Glutamine: a Nitrogen Source in Urea Synthesis

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In early studies on the synthesis of urea in the mammalian liver the amino groups of the protein constituents had been recognized as the nitrogenous source of urea, and the action of arginase was considered the last step in the synthesis. Krebs & Henseleit (1932) showed that the supply of arginine was ensured by its continuous resynthesis and that the latter was catalysed by the ornithine-citrulline cycle. However, the details of the partial reactions in this cyclic scheme are still under investigation, and in particular the link between the latter and the amino acid source awaits a full explanation. According to Bach (1939), direct participation of aminodicarboxylic acids in the urea-synthesizing mechanism occurs without the liberation of free ammonia. Glutamine amide nitrogen was considered to represent a direct source of urea nitrogen and a mechanism was suggested for its resynthesis to allow the continuous yield of urea. The author's views were inspired by the observation of Leuthardt (1938*a*) that urea synthesis can take place from glutamine in guinea-pig liver in the presence or absence of ammonia. Later, the special significance of the aminodicarboxylic acids in urea synthesis became apparent when Cohen & Hayano (1948) attributed to glutamic acid an essential role in the formation of citrulline, and when aspartic acid was found by Ratner & Pappas (1949) to participate in the synthesis of arginine. However, in the first step of urea synthesis, glutamic acid, or its *N*-acyl derivatives, were thought to act as catalysts rather than as sources of urea nitrogen and the presence of ammonia was still considered necessary to initiate the ornithine cycle (Grisolia, Burris & Cohen, 1952).

It is this initial step in the synthesis of urea that forms the subject of this work. The role of intact glutamine as the source of urea nitrogen was investigated and evidence was obtained that hydrolysis of glutamine is not a prerequisite for its participation in urea synthesis. The earlier work of Leuthardt (1938*a, b*; 1940), Leuthardt & Glasson (1942, 1944) and Leuthardt, Müller & Nielson (1949) supported this concept. The importance of intact glutamine as the principal nitrogen donor for urea is also borne out by the work of Kamin & Handler (1951), who observed in experiments *in vivo* that glutamine and arginine caused by far the greatest urinary output of urea, glutamine being the more

effective of the two. The results of Hirs & Rittenberg (1950), which were not obtained under conditions comparable with those in the experiments reported here, could not be confirmed in the present work. These authors observed only a small utilization of the amide nitrogen of glutamine for urea synthesis, but evidence has now been obtained that the urea nitrogen can be entirely accounted for by an equal utilization of the two nitrogenous groups of glutamine (Bach & Smith, 1955). Observations made in this work justify the suggestion that this utilization may be made possible through a reaction of these nitrogenous groups with bicarbonate.

METHODS

Animals. Albino rats 5-7 months old (200-300 g.) were fed on rat cake supplied by the Aberdeen Flour Millers; the composition was that recommended by Parkes (1946).

Tissue slices. The rats were killed by a sharp blow on the neck, the liver was removed rapidly and sliced into 200 mg. batches immediately. Each batch was suspended in three changes of 15 ml. of 0.9% NaCl for 3 min. After the last washing each 200 mg. of slices was drained and transferred to a Warburg vessel which contained 4 ml. of the incubation mixture. When Krebs bottles were used, as in the experiments shown in Tables 3 and 4, 1.2 g. of slices were suspended in 24 ml. of incubation mixture. For comparison all results are referred to incubation mixtures of 4 ml. containing 200 mg. of slices.

Tissue homogenates. The washed liver slices were transferred to a Potter homogenizer and homogenized with a minimal amount of Ringer solution (see below), after which more Ringer solution was added so that 1 ml. of the homogenate contained 200 mg. of tissue. A volume of the homogenate (1 ml. or 6 ml.) was then transferred to Warburg vessels or Krebs bottles, respectively.

Incubation. The Warburg flasks contained 0.4 ml. of neutralized substrate solutions which were diluted with buffered Ringer solution to 4 ml. and subsequently gassed for 10 min. with an $O_2 + CO_2$ mixture (except when bicarbonate was absent from the buffer solution). When Krebs bottles were used the quantities were proportionately increased for a total volume of 24 ml. The vessels were shaken at 38° for 90 min.

Three types of Ringer solution were used: (a) Bicarbonate-Ringer according to Krebs & Henseleit (1932). (b) Amino-trihydroxymethylmethane (tris)-Ringer, in which the bicarbonate component of the Krebs-Ringer solution was replaced by 12 parts of 0.1M acidified tris solution so that the pH of the final mixture was 7.4. (c) Tris-bicarbonate Ringer, which consisted of 121 ml. of tris-Ringer to which

9 ml. of a solution containing 0.273 g. of NaHCO_3 was added. Solutions (a) and (c) were equilibrated with a gas mixture containing $\text{O}_2 + \text{CO}_2$ (95:5, v/v) for 40 min. at room temp. before placing them in the Warburg vessel. The complete incubation mixture was regassed at 38° for 10 min. preceding the experimental period.

Reagents. L-Glutamine and L-glutamic acid were supplied by L. Light and Co. Ltd. The purity of the glutamine with respect to amide N was found to be 92% as determined by acid hydrolysis or by the decarboxylase method of Krebs (1948). With respect to the glutamic acid moiety the purity was 98%. By inference the main impurity would seem to be glutamic acid. The absence of arginine was verified by incubation of the glutamine solution with a purified arginase solution, followed by urea estimation.

Estimation of nitrogenous compounds

Urea. The manometric method of Krebs & Henseleit (1932) was used. In the experiments where urea was the only metabolite to be estimated the acetate buffer was added to the Warburg vessel containing the tissue suspension at the end of the incubation period, and then the estimation of urea CO_2 by the urease method was carried out. When other metabolites were estimated as well, Krebs bottles were used for the incubation, and 3 ml. portions of the suspension fluid were removed for urea estimation. The values for urea initially present in the washed slices before incubation were estimated on several occasions and found to be negligible.

Ammonia. This was estimated on 2 ml. portions of the incubation mixture (except in the experiments shown in Tables 1 and 2 where the estimation was on 1 ml. portions) by the method of Parnas & Heller (1924); 0.01 N-HCl was used in the receiving vessel, and 0.01 N-NaOH was used for the titration.

Amide nitrogen. This was estimated by acid hydrolysis. The results were checked on various occasions by enzymic hydrolysis (Krebs, 1948) and a good agreement was found between the results of the two methods. For the acid hydrolysis 1.5 ml. portions of the incubation mixture were deproteinized with 5% of trichloroacetic acid (final concentration). The protein precipitate was washed twice with 5% trichloroacetic acid and the washings were added to the protein-free solution. This was hydrolysed by boiling on a water bath for 5 min. with 2N- H_2SO_4 (final concentration), and the ammonia liberated was distilled in the Markham (1942) apparatus and estimated by titration.

α -Amino nitrogen in the presence of glutamine. The disappearance of amino N in an incubation mixture containing glutamine was determined from the difference between the amino N content of a mixture incubated in absence of glutamine to which glutamine was added at the end of the incubation period, and one in which the same amount of glutamine had been added at the beginning of the incubation period.

Recovery experiments with glutamine were carried out both in the presence and absence of liver slices. In the latter case the solutions containing glutamine and bicarbonate-Ringer were treated as described below, the urease and deproteinization treatments being omitted. In this case the recovery of glutamine amino N amounted to 101 and 104%, allowance being made for the purity of the material. For the recovery experiments in the presence of tissue, the amino N content of a mixture that had been incubated with

200 mg. of liver slices and to which glutamine had been added at the end of the incubation period, was compared with a mixture incubated with 200 mg. of slices without further addition of glutamine. The treatment of the mixtures was the same as described below, and the recovery obtained was 97%.

The procedure for the metabolic experiments was as follows: two batches of 800 mg. of slices were incubated, one with bicarbonate-Ringer and the other with bicarbonate-Ringer plus glutamine, the total volume in each being 16 ml. To part of the first batch, glutamine was added after incubation in the same concentration as in the second batch. Several 4 ml. portions from the three mixtures were transferred to Warburg flasks, where, after urease treatment, urea was estimated and at the same time removed. The urea-free solutions were deproteinized with trichloroacetic acid as described above and the protein-free solutions were subjected to acid hydrolysis with 2N- H_2SO_4 (final concentration) for an hour. The hydrolysates were adjusted to pH 10 with 8N-NaOH and the ammonia was removed by distillation *in vacuo* at 40° ; distillation was continued until the volume was approximately 10 ml. The protein-free solutions, which now contained glutamine as glutamic acid, were acidified with acetic acid and made up to 25 ml. Portions (5 ml.) were taken for the estimation of amino N according to the method of Peters & Van Slyke (1932). The removal of the amide group, urea and ammonia was necessary since these three components would otherwise have reacted with the nitrous acid used in the subsequent treatment. The period of hydrolysis of the amide group was prolonged to 1 hr. to avoid formation of pyrrolidonecarboxylic acid (Hamilton, 1945).

Glutamic acid. The glutamic acid moiety of glutamine and glutamic acid was estimated by a slightly modified method of Krebs (1948). A preparation of the decarboxylase from *Clostridium welchii* SR 12 was prepared as described by Gale (1945) and was subsequently freeze-dried. Portions (4 ml.) of the incubation mixtures were diluted to 25 ml. and estimations carried out on 4 ml. portions of these solutions in Warburg flasks. A volume (0.5 ml.) of Cetavlon (cetyltrimethylammonium bromide)-acetate buffer (4 ml. of 1.5M acetate buffer, pH 4.9, 2 ml. of 1% Cetavlon) was added to the main compartment of each flask. Into the side bulb 0.5 ml. of enzyme suspension (100 mg. of freeze-dried enzyme preparation, 3.6 ml. of water, 0.4 ml. of 1.5M acetate buffer, pH 4.9) was introduced. The vessels were shaken at 30° , and the CO_2 evolution was measured.

Paper chromatography

Liver slices were incubated as described, with and without ammonium chloride, the volumes being 8 and 4 ml. respectively. After incubation the mixtures were transferred to centrifuge tubes and deproteinized with 0.25 vol. of a solution of colloidal iron hydroxide containing 5% of Fe_2O_3 . After centrifuging, the ammonia-containing mixture was diluted with water to 20 ml., of which 10 ml. was desalted as follows: The mixture was allowed to permeate an Amberlite IR 120 (H) cation-exchange column, 15 cm. long with a diameter of 1 cm., at a rate of 1 drop/5 sec. The column was then washed with about 50 ml. of water to ensure complete removal of anions, and the amino acids were finally eluted with N- NH_3 soln. at a rate of approximately 2 drops/5 sec.; 50 ml. of eluate was collected. The ammonia-free mixture was desalted similarly. The second

10 ml. portion of the deproteinized ammonia-containing mixture was hydrolysed by boiling with 2N-HCl (final concentration) and then desalted. The three desalted mixtures were evaporated to dryness *in vacuo* at a temperature not exceeding 40°; the residues were taken up in 1 ml. of water. The three concentrated desalted solutions were filtered and 7 μ l. portions of each mixture were applied to Whatman no. 1 chromatography paper for two-dimensional paper chromatography in phenol-water (in presence of HCN and NH_3) and in *n*-butanol-acetic acid-water (250:60:250, by vol.). The papers were dried at 50°, sprayed with 2% of ninhydrin in water-saturated *n*-butanol and left for another hour at 60°.

Radioautography of incubation mixtures in presence of $\text{NaH}^{14}\text{CO}_3$

The incubation was carried out in several Warburg flasks, each containing 200 mg. of washed liver slices, 3.1 ml. of bicarbonate-Ringer, 0.4 ml. of 0.206M glutamine and in the side bulb 0.5 ml. of a solution of $\text{NaH}^{14}\text{CO}_3$ containing 1.18 mg. of bicarbonate with a radioactivity of 21.5 μC . After equilibration the contents of the side bulbs were tipped and the side bulbs washed out with the incubation mixture. After incubation the contents of the flasks were combined and subjected to paper chromatography as described in the section above. Before spraying, the two-dimensional chromatograms were placed in X-ray cassettes and exposed to either Ilfex X-ray paper or Kodak Blue Brand X-ray film. The films were developed, in some cases after 3 days and in others after 23 days. After the exposure the papers were sprayed with ninhydrin or with *p*-dimethylaminobenzaldehyde reagent (as used by Partridge (1948) for the detection of hexosamines) to locate the deposition of glutamine and urea, respectively, for comparison with the exposed films.

RESULTS

Ammonia and glutamine in urea synthesis

In a series of approximately 200 experiments with washed liver slices from fed rats, urea synthesis from ammonium chloride was compared with that from glutamine at various substrate concentrations. Except for concentrations below 0.01M, a considerably faster rate of urea synthesis from glutamine than from ammonium chloride was observed (Fig. 1). The figure also demonstrates a close proportionality between the concentration of glutamine and the rate of urea synthesis. The positions of the lines in Fig. 1 were calculated as those best fitting the scatter of points.

The results with glutamine and with ammonia indicate that at higher substrate concentrations glutamine was more effective than ammonia and that the former possibly represented the more direct source of urea (see Discussion). It has been suggested (Krebs, 1942) that the intracellular effect of glutamine on urea synthesis is due to the liberation of ammonia and glutamic acid by glutaminase action. Therefore, further experiments were undertaken to determine the role of glutaminase in urea

synthesis. There is little activity of this enzyme in the liver with low substrate concentrations, as demonstrated in cell-free extracts (Greenstein & Leuthardt, 1948; Greenstein & Price, 1949) and in the intact liver (Kamin & Handler, 1951). For a study of the enzyme action, experiments with liver homogenates were carried out in which a considerable hydrolysis of glutamine was observed when it was added at higher concentrations (Table 1). By analogy with the observations made by Hughes & Williamson (1952) in their experiments with *Clostridium welchii*, it was found that liver glutaminase was strongly inhibited by bromocresol purple and bromocresol green. Further, in confirmation of the experiments of Greenstein & Leuthardt (1948) with liver homogenates, the enzyme was shown to be strongly activated when pyruvate was added in sufficiently high concentrations (Table 2). When in further experiments bromocresol purple or bromocresol green was added to liver slices in the presence of glutamine, urea synthesis proved to be unaffected by the presence of the inhibitors (Table 1). That the liver cells were

Fig. 1. Effect of ammonium chloride and glutamine on urea synthesis in rat-liver slices. O, Ammonium chloride; ●, glutamine; incubation time, 90 min.; 200 mg. of slices in bicarbonate-Ringer; total volume, 4 ml. Vertical lines represent the standard deviation of 25 experiments with ammonium chloride (A) and 95 experiments with glutamine (B).

permeable to the inhibitors to some degree was verified by microtome sections of the tissue after incubation. From the observations recorded in Table 1, it is concluded that glutaminase does not participate in urea synthesis. On the contrary, when the hydrolysis of glutamine is enforced by a strong activation of the enzyme (Table 2), the synthesizing mechanism is grossly impaired.

Table 1. *Effect of sulphophthaleins on the deamidation of glutamine in homogenates and on urea synthesis in slices*

Liver tissue, 200 mg.; bicarbonate-Ringer; total volume, 4 ml. Figures in parentheses represent percentage deamidation.

Glutamine (10^{-2} M)	Bromocresol purple (10^{-4} M)	Bromocresol green (10^{-4} M)	Ammonia N formed (μ g.)
Homogenates			
0.35	—	—	9 (4)
1.38	—	—	92 (13)
1.73	—	—	234 (26)
1.73	3.71	—	97 (11)
1.73	—	6.5	80 (9)
2.06	—	—	526 (51)
2.06	7.4	—	108 (10)
2.06	—	3.25	103 (10)
2.76	—	—	824 (58)
Slices			
			Urea N formed (μ g.)
1.73	—	—	230
1.73	3.71	—	272
2.06	—	—	284
2.06	7.4	—	265
2.06	—	6.5	254
2.76	—	—	326
2.76	3.71	—	314

Table 2. *Effect of pyruvate on the deamidation of glutamine in homogenates and on urea synthesis in slices*

Liver tissue, 200 mg.; bicarbonate-Ringer; total volume, 4 ml.

Glutamine (10^{-2} M)	Sodium pyruvate (10^{-2} M)	Ammonia N formed (μ g.)
Homogenates		
0.34	—	0
0.34	1.03	63
1.73	—	234
1.73	5.12	456
2.06	—	496
2.06	6.18	670
2.74	—	936
2.74	8.23	1124
Slices		
		Urea N formed (μ g.)
2.06	—	$289 \pm 24.9^*$
2.06	6.18	$77 \pm 8.4^*$

* Mean of thirteen experiments.

isoGlutamine. In preliminary liver-slice experiments carried out with the small amounts of *iso*-glutamine available, the observation was made that *isoglutamine* promoted urea synthesis to a much greater extent than glutamine. After incubation for 90 min. at 37° and at a substrate concentration of 3.4×10^{-3} M the synthesis from *isoglutamine* amounted to 199 and 171 μ g. as compared with 89 and 59 μ g. from glutamine. From this it seems possible that *isoglutamine* rather than glutamine may represent the more direct source of urea nitrogen, a hypothesis which is referred to below.

Synthesis of glutamine from ammonium chloride

While it appears from the experiments described above that intact glutamine, as distinct from ammonium glutamate, represents the principal substrate for the synthesis of urea, there is also no doubt that urea formation can occur to some extent from ammonia alone. This could be due to a transitory synthesis of glutamine from added ammonium salts and tissue glutamic acid. For the purpose of investigation ammonium chloride (5.6×10^{-3} M) was added to washed liver slices and the mixture was subjected to paper chromatography. A notable amount of glutamine was detected in the mixture after incubation. No glutamine could be detected when the tissue was incubated in the absence of ammonium chloride. When the same mixture after incubation with ammonium chloride was hydrolysed with 2N-HCl for 5 min. paper chromatography revealed no trace of glutamine and a considerable increase in the amount of glutamic acid. The position of the other ninhydrin-reacting substances on the paper chromatogram remained unchanged.

Role of bicarbonate in urea synthesis from glutamine

Experiments with $\text{NaH}^{14}\text{CO}_3$. It has been known for some time that bicarbonate carbon represents the source of urea carbon (Rittenberg & Waelsch, 1940; Evans & Slotin, 1940; Mackenzie & du Vigneaud, 1948). Confirmation of this was obtained in experiments carried out in this work when $\text{NaH}^{14}\text{CO}_3$ was incubated with liver slices in the presence of glutamine. After the glutamine and urea components of the experimental mixture had been separated by paper chromatography, the compounds were tested for the presence of radioactive carbon by radioautography. Although no activity could be detected in the glutamine, the urea was found to be radioactive. This result eliminated the possibility that bicarbonate acted as a carbon source of urea after its incorporation into the glutamine molecule. The effect of glutamine on urea synthesis must therefore be due to its nitrogenous groups (see Discussion).

Effect of bicarbonate on the utilization of glutamine. When glutamine is incubated with liver slices in the presence of bicarbonate-Ringer a notable part of the amide nitrogen and of the glutamic acid moiety, as determined by the decarboxylase method, is utilized during the synthesis of urea. On the other hand, in the absence of added bicarbonate there is very little disappearance of either of the two glutamine components (Table 3). These findings show that the utilization of glutamine for urea synthesis occurs simultaneously with that of bicarbonate and possibly suggest a reaction of the latter with both the amide group and the glutamic acid moiety. For, in the absence of bicarbonate the amide group as well as the amino group of glutamine remains intact.

Contribution of amide nitrogen and amino nitrogen of glutamine to urea nitrogen

Liver slices were incubated with glutamine and amide nitrogen; ammonia nitrogen, urea nitrogen as well as glutamic acid were determined before and after incubation. Again it will be seen from these experiments, recorded in Table 4, that a significant proportion of the amide nitrogen and of the glutamic

acid moiety originally present was utilized during the reaction.

However, the disappearance of the glutamic acid moiety, as ascertained by the method of bacterial decarboxylation, gives no indication as to the nature of the group or groups within the molecule which may have reacted during incubation with liver slices. Therefore an attempt was made to estimate the change in α -amino groups during the experimental period. Special precautions were taken to prevent the formation of pyrrolidonecarboxylic acid from glutamine during the estimation, and to remove nitrogenous compounds other than amino acids which could interfere with the Van Slyke method. It will be seen from Table 4 that the disappearance of amino nitrogen was of the same order of magnitude as that of the disappearance of the intact glutamic acid moiety. This suggests that the changes observed with the decarboxylase method are due to, or paralleled by, the utilization of the α -amino groups during the synthesis of urea.

A comparison of the disappearance of glutamic acid or of the α -amino nitrogen with that of the amide nitrogen reveals that the contribution of the two nitrogenous groups of glutamine was approximately equal, and further, that the utilization of these two groups was accounted for by the formation of urea nitrogen. For, when the amount of nitrogen utilized is corrected for the small quantities of ammonia found in the mixture after incubation, the quotient glutamine nitrogen utilized/urea nitrogen synthesized approaches unity.

Table 3. *Utilization of glutamine during incubation with liver slices in the presence and absence of bicarbonate*

Liver slices, 1200 mg.; tris-Ringer, pH 7.4; glutamine concentration, $2.06 \times 10^{-2}M$; total volume, 24 ml. Results are calculated for 4 ml. of incubation mixture.

	In presence of HCO_3^- ($\mu g.$)	In absence of HCO_3^- ($\mu g.$)
Utilization of amide N	160	49
Utilization of glutamic acid moiety (expressed as amino N)	145	25
Urea N formation	320	27
Ammonia N formation	25	28

DISCUSSION

Effect of glutamine and ammonia on synthesis of urea

The results of numerous experiments in this work demonstrate that, except at very low substrate concentrations, the rate of urea formation from

Table 4. *Nitrogen balance in urea synthesis from glutamine in liver slices*

Liver slices, 1200 mg.; bicarbonate-Ringer; glutamine concentration, $10^{-2} \times 2.06M$; total volume, 24 ml. Results are calculated for 4 ml. of incubation mixture.

Change in amide N ($\mu g.$) (a)	Change in ammonia N ($\mu g.$) (b)	Change in amino N ($\mu g.$) (c)	Change in urea N ($\mu g.$) (d)	$\frac{(a+c)-b}{d}$
-212	+60	-185*	+308	1.09
-134	+42	-174*	+222	1.20
-160	+25	-145*	+320	0.88
-192	+35	-125*	+284	0.99
-191	+51	-168*	+245	1.26
-180	+50	—	+253	—
—	—	-145†	+300	—
Average -178	—	-159	—	1.08

* Estimated by the decarboxylase method and expressed as amino N.

† Estimated as amino N.

glutamine in liver slices was much faster than that from ammonium chloride (Fig. 1). Moreover, the rate of urea synthesis from glutamine was directly proportional to the substrate concentration, whereas with ammonia the rate declined when higher concentrations were used (Fig. 1). Thus there is evidence for the greater efficiency and for the more direct action of glutamine as compared with ammonia. The higher efficiency of ammonia compared with glutamine at very low concentrations could be explained by the presumably faster rate of diffusion of ammonium ions into the cell. Since, as discussed below, urea formation from ammonia may be due to glutamine synthesis, it can be argued that at low substrate concentrations more glutamine can arise in the cells through synthesis from ammonia than through the diffusion of extracellular glutamine. At higher ammonia concentrations the synthesis of glutamine may become the limiting factor, which would lead to a 'plateau' in the rate of synthesis. Such an explanation would be compatible with the results shown in Fig. 1.

Ammonium glutamate. The effect of glutamine on urea synthesis has been explained by the hydrolysis of glutamine to glutamic acid and ammonia in the liver cell (Krebs, 1942). It is difficult to obtain direct proof for this hypothesis through experiments with liver slices, in which urea synthesis from glutamic acid and ammonia is compared with that from glutamine, for it is known that the diffusion of glutamic acid into liver cells is very slow or even negligible (Cohen & Hayano, 1946; Schwerin, Benman & Waelsch, 1950; Kamin & Handler, 1951). It may be argued from a different aspect that if the liberation of ammonia from glutamine were a prerequisite for urea synthesis then, in view of the very considerable production of urea in the liver, at least some accumulation of ammonia would be expected. However, only relatively small amounts of free ammonia are found in the incubation mixtures after urea synthesis, as seen from the experiments of Table 4. Similar observations have been made by other workers (Leuthardt, 1938*a, b*, 1940). In considering this evidence as well as the high rate of utilization of glutamine, Leuthardt & Glasson (1944) came to the conclusion that hydrolysis of glutamine to ammonia could not have occurred during the synthesis. Moreover, it has been accepted by many that any formation of free ammonia, even as a result of the deamination of amino acids, is improbable, and this concept is well supported by the experiments *in vivo* of Kamin & Handler (1951). If the opposite view is taken, namely that the deamidation of glutamine should precede urea synthesis, an inhibition of glutaminase should prevent urea formation and an activation should enhance it. As seen from Tables 1 and 2, urea synthesis was unaffected when glutaminase

was inhibited, and a strong suppression was observed when the enzyme was activated by pyruvate. The interpretation of the inhibitory effect of pyruvate on urea synthesis is, however, not without difficulty since pyruvate also acts as a strong acceptor of the amino group of glutamine (Meister & Tice, 1950). Part of the suppressing effect on urea synthesis could therefore be due to a deprivation of α -amino nitrogen, since this is equally concerned in urea formation, as seen in the experiments of Table 4.

Synthesis of glutamine from ammonia

It has already been suggested that the effect of ammonia on urea synthesis depended upon glutamine formation. This concept, which has been upheld also by other workers (Leuthardt & Glasson, 1944; Leuthardt, Müller & Nielson, 1948; Kamin & Handler, 1951), gains probability by the finding of notable quantities of glutamine in the incubation mixture to which ammonium salts had been added. The glutamic acid required for the synthesis of glutamine could well arise by endogenous synthesis, an indication of which is the relatively high glutamic-acid content of the liver [0.6 mg./g. of fresh tissue (Awapara, 1949)]. If glutamine can thus be regarded as a direct source of urea nitrogen, it is not surprising that when the organism is deprived of glutamine through the administration of phenylacetic acid, which is followed by excretion of phenylacetylglutamine, urea formation is greatly reduced (Shiple & Sherwin, 1922).

Contribution of amide and amino nitrogen to synthesis of urea

One of the principal findings in this work was the apparently equal contribution of amino nitrogen and amide nitrogen of glutamine to the formation of urea. This relationship was shown in balance experiments (Table 4) where the disappearance of amide nitrogen and of amino nitrogen or of the glutamic acid moiety were determined side by side with the appearance of urea and ammonia. More urea nitrogen was formed than could be accounted for by the disappearance of the former two fractions; the excess disappearance of nitrogen was of approximately the same order as the small amount of ammonia which was found in the mixture after the incubation. Since this appearance of ammonia was unaffected by conditions which suppress urea synthesis, as for instance by the absence of bicarbonate (Table 3), it appears to be unconnected with the urea-synthesizing mechanism.

The carbon source of urea

Since the pioneer experiments of Krebs & Henseleit (1932) bicarbonate has been accepted as the source of urea carbon. During the course of the

present work the possibility was envisaged that bicarbonate carbon could be incorporated into the γ -position of the glutamine molecule and that the peptide-like CO.NH_2 group could, through group-transfer, be incorporated into the urea molecule. However, on incubation of glutamine in the presence of $\text{NaH}^{14}\text{CO}_3$ with liver slices, by subsequent isolation of glutamine the latter was found to be isotope-free. It appears therefore that the contribution of glutamine to urea synthesis is limited to that of its nitrogenous groups.

The absence of bicarbonate from the incubation mixture not only prevented urea formation but also impeded to a great extent the utilization of both the amide nitrogen and the amino nitrogen. In the presence of bicarbonate the amide nitrogen and the amino nitrogen of glutamine were utilized. The coincidence of these phenomena may find its explanation in a reaction of bicarbonate with the two nitrogenous groups of glutamine as the first step in urea synthesis.

The possible nature of the product of reaction in this step is discussed below. Though it is realized that the utilization of the nitrogenous groups of glutamine and of bicarbonate could be explained in different ways, for instance by transamination or transamidation to acceptors which in turn could react with bicarbonate, it is not easy to see why the occurrence of the two first-named reactions should depend on the presence of bicarbonate.

*Ureido compounds as possible intermediates
in the synthesis of urea*

If a direct reaction between bicarbonate and the two nitrogenous groups of glutamine is assumed, the transitory formation of a ureido compound of type (I) can be envisaged. If, on the other

hand, as indicated by the preliminary experiments mentioned, *isoglutamine* (III) takes part in the initial reaction in place of glutamine, the formation of an analogous ureido compound, hydantoinpropionic acid (II), could be envisaged. Through a hydrolytic split of the amide linkage either of the compounds would be converted into carbamylglutamic acid (IV), and an oxidative split of the amino linkage in the ureido compounds (I) and (II) would give rise to the keto analogues of carbamylglutamine and carbamyl*isoglutamine* (V) and (VI).

The significance of carbamylglutamic acid for urea synthesis has been brought to light through the work of Cohen & Hayano (1948) who, after confirming the promoting effect of formate on the synthesizing system previously observed by Bach (1939), came to the conclusion that carbamylglutamic acid acted as a catalyst in the synthesis of citrulline from ornithine (Cohen & Grisolia, 1948; Grisolia & Cohen, 1952).

A concurrent hydrolytic and oxidative split of compounds (I) or (II) would lead directly to urea formation. Alternatively, one could envisage a utilization of the suggested carbamyl compounds (IV), (V) or (VI) for the synthesis of citrulline, with carbamyl phosphate (Jones, Spector & Lipmann, 1955) acting as an intermediate.

A consideration of the metabolism of histidine (Borsook & Jeffreys, 1935; Tesar & Rittenberg, 1947) encourages belief in the occurrence of some of the proposed metabolites in urea synthesis, for *isoglutamine* (III) and α -formyl*isoglutamine* (VII) (Oyamada, 1944; Abrams & Borsook, 1952), as well as the closely related hydantoinpropionic acid (II) (Uchida, Itagaki & Wachi, 1952; Matsuda, Itagaki, Wachi & Uchida, 1952), were found in incubation mixtures during the degradation of histidine.

The proposed interrelationship of the suggested metabolites is outlined as shown in the scheme below:

SUMMARY

1. Glutamine and, from the results of preliminary experiments, *isoglutamine* were found to be far more effective nitrogen sources of urea than ammonia when incubated with rat-liver slices.

2. It was shown that the effect of glutamine was not due to its hydrolysis to ammonium glutamate. Evidence for this view was obtained by experiments in which glutaminase action was inhibited in one case and activated in another. The inhibition of the enzyme had no effect on urea synthesis from glutamine, and activation of glutaminase almost completely suppressed urea formation.

3. The synthesis of urea which is observed when ammonium chloride is added to liver slices is believed to be due to the intermediary formation of glutamine from ammonia and tissue glutamic acid. Evidence for a notable glutamine synthesis from ammonia during urea formation was obtained by paper chromatography.

4. In the absence of bicarbonate not only was urea synthesis suppressed but both the nitrogenous groups of glutamine remained intact. In the presence of bicarbonate the amide and amino nitrogen of glutamine were utilized for urea synthesis to approximately the same extent, and the whole of the urea nitrogen could be accounted for by the disappearance of the two nitrogenous groups during the synthesis. Therefore, the following overall reaction is suggested:

Glutamine amide nitrogen

+ glutamine amino nitrogen → urea nitrogen.

5. From the observation stated in (4) it is suggested that the first step in the synthesis of urea from glutamine may consist of a reaction between bicarbonate and the two nitrogenous groups of glutamine or *isoglutamine*, which results in the formation of ureido compounds of two types, and which through oxidative or hydrolytic fission would yield carbamyl compounds.

6. The carbamyl groups of the above compounds may take part in the synthesis of citrulline from ornithine. Alternatively, if the oxidative and the

hydrolytic split of the ureides occurred concurrently, a direct formation of urea could be visualized.

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Glutathione Metabolism

1. THE GLYOXALASE ACTIVITY OF MATURE MAMMALIAN ERYTHROCYTES

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The glyoxalase system is frequently used in the assay of reduced glutathione and is considered by some (Bhattacharya, Robson & Stewart, 1955) to be one of the most reliable of the available methods. It has been noted on several occasions (Lohmann, 1932; Platt & Schroeder, 1934; Woodward, 1935) that the method is applicable only in the presence of relatively low concentrations of glutathione. At high glutathione levels, the glyoxalase activity is no longer proportional to the glutathione concentration. Thus with acetone-dried yeast, Platt & Schroeder (1934) observed that a steady increase in the glutathione concentration above a critical level resulted in a progressively smaller increment in glyoxalase activity until a maximum had been reached. Indeed, a further increase in glutathione

In the course of an investigation carried out in this laboratory on factors affecting the oxidation and reduction of glutathione in the mammalian erythrocyte, it became evident that in this tissue, also, the behaviour of the glyoxalase system in the presence of relatively large concentrations of glutathione was not easily understandable in terms of ordinary enzyme kinetics. Jowett & Quastel (1933) were led earlier to a similar conclusion. Recently, the glyoxalase system has been shown to consist of two distinct enzymes, glyoxalase I, which catalyses the formation of an addition compound between methylglyoxal (and other glyoxals) and glutathione, and glyoxalase II, which catalyses the breakdown of the addition compound to form lactic acid and glutathione. The addition compound also

concentration beyond the level required to produce a maximum glyoxalase activity resulted in a slight decrease in activity. These investigators noted further that the maximum glyoxalase activity produced with optimum glutathione levels was not constant for a particular enzyme concentration, but was raised still further by an increase in the methylglyoxal concentration. This latter observation suggests that factors which are non-enzymic in nature may influence the glyoxalase activity at high glutathione concentrations.

breaks down spontaneously under the influence of a number of factors (Racker, 1951; Crook & Law, 1952). In view of the recent findings, a reinvestigation of the conversion of methylglyoxal into lactic acid in intact and lysed erythrocytes was undertaken.

EXPERIMENTAL

Material. Venous blood was freshly drawn from normal human subjects, heparinized [1 drop of 1:1000 solution of heparin (Connaught Laboratories)/10 ml. blood], the plasma and buffy coat were removed by centrifuging, and the erythrocytes washed three times with 0.16M-NaCl.

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