CXXII. THE SYNTHESIS OF GLUTAMINE IN PIGEON LIVER

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It has previously been shown [Edson *et al.* 1936] that pigeon liver utilizes added NH_3 more rapidly than can be accounted for by the rate of hypoxanthine synthesis. Under some conditions, for instance in the presence of 0.02 to 0.04 *M* ammonium pyruvate, the amount of NH_3 converted into hypoxanthine is less than 20 % of total NH_3 metabolized; the bulk of the NH_3 is converted into substances which remained unidentified. We have now identified glutamine in this fraction. Its quantity accounts for about 40 to 50 % of the total NH_3 metabolized.

EXPERIMENTAL

As in the previous experiments slices of pigeon liver suspended in the saline medium of Krebs & Henseleit [1932] were used. Small scale experiments were carried out in manometric flasks, large scale experiments in "metabolism flasks", as described by Krebs [1933]. These flasks have a diameter of about 11 cm. and they contained not more than 100 ml. medium in order to secure sufficient oxygenation. The temperature was 40°. Ammonia was determined according to Parnas & Heller [1924], amino-N according to Van Slyke [1929] after the removal of NH_3 from the solution.

Formation of "amino-nitrogen" in pigeon liver

Data concerning the rate of NH_3 uptake have already been given [Edson *et al.* 1936] and it has also been mentioned that part of the NH_3 is converted into amino compounds. In a series of further experiments we compared the rates of NH_3 uptake and of hypoxanthine and "amino-N" formation. The highest rates of NH_3 uptake and amino-N formation were observed in the presence of pyruvate. An example is given in Table I. In this experiment the amino-N formed accounts for most of the metabolized NH_3 . "Amino-N", as determined by Van Slyke's method, it should be remembered, does not necessarily imply $=\text{CHNH}_2$ groups but would include among other groupings the $-\text{CONH}_2$ group of glutamine.

Table I. *Utilization of added NH_4Cl by pigeon liver*

The medium contained 0.04 *M* pyruvate and 0.01 *M* NH_4Cl ; 44 mg. liver; 2 hr.; 40°.

mg. NH_3 -N added	0.840
mg. NH_3 -N used	0.795
mg. hypoxanthine-N formed	0.110
mg. "amino-N"	0.658

Data for control to which no NH_4Cl was added calculated for same quantity of tissue.

mg. NH_3 -N formed	0.021
mg. hypoxanthine-N formed	0.081
mg. amino-N formed	0.163

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Formation of amide-nitrogen

If the solution from which NH_3 has been removed by pigeon liver is heated for 10 min. to 100° in the presence of 5 % H_2SO_4 , 15 to 25 % of the NH_3 metabolized returns to the solution. Examples are given in Table II. Heating for

Table II. *NH_3 -consumption and amide formation in pigeon liver*

No.	Substrate added (total volume of medium 3-4 ml.)	mg. tissue	Time min.	$\mu\text{l. NH}_3$ used	$\mu\text{l. amide-N}$ formed	Q_{NH_3}	$Q_{\text{amide-N}}$
1	—	29.9	90	+ 26	39.6	+ 0.58	+ 0.88
	0.01 M NH_4Cl ; 0.04 M pyruvate	29.6	90	— 792	131	— 17.9	+ 2.95
	0.01 M NH_4Cl ; 0.02 M $l(+)$ glutamate	40.2	90	— 412	115	— 6.8	+ 1.91
	0.01 M NH_4Cl ; 0.01 M α -ketoglutarate	36.0	90	— 447	113	— 8.3	+ 2.09
2	0.0066 M NH_4Cl	16.1	60	— 118	30	— 7.35	+ 1.87
	0.0066 M NH_4Cl ; 0.02 M pyruvate	13.0	60	— 267	42.5	— 20.5	+ 3.28
	0.0066 M NH_4Cl ; 0.02 M oxaloacetate	10.9	60	— 186	31.7	— 17.1	+ 2.92
3	0.01 M NH_4Cl	32.5	90	— 304	— 76.5	— 6.25	+ 1.57
	0.01 M NH_4Cl ; 0.02 M α -ketoglutarate	30.7	90	— 327	100	— 7.1	+ 2.17
	0.01 M NH_4Cl ; 0.02 M $l(+)$ glutamate	27.8	90	— 266	85.5	— 6.4	+ 2.05
	0.01 M NH_4Cl ; 0.02 M pyruvate	28.7	90	— 618	145	— 14.4	+ 3.37
4	0.02 M NH_4Cl	68.7	120	— 182	98	— 1.3	+ 0.71
	0.02 M NH_4Cl ; 0.02 M pyruvate	73.2	120	— 1070	198	— 7.3	+ 1.35
	0.02 M NH_4Cl ; 0.02 M oxaloacetate	61.0	120	— 800	157	— 6.6	+ 1.28
5	0.02 M NH_4Cl	52.5	100	— 658	144	— 7.5	+ 1.6
	0.02 M NH_4Cl ; 0.02 M pyruvate	38.4	100	— 1090	257	— 17.0	+ 4.0
	0.02 M NH_4Cl ; 0.04 M pyruvate	37.3	100	— 1122	313	— 18.1	+ 5.0
	0.02 M NH_4Cl ; 0.04 M $l(+)$ lactate	47.6	100	— 419	146	— 5.4	+ 1.84
	0.02 M NH_4Cl ; 0.04 M $l(+)$ glutamate	42.0	100	— 589	248	— 8.4	+ 3.55
	0.02 M NH_4Cl ; 0.04 M α -ketoglutarate	44.0	100	— 574	148	— 7.0	+ 2.28

more than 10 min. does not significantly increase the yield of NH_3 . Alkaline hydrolysis (10 min., 100° , 2*N* NaOH) liberates the same quantity of NH_3 as acid hydrolysis. The solutions thus contain a compound which behaves like glutamine or similar acid amides [*vide* Krebs, 1935, 1].

The yield of amide-N is greatest in the presence of pyruvate. There is only a small increase over the blank when $l(+)$ glutamate or α -ketoglutarate is added. In this respect pigeon liver behaves differently from a group of tissues comprising mammalian brain and retina and the kidney of guinea-pig and rabbit in which a synthesis of glutamine was previously found to take place [Krebs, 1935]. In these tissues a rapid synthesis of glutamine occurs if ammonium glutamate is added. Ammonium pyruvate (and alanine) are also converted into glutamine but the rate of the synthesis is in this case no more than 10 or 20 % of that observed in the presence of ammonium glutamate (Table III).

Table III. *Formation of glutamine in guinea-pig kidney*

40° ; bicarbonate saline; 0.01 M NH_4Cl . 5 % CO_2 in O_2 .

Substrates (final concentration)	$Q_{\text{amide-N}}$
—	1.31
0.02 M pyruvate	2.40
0.02 M $l(+)$ glutamate	12.9
0.02 M $l(+)$ alanine	4.7
0.03 M α -ketoglutarate	3.6
0.02 M α -ketoglutarate; 0.02 M $l(+)$ alanine	10.3

Isolation of the acid amide

The determination of amide-N on samples containing 0.05–0.1 mg. amide-N proved a convenient guide in the isolation of the amide. The amide compound is not precipitated by phosphotungstic acid, Cu or Ag salts, but forms an

insoluble compound with mercuric salts in weakly acid, neutral or weakly alkaline solution. After many preliminary tests the isolation was finally carried out in the following way.

Two or three livers of well fed, freshly killed pigeons are roughly weighed and then sliced with a razor. For each g. fresh weight 7 ml. saline [Krebs & Henseleit, 1932], 0.7 ml. 0.2 *M* NH_4Cl and 1.4 ml. 0.2 *M* sodium pyruvate are measured into a metabolism flask [Krebs, 1933]. The proportion of NH_4Cl to tissue chosen allows an almost complete utilization of the added NH_3 during an experimental period of 3 hr. The removal of NH_3 simplifies the purification of the substance.

The flasks are filled with 5% CO_2 in O_2 and shaken at 40° for 3 hr. at the rate of 40–60 periods per min. After shaking 0.2 vol. 30% trichloroacetic acid is added and the mixture is cooled in ice. It is filtered after about 30 min. A slight turbidity of the filtrate does not interfere with the later stages of the isolation and may be neglected. The filter is washed with 3% trichloroacetic acid.

The acid amide is then precipitated with the mercuric nitrate reagent introduced by Schulze [1882]. To each 50 ml. of the combined filtrate and washings are added 5 ml. of mercuric oxide, dissolved in nitric acid (160 ml. concentrated HNO_3 , 300 ml. H_2O and 220 g. HgO are boiled under reflux until the oxide is dissolved. After cooling *N* NaOH is added to produce a faint opalescence. The volume is made up to 1 l. with H_2O).

A 30% NaOH solution is slowly added under constant shaking until the solution is distinctly alkaline (litmus). It is then placed in the refrigerator overnight. The precipitate is centrifuged and the supernatant is tested with a drop of NaOH . If a yellow precipitate is formed more NaOH is added. The precipitate which contains among other substances the mercury salts of the "amide" is washed twice on the centrifuge with distilled water. After the last washing the precipitate is suspended in water and treated with H_2S . The mercuric sulphide is filtered off and washed with water. The filtrate is concentrated *in vacuo* to about 2 or 3 ml., transferred into a centrifuge tube and dried in a vacuum desiccator.

The oily or dry residue is stirred with about 0.5 ml. water, 0.1 ml. conc. HCl and 3 ml. methyl alcohol; if an insoluble fraction remains, it is removed by centrifuging; the supernatant is mixed with sufficient ether to produce a turbidity. Crystals appear soon when the mixture is placed in the ice box. Addition of more ether increases the yield, but sometimes lowers the purity of the product. Yield: 30–50 mg. per 10 g. wet liver. The substance is recrystallized by dissolving it in a few drops of water, adding 4 vol. methyl alcohol and ether as described above.

The combined yields from several experiments were recrystallized until the melting point remained constant at $171\text{--}173^\circ$ (uncorrected). The compound begins to darken a few degrees below this temperature and decomposes when melting. The analytical data (Dr Weiler and Dr Strauss) agree with those for glutamine hydrochloride ($\text{C}_5\text{H}_{11}\text{N}_2\text{O}_3\text{Cl}$):

C found: 32.91%; 33.05%; calculated for $\text{C}_5\text{H}_{11}\text{N}_2\text{O}_3\text{Cl}$: 32.86%			
H	„	: 5.68%; 6.13%;	„ : 6.07%
N	„	: 15.2%;	„ : 15.39%
Cl	„	: 18.8%;	„ : 19.4%

Treatment with chloramine T under the conditions described by Cohen [1939] for the determination of glutamic acid yielded succinic acid. From 2.87 mg. of

the substance 1.1 mg. succinic acid were obtained. The yield is not quantitative but of the same order as that obtained with pure glutamine (60–70%).

A preparation of glutamine hydrochloride obtained by dissolving glutamine from mangel-wurzels in a small volume of 10% HCl and precipitation with alcohol-ether was identical in every respect with the substance isolated from pigeon liver. The mixed melting points were unchanged.

The quantities of glutamine isolated in the pure state varied from 40 to 60% of the total "amide" present in the starting material. A considerable loss during the preparation is almost unavoidable owing to the instability of glutamine, and we therefore assume that glutamine represents most, if not all, of the amide synthesized in pigeon liver.

Glutamine synthesis in other species

Fowl and duck livers also form glutamine from added ammonium pyruvate and the rate was found to be about the same as in pigeon liver. Since the isolation of glutamine was carried out in the same way, these experiments are not described in full.

Rat liver, too, produces an acid amide, but in this case the rate is not appreciably increased over the blank when ammonium pyruvate is added. The maximum rate observed in rat liver was 1.0–1.5 ($Q_{\text{amide-N}}$), against values up to 5.0 in pigeon liver. The amide formed in rat liver has not yet been isolated and identified.

DISCUSSION

Synthesis of glutamine from 3-carbon compounds. The synthesis of glutamine from ammonium pyruvate involves the formation of a 5-carbon chain. It is probable that the synthesis of α -ketoglutaric acid is the primary step in the formation of glutamine. Several mechanisms have previously been discussed for the synthesis of this compound [*vide* Krebs, 1935, 2; Weil-Malherbe, 1936; Krebs & Johnson, 1937] but it is yet too early to decide between the various possibilities.

Glutamine synthesis in guinea-pig kidney and bird's liver. It was pointed out that guinea-pig kidney (and some other tissues) synthesize glutamine more rapidly from ammonium glutamate than from ammonium pyruvate, whilst pigeon liver yields glutamine more rapidly when ammonium pyruvate is the substrate. Such differences in rates, in our view, do not necessarily indicate entirely different mechanisms; they may be explained on the assumption that the factors limiting the rate of glutamine synthesis are different in the two groups of tissues.

Significance of the glutamine synthesis. The significance of the synthesis of glutamine remains obscure as long as the role of glutamine in tissue metabolism is unknown. Some observations mentioned in the discussion of the preceding paper and the facts reported in this paper leave no doubt that glutamine is a factor of general importance in cell metabolism, but the nature of its function is not yet clear.

SUMMARY

Slices of pigeon, fowl or duck liver form glutamine from ammonium pyruvate. The substance was isolated as glutamine hydrochloride; 1 g. of liver (dry weight) produces up to 30 mg. glutamine per hour.

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