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Studies in Biochemical Adaptation. The Origin of Urinary Ammonia as Indicated by the Effect of Chronic Acidosis and Alkalosis on Some Renal Enzymes in the Rat

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The origin of urinary ammonia, which is an important part of the mechanism for maintaining the acid-base equilibrium, has been the subject of much study. The early view was that it was derived from urea (Barnett & Addis, 1917; Nash & Benedict, 1921; Bollman & Mann, 1930). More recent work has shown this view to be incorrect (Van Slyke *et al.* 1943; Pitts, 1936). It is now believed that the ammonia is derived from glutamine and from other amino-acids. Whereas the kidney contains no urease, it does contain glutaminase and a variety of deaminating and transaminating enzymes acting upon amino-acids (Krebs, 1935*a, b*; Blanchard, Green, Nocito & Ratner, 1944; Mylon & Heller, 1948).

Support for this is found from two types of experiments with dogs *in vivo*. In the first, possible precursors of urinary ammonia were administered intravenously, and the effect on ammonia excretion

determined by estimation of ammonia either in the urine or in the renal artery and vein. By such experiments, Polonovski, Boulanger & Bizard (1934), Polonovski & Boulanger (1938), Bliss (1941) and Lotspeich & Pitts (1947) suggest that glycine, D- or L-alanine, D- or L-leucine and DL-aspartic acid may act as precursors of urinary ammonia, whereas L-arginine, L-lysine and L-glutamic acid apparently do not. Such results are not conclusive evidence of the normal mechanism in the living organism. This is obvious in regard to the unnatural (D) forms of amino-acids which are certainly not the normal precursors, though their administration increases the excretion of urinary ammonia. It may also be true of the natural acids, since they do not normally occur in the high concentrations which result from the intravenous injections used in the experiments.

In the second type of *in vivo* experiment, estimations were made of the rate of removal from the blood of the possible precursors themselves, already present in the blood. In this way, Van Slyke *et al.* (1943) showed that L-glutamine was removed from the renal blood in amounts which account for 60%

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or more of the ammonia excreted. The rest of the ammonia was accounted for by a decrease in α -amino-acid nitrogen in the renal blood.

Our interest in adaptive enzymes suggested to us another approach to this problem (Yudkin, 1952). Since acidosis results in more urinary ammonia production and alkalosis results in less, it is possible that, in time, these conditions might produce an increase or a decrease in some of the renal enzymes concerned in ammonia production. The possibility that such an adaptive response occurs is supported by the fact that the increase in urinary ammonia induced by chronic acidosis reaches its maximum only gradually. The experiments to be described show that adaptation does in fact occur; of five renal enzymes examined, three showed an appreciable increase in chronic acidosis and an appreciable decrease in chronic alkalosis.

EXPERIMENTAL

Enzyme activity was measured in kidney slices from normal rats and from rats rendered chronically acidotic and alkalotic.

Animals and diets. Albino rats of the Wistar strain were used throughout. Litter mates of the same sex were distributed into three groups of six animals. Each group was housed in one cage and fed and watered *ad lib*. One group received water only, one received 0.05N-HCl and the third 0.1N-NaHCO₃ (see below). Apart from the experiments with L-glutaminase, the rats were fed from weaning on a stock diet consisting of cubes, with supplements of milk and greens (see Wiesner & Yudkin, 1951). The experiment with L-glutaminase was the first of these series and the rats were given diet AX, consisting of national bread 30%, sucrose 35%, light white casein 20%, dried baker's yeast 5%, arachis oil 3%, cod-liver oil 0.5% and salt mixture 6.5%. The animals received this from the age of 12 weeks, together with water, acid or alkali. By the time this experiment was completed, it had been established that growth on this diet was slightly, though not significantly, inferior to that on the laboratory stock diet; in all other experiments therefore the stock diet was used.

The animals were maintained on water, acid or alkali for at least 3 months, to ensure adequate time for adaptation to occur. For practical reasons, it was not possible to estimate enzyme activity in each experiment after equal times, and these were carried out from 3 to 8 months from the beginning of the experiment. Again, for practical reasons, male animals were used in some experiments and female animals in others. Table 4 summarizes some of the conditions in the experiments performed. Although these different conditions might have affected the absolute values found, they do not affect the conclusions drawn in each experiment from comparison of the groups receiving water, acid or alkali.

Induction of acidosis and alkalosis. The method chosen for induction of acidosis and alkalosis was the substitution of dilute solutions of HCl or of NaHCO₃ for the drinking water, to which the animals had constant access. A strength of 0.1N-HCl proved unpalatable so that this was reduced to 0.05N; NaHCO₃ was given as 0.1N. That this method produced substantial changes in the production of urinary

ammonia was determined as follows. After 3 months on this regime, six rats given water, acid or alkali were transferred to individual metabolism cages, the urine collected for 24 hr. and ammonia estimated by the aeration method of Van Slyke (see Hawk, Oser & Summerson, 1947). Since it was desired to ascertain only the existence of obvious differences in ammonia production, no washings of the cages and funnels were made for addition to urine. Significant differences occurred in urinary ammonia in both male and female animals (Table 1). It is interesting to observe that appreciable differences exist in ammonia excretion in male and in female animals. This may be due to the differences in weight; the average weight of the males was 285 g. and of the females 175 g.

Table 1. *Excretion of urinary ammonia in normal, acidotic and alkalotic animals*

(Six animals in each group, fed stock diet and given water, acid or alkali for 3 months.)

Sex of animals	Urinary ammonia produced in 24 hr. (mean mg. NH ₃ -N \pm s.e.)		
	Normal	Acid	Alkali
Male	11.1 \pm 0.86	21.6 \pm 0.77	2.3 \pm 0.10
Female	8.7 \pm 0.13	12.1 \pm 0.48	2.0 \pm 0.13

Table 2 shows that the excretion of ammonia varied little from day to day. As a routine in later experiments therefore, one rat from each group was tested a day or two before the estimation of enzyme activity, to ensure the persistence of acidosis or alkalosis in the group.

Table 2. *Constancy of urinary ammonia*

(One male rat from each group, fed stock diet and given water, acid or alkali for 3 months; urinary ammonia measured for 3 consecutive days.)

Group	Urinary ammonia in 24 hr. (mg. NH ₃ -N)		
	Day 1	Day 2	Day 3
Water	12.4	12.1	12.3
Acid	24.4	23.9	24.1
Alkali	2.9	3.0	2.9

Enzymes. Ammonia may arise from amino-acids by deamidation, oxidative deamination or transamination. In order to examine possible adaptation in all these processes, the enzymic production of ammonia from the following five substances was studied: L-glutamine, glycine, L-leucine, L-aspartic acid and L-alanine. The pertinent data determining the selection may be summarized as follows: Van Slyke *et al.* (1943) claimed that L-glutamine is the chief source of urinary ammonia; their evidence suggests that 60% or more is derived from this substance. Glycine is one of the amino-acids which increase ammonia excretion when administered to acidotic dogs (Lotspeich & Pitts, 1947). Glycine oxidase is specific for this substance (Ratner, Nocito & Green, 1944). The experiments of Lotspeich & Pitts (1947) suggest that L-leucine, a monoamino monocarboxylic acid, may also be a precursor of urinary ammonia. It is deaminated by L-amino-acid oxidase and its deamination *in vitro* is at a greater rate than any other monoamino

monocarboxylic acid (Blanchard *et al.* 1944). Lotspeich & Pitts (1947) showed that DL-aspartic acid increased urinary ammonia in acidotic dogs. The rate of deamination *in vitro* is greater than that of any other amino-acid (Krebs, 1935a). L-Aspartic acid is not affected by L-amino-acid oxidase (Blanchard *et al.* 1944) but appears to be deaminated by a transaminase system (Braunstein, 1947).

The four preceding enzymes cover the processes of deamidation, oxidative deamination of glycine, oxidative deamination of other naturally occurring (L) amino-acids, and transamination. Nevertheless, it was thought worth while also to examine the deamination of L-alanine, since this may be effected both by L-amino-acid oxidase (Blanchard *et al.* 1944) and by transamination (Braunstein, 1947; Krebs, 1948).

Enzyme assay. The selected rat was killed by a blow on the head and the kidney rapidly removed, decapsulated and weighed. A portion was removed, weighed and again weighed after drying at 110°. Slices of 0.3 mm. were cut by the method of Deutsch (1936) from material kept ice-cold and moistened with oxygenated Ringer-phosphate solution of pH 7.4. Ammonia production was measured in the presence of O₂ in Warburg manometers at 38°. The flasks contained 2.5 ml. Ringer-phosphate solution and the side arm 0.5 ml. neutralized substrate solution. Ammonia production from kidney slices in the absence of substrate was examined at the same time in flasks containing 3.0 ml. Ringer-phosphate and these control values were subtracted from the values obtained in the presence of substrate. At the end of the experiment, the tissue slices were removed, dried and weighed. The ammonia produced was measured either by Van Slyke's aeration method or by the HCl-Ba(OH)₂ method of Conway (Conway, 1947). Enzyme activity was expressed as $\mu\text{g. NH}_3\text{-N}$ produced from the substrate/hr. in these conditions by selected dry weights of kidney tissue and by the whole kidney. Control values are reported for all experiments except for those with L-glutamine, where the blanks were less than 10% of the effect. With each enzyme, preliminary experiments were carried out to ascertain optimal substrate concentration, time of incubation and appropriate amounts of tissue to be used.

Glutamine. It was found that slices of cortex had the same enzyme activity as slices of cortex with medulla, so that the experiments with this enzyme were carried out with slices including both. From initial experiments, it was decided to use 4 mg. L-glutamine (final concentration 0.01 M approx.) and an incubation time of 30 min.; this gave reasonable yields of ammonia which, even in the most active tissues, were appreciably below the theoretical yield of 381 $\mu\text{g.}$ As the relation between ammonia produced and tissue used was not quite linear, four different amounts of tissue, with dry weights assessed at between 5 and 14 mg., were used for each rat, and an enzyme activity curve constructed. From this, enzyme activity was calculated by interpolation for dry weights of tissues of 6, 8, 10 and 12 mg.

Other substrates. Slices of renal cortex were used with these substrates. Trial experiments led to the adoption of the following final concentrations of substrate and times of incubation: glycine, 0.01 M and 90 min.; L-leucine, 0.01 M and 60 min.; L-aspartic acid, 0.005 M and 60 min.; L-alanine, 0.01 M and 120 min. These conditions were determined partly by the solubility of the substrate and partly by the need to have maximal production of ammonia without exhaustion of substrate or sensible deterioration of enzyme activity.

For these four substrates, calibration curves were constructed relating enzyme activity and amount of tissue. Each curve was linear up to about 15 mg. dry weight of tissue. In the estimation of enzyme activity, therefore, kidney slices were rapidly weighed before placing in the manometers and amounts used which would give dry weights between 10 and 15 mg. For each animal, these experiments and their controls were carried out in duplicate.

RESULTS

Growth of animals

In each of the five experiments, there were slight differences in weight in the normal, acidotic and alkalotic groups (Table 3). The differences between the normal and alkalotic animals were slight and variable; the differences between either of these and the acidotic animals were greater and consistently in favour of the normal and alkalotic groups. Although the differences were statistically significant in Exp. 2 only, it seems that chronic acidosis over a long period may produce slight impairment of growth.

Table 3. *Effect of chronic acidosis and chronic alkalosis on growth of rats*

(Six animals in each group. Diets and duration of experiment as in text. Mean wt. \pm s.e.)

Exp. no.	Substrate investigated	Wt. of animals at end of adaptation (g.)		
		Normal	Acid	Alkali
1	L-Glutamine	194 \pm 4.1	193 \pm 1.1	194 \pm 3.4
2	Glycine	361 \pm 4.4	337 \pm 3.2	368 \pm 6.4
3	L-Leucine	215 \pm 5.8	204 \pm 2.7	217 \pm 6.2
4	L-Aspartic acid	352 \pm 4.4	330 \pm 11.6	342 \pm 8.5
5	L-Alanine	346 \pm 12.2	340 \pm 10.5	347 \pm 8.7

(Differences between 'normal' and 'acid' groups and 'acid' and 'alkali' groups in Exp. 2 are significant ($P=0.01$). Differences in other experiments are not significant ($P>0.05$).

Enzyme activity

Glutamine. Acidosis resulted in a significant increase in glutaminase and alkalosis in a significant decrease (Table 5).

Other substrates. Since these experiments were made with renal cortex only, it is not possible to calculate the absolute amounts of enzyme in the whole kidney. If, however, it is assumed that the relative proportions of cortex and medulla were the same in all the groups, it is possible to calculate the relative amounts of total enzyme from the measured activity, the weight of the tissue used and the weight of the kidney. We have therefore given the arbitrary figure of 100 as the total activity of the kidneys from normal animals; the activities of acidotic and alkalotic animals are given as a proportion of this.

Table 4. *Summary of some of the experimental procedures*

Exp. no.	Substrate investigated	Sex of animals	Age of animals (weeks)		Duration of adaptation (weeks)	Concentration of substrate (M)	Time of incubation (min.)
			Beginning of adaptation	End of adaptation			
1	L-Glutamine	Female	13	26	13	0.01	30
2	Glycine	Male	3	33	30	0.01	90
3	L-Leucine	Female	3	21	18	0.01	60
4	L-Aspartic acid	Male	3	24	21	0.005	60
5	L-Alanine	Male	3	33	30	0.01	120

Acidosis resulted in a significant increase, and alkalosis in a significant decrease, in the enzymes producing ammonia from glycine and from L-leucine (Table 6). When calculated on the basis of unit weight of kidney, acidosis resulted in a slight

Thus, increase or decrease in ammonia production induced by acidosis or alkalosis results in an adaptive response in three of the five enzyme systems studied—those concerned in ammonia production from L-glutamine, glycine and L-leucine.

Table 5. *Effect of chronic acidosis and chronic alkalosis on renal ammonia production from L-glutamine*

(Slices incubated for 30 min. with 0.01M-L-glutamine. Kidney wt.: differences between any two groups are not significant ($P > 0.05$). Enzyme activity: in sections (a) and (b) differences between any two groups are significant ($P < 0.01$.)

(a) Activity/unit weight of kidney cortex. Values, corrected for blanks, were calculated by interpolation from experimental values and are expressed as $\mu\text{g. NH}_3\text{-N/mg. dry wt. of tissue/hr.} \pm \text{s.e.}$ for six animals in each group.

Dry wt. of tissue (mg.)	Group		
	Normal	Acid	Alkali
6	22 \pm 0.9	42 \pm 5.1	14 \pm 1.4
8	22 \pm 0.8	40 \pm 4.4	16 \pm 1.3
10	19 \pm 0.6	36 \pm 3.3	15 \pm 0.8
12	17 \pm 0.6	33 \pm 2.6	13 \pm 0.8

(b) Activity for whole kidney. Calculated for intrapolated values for 10 mg. dry wt.

Group	Dry wt. of total kidney (mg.)	Ammonia produced \pm s.e.	
		($\mu\text{g. NH}_3\text{-N/mg. dry wt./hr.}$)	($\mu\text{g. NH}_3\text{-N/total kidney/hr.}$)
Normal	380 \pm 18	19 \pm 0.6	7 280 \pm 450
Acid	370 \pm 7	36 \pm 3.3	13 400 \pm 1 330
Alkali	380 \pm 10	15 \pm 0.8	5 820 \pm 380

though significant increase in the enzyme producing ammonia from L-aspartic acid. This small change is probably fortuitous, since it disappeared when the total activity of the kidney was calculated. It seems legitimate to conclude that, as far as the whole kidneys are concerned, neither acidosis nor alkalosis resulted in a significant change in the enzymes producing ammonia from L-aspartic acid or from L-alanine.

DISCUSSION

This work on enzyme adaptation provides evidence that the sources of urinary ammonia include L-glutamine, glycine and L-leucine. This is in accord with the results referred to earlier, in which it was shown that the intravenous administration of these substances increase the amount of ammonia excreted.

The present work provides no new evidence concerning the participation of L-alanine or L-aspartic acid in ammonia production. No adaptive changes were found, but, as has been pointed out elsewhere (Yudkin, 1952), not all enzymes are capable of showing adaptation. It would appear from this, therefore, that the enzymes concerned in the deamination of these two amino-acids either are not concerned in ammonia production or are not adaptive enzymes.

This question is not answered by the results of other workers. We have seen that Bliss (1941) and Lotspeich & Pitts (1947) found that the administration of D- or L-alanine or of DL-aspartic acid increased urinary ammonia. The latter authors have themselves questioned whether ammonia could be formed from the L-isomer in the experiments with DL-aspartic acid, since the injection of another L-monoamino dicarboxylic acid, L-glutamic acid, failed to augment urinary ammonia. In addition, as we have pointed out, the concentrations of the amino-acids following injection are abnormally high. It remains therefore that no decision can be reached at this stage concerning the participation or otherwise of L-alanine and L-aspartic acid in renal ammonia production.

It is interesting to examine more closely the enzymes involved in ammonia production from L-alanine and L-aspartic acid. Both may be acted upon by the transaminase system, whilst L-alanine may also be acted upon by L-amino-acid oxidase.

Our results with L-leucine have shown that acidosis or alkalosis leads to a considerable increase or decrease in L-amino-acid oxidase. Since, however, these conditions do not affect ammonia production from L-alanine, it must be concluded that this amino-acid is acted upon very little, if at all, by the L-amino-acid oxidase of kidney slices. In this connexion, it is relevant to recall that alanine is the least rapidly deaminated amino-acid of the 13 acted upon by purified L-amino-acid oxidase; the rate is, for example, only 3% of that of L-leucine (Blanchard *et al.* 1944).

enzyme adaptation support the participation of the first three of these but can provide no evidence for or against the participation of the last.

Whilst the work described here was in progress, Handler, Bernheim & Bernheim (1949) reported experiments along similar lines. They found no change in ammonia production from L-alanine, L-glutamine or L-leucine by kidney slices of normal rats compared with those of rats rendered acidotic or alkalotic. Though their results with L-alanine agree with ours, their results with L-leucine and L-glutamine do not. However, examination of their paper

Table 6. *Effect on chronic acidosis and chronic alkalosis on renal ammonia production from glycine, L-leucine, L-aspartic acid and L-alanine*

(Mean values, corrected for blanks for six animals in each group \pm s.e. Figures in brackets are mean control values. Measurements with different substrates were made under different conditions, as given in Table 4. Comparisons may therefore be made horizontally, but not vertically.)

Substrate	Ammonia produced (μ g. $\text{NH}_3\text{-N}$ /mg. dry wt./hr.)			Dry wt. of total kidney (mg.)			Ammonia produced by total kidney (Arbitrary units; ammonia produced by normal rats = 100)		
	Normal	Acid	Alkali	Normal	Acid	Alkali	Normal	Acid	Alkali
Glycine	0.64 \pm 0.01 (1.05)	1.59 \pm 0.07 (1.09)	0.43 \pm 0.01 (0.99)	450 \pm 9	430 \pm 17	430 \pm 11	100 \pm 2.5	236 \pm 14.1	63 \pm 2.1
L-Leucine	1.94 \pm 0.05 (1.15)	3.01 \pm 0.03 (1.14)	1.02 \pm 0.03 (1.16)	330 \pm 11	340 \pm 10	340 \pm 21	100 \pm 1.9	157 \pm 5.2	53 \pm 4.2
L-Aspartic acid	3.53 \pm 0.05 (1.16)	3.81 \pm 0.06 (1.13)	3.73 \pm 0.11 (1.15)	520 \pm 25	470 \pm 14	460 \pm 7	100 \pm 4.7	98 \pm 3.5	94 \pm 3.4
L-Alanine	0.28 \pm 0.01 (1.01)	0.28 \pm 0.01 (1.08)	0.28 \pm 0.01 (1.02)	480 \pm 24	490 \pm 30	460 \pm 16	100 \pm 4.4	101 \pm 6.0	93 \pm 4.5

Kidney wt.: difference between any two groups not significant ($P > 0.05$), except difference between 'normal' and 'alkali' groups with L-aspartic acid ($P = 0.04$). Enzyme activity: with glycine and L-leucine, differences between any two groups significant ($P < 0.01$); with L-aspartic acid and L-alanine, differences between groups not significant ($P > 0.05$), except for difference between 'normal' and 'acid' groups with L-aspartic acid, calculated on basis of unit wt. of kidney ($P = 0.01$).

It seems then that L-alanine is deaminated chiefly, if not entirely, by the transaminase system and our results therefore suggest that chronic acidosis or alkalosis produce no change in this system. This may be because it plays no part in the formation of urinary ammonia. It might, on the other hand, be that the transaminase system is not adaptive; L-alanine and L-aspartic acid may in fact be precursors of urinary ammonia, but adaptation of this system does not occur. Since the transaminase system consists of more than one enzyme (probably a dehydrogenase and a transaminase), it is possible to imagine that one of them does in fact respond adaptively but that the other does not, and limits the rate of ammonia production. The effect would be the same in that the system as a whole would not appear to be adaptive.

This discussion may now be summarized in the following way. Experiments *in vivo* suggest that urinary ammonia may be produced by four enzyme systems: L-glutaminase, glycine oxidase, L-amino-acid oxidase and transaminase. The experiments on

makes it difficult to accept their findings without criticism. With L-leucine, they used only one normal and one acidotic animal. In addition, the rate of deamination reported with this substrate was lower than with any other, which is not in conformity with the results of other workers. With L-glutamine, they used only one normal, one acidotic and one alkalotic animal. More important, however, the conditions under which they measured ammonia production were such that about 90% of the possible maximal amount of ammonia was produced from the glutamine. It is clear that, in these circumstances, it might prove quite impossible to detect differences in enzyme activities.

Our experiments also provide evidence in another matter which has been the subject of discussion. This is whether an increase in urinary ammonia is caused by an increase in actual production of ammonia or, as suggested by Krebs (1935*a*) and Polonovski, Boulanger & Bizard (1935), an increase merely in the amount removed from the blood. The fact that chronic acidosis is accompanied by an

increase in enzymes responsible for ammonia production favours the view that increased urinary ammonia is caused, at least in part, by an increased formation of ammonia.

SUMMARY

1. Changes in ammonia excretion were produced in rats by the induction of chronic acidosis and chronic alkalosis. After 3–8 months, some selected renal enzymes were assayed in these animals by determining ammonia production, by kidney slices, from L-glutamine, glycine, L-leucine, L-aspartic acid and L-alanine. These substrates were selected to investigate possible adaptation of four enzymes which might be concerned in ammonia production: L-glutaminase, glycine oxidase, L-amino-acid oxidase and the transaminase system.

2. Chronic acidosis resulted in an appreciable increase, and chronic alkalosis in an appreciable

decrease, in ammonia production from L-glutamine, glycine and L-leucine. There was no significant change in ammonia production from L-aspartic acid and L-alanine.

3. These results suggest that urinary ammonia is produced, at least in part, by (1) deamination of glutamine, (2) deamination of glycine by glycine oxidase, (3) deamination of other monoamino monocarboxylic acids by L-amino-acid oxidase.

4. Reasons are given for supposing that deamination of L-alanine is effected chiefly, if not entirely, by the transaminase system which also acts upon L-aspartic acid. It would then appear either that this system plays no part in the normal mechanism of urinary ammonia formation, or that one or more of its component enzymes is not adaptive.

5. The adaptive changes found in three renal enzymes indicate that acidosis and alkalosis cause a change in ammonia formation and not merely in ammonia elimination.

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Kinetic Studies of the Metabolism of Foreign Organic Compounds

2. THE FORMATION OF PHENOLS FROM CERTAIN PRECURSORS

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The introduction of a hydroxyl group into the benzene nucleus is recognized as a common metabolic process. Compounds which are hydroxylated in this way may be regarded as phenol precursors. The present investigation deals with phenol precursors which are hydroxylated to give predominantly one isomer, namely anisole, which gives *p*-methoxyphenol (Bray, Thorpe & Wasdell, 1951a),

N-phenylurea, which gives *N*-(*p*-hydroxyphenyl)-urea (Bray, Lake & Thorpe, 1949) and benzoxazolone, which is excreted as a hydroxybenzoxazolone, the configuration of which has not yet been established (Bray, Clowes & Thorpe, 1952a). The overall plan was as described previously (Bray, Thorpe & White, 1951b). In some experiments the metabolites were estimated as phenols, in the