, but the changes were slight. The lymphatic follicles of the spleen exhibited irregularity in shape, slight atrophy, and an increase in fibrous components. The sinuses of the spleen were distended with blood, and relatively severe accumulation of hemosiderin deposit was noted. However, the splenic changes were minimal.

For the adrenals, 2 out of 5 revealed infiltration of small round cells in the fascicular zone of the cortex. One of them showed hydropic degeneration of parenchymal cells. In view of the fact that adrenal infiltrationoof small round cells is extremely rare in toxicosis, the presence of such change in this case seems to suggest the significance of the part played by Cl ions in this form of intoxication, but conclusive words in this respect are not permittedat the present stage of investigation. The testis and pancreas were normal. As stated above, the inhalation of NH_4Cl mist at 10, 20, and 30% concentrations caused slight changes in the respiratory system, liver and spleen in the present experiment. The catarrhal inflammation in the lungs observed in this experiment can be related to the following action of the compound. NH_4Cl is often used as a sedative expectorant /_12_/, exerting its effective action in the treatment of dry cough at oral doses ranging from 0.3 to 0.5 g at 1 to 2 hour intervals. It stimulates the secretory function of the respiratory tract and makes the persistant, viscous phlegm water-soluble. Basch et al. /_18_/ and Perry and Boyd /_19_/ observed increases in the amount of phlegm upon administration of this compound in their clinical and animal experiments, respectively. Although the mechanism of action is yet to be determined, some investigators interprete the situation as that the stomach reflectively enhances the bronchial secretary function.

The above-described action of NH_4Cl might have caused the slight catarrhal inflammation in the respiratory system. In any event, the extremely severe circulatory disturbance and the degeneration of parenchymal cells in the lungs and liver following large-dose hypodermic injection was non-existent in the mist inhalation experiment. Thus, it seems that the toxicity of NH_4Cl is lower by way of the respiratory route than by the hypodermic route. On the other hand, the inhalation of a 10% $ZnCl_2$ solution mist caused extremely severe respiratory disturbance, characterized by hemorrhagic and exudative pneumonia over the entire

lobes in 2 out of 4 cases. As compared to the inhalation of NH_4Cl solution mist, the experiment with the mist of $ZnCl_2$ solution revealed highly acute and toxic effects of the compound.

As stated above, NH_4Cl exhibited its toxic effect when administered at a large dose, but the toxicity was low when it was administered in the form of mist by the respiratory tract. On the other hand, the mist of a $ZnCl_2$ solution is of extremely high toxicity, as has been confirmed by many investigators. The seriousness of the effect was demonstrated by the abnormal elevation of leukocyte count, short survival period, and pathological changes in this experiment. Thus, the death of the factory worker in a zinc depositing plant, discussed in the beginning of this paper, is attributed to the toxicity of $ZnCl_2$ rather than NH_4OH, although the toxic material responsible in this case is fume, not mist.

V. CONCLUSION

Ammonium chloride was administered hypodermically to mice at total doses ranging from 242 mg to 415 mg over periods from 33 to 102 days, and general symptoms, changes in body weight, and histopathological changes were observed. At the same time, 10, 20, and 30% ammonium chloride and 10% zinc chloride solutions, 50 cc daily, were administered to rabbits in the form of mist through inhalation. The ammonium chloride mist experiment lasted for 55 days, and the zinc chloride experiment, for 11 days. The changes in erythrocyte and leukocyte counts, and histopathological changes were studied on a comparative basis. The results are summarized as follows.

1. Sudden loss of weight was noted in mice following a single large dose hypodermic administration of NH_4Cl. Death occurred at 0.25 cc of a 5% NH_4Cl solution, the lethal dose being approximately 600 mg/g of body weight.

2. In prolonged administration of NH_4Cl solutions to mice, high concentration solutions caused a drop in body weight with an average survival period of 36.2 days, as compared to an average of 94.6 days with low concentration solutions. Histopathological changes included slight congestion of the heart, localized hemorrhagic, exudative, exfoliating pneumonia, pronounced infiltration of small round cells around blood vessels and bronchia of the lungs. The liver revealed necrobiosis of liver cells, lobular infiltration of small round cells, localized hemorrhage and proliferation and aggregation of star cells, and infiltration of small round cells and hemosiderin deposit in Glisson's capsule. The changes in the kidneys comprised adhesion of glomeruli to Bowman's capsule with small round cells around the area, turbidity swelling and destruction of the epithelia of convoluted uriniferous tubules, and interstitial infiltration of small round cells. The spleen revealed well developed reticulo-endothelial system and connective tissue in the lymphatic follicles and the sinuses of the spleen, giant nuclei in spleen cells, and deposit of hemosiderin. The adrenals were marked with hydropic degeneration and infiltration of small round cells in the fascicular and reticular zones of the cortex. Hypodermic injections of NH_4Cl solutions at high concentrations caused, primarily, pneumonia, and parenchymal degeneration of the liver, whereas at low concentrations, parenchymal degeneration of the liver was more notable than pulmonary damage.

3. Rabbits showed no change in erythrocyte count after inhalation of the mist of 10, 20, and 30% NH_4Cl solutions, but 5 rabbits developed hyperleukocytosis, with values rising from the initial 7,200 to 10,700/mm^3 in average, after inhalation of 30% NH_4Cl solution mist. There was neither abnormality in general condition or body weight, nor death during the period of 55 days with 52 times of mist inhalation. Histopathological findings comprised slight catarrhal inflammation of the lungs with infiltration of small round cells, slight congestion and vacuolar degeneration of liver lobules, and infiltration of small round cells in hepatic lobules and Glisson's capsule, epithelial destruction and infiltration of small round cells in renal convoluted tubules, slight atrophy of lymphatic follicles and fibroid degeneration of the sinuses of the spleen, distortion of the reticular zone and infiltration of small round cells in the adrenals, etc. These changes exhibited individual variation. The changes were generally slight as compared to those following a large dose hypodermic injection of NH_4Cl.

4. Inhalation of the mist of a 10% zinc chloride solution developed hyperleukocytosis, with the value rising from the initial 9,000 to 14,500/mm^3, in rabbits 24 hours later, and the leukocyte count remained close to 15,000/mm^3. Two out of 4 rabbits died on the 2nd and 5th days, showing cyanotic signs in the eyeballs, palpebral mucuous membrane and lips, dyspnea, and wheezing. The autopsy of the animals revealed hemorrhagic, exudative pneumonic lesions in all the lobes of the lungs.

Thus, the inflammatory action of the mist of ammonia chloride solutions was found to be weaker than that of the mist of a zinc chloride solution. However, a large dose hypodermic injection of ammonium chloride produced toxic symptoms of extremely high severity.

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EXPLANATION OF PHOTOGRAPHS

1. MOUSE GROUP A-NO. 7, FEMALE. LUNG. MAGNIFICATION, 72.
   (H-E PREPARATION)
   Hemorrhagic and exudative bronchopneumonia, with perivascular and peribronchial infiltration of small round cells.

2. MOUSE GROUP A-NO. 10, FEMALE. LUNG. MAGNIFICATION, 182.
   (H-E PREPARATION)
   Loop-like infiltration of small round cells around the bronchius and proliferation of its epithelial cells, noted; the alveoli reveal hydropic degeneration and swollen cells.

3. RABBIT NO. 1., MALE. LUNG. MAGNIFICATION, 115.
   (H-E PREPARATION)
   Bronchial epithelial cells, swollen and proliferating; the lymphatic follicle, well developed.

4. RABBIT NO. 4, MALE. LUNG. MAGNIFICATION, 152.
   (H-E PREPARATION)
   Nodular aggregation of wandering cells and small round cells; the alveoli appear edematous and hypertrophic.

5. MOUSE GROUP B-NO. 3. MALE. LIVER. MAGNIFICATION, 100
   (H-E PREPARATION)
   Liver cells are necrobiotic and irregular in size. The sinusoids contain small round cells. Star cells grew in number.

6. MOUSE GROUP A-NO. 5, FEMALE. LIVER. MAGNIFICATION, 310.
   (H-E PREPARATION)
   There is a small necrotic image of liver cells, surrounded by small round cells; star cells are enlarged.

7. MOUSE GROUP A-NO. 7, FEMALE. LIVER. MAGNIFICATION, 104.
   (H-E PREPARATION)
   Proliferation of star cells, necrobiosis of liver cells, and severe infiltration of small round cells in Glisson's capsule, shown.

8. MOUSE GROUP A-NO. 10, FEMALE. LIVER. MAGNIFICATION, 154.
   (H-E PREPARATION)
   Liver lobules show coagulation necrosis, with infiltration of small round cells.

9. RABBIT NO. 3, MALE. LIVER. MAGNIFICATION, 108.
   (H-E PREPARATION)
   Glisson's capsule contains many small round cells.

10. RABBIT NO. 4, MALE. LIVER. MAGNIFICATION, 108.
    (H-E PREPARATION)
    The hepatic lobule shows slight infiltration of small round cells

11. MOUSE GROUP A-NO. 5, FEMALE. KIDNEY. MAGNIFICATION, 310.
(H-E PREPARATION)
The cortex and glomeruli are slightly atrophic, with severe infiltration of small round cells.

12. MOUSE GROUP A-NO. 7, FEMALE. KIDNEY. MAGNIFICATION, 310.
(H-E PREPARATION)
Small round cells infiltrate around the glomerulus which shows partial loss of epithelial cells.

13. RABBIT NO. 2, MALE. KIDNEY. MAGNIFICATION, 120.
(H-E PREPARATION)
Interstitial infiltration of small round cells and partial loss of epithelial cells of a uriniferous tubule are noted.

14. MOUSE GROUP A-NO. 5, FEMALE. SPLEEN. MAGNIFICATION, 350.
(H-E PREPARATION)
Proliferation of connective tissue in the lymphatic follicle is noted, with a tendency of hyaline degeneration.

15. MOUSE GROUP A-NO. 8, FEMALE. SPLEEN. MAGNIFICATION, 110.
(H-E PREPARATION)
The roundccells in the lymphatic follicles, reticulo-endothelial system, and connective tissue show notable growth. Spleen cells with giant nucleus grew in number.

16. RABBIT NO. 5, MALE. SPLEEN. MAGNIFICATION, 65.
(H-E PREPARATION)
Lymphatic follicles exhibit distortion; reticulo-endothelial system and large roundcells, well developed.

17. MOUSE GROUP B-NO. 8, FEMALE. ADRENAL. MAGNIFICATION, 230.
(H-E PREPARATION)
The cells of the fascicular zone of the cortex are swollen and show hydropic degeneration.

18. MOUSE GROUP B-NO. 8, FEMALE. ADRENAL. MAGNIFICATION, 230.
(H-E PREPARATION)
Cells of the fascicular zone are swollen with infiltration of small round cells.

Yoshida, J., K. Nakame, and R. Nakamura: STUDIES ON THE TOXICITY OF UREA AND ITS CONTROL. II. TOXICITY OF AMMONIUM SALTS AND UREA IN RABBITS AND GOATS. Nippon Chikusangaku Kaiho, Vol. 28, pp. 185-91, 1957. Department of Agriculture, Ibaragi University.

I. INTRODUCTION

Urea can be effectively utilized by ruminants as a protein substitute, but, as is well known, an excessive amount of it could lead to urea intoxication or even fatality. In order to investigate the cause and mode of urea intoxication, the authors produced experimental, acute urea intoxication in goats by administering urea mixed in the feed. They also induced acute intoxication by the similar method and chronic intoxication by prolonged administration of urea in rabbits /1/. As Hirose /2/ had reported, there was a marked difference in acute toxic dose between goat and rabbit. In both experiments, marked increases were noted in the amount of ammonia (the substance quantitatively determined as ammonia by the diffusion procedure), as well as urea in gastrointestinal contents, blood and internal organs, which suggests that the intoxication is caused by ammonia, a decomposition product of urea, rather than urea itself.

Based on the experimental results, the authors assumed that urea poisoning is a form of ammonia intoxication. In order to substantiate this assumption, toxicity tests of ammonium salts were performed using mice, rabbits and goats, and the clinical symptoms of intoxication and changes in the amount of N(NH_3-N) and N (urea-N) were compared with those in urea intoxication.

II. EXPERIMENTAL PROCEDURE AND RESULTS

1. NORMAL BLOOD LEVELS OF NH_3-N AND UREA-N IN LABORATORY ANIMALS

Five mice and twenty rabbits weighing 200 to 300 g and 1.5 to 2.2 kg, respectively, were used. Blood samples were collected from the heart. Blood was drawn from the jugular veins of 11 goats, weighing 11 to 17 kg. The normal values of NH_3-N and urea-N were measured by Conway's micro diffusion procedure /3/, and the critical regions at 5% significant level were determined. See Table 1.

TABLE 1. NH_3-N AND UREA-N CONCENTRATIONS IN NORMAL BLOOD OF MICE, RABBITS AND GOATS.

	例数	体重範囲	NH_3-N mg/100cc	urea-N mg/100cc	採血部位
白鼠	5	200～300g	0.48±0.40	7.8±20.9	心臓
家兎	20	1.5～2.2kg	0.35±0.46	11.2±13.2	〃
山羊	11	11～17kg	0.35±0.38	9.5±13.7	頸静脈

Keys: a, mice; b, rabbits; c, goats; d, no. of cases; e, body weight; f, site of blood collection; g, heart; h, jugular vein.

Considerable variation was noted between individual animals, but the NH_3-N and urea-N concentrations below 0.9 mg and 30 mg/100 cc, respectively, were found to be within the normal range.

2. VASCULAR INJECTION OF AMMONIUM CARBONATE AND UREA TO MICE

In the previous study, the blood samples of the rabbits and goats which died of intoxication showed 4 mg and 60 mg/100 cc or more of NH_3-N and urea-N, respectively. Ammonium carbonate and urea were dissolved in water in the amounts that would produce the above concentrations under the condition that the amount of blood was regarded as 1/13 of the body weight, and the solutions were injected into the heart. The amount injected was 0.5 cc, and the concentration was increased with larger amount of the injected material. Using ammonium carbonate as the source of NH_3-N, the concentration was so adjusted that 2, 4, and 8 mg of NH_3-N would be contained in each 100 cc of blood. Mild toxic symptoms appeared at 4 mg, and fatality occurred at 8 mg. The amount of NH_3-N in the blood upon death was 2.62 mg, blow the expected level. Urea was injected at 10 dosages between 30 mg and 2000 mg per 100 cc of blood. Toxic symptoms began to appear at 1500 mg, except for one animal which died at a dose of 500 mg presumably due to a technical error during injection process. In one fatal case, the concentration of NH_3-N per 100 cc of blood was 1.43 mg, and that of urea-N, 65 mg, the values being considerably lower than expected. The results are summarized in Table 2.

TABLE 2. CHANGES FOLLOWING VASCULAR INJECTION OF AMMONIUM CARBONATE AND UREA SOLUTIONS IN MICE

	c 体重 (g)	d 性別	溶液注入後NH_3-Nまたはurea-Nが血液100cc中に占めるべき量 e	f 結果	g 摘要
a 炭酸アンモニウム	325	♂	2mg h	耐過	
	320	♀	4	〃	軽症状 j
	300	♀	8 i	死	2分で死亡、死後血中、NH_3-N 2.62mg/100cc j
b 尿素	324	♂	30mg h	耐過	
	325	♂	80	〃	
	329	♂	130	〃	
	383	♂	180	〃	
	142	♀	230	〃	
	118	♀	260	〃	
	163	♀	500 j	死	3分で死亡(注入の失敗と思われる) k
	152	♀	500 h	耐過	
	128	♀	1000	〃	
	155	♀	1500 i	死	死後血液10cc中NH_3-N 1.43mg, urea-N 65mg
	148	♀	1500 h	耐過	軽症状 m
	146	♂	2000 i	死	

Keys: a, ammonium carbonate; b, urea; c, body weight; d, sex; e, amount of NH_3-N or urea-N in 100 cc of blood after injection; f, result; g, remarks; h, survived; i, died; j, mild symptoms; j', died in 2 min; NH_3-N concentration, after death, 2.62 mg/100 cc; k, died in 3 min (presumably due to technical error); l, NH_3-N concentration after death, 1.43 mg/100 cc; urea-N concentration, 65 mg; m, mild symptoms.

3. ADMINISTRATION OF AMMONIUM SALTS TO RABBITS AND GOATS

If NH_3-N is the cause of intoxication, the administration of an ammonium salt should develop symptoms similar to those of urea intoxication. The following experiment was conducted based on this assumption. Ammonium

carbonate was employed as alkaline salt, and ammonium chloride, as acidic salt. The sample solutions of these salts without adjustment were pH 9.6 or lower and pH 5, respectively, when tested with a test paper. The animals were refrained from feed for a day, and the salts were administered on the following day. The salts were dissolved in 20 - 400 cc (rabbits) and 50 - 100 cc (goats) of water at urea concentrations varying from 0.2 g to 10 g, calculated in terms of the amount of N. The solutions were injected into the stomach or the first chamber with a catheter without adjustment, or after alkalized, neutralized, or alkalized with acetic acid or caustic soda. The results are shown in Table 3. In the experiment with ammonium salts, the rabbits developed symptoms similar to those of urea intoxication such as clonic spasms, dyspnea, tonic spasms, etc., and finally died at doses equivalent to 1 g per kg of body weight which normally develops toxic symptoms in goats.

TABLE 3. CHANGES FOLLOWING THE ADMINISTRATION OF AMMONIUM SALTS TO RABBITS AND GOATS

動物種類	家畜	試験番号	個体番号 No.	性別	体重 (kg)	体重1kgあたりの尿素当量(g)	投与条件	結果	死亡または屠殺までの時間(分)	血液100cc中 NH_3-N(mg) 投与前	投与後	血液100cc中 urea-N(mg) 投与前	投与後	摘要
1. 炭酸アンモニウム	兎	A	1	♂	2.55	10.0	非	死	19	0.44	12.40			投与液pH8.6
			2	♂	3.20	7.7	pH7.8	〃	7	0.47	3.61			
		B	3	♀	2.64	1.0	pH7.4	〃	49	0.65	8.94			投与液pH5.6, NH_3-N 経過35分3.58mg/100cc血液
		C	4	♂	1.73	0.2	pH7.2	痙攣	90	0.30	0.26	16	35	
			5	♀	1.43		非	〃	90	0.35	0.22	20	8	
		D	6	♂	1.81	0.4	pH7.6	〃	40	0.51	0.43	11	16	
			7	♀	1.68		非	〃	60	0.74	0.61	10	14	
		E	8	♂	1.73	0.8	pH7.2	〃	27	0.56	0.87	17	18	
			9	♀	1.73		非	痙攣状	30	0.30	1.12	4	5	
		F	10	♀	1.44	0.8	pH7.0	〃	45	0.19	0.71	4	4	
			11	♀	1.55		非	〃	45	0.14	0.67	2	3	
		G	12	♀	1.52	1.0	pH6.8	〃	36	0.33	0.39	16	19	
			13	♀	1.70		非	死	36	0.89	4.78	7	11	
		H	14	♀	1.47	1.0	非	〃	371	0.08	2.03			NH_3-N 経過140分1.12mg/100cc血液 [illegible]
			15	♂	1.60		pH8.4	〃	135	0.13	8.50			
			16	♀	1.44		pH7.0	痙攣状	125	0.18	1.26			
	山羊	I	1	♂	12.9	0.5	非	痙攣	30	0.93	0.55			
			2	♂	12.1		pH7.0	痙攣状	30	0.85	1.70			
		J	3	♂	17.5	0.8	非	死	27	0.42	4.99			NH_3-N 経過16分4.82mg/100cc血液, 第1胃内6.84 g投与 NH_3-N 経過 22分1.08mg/100cc血液, 第1胃内3.43 g投与
			4	♂	11.3		pH7.0	〃	151	0.41	3.88			
2. 塩化アンモニウム	家兎	K	17	♀	1.64	1.0	pH9.6以上	〃	23	0.23	8.57			
			18	♀	1.62		pH8.4	〃	21	0.16	8.52			
			19	♀	1.73		非	〃	40	0.15	9.38			
	山羊	L	1	♂	12.4	0.4	pH9.6以上	痙攣	35	0.47	1.29			
			2	♂	12.2		非	痙攣状	55	0.65	0.65			

* Key (s) indicates that the solution was not pH-adjusted. Other pH values indicate values after adjustment with CH_3COOH or NaOH.

Keys: a, ammonium carbonate; b, ammonium chloride; c, type of animal; d, rabbit; e, goat; f, experiment number; g, animal number; h, sex; i, body weight; j, urea-equivalent dose per kg of body weight; k, conditions of administration; l, result; m, time up to death or blood collection (min); n, NH_3-N per 100 cc of blood; o, urea-N per 100 cc of blood; p, remarks; q, before injection; r, after injection; s, not adjusted; t, or higher;

TABLE 3 (Keys) - continued.

u, died; v, survived; w, mild symptoms; x, gastric contents, pH 8.0; y, gastric contents, pH 5.6; NH_3-N 35 min later, 3.58 mg/100 cc of blood; z, NH_3-N 140 min later, 1.12 mg/100 cc of blood; the animals in Experiment H were fed on the day before the experiment; z', NH_3-N 16 min later, 4.82 mg/100 cc; 6.84 g retained in the 1st chamber; z", NH_3-N 22 min later, 1.09 mg/100 cc of blood, 3.43 g retained in the 1st chamber.

The neutralization and acidification of the salt solutions seemed to have mitigated toxic manifestation or prolonged the time to death, as compared to the alkalization of the solutions. Generally, the symptoms began to appear as the blood NH_3-N level rose, subsequently resulting death, as in urea intoxication. The blood NH_3-N level at the time of death was over 4 mg/100 cc, the level normally attained in urea intoxication. On the other hand, no increase in urea-N was observed in Experiments C to G and L. As in urea intoxication, autopsy revealed hyperemia of the renal cortex and a large amount of gas in the gastrointestinal tract of those given ammonium carbonate. The gastric contents indicated considerable influence of the pH of the injected solution, as evidenced in the data from Experiment A-2 and Experiment B-3. In the experiment using goats, death occurred at 0.8 g per kg of body weight (Experiment J) of ammonium carbonate. As with rabbits, the neutralization or acidification of the solution reduced toxic symptoms and rpolonged the time to death, and the results were generally similar to those of rabbits, with the symptoms prior to death, changes in the amount of NH_3-N in the blood, and gas formation in the first chamber following the same patterns. With ammonium chloride, mild symptoms began to appear at a dose equivalent to 0.4 g per kg of urea, and it was assumed that the results be similar to those of the experiment with ammonium carbonate. In Experiment H, the rabbits were fed on the day before the experiment, which resulted in considerable delay of toxic manifestation and death. Large amounts of NH_3-N were found in the gastrointestinal contents of the dead animals.

4. ADMINISTRATION OF UREA TO RABBITS.

The difference in toxic dose between rabbit and goat is considerable. In order to investigate the reason for the difference, the toxic dose for rabbit was determined and the effect of the change in the conditions of administration studied. Experiments A and E to G in Table 4 employed solutions of urea dissolved in 20 or 40 cc of water, and Experiment B to D, the solutions of urea dissolved in phosphate buffer and and phosphate buffer with crude urease so that the effects of gastric pH adjustment and urease be examined. The solutions were injected to the stomach with a catheter. In Experiment H, 1 g of phosphate powder, 6 g of wheat powder, and 0.5 g of sugar for the improvement of palatability were mixed with urea and the mixture was formed in patties. The patties, prepared for the purpose of prolonging the retention of urea in the stomach, were forced on the animals. The amount of urea thus administered varied from 1 g to 16 g per kg of body weight, graded in 8 steps. Experiments A to D with a dosage of 1 g per kg revealed no effect of buffer or urease, or abnormality. In Experiment E, the animals died at 6 g per kg. In Experiment F, no death occurred even at 10 g per kg, and only mild toxic symptoms were observed. It must be noted, however, that the rabbits had already received urea several times prior to this experiment. Those in Experiment H showed no

abnormality. With the exception of the fatal cases in Experiments E and G, no appreciable increase in the blood NH_3-N level was noted, the values being under 1 mg/100 cc, but in the presence of toxic symptoms, the level was higher than 3.6 mg/100 cc, the results being similar to those in the previous study. Urea-N always indicated increase after the administration of the salt, the values reaching approximately 60 mg/100 cc, the level of the fatal cases, in many of the surviving animals including A-No. 23, B-No. 23, F-No. 23, and H-No. 24. The toxic symptoms resembled those in the experiment with ammonium salts.

5. CHANGES IN BLOOD NH_3-N AND UREA-N LEVEL BY SITE OF BLOOD COLLECTION

In Experiments E and F, the blood NH_3-N level in the presence of toxic symptoms was considerably higher than that without such symptoms. An experiment was performed to pursue whether the NH_3-N level varied between the portal blood and the circulating blood. Immediately after death, the animals were subjected to autopsy, and blood samples were drawn from the heart, portal vein, and hepatic vein. The experimental conditions and results are shown in Table 5 and Figure 1.

TABLE 4. CHANGES FOLLOWING THE ADMINISTRATION OF UREA TO RABBITS

a 実験	b No.	性別	c 体重 (kg)	d 体重1kg当り給与尿素(g)	e 給与条件	f 結果	g 死又は血液採取時間 (分)	h 血液100cc中 NH_3-N(mg) 給与前	給与後	i 血液100cc中 urea-N(mg) 給与前	給与後
A	21	♀	1.65	1	水200ccに溶解	崩壊	69	0.21	0.65	—	—
	22	♀	1.69		〃	〃	85	0.13	0.25	—	—
	23	♀	1.51		〃	〃	150	0.57	0.37	23	59
B	23	♀	1.58	1	緩衝液5ccに溶解	〃	150	0.48	0.27	12	54
C	22	♀	1.60	1	ウレアーゼ0.7gと緩衝液10ccに溶解	〃	150	0.40	0.56	7	36
D	24	♀	1.86	1	ウレアーゼ0.7gと緩衝液50ccに溶解	〃	60	0.40	0.71	6	11
	25	♂	1.90		緩衝液50ccに溶解	〃	60	0.43	0.57	6	10
E	26	♀	1.50	2	水40ccに溶解	〃	85	trace	0.37	12	40
	27	♂	1.57	4	〃	後肢麻痺	94	0.11	0.28	18	43
	28	♂	1.50	6	〃	死	99	0.10	6.39	12	51
	29	♂	1.39		〃	〃	201	0.42	6.25	15	105
F	23	♀	1.76	6	〃	崩壊	80	trace	0.14	3	63
	25	♂	2.28	8	〃	後肢麻痺	70	0.16	0.16	15	33
	24	♀	1.93	10	〃	麻痺	149	—	0.23	—	44
G	51	♂	1.45	14	〃	死	150	—	3.84	—	48
	52	♂	1.25	16	〃	〃	180	—	3.58	—	67
H	24	♀	1.92	1	固線にして強制食下	崩壊	80	0.70	0.87	7	12
	24	♀	1.71	2	〃	〃	80	0.38	0.32	18	29
	24	♀	1.61	4	〃	〃	150	0.13	0.27	26	50
	24	♀	1.65		〃	〃	95	0.47	0.30	30	28

* The buffer solution used was phosphate buffer at pH 7.4.

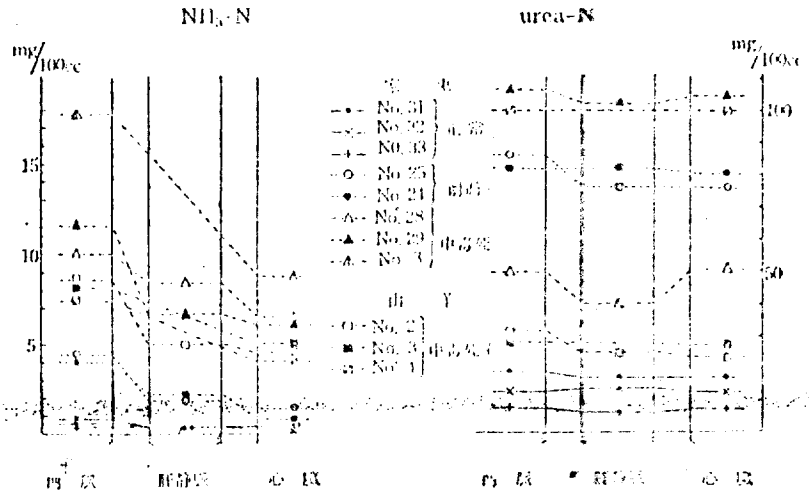
Keys: a, experiment; b, sex; c, body weight; d, amount of urea administered per kg of body weight; e, conditions of administration; f, result; g, time to death or blood collection (min); h, amount of NH_3-N per 100 cc of blood; i, amount of urea-N per 100 cc of blood; j, before administration; k, after administration; l, dissolved in 200 cc of water; m, dissolved in 5 cc of buffer solution; n, dissolved in 0.7 g of urease and 10 cc of buffer solution; o, dissolved in 0.7 g of urease and 50 cc of buffer solution; q, dissolved in 40 cc of water; r, shaped in patties and fed; s, survived; t, mild symptoms; u, died; v, mild symptoms.

TABLE 5. EXPERIMENTAL CONDITIONS

		動物 No.	体重 (kg)	性別	体重1kg当り尿素または尿素換算尿素量 (g)	中毒死または殺害までの経過時間 (分)
家	正常	31	1.10	♂	0	0
		32	1.50	♂	0	0
		33	2.20	♂	0	0
	耐過	25	2.24	♂	8	51
		24	2.20	♀	4	49
兎	中毒死	28	1.50	♂	6	99
		29	1.39	♂	6	201
		3	2.61	♀	$(NH_4)_2CO_3$ 1	49
山羊	中毒死	2	13.0	♂	1	83
		103	22.0	♂	1	
		104	24.0	♂	3	

Keys: a, rabbit; b, goat; c, normal; d, survived; e, died of intoxication; f, animal no.; g, body weight; h, sex; i, amount of urea, or urea-equivalent dose, g/kg of body weight; j, time to death due to intoxication or sacrifice (min).

FIGURE 1. DIFFERENCE IN NH_3-N AND UREA-N CONCENTRATION BY SITE OF BLOOD COLLECTION



Keys: a, rabbit; b, normal; c, survived; d, died of intoxication; e, goat; f, portal vein; g, hepatic vein; h, heart

The rabbits of Experiment F which were likely to have developed tolerance, were given 8 g per kg of body weight, a toxic dose, and 4 g per kg of body weight, a potentially toxic dose. After the absence of toxic symptoms was confirmed, the animals were sacrificed. The normal feed mixed with urea at 1 g per kg of body weight was given to the goats upon demand, as a result of which acute intoxication developed. In both intoxicated animals and surviving animals, the amounts of NH_3-N in the portal blood were 1.5 – 2 times greater than those of the cardiac blood, but these in the hepatic vein were considerably less, close to the concentration at the heart. The urea-N concentrations in blood indicated no appreciable difference by site, the values generally being close to the concentration in the portal vein. In surviving animals, the portal NH_3-N level was considerably

lower than that of the animals with toxic manifestations, but the values were larger than those of the concentration in the cardiac blood of the rabbits which died of intoxication in Experiment G in Experiment 4, and those in the previous study. The concentrations became lower from the hepatic vein to the heart, the values at the heart being close to normal.

TABLE 6. CHANGES IN BLOOD NH_3-N AND UREA-N CONCENTRATION BY TOXIC SYMPTOMS

動物	山羊							家兎	
No.	103		104		104		2	29	
結果	死		耐過		耐過		死	死	
体重1kg当り尿素(g)	1		1		1		1	6	
血液100cc中	NH_3-N mg	urea-N mg	NH_3-N mg	urea-N mg	NH_3-N mg	NH_3-N mg	urea-N mg	NH_3-N mg	urea-N mg
給与前	0.20	16	0.08	17	0.16	15	0.21	0.42	15
症状発現前								0.85	62
症状発現時	1.38	18	2.62	36	(異状なし)		4.20		
症状悪化	2.03	22	(軽症状)						
死または耐過後	5.09	27			0.12	60	6.08	6.25	105

Keys: a, animal; b, goat; c, rabbit; d, result; e, died; f, survived; g, amount of urea administered, g/kg of body weight; h, per 100 cc of blood; i, before administration; j, before toxic manifestation; k, at the time of toxic manifestation; l, aggravation of symptoms; m, after death or toxic manifestation; n, mild symptoms; o, no abnormality

The urea-N concentration was high at the portal vein and the heart, without variation as in the cases which developed no toxic symptoms.

6. CHANGES IN BLOOD NH_3-N AND UREA-N LEVELS BY SYMPTOM

In urea intoxication, the blood NH_3-N level and urea-N level exhibited notable and slight variations, respectively, according to the severity of the symptoms. Assuming that there is a correlation between the severity of symptoms and the concentration of NH_3-N or urea-N, the data on 4 goats and 1 rabbit are introduced in Table 6. The concentration rose with elapsed tiame and the severity of symptoms. Since a similar tendency was noted in Experiment 3 with ammonium salts, the concentration at the time of death is considered to be at the peak. It was also confirmed that, if NH_3-N concentration did not increase, the symptoms did not occur even with the elevation of urea-N concentration.

7. NH_3-N AND UREA-N CONCENTRATIONS IN INTERNAL ORGANS

The NH_3-N and urea-N retained in the gastrointestinal contents, etc. were measured and the results are shown in Table 7. When ammonium chloride was given to rabbits, considerable amounts of NH_3-N were found in the gastric, intestinal, and appendiceal contents. With urea, the gastric NH_3-N concentrations were more than 10 times that of normal concentration. Goats also showed large amounts of NH_3-N in the first chamber, as in the previous study.

III. DISCUSSION

When ammonium carbonate and urea were injected into the blood of mice, their blood concentrations did not quite reach the expected levels, but when the concentration of ammonium salt was so adjusted that the NH_3-N

TABLE 7. NH_3-N AND UREA-N CONCENTRATIONS IN INTERNAL ORGANS (mg)

	No.	給与物質	尿素または尿素換算給与量 (g)	胃または第1胃内容		十二指腸または第4胃内容		腸内容		血尿		肝臓		腎臓		摘要
				NH_3-N	urea-N	NH_3-N	urea-N	NH_3-N	urea-N	NH_3-N	urea-N	NH_3-N	urea-N	NH_3-N	urea-N	
実験家	17	NH_4Cl	(pH9.6) 1	500	—	48	—	102	—	—	—	—	—	—	—	
	18	〃	(pH8.4) 1	370	—	133	—	98	—	—	—	—	—	—	—	
	19	〃	(pH5.0) 1	511	—	—	—	101	—	—	—	—	—	—	—	
	28	尿素	6	94	46	133	5	6	9	(8.7)	—	—	—	—	—	
	29	〃	6	42	291	82	3	6	27	—	—	(41.2)	(126)	(19)	(117)	
	25	〃	8	239	135	36	194	12	34	(1.0)	(149)	(5.5)	(73)	—	—	耐過
	24	〃	4	50	—	126	1	4	11	(3.0)	(70)	(9.6)	(80)	—	—	耐過
	51	〃	14	82	1720	—	—	—	—	—	—	(13.0)	(424)	(55)	(389)	
	52	〃	16	45	1100	—	—	—	—	—	—	(34.0)	(297)	(21)	(416)	
見本	31	正常	0	2	trace	(24)	trace	0.7	trace	(0.7)	—	(2.2)	(16)	trace	(26)	
	32	〃	0	2	〃	(18.6)	〃	1.5	〃	(0.7)	(87)	(4.4)	(8)	(2)	(23)	
	33	〃	0	6	〃	(3.6)	〃	1.8	〃	(0.3)	(85)	—	—	—	—	
山羊	2	尿素	1	2600	39	55	—	15	4	(162)	(90)	(34.0)	—	—	—	
		正常	0	40	trace	15	—	—	—	—	—	(3.0)	—	(3)	(3)	

* The amount of administration is expressed in grams per kg of body weight.

** The values in parentheses indicate the amounts per 100 g.

Keys: a, rabbit; b, goat; c, material administered; e, contents of the stomach or the 1st chamber; f, contents of the appendix or the 4th chamber; g, intestinal contents; h, urine; i, liver; j, kidney; k, remarks; l, urea; m, normal; n, tolerated.

concentration in the blood would reach the lethal level, the injection of such solution caused death or the animals. On the other hand, neither fatality nor toxic manifestation occurred in the administration of urea until the concentration of urea was so adjusted that the blood urea-N concentration would reach 10 to 20 times the lethal dose, 1500 mg/100 cc of the blood. Umishio et al. /4/ injected ammonium carbamate, an intermediary decomposition product of urea, to goats by the intravenous route, and created symptoms similar to those due to urea intoxication, but the amount of urea injected in their experiment was considerably smaller than that causing death of mice in the vascular injection in the authors' experiment. These experimental results suggest that urea poisoning is not caused directly by the urea in the blood.

The administration of ammonium salt in an amount equivalent to 1g and 0.8 g of urea per kg of body weight induced symptoms almost identical to those of urea intoxication in rabbits and goats, respectively, which subsequently died, and the changes in blood NH_3-N concentration relative to the severity of symptoms followed exactly the same pattern as in urea intoxication. The results obtained in the present experiment were in fairly good agreement with the data published by Repp /5/ who studied the toxicity of ammonium formate and urea in lambs. The acidification of the injection solution prolonged the time up to the manifestation of toxic symptoms or mitigated the symptoms. This is presumably due to the fact that the liberation of ammonium is inhibited. Such interpretation agrees with Rapp's observation /5/ that an adequate dose of acetic acid minimized acute intoxication. The notably delayed manifestation of toxic symptoms in the rabbits in Experiment H which were fed the day before the experiment indicates that the absorption of the toxic substance is influenced by the amount of gastric contents.

With urea, the rabbits and goats died at 6 g per kg and 1 g per kg, respectively, but Experiment 4-F suggests that the rabbits which had

previously received urea developed tolerance. Furthermore, the toxic symptoms and changes in blood NH_3-N concentration exhibited strong resemblance to those following the administration of ammonium salt, which suggests that the intoxication by these different substances is of the same origin. The failure to observe any effect of the urease or buffer added in small quantities, or the formation of the salt and additives in patties is attributed to the gastric acidity.

The concentration of NH_3-N in the blood showed considerable variation by the site of sample collection. The portal vein indicated an extremely high concentration, but the hepatic vein beyond the liver showed concentrations similar to those of the cardiac vein. The concentration of urea-N remained without appreciable difference from the portal to the cardiac blood, indicating that the decomposition of part of the urea occurred before it entered the portal vein. The rabbits which did not develop toxic symptoms presumably due to the tolerance gained during previous urea treatments were subjected to autopsy. In these animals, the portal NH_3-N concentration was similar to the concentration in the cardiac blood of the animals which developed toxic symptoms, but the concentration in the cardiac blood, beyond the hepatic vein, was almost normal. Thus, the concentration of urea-N exhibited no appreciable variation by site, and no symptom was indicated even when the concentration was equal to or higher than that of the animals with toxic manifestations. This is indicative of the fact that the NH_3-N in the circulating blood takes a greater part in the development of toxic symptoms than urea-N. Thus, the following assumption is made. The NH_3-N in the portal blood is suppressed by the liver. Toxic symptoms develop when NH_3-N is introduced to the liver in an amount beyond its capacity to suppress, and enters the circulating blood through the hepatic vein, increasing its concentration. The change of urea-N in the liver is unknown, but it apparently passes through the organ as it is.

The increase of NH_3-N and urea-N in the blood was proportional to the severity of toxic symptoms, reaching a peak at the time of death. However, it was confirmed that an increase in urea-N did not accompany the symptoms unless the amount of NH_3-N reached the toxic dose.

It is of significant interest to note that the concentration of NH_3-N in the gastric contents exhibited a marked increase following the administration of urea to rabbits.

Despite the fact that the toxic dose of ammonium salt shows no significant difference between goat and rabbit and that of urea for rabbit is approximately 6 times that for goat, the concentrations of NH_3-N and urea-N at the time of toxic manifestation were approximately the same. This is due to the difference in the capacity of the entire gastrointestinal tract to decompose urea, and the experimental facts discussed above indicate that the direct cause of intoxication is the NH_3-N which can be quantitatively determined by the micro-diffusion procedure. However, since the object of measurement was limited to urea and ammonia, and the location of urea decomposition is not known, further investigation is necessary for any conclusive statement.

IV. SUMMARY

1. The concentrations of NH_3-N and urea-N in the blood of normal mice, rabbits and goats were measured. In any of these animals, values below 0.9 mg and 30 mg/100 cc, respectively, were considered to be within normal range.

2. The toxicity of NH_3-N and urea-N was determined by injecting ammonium carbonate and urea into the blood of mice. Ammonium carbonate caused fatality at a dose equivalent to 8 mg of urea-N per 100 cc of blood, and urea, at a dose equivalent to 1500 mg of urea-N per 100 cc of blood.

3. Ammonium carbonate and ammonium chloride solutions were injected to the stomach of rabbits and goats with a catheter. The rabbits and goats died at doses equivalent to 1 g and 0.8 g of urea per kg of body weight, respectively, with the symptoms and blood NH_3-N concentration similar to those in urea intoxication. There was no appreciable difference between rabbits and goats with regard to the amount of ammonium salt administered at the time of death. No increase in blood urea-N concentration was observed.

4. The acidification of the ammonium salt solutions prolonged the time to toxic manifestation or minimized the symptoms. The amount of gastric contents also influenced such factors.

5. When a urea solution was injected to the stomach of rabbits with a catheter, the animals died at 6 g per kg of body weight with symptoms resembling those in ammonium salt poisoning. However, the rabbits which had previously received urea indicated tolerance against urea. The combined use of phosphate buffer or the formation of urea mixture in patties showed no effect.

6. Comparing the NH_3-N concentrations in the portal, hepatic and cardiac blood of the normal, surviving, and dead animals, the concentrations at the portal vein were approximately 2 times that at the cardiac blood, and the concentration was lower in the above order. However, the concentration of urea-N remained without appreciable variation. Thefollowing assumption is made based on the experimental results. Urea is decomposed before it enters the portal vein, and the N is changed to NH_3-N. The liver normally prevents the NH_3-N to be released directly into the circulatory system, but the urea apparently passes the liver without being changed.

7. In goats and rabbits, the blood NH_3-N and urea-N concentrations increased with the severity of the symptoms, reaching peaks at the time of death. An increase in urea-N did not accompany toxic symptoms unless NH_3-N reached a certain level.

8. When urea was given to rabbits, the NH_3-N concentrations in gastrointestinal contents also increased.

9. The above experimental results strongly suggest that the direct cause of intoxication is the NH_3-N increasing in the blood.

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