To set the pipette for use again, open *X* to connect *A* and *B* and allow mercury to rise to the zero mark in *B*.

It is not usually necessary to empty the pipette. If a different reagent is to be put into it, the aqueous contents may be removed by placing the pipette on the Van Slyke apparatus, opening *X* to connect *A* to *E* and *Y* to connect *C* to *F* and drawing them down into *G*. The fluid is then expelled from *G* to waste, and *G* refilled with water which is passed up into the pipette, this process being repeated until the pipette is clean. Should it become necessary to

empty mercury out of the pipette this is most conveniently done by holding the pipette face downwards over a suitable wide container and withdrawing the plug of cock *X*.

SUMMARY

A special pipette is described for use with the Van Slyke manometric apparatus. It allows storage of reagents out of contact with air for indefinite periods and measurement of any required quantity of reagent into the manometric apparatus without its coming into contact with air.

Synthesis of Glutamic Acid in Animal Tissues*

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In earlier experiments (Krebs & Henseleit, 1932) it was noted that slices of rat liver, suspended in a saline medium containing ammonium salts, often removed more ammonia from the medium than could be accounted for by the formation of urea. The present work arose from a study of the reactions other than the synthesis of urea, which cause a disappearance of ammonia in liver tissue.

EXPERIMENTAL

Analytical methods. Urea was determined manometrically with urease (Krebs & Henseleit, 1932; Krebs, 1942); NH_3 was steam-distilled *in vacuo* according to Parnas and Heller, as described by Krebs (1935), and measured photoelectrically after nesslerization. Amino N was determined with Van Slyke's (1929) manometric nitrous acid method, after deproteinization with trichloroacetic acid and removal of NH_3 . Glutamic acid was determined according to Gale (1945) with the decarboxylase of *Clostridium welchii*, strain S.R. 12. Succinic and α -ketoglutaric acids were determined by the succinic dehydrogenase method (Krebs & Eggleston, 1948a), acetoacetate with aniline citrate (Edson, 1935), and oxaloacetate with $\text{Al}_2(\text{SO}_4)_3$ (Krebs & Eggleston, 1945).

Tissue preparations. Homogenates were made in the glass apparatus of Potter & Elvehjem (1936), and in a similar instrument made from stainless steel. The medium used for the dilution of the tissue was 'phosphate saline without Ca' (Krebs & Eggleston, 1940) or phosphate bicarbonate saline without Ca. Lower rates of reaction were found when Ca was present in physiological concentrations. A medium simulating the ionic composition of liver cells (containing 6 ml. of 0.1 M-CaCl₂, 18 ml. of 0.1 M-MgCl₂, 15 ml. of 0.1 M-K₂HPO₄, 10 ml. of 0.1 M-KH₂PO₄, 10 ml. of 0.155 M-KCl

and 41 ml. of 0.155 M-NaCl) gave rates of about 25% and, when Ca was omitted, of about 90% of those in 'phosphate saline without Ca'. The homogenates were used as freshly as possible. On storage their activity, as measured by their capacity to form glutamic acid, fell fairly rapidly, e.g. by c. 50% when kept in the refrigerator for 24 hr.

Slices were suspended in phosphate saline or phosphate bicarbonate saline.

Adenosine triphosphate (ATP). Ba-ATP (64.7 mg.) was dissolved in 3.3 ml. of 0.1 N-HCl. Ba was precipitated by the addition of 0.1 ml. of M-Na₂SO₄ and the solution was neutralized with 0.15 ml. of 2N-NaOH. Water was added to 5 ml. The precipitate was removed by filtration or centrifugation. The solution contained 0.02 M-ATP.

General. The material was incubated in conical Warburg vessels. The substrates were placed in the side arm and added to the tissue preparation at zero experimental time, usually when the manometers were attached to the water bath. The temperature was 40°. All substrate concentrations mentioned in the tables refer to the final concentrations in the medium. In anaerobic experiments yellow P was placed in the centre well. Quantities of metabolites are expressed in μl . and rates of metabolic processes in the usual *Q* values. When homogenates were used the dry weight for calculating the *Q* values was assumed to be 30% of the fresh weight in the case of liver and 20% in the case of brain cortex, kidney cortex or muscle.

To stop the metabolic processes in homogenates after a specified time the flasks were detached from their manometers and 1 ml. of 2N-HCl/4 ml. suspension was added as quickly as possible. If the analyses could not be carried out immediately the solutions were stored in the refrigerator. Of the acidified suspension usually 1 ml. was used for the determination of glutamic acid, 1 ml. for NH_3 , 3 ml. for succinic acid. If a larger amount of material was required for further analyses, duplicate Warburg cups were set up and the contents were combined after completion of the incubation.

* Preliminary account, *Nature, Lond.*, 159, 808 (1947).

Homogenates often contained significant amounts of NH_3 , glutamic acid and other metabolites. These initial concentrations were determined in a sample acidified with 2N-HCl (0.25 vol.) at the start of the experiment.

RESULTS

Excess of ammonia consumption over urea formation. Examples showing the quantities of ammonia removed by rat-liver slices in relation to urea formation are shown in Table 1. The amount which cannot be accounted for by the urea formation varied with the presence of other substrates and was of the same order of magnitude as those converted into urea. In these experiments no ornithine or citrulline was added. In the presence of these substances the excess of ammonia consumption over urea formation was usually small.

rat liver was only a small fraction, usually less than 10%, of the rate observed in slices, unless ornithine or citrulline was added. For example, Q_{urea} was 7.1 in slices and 0.25 in homogenized rat liver (substrate 0.01M-ammonium chloride). Similar results have been reported by Cohen & Hayano (1946). Homogenates, however, utilize ammonia if suitable substrates are present (Table 2). Of the substrates tested α -ketoglutarate had the greatest effect on the utilization of ammonia; citrate was slightly less effective, pyruvate and oxaloacetate still less. No appreciable effects were found with fumarate, lactate, glucose and acetoacetate. With α -ketoglutarate the rate observed in this experiment was of the same order as in slices.

Effect of tissue dilution. In the preceding experiments 1 part of the tissue was diluted with medium to about 7 parts of suspension. Dilution to 15 parts

Table 1. Urea formation and ammonia consumption in rat-liver slices

(Slices suspended in 4 ml. phosphate saline. Incubation 1 hr.)

Substrates	Tissue (mg.)	Urea ($\mu\text{l.}$)	Q_{urea}	NH_3 left ($\mu\text{l.}$)	NH_3 removed ($\mu\text{l.}$)	Q_{NH_3}	Amount of NH_3 ($\mu\text{l.}$)	
							Accounted for by urea	Not accounted for by urea
None	12.60	13	1.03	Nil	Nil	0	—	—
NH_4Cl (0.005M)	13.30	53	3.98	250	-180	-13.52	80	100
+L-lactate (0.02M)	8.65	35.5	4.10	358	-72	-8.32	45	27
+pyruvate (0.02M)	8.50	34	4.00	328	-102	-12.00	42	60
+oxaloacetate (0.02M)	13.80	57	4.13	250	-180	-13.04	88	92
+glucose (0.02M)	11.80	57	4.84	285	-145	-12.29	88	57

Table 2. Removal of ammonia in rat-liver homogenate

(One part of liver homogenized with 4 parts of phosphate bicarbonate saline; 3 ml. diluted with 1 ml. of substrate solution or saline; 5% CO_2 ; 95% O_2 ; 40°; incubation 20 min.; concentration of other substrates 0.01M; NH_4Cl added, 430 $\mu\text{l.}$; NH_4Cl found in suspension at start, 63 $\mu\text{l.}$)

Substrate added (in addition to NH_4Cl)	NH_3 found ($\mu\text{l.}$)	Change in NH_3 ($\mu\text{l.}$)	Q_{NH_3}
α -Ketoglutarate	116	-377	-6.3
Citrate	266	-227	-3.8
Pyruvate	318	-175	-2.9
L-Lactate	482	-11	-0.2
Acetoacetate	469	-24	-0.4
Fumarate	486	-7	-0.1
Glucose	512	+19	+0.3
Oxaloacetate	395	-98	-1.6
None	512	+19	+0.3

Ammonia utilization in liver homogenates. It was thought advantageous for the present purpose to work under conditions in which the urea synthesis was suppressed whilst other reactions involving the utilization of ammonia still proceeded. This object was largely realized by the use of homogenized liver. After homogenization the rate of urea synthesis in

decreased the initial rate of ammonia removal in proportion to the tissue concentration; in other words Q_{NH_3} remained constant. In the subsequent experiments a final tissue concentration of 1 part of tissue in 9 parts of medium was used unless otherwise stated.

Effect of oxygen and of saline media. Rat-liver homogenates, in the presence of α -ketoglutarate, also remove ammonia anaerobically. The data in Table 3 show that the anaerobic rate can even be higher, by about 15%, than the aerobic rate. The presence of bicarbonate and carbon dioxide, which is of importance for the formation of urea, did not affect the rate of the reaction responsible for the removal of ammonia in the present experiment.

Since anaerobic conditions reduce the number of reactions that occur in tissue preparations, and thus simplify the interpretation of results, the majority of the following experiments were carried out anaerobically.

Formation of glutamic acid. Determinations of amino N in the solutions of the experiment recorded in Table 2 showed an increase in amino N approximately equivalent to the loss of ammonia. The fact that α -ketoglutarate was the most effective substrate in removing ammonia and forming amino N

Table 3. *Aerobic and anaerobic removal of NH₃ in the presence of α-ketoglutarate in rat-liver homogenate*
(One part of liver homogenized with 4.5 parts of saline; each cup contained 3 ml. of homogenate and 1 ml. of additional solution (substrate or saline). Incubation 30 min. The homogenate contained 90 μl. NH₃ per cup from internal sources at the start of the experiment. The data refer to 4 ml. of suspension.)

Medium	...	...	Phosphate saline				Phosphate bicarbonate saline			
			O ₂		N ₂		5% CO ₂ in O ₂		5% CO ₂ in N ₂	
			0	914	0	914	0	914	0	914
Gas	...	...	0	1792	0	1792	0	1792	0	1792
NH ₄ Cl added (μl.)			71	264	124	242	102	322	150	276
α-Ketoglutarate added (μl.)			-19	-650	+34	-678	+12	-592	+60	-638
NH ₃ found after incubation (μl.)				-631		-712		-604		-698
Change in NH ₃ (μl.)										
Same corrected for suspension without added NH ₄ Cl										
Q _{NH₃}				-7.7		-8.7		-7.4		-8.5

suggested that the reaction under investigation was the reductive amination of α-ketoglutarate, and the solutions were therefore tested for glutamic acid by Gale's (1945) method. It will be seen from Table 4 that the quantities of ammonia removed and

Formation of succinic acid. The reductive amination of α-ketoglutarate is necessarily coupled with an equivalent oxidative process. Previous observations in this laboratory (Krebs & Cohen, 1939) suggested that the oxidative decarboxylation of α-ketoglutarate might be the equivalent reaction. The experimental data recorded in Table 5 show

Table 4. *Formation of glutamic acid in rat-liver homogenate*

(One part of liver, 9.5 parts of phosphate saline; N₂; 40°; 40 min. incubation. The corrected values were obtained by deducting the values found in the absence of substrate.)

Substrates added (final concentration)	None	α-Ketoglutarate (0.01 M)
		NH ₄ Cl (0.01 M)
NH ₃ change (μl.) (observed)	+45	-318
(corrected)	—	-363
Glutamic acid (μl.) (formed)	+40	+395
(corrected)	—	+355

glutamic acid formed agree within the limits of error if corrections are made for changes in the tissue which occur without the addition of substrates. Other examples showing this agreement are found in Tables 5 and 6; in some experiments the formation of glutamic acid was rather smaller than the removal of ammonia.

Table 5. *Anaerobic formation of succinic acid*

(Conditions as in Table 4.)

Substrates added	None	α-Keto-glutarate (0.01 M)
		NH ₄ Cl (0.01 M)
NH ₃ change (μl.) (observed)	+58	-322
(corrected)	—	-380
Glutamic acid found (μl.) (observed)	+84	+415
(corrected)	—	+331
Succinic acid found (μl.) (observed)	+18	+394
(corrected)	—	+376

that this is essentially true: the yields of glutamic and succinic acids and the amounts of ammonia used in this experiment are roughly in accordance with the equation

Table 6. *Comparison of changes in NH₃, glutamic acid and succinic acid in liver homogenates*

(Final tissue concentration 10%; α-ketoglutarate and NH₄Cl 0.01 M; 'blank' refers to tissue incubated without substrate.)

Species	Period of incubation (min.)		NH ₃	Glutamic	Succinic
			(μl.)	acid (μl.)	acid (μl.)
Guinea pig	40	Blank	+ 42	+ 95	+ 35
		With substrates	-287	+375	+222
		Data corrected for blank	-329	+280	+187
Guinea pig	40	Blank	+ 37	+ 93	+ 20
		With substrates	-336	+430	+398
		Data corrected for blank	-373	+337	+378
Rat	60	Blank	+106	+ 65	+ 15
		With substrates	-236	+305	+205
		Data corrected for blank	-342	+240	+190
Rat	20	Blank	+ 21	+ 17	0
		With substrates	-353	+253	136
		Data corrected for blank	-374	+236	136

In other experiments, however, the yield of succinate was low and the disappearance of ammonia high (Table 6). The former observation was taken to indicate that other oxidative reactions can be coupled with the synthesis of glutamic acid; the latter suggests that additional reactions causing the removal of ammonia occur in liver homogenates.

Effects of various metabolites on the synthesis of glutamic acid. In order to find the other reactions which might be coupled with the synthesis of glutamic acid, various substrates were added together with α -ketoglutarate and ammonium chloride under anaerobic conditions. No definite effects on the yield and rate of formation of glutamic acid were found with glucose, glycogen, glucose-1-phosphate, acetate, crotonate, acetoacetate, glycerol, α -glycerophosphate, ethanol, stearate and choline. Three sets of substances (Table 7) ((a) citrate and the two related tricarboxylic acids, (b) β -hydroxybutyrate, (c) fumarate and L-malate) increased the rate of glutamic acid synthesis as well as the final yield, which is 50 % of the added α -ketoglutarate if reaction (1) is the only mechanism leading to glutamic acid. Major inhibitions of both glutamic acid synthesis and ammonia consumption were found with L-lactate and hexosediphosphate (Table 8). Acetate and butyrate caused slight inhibitions in some experiments. L-Alanine increased, and pyruvate decreased, the yield of glutamic acid owing to transamination.

Examples showing the effects of citrate, β -hydroxybutyrate and fumarate in guinea-pig liver are given in Table 7. In this experiment, as in all similar ones,

citrate had the greatest effect. It increased the removal of ammonia and the formation of glutamic acid almost equally without appreciably affecting the formation of succinate. The sum of the α -ketoglutarate, succinate and glutamate found exceeded by 440 μ l. the amount of α -ketoglutarate added. This can be explained by the assumption that citrate was converted into α -ketoglutarate under the conditions of the experiment. The yields and the calculations concerning the oxido-reduction balance are in agreement with the view that the reductive amination of α -ketoglutarate (found 782 μ l.) was coupled with the following two oxidative reactions:

Citrate $\rightarrow$ α -ketoglutarate (found 440 μ l.).

α -Ketoglutarate $\rightarrow$ succinate (found 350 μ l.).

The effect of β -hydroxybutyrate was much smaller than that of citrate but was also definite. The yield of glutamate, which in the absence of added substrate does not exceed 50 % of the α -ketoglutarate (as expected from equation 1), reached 59 %, while the yield of succinate showed a slight decrease. The presence of acetoacetate in the solution indicates that the additional oxidative reaction coupled with the glutamate formation was the oxidation of β -hydroxybutyrate to acetoacetate.

In the case of fumarate the yield of glutamate also exceeded 50 %; it was 64 %. The fact that oxaloacetate appeared indicates that the reaction fumarate $\rightarrow$ oxaloacetate was in this case one of the oxidative equivalents of the glutamate formation. That the amount of oxaloacetate found was re-

Table 7. *Effect of citrate, β -hydroxybutyrate and fumarate on the synthesis of glutamic acid in guinea-pig liver homogenate*

(General conditions as in Table 6; incubation 40 min.; substrate concentrations all 0.01 M except DL- β -hydroxybutyrate which was 0.02 M. Controls showed that in the absence of α -ketoglutarate and NH_4Cl citrate yielded no α -ketoglutarate, β -hydroxybutyrate no acetoacetate and fumarate no oxaloacetate.)

Substrates added	...	{ None	α -Keto- glutarate, NH_4Cl	α -Keto- glutarate, NH_4Cl , citrate	α -Keto- glutarate, NH_4Cl , DL- β -hydroxy- butyrate	α -Keto- glutarate, NH_4Cl , fumarate
NH_3 ($\mu\text{l.}$) change	+37		-336	-754	-496	-612
Corrected		-373	-791	-533	-649	
Glutamic acid formed ($\mu\text{l.}$)	+93	+430	+875	+600	+663	
Corrected		+337	+782	+507	+570	
Succinate formed ($\mu\text{l.}$)	+20	+398	+370	+380	+438	
Corrected		+378	+350	+360	+418	
Acetoacetate formed ($\mu\text{l.}$)	0	0	0	+210	0	
Oxaloacetate formed ($\mu\text{l.}$)	0	0	0	0	+38	
α -Ketoglutarate found ($\mu\text{l.}$)	0	104	204	49	0	
α -Ketoglutarate added ($\mu\text{l.}$)	0	896	896	896	896	
Sum of α -ketoglutarate, succinate and glutamic acid recovered (corrected)		819	1336	916	988	
Oxido-reduction balance						
Reductions:						
Formation of glutamic acid		337	782	507	570	
Oxidations:						
Formation of succinate		378	350	360	418	
Formation of acetoacetate or oxaloacetate			790	210	38	
Formation of α -ketoglutarate						

latively small is not unexpected because of its rapid secondary reactions. Some oxaloacetate must have reacted with glutamate by transamination; hence the amount of glutamate found was smaller than that of the ammonia removed. Another fraction of oxaloacetate probably reacted to form the tri-carboxylic acids, α -ketoglutarate and malate (Krebs, Eggleston, Kleinzeller & Smyth, 1940); hence the sum of α -ketoglutarate and succinate exceeded the amount of α -ketoglutarate added. In all experiments on guinea-pig liver the effect of fumarate was of a similar magnitude; in rat liver the effect was smaller, but also marked.

The effects of citrate, β -hydroxybutyrate and fumarate suggest that the reductive amination of α -ketoglutarate can be coupled with four different oxidative reactions (a) α -ketoglutarate $\rightarrow$ succinate, (b) isocitrate $\rightarrow$ α -ketoglutarate, (c) β -hydroxybutyrate $\rightarrow$ acetoacetate, (d) fumarate (or L-malate) $\rightarrow$ oxaloacetate.

Examples of the inhibitory effect of lactate, hexosediphosphate, acetate and butyrate are given in Table 8. The inhibition of glutamic acid formation by 0.01 M-L-lactate in guinea-pig liver was 73%. The inhibition of the ammonia uptake was of the same order. No explanation for the mechanism of this inhibition can be offered.

Effect of adenosinetriphosphate. If the experimental periods were short (less than 10 min.) and the tissue concentrations in the homogenate high (10% or more), addition of ATP had little or no effect on the rate of glutamic acid synthesis. In longer experiments with more dilute suspensions, ATP had definite effects in that it delayed the decline in the rate. These effects were larger if sodium fluoride, which inhibits the hydrolysis of ATP, was also present (see Table 9). The effects may be taken to indicate that ATP is a component of the system responsible for the synthesis of glutamic acid.

Effect of substrate concentration. As Table 9 indicates, the rate of glutamic acid synthesis in homogenates falls quickly with time. In further experiments, designed to measure the initial rates, a short incubation period of 10 min. was therefore chosen. When the concentration of α -ketoglutarate was varied between 0.005 and 0.04 M, the highest rates were found at 0.02 M. Variations of the concentration of ammonium chloride between 0.05 and 0.02 M, with a concentration of α -ketoglutarate of 0.02 M, had no effect on the rates.

Rate of glutamic acid synthesis in homogenates and slices of various tissues. Table 10 compares the rates of the synthesis of glutamic acid in homogenates with those in slices of liver and kidney. In the case

Table 8. *Inhibition of glutamic acid synthesis by lactate and other substances in liver homogenates*

(Concentration of α -ketoglutarate and NH_4Cl , 0.01 M; N_2 ; data refer to 4 ml.)

Species	Period of incubation (min.)	Additional substrates	Glutamic acid found ($\mu\text{l.}$)	NH_3 removed ($\mu\text{l.}$)
Rat	20	—	237	375
		L-Lactate, 0.02 M	68	86
		Na acetate, 0.01 M	180	165
Guinea pig	15	—	201	—
		L-Lactate, 0.01 M	55	—
		Hexosediphosphate, 0.01 M	33	—
		Na butyrate, 0.01 M	150	—

Table 9. *Effects of adenosine triphosphate and NaF on the rate of glutamic acid synthesis in rat-liver homogenates*

(Medium: phosphate saline; 40°; N_2 ; 0.02 M- α -ketoglutarate; 0.01 M- NH_4Cl . The initial values for glutamic acid in the homogenates have been deducted.)

No.	Tissue concentration (%)	Additional substrates added	Glutamic acid formed ($\mu\text{l.}$)	
			10 min.	20 min.
1	10	None	255	263
		ATP, 0.001 M	277	295
		NaF, 0.02 M	223	273
		ATP, 0.001 M; NaF, 0.02 M	281	368
			15 min.	30 min.
2	5	None	99	126
		ATP, 0.001 M	141	166
		NaF, 0.02 M	78	101
		ATP, 0.001 M; NaF, 0.02 M	125	206

Table 10. *Comparison of the rates of glutamic acid synthesis in homogenates and in slices*

(Data for homogenates and slices given in the same horizontal line refer to the same tissue except where an asterisk is added; 0.02M- α -ketoglutarate; 0.01M-NH₄Cl. Incubation period for homogenates 10 min.; N₂.)

Tissue	Homogenate $Q_{\text{glutamic acid}}$	Slices	
		Period of incubation (min.)	$Q_{\text{glutamic acid}}$
Liver, rat	12.8	20	1.0
Liver, rat	12.3	20	2.0*
Liver, rat	9.3	30	4.8*
Liver, rat	10.5	30	4.1*
Liver, guinea pig	9.8	20	4.5
Liver, sheep	3.3	15	1.0
Liver, pigeon	15.5	30	2.7*
Liver, pigeon	17.6	—	—
Kidney cortex, rat	7.1	30	4.7*
Kidney cortex, rat	2.0	30	2.7*
Kidney cortex, rat	5.2	—	—
Kidney cortex, sheep	3.3	15	3.2
Kidney cortex, sheep	4.2	15	2.6

of homogenates the values given are corrected for the initial amount of glutamic acid, and with slices for the amount of glutamic acid found on incubation without addition of substrates. It will be seen that in rat-liver homogenates $Q_{\text{glutamic acid}}$ was between 9.3 and 12.8, against 1.0–4.8 in slices. For the livers of the guinea pig, sheep and pigeon the rates were likewise much greater in homogenates. On the other hand, the differences between sliced and homogenized material were small in kidney cortex of the rat and sheep.

In slices the rate of glutamic acid synthesis increased with time, in contrast to the fall that occurred in homogenates. Examples are given in Table 11. The accelerating effect of citrate already

reported for homogenates was also found in slices. In the liver and kidney of sheep and rats citrate approximately doubled the rate of synthesis; in homogenates the increase was usually about 50%. The highest rates of glutamic acid synthesis in the presence of citrate were, in terms of $Q_{\text{glutamic acid}}$, 27 in pigeon liver and 20 in rat liver.

Table 11. *Time course of glutamic acid synthesis in sliced tissue*

(0.02M- α -ketoglutarate; 0.01M-NH₄Cl; N₂.)

Tissue	Time of incubation	$Q_{\text{glutamic acid}}$
Liver, sheep	First 15 min.	1.0
	Second 15 min.	2.1
Kidney, sheep	First 15 min.	3.2
	Second 15 min.	6.5

In brain, minced heart muscle and pigeon breast muscle, small but measurable quantities of glutamic acid were always found on incubation with α -ketoglutarate and ammonium chloride. Addition of citrate usually caused a considerable increase (Table 12). Negligible rates ($Q_{\text{glutamic acid}} < 1$) were found in sheep spleen and guinea-pig lung.

Synthesis of glutamic acid in relation to the removal of ammonia in sliced liver. The question arises whether the synthesis of glutamic acid is one of the reactions other than urea synthesis causing a disappearance of ammonia in liver slices. To test this, liver slices were shaken aerobically under conditions similar to those stated in Table 1, and the amounts of ammonia removed, and of urea and glutamic acid formed, were measured (Table 13). Only a small proportion of the ammonia removed which was not accounted for by the formation of urea was recovered as glutamic acid. The proportion of the latter was largest when citrate was the substrate, but even in this case amounted to only 23% of the missing nitrogen. There was no formation of glutamine under

Table 12. *Rate of glutamic acid synthesis in various tissues*

(0.02M- α -ketoglutarate; 0.01M-NH₄Cl; 0.01M-citrate; N₂.)

Tissue	Substrates added	Period of incubation (min.)	$Q_{\text{glutamic acid}}$
Brain cortex, sliced, sheep	α -Ketoglutarate, NH ₄ Cl	30	0.8
	α -Ketoglutarate, NH ₄ Cl, citrate	30	1.5
Brain cortex, sliced, guinea pig	α -Ketoglutarate, NH ₄ Cl	30	0.9
	α -Ketoglutarate, NH ₄ Cl, citrate	30	2.3
Brain cortex, sliced, guinea pig	α -Ketoglutarate, NH ₄ Cl	30	1.3
	α -Ketoglutarate, NH ₄ Cl, citrate	30	4.8
Brain cortex, sliced, pigeon	α -Ketoglutarate, NH ₄ Cl	30	1.0
	α -Ketoglutarate, NH ₄ Cl, citrate	30	1.4
Brain, minced, sheep	α -Ketoglutarate, NH ₄ Cl	20	0.4
	α -Ketoglutarate, NH ₄ Cl, citrate	20	0.4
Heart, minced, sheep	α -Ketoglutarate, NH ₄ Cl	20	1.4
	α -Ketoglutarate, NH ₄ Cl, citrate	20	2.6
Striated muscle, minced, pigeon	α -Ketoglutarate, NH ₄ Cl	20	1.5
	α -Ketoglutarate, NH ₄ Cl, citrate	20	1.8

Table 13. *Formation of glutamic acid in rat-liver slices in the presence of NH_4Cl and other substrates*

(Phosphate saline; O_2 . The data refer to 30 mg. (dry) tissue suspended in 4 ml. of saline, and are corrected for amounts formed by tissue without added substrate; concentration of NH_4Cl , 0.005 M; of other substrates, 0.01 M.)

Exp. no.	Substrates added	Period of incubation (min.)	NH_3 removed ($\mu\text{l.}$)	NH_3 removed not accounted for by urea ($\mu\text{l.}$)	Glutamic acid found ($\mu\text{l.}$)
1	NH_4Cl	60	167	161	27
2	NH_4Cl	90	96	71	0
	NH_4Cl , citrate	90	51	43	10
	NH_4Cl , glucose	90	9	9	0
	NH_4Cl , fumarate	90	0	0	0

Table 14. *Removal of glutamic acid by rat-liver slices*

(Phosphate saline; O_2 . The data refer to 40 mg. (dry) tissue suspended in 4 ml. phosphate saline, and are corrected for amounts formed by tissue without added substrate.)

Exp. no.	Substrates added	Period of incubation (min.)	Glutamic acid removed ($\mu\text{l.}$)	Urea + NH_3 formed (as NH_3) ($\mu\text{l.}$)
1	L-Glutamate, 0.01 M	60	172	6
	L-Glutamate, 0.01 M; NH_4Cl , 0.005 M	60	161	51
2	L-Glutamate, 0.005 M	90	93	19
	L-Glutamate, 0.005 M; NH_4Cl , 0.005 M	90	93	40
3	L-Glutamate, 0.005 M	90	140	65
4	L-Glutamate, 0.005 M	90	74	34

the conditions of the experiments. For the interpretation of this result it is of importance, however, that liver tissue is capable of removing considerable quantities of glutamic acid without forming equivalent quantities of urea or ammonia, as is shown in Table 14. Accumulation in the slices did not account for an appreciable amount of the glutamic acid removed from the medium. The non-appearance of glutamic acid is therefore no proof of its non-formation. Glutamic acid may be used up in secondary reactions, and it is therefore possible that the synthesis of glutamic acid accounts for a greater proportion of the missing nitrogen than the data in Table 13 suggest.

DISCUSSION

The occurrence of a synthesis of glutamic acid in animal tissues was first recorded by Euler, Adler, Günther & Das (1938) and Dewan (1938). The main new contribution of the present work is the measurement of the rate of this reaction under varying conditions, and the observation that the reductive amination of α -ketoglutarate can be coupled with the reaction L-malate $\rightarrow$ oxaloacetate, as an alternative to the three reactions α -ketoglutarate $\rightarrow$ succinate + CO_2 (Krebs & Cohen, 1939), isocitrate $\rightarrow$ α -ketoglutarate + CO_2 (Adler, Euler, Günther & Plass, 1939), and β -hydroxybutyrate $\rightarrow$ acetoacetate (Dewan, 1938).

Physiological significance of the synthesis of glutamic acid. There are many indications that glutamic acid has a special function in the metabolism

of various cells, though so far little information is available on this point. The rapid synthesis of glutamic acid in liver may serve partly to supply the requirements of this acid for the whole body, but the major part probably reacts by 'transamination', according to Cohen & Hayano (1946), and is thus a link in urea synthesis. The rates of transamination and of glutamic acid synthesis are of the same order and sufficient to account for the formation of urea, the highest value for Q_{urea} observed in rat liver being about 20 (Krebs & Henseleit, 1932) and for $Q_{\text{glutamic acid}}$ about 20. The stage citrulline + $\text{NH}_3 \rightarrow$ arginine of the ornithine cycle can therefore be resolved into the two stages:

It has always been obvious that the synthesis of urea through the ornithine cycle, being an endergonic process, must be coupled with exergonic reactions, but no information on the nature of the exergonic reactions was previously available. It is now clear that the oxidative reactions which are coupled with the amination of α -ketoglutarate are components of the system of exergonic reactions supplying the energy for the synthesis of urea.

Several facts indicate that reaction (2) is a complex process. It requires the presence of oxygen (Borsook & Dubnoff, 1941) and is inhibited by low concentrations (0.005 M) of malonate (Cohen & Hayano, 1946). The malonate inhibition is abolished

by fumarate (Fahrländer, Favarger, Nielsen & Leuthardt, 1947). This effect of fumarate is a catalytic one; under suitable conditions one molecule of fumarate (or L-malate) can cause the formation of many more than five molecules of arginine (Krebs & Eggleston, 1948b). Ratner (1947) suggests that reaction (2) consists of two stages:

Glutamic acid + oxaloacetate $\rightarrow$ aspartic acid + α -ketoglutarate.

Aspartic acid + citrulline $\rightarrow$ L-malate + arginine.

Whilst the effect of fumarate on the malonate inhibition would be explained by this scheme it is difficult to account for the following four observations on rat liver homogenates (Krebs & Eggleston, 1948b):

(1) Aspartate and citrulline yield arginine very slowly if the conditions are anaerobic, the rate being less than 5% of that observed aerobically with glutamate and citrulline.

(2) Aerobically, glutamate and citrulline react about five times faster to yield arginine than aspartate + citrulline.

(3) Aspartate removes the malonate inhibition of reaction (2) less effectively than fumarate, the restoration being 50% with 0.005M-aspartate against 100% with 0.005M-fumarate.

(4) Malonate (0.005M) completely abolishes the interaction between aspartate and citrulline in oxygen; glutamate or α -ketoglutarate restores the capacity of liver homogenates to form arginine.

Differences between sliced and homogenized material. It would not be surprising to find that the rate of metabolic processes taking place in slices of animal tissues is greatly reduced, even to zero, by homogenization of the tissue, but it is somewhat unexpected to observe the reverse, i.e. a rapid rate in homogenates against an almost negligible rate in slices. The first to report such a phenomenon were Cohen & Hayano (1946) working on transamination in rat liver. The present observations on the reductive amination of α -ketoglutarate in sliced and homogenized rat material are in general similar to those on transamination. In both cases there is no major difference in sliced and homogenized kidney, whilst in rat liver the rates in homogenates are about three to fifteen times greater than in slices. To explain the relatively low rate of reaction in liver slices one may assume that either the enzyme systems concerned are largely inactive in the intact cell, owing to the presence of intracellular inhibitors, or that permeability barriers prevent the access of the substrates to the enzymes. Whatever the explanation of the phenomenon it is clear that major differences exist in the behaviour of kidney and liver cells.

SUMMARY

1. A search has been made for reactions, other than urea synthesis, causing a disappearance of added ammonia in isolated liver preparations. Homogenization of liver was found to abolish, under certain conditions, the conversion of ammonia into urea, whilst ammonia was still removed when pyruvate, oxaloacetate, α -ketoglutarate, or citrate were added. The main reaction responsible for the removal of ammonia in homogenates, on addition of the above substrates, was found to be the synthesis of glutamic acid.

2. The synthesis of glutamic acid in the presence of α -ketoglutarate and ammonia occurred both anaerobically and aerobically, the anaerobic rates being somewhat higher than the aerobic rates.

3. The reductive amination of α -ketoglutarate by liver can be coupled with the following four oxidative reactions: α -ketoglutarate $\rightarrow$ succinate + CO₂ (Krebs & Cohen, 1939); isocitrate $\rightarrow$ α -ketoglutarate + CO₂ (Adler *et al.* 1939); β -hydroxybutyrate $\rightarrow$ acetoacetate (Dewan, 1938); L-malate $\rightarrow$ oxaloacetate (not recorded previously).

4. L-Lactate and hexosediphosphate, and to a less extent acetate and butyrate, inhibited the formation of glutamic acid from α -ketoglutarate and ammonia.

5. Adenosine triphosphate delayed the fall in the rate of glutamic acid synthesis which occurred in homogenates when the experimental period exceeded 10 min.

6. The rate of glutamic acid synthesis in liver homogenates was three to fifteen times greater than in liver slices. In kidney cortex the differences in the rates observed in homogenized and sliced material were small.

7. The highest recorded rates of glutamic acid synthesis, in terms of $Q_{\text{glutamic acid}}$, were 20 for rat liver, 27 for pigeon liver, 8 for kidney cortex (sheep, rat), 5 for brain cortex, 3 for heart muscle, 2 for pigeon breast muscle, <1 for spleen and lung.

8. Glutamic acid was removed by liver slices without forming ammonia or urea, an indication of the occurrence of unknown reactions in which glutamic acid is utilized.

9. The synthesis of glutamic acid is responsible for a part, but probably not for the whole, of the ammonia which is removed by liver slices and not accounted for by urea formation.

10. The physiological significance of the synthesis of glutamic acid is discussed. One of the reactions for which glutamic acid is required in liver tissue is presumably the conversion of citrulline into arginine in the course of the synthesis of urea (Cohen & Hayano, 1946).

REFERENCES

- Adler, E., Euler, H. v., Günther, G. & Plass, M. (1939). *Biochem. J.* **33**, 1028.
- Borsook, H. & Dubnoff, J. W. (1941). *J. biol. Chem.* **141**, 717.
- Cohen, P. P. & Hayano, M. (1946). *J. biol. Chem.* **166**, 251.
- Dewan, J. G. (1938). *Biochem. J.* **32**, 1378.
- Edson, N. L. (1935). *Biochem. J.* **29**, 2082.
- Euler, H. v., Adler, E., Günther, G. & Das, N. B. (1938). *Hoppe-Seyl. Z.* **254**, 61.
- Fahrlander, H., Favarger, F., Nielsen, H. & Leuthardt, F. (1947). *Helv. physiol. pharmacol. Acta*, **5**, 202.
- Gale, E. F. (1945). *Biochem. J.* **39**, 46.
- Krebs, H. A. (1935). *Biochem. J.* **29**, 1620.
- Krebs, H. A. (1942). *Biochem. J.* **36**, 758.
- Krebs, H. A. & Cohen, P. P. (1939). *Biochem. J.* **33**, 1895.
- Krebs, H. A. & Eggleston, L. V. (1940). *Biochem. J.* **34**, 442.
- Krebs, H. A. & Eggleston, L. V. (1945). *Biochem. J.* **39**, 408.
- Krebs, H. A. & Eggleston, L. V. (1948a). *Biochem. J.* **42**, 17.
- Krebs, H. A. & Eggleston, L. V. (1948b). *Biochem. Biophys. Acta*. In the Press.
- Krebs, H. A., Eggleston, L. V., Kleinzeller, A. & Smyth, D. H. (1940). *Biochem. J.* **34**, 1234.
- Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
- Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
- Ratner, S. (1947). *J. biol. Chem.* **170**, 761.
- Van Slyke, D. D. (1929). *J. biol. Chem.* **83**, 425.

The Determination of Quinine Degradation Product in Blood, and its Absorption in the Chick

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In a previous publication (Marshall, 1945a) we reported that quinine degradation product (QDP) showed no antimalarial action against *Plasmodium gallinaceum* in young chicks, when given orally in amounts equivalent to an active dose of quinine. Subsequently, Kelsey, Oldham, Cantrell & Geiling (1946) reported that, when given intravenously, the compound showed one third to one fifth the activity of quinine against chick malaria, and less than one twentieth that of quinine against *P. lophurae* when given orally to ducks. Pointing out that QDP was less toxic than quinine, thus making its therapeutic index nearly equal to that of quinine, these workers disagreed with our view that the metabolism of quinine results in an inherently less efficient drug. They suggested that the reduction of both antimalarial activity and toxicity might be due to a decreased rate of absorption in the host.

In order to test this theory, we decided to compare the concentrations of quinine and QDP attained in the red cells of chicks following oral doses of the compounds, and this necessitated the designing of a method of determination of small quantities of QDP. The fluorescence of QDP in ultraviolet light was too feeble for the determination of very small amounts, though it has been used to determine large concentrations (Knox, 1946). We found, however, that the bromothymol blue method (Marshall & Rogers, 1945) was almost as sensitive with QDP as with quinine.

The degradation product, which is prepared by incubating quinine with rabbit liver suspension, was shown by Mead & Koepfli (1944) to be quinine carbostyryl, i.e. quinine with a hydroxyl group substituted in position 2 of the quinoline ring. This confers phenolic properties on the compound, making it unextractable from sodium hydroxide solution, thus providing a ready method for the separation of QDP from quinine. The degradation product can be extracted from ammoniacal solution (Kelsey, Geiling, Oldham & Dearborn, 1944). We have used the basic principles of this method, but emulsification problems made quantitative extraction difficult. However, by modifying the technique suitably, and carefully controlling the shaking operations, a method giving satisfactory recoveries of small amounts of QDP was produced.

METHODS

Quinine degradation product. This was prepared by incubating quinine with rabbit-liver suspension, and extracting and purifying the compound according to the method of Kelsey *et al.* (1944). The melting point of the final product was 250°.

Chicks and dosing. The chicks used were 7–14 days old, and their body weights ranged between 50 and 80 g. A single dose of 200 mg./kg. of quinine or QDP base was given into the crop by a catheter tube. The bases were dissolved in a minimum amount of HCl. Pairs of birds from the quinine and QDP groups were killed at suitable intervals by placing