THE CONVERSION OF CITRULLINE TO ARGININE IN KIDNEY*

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Glycocyamine is formed in the kidney by the transfer of the amidine group of arginine to the nitrogen atom of glycine. In the study of this reaction it was observed that glycocyamine was also formed from citrulline and glycine. No other donor or precursor of the amidine group was found (1).

The most probable explanation of this effect of citrulline was that it was first converted to arginine, from which the amidine group was then transferred to glycine. This has now been proved.

This formation of arginine consists in an interaction of citrulline with either glutamic acid or aspartic acid. Its speed is of the same order of magnitude as that of oxidative deamination in the kidney. The two dicarboxylicamino acids are equally effective in this respect. An oxidation is involved in the reaction; it is nearly completely inhibited by such oxidation inhibitors as KCN, As_2O_3, and As_2O_5 in low concentration. The KCN inhibition is partly relieved by hydrogen acceptors; that of As_2O_3 and As_2O_5 is not.

There are in general three possible types of mechanism for this reaction, the essential differences in them consisting in the point at which the oxidation, *i.e.* the dehydrogenation, occurs: (*a*) at the dicarboxylicamino acid before it reacts with the citrulline, (*b*) at an intermediate compound consisting of the citrulline and the dicarboxylicamino acid, or (*c*), after the cleavage of this hypothetical intermediate compound, at a derivative of the citrulline which is the immediate precursor of the arginine.

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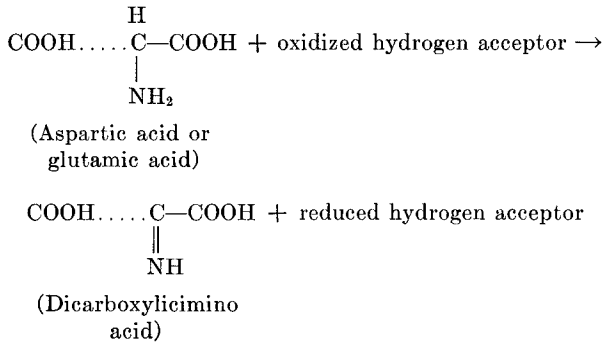
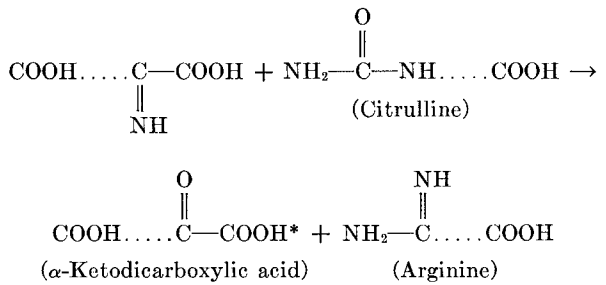
The first of the three possibilities is depicted by the following equations,

(1, *a*)



(1, *b*)



* Here, as in Equations 2 to 4, the dicarboxylic acid is written as being converted to the corresponding keto acid derivative. This is done only for convenience. We have obtained no evidence regarding the nature of the product arising from the deaminized dicarboxylicamino acid. It is possible that it is also decarboxylated in the oxidation; *i.e.*, the reaction mechanism is that suggested by Herbst (2).

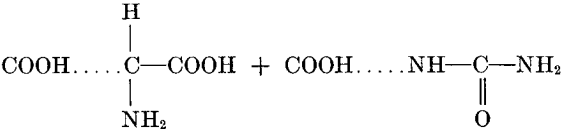
This mechanism was eliminated by the finding that arginine formation is inhibited under conditions in which oxidative deamination of the dicarboxylicamino acids proceeds unchecked.

Arginine is also formed from citrulline, ammonia, and α-ketoglutarate or oxalacetate. These reactions are also inhibited by KCN or As_2O_3. The interpretation we have placed on these results is that here also the reaction proper (*i.e.*, arginine formation) is between citrulline and glutamic acid or aspartic acid; that the dicarboxylicamino acids were formed by reductive amination prior to their combination with citrulline. It follows that the imino

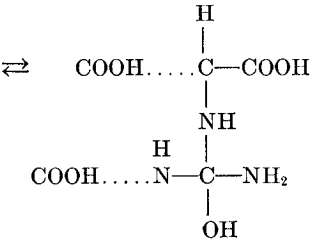
acid derivatives of the dicarboxylicamino acids (which are formed spontaneously and are in equilibrium in water with their corresponding α-keto acids and ammonia) do not react with the citrulline. This evidence also therefore excludes the reaction mechanism of Equations 1, *a* and 1, *b*.

The other two possible mechanisms are given by Equations 2, 3, and 4.

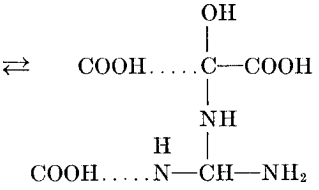
(2, *a*)



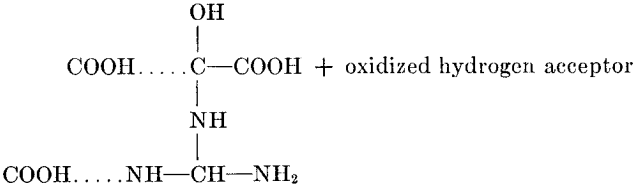
(2, *b*)



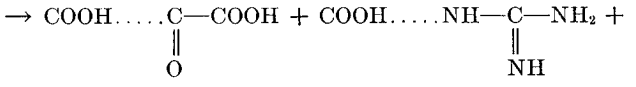
(2, *c*)



(3, *a*)



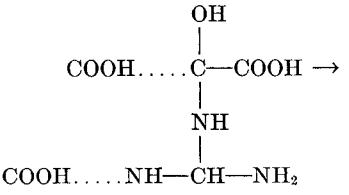
(3, *b*)



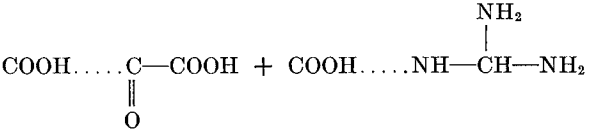
reduced hydrogen acceptor

or

(4, *a*)

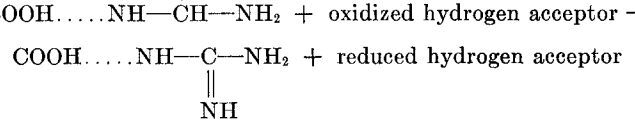


(4, *b*)



(4, *c*)

(4, *d*)



In both mechanisms an intermediate compound between the citrulline and the dicarboxylic acid is postulated. According to the mechanism of Equations 3, *a* and 3, *b* this compound is decomposed by dehydrogenation into arginine and the α-keto acid derivative of the dicarboxylicamino acid.

In the mechanism of Equations 4, *a* to 4, *d* the hypothetical intermediate compound is decomposed first and the resulting diamine group undergoes dehydrogenation to form arginine.

If the mechanism of Equations 4, *a* to 4, *d* were correct, when citrulline and the dicarboxylicamino acid are present but arginine formation is prevented by As_2O_3, the keto acid should be formed nevertheless, but without an equivalent amount of ammonia. Ammonia and keto acid will be formed in addition from the concurrent and independent oxidative deamination of the dicarboxylicamino acid. Accordingly the ratio Δ NH_3 to Δ keto acid should be lower and the absolute amount of keto acid higher in solutions containing citrulline, dicarboxylicamino acid, and As_2O_3 than in the absence of citrulline; *i.e.*, the reaction would proceed as far as Equation 4, *b*. It was found that the ratio Δ NH_3 to Δ keto acid in the presence of As_2O_3 was the same with and without citrulline; nor was there an absolute increase in keto acid in the presence of citrulline.

The evidence appears to lead by exclusion to a mechanism of the type of Equations 2 and 3. Braunstein (3) postulated an intermediate compound analogous to that of Equation 2 as a preliminary to transamination. It must be emphasized that in both cases the existence of this hypothetical compound is, as yet, unsupported by any direct evidence. It is an inference only.

Positive results were obtained when proline, hydroxyproline, ornithine, or lysine was used in place of aspartic acid or glutamic acid. All the other amino acids were negative. There is independent evidence that the first three amino acids named are readily convertible to glutamic acid in the rat (4–7). It is reasonable to conclude, therefore, that lysine also is converted to glutamic acid (or aspartic acid). This is the first clue, as far as we are aware, regarding the path of degradation of the carbon skeleton of lysine in the animal body.

In the ornithine cycle by which urea is formed in the liver from ammonia and carbon dioxide, one of the steps proposed is the conversion of citrulline to arginine (8). The mechanism for this conversion which we have found in kidney, *i.e.* the interaction of citrulline with aspartic acid or glutamic acid, does not occur in liver. A study of arginine formation in liver is in progress and will be reported later.

Procedure and Preparations Used

Most of the experiments were carried out with surviving rat kidney slices. In a few experiments guinea pig kidney was used. The details of the technique have been described (9). Two or three small slices were suspended in 4 ml. of bicarbonate-Ringer's solution containing the substances under investigation. The pH of the solution was 7.4 after equilibration with 95 per cent oxygen and 5 per cent CO_2. All the experiments were carried out at 38° for 1 or 2 hours.

At the end of the experimental period the contents of the reaction vessels, acidified to pH 6.0 with a drop of 0.5 N HCl, the slices, and two 2.5 ml. washings with water were transferred to test-tubes graduated at 10 ml. After 10 minutes in a boiling water bath the test-tubes were cooled, and the contents made up to the mark with water, mixed by shaking, and filtered. The tissue slices were dried at 100° and weighed.

Glycocyamine and arginine were then determined in the protein-free filtrates. A 5 ml. aliquot was passed through a column of permutit and the traces of glycocyamine remaining in the column removed with 5 ml. of 0.3 per cent NaCl. The glycocyamine was determined in the combined filtrate by the method we have described (10). The arginine was then eluted from the permutit and determined as described by Dubnoff.[1]

Ammonia and keto acids were also determined in some experiments. These analyses were carried out on the protein-free filtrates which were not passed through permutit. The analytical method used for ammonia has been described (11).

The keto acids were determined as follows: To 2 ml. of protein-free filtrate 0.3 ml. of a saturated solution of 2,4-dinitrophenylhydrazine in 1 N HCl was added, followed after standing for a few minutes by 1 ml. of 1 per cent NaOH. An intense, deep red color appears immediately. This quickly fades and after 20 minutes a stable red color remains whose intensity is proportional to the concentration of pyruvic acid, oxalacetic acid, or α-ketoglutaric acid present. A linear relationship is obtained between the concentration of keto acid and the depth of color when it is measured at a wave-length of 0.525 μ, in the concentration range from 0 to 2.0 mg. per cent. The color of higher concentrations is too deep to be measured accurately.

The use of 2,4-dinitrophenylhydrazine in alkaline solution for the colorimetric determination of pyruvic and other keto acids has recently been reviewed by Klein (12). The simple procedure we have employed, without preliminary extraction of the hydrazone, would give positive values with such substances as acetone and acetoacetic acid. Under our experimental conditions the latter two substances are not formed in measurable amounts. The controls (Table VII) showed that for our limited purposes the simplified procedure was satisfactory.

l(+)-Citrulline was used throughout. It was prepared by a combination of the methods described by Kurtz (13) and by Gornall and Hunter (14). The starting material was a commercial preparation of *l*(+)-ornithine. All the other amino acids used were prepared or purified either by Amino Acid Manufactures, University of California at Los Angeles, or in this laboratory. We are in-

[1] Dubnoff, J. W., *J. Biol. Chem.*, **141**, 711 (1941).

debted to Professor H. B. Vickery for a generous donation of glutamine. The naturally occurring isomers of the amino acids were used except when the *dl* form is specified.

Pyruvic acid was prepared by distillation of a commercial product; the distillate was neutralized by NaOH, and the sodium salt crystallized and dried.

α-Ketoglutaric acid was prepared by the method of Neuberg and Ringer (15). We are indebted to Mr. Werner Baumgarten for this preparation.

Oxalacetic acid was prepared by the method of Simon (16).

The α-keto acid derivative of methionine was obtained by oxidation of *dl*-methionine with *d*-amino acid oxidase prepared from hog kidney, ethereal extraction of the acid from the acidified, deproteinized solution, evaporation of the ether, solution of the acid in water, neutralization with NaOH, and recrystallization from water.

The acetaldehyde was a commercial preparation.

Coenzyme I was prepared by the method of Ohlmeyer (17), and coenzyme II and adenosine triphosphate according to the prescription given by Warburg and Christian (18). The coenzyme preparations, as used, were 60 per cent pure. These preparations were made by Dr. Norman H. Horowitz.

Results

Table I is a typical protocol showing the formation of arginine from citrulline and glutamic acid or aspartic acid. There was always a significant increase in arginine over the blank value when citrulline alone was added to the Ringer's solution. This arginine was formed, presumably, from the added citrulline and aspartic acid or glutamic acid (see Table IV) which was present in the kidney slices at the outset or formed in the slices during the experiment. Nearly 3 times as much arginine was formed, however, when either aspartic acid or glutamic acid was added with the citrulline. The two dicarboxylicamino acids were equally effective.

There was a small increase in glycocyamine whenever arginine formation occurred. It may be inferred from previous observations (1) that the amidine group of the arginine was transferred to glycine which was present in the slices initially or formed in the course of the experiment. The quantity of available glycine was

small and also limited because no more glycocyamine was formed when the arginine was increased 3-fold.

It was not feasible, because of the high solubility of arginine and its salts, to attempt to prove by isolating one of them that the substance we were measuring colorimetrically was arginine. This was

TABLE I

Typical Protocol Showing Formation of Arginine by Surviving Rat Kidney Slices from Citrulline and Glutamic Acid or Aspartic Acid

Ringer's solution; 38°; 1 hour.

Solution No.	Tissue weight, dry	Citrulline, 0.0025 M	Glutamic acid, 0.005 M	Aspartic acid, 0.005 M	Arginine found		Glycocyamine found	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
	mg.				*mg. per cent*	*mg. per gm. fresh tissue*	*mg. per cent*	*mg. per gm. fresh tissue*
1	3.2	−	−	−	0.02	0.3	0.02	0.3
2	7.3	−	−	−	0.02	0.1	0.04	0.2
3	7.2	+	−	−	0.33	1.8	0.07	0.4
4	6.7	+	−	−	0.31	1.9	0.07	0.4
5	11.7	−	+	−	0.04	0.1	0.04	0.1
6	11.1	−	+	−	0.04	0.1	0.06	0.2
7	8.3	+	+	−	1.07	5.2	0.08	0.4
8	6.2	+	+	−	0.89	5.7	0.07	0.4
9	10.3	−	−	+	0.03	0.1	0.04	0.2
10	11.9	−	−	+	0.04	0.1	0.04	0.2
11	6.1	+	−	+	0.89	5.8	0.06	0.4
12	7.5	+	−	+	1.02	5.4	0.07	0.4

The figures in Columns 6 and 8 give the concentrations of arginine and of glycocyamine in the protein-free solutions after they had passed through the columns of permutit. The figures in Columns 7 and 9 are obtained by multiplying those in Columns 6 and 8 respectively by the factors, $4/100 \times 10/4 \times 2 \times 1000/(5 \times W) = 40/W$, where W is the dry weight of tissue in mg. The basis of these factors is as follows: The volume of the original, undiluted Ringer's solution was 4 ml.; it was diluted to 10 ml. for deproteinization; in the course of separation of the glycocyamine and elution from permutit the aliquot used was diluted with an equal volume of salt solution; the fresh weight of the slices is 5 times the dry weight.

established by three independent pieces of evidence: the specificity of the color reagent under the conditions in which we used it, the disappearance of this chromogenic material when subjected to the action of arginase, and the formation of glycocyamine in the highly specific transamidination reaction with glycine.

The color reagent, the Sakaguchi reagent, gives a positive test with only three common biological substances. These are glycocyamine, histidine, and arginine. Glycocyamine is separated quantitatively from arginine and histidine by the adsorption and elution procedure employed. Histidine, however, remains on the permutit and is removed with arginine by the 3 per cent sodium chloride solution used for eluting the arginine from permutit. Histidine has about 1 per cent the chromogenic power of arginine. Even if all the added citrulline had been converted to histidine, most of the color developed would still remain to be accounted for.

Table II presents direct evidence that the material in question was arginine. Digestion with arginase removed nearly all of the chromogenic material designated as arginine. The details of the experimental procedure are given in the foot-note to Table II.

The transamidination reaction (*i.e.*, formation of glycocyamine from arginine and glycine) is a highly specific test for arginine when an extract of kidney (instead of kidney slices) is used to provide the enzyme. The arginine formed from any citrulline present is negligible for the purposes of this test. The test consists in incubating the material in question with an excess of glycine in the presence of the enzyme. A positive result is indicated by the formation of glycocyamine, and the rate of formation of glycocyamine is proportional to the initial concentration of arginine.

Table III shows that this criterion also afforded clear evidence that we were dealing with arginine. If the chromogenic substance in experiments such as those in Table I was arginine, it was to be expected that there would be (*a*) little or no glycocyamine in Solutions 1, 3, and 4 of Table III, (*b*) large increases in Solutions 2, 5, and 6, with 2 to 3 times as much in Solutions 5 and 6 as in Solution 2, and (*c*) less arginine remaining in this experiment than in the experiment of Table I because of the conversion of some of the arginine to glycocyamine; the "total"[2] arginine formed per hour would, however, be the same in Solutions 5 and 6 of Table III as in Solutions 7 and 8 and 11 and 12 of Table I. The results obtained were in complete accord with these predictions.

Table IV summarizes our findings on the relative effectiveness of

[2] The "total" arginine is the sum of the arginine found and of the amount of glycocyamine multiplied by 1.49 (the ratio of the molecular weight of arginine to that of glycocyamine).

different amino acids and of some other nitrogen compounds as contributors of the =NH group in the conversion of citrulline to arginine. Glutamine was the only compound which was as effective as aspartic acid or glutamic acid. This may be taken to indicate the rapid hydrolysis of the added glutamine rather than that the amide itself is effective. An active glutaminase is present in

TABLE II

Proof from Action of Arginase That Arginine Was Main Chromogen Measured by Colorimetric Method Employed

The arginine values are measured in mg. per gm. of fresh tissue.

Citrulline, 0.0025 M	Aspartic acid, 0.005 M	Glutamic acid, 0.005 M	Arginine present measured colorimetrically	
			Before action of arginase	After action of arginase
−	−	−	1.4	0.2
+	−	−	3.3	0.2
+	+	−	11.0	0.6
+	−	+	8.8	0.5
−	+	−	1.2	0.2
−	−	+	1.0	0.2

Ringer's solution, 38°; 1.5 hours. Each of the six mixtures was carried through in quadruplicate to the end of the period of incubation with kidney slices; pairs of similar solutions were then combined. Each of the values given above therefore is an average of two completely separate but identical experiments run simultaneously from the initial incubation with kidney slices through the digestion with arginase to the final colorimetric determination. The arginine was determined before and after digestion of the protein-free filtrates with arginase. Dry arginase powder was prepared by the method of Hunter and Dauphinee (19). 4 ml. of the protein-free filtrate were digested at pH 8.7 for 1 hour at 38° with 0.2 ml. of a 3 per cent solution of the arginase powder. At the end of the hour the solutions were acidified to pH 6.0, boiled, and filtered. The arginine in the filtrates was then determined in the usual manner after removal of the glycocyamine with permutit.

kidney (20). Asparagine was much less active than aspartic acid (the asparaginase activity of kidney is very low), while acetamide, formamide, and nicotinamide were negative.

The positive results with proline, hydroxyproline, and ornithine were to be expected. Weil-Malherbe and Krebs (4) and Neber (5) have proved that proline and hydroxyproline are converted to

glutamic acid by rat kidney slices. The figures in Table IV show that the formation of arginine from citrulline was approximately twice as fast with proline as with hydroxyproline. This is in accord with the findings of Weil-Malherbe and Krebs that glutamic acid is formed twice as quickly from proline as from hydroxyproline.

The conversion of *l*(+)-ornithine to glutamic acid was first suggested by Krebs (6) on the basis of indirect evidence. *d*(+)-Proline and *d*(−)-ornithine gave the same oxidation product, α-keto-δ-aminovalerianic acid, after treatment with *d*-amino

TABLE III

Glycine Transamidination with Arginine Formed from Citrulline

The results are expressed as mg. per gm. of fresh tissue.

Solution No.	Metabolites used	Glyco-cyamine	Arginine	"Total" arginine
1		0	0.4	0.4
2	Citrulline	1.2	2.0	3.8
3	Aspartic acid	0.2	0.8	1.1
4	Glutamic "	0.2	0.6	0.9
5	Citrulline + aspartic acid	2.6	5.9	9.8
6	" + glutamic "	2.7	6.6	10.6

The different mixtures of metabolites were first incubated at 38° with rat kidney slices for 2 hours. The citrulline, aspartic acid, and glutamic acid were initially 0.01 M. A 3 ml. aliquot of each mixture was then removed and added to 1 ml. of a cell-free extract of rat kidney to which glycine had been added to a concentration of 0.04 M. These mixtures were then incubated at 38° with shaking for 6 hours. They were then deproteinized and analyzed for glycocyamine and arginine.

acid oxidase. *l*(−)-Proline was proved to be converted to glutamic acid. It was a reasonable surmise then that glutamic acid was formed also from *l*(+)-ornithine via α-keto-δ-aminovalerianic acid in the same manner; *viz.*, oxidation at the δ-carbon atom to form the acid and amination of the α-carbon atom. Direct evidence of this conversion was obtained by Roloff, Ratner, and Schoenheimer (7). These workers fed deutero ornithine to normal adult mice and later found not only deutero arginine but also deutero proline and deutero glutamic acid. Data such as those in Table IV reveal that the conversion of *l*(+)-ornithine to glutamic acid occurs rapidly in rat kidney.

In view of the findings with proline, hydroxyproline, and ornithine it is a reasonable interpretation of the positive result with lysine (Table IV) that this amino acid also is converted to glutamic acid.[3]

TABLE IV

Relative Effectiveness of Different Amino Acids and Other Nitrogen Compounds As Contributors of =NH Group in Conversion of Citrulline to Arginine

Ringer's solution; 38°; 1 hour. The citrulline was initially 0.0025 M; all other compounds 0.005 M. Except where the *dl* form is specified, the naturally occurring isomer was used.

The results are expressed as per cent of "total" arginine formed from citrulline and glutamic acid. "Total" arginine = arginine + glycocyamine × 1.49.

Substance	Relative speed of arginine formation	Substance	Relative speed of arginine formation
Acetamide	−7	*dl*-Isoleucine	−8
Alanine	−8	Leucine	−8
Ammonia	1	Lysine	47
Asparagine	55	*dl*-Methionine	−8
Aspartic acid	108	Nicotinic acid amide	−21
Cysteine	−4	*dl*-Norleucine	−18
Formamide	−13	Ornithine	42
Glutamic acid	100	*dl*-Phenylalanine	−12
Glutamine	101	Proline	51
Glutathione	46	*dl*-Serine	−3
Glycine	1	Threonine	−14
Histidine	0	Tryptophane	−16
Hydroxyproline	23	Tyrosine	−6
		Valine	5

Table V shows the inhibition of the interaction of citrulline and aspartic acid or glutamic acid by low concentrations of KCN, As_2O_3, or As_2O_5. This inhibition is about 95 per cent complete.

[3] It may be expected from their structural similarity that the conversion of lysine to glutamic acid follows a course analogous to that of ornithine. One pathway for which there is some experimental evidence is as follows: oxidative deamination of the α-amino group, followed by oxidative deamination of the ϵ-amino group, β oxidation at the carbon atom to form α-ketoglutaric acid, which is reduced with the addition of ammonia to glutamic acid.

The cyanide inhibition is relieved to a considerable extent by the α-keto acid derivative of methionine, by oxalacetate, and by

TABLE V

Inhibition of Arginine Formation by Oxidation Inhibitors

Ringer's solution; 38°; 2 hours. The initial concentration of citrulline was 0.0025 M; of =NH donor 0.005 M.

The results are expressed as mg. of "total" arginine per gm. of fresh tissue.

	Uninhibited	KCN 0.001 M	As_2O_3 0.001 M	As_2O_5 0.0036 M
No amino acids added	1.3	0.9	0.9	0.8
Citrulline	2.7	1.1	1.1	1.1
" + aspartic acid	12.2	1.8	1.8	1.7
" + glutamic "	11.2	1.7	1.8	1.7

TABLE VI

Counteraction of Hydrogen Acceptors on Inhibition of Arginine Formation by KCN and As_2O_3

Ringer's solution; 38°; 2 hours. The citrulline was initially 0.0025 M; =NH donor and other metabolites 0.005 M.

=NH donor	Aspartic acid			Glutamic acid		
	Uninhibited	0.001 M KCN	0.001 M As_2O_3	Uninhibited	0.001 M KCN	0.001 M As_2O_3
No amino acids added	1.1	0.9	0.9	1.1	0.9	0.9
Citrulline alone	3.0	1.0	1.1	3.1	1.0	1.0
" + =NH donor	12.2	1.6	1.0	12.0	1.3	1.6
" + " " + acetaldehyde		0.9			0.6	
Citrulline + =NH donor + α-keto acid derivative of methionine		5.5			7.0	
Citrulline + =NH donor + oxalacetate		3.1	1.1		4.3	1.9
Citrulline + =NH donor + pyruvate	7.2	3.1	1.2	6.7	2.2	1.3

pyruvate. These metabolites do not relieve the inhibition by arsenite (Table VI).

Our interpretation of these findings is that both cyanide and

As_2O_3 (and As_2O_5) inhibit the formation of arginine by preventing the necessary dehydrogenation of the citrulline-aspartic acid (or glutamic acid) complex. The effect of cyanide is indirect; it prevents the oxidation of cytochrome. The flow of electron and of hydrogen transfer is thus blocked by the lack of an available acceptor. Arginine formation can be reinstituted, however, by the provision of electron and hydrogen acceptors other than the cytochrome-oxygen system which can function even in the presence of cyanide. They must be provided in sufficient concentration because their reoxidation is also blocked by cyanide and they cannot therefore function in a cyclic manner. The α-keto acid derivative of methionine, oxalacetic acid, and pyruvic acid served this purpose. Acetaldehyde did not relieve the cyanide inhibition. This would exclude complex formation with the cyanide as an explanation of the positive results with the above keto acids.

The arsenite inhibition, on the other hand, is not relieved by these metabolites because this inhibitor interferes with the process of dehydrogenation; *i.e.*, the initial process of donation of electrons and hydrogens is blocked. Hence the provision of additional hydrogen acceptors affords no relief.

In view of the participation of the pyridine coenzymes in the oxidative deamination of *l*(+)-glutamic acid (21) and the evidence that an oxidation of aspartic acid or glutamic acid is involved in this formation of arginine, we tested the effect of coenzymes I and II and of adenylic acid pyrophosphate on the reaction. These substances neither relieved the arsenite inhibition nor increased the rate in the absence of an oxidative inhibitor.

The enzymatic activity of kidney slices is largely lost (about 90 per cent) when the kidney is homogenized into a cell-free suspension. One possible explanation for this loss of activity was that the pyridine coenzymes were dissociated from the protein of the enzyme as a result of the dilution consequent on the preparation of the cell-free suspension. If this explanation were correct, addition of one of the two pyridine coenzymes or of adenylic acid pyrophosphate might be expected to restore some of the lost enzymatic activity. This was not the case. It is possible that some other coenzyme is operative in this reaction.

Another piece of evidence against the above explanation of the loss of enzymatic activity is that kidney tissue homogenized and

tested without any added fluid is no more active than when homogenized and suspended in 4 times its volume of buffer solution.

Table VII shows the separation of oxidative deamination of the dicarboxylicamino acids from the process in which citrulline is converted to arginine. The latter reaction is inhibited by arsenite; the former is not. In fact an inhibitor such as As_2O_3 which retards the disappearance of the products of deamination, the ammonia and keto acids, is necessary for the demonstration of oxidative deamination by tissue slices.

TABLE VII

Separation of Conversion of Citrulline to Arginine from Oxidative Deamination

Guinea pig kidney slices were used; Ringer's solution; 38°; 2 hours. The citrulline was initially 0.0025 M; aspartic acid either 0.005 or 0.05 M as indicated; As_2O_3 0.001 M.

The results are expressed as micromoles per gm. of fresh tissue.

Citrulline	Aspartic acid, initial molality	As_2O_3	Arginine found	Keto acid as pyruvic acid	Ammonia	$\frac{\Delta \text{ ammonia}}{\Delta \text{ pyruvic acid}}$
−		−	0.9	0	4.3	
−		+	1.0	0	10.4	
+		−	1.9	0	7.1	
+		+	0.9	0	10.9	
+	0.005	−	12.1	0	8.7	
+	0.05	−	6.3	1.8	13.6	
+	0.005	+	1.7	13.5	25.5	1.6
+	0.05	+	1.3	36.9	54.2	1.4
−	0.005	−	1.0	0	9.0	
−	0.05	−	1.2	0	20.2	
−	0.005	+	1.0	10.4	23.7	1.9
−	0.05	+	1.0	35.1	54.1	1.4

Another piece of evidence which indicated that the two oxidative processes, arginine formation and deamination, are different, although they involve the same substrate (aspartic acid or glutamic acid), was obtained from a comparison of the effects of two different concentrations of aspartic acid, 0.005 and 0.05 M. The higher concentration of aspartic acid inhibited arginine formation, but the rate of deamination, in an absolute sense, was more than 3 times greater. If the reaction consisted in an exchange of the oxygen on the ϵ-carbon atom of citrulline for the =NH group of

the imino acid derivative of aspartic or glutamic acid, the reaction should proceed with oxalacetic acid and ammonia or ketoglutaric acid and ammonia, even in the presence of cyanide, because the imino acids are formed spontaneously from the latter keto acids and ammonia.

Table VIII shows that arginine is formed from citrulline and the products of deamination of the dicarboxylicamino acids under normal conditions; but the reaction is completely inhibited by cyanide.

TABLE VIII

Aspartic Acid or Glutamic Acid Formation in Rat Kidney Slices; Attested to by Formation of Arginine from Citrulline

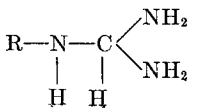
Ringer's solution; 38°; 2 hours. The initial concentration of citrulline was 0.0025 M; of glutamic acid or other metabolites and ammonia 0.005 M.

The results are expressed as "total" arginine formed per gm. of fresh tissue.

Metabolites in addition to citrulline	"Total" arginine found
None	2.4
Citrate + ammonia	2.9
Fumarate + "	4.8
Glucose + "	3.7
Glutamate	10.5
α-Ketoglutarate + ammonia	7.9
Oxalacetate + ammonia	4.8
Pyruvate + ammonia	2.6
Succinate + "	4.5

Three independent lines of evidence concur, therefore, in excluding prior oxidative deamination of the aspartic acid or glutamic acid as part of the reaction mechanism in the formation of arginine from citrulline.

As stated above, another possible reaction mechanism would locate the oxidation on a derivative of the citrulline after it had undergone transamination with the dicarboxylic acid to form a compound of the type



Dehydrogenation of the diamine group would then yield arginine. If this were the reaction mechanism, the addition of citrulline in the presence of As_2O_3 and aspartic acid or glutamic acid would, as discussed above, decrease the ratio Δ ammonia to Δ keto acid, because of an absolute increase in the keto acids without a corresponding increase in ammonia. The data in Table VII eliminate this hypothesis. In the presence of As_2O_3 the absolute amount of keto acids and the ratio of Δ ammonia to Δ keto acid were the same in the presence as in the absence of citrulline.

The evidence, by exclusion, therefore points to the locus of the oxidation on a hypothetical intermediate compound of citrulline and the dicarboxylicamino acid.

The reason that the ratio Δ ammonia to Δ pyruvic acid is greater than 1 is that As_2O_3 does not completely inhibit the oxidation of keto acids. Even in unpoisoned tissue the removal of ammonia is less complete than that of the keto acids. Similar observations were made by Krebs (6).

The formation of arginine from citrulline by surviving kidney slices can be used as a test for potential sources of aspartic acid or glutamic acid. It was shown by this method that certain amino acids are converted in the kidney to one or the other of these two dicarboxylicamino acids (Table IV). The action of tissue slices in this respect parallels closely that of the whole animal. This parallel was also seen in the synthesis of hippuric acid (22) and of creatine (23). The same method can be used as a test for precursors of the carbon skeleton of the dicarboxylicamino acids. The reagents are citrulline, an excess of ammonia, and the metabolite in question. Some examples are shown in Table VIII. Fumarate, glucose, α-ketoglutarate, oxalacetate, and succinate were definitely positive. A dubiously slight positive result was obtained with citrate. Pyruvate was negative.

The mechanisms by which these non-nitrogenous metabolites may be converted to the dicarboxylicamino acids are well known and need not be discussed here. The data in Table VIII are positive evidence that these changes occur readily in the kidney. The citric acid cycle appears to be quantitatively less important than simple oxidation of succinic acid and its derivatives.

The formation of arginine may be used as an indicator to ascertain the "preferred" directions of some of the reversible processes

in the dynamic steady state of metabolic reactions in kidney slices. This test is based on the fact that, in the presence of an excess of citrulline, the formation of arginine is faster the greater the concentration of aspartic acid or glutamic acid (at concentrations below 0.005 M). The following are two examples: The amount of arginine formed from citrulline in the presence of different amino acids and α-ketoglutarate is an indication of the speed of transamination in the direction of glutamic acid formation; the difference in the amount of arginine formed from citrulline, α-ketoglutarate, and ammonia in the presence and in the absence of

TABLE IX

Transamination in Rat Kidney Slices

Ringer's solution; 38°; 2 hours. The initial concentration of citrulline was 0.0025 M; of other amino acids, keto acids, and ammonia 0.005 M.

The results are expressed as "total" arginine formed per gm. of fresh tissue.

Metabolites in addition to citrulline	"Total" arg nine found
None	2.0
Glutamate	10.9
" + pyruvate	7.8
α-Ketoglutarate	3.1
Ammonia	2.2
α-Ketoglutarate + ammonia	4.6
" + pyruvate + ammonia	1.9
Alanine + α-ketoglutarate	2.8
dl-Alanine + α-ketoglutarate	2.1
Valine + α-ketoglutarate	1.6

pyruvate indicates whether ammonia nitrogen available for amino acid synthesis is bound preferentially as glutamic acid or as alanine.

Table IX is a summary of some experiments along these lines. It is seen that glutamic acid contributes its amino group to pyruvate. On the other hand, amino nitrogen does not pass readily from *l*(+)-alanine, *dl*-alanine, and *l*(+)-valine to α-ketoglutarate. In this respect ammonia is more effective. We may infer then that the formation of glutamic acid from α-ketoglutarate is faster by reductive amination than by transamination.

Similarly, in the presence of ammonia, α-ketoglutarate, and pyruvate there was no evidence of the formation of any glutamic acid available for arginine formation; presumably the nitrogen was bound preferentially as alanine.

The dynamic steady state in kidney slices appears therefore to include a cycle which favors the formation of glutamic acid by reductive amination, followed, if suitable keto acids are available, by transamination to form other amino acids with the regeneration of the α-ketoglutarate.

TABLE X

Inhibition by Pyruvate of Arginine Formation from Citrulline

Ringer's solution; 38°; 1 hour. The initial concentration of citrulline was 0.0025 M; of other amino acids and pyruvate 0.005 M.

The results are expressed as "total" arginine formed per gm. of fresh tissue.

Metabolites added in addition to citrulline	"Total" arginine formed
None	1.3
Aspartate	5.6
" + pyruvate	2.8
Glutamate	4.6
" + pyruvate	2.3
Lysine	3.3
" + pyruvate	1.8
Ornithine	3.7
" + pyruvate	2.4
Proline	2.8
" + pyruvate	1.1

The situation in kidney slices appears to be similar to that in the whole animal. Schoenheimer and his colleagues (24) observed that the most extensive exchange of normal nitrogen for N^{15} occurred in the nitrogen of aspartic acid and glutamic acid whether the N^{15} was introduced as ammonia or as a single amino acid.

The formation of arginine from citrulline was inhibited by pyruvate whether the donor of the =NH group was aspartic acid, glutamic acid, or a precursor of these dicarboxylicamino acids (Table X). This is in accord with the findings in Table IX that pyruvate is converted to alanine at the expense of glutamic acid. It is interesting that pyruvate is inhibitory under the normal cir-

cumstances of the experiment of Table X; yet in the presence of cyanide it permits arginine formation to occur, neutralizing to some extent the cyanide inhibition (Table VI). This latter fact suggests strongly that pyruvate does not inhibit the oxidation necessary for the formation of arginine from citrulline, that it is inhibitory under normal circumstances because it competes effectively with the citrulline for the amino group of aspartic acid or glutamic acid. More direct evidence is required, of course, to determine whether or not this hypothesis is correct.

DISCUSSION

The work of Braunstein and Kritzmann (3, 25), of von Euler *et al.* (21), and of Cohen (26) established by indirect but strong evidence the central position of the dicarboxylicamino acids in the continual and rapid interchange of amino nitrogen which Schoenheimer and his coworkers demonstrated to occur in the body by direct evidence (24).[4] The mechanism of this interchange involves transamination and reductive amination.

Another mechanism is involved in the formation (in the kidney) of arginine from citrulline in which the dicarboxylicamino acids also participate. Instead of transamination, the reaction might be designated as transimination, except that the dehydrogenation of the amino group of the dicarboxylicamino acid does not occur prior to its reaction with the citrulline but while it is in combination with it.

The discovery of an extremely active mechanism for converting citrulline to arginine in the kidney suggests a hitherto unsuspected source of the arginine which the kidney requires for, among other purposes, the transamidination reaction with glycine to form glycocyamine.

It seems improbable that a mechanism which can transform citrulline to arginine so quickly and act upon low concentrations of metabolites, less than 0.001 M, is inoperative *in vivo*, teleological as the argument is. The question then arises, what is the source of the citrulline? The kidney cannot convert ornithine to citrulline.

[4] It is interesting in retrospect that this interchange of amino nitrogen could have been inferred from the fact that ammonia exerts considerable nitrogen sparing action even in man. The continual and extensive synthesis and breakdown of protein was deduced from this and other data (27).

If one accepts the ornithine-urea cycle in the liver as proposed by Krebs and Henseleit (8), the liver can hardly supply the citrulline, because in that cycle the existence of the citrulline is only transitory on its way to arginine.

SUMMARY

1. Citrulline is converted to arginine at a rapid rate by rat and guinea pig surviving kidney slices. This property is almost completely lost when the cell structure is destroyed.

2. Either aspartic acid or glutamic acid is necessary for this reaction (in addition to citrulline).

3. Proline, hydroxyproline, lysine, and ornithine may replace the dicarboxylicamino acids in this reaction. Evidence is adduced that they do so by being converted first to glutamic acid (or, possibly but less likely, to aspartic acid).

4. Arginine is formed from citrulline and α-ketoglutaric acid and ammonia or oxalacetic acid and ammonia. Evidence is presented that these products of oxidative deamination are reduced by kidney slices to form the parent dicarboxylicamino acids.

5. This formation of arginine from citrulline is nearly completely inhibited by oxidative inhibitors, KCN, As_2O_3, and As_2O_5, indicating an oxidative step in the reaction mechanism. The cyanide inhibition is relieved in part by the α-keto acid derivative of methionine, by oxalacetate, and by pyruvate. The inhibition by As_2O_3 and As_2O_5 is not relieved by these metabolites. An interpretation of these findings is presented.

6. The oxidative step is not a dehydrogenation of the amino group of the dicarboxylicamino acid (to form the imino group) prior to its reaction with citrulline. Evidence is presented that this oxidation may be located at a hypothetical intermediate compound of citrulline and the dicarboxylicamino acid.

7. Arginine formation from citrulline can be used as an indicator of the "preferred" direction of some of the reversible processes in the metabolism of the cells. Some examples are presented.

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