CCXLVI. METABOLISM OF AMINO-ACIDS.

V. THE CONVERSION OF PROLINE INTO GLUTAMIC ACID IN KIDNEY.

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I. *Formation of amide-nitrogen from proline and hydroxyproline.*

IT was shown in the preceding paper of this series [Krebs, 1935, 2] that kidney of rabbit and guinea-pig converts ammonium glutamate into glutamine. When experiments were carried out to determine whether other amino-acids could form amides, it was found that only proline and hydroxyproline behaved similarly to glutamic acid.

If proline or hydroxyproline and ammonium salts are added to kidney, ammonia disappears and the ammonia which has disappeared is found in the solution as amide-nitrogen (Table I). The rate of amide-nitrogen formation is smaller in the presence of proline or hydroxyproline than it is in the presence of glutamic acid. With proline the rate of amide-nitrogen formation is 20–30 %, whilst with hydroxyproline it is 5–10 %, of the rate obtained with *l*-(+)-glutamic acid.

When proline or hydroxyproline (without ammonia) is added to kidney slices, less ammonia is formed than in their absence; instead of ammonia amide-nitrogen is found in the solution (Table I).

Proline and hydroxyproline also cause an increase in the oxygen uptake of kidney as previously observed [Krebs, 1933, 1; Bernheim and Bernheim, 1934]. This increase amounts to 60–100 % and is about the same with proline and hydroxyproline (Tables I and II).

II. *Formation of amino-nitrogen from proline and hydroxyproline.*

The amide formed in the presence of proline and hydroxyproline behaves like glutamine on acid hydrolysis (complete hydrolysis in 5 % sulphuric acid at 100° in 5 min.). This makes it probable that the amide formed from proline and hydroxyproline is glutamine. A conversion of proline into glutamine is conceivable, the primary step being the oxidation of proline to glutamic acid according to the equation:

$$\underset{\text{proline}}{C_5H_9O_2N} + O_2 \rightarrow \underset{\text{glutamic acid}}{C_5H_9O_4N} \qquad \text{.....(1).}$$

In this reaction amino-nitrogen would be formed; we determined the amino-nitrogen by Van Slyke's method and found an amount of the expected order of magnitude (Table II). This shows that the oxidation of proline and hydroxyproline actually yields an amino-compound.

Under anaerobic conditions no amino-nitrogen is formed from proline or hydroxyproline (Table II). Only by oxidation can the pyrrolidine ring be opened by kidney tissue.

Table I. *Disappearance of ammonia and formation of amide-nitrogen in the presence of proline and hydroxyproline in kidney.*

(Phosphate saline, 37·5°; for experimental details see Krebs [1935, 1, 2].)

Animal	mg. tissue	Substrate added	Time min.	Q_{O_2}	Amount of NH_3 (μl.) Initial	Final	Q_{NH_3} μl.	Amide-N found μl.	$Q_{Amide\text{-}N}$
Guinea-pig	13·81	*M*/50 *l*-(−) proline	120	−20·6	0	5·5	0·20	55·8	2·0
	13·95	*M*/50 *l*-(−) hydroxyproline		−19·6	0	17·1	0·61	28·3	1·0
	9·31	*M*/50 *l*-(+) glutamic acid		−25·9	0	6·4	0·34	98·6	5·3
	12·52	0		−14·3	0	31·0	1·2	19·2	0·8
Guinea-pig	12·54	*M*/50 *l*-(−) proline	180	−20·0	224	37·8	−5·0	249	6·6
	10·04	,,		−21·1	0	1·65	0·05	80·0	2·6
	10·01	*M*/50 *l*-(−) hydroxyproline		−22·6	224	178	−1·5	66	2·2
	12·78	,,		−20·2	0	8·2	0·25	38·0	1·0
	15·36	0		−11·8	224	231	0·15	13·5	0·3
	12·47	0		−14·1	0	27·2	0·73	17·8	0·5
Guinea-pig	16·14	*M*/50 *l*-(−) hydroxyproline*	120	−19·5	74·5	23·2	−1·6	70·6	2·2
	14·23	,,		−20·5	0	6·2	0·22	37·2	1·3
	17·59	0		−14·0	74·5	87·5	0·37	19·3	0·55
	13·83	0		−15·4	0	10·2	0·37	24·9	0·90
Guinea-pig	13·05	*M*/50 *l*-(−) proline	120	−20·9	228	30·8	−7·6	240	9·2
	9·28	*M*/50 *l*-(−) hydroxyproline		−23·7	228	211	−0·9	47·5	2·6
	12·57	0		−15·3	228	218	−0·4	16·3	0·7
Guinea-pig	11·38	*M*/50 *l*-(−) proline	30	−24·9	224	167	−10·0	38·5	6·7
	9·82	,,	60	−25·7	224	136·5	−8·9	63·2	6·4
	16·27	,,	120	−25·2	224	38	−5·8	113	3·5
	10·42	0	120	−17·8	224	207	−0·8	16·4	0·8
Guinea-pig	9·77	*M*/50 *l*-(−) proline	120	−25·2	224	87	−7·0	120	6·2
	8·13	0		−16·5	224	217	−0·5	14·5	0·9
Rabbit	18·62	*M*/50 *l*-(−) proline	80	−20·4	224	161	−2·54	65·5	2·6
	9·33	*M*/50 *l*-(+) glutamic acid		−30·3	224	70	−12·4	186	14·9
	17·10	0		−11·1	0	28·8	1·26	9	0·4

* The hydroxyproline used for this experiment was twice recrystallised, in order to free the substance completely from proline.

Table II. *Formation of amino-nitrogen from proline and hydroxyproline in kidney.*

(Phosphate saline, 37·5°.)

Animal	mg. tissue	Substrate added	Time min.	Q_{O_2}	Amino-N formed μl.	$Q_{Amino\text{-}N}$
Rat	12·18	*M*/50 *l*-(−) proline	120	−45·0	182·6	7·5
	13·98	0	120	−25·1	98·2	3·5
Rat	5·19	*M*/50 *l*-(−) proline	120	−38·6	144	13·9
	5·82	0	120	−23·5	51	4·4
Guinea-pig	13·74	*M*/50 *l*-(−) proline	60	−25·6	147	12·0
	8·80	*M*/50 *l*-(−) proline	120	−19·9	211	10·7
	11·57	0	60	−17·3	74	6·4
	11·46	0	120	−18·7	72·3	3·6
Guinea-pig	16·07	*M*/50 *l*-(−) hydroxyproline	180	−19·6	347	7·2
	15·36	0	180	−13·8	84	1·8
Guinea-pig*	20·60	*M*/50 *l*-(−) proline	120	0	119	2·9
	19·69	0	120	0	92	2·4
Guinea-pig	16·75	*M*/100 *l*-(−) proline	120	−27·7	433	12·9
	15·14	*M*/100 *l*-(−) hydroxyproline	120	−22·7	274	9·1
	18·04	0	120	−14·9	91	2·5

* Anaerobic experiment.

III. *Isolation of α-ketoglutaric acid.*

Since amino-acids are oxidised by kidney slices, it is not possible to accumulate the amino-acid which is formed from proline in sufficient quantity for isolation. It was possible however, by adding arsenious oxide [Krebs, 1933, 2], to check the oxidation of the amino-acid at the stage of the ketonic acid and to identify the latter as α-ketoglutaric acid.

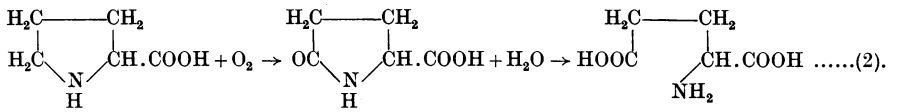
Slices of rabbit kidney cortex (1·3 g. dry weight) were suspended in 150 ml. bicarbonate saline containing *M*/25 *l*-(−) proline and *M*/380 arsenious oxide. The suspension was placed in four flasks of the shape previously described [Krebs, 1933, 1], the gas space being filled with 5 % CO_2 in O_2. The vessels were shaken for 4 hours at 37·5° after which the slices were removed and the protein was precipitated with trichloroacetic acid The clear solution was concentrated *in vacuo* to about 60 ml. and 5 ml. of a solution of 2:4-dinitrophenylhydrazine (0·7 % in 2*N* HCl) added. After a few minutes a precipitate began to separate. The fluid was kept on ice and on the following day the precipitate was removed by centrifuging and washed with dilute HCl and water. The dried precipitate weighed 63·7 mg.; it showed two different forms of crystals—fine needles and whetstone crystals, arranged in rosettes. The melting-point of this crude product was 209·5° (uncorrected). The crystals were very readily soluble in *N* sodium bicarbonate, giving a pale brown solution. On acidification with HCl 58 mg. separated out (M.P. 211·5°, uncorrected). This material was not completely soluble in ethyl alcohol. On dilution of the alcoholic solution with water crystals of both types were obtained again (48·2 mg., M.P. 215°). The product was now recrystallised from ethyl acetate. Again a small amount of insoluble residue remained. This time the recrystallised material was uniform and consisted of fine needles. The colour was lemon yellow whereas the former products were orange; M.P. 222° (uncorrected). The dinitrophenylhydrazone of pure α-ketoglutaric acid has the same colour, the same melting-point and the same solubility in *N* sodium bicarbonate [Krebs, 1933, 2]. Mixed melting-point 222°. (Found (Dr Weiler, Oxford): C, 40·59 %; H, 3·42 %; N, 16·71 %. $C_{11}H_{10}O_8N_4$ requires C, 40·44 %; H, 3·09 %; N, 17·18 %.)

In the mother-liquor of the first dinitrophenylhydrazone precipitate 2·73 mg. ammonia-N and 2·04 mg. amide-N were found. 63·7 mg. dinitrophenylhydrazone of α-ketoglutaric acid are equivalent to 2·77 mg. amino-N. Thus less α-keto-glutaric acid is found than is calculated from the amount of ammonia and amide-N. This is explained by the fact that arsenious oxide does not completely inhibit the breakdown of the ketonic acid formed.

When hydroxyproline was added as substrate instead of proline no appreciable amounts of a dinitrophenylhydrazone were found.

IV. *Pyrrolidonecarboxylic acid.*

It might be assumed that the first step in the oxidation of proline is the formation of pyrrolidonecarboxylic acid, according to the equation:



(2).

We therefore studied the behaviour of this supposed intermediate in guinea-pig kidney. *l*-(−) Pyrrolidonecarboxylic acid was prepared from *l*-(+) glutamic acid

according to Abderhalden and Kautzsch [1910]. The substance was free from glutamic acid (no amino-N).

Pyrrolidonecarboxylic acid, when added to guinea-pig kidney in neutral solution, had no effect on the oxygen uptake, on the ammonia consumption or on the formation of amino- and amide-nitrogen. This leads to the conclusion that pyrrolidonecarboxylic acid is not the intermediate, and that scheme (2) does not apply to the oxidation of proline in kidney.

Abderhalden and Hanslian [1912] observed that *l*-(−) pyrrolidonecarboxylic acid is metabolised when fed to a rabbit. The path of the breakdown is unknown however: we were unable to detect an enzyme which hydrolyses pyrrolidonecarboxylic acid to glutamic acid either in slices or in extracts of guinea-pig or rabbit kidney.

V. *Inhibition of the oxidation of proline by oxidisable substances.*

It was shown in section II that amino-nitrogen is formed when proline is oxidised. The formation of amino-nitrogen can be used to follow the oxidation of proline under various conditions. The formation of amino-nitrogen from proline is depressed when lactate or pyruvate is added (Table III), because these

Table III. *Oxidation of proline in the presence of lactate and pyruvate.*

Guinea-pig kidney, 37·5°, 2 hours.

mg. tissue	Substrate added (final concentrations)	Q_{O_2}	Amino-N formed (μl.)	$Q_{\text{Amino-N}}$
18·04	0	−14·9	90·8	2·5
18·36	*M*/100 *dl*-lactate	−23·2	103	2·8
14·79	*M*/100 pyruvate	−23·2	—	—
16·75	*M*/100 *l*-(−) proline	−27·7	433	12·9
18·19	*M*/100 *dl*-lactate + *M*/100 *l*-(−) proline	−24·3	272	7·5
17·22	*M*/100 pyruvate + *M*/100 *l*-(−) proline	−24·6	319	9·2

substances are oxidised instead of proline. Each substrate—proline, lactate or pyruvate—causes an increase in the oxygen uptake; but two substrates together do not yield a summation of the separate increments [see Krebs, 1935, 1]. These experiments seem to indicate that the activation of oxygen is the same for proline and for the other substrates; but it is still an open question whether the activation of proline (by a "dehydrogenase") is specific or whether it is the same as for the *l*-amino-acids. The oxidation of proline occurs only in tissues which oxidise *l*-amino-acids, that is in kidney and liver, and the rates of oxidation seem to run parallel: proline and *l*-amino-acids are oxidised about ten times more rapidly in kidney than in liver. We found no oxidation of proline in brain, muscle, intestine or spleen of the guinea-pig.

The oxidation of proline is inhibited to the same extent as the oxidation of *l*-amino-acids by cyanide and by octyl alcohol. In the minced kidney (Latapie mincer) proline is still oxidised if the "brei" is suspended in a small volume of liquid, but the oxidation ceases if more than four volumes of liquid are used for dilution [see also Bernheim and Bernheim, 1932].

VI. *Conclusions.*

Three products of oxidation of proline have been found in this investigation: α-ketoglutaric acid and ammonia appear when the kidney is poisoned with arsenious oxide; an acid amide which reacts like glutamine is found when

ammonia is added. The metabolism of proline in kidney may therefore be formulated in the following way:

$$\text{proline} + O_2 \rightarrow \text{glutamic acid} \begin{cases} + NH_3 \rightarrow \text{glutamine} \\ + \tfrac{1}{2}O_2 \rightarrow \alpha \text{ ketoglutaric acid} + \text{ammonia} \end{cases}$$

The intermediate stages between proline and glutamic acid are obscure. Pyrrolidonecarboxylic acid is not the intermediate, since it is not metabolised to a measurable extent.

Hydroxyproline also causes the formation of an acid amide which reacts like glutamine. Although one might hesitate to postulate the formation of glutamine from hydroxyproline, since this would necessitate the reduction of the γ-hydroxy-group, it is possible that this reduction is the first step in the breakdown of hydroxyproline, proline thus being an intermediate. Hydroxyproline is certainly not the intermediate in the oxidation of proline since proline reacts more rapidly than hydroxyproline in respect of the formation of amino-nitrogen and of amide-nitrogen.

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