

CCXXVIII. ON THE MECHANISM OF UREA FORMATION

By STEFAN JOSEPH BACH

From the Biochemical Laboratory, Cambridge

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CURRENT views on the mechanism of urea formation are based on two outstanding discoveries, one due to Krebs & Henseleit [1932] and the other to Leuthardt [1938].

Krebs & Henseleit observed that ornithine and citrulline catalyse urea synthesis from NH_3 and energy-supplying substances such as lactic acid. They explained this catalytic effect by the assumption of a cyclic reaction leading from ornithine via citrulline and arginine to urea, and thus back to ornithine. The evidence for this suggestive theory consists in the demonstration of the catalytic effect of ornithine and citrulline. The intermediates, citrulline and arginine, have not yet been isolated. Leuthardt demonstrated urea formation from glutamine and NH_3 . The presence of energy-supplying substances was not necessary for this reaction. Furthermore, the synthesis was not catalysed by ornithine. Thus two different ways of urea formation seem to be possible.

The following experiments started with the investigation of the role of citrulline in the urea cycle of Krebs & Henseleit [1932] and led to observations which seem to connect the results obtained by the above workers with those of Leuthardt [1938].

Methods

Animals. Young rats, chiefly albinos, were used; all were males with an average weight of 200 g., normally fed on ordinary stock laboratory diet.

Slice technique. The liver was removed and slices were cut with a razor moistened in Ringer solution from time to time and then blotted. The slices were accumulated in a moist chamber. As they were not wetted with Ringer solution, they could be weighed and adjusted to 100 mg. wet weight.

In Exps. XIV-XXI, where unwashed tissue was used, the slices were placed, after weighing, in the vessels used for incubation. In all other experiments they were placed instead in beakers containing 20 ml. of Ringer solution, where they were left for 3 min., after which the fluid was decanted and the beakers refilled with fresh Ringer solution. This procedure was twice repeated, after which the slices were placed in the vessels.

Incubation of slices. Barcroft vessels were used fitted with a side-tube for irrigation with gas mixture. They contained in all experiments 4.9 ml. of fluid. The latter was NaHCO_3 -Ringer [Krebs & Henseleit, 1932] except for the experiments with phosphate buffer (Table II). The *pH* was 7.3. In experiments with added substrates, 0.5 ml. Ringer solution was replaced by 0.5 ml. of the respective substrate, brought to *pH* 7.3 before being added to the vessel content. The vessels were shaken in a water thermostat at 38° for 10 min. at a rate of 120 times per min. during which the Ringer-bicarbonate solution was equilibrated with a gas mixture containing 95% O_2 and 5% CO_2 . After equilibration the

taps were closed and the vessels shaken for the experimental period which included the period of equilibration.

Preparation for N estimations. After the period of incubation 4 ml. of the vessel contents were placed in a centrifuge tube graduated at 10 ml., followed by acidification with 1 ml. of 40% trichloroacetic acid and centrifuging. The fluid was neutralized with NaOH to pH 6.5, and made up to mark (solution A). 3 ml. were used for NH_3 estimation. The remainder of the fluid was treated with urease and incubated at 38° for 2 hr. (solution B). 3 ml. of solution B were then used for the estimation of $\text{NH}_3\text{-N} + \text{urea-N}$. 3 ml. of the remaining solution were placed in a tube graduated at 10 ml., H_2SO_4 (final concentration 5%) was added and the fluid was then heated for 7 min. on a boiling water bath, neutralized and made up to mark (solution C). 5 ml. of solution C were used for the estimation of amide-N. To 4 ml. of the remaining solution C, 10 drops of 20% $\text{Mg}(\text{OH})_2$ suspension were added, and the mixture was placed on the boiling water bath for 25 min. The fluid after acidification with a few drops of glacial acetic acid was then used for amino-N estimation. When amide-N was not estimated the treatment with H_2SO_4 was omitted. The latter did not affect the results of amino-N estimation.

Estimation of NH_3 , urea and amide-N. The NH_3 estimation was carried out by distillation according to Parnas & Heller [1924] with collection in 0.01 N HCl and titration with 0.01 N NaOH using a microburette. Solution A gave the $\text{NH}_3\text{-N}$ content, B minus A the urea-N content and C minus B the amide-N content. Recovery experiments for $\text{NH}_3\text{-N}$, urea-N and amide-N yielded 97, 99.5 and 92% respectively of the theoretical values.

Estimation of amino-N. The amino-N was estimated with the Van Slyke apparatus. The amino-N obtained in recovery experiments with arginine represented 88% of its α -amino-N; 85% of the two amino-N atoms were recovered from ornithine [cf. Edlbacher & Burchard, 1931]. 110% of an amount equivalent to one amino-N per mol. were recovered in amino-N estimations with citrulline. The yields given above were obtained in recovery experiments in presence of tissue and after the preparatory treatment of the amino-acid solutions as described earlier.

Reagents. *dl*-Citrulline was prepared in the following way: arginine flavianate was prepared from gelatin, from the former benzylidenearginine was obtained and finally arginine HCl [Whitmore, 1932]. Citrulline was made from arginine HCl according to the method of Fox [1938].

In the later experiments a preparation of *dl*-citrulline was used which was kindly supplied by Bayer Products Ltd., London, W.C. 2, to whom I wish to express my gratitude. Further, I am greatly indebted to Dr R. Duschinsky, Hoffmann La-Roche & Co., Fontenay-Sous-Bois (Seine) for a gift of 1 g. *l*(+)-citrulline, to Mr S. Williamson for a preparation of α -ketoglutaric acid, to Mr R. L. M. Synge for a preparation of glutamine, and finally to Dr L. Zerfas for a gift of *l*(+)arginine, *l*(+)ornithine and *l*(+)glutamic acid. The three last-named preparations were the products of Hoffmann La-Roche & Co., Basle. All the other reagents were obtained from British Drug Houses Ltd.

EXPERIMENTAL

According to Krebs & Henseleit [1932] ornithine and citrulline, when added to liver slices of rat in presence of NH_4Cl and lactate, give rise to urea formation and cause partial disappearance of NH_3 . The experiments in Table I were carried out with citrulline, for which only few experimental results were given by the earlier workers.

Table I. *The effects of ammonium lactate and dl-citrulline on urea formation*

Exp. period: 90 min. Final concentrations of substances added: *dl*-citrulline, 0.2%; lactate, 0.2%; NH₃, 0.012%.

No. of exp.	Substances added to 100 mg. liver slices	μg. NH ₃ -N		μg. urea-N		A Change NH ₃ -N μg.	B Change urea-N μg.	Ratio $\frac{A}{B}$
		Before incubation	After incubation	Before incubation	After incubation			
X	None	20	0	0	10	- 20	+ 10	—
	<i>dl</i> -Citrulline	20	0	10	10	- 20	0	—
	Ammonium lactate	430	310	20	140	- 120	+ 120	—
	<i>dl</i> -Citrulline + ammonium lactate	390	210	110	285	- 180	+ 175	1.03
I	None	37	37	28	47	0	+ 19	—
	Citrulline	32	32	33	52	0	+ 19	—
	Ammonium lactate	455	325	0	159	- 130	+ 159	—
	<i>dl</i> -Citrulline + ammonium lactate	473	260	11	200	- 213	+ 189	1.12
III	None	0	0	14	33	0	+ 19	—
	Citrulline	0	0	14	47	0	+ 33	—
	Ammonium lactate	440	300	0	80	- 140	+ 80	—
	<i>dl</i> -Citrulline + ammonium lactate	430	130	10	310	- 300	+ 300	1.0

The results of Table I confirm the effect of citrulline on urea formation observed by these workers inasmuch as urea synthesis was markedly increased by citrulline in presence of ammonium lactate. The ratio of NH₃-N disappearing (A) to urea-N formed (B), on the other hand, was approximately 1 in all cases, whereas it should be 0.5 according to Krebs & Henseleit [1932] if urea formation from citrulline were necessarily preceded by arginine synthesis, in which 1 atom of NH₃-N is needed for every 2 atoms of urea-N formed. Further results in Table II showing amino-N determinations performed simultaneously with the experiments of Table I demonstrate that no significant changes in amino-N occurred during urea synthesis in presence of citrulline, whereas an increase in amino-N, owing to formation of ornithine (see methods), would be expected according to Krebs & Henseleit [1932]. When however arginine was used as a catalyst, amino-N markedly increased.

Table II. *Amino-N changes during urea synthesis catalysed by citrulline or arginine*

Exp. period: 90 min. Final concentrations of substances added: *dl*-citrulline, 0.2%; α-ketoglutaric acid, 0.2%; *l*(+)arginine, 0.1%.

No. of exp.	Subs. added to 100 mg. liver slices	μg. amino-N			μg. urea-N formed
		Before	After	Change	
I	<i>dl</i> -Citrulline + ammonium lactate	950	961	+ 11	189
II	„ „	922	945	+ 23	240
IV	„ „	920	905	- 15	200
XVI	<i>dl</i> -Citrulline + α-ketoglutaric acid + NH ₄ Cl	985	920	- 65	170
XX	<i>l</i> (+)arginine + α-ketoglutaric acid + NH ₄ Cl	354	554	+ 200	550

Further, it will be seen from Table I that only insignificant amounts of urea were formed when citrulline alone was added to the washed tissue. In some cases, on the other hand, there was a definite synthesis when ammonium lactate

was added in the absence of citrulline. The addition of liver *Kochsaft* and liver extract from amounts of tissue greater than those used in the experiments failed to increase urea formation to the level obtained in the presence of citrulline (Table III), thus suggesting that urea formation from ammonium lactate is not determined by water-soluble or heat-stable constituents of the tissue such as citrulline.

Table III. *The effects of liver Kochsaft and liver extract on urea formation from ammonium lactate*

Exp. period: 90 min. Final concentrations of substances added: as in Table I.

No. of exp.	Subs. added to 100 mg. liver slices	µg. urea-N			Effect of citrulline	Effects of liver <i>Kochsaft</i> and liver extract
		Before	After	Change		
III	Ammonium lactate	0	80	+ 80	—	—
	Ammonium lactate + dl-citrulline	10	310	+300	+220	—
II	Ammonium lactate	0	146	+146	—	—
	Ammonium lactate + dl-citrulline	0	240	+240	+ 94	—
VI	Ammonium lactate	0	72	+ 72	—	—
	Ammonium lactate + liver <i>Kochsaft</i> *	11	103	+ 91	—	+19
VIII	Ammonium lactate	0	52	+ 52	—	—
	Ammonium lactate + liver extract†	83	158	+ 75	—	+23

* Liver *Kochsaft*: 1 g. of minced liver boiled in 1.6 ml. Ringer sol. for 7 min., 0.3 ml. of centrifugate added.

† Liver extract: 2.5 g. minced liver ground with sand and 2.5 ml. Ringer sol., centrifuged and 0.3 ml. of centrifugate added.

As the effect of added lactate was found not to be very consistent, the question arose as to whether substances derived from lactate, such as keto-acids, might play an active part in the formation of urea in the absence of citrulline. α -ketoglutaric acid was chosen for the following experiments.

Table IV. *Effects of α -ketoglutaric acid, NH_4Cl and dl-citrulline on urea synthesis*

Exp. period: 90 min. Final concentrations of substances added: dl-citrulline, 0.2%; α -ketoglutaric acid, 0.20%; NH_3 , 0.012%.

No. of exp.	Subs. added to 100 mg. liver slices	µg. NH_3 -N		µg. urea-N		µg. changes in		Ratio $\frac{A}{B}$
		Before	After	Before	After	NH_3 -N (A)	Urea-N (B)	
XVIII	α -Ketoglutaric acid	0	40	0	0	+ 40	0	—
XIII	NH_4Cl	490	482	18	0	- 8	- 18	—
XI	NH_4Cl	460	440	30	42	- 20	+ 12	—
XIV	NH_4Cl + ketoglutaric	490	347	0	118	-143	+118	—
XVII	" "	486	328	27	123	-158	+ 96	—
XI	" "	480	366	0	74	-114	+ 74	—
XX	NH_4Cl + ketoglutaric + citrulline	503	254	9	266	-249	+257	0.97
XII	NH_4Cl + ketoglutaric	508	399	0	79	-109	+ 79	—
	NH_4Cl + ketoglutaric + citrulline	508	324	0	166	-184	+166	1.11

It will be seen from Table IV that urea formation took place regularly in the washed tissue in presence of α -ketoglutaric acid + NH_3 but in absence of citrulline. Both NH_3 disappearance and urea formation were of about the same

order as when ammonium lactate was added (Table I), and both phenomena were intensified by the addition of citrulline to a similar extent as was earlier observed in presence of ammonium lactate (Table I). No significant N changes were observed with either NH_4Cl alone, or with α -ketoglutaric acid alone. Again, the quotient $\frac{\text{NH}_3\text{-N disappeared}}{\text{Urea-N formed}}$ was found to be approximately 1 when citrulline was added.

The important role of α -ketonic acids was further shown by experiments on urea formation from lactate and ketoglutaric acid in presence of alanine and glycine. Since glycine condenses with ketonic acids [Bach, 1939] an inhibiting effect of glycine on urea formation was to be expected in presence of ketoglutaric acid. Table V demonstrates this inhibition and a similar effect of *dl*-alanine.

Table V. *The effects of dl-alanine and glycine on urea formation from α -ketoglutaric acid + NH_4Cl*

Exp. period: 90 min. Final concentrations of substances added: α -ketoglutaric acid, 0.2%; NH_3 , 0.012%; *dl*-alanine and glycine, 0.2%.

No. of exp.	Subs. added to 100 mg. liver slices	$\mu\text{g. NH}_3\text{-N}$		$\mu\text{g. urea-N}$		$\mu\text{g. changes in}$		
		Before	After	Before	After	$\text{NH}_3\text{-N}$	Urea-N	Amino-N
XVI	α -Ketoglutaric acid + NH_4Cl	495	345	0	115	- 150	+ 115	+ 50
	α -Ketoglutaric acid + NH_4Cl + glycine	495	447	25	13	- 48	- 12	- 230
XIV	α -Ketoglutaric acid + NH_4Cl	490	347	0	118	- 143	+ 118	+ 140
	α -Ketoglutaric acid + NH_4Cl + <i>dl</i> -alanine	490	410	0	55	- 80	+ 55	- 170
	<i>dl</i> -Alanine	0	18	0	116	+ 18	+ 116	- 120
XV	<i>dl</i> -Alanine	0	0	18	83	0	+ 65	—
	<i>dl</i> -Alanine + α -ketoglutaric acid + glycine	9	9	9	62	0	+ 53	—

Exp. XVI in Table V shows urea formation and NH_3 disappearance of the same order as usually obtained from ketoglutaric acid and ammonia, while the appearance of $\text{NH}_2\text{-N}$ suggested amination of ketoglutaric acid to glutamic acid. Urea formation was entirely inhibited when glycine was added, while a considerable disappearance of amino-N indicated condensation. *dl*-Alanine, which, unlike glycine, is known to give rise to urea in liver slices [Krebs, 1933; Bach & Holmes, 1937] had a similar effect though to a smaller extent, urea formation being only partly inhibited from both ammonium lactate and ketoglutaric acid and NH_3 . Again amino-N disappeared. If, on the other hand, *dl*-alanine was added alone, urea was synthesized and the disappearance of amino-N indicated deamination. If glycine was added, however, to alanine and ketoglutaric acid the latter seemed to react preferentially with glycine, thus preventing the removal of the alanine by the ketoglutaric acid. The effect of the keto-group in the mechanism of urea formation is thus evident.

The following experiments were devoted to the question as to whether ketoglutaric acid or pyruvic acid exerted an effect on citrulline in absence of NH_3 .

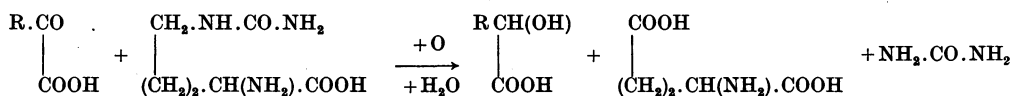
Table VI demonstrates urea formation in five successive cases from citrulline in presence of ketoglutaric acid or pyruvic acid, but in absence of added NH_3 .

Table VI. *The effects of α -ketoglutaric acid and pyruvate on urea formation from citrulline in absence of ammonia*Exp. period: 90 min. Final concentrations of substances added: *l*(+)citrulline, 0.1%; pyruvate, α -ketoglutaric acid and *dl*-citrulline, 0.2%.

No. of exp.	Subs. added to 100 mg. liver slices	$\mu\text{g. NH}_3\text{-N}$		$\mu\text{g. urea-N}$		$\mu\text{g. changes in}$	
		Before	After	Before	After	NH ₃ -N	Urea-N
XXII	<i>l</i> (+)Citrulline	0	0	0	0	0	0
	<i>l</i> (+)Citrulline + α -keto-glutaric acid	0	0	0	44	0	+44
	<i>l</i> (+)Citrulline + pyruvate	0	0	0	53	0	+53
XVI	<i>dl</i> -Citrulline + α -keto-glutaric acid	0	9	0	80	+9	+80
XVIII	<i>dl</i> -Citrulline	0	0	0	9	0	+9
	<i>dl</i> -Citrulline + α -keto-glutaric acid	0	0	0	53	0	+53
XXIII*	None	0	12	35	23	+12	-12
	Pyruvate	0	0	35	35	0	0
	<i>l</i> (+)Citrulline + pyruvate	0	0	35	130	0	+95

* 500 mg. liver slices used.

There was practically no NH₃ present in the tissue and only negligible changes in the NH₃ content were observed during the experimental period; there was no change in NH₂-N. Less urea was synthesized than in presence of NH₃. In view of the absence of other nitrogenous substances and the unchanged NH₂-N it can be concluded that the urea formed originated from the citrulline itself. As in absence of ketoglutaric acid citrulline gives rise to only insignificant amounts of urea, while on the other hand considerable amounts of urea are formed in the presence of a keto-acid the role of α -ketonic acids in this reaction had to be explained. The following experiments were based on the hypothesis that ketonic acids might act as oxidizing agents inducing oxidation of the citrulline, followed by hydrolysis to glutamic acid and urea. This mechanism may be pictured as follows:



The role of glutamic acid in urea synthesis was, therefore, next investigated.

Table VII. *The effect of glutamic acid on urea formation*Exp. period: 90 min. Final concentrations of substances added: *dl*-glutamic acid, α -ketoglutaric acid, 0.2%; NH₃, 0.012%; *l*(+)glutamic acid, 0.1%.

No. of exp.	Subs. added to 100 mg. liver slices	$\mu\text{g. NH}_3\text{-N}$		$\mu\text{g. urea-N}$		$\mu\text{g. changes in}$			
		Before	After	Before	After	NH ₃ -N	Urea-N	NH ₂ -N	Amide-N
XVI	<i>dl</i> -Glutamic acid	0	0	0	0	0	0	-20	—
XVII	<i>l</i> -Glutamic acid	0	0	26	26	0	0	—	—
XVII	<i>dl</i> -Glutamic acid + keto-glutaric acid + NH ₄ Cl	486	363	27	88	-123	+61	-60	—
	<i>l</i> (+)Glutamic acid + keto-glutaric acid + NH ₄ Cl	486	310	27	97	-176	+70	0	—
XXI	<i>l</i> (+)Glutamic acid + NH ₄ Cl	525	387	0	100	-138	+100	0	+40

The results given in Table VII show that no urea was formed if either *l*(+) or *dl*-glutamic acid was added alone, but considerable amounts were synthesized when NH_3 was also added. The addition of ketoglutaric acid also resulted in a smaller urea synthesis than was found in the absence of the keto-acid. The amino-N content remained unchanged when *l*-glutamic acid was used while a small disappearance was observed in two cases where *dl*-glutamic acid was added.

The fact that urea was formed from glutamic acid in presence of NH_3 suggested a possible formation of glutamine, especially as Exp. XXI of Table VII revealed the formation of amide-N. The action of glutamine was therefore tested.

Table VIII. *The effect of glutamine on urea formation*

Exp. period: 90 min. Final concentrations of substances added: glutamine, 0.1%; α -ketoglutaric acid, 0.2%; NH_3 , 0.012%.

No. of exp.	Subs. added to 100 mg. liver slices	$\mu\text{g. NH}_3\text{-N}$		$\mu\text{g. urea-N}$		$\mu\text{g. amide-N}$		$\mu\text{g. changes in}$		
		Before	After	Before	After	Before	After	$\text{NH}_3\text{-N}$	Urea-N	Amide-N
XVIII	Glutamine	—	—	8	53	—	—	—	+ 45	—
	Glutamine + ketoglutaric acid	—	—	8	52	—	—	—	+ 44	—
XIX	Glutamine + NH_4Cl	550	486	0	144	450	410	- 64	+ 144	- 40
	Glutamine + NH_4Cl + ketoglutaric acid	514	363	53	167	430	315	- 151	+ 114	- 115
	Ketoglutaric acid + NH_4Cl	505	407	17	79	46	82	- 98	+ 62	+ 36

According to Table VIII glutamine when added alone to the liver slices gave rise to only small amounts of urea, possibly owing to hydrolysis of the amide, while the addition of NH_3 to glutamine increased the yield by 200%. An appearance of small amounts of amide-N from ketoglutaric acid and NH_3 indicated glutamine formation which in turn could explain urea synthesis from ketoglutaric acid and NH_4Cl in absence of other urea precursors.

As glutamine is known to be partly converted into α -pyrrolidonecarboxylic acid [Chibnall & Westall, 1932] the estimated values for ammonia-N, urea-N and amide-N could not be expected to balance as some of the amide- or amino-N may have escaped estimation.

The reactions here proposed for citrulline should equally apply to ornithine except for the initial stage of oxidation in presence of keto-acid, by means of which ornithine should be oxidized to glutamine.

Evidence for this concept is produced in Exp. XXII of Table IX in which small amounts of amide-N were formed from ornithine and ketoglutaric acid, while practically no urea appeared. When the experiment was carried out, however, in presence of ammonium lactate approximately 160 $\mu\text{g.}$ of urea were formed as compared with an average formation of approximately 250 $\mu\text{g.}$ from citrulline according to Table I. The difference can be explained. Citrulline and ornithine both give rise to glutamine which acts as catalyst in the formation of urea. In addition to this effect, citrulline yields urea by oxidation and hydrolysis. The amount is of the order of 60 $\mu\text{g.}$ according to Table VI. Although in the experiments with ornithine and ketoglutaric acid (Table IX) no trace of ammonia could be detected, the possibility remains that the glutamine found might have been derived from ketoglutaric acid and an unknown ammonia source.

Table IX. *Effects of ketoglutaric acid and ammonium lactate on urea formation from ornithine*

Exp. period: 90 min. Final concentrations of substances added: ketoglutaric acid, 0.2%; ammonium lactate, 0.2%.

No. of exp.	Subs. added	μg. NH ₃ -N		μg. urea-N		μg. amide-N		μg. changes in		
		Before	After	Before	After	Before	After	NH ₃ -N	Urea-N	Amide-N
XXII	<i>l</i> -Ornithine 0.1%	0	0	0	8	0	0	0	8	0
	<i>l</i> -Ornithine 0.1% + pyruvate	0	0	0	0	0	0	0	0	0
	Ornithine 0.1% + ketoglutaric acid	0	0	0	10	0	23	0	+ 10	+23
V	None	0	0	42	105	—	—	0	+ 63	—
	<i>dl</i> -Ornithine 0.2%	0	0	63	94	—	—	0	+ 31	—
	<i>dl</i> -Ornithine 0.1%	0	0	42	94	—	—	0	+ 52	—
	Ammonium lactate	450	334	85	128	—	—	-116	+ 43	—
	Ammonium lactate + 0.2% ornithine	460	220	95	253	—	—	-240	+158	—
	Ammonium lactate + 0.1% ornithine	530	270	0	161	—	—	-260	+161	—

Table X. *Urea formation from ornithine, citrulline and arginine*Exp. period: 90 min. Final concentrations of substances added: amino-acids, 0.1%; ketoglutaric acid, 0.2%; NH₃, 0.012%.

No. of exp.	Subs. added to 100 mg. liver slices	μg. NH ₃ -N		μg. urea-N		μg. amino-N		μg. changes in		
		Before	After	Before	After	Before	After	NH ₃ -N	Urea-N	Amino-N
XX	Ketoglutaric acid + NH ₄ Cl + <i>l</i> -ornithine	484	262	54	233	580	580	-222	+169	0
	Ketoglutaric acid + <i>l</i> -citrulline + NH ₄ Cl	503	254	9	266	483	548	-249	+257	+ 65
	Ketoglutaric acid + NH ₄ Cl + <i>l</i> -arginine	503	272	67	621	354	554	-231	+554	+200

Table X presents a comparison of the effects of *l*-ornithine, *l*-citrulline and *l*-arginine on urea formation in presence of ketoglutaric acid and NH₄Cl. Urea formation from arginine takes place about three times as fast as from ornithine and about 1.5 times as fast as from citrulline. The amino-N is only little increased with citrulline, remains unchanged with ornithine and is markedly increased with arginine. Finally, the NH₃ consumption is approximately the same in all three cases.

The changes in amide-N observed in Tables VII, VIII and XI led to the assumption of a continuous synthesis and breakdown of the amide group and strengthened the idea that glutamine, like ornithine and citrulline, may act as a catalyst for urea synthesis. It was therefore necessary to reinvestigate the role of CO₂ and CO₂ precursors as a source of carbon for urea formation. Preliminary experiments in this direction are recorded in Table XI which shows a marked increase in urea formation from *l*-glutamic acid if Na formate + NH₄Cl were added. A full N-balance sheet is given in Exp. XXI, Table XI, showing that NH₃ was used both to increase amide-N and urea, while amino-N remained unaltered. Owing to the difficulty caused by the formation of pyrrolidone-carboxylic acid from glutamine, a similar balance could not be set up in Exp. XXIV. This experiment, however, revealed again a very marked increase in urea formation from glutamine + NH₄Cl when Na formate was added to the

Table XI. *The effect of Na formate on urea formation from glutamic acid and glutamine*

Exp. period: 90 min. Final concentrations of substances added: *l*-glutamic acid, 0.1%; glutamine, 0.1%; Na formate, 0.1%.

No. of exp.	Subs. added to 100 mg. liver slices	$\mu\text{g. NH}_3\text{-N}$		$\mu\text{g. urea-N}$		$\mu\text{g. amide-N}$		$\mu\text{g. changes in}$			
		Before	After	Before	After	Before	After	$\text{NH}_3\text{-N}$	Urea-N	Amide-N	Amino-N
<i>Unwashed tissue</i>											
XXI	<i>l</i> -Glutamic acid + NH_4Cl	525	387	0	100	35	75	-138	+100	+40	0
	<i>l</i> -Glutamic acid + NH_4Cl + Na formate	525	343	0	147	35	68	-182	+147	+33	0
<i>Washed tissue</i>											
XXIV	Glutamic + NH_4Cl (NaHCO_3 buffer)	575	648	10	12	—	—	+73	+2	—	—
	Glutamic + NH_4Cl + Na formate (NaHCO_3 buffer)	575	531	10	181	—	—	-56	+171	—	—
	Glutamic + NH_4Cl (phosphate buffer)	575	637	10	42	—	—	+62	+32	—	—
	Glutamic + Na formate	575	478	10	252	—	—	-97	+242	—	—

washed tissue. The urea synthesis was even greater when the experiment was carried out in phosphate buffer, probably indicating that bicarbonate was not a necessary substrate for the synthesis while Na formate seemed to serve this purpose.

DISCUSSION

In the experimental part the suggestion was tentatively put forward as a working hypothesis, that citrulline forms urea in two stages. The first can be described as oxidative hydrolysis, giving glutamic acid and urea; in the second the product of oxidation, glutamic acid, forms glutamine which in turn gives urea in presence of NH_3 and of CO_2 or a precursor of CO_2 .

The evidence for the first is based mainly on the experiments recorded in Table VI, showing the formation of urea in presence of a ketonic acid and in absence of NH_3 . Whether the ketonic acid is the H acceptor in this reaction, as suggested, or whether the oxidation of the ketonic acid in turn induces the oxidation of the terminal CH_2 -group of citrulline, has not been investigated so far. No direct proof is at present available for the assumed formation of glutamic acid or of glutamine arising from this reaction. An alternative explanation can be based on the results of Ikeda [1938], who found in perfusion experiments with citrulline in dog's liver that 136.8 mg. citrulline had disappeared from 189.9 mg. citrulline added to the perfused blood, whilst urea and ornithine were formed in considerable quantities. In the experiments here described with liver slices of rats however no ornithine was found as shown in Table II.

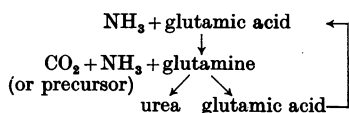
That ketoglutaric acid acts as " NH_3 acceptor" has been brought to light by the work of Braunstein & Kritzmann [1937] and of Kritzmann [1939]. It can therefore be assumed that under the experimental conditions the formation of urea from ketonic acids such as ketoglutaric acid in presence of NH_3 proceeds through glutamic acid, which in turn was shown to yield urea in presence of NH_3 .

Thus, in these experiments, the part played by the ketonic acid which is necessary for the formation of urea from citrulline in the absence of NH_3 does

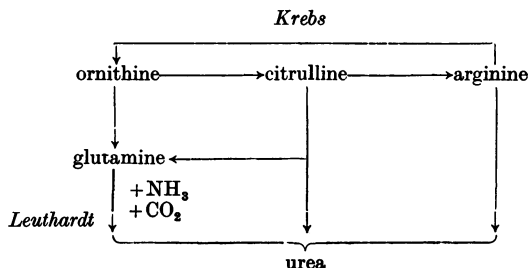
not appear to be merely that of a source of energy, as assumed by previous workers, but may be twofold. In the first place, in the presence of citrulline or ornithine, it may act as an oxidizing agent, as suggested above. For instance, in the case of ornithine, small amounts of amide-N could be detected, which according to the analytical methods applied must be rightly ascribed to glutamine. Secondly the ketonic acid behaves as a precursor of glutamine as shown by the rise in amino-N and the formation of amide-N in the experiments with NH_4Cl in the absence of amino-acids (Tables V and VIII).

The formation of glutamine from glutamic acid, as shown by Krebs [1935] and here assumed to occur in the course of the oxidative synthesis of urea, finds significant support in the experiments of Tables VII and XI, in which a clear N-balance sheet shows the formation of amide-N simultaneously with the urea. In these experiments almost 100 % of the N-change could be accounted for in the fractions: NH_3 , amino-N, amide-N and urea-N.

The amide group thus appears to be essential for urea formation from glutamic acid and glutamine, as amide nitrogen is formed in presence of the former (Table VII) and partly disappears in presence of the latter (Table VIII). Further, urea synthesis fails to take place from glutamic acid in the absence of NH_3 . A cyclic formation and disappearance of amide-N from glutamic acid and NH_3 , which leads to the formation of urea, and which is set out below, appears to be an explanation.



The synthesis of urea from glutamine has been extensively proved by the work of Leuthardt [1938] who found that urea formation from glutamine was independent of the addition of "substrates for respiration" and of ornithine. The above investigations point to a possible connexion between the work of Krebs & Henseleit [1932] and that of Leuthardt [1938]. The following scheme gives expression to this view:



The experiments discussed above thus suggest a possibility of another pathway from ornithine and citrulline to urea in addition to that discovered by Krebs & Henseleit. The four different mechanisms may be pictured as follows:

1. The mechanism of Krebs.
2. The formation of urea from glutamine (Leuthardt).
3. Urea synthesis from citrulline by oxidative hydrolysis.
4. The possible oxidative conversion of ornithine and citrulline into glutamic acid and finally glutamine, where the pathway joins that suggested by Leuthardt.

SUMMARY

1. Citrulline, when added to liver slices, gives rise to urea in presence of ammonium lactate. The ratio $\text{NH}_3\text{-N}$ disappearance to urea-N formation was found to be approximately 1. Amino-N remained practically unchanged during the synthesis and was markedly increased when citrulline was replaced by arginine, showing that no ornithine was formed from citrulline. A marked urea formation was also obtained in presence of ammonium lactate but in the absence of citrulline or any other amino-acid.

2. When ammonium lactate was replaced by α -ketoglutaric acid and NH_4Cl the urea formed in absence of citrulline was accompanied by a rise in amino-N and of amide-N, interpreted as a synthesis of glutamine.

3. The role of ketonic acids in the mechanism of urea synthesis was further shown by experiments in presence of glycine and alanine. Urea formation from ketoglutaric acid and NH_4Cl was inhibited in presence of glycine and partially inhibited in presence of alanine, owing to the action of the amino-acids on α -ketoglutaric acid, during which amino-N disappeared.

4. A small but distinct synthesis of urea was observed in presence of citrulline and α -ketoglutaric acid or pyruvic acid in absence of NH_3 . This result suggested an oxidation of citrulline by the ketonic acids leading to the formation of urea and glutamic acid. A scheme for this reaction is outlined.

5. Glutamic acid and glutamine both give rise to urea formation in presence of NH_3 , but not in its absence. A satisfactory N-balance sheet was set up. It reveals a marked formation of glutamine from glutamic acid and a disappearance of amide-N from glutamine. These observations led to the concept of a cyclic formation and disappearance of glutamine, catalysing urea synthesis.

6. Ornithine, like citrulline, gives rise to urea formation in presence of α -ketoglutaric acid and NH_4Cl , but not in absence of the latter. This is in agreement with the hypothesis of its oxidation to glutamine which was supported by the appearance of amide-N, ascribed to glutamine according to the methods applied.

7. Urea synthesis from arginine in presence of ketoglutaric acid and NH_4Cl was found to be three times as fast, and from citrulline 1.5 times as fast, as from ornithine. Again, little change in amino-N was found with citrulline and none with ornithine whereas amino-N markedly increased with arginine. The disappearance of NH_3 was of about the same order in all three cases.

8. The role of CO_2 or CO_2 precursors as a source of carbon for urea formation was investigated. Na formate markedly increased urea formation in presence of NH_4Cl from glutamic acid or glutamine.

9. A scheme was outlined suggesting urea synthesis from ornithine, citrulline and also by hydrolysis from arginine, thus connecting the mechanism shown by Krebs [1933] with that observed by Leuthardt [1938].

I would like to express my thanks to Dr E. Friedmann for many inspiring and valuable discussions and also to Prof. Sir Frederick G. Hopkins for his interest and advice. I am indebted to Mr S. Williamson for carrying out the greater part of the amino-N determinations.

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