CHEMICAL CHARACTERISTICS AND PHYSIOLOGICAL RÔLES OF GLUTAMINE

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Plant physiologists for the past seventy-five years have attached considerable importance to their observations that the amides of the dicarboxylic amino acids were present in considerable concentration in plant tissue in free form, and postulated that the amides were present also, combined with other amino acids, in the protein molecule. Nevertheless, there grew a tendency among many protein chemists and physiologists to focus attention on the more stable amino acid residues in hydrolysates. This became increasingly true after 1873 when Hlasiwetz and Habermann (97) introduced the use of hydrochloric acid with stannous chloride in place of sulfuric acid, and thereby made acid hydrolysis more popular as a tool for the study of the units of protein structure. Labile amides destroyed by hydrolysis with strong acids were absent from the hydrolysates under investigation and for this reason received decreasing attention. While it was still commonly acknowledged that the ammonia liberated by the hydrolysis of protein probably arose from labile amide groups, it has been only recently that the rôle of these amides again began to receive due consideration.

The biological significance of amides has been reviewed extensively in Russian (67), but no comprehensive treatment of the rôle of glutamine has been available in English. The recent crescendo of interest in the rôle of glutamine (6, 7, 57, 58, 68, 69, 85, 86, 87, 89, 153, 268), in animal physiology especially, makes timely both a review of the literature on the subject and an attempt to correlate observations in the light of present knowledge.

I. Chemical Characteristics of Glutamine

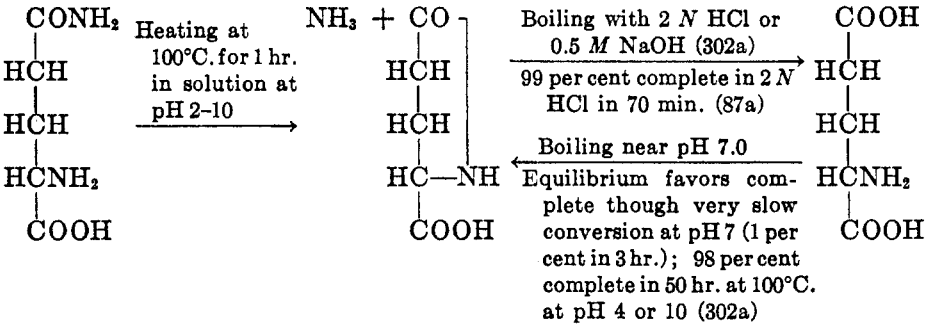
So that the rôle of glutamine in metabolic processes may be better understood, let glutamine be considered first as a chemical substance, apart from its physiological rôles. As an amide of glutamic acid it is a neutral reservoir and precursor of ammonia, glutamic acid, or α-ketoglutaric acid. Obviously, consideration of the rôle of glutamine will involve simultaneous regard for the rôles of glutamic acid and ammonia as well as of the closely related asparagine. For this reason considerable attention will be devoted in the following review to the function of glutamic acid and asparagine.

A. Properties and reactions of glutamine

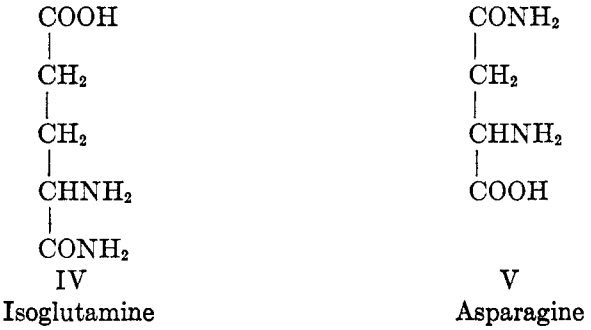
Glutamine has a molecular weight of 146.15 and an elementary composition of C = 41.09 per cent, H = 6.90 per cent, O = 32.84 per cent, and N = 19.17 per cent. Isoglutamine and ammonium pyrrolidonecarboxylate have the same molecular weight and elementary composition as glutamine. Glutamine is soluble in water (3.6 parts in 100 of water at 18°C.) but only very slightly soluble in absolute methyl alcohol (about 3.5 mg. per 100 cc. at 25°C. (87a))

or ethyl alcohol (0.46 mg. per 100 cc. at 23°C.) and almost insoluble in ethyl acetate, chloroform, ethyl ether, and acetone.

Bergmann *et al.* (15) give the melting point of synthesized and natural (beet) glutamine as 184–185°C. McIlwain *et al.* (153) report that glutamine isolated from horse meat melts at 205°C. The melting point of ammonium pyrrolidonecarboxylate is 185–186°C. (9,138). It is of interest in this connection that glutamine (from beets), when heated under the conditions of melting-point determination to 180°C. for 2 min., undergoes less than 5 per cent decomposition, whereas glutamine, similarly heated to 186°C. for 30 sec. (until it is all melted), is quantitatively converted to ammonium pyrollidonecarboxylate (9). Glutamine is known to be capable of ring condensation to form pyrollidonecarboxylic acid, while severe acid hydrolysis yields glutamic acid. A quantitative study of these reactions showed that they could be used both to characterize and to determine glutamine in solution. The reactions are:

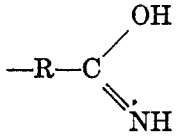


For purposes of comparison, the formulas of isoglutamine (IV), which is an isomer of glutamine (I) and of pyrrolidonecarboxylic acid (II), and of the homologous amide, asparagine (V), are given below.



The nitrogen evolved by reaction with the amino groups of I and III can be determined by Van Slyke's nitrous acid method for amino nitrogen, and the carbon dioxide from amino acid carboxyl groups of I and III by the ninhydrin reaction recently published from this laboratory (85, 86). Glutamine is believed to be the only substance known which shows the peculiar decrease in amino nitrogen and "carboxyl" (87) carbon dioxide on heating at pH 2, and the subsequent increase on boiling with hydrochloric acid. The decrease in carboxyl carbon dioxide caused by heating at pH 2 can be used for quantitative estimation of the glutamine.

Free glutamine is unique as an amide. As suggested by Chibnall (46), it might be considered a hybrid between a true amide and ammonium glutamate. The amide group is much more labile than that of other common amides. Glutamine reacts with nitrous acid in acetic acid, giving nitrogen equivalent to 180 per cent of the α-amino nitrogen (50, 111, 232, 261, 280), whereas asparagine yields nitrogen equivalent to only 100 per cent of the α-amino nitrogen, and amides in which there is no amino group in the α-position to a carboxyl group yield no nitrogen (265). Indeed Schulze and Bosshard (232), who first isolated glutamine, had shown in 1883 that the nitrous acid method of Sachsse and Kormann (211) (KNO_2 + HCl), a forerunner of Van Slyke's method (265), gave nearly double the expected yield of nitrogen when applied to glutamine. As pointed out by Plimmer (181), the nitrogen of any amide reacts completely with nitrous acid in the presence of a strong acid such as 2*N* hydrochloric acid. This suggests that in solutions of weaker acid most amides exist as the enol form:



This theory is used by Chibnall and Westall (50) to explain the unique behavior of glutamine in nitrous acid. They assume that glutamine in acetic acid exists as $R\overset{\overset{O}{\|}}{C}NH_2$. Lichtenstein (140) observes that α-alkylamides of glutamic acid also react in the Van Slyke amino method to give 90 per cent of their total nitrogen, and argues that since no primary amido group is present in the alkylamides, the explanation of Chibnall and Westall cannot hold. Lichtenstein suggests that the unique behavior of glutamine is due to preliminary reaction of nitrous acid with the α-amino group, followed by lactone closure and reaction with the amide group as it is liberated. This explanation would stand only on the assumption that nitrous acid will react to the extent of 80 per cent with amide nitrogen *at the moment* it is being hydrolyzed. Once ammonia is formed, nitrogen equivalent to only 25 per cent is formed under the conditions of the analysis (265).

Thierfelder *et al.* (261) showed in 1919 that di- and tri-peptides of glutamine, acetyl derivatives, and gliadin split off ammonia much less readily than did glutamine in 1 *N* sulfuric acid at 20° and 100°C. These findings were confirmed

by Chibnall and Westall (50), using Thierfelder's preparation of leucylglutamine. Nevertheless, Melville (148) reports that in the glutamine peptides which he synthesized the amide group was very labile.

Chibnall (46) is of the opinion that once glutamine is combined with other amino acid residues, as is the case in a protein, its amide group becomes more stable and more closely simulates in properties other amides.

The similarity of structure of amides and peptides is noteworthy. Peptides are indeed substituted amides (in most cases monosubstituted amides). That one carboxyl group of glutamic (or aspartic) acid should form a "peptide" with ammonia is not surprising, in view of this similarity of structure. The similarity of the peptide and amide bonds is indicated also by the similar behavior of the two bonds when subjected to hydrolysis catalyzed by sulfate or sulfonate groups. This type of hydrolysis will be discussed below under the heading "Presence of glutamine in the protein molecule" (see page 169).

It is worthy of note that several investigators, in their attempt to develop methods of analysis for glutamine, have been unable to obtain theoretical yields when they analyzed what they believed to be pure glutamine. The yields have ranged from 80 to 95 per cent of the expected values, suggesting either that glutamine was participating in some unrecognized side reaction or that it existed, in part, in some form the properties of which are not indicated by the recognized structural formula which Thierfelder (258) showed was consistent with most of the properties of glutamine.

Thus Cohen (51), analyzing for glutamic acid by conversion to succinic acid and subsequent use of succinoxidase, obtained almost theoretical yields (94 per cent) with glutamic acid but only 70 to 80 per cent of the expected succinic acid yield with glutamine. The yield was not increased by preliminary hydrolysis of glutamine with acid or alkali. These observations were confirmed by Örström *et al.* (170).

Assuming that in the nitrous acid method of Van Slyke (265) reaction with the α-amino nitrogen of glutamine is complete, only 84 per cent of the amide nitrogen is liberated (50) in 10 min. at room temperature and 95 per cent after 2.5 hr. Vickery *et al.* (280) report that in 4 min. at 22.5°C. 90 per cent of the total nitrogen (80 per cent of the amide) is liberated in the nitrous acid reaction.

Failure of other methods to give theoretical yields with glutamine is due probably to the use of impure preparations, or, in the case of some methods involving hydrolysis, to incomplete reaction under the conditions employed.

Glutamine when heated in weak acid, neutral, or alkaline solution is converted to pyrrolidonecarboxylic acid or its salt much more rapidly than is glutamic acid. At pH 6.5, 99 per cent of the glutamine is converted in 1.5 hr. at 100°C. to pyrrolidonecarboxylate, and the other 1 per cent is converted to ammonium glutamate (86). The main product obtained by non-enzymatic decomposition of glutamine differs from that (ammonium glutamate) obtained by enzymatic hydrolysis, as shown by Krebs (112) and Leuthardt (131) using the nitrous acid technique and, as confirmed in this laboratory, by use of the ninhydrin manometric method (10). The effect of pH and temperature on the rate of

conversion of pyrrolidonecarboxylic acid to glutamic acid (and *vice versa*), as well as the composition of the equilibrium mixtures, have been studied by Wilson and Cannan (302a), Vickery *et al.* (280), Olcott (168a), and Hamilton (87a).

Lichtenstein's (138, 139) claim for conversion of ammonium pyrrolidonecarboxylate to glutamine was later (140) rescinded. He states, however, that alkylamines in aqueous solution react with pyrrolidonecarboxylic acid to yield α-alkylamides of glutamic acid. Besides yielding 90 per cent of total nitrogen in the Van Slyke apparatus, these products were stated to give a strong ninhydrin (colorimetric?) reaction.

It was of interest to note that the relatively stable amide, asparagine, was known sixty-three years before Ritthausen (203) in 1869 isolated the corresponding free acid by acid hydrolysis of conglutin. That same year he postulated the presence of the amide in protein. Glutamic acid, however, was isolated by Ritthausen (202) in 1866 from gliadin. Not until seven years later (97) was the suggestion made that the corresponding amide occurred in protein, and it was seventeen years later before this labile amide was recognized and isolated by Schulze and Bosshard (232) from beet juice.

The structural similarity of pyrrolidonecarboxylic acid to proline makes the observed biological conversion of proline to glutamic acid and glutamine in kidney and perhaps also in liver (164) less surprising. Weil-Malherbe, and Krebs (302) noted that guinea pig and rabbit kidney oxidized proline (although not pyrrolidonecarboxylic acid) to glutamic acid. When ammonium salts were present glutamine was a product. Hydroxyproline gave rise to a small amount of a glutamine-like substance. Krebs (115) showed that proline and ornithine give the same oxidation product after treatment with *d*-amino acid oxidase and are converted to glutamic acid *in vivo*. Roloff, Ratner, and Schoenheimer (206), after feeding deuteroörnithine to mice, recovered deuteroproline and deuteroglutamic acid (*cf.* also 236). Borsook and Dubnoff (22) indicate that ornithine is rapidly converted to glutamic acid in rat kidney. Pedersen and Lewis (174) have observed rapid formation of urea after feeding either glutamic acid or proline and quote the previous findings of other authors, supposedly to imply that they feel that proline is converted to glutamic acid.

The similarity in structure of citrulline and glutamine has been discussed (7); in this connection it is interesting to note that Wada (293) claims that boiling citrulline in concentrated hydrocholoric acid for 8 hr. gives rise to proline.

Although at least 95 per cent of the glutamine obtained by the synthetic method of Bergmann, Zervas, and Salzmann (15) is the isomer corresponding to the glutamic acid used as starting material (9), nearly all the glutamine available commercially in this country has so far been prepared from beets by the method of Vickery, Pucher, and Clark (278). Owing to the recent heavy demands for glutamine, a number of commercial houses have undertaken to prepare the material. Some, not appreciating the instability of this amide or misled (140) by early papers by Lichtenstein (138, 139), have offered as glutamine a product over 95 per cent of which is ammonium pyrrolidonecarboxylate. Because glutamine and its decomposition product, ammonium pyrrolidonecarboxy-

late, have identical empirical formulas, elementary analysis gives no clue as to the purity of such preparations. Heretofore there has been published no simple procedure for testing roughly the purity of glutamine preparations, or the concentration of its most common contaminant, ammonium pyrrolidonecarboxylate. Gradual formation of color with Nessler's reagent was observed by Schlenker (219). If to a solution of several milligrams of a preparation in 2 cc. of water one adds 0.5 cc. of the Nessler reagent, one may quickly obtain information as to the quality of the glutamine (9). A good preparation (i.e., one in which there is little or no preformed ammonia) gives no immediate color, but because of the alkalinity of the Nessler reagent glutamine amide nitrogen will split off as ammonia at the rate of about 1 per cent every 5 min. (6). If ammonium pyrrolidonecarboxylate alone is present, maximum color will be obtained immediately on addition of the Nessler reagent. Accurate methods of analysis are included among the following.

B. METHODS FOR THE DETERMINATION OF GLUTAMINE

The methods of glutamine assay available for study of the rôle of this amide can be reviewed briefly. With the exception of Cohen's method, all of these methods involve hydrolysis of the amide linkage. One group of methods depends on the determination of the ammonia liberated by mild hydrolysis with dilute hydrochloric, sulfuric, or trichloroacetic acid, or with alkali, or with heat at neutral pH, or by the action of the specific enzyme glutaminase. Other methods measure the decreased reactivity with nitrous acid or ninhydrin that occurs when glutamine undergoes hydrolysis and condensation to ammonium pyrrolidonecarboxylate.

1. Methods involving measurement of ammonia

(*a*) *Non-enzymatic hydrolysis:* The oldest method involves measurement of ammonia after mild acid hydrolysis with hydrochloric or sulfuric acid and is dependent on the greater lability of the amide group of glutamine as compared with that of asparagine. Chibnall and Westall (50) and Vickery *et al.* (280) have worked out this method in greatest detail. Krebs (112) and Ferdman *et al.* (69) used ammonia production on 5-min. hydrolysis at 100°C. with 5 per cent sulfuric acid as an index of glutamine. Krebs pointed out that this procedure, besides liberating all the glutamine amide nitrogen, liberated 23.2 per cent of the amide nitrogen of asparagine. Örström *et al.* (170) had used the same hydrolysis for 10 min. Schlenker (219) reviewed methods of glutamine determination up to 1932. After removing preformed ammonia with sodium permutit (218, 219), he heated plant extracts for 2 hr. at 100°C. in 0.2 *M* phosphate buffer (pH 6.0–6.5) to distinguish between glutamine and asparagine. Wood *et al.* (303, 304, 305) used similar conditions. No correction for ammonia liberated from urea was made by any of these workers, although Chibnall and Westall (50) noted that 22.9 per cent of urea was hydrolyzed in 1 *N* sulfuric acid in 1 hr. at 100°C. Harris (89) has measured the *rate* of ammonia liberation on hydrolysis with 10 per cent trichloroacetic acid at 50° to 80°C., and has used

this as a qualitative test for, and as a measure of, glutamine amide nitrogen in plasma and spinal fluid. After 1¼ hr. at 70°C. glutamine was completely hydrolyzed, whereas only 12–15 per cent of added asparagine was hydrolyzed. Harris recognized the importance of correcting for the ammonia split off from urea during acid hydrolysis and selected his conditions to minimize urea breakdown. Steinhardt and Fugitt (248), by the use of catalysts such as dodecyl sulfate, have extended the acid hydrolysis method to measure glutamine amide nitrogen as well as of asparagine amide nitrogen in proteins. A slight modification of this method and the use of commercially available detergents have provided the author (9) with similar data on other proteins.

Efimenko and Naugolnaya (61) used alkali (sodium carbonate) to decompose amines and amides, but this method is less specific. Possible decomposition of arginine, citrulline, and canavanine would have to be considered. Mendel and Vickery (149) determined glutamine by hydrolysis at 100°C. in a buffer at pH 6.5. Mothes (159) and Schlenker (219) used similar conditions. Chibnall and Westall (50) observed that glutamine was almost completely hydrolyzed after 3 hr. at 100°C. and pH 8. Asparagine, however, was very little affected by such conditions.

(*b*) *Enzymatic methods—glutaminase:* The author (5, 6, 7) has found the measurement of ammonia formed by glutaminase action most convenient for measuring glutamine in dog and human blood plasma. This procedure has the advantage over methods involving acid hydrolysis in that it is more specific in the presence of urea and asparagine; the method is one of promise for the determination of free glutamine in enzymatic hydrolysates of proteins (9, 55). The weakness of the method rests on the fact that the glutaminase preparations liberate ammonia from the adenosine and its derivatives present in tissues. It is probable, however, that the glutamine amide nitrogen of tissues can be determined enzymatically as the difference between the ammonia nitrogen liberated after digestion with the glutaminase preparation and that liberated after digestion with the glutaminase preparation + bromsulfalein. Bromsulfalein inhibits glutaminase (7) but not adenosine deaminase (9).

2. Chloramine T and succinoxidase

Cohen (51) oxidized glutamine or glutamic acid to succinic acid and then measured the latter, using succinoxidase. This method, however, does not distinguish between glutamic acid and its amide.

3. Methods involving measure of decreased reactivity on ring closure

(*a*) *Nitrous acid:* When glutamine is heated at a pH near 7 it is converted rapidly to ammonium pyrrolidonecarboxylate. The nitrogen in the ring of this product reacts inappreciably in the Van Slyke nitrous acid method for amino nitrogen and only 25 per cent of the nitrogen in the ammonium radical reacts. When the pyrrolidonecarboxylate is heated with strong acid, the ring is hydrolyzed with the formation of glutamic acid.

The decrease in the nitrous acid amino value, resulting from heating glutamine at a pH near 7 (280), and partial restoration of this value following hydrolysis of pyrrolidonecarboxylate to glutamic acid by heating (100°C. for 2 hr.) in 2 *N* hydrochloric acid, have been used by Pucher and Vickery (192) as an index of glutamine content. Pucher and Vickery separated the pyrrolidonecarboxylic acid from ammonia and urea by extracting the acid with ethyl acetate.

(*b*) *Ninhydrin:* Glutamine reacts with ninhydrin, liberating carbon dioxide equivalent to the α-amino nitrogen. Pyrrolidonecarboxylic acid does not react. Neuberger and Sanger (165) measured the carbon dioxide produced in the ninhydrin reaction before and after heating for 3 hr. at pH 6.8 and considered the drop in the carbon dioxide production as a measure of glutamine. This method was developed independently (85, 268) and worked out in detail by Hamilton (86). Hamilton has drawn attention to the instability of glutamine in the presence of such anions as phosphate, and has made his method specific for glutamine in blood and tissues by removing such interfering substances as ascorbic acid and glutathione by preliminary precipitation at pH 6.5 with neutral lead acetate. Under the conditions employed by Hamilton, less than 0.1 per cent of glutamic acid is converted to pyrrolidonecarboxylic acid and less than 0.1 per cent of asparagine is hydrolyzed. The fact that asparagine does not interfere gives the ninhydrin method an advantage over the glutamine methods, which measure ammonia produced on acid hydrolysis. This advantage is of particular importance in dealing with plant tissues. The fact that adenosine and its derivatives do not interfere gives the method an advantage over the glutaminase method and permits measurement of glutamine in animal tissues.

C. PRESENCE OF GLUTAMINE IN THE PROTEIN MOLECULE

Ritthausen in 1869 (204, 205) recognized a product of acid hydrolysis of conglutin (from lupin seeds) to be the same (aspartic acid) as that given by hydrolysis of asparagine and postulated that asparagine was probably present in the protein molecule. Nasse (161, 162, 163) in the same year concluded that the ammonia produced on acid hydrolysis of proteins came from acid amide groups like that in asparagine.

Hlasiwetz and Habermann in 1873 (97), from experiments on acid hydrolysis, concluded that casein was composed of units including aspartic acid and asparagine, glutamic acid, and glutamic acid amide. Their suggestion that the protein contained glutamine preceded by ten years the discovery of glutamine by Schulze and Bosshard (232).

In 1877 Schulze (223) postulated that since glutamic acid could be separated from beet juice and pumpkin seedlings (231) only after an acid hydrolysis which was accompanied by formation of ammonium ion, glutamic acid existed in beets (or pumpkin seedlings) in the form of glutamine. He suggested later that glutamine, which he found free in germinating plants, was derived from the protein, and that after germination the glutamine was reconverted to protein. In 1883 Schulze and Bosshard (232) isolated glutamine from beets.

In 1904 Emil Fischer (72) and in 1908 Osborne, Leavenworth, and Brautlecht

(171, 172) agreed that most of the ammonia produced on acid hydrolysis of proteins came from combined glutamine and asparagine. Indeed, Osborne *et al.* stated that the ammonia production observed was equivalent, in the case of most proteins, to the sum of the aspartic and glutamic acids which could be isolated from the hydrolysates. They concluded that these amino acids in the protein molecule exist as peptides of asparagine and glutamine. They noted (172) that on boiling a solution of gliadin in 20 per cent hydrochloric acid ammonia production was complete in 30 min., as was the case also with asparagine. Since the amounts of dicarboxylic amino acid isolated were somewhat smaller than the amounts present in the proteins or their hydrolysates, it might be concluded that a small part of these dicarboxylic acids existed as such rather than as amide in the peptide chain. This would seem to be especially true in the case of three pea proteins studied by Osborne *et al.* In the case of cereal proteins, however, the yield of ammonia was so much more than the amount equivalent to the sum of the two dicarboxylic acids isolated that Osborne *et al.* postulated the existence of a third dicarboxylic amino acid in the protein molecule. The contention that amides existed in the protein molecule was supported in 1912 by Van Slyke (266), who showed also that the ammonia produced was greater than could be expected from cystine, which was the only non-amide amino acid giving appreciable ammonia on acid hydrolysis at 100°C. (see also 168a). Thierfelder *et al.* (261) in 1919 prepared some glutamine peptides and postulated their occurrence in proteins. Actual proof that the ammonia produced on acid hydrolysis arose from amides of amino acid came in 1932, when Damodaran (54) isolated asparagine from edestin and later with Jaaback and Chibnall (55) obtained glutamine from the enzymatic digest of gliadin. In 1935 Shore (242) showed that on acid hydrolysis of protein there was first a rapid rise in ammonia production, followed by a slower rise and humin formation. He calculated that on the assumption of a molecular weight of 34,500 for egg albumin, twenty-four amide groups per mole were present.

In 1935 Melville (148) synthesized glutaminyl peptides and postulated their natural occurrence. The next year the presence of glutamine in insulin was suspected by Harington and Mead (88).

Wormell and Kaye (307) report that animal and plant caseins are deamided by the action of 1 per cent sodium hydroxide for 40 hr. at 45°C. No data are given to indicate whether this technique splits off all amide groups or whether it causes any liberation of ammonia from combined arginine or citrulline. They claim that in neutral solutions (near the isoelectric point of protein) formaldehyde combines with the free amino groups of protein (especially of lysine). In acid solution formaldehyde combines also with amide groups. In view of this finding it is pertinent to enquire whether or not formaldehyde was used by Miles and Pirie during the preparation of the antigen of *B. melitensis*. As noted below, Philpot (177) reported that formic acid was split off from this antigen by the action of detergents.

Fraenkel-Conrat *et al.* (73a) likewise conclude that formaldehyde combines with the primary amino groups and primary amide groups of proteins and poly-

peptides but not with the secondary amide linkages of the peptide chain. This conclusion was reached partly by comparing the amount of formaldehyde bound by (*a*) polyglutamic acid synthesized by *B. subtilis* and by (*b*) polyglutamine synthesized *in vitro* from polyglutamic acid methyl ester.

Synge (254) subjected glutamine in the protein molecule (gliadin) to Hofmann degradation by treating with cold hypobromite, heating to 80°C., then hydrolyzing the peptide bonds by refluxing with hydrochloric acid. He obtained α,γ-diaminobutyric acid equivalent to 16.5 per cent of the glutamic acid residue and concluded that at least this much glutamic acid existed as the amide in the protein.

Chibnall (47, 48) in 1942 listed the amide nitrogen content of thirteen proteins. Gliadin has 25.78 per cent of its total nitrogen in the form of amide nitrogen and corresponding figures for other proteins are zein 18.3 per cent; edestin 9.49 per cent; casein 9.05 per cent; gelatin 0.5 per cent. By reasoning on the grounds of a *revised* Bergmann–Niemann hypothesis, Chibnall concludes from the total ammonia liberated on acid hydrolysis and the number of glutamine and aspartic residues, that only a portion of the dicarboxylic acids exist as the amides. In the case of edestin, for every unit containing 432 amino acid residues, 72 were glutamic acid, of which 36 were believed to be present in the protein as a peptide of glutamine, and 45 were aspartic acid, of which 27 were believed to preëxist as peptides of asparagine. From the amount of ammonia liberated on acid hydrolysis it was concluded that in β-lactalbumin only 32 of the 93 dicarboxylic acid residues could exist as amide in the protein, whereas for egg albumin only 31 of 75 dicarboxylic acid units were amides. Clearly, then, the statement by Everett (65) that "Distillation of protein hydrolysates with magnesium oxide *in vacuo* and determination of the ammonia in the distillate provides a quantitative measure of the dicarboxylic content of proteins" can be true only in so far as the result indicates, in some cases, a minimal value for dicarboxylic acid, and in other cases includes some of the nitrogen of citrulline.

Brand *et al.* (29a), in the most complete analysis of a protein yet recorded, account for 99.3 to 99.6 per cent of the amino acid present. They state that a mole of crystalline β-lactoglobulin (mol. wt. 42,020), found in whey of cow's milk, contains 36 moles of aspartic acid and 56 moles of glutamic acid. The amide nitrogen found was adequate to account for only 32 (of the 92) dicarboxylic acid residues existing preformed as amides in the protein molecule. Hence in this protein (unlike those described by Osborne *et al.*), most of the dicarboxylic acid residues are not present as amides.

Bergmann and Fruton (14) have considered the possibility that glutamine reacts with keto acids to form CO—NH— bonds involving the γ-carboxyl of glutamic acid, similar to a bond already recognized in glutathione.

Protein denaturation by detergents has been discussed in a review (166) by Neurath *et al.* The action on proteins of cationic detergents such as aryl alkyl substituted ammonium halides has been described by Kuhn *et al.* (125) and Schmidt (220). The effect of only the anionic detergents will be considered here.

D. CATALYTIC HYDROLYSIS OF AMIDE GROUPS IN PROTEINS BY ANIONIC DETERGENTS

Steinhardt and Fugitt (248) provided a simple technique for measuring the amount of glutamine (also of asparagine) present in proteins by plotting, against time, the amount of ammonia liberated from a protein when heated to 65°C. with 0.05 *M* hydrochloric acid and 0.03 *M* neutral salt of a catalyst such as dodecyl sulfate. Thus the rate of ammonia production from wool protein at 65°C. by hydrochloric acid is less than half the rate found in the presence of sulfuric acid, and this in turn is much less than the rate in the presence of ions of dodecyl sulfuric acid. The anions, much more than H^+, catalyze hydrolysis by being adsorbed preferentially on the amide groups, and then on the amino groups, of the protein. Normal chains of twelve to fourteen carbon atoms in the case of sulfate half-esters and of twelve or more carbon atoms in the case of sulfonates were shown to have greater affinity for proteins and greater catalytic action on glutamine amide hydrolysis than other isomers or homologues. Steinhardt and Fugitt showed that for wool protein the rapid production of ammonia by this technique corresponded in amount to the glutamic acid present, suggesting that nearly all, if not all, the glutamic acid in this protein existed in the form of its amide, glutamine. In the case of egg albumin the shape of the curve suggested that nearly all the amide present was glutamine. Though Krebs (112), Chibnall and Westall (50), Vickery *et al.* (280), Schlenker (219), and Ferdman (69) had previously determined *free* glutamine and asparagine by measuring the ammonia produced by varying conditions of acid hydrolysis, the technique of Steinhardt and Fugitt measures amides *in* the protein molecule without preliminary hydrolysis. The method of Steinhardt and Fugitt has been extended (9) by the use of a lower temperature and commercially available detergents (Aerosol OT and dodecyl benzenesulfonate) to permit more accurate determination of glutamine and asparagine in proteins such as dog heart muscle proteins, dog plasma proteins, dog hemoglobin, crystalline apoferritin, crystalline catalase, crystalline pepsin, and crystalline trypsin. The results parallel in general those given by Steinhardt and Fugitt (248) for wool protein and egg albumin. Asparagine amide nitrogen, for the time being, was considered to be the difference between total ammonia liberated on acid hydrolysis and the glutamine ammonia determined by the modification of Steinhardt's method. It is probable, however, that this fraction includes at least one and perhaps two sources of ammonia other than asparagine amide nitrogen. Citrulline has been found by Wada (293) in enzymatic digest of casein and by the author (8, 9) in enzymatic digests of plasma protein. Unless this citrulline was synthesized during the enzymatic digestion (as, for example, by enzymatic breakdown of arginine), citrulline must be present in protein. On prolonged hydrolysis of citrulline in boiling strong acid, ammonia is split off. Consequently, citrulline is almost absent from acid hydrolysates of protein. This is one reason why citrulline, like glutamine, has received so little consideration as a component of the protein molecule. It is probable, therefore, that part of the ammonia split off from proteins during hydrolysis by strong acid is derived from citrulline and

does not represent true amide nitrogen. The similarity in structure of the NH_2CONH— group of citrulline and the NH_2COCH_2— group of glutamine has been discussed by the author (7) in connection with a discussion of the specificity of glutaminase. Despite this resemblance of the terminal amino group of citrulline to amide nitrogen, it does not seem advisable to include this with true amide nitrogen.

During the early stages of the sulfonate-catalyzed hydrolysis of protein at room temperature, the ammonia liberated is almost entirely from combined glutamine. Hydrolysis of peptide bonds, however, is also accelerated, especially in the presence of a high concentration of detergent. The increasingly widespread use of detergents in the study of proteins (264) and in the commercial processing of materials containing protein makes timely a consideration of the rôle of such reactions as were studied by Steinhardt and Fugitt in bringing about the observed alteration in properties of proteins. Sreenivasaya and Pirie (246), working with tobacco mosaic virus protein in the presence of detergents, and Miles and Pirie (155) with the antigen of *B. melitensis*, probably hydrolyzed labile amide as well as peptide groups. After mild hydrolysis with sodium dodecyl sulfate the former workers could no longer precipitate their protein with immune serum. The latter group could no longer precipitate their protein by centrifugation at 16,000 R.P.M. but could precipitate it with immune serum. As pointed out earlier in this paper, a peptide is structurally a substituted amide. Philpot (177) has shown that the hydrolysis reported by Miles and Pirie liberates formic acid and an $—NH_2$ group which reacts with nitrous acid. As indicated by Lundgren, Elam, and O'Connell (143), alkyl benzenesulfonates form complexes with native and heat-denatured egg albumin on the alkaline side of the isoelectric point. Putnam and Neurath (197) report that horse serum albumin gives anomalous electrophoresis while in complex formation with alkaline dodecyl sulfate. After removal of the detergent the proteins, though electrophoretically homogeneous, had an altered mobility and viscosity. Complex formation of detergents with protein and denaturation occur on both sides of the isoelectric point, but precipitation occurs only on the acid side. The production of fibers from globular protein detergent mixtures (142), the precipitation of soluble proteins by detergent acids (154), the reversible formation of a heme from cytochrome C (106), the dissociation of the chlorophyll–protein complex (244), and the alteration of insulin by dodecyl sulfate (156) are probably all associated to some extent with hydrolysis of amide and peptide bonds and result in a greater or lesser degree of denaturation similar to that demonstrated by Anson (4).

Having now seen that glutamine (and asparagine) are building blocks of proteins, just as we have been accustomed to consider simple amino acids in the past, and having realized that much of the glutamic acid said to be present in proteins is probably present as glutamine, we can understand that there is (*a*) a plentiful supply of glutamine stored in body proteins, and (*b*) a need for glutamine or the ability to synthesize it in building body proteins.

Now let us consider the active rôle of glutamine in metabolic processes other than protein synthesis or hydrolysis.

II. The Rôle of Glutamine in Plants

A. THE HIGHER PLANTS

1. Origin of nitrogen of amides

Since the discovery of asparagine in 1806 by Vauquelin and Roubiquet (269) and the recognition of this substance by Pelouze (175) in 1833 as the amide of aspartic acid, plant physiologists have speculated as to the rôle played by amides in plant metabolism. Their theories and the data from their experiments are worthy of serious consideration by physiologists seeking leads to the study of the rôle of amides in animal metabolism. Studies so far have been based chiefly on the rôle of amides in (*a*) seedlings and (*b*) detached leaves. An excellent review on this subject has been published by Chibnall (45). An earlier work by Tauböck and Winterstein (257) reviews not only the physiological rôle of amides in plants but also methods of determination of amides.

As asparagine was isolated seventy-seven years before glutamine, all the earlier work and much of the later work dealt with the more easily recognized asparagine. Since, however, such data as are available point to *similarity of rôles for the two amides in plants, it seems advisable, in consideration of the rôle of glutamine, to review in fair detail the rôle played by asparagine.* This amide was identified and assayed by the method of Hartig and Pfeffer (see 45, page 28). This consisted of dabbing with alcohol a section of tissue on a microscope slide. After the alcohol had evaporated (2 hr.), crystals of the monohydrate of asparagine could be seen under the microscope. Their identity was checked by watching efflorescence at 100°C. and by noting their insolubility in a saturated solution of asparagine.

Piria (178, 179) in 1844 followed the concentration of asparagine in vetch and noted a sharp increase on germination and a decline as the plant matured to and through the fruit-bearing stage. He concluded that the asparagine was derived from a nitrogenous reserve in the plant, but he did not specify protein.

Sullivan (251) in 1858 noted that asparagine accumulated in large amounts in seedlings grown in the dark, and that it disappeared when the plants were exposed to light. Boussingault (26) confirmed these findings in 1868 with the root, stems, and leaves of pea, wheat, maize, and bean. He reasoned that just as animals metabolized protein nitrogen to urea, which they excrete, so plants in the dark metabolize protein nitrogen to asparagine, which remains in the sap until the plant has access to light, which is necessary for utilization of amide nitrogen.

In 1876 Schulze (with Umlauft) published the first of a thirty-five-year series of papers which reflect skillfully conducted experiments, logical reasoning, and conservative speculation. These authors (234) provided the first chemical proof that asparagine nitrogen found in seedlings was derived from protein nitrogen reserves of the seed. Of the protein nitrogen lost from lupin seedlings on germination, 63.5 per cent was present as asparagine nitrogen and free asparagine accounted for 22.3 per cent of the dry weight of the seedlings. Later Schulze and Castoro (233) and Wassilieff (297) found that of the protein nitrogen lost

from *Lupinus albus* during 14 days' growth in light, 86 per cent was present in free asparagine.

Borodin in 1876–78 (21) maintained that while asparagine was formed in all parts of plants it was formed most abundantly and therefore was most easily demonstrated in developing buds and germinating seeds. He concluded that asparagine was produced at a place where protein was decomposed and that its accumulation depended on an inadequate carbohydrate supply, such as would be maintained in buds on cut branches. He regarded asparagine as a transition substance between reserve protein and the cells of the bud or seedling and believed that while asparagine was always being formed it was as rapidly synthesized into protein when an adequate amount of soluble carbohydrate (glucose) was available. Starch in the form of insoluble granules was insufficient, and the amount of monosaccharide required for resynthesis of protein from asparagine was over and above that required by other respiratory processes.

In 1877 von Gorup-Besanez (81) postulated that on germination, enzymatic digestion of reserve proteins of vetch seedlings liberated amino acids and amides. He extracted (80; see also 84) from these seedlings an enzyme which converted albumin to peptone, and he detected leucine, tyrosine, asparagine, and glutamic acid in the germinating seedlings. This list of amino acids was later supplemented by Schulze.

Schulze in 1879 (225) postulated and later (226) proved that glutamine was equivalent to asparagine and played the rôle of asparagine in those plants in which asparagine could not be demonstrated. He recognized (224) also that the proportion of amino acids found free in seedlings was different from that obtained by acid hydrolysis of seed protein, and that the amounts of free amides formed in seedlings were far greater than could exist preformed in seed proteins.

Palladin in 1888 (173) showed that asparagine was not formed when plants were deprived of oxygen. This gave experimental backing to the previous supposition of Pfeffer (176) and Sachsse (210) that asparagine formation from protein involved oxidation. Schulze then argued (227) in 1888 that while part of the asparagine formed in germinating seedlings was probably formed by simple hydrolysis of reserve protein, another part, and probably the largest portion of the asparagine, derived its amide nitrogen from other amino acids liberated by this hydrolysis. That is, he suggested that amino acids liberated by enzymatic hydrolysis of reserve proteins were further degraded in the plant and that their nitrogen was used in asparagine synthesis. In 1892 (228) he suggested that this might be due to oxidation. Prianischnikow (187) in 1899 more eagerly accepted the oxidation hypothesis and pointed out, as had Boussingault (26), that amide synthesis was analogous to production of urea in animals. In 1904 (188) he was convinced that oxidation was involved and gave as evidence Demjanow's *in vitro* oxidation of leucine by permanganate with formation of valeric acid, carbon dioxide, and ammonia, and Butkewitsch's (42) statement that toluene vapor caused cessation of synthetic processes in seedlings while protein decomposition continued with accumulation of ammonia instead of asparagine. By 1906 Schulze (230) conceded that plants oxidatively deaminated amino acids,

and that ammonia, one of the resulting products, then condensed with organic acids to give asparagine or, in certain species, glutamine.[2, 3, 4] These amides were then used preferentially by the plant in protein synthesis. The extent of amide accumulation depended on the concentration of glucose available. As had Hlasiwetz and Habermann before him, Schulze believed (230) that both asparagine and glutamine were present in the protein molecule.

Schulze (see 45, page 58) has shown that in seedlings of the orders *Caryophyllaceae*, *Chenopodiaceae*, *Cruciferae*, *Polygonaceae*, *Polypodiaceae*, and *Umbelliferae*, glutamine rather than asparagine predominates as the product of protein metabolism, though the ratio of the two amides varies with age and environment. Oil-bearing seeds (e.g., castor bean) more often yield glutamine. Such seeds have low protein stores, and hence yield little glutamine (229) on germination (2.5 per cent of dry weight). Stieger (249) has indicated the predominating amide in a wide variety of plants and has added iris and carrots to the list of those rich in glutamine. The ratio of glutamine and asparagine in many seedlings has been determined and recorded by Schwab (235). Westall (see 45, page 60) showed that as castor bean seedlings developed, asparagine nitrogen decreased and glutamine nitrogen increased, and that (see 45, page 61) more glutamine is formed than could preëxist in the seed proteins lost. Vickery and Pucher (276) have recently shown that seedlings of summer squash, *Cucurbita pepo*, produce glutamine to the extent of 3 per cent of the original seed weight together with half as much asparagine, and hold out some hope that this may prove a laboratory source of glutamine.

2. Origin of the carbon skeleton of amides

So far we have considered only the nitrogenous precursors of the amides. The carbon skeleton of amides comes usually only in small part from the corresponding amino acids. Amides are often formed from ammonia and a nitrogen-free carbon skeleton derived from glucose. Damodaran and Nair (56) showed that legume seedlings contain *l*-glutamic acid dehydrogenase, through the action of which the glutamic acid formed on hydrolysis of reserve protein is converted to α-ketoglutaric acid. Oxidative decarboxylation gives rise to succinic acid, which may be metabolized through oxalacetic to asparagine.

Chibnall (45, page 95) suggests that arginine, proline, and histidine might also be metabolized to succinic acid, and thence to asparagine. Proline might

[2] Free arginine, such as occurs in considerable concentration in conifer seedlings, is also a reserve of nitrogen and may be further metabolized if necessary (Mothes (157)). Nearly all the arginine in the seed protein is rapidly liberated to the free form in seedlings (107). There is no good evidence, however, to indicate that it is synthesized by seedlings in preference to amides, as suggested by Suzuki (253). Stieger pointed out (249) that arginine more often occurs with asparagine than with glutamine.

[3] Some plants with acid sap (pH 1.5–3.0) contain moderately high concentrations of ammonium salts of organic acids as well as varying amounts of amides (126, 207, 208, 209, 235, 281). As pointed out by Vickery *et al.* (281), formation of amides in such plants can scarcely be considered a mechanism for detoxification of ammonia.

[4] Schulze's ideas on the mechanism of protein metabolism in seedlings are essentially identical with those which are accepted today (see reference 274).

be metabolized either to α-ketoglutaric acid, as by the mechanism shown by Weil-Malherbe and Krebs (302) to occur in kidney, or to succinic acid. Thus either amide could derive from proline. In the case of histidine, the formation of glutamic and succinic acids might proceed according to the path suggested by Edlbacher (59, 60).

Suzuki (252) showed that plants utilized ammonia from their culture medium for asparagine formation more rapidly if glucose was also present in the medium. His findings were confirmed with barley seedlings in 1910 by Prianischnikow and Schulow (190), who added that the ammonia taken up had provided both the amino and the amide groups in the asparagine. Vickery, Pucher, and Clark (278) found the same to be true with respect to glutamine formed in beets grown in plots dressed with ammonium sulfate. These experiments showed for the first time that amide formation was not always secondary to protein hydrolysis. In these cases the amides were derived from nitrogen-free carbon skeletons.

Prianischnikow (189) then showed that plants (lupin) which ordinarily did not form asparagine and which have very low carbohydrate reserves could be made to synthesize amide if the plants were exposed to light so that photosynthesis could supply the necessary carbohydrate. Amide formation also took place (Smirnow (243)), together with protein synthesis, when the lupins were grown in the dark in a medium containing glucose and ammonium salt. Likewise (189), seeds with a high carbohydrate reserve which ordinarily formed asparagine formed none after removal of the cotyledons or endosperms containing this carbohydrate reserve.

Prianischnikow regarded amide formation in plants as a process of detoxification of ammonia. The amides were a non-toxic form in which fixed nitrogen could be stored yet held in readiness for protein synthesis. Ammonia formation he regarded as the first step in protein and amino acid synthesis and the last step in their catabolism.

We have reviewed, so far, data which show that amides are synthesized from a number of amino acids derived from proteins in germinating seedlings. As the protein stores in these seedlings become depleted, the plant relies more on formation of amide (and subsequently on protein) derived from exogenous sources such as ammonia and products derived from glucose. Damodaran and Nair (56) have shown that in some legumes under the influence of an aerobic dehydrogenase *l*-glutamic acid is converted to α-ketoglutaric acid and ammonia. That these products can be converted to glutamine (see 45, page 209) in blades of perennial rye grass and beet roots indicates that the reverse process occurs also in plants. Adler *et al.* (1) have shown that cozymase was the activator of this transamination occurring in higher plants. The fact that protein synthesis (which in growing plants not absorbing amino acids implies amino acid synthesis) takes place readily at the expense of nitrogen stored in amides suggests strongly that the transamination mechanism described (*q.v.*) by Braunstein and Kritzmann (34, 35) for animal tissues, operates also in plants. Euler *et al.* (63) observed that exchange amination took place *in vitro* with glutamic or α-ketoglutaric acid when he used enzyme solutions prepared from higher plants.

Virtanen and Laine (289) demonstrated that aspartic acid (the only α-amino acid found to be produced during nitrogen fixation by bacteria of legumes) transferred its amino nitrogen to pyruvic acid with formation of alanine in the presence of crushed peas. They (285, 286, 288, 289, 290) showed that legume bacteria of peas planted in nitrogen-free sand excreted aspartic acid and its decarboxylation product, β-alanine, from root nodules. Virtanen and Laine (287) postulated that nitrogen fixation took place through formation in series of hydroxylamine, oxalacetic acid (later (289) shown to be present), oxalacetic acid oxime, and *l*-aspartic acid. *Azotobacter chroöcoccum* and *Bejerinckie* were shown to yield oxime nitrogen (62, 287) and aspartic acid (287). These authors concluded that other amino acids may then be synthesized at the expense of aspartic acid or asparagine.

Let us consider again the possible and likely precursors of the nitrogen-free skeletons of the amides. One may postulate from analogy (as has Chibnall (45, page 190)) that, as in muscle metabolism (3, 83, 306), enzyme systems are present which convert succinic acid through fumaric and *l*-malic acids to oxalacetic and aspartic acids and that each of the four nitrogen-free acids (as is the case in muscle) (3, 247, 255) catalyzed oxidation.

Just as such reactions could provide the precursors of asparagine, so citric acid or *cis*-aconitic (38, 117) through the cycle of Krebs (114) and Johnson (120) could lead to the formation of α-ketoglutarate (146, 147), glutamic acid, and glutamine. Indeed, some of the suggested precursors of asparagine (e.g., oxalacetic acid) are believed to be converted by muscle to citric or *cis*-aconitic acid through oxidation of carbohydrate according to the mechanism of Knoop and Martius (109) and might therefore act also as precursors of glutamic acid and glutamine.

Buchanan *et al.* (40) have recently found that heavy carbon in acetoacetic acid after treatment with homogenized kidney cortex can be recovered in α-ketoglutaric and fumaric acids. This suggests that in animals, acetoacetic acid and substances which are metabolized to it are to some extent at least precursors of α-ketoglutaric acid and therefore also precursors of glutamic acid and glutamine.

Experimental data to indicate whether or not such a mechanism operates in plants are still meagre and fragmentary. Virtanen and Tarnanen (291), however, have claimed that there is aspartase activity in germinating peas and in leaves of red clover.

Chibnall suggests that the nitrogen-free precursors of the dicarboxylic amino acids and their amides might be derived from carbohydrate, fat, or protein (45, page 193). Thus proteins, after hydrolysis to amino acids and deamination, give rise to α-keto acids which may condense with oxalacetic acid to give citric acid or be oxidized to succinic acid. Fats would give rise to succinic acid (270), and carbohydrates could provide the necessary precursors through the action of Krebs's cycle. Conversely, Chibnall postulates that asparagine and glutamine are reservoirs of oxalacetic acid and α-ketoglutaric acid for use by plants during seasons of extreme starvation. Suggestive, but as Chibnall (45, page 197)

points out, not conclusive evidence that these organic acids were precursors of amides was given by Mothes (158) in 1933, using vacuum infiltration of leaves with ammonium salts of organic acids by the method of Björksten (18). However, the work of Schwab (235) and of Chibnall (45, page 205) failed to confirm these observations. Chibnall believes, nevertheless, that when solutions of ammonium α-ketoglutarate are infused, both ions are used for glutamine synthesis by rye grass. However, definite proof of the exact nature of the nitrogen-free precursors of the amides will probably wait for application of a new technique, such as the use of metabolites containing tagged atoms. It seems reasonable to assume, however, that when ammonium ions and oxalacetic or α-ketoglutaric acid are present they would be used for synthesis of asparagine and glutamine equally well, whether the nitrogen-free precursor be derived from carbohydrate, fat, or protein, or whether it be poured preformed into the system from a test tube.

Whether the nitrogen-free precursors of amides derive from carbohydrate, fat, or protein, or whether they are preformed in the plant, would seem to depend on the history and environment of the organism. Under given conditions one food type may supply the precursor and under others another may provide the carbon skeleton of the amides. It would seem that there was a dynamic equilibrium of metabolites and products, and whether amide formation took place at the expense of exogenous nitrogen or ammonia from breakdown of proteins on the one hand, and on the other hand from carbon skeletons from proteins, carbohydrates, or fat, would depend on the age of the plant, its rate of growth, the relative and absolute concentrations of different types of food stores, and physical conditions such as light, temperature, humidity, etc. If the experimenter were to raise the concentration of an exogenous amide precursor and obtain increased yields of amide, he would indicate thereby neither whether this mechanism were one by which the organism ordinarily synthesized the major portion of its amide, nor whether the nitrogen-free precursor ordinarily was derived from carbohydrate, fat, or protein.

3. Amide metabolism in detached leaves

In barley leaves which have been detached from part of the plant, part of the leaf protein disappears. Simultaneously the concentration of glutamine increases. Glutamine nitrogen in detached barley leaves comes probably in part from direct hydrolysis of protein. However, the increase in free glutamine nitrogen is so much greater than the amount of amide nitrogen which is present in the leaf protein which is hydrolyzed that at least 75 per cent of the free glutamine formed must derive from other amino acids (308). As the concentration of carbohydrate decreases, first a stable amide and then free ammonia forms. In detached tobacco leaves, Pucher, Vickery, *et al.* (193, 194, 195) noted that asparagine formation was rapid in the dark but retarded in the light. Glutamine formation, however, was rapid in the light and very slow in the dark. Vickery (273) and Chibnall (45, page 224) have shown that the nitrogen for amide formation was made available through oxidation of amino acid to give ammonia.

Vickery (271) and Vickery *et al.* (282) showed that in detached tobacco leaves glutamine was formed in the light only when products of photosynthesis (carbohydrate) were available, suggesting that carbohydrate was the precursor of the carbon skeleton of glutamine. Asparagine, however, was formed whether or not light and carbohydrate were available, and since in the light the concentration of free malic, citric, and oxalic acids in the leaves remained unchanged, Vickery *et al.* felt that these acids were not the nitrogen-free precursors of asparagine. In the dark (when carbohydrate was used up), however, the concentration of malic acid decreased, and they felt that malic acid might have been utilized in asparagine formation, despite the fact that the malic acid loss was associated with an increase in citric acid in the dark only (271).

Rhubarb leaves (272, 275, 283, 284), on the other hand, after 114 hr. in the dark exhibited marked glutamine synthesis without formation of asparagine. Addition of glucose did not stimulate amide synthesis, and the same yield of glutamine (7 per cent of organic solids in some specimens (281)) was formed on exposure to light (277). Fifty per cent of the soluble nitrogen was present as ammonia. Vickery concludes that in excised leaves protein is metabolized through amino acids and ammonia to the amides.

When *Lupinus augustifolius* seedlings are grown in the dark, asparagine concentration reaches a maximum (11 per cent of original seed weight) after 12 days, and then drops rapidly with production of ammonia. Since no invasion by microörganisms could be demonstrated, Vickery and Pucher (276) concluded that this change reflected an exhaustion of non-nitrogenous components essential for synthesis of asparagine.

Mothes (159) notes that although starved plants do not either make or contain glutamine they maintain the mechanism for synthesizing it. As long as carbohydrate is present, a given plant maintains a constant glutamine/asparagine ratio.

4. Presence of amide hydrolase in plants

The enzymes involved in transfer of amide nitrogen to and from the carboxyl group of dicarboxylic acids have been demonstrated in several plant tissues. Chibnall and Grover (49) prepared asparaginase from germinating barley. Mothes (158) showed that leaves synthesize asparagine from ammonium aspartate. Vickery *et al.* (277, 279) and Chibnall (45, page 205) have shown that tomatoes, beets, and perennial rye grass (*Lolium perenne*) synthesize glutamine (the latter two at least from ammonium glutamate), and concluded that roots of these plants contain a rapidly synthesizing glutaminase. Vickery *et al.* (279) note that in beets grown in soil dressed with ammonium sulfate the increase in soluble nitrogen corresponds to both nitrogens of glutamine. Hence ammonia appeared to serve as a source of nitrogen for both the α-amino and the acid amide nitrogen of the synthesized glutamine. These workers nevertheless assumed that the synthesis of glutamine took place in two steps: (*1*) formation of glutamic acid from a non-nitrogenous compound; (*2*) dehydration of ammonium glutamate to glutamine by a mechanism such as that shown by Krebs to take place in some

animal tissues. Chibnall and Schwab interpreted this as indicating that beets contained glutaminase. Indeed, Schwab (235) suggested that plants be characterized as glutaminase or asparaginase plants, rather than as glutamine- or asparagine-forming plants.

Wood and his colleagues (303, 304, 305) have shown that glutamine rapidly disappears from fasting leaves of Kikuyer grass and that asparagine accumulates. In leaves of Algerian oats (*Avena sterilis* L.) glutamine accumulates. Wood assumes that the synthesizing enzyme for the amides is present in the leaves of these plants.

The author has found (7, 9) that tomato, zuccini, and perennial rye grass contain small but demonstrable concentrations of an enzyme which *hydrolyzes* glutamine to ammonia *in vitro*. The concentration of this enzyme is increased greatly if the plants are grown in soil dressed with ammonium glutamate. The enzyme is localized to the roots. Geddes and Hunter (74) claim that yeast extract can hydrolyze either amide, although Nielsen (167) and Schwab (235) report that it acts only on asparagine. Grassmann and Mayr (82) and Luck (141) claim that yeast extract hydrolyzes glutamine. The author, using a baker's yeast or brewer's yeast, found no action on glutamine and very little on asparagine until the yeast had been cultured in a medium containing asparagine (9).

In summary, we may conclude that reserve proteins of plants (as, for example, in germinating seedlings) are capable of being hydrolyzed enzymatically to amides and amino acids. The amino acids may be used in part for resynthesis of protein, but in part are deaminated, and from their products new amino acids, asparagine and glutamine, are formed, all these products being capable of use for protein synthesis. Amides tend to serve as a neutral store of fixed nitrogen, especially (*a*) when conditions are such as to favor inadequate concentrations of nitrogen-free precursors of amino acids, as, for example, when the light intensity is low enough to prevent appreciable photosynthesis of carbohydrate, and (*b*) when plants starve and proteins must be metabolized to supply energy to the plant. On the other hand, proteins of growing parts have different amino acid compositions from the proteins of reserve organs; hence in the absence of adequate external sources of nitrogen, the requisite amounts and kinds of amino acids tend to be synthesized by the plant from reserves, presumably with involvement of amide nitrogen. This synthesis of new amino acid and protein from amide is favored when adequate carbohydrate is made available from "stores" or by photosynthesis.

The direction of these several reversible reactions—hydrolysis, synthesis, transamination, etc. (catalyzed by enzymes)—and the position of the dynamic equilibrium established are governed by mass-action laws and relative concentrations of the reactants. These concentrations in turn vary with physical and chemical characters of the environment. As Chibnall has pointed out (45, page 112), the observation by Braunstein and Kritzmann that aspartic and glutamic acids can donate amino nitrogen to α-ketonic acids serves to emphasize the ability of the corresponding amides to serve as reserve agents for protein synthesis.

A relation between carbohydrate metabolism and protein synthesis is seen when it is considered that carbohydrate provides the necessary α-keto acids for formation of amino acid units of protein structure as well as the energy necessary for amide and protein synthesis.

B. THE LOWER PLANTS

1. Glutamine in the physiology of bacteria

Since 1881, when Loeffler introduced the use of meat extract in culture media, bacteriologists have been aware that the extract contained an elusive factor which was necessary for the growth of certain pathogenic organisms. McIlwain reported that the factor in this extract was glutamine and isolated glutamine from horse meat. In 1939 McIlwain *et al.* (153) reported that most, though not all, of the pathogenic strains of *Streptococcus hemolyticus* required glutamine for their growth. As little as $M/500$ glutamine added to the inadequate basal culture medium permitted full growth in 16 hr. In the same year Fildes and Gladstone (70, 71) reported that Group A hemolytic streptococcus as a rule required glutamine, while Group B streptococcus usually grew as rapidly without its addition. Most of the strains of Groups C, E, F, and G required glutamine. McIlwain (152) showed that no compound, even though closely related chemically, could replace glutamine. He reasoned that since insulin is not a substitute for glutamine in the growth of *Streptococcus*, the organisms do not break down glutamine peptides to glutamine, and that it is unlikely that the organisms have (reversible) enzymes present capable of synthesizing these peptides (unless the enzymes present are specific for the peptides involved). McIlwain felt that the function of glutamine is for ammonia transference rather than for protein synthesis. This is in contrast to the belief of Pollack and Lindner (183) regarding the function of glutamine in the case of lactic acid bacteria (*q.v.*). McIlwain showed that growth of streptococci took place, though less actively, if glutamate (in concentration 100 times greater) were added instead of glutamine, and assumed that glutamine was synthesized from glutamate. Landy (127) confirmed McIlwain's findings but added that in peptone there is present another factor necessary for optimum growth of *Streptococcus hemolyticus*. Fildes and Gladstone in 1939 (71) reported that of six strains of *B. anthracis* tested, five grew more rapidly in the presence of glutamine. All of four strains of *Streptococcus viridans* required glutamine. Glutamine and glutamic acid produced equal but marked stimulation of growth of *Proteus* but asparagine had no effect. Glutamine stimulated growth of *E. coli* when the inoculum was a 14-day-old culture, but not when it was only 24 hr. old. Glutamine had little if any stimulating effect on the growth of *H. influenzae* or *C. diphtheriae* and none on *N. gonorrheae*.

Lankford and Snell (129), however, report that glutamine is necessary for certain strains of *Neisseria*. Fildes and Gladstone claim that *Staphylococcus* showed some acceleration of growth on addition of glutamine. One strain of *Pneumococcus* tested gave much more rapid growth when glutamine was present. A high concentration of glutamate, however, caused good growth in the absence

of glutamine. Bernheimer and Pappenheimer (16, 17) in 1942 showed that a concentration of 10 mg. per cent glutamine gave 75 per cent of maximum growth of *Pneumococcus*, and used 20 mg. per cent glutamine in their medium for *Streptococcus hemolyticus*. Gibert (76) has studied the effect of varying concentrations of glutamine and asparagine on the growth of *Pneumococcus*, and has noted that 10 mg. of glutamine per 100 cc. inhibits some strains and favors the growth of other strains of *Pneumococci*. Strong, Feeney, and Earle (250) and Feeney and Strong (66) reported that glutamine in a concentration of 0.01 mg. per 100 cc. was effective as a growth factor for *Lactobacillus casei* and assumed that the organisms are capable of synthesizing glutamine from glutamic acid. Asparagine was also an effective stimulant but chiefly when glutamic acid was present to accept the amide nitrogen.

Pollack and Lindner (182, 183) reported the stimulating action of glutamine and many plant and animal extracts on the growth of lactic acid bacteria (including *Streptococcus lactis* and *Lactobacillus arabinosus*). Addition of other amino acids was without effect. Known vitamins were present. Glutamine gave a response ten times as strong as did peptone. Lewis and Olcott (137) found that glutamic acid or glutamine was necessary for the growth of *L. arabinosus* 17-5. Glutamine was 140 per cent as effective as glutamic acid. Lyman *et al.* (144) observed that production of lactic acid by this microörganism (used as a measure of growth of the bacteria) did not increase with increasing amounts of glutamic acid until a concentration of 0.8 mg. of glutamic acid per 100 cc. of medium had been reached. When 0.2 mg. of glutamine was present in 100 cc. of medium the *increase* in the lactic acid formed in 72 hr. was almost proportional to the concentration (from 0.0 to 1.2 mg. per 100 cc. of medium) of glutamic acid added. They conclude that glutamic acid is first converted to glutamine by the organism. This contention is supported by their observation that in the presence of glutamic acid, but only in the absence of glutamine, addition of ammonium salts increases the production of lactic acid. This further suggests that the organism contains an enzyme system capable of synthesizing glutamine from glutamic acid and that the chief use of glutamic acid, in the economy of this organism's metabolism, is for the synthesis of glutamine; probably the glutamine is used for synthesis of cellular proteins (*cf.* 84a). Pollack and Lindner (182, 183) believe that there is also an unknown stimulating factor other than glutamine in natural products. This unknown has an isoelectric point between 3.5 and 4.5, is not inactivated by proteolytic enzymes, and is less easily hydrolyzed than glutamine. Unlike glutamine, it can withstand heating for 2 hr. at 100°C. at pH 2–11. They believe that the material is not a peptide of glutamine, since enzymatic digests of the peptone factor grow even more rapidly when glutamine is added. Later Pollack and Lindner stated that nine strains of lactic acid bacillus require either glutamic acid or its amide. Four require glutamine (or eleven times as much glutamic acid). Ammonium chloride was not sufficient. They believed that the glutamate or glutamine is required simply as building blocks for the formation of cell protein.

Niven (168) notes that for maximum growth of *Streptococcus lactis* and *Strep-*

tococcus cremoris either glutamine or asparagine (and also valine, leucine, isoleucine, methionine, and arginine) is required.

Hill and Mann (96) reported that the inhibitory effect of sulfanilamide on *E. coli* growth *in vitro* was counteracted by glutamine or glutamate concentrations ranging from 15 mg. per cent to 150 mg. per cent. Harris and Kohn (93) had previously reported that methionine (especially in the presence of xanthine and hypoxanthine) offered similar protection of *E. coli* from sulfanilamide.

Bovarnick (27, 28, 29) has demonstrated that when glutamate and asparagine (0.007 *M*) react at 100°C. and pH 7.0 for 24 hr. some nicotinamide is formed. The reaction is catalyzed by 7×10^{-5} *M* ferrous sulfate. The same reaction takes place though to a smaller extent when glutamine is heated. One wonders whether or not the synthesis of nicotinamide from amide and glutamic acid accomplished *in vitro* by such drastic means can be catalyzed enzymatically *in vivo*.

The phenomenon reminds one of the rather surprising claim of Fildes *et al.* (see 152) that glutamine in some bacteriologic culture media containing agar was stable to autoclaving at 120°C. and pH 7.2 for 20 min., and that only a little loss of activity occurred when meat concentrate was heated at pH 5, 6, or 7 at 100°C. for 1 hr. It is likely that if glutamine is added to the culture medium before autoclaving a small amount of nicotinamide is formed during the autoclaving. It is unlikely, however, that such a reaction accounts for the activity of autoclaved glutamine, when nicotinamide is added to the basal medium. On the other hand, it is possible that glutamine in the presence of the components of the culture medium or at the pH of the medium is adequately (though only partially) protected from decomposition by heat. Niven (168) notes that asparagine and glutamine are more effective if they are sterilized by filtration rather than by autoclaving, and Lankford *et al.* (128) note that the factor necessary for growth of gonococci is destroyed on autoclaving. This is to be anticipated in view of the heat lability of glutamine especially.

Tatum *et al.* (256) noted that glutamine or asparagine was necessary for the fermentation of starch by butyric acid bacteria.

2. Yeast

Nielsen (167) has shown that glutamine is not an essential component of media for the strains of yeast he tested. Smythe (245) has shown that glutamine increases the rate of fermentation by yeast.

The above observations indicate that glutamine is an essential metabolite in the more specialized of even the lowest forms of plant life. Some forms cannot grow unless glutamine is already present. Others appear to be able to synthesize the necessary glutamine if the proper precursors are present. Whether this glutamine is used merely in building proteins of the bacterial cell, or whether it is used also, or only, as a carrier of labile $—NH_2$, is a matter which must await further study.

III. The Rôle of Glutamine in Animal Metabolism

Krebs (112) reported that there were present in the liver, kidney, brain, and retina enzymes which were capable, on the one hand, of synthesizing glutamine

from glutamic acid, and on the other, of hydrolyzing glutamine to glutamic acid. From this it seemed reasonable to suppose that glutamine played an essential rôle in animal metabolism in each of these three sites, and that at least part of these essential reactions was localized to the organs mentioned and that the glutamine might play different rôles in the several organs.

It would not be surprising then to find considerable conentrations of glutamine either in these organs or in the circulating fluids, including blood, or in both. The concentrations expected would vary depending upon the summation of the relative rates of hydrolysis and synthesis under the several conditions to which the tissue had been subjected prior to analysis. The concentration would tend to be high in those organs the enzyme systems of which favored synthesis or storage and low in the organs which favored glutamine hydrolysis. Let us consider the rôles in blood and in each organ named.

A. PRESENCE OF GLUTAMINE IN BLOOD

Glutamine, or a substance very closely resembling it in properties, has been reported by Ferdman, Frenkel, and Silakova (69) to be present in dog and pigeon blood in concentrations of 14 and 20 mg. per 100 cc., respectively. These authors measured glutamine by determining the amount of ammonia set free on heating for 5 min. at 100°C. with 5 per cent sulfuric acid. After these findings had been published and just before they became available in this country, Hamilton and the author, working in the same laboratory but using different methods, concluded that 5–12 mg. of glutamine was present in 100 cc. of fasting dog or human plasma. Hamilton (85, 268) used the ninhydrin carbon dioxide method. The author (6, 7, 268) used a glutaminase method. Hamilton concludes that red cells (86, 87) have approximately the same concentration of glutamine as the surrounding plasma. Harris *et al.* (89, 91) have followed the rate of ammonia liberation on acid hydrolysis at various temperatures and concluded that the glutamine levels reported by Hamilton hold for both human plasma and spinal fluid, and that no appreciable concentration of asparagine is present. The fact that Cohen (51) finds human plasma has a total of 3 mg. per 100 cc. glutamic acid plus glutamine by a method which does not distinguish between the two compounds, and that 5–10 mg. per 100 cc. of glutamine is found by several laboratories and methods indicates that nearly all, if not all, the glutamic acid in blood is present as the amide.

It is our belief (6) that spontaneous breakdown of glutamine in plasma is responsible for part of the "γ-ammonia" which Conway and Cooke (53) report is liberated from plasma. Whether or not glutamine is the precursor of the ammonia formed in the kidney and liberated into the renal venous blood is still undetermined. It would not be surprising, however, if this ammonia were formed by the same mechanism that provides most of the urinary ammonia in dogs (268).

The above-mentioned workers (6, 7, 69, 85, 89) and Gerard (75) favor the hypothesis suggested by Leuthardt (109) that glutamine serves as an ammonia carrier in cell metabolism. Örström, Örström, and Krebs (169) suggested that it acted specifically as an ammonia carrier in formation of hypoxanthine in pigeon liver.

B. PRESENCE OF GLUTAMINE IN CEREBROSPINAL FLUID

Values given by Ferdman *et al.* (69) indicate a concentration in the human spinal fluid of 1.0 mg. of glutamine amide nitrogen per 100 cc. This figure corresponds to the value of 5–10 mg. of glutamine per 100 cc. of spinal fluid given by Harris (89).

C. PRESENCE OF GLUTAMINE IN JOINT FLUID

Glutamine concentration in human joint fluid (6) appears to parallel that in blood plasma.

D. PRESENCE OF GLUTAMINE IN URINE

So far there are few data to indicate the concentration of glutamine in urine. Cohen (51), using a technique which does not distinguish between glutamine and glutamic acid, found that the sum of the two amounted to 4.3 mg. per 100 cc. in human urine. McIlwain (153) concludes that because urine supports the growth of *S. hemolyticus* it contains glutamine, but he does not indicate the concentration. Ferdman *et al.* (69) report that 1.4–2.4 g. of glutamine per 24 hr. is excreted in human urine. As pointed out by Hamilton (86, 87), this figure is probably too high, as Ferdman *et al.* estimated glutamine amide nitrogen as the ammonia liberated by hydrolysis for 5 min. at 100°C. with 5 per cent sulfuric acid. The relatively high concentration of urea in urine would contribute appreciable ammonia under these conditions. Enzymatic determination (6, 7, 9) of glutamine in one normal human urine (sp. gr. 1.026) indicated a concentration of 4.7 mg. of free glutamine per 100 cc., a figure which is very close to that given by Cohen for glutamic acid (plus glutamine). Such data as are available (6, 9, 22, 51) indicate that arginine, glycocyamine, glutamic acid, and glutamine spill through into the urine in roughly the same relative proportions as exist in plasma. Some glutamine may be present in human urine combined with phenylacetic acid.

E. PRESENCE OF GLUTAMINE IN EGG

The "white" of unincubated hen's egg contains only a very low concentration of glutamine. The yolk, however, contains relatively large amounts of free glutamine (6). On incubation of eggs the total amount of free glutamine decreases and the concentration of glutamine outside the yolk increases (9). Ultraviolet-absorption curves of dialysates (9) of portions of incubated and unincubated eggs indicated the absence of appreciable concentrations of interfering substances such as adenosine or adenylic acids which, if present, would increase the apparent concentration of glutamine.

The presence of a high concentration of free glutamine in egg and the maintenance of a moderately high concentration during incubation of fertile eggs is probably one reason why the egg is such an excellent medium for the growth of certain microörganisms. Presumably the free glutamine concentration in in-

cubating eggs is maintained as part of a dynamic equilibrium between the bound glutamine of protein reserves and of embryo protein. Whether or not there occurs in the egg a synthesis of glutamine from other amino acids similar to that which takes place in seedlings has not yet been determined.

F. RÔLE OF THE GLUTAMINE–GLUTAMIC ACID–GLUTAMINASE SYSTEM IN THE CENTRAL NERVOUS SYSTEM

A better understanding of the significance of glutamine as a metabolic product and constituent of nervous tissue will be obtained if preliminary consideration is given to the rôle of a metabolite which probably stimulates glutamine formation, *viz.*, ammonia.

Work on the physiology of ammonia in nervous tissue up to 1935 has been reviewed by Schneller (221) and will not be discussed further in the present review. As shown by Pugh and Quastel (196), ammonia is formed by isolated brain from butyl-, amyl-, isoamyl- and heptyl-amines, and amines decrease the oxygen consumption of brain.

In 1935 Kahn and Chekoun (104) showed that by partial asphyxiation of fish, the ammonia production by brain was increased. Increased ammonia production occurred also *in vitro* on stimulation of brain. Formation of unusually large amounts of ammonia in nerve tissue of molluscs was noted by Kahn *et al.* (77, 103).

Sapirstein *et al.* (215, 216) reported that 0.5 g. of ammonium salts given by mouth to humans increased "after-contractions" which they regarded as a measure of cerebral cortex excitability. Bruhl (39) stated that the ammonia content of brain increased as the adenylic acid content decreased in convulsive states.

Krebs (112) in 1935 showed that brain contained glutaminase and that this enzyme *in vitro* synthesized glutamine from ammonia and glutamic acid when an energy source such as glucose was present. The following year he showed that addition of *l*-glutamic acid to brain doubles the QO_2 and the brain converts free ammonia nitrogen to amide nitrogen. Grey cortex and retina can bind ammonia at the rate of 0.8 per cent of the tissue dry weight per hour if glutamic acid is present.

Weil-Malherbe (299, 300) later in the same year reported that of twelve amino acids tested, glutamic acid was the only one which caused increased uptake of oxygen by brain tissue *in vitro*. Both *l*- and *d*-forms were oxidized. Later (301) he stated that in brain (and spleen) 0.033 *M* ammonium ion inhibits anaerobic glycolysis but increases aerobic glycolysis. Glutamate, hydroxyglutamate, and glutamine have the same effect on brain (only), especially in the presence of 0.01 *M* succinate or lactate. Harris, Blalock, and Horwitz in 1938 (92) suggested that the drop in blood amino acid level noted by them during insulin hyperglycemic shock therapy of mental patients had an effect on the mechanism referred to by Krebs. No data were given in support of this view. Harris (90) in 1943 showed that glutamine participated in this fall of blood amino acid level during insulin shock.

Folch-Pi (73) in 1942 postulated that glutamic acid under the influence of

glutaminase detoxified the ammonia formed in nerve tissue and that in the brain glutamine was a by-product of this detoxification. Sapirstein (214) in 1943 evoked convulsions in twelve rabbits by injecting 15-25 cc. of 2.5 per cent ammonium chloride at the rate of 1 cc. per kilogram per 5 min. Complete protection from these convulsions was given by a preliminary injection of 50 cc. of 5 per cent *l*-glutamic acid in 3 per cent sodium bicarbonate, even when 50 cc. of 2.5 per cent ammonium chloride was given. Aspartic acid failed to offer protection.

Price, Waelsch, and Putnam (191) report that on feeding 12 g. of *dl*-glutamic acid hydrochloride to persons who are subject to frequent attacks of psychomotor disturbance or petit mal, the number of attacks is decreased from 25–50 per day to 5–25 per day, as long as the medication is continued. They attribute the effect chiefly to acidification of the fluid about the brain cells, but used glutamic acid because of the unique rôle which the *l*-form is believed to play in brain metabolism. Later Waelsch and Price (295) concluded that *l*(+)-glutamic acid was the chief factor favoring improvement. Decision as to whether the phenomenon produced by administration of glutamic acid was the result of an increased rate of detoxification of ammonia and synthesis of glutamine (a reaction which is retarded *in vitro* by the addition of either *d*- or *l*-glutamic acid) or whether it is a result of activation of the synthesis of acetylcholine by *l*(+)-glutamic acid (294) must await further work. Atebrin strongly inhibits glutaminase (7) and choline esterase (294). Whether its action on the esterase is due to inhibition of glutamine hydrolase and a decreased concentration of glutamic acid, is undetermined.

Since glutamine is less than half as active as glutamic acid as an accelerator of acetylcholine synthesis by rat brain extract (160, 294), it is conceivable that the glutamine–glutaminase system in brain (by controlling the relative concentrations of glutamic acid and glutamine) is involved in a control of choline acetylase activity and therefore also in the control of the potential of nerve action.

Hueber (98) has reported that administration of 10 cc. of 10 per cent magnesium glutamate three times a day to hyperthyroid patients having a basal metabolic rate 25–60 per cent above average reduced the basal metabolic rate until it was only 7–13 per cent above average. No clinical improvement accompanied this fall. Decision as to whether or not this observation is dependent on the action of glutamate on the nervous tissue must await further investigation.

G. RÔLE OF GLUTAMINE AND GLUTAMIC ACID IN MUSCLE METABOLISM

Riabinovskaja (199) reports that in a medium of 0.01 *M* sodium glutamate the indirect excitability (i.e., through the nerve) of frog sartorius-nerve preparations is eliminated and the direct excitability is decreased. The presence of high concentrations of both glutamine (69, 87, 153) and adenosine derivatives in muscle has not yet been shown to be more than fortuitous but suggests that the two components may function in the same system. The subject of transamination in muscle (52) will not be discussed here, since similar processes in the liver are considered in the next paragraph.

H. RÔLE OF GLUTAMINE IN LIVER

1. Deamination-transamination

The liver has long been regarded as the chief center of deamination of amino acids. The subject of transamination has been discussed thoroughly by Herbst (95) and is reviewed here only insofar as it relates to the dicarboxylic acids and their amides. While the reactions of transamination require glutamic or ketoglutamic or the corresponding aspartic (31) compounds and have not been shown to require participation of the amides, the significance of glutamine in relation to transamination and deamination is that it can act either as a storage or transport form of α-amino nitrogen, glutamic acid, or α-ketoglutaric acid. That is, glutamic acid may accept the $—NH_2$ made available by the deamination of other molecules, thus forming glutamine (112). This glutamine is in part then transported from the liver to other parts of the body *via* blood to perform other functions elsewhere and may be, in part, utilized directly in the liver to form urea (116, 130, 131, 135, 136).

In 1939 Karyagina (105) showed that *in vitro* skeletal and heart muscle, liver, kidney, and brain, the amino groups of glutamic and aspartic acids in the presence of pyruvic acid were transferred, giving rise to alanine and α-ketoglutaric and oxalacetic acids. Kritzmann (123) showed that this and the reverse action occurred also *in vivo* in rabbits, pigeons, and white mice. Bychkov (43) showed that where the carboxyl group of a monocarboxylic amino acid is supplemented by a second acid group ($—SO_3H$ as in cysteic acid or $—OPO_3H_2$ as in phosphoserine), the resulting acid is capable of donating $—NH_2$ to a keto acid just as do either of the two dicarboxylic amino acids, but cannot catalyze the transfer of $—NH_2$ between two monocarboxylic acids such as lysine and pyruvic acid. Braunstein and Kritzmann (36, 37) showed that there is no transfer of $—NH_2$ from monocarboxylic acids to keto acids unless $M/16{,}000$ of either aspartic or glutamic acid or their precursors (keto acids or citric, succinic, fumaric, or maleic acid) were present. Braunstein and Bychkov (33) maintained that deamination of *l*-monocarboxylic amino acids involved first a transfer of the α-amino group to ketoglutaric or oxalacetic acid by the corresponding enzyme, glutamic or aspartic "aminopherase"[5] (31), and that aspartic or glutamic dehydrogenase then deaminated the dicarboxylic amino acids (see also 32). Both aminopherases have been purified and require a coenzyme (121, 122, 124). Kritzmann (36) reported that of the amino acids tested all except glycine are deaminated by this mechanism. Cohen (52), on the other hand, reports that in pigeon breast muscle only aspartic acid and alanine readily donate their $—NH_2$ group to α-ketoglutaric acid to form glutamic acid. Conversely, the corresponding deamination products, oxalacetic and pyruvic acids, most readily accepted $—NH_2$ from glutamic acid. α-Aminobutyric acid and valine were slightly active as $—NH_2$ donors to ketoglutaric acid, but seventeen other amino acids were inactive.

Braunstein and Bychkov (33) accomplished *in vitro* aerobic deamination of

[5] The term "transaminase" has been used by some English and American writers to include both the aminopherase and dehydrogenase referred to by Braunstein.

l-alanine with the cell-free system including glutamic aminopherase and dehydrogenase, cozymase and ketoglutaric acid and an autoxidizable hydrogen carrier such as methylene blue. The aminopherase was inhibited by 0.001 *M* potassium cyanide, quinone, or glutathione.

Cedrangolo and Carandante (44) are reported (44b) to have stated that *l*-amino acid oxidase may be identical with glut*amine* aminopherase and that the indirect oxidation of *l*-amino acids which involves amination of ketoglutaric acid occurs chiefly in the kidney, while the main oxidation is accomplished directly by another enzyme. According to Neber in 1936 (164) proline is broken down by a different mechanism and in the liver is oxidized to glutamic acid. He showed that in the presence of hydrogen peroxide proline is oxidized to glutamic acid.

I. RÔLE OF GLUTAMINE IN UREA FORMATION

From data available at present it would appear that (*1*) some urea is synthesized by operation of the ornithine–arginine pathway suggested by Krebs but that one step—the conversion of citrulline to arginine—can take place in the kidney, and (*2*) urea can be synthesized in the liver by a mechanism which utilizes amide nitrogen and which is independent of the ornithine mechanism.

In the present review Krebs's mechanism will be considered only to such an extent as will facilitate interpretation of experiments dealing with the rôle of glutamine in urea synthesis.

1. Krebs's mechanism

Since Krebs (119) postulated the ornithine–citrulline system for urea formation, there has been considerable doubt as to whether the mechanism, as he outlined it, represents the main or whole pathway of *in vivo* synthesis of urea. Such evidence as there is to support Krebs's theory can be summarized as follows: (*1*) The formation of urea from ammonia by liver slices or perfused liver (263) is catalyzed by ornithine, and the process has been shown by isotopic studies (64, 201) to be accompanied by an uptake of carbon dioxide. (*2*) When isotopic nitrogen was fed to animals as ammonia and amino acid, more was recovered in the amidine group of arginine in liver proteins than in any other form (though much isotopic nitrogen turned up in the "amide nitrogen" liberated as ammonia during acid hydrolysis of the tissue proteins) (222). It is conceivable that free liver arginine and arginine in liver proteins continuously exchange places (222). (*3*) Citrulline, which according to the theory is an intermediary product, has been isolated from liver (78) and has been found in blood (8). (*4*) Arginase, which hydrolyzes arginine to urea and ornithine, is found only in livers of those animals whose main end product of nitrogen catabolism is urea (100, 101).

Gornall and Hunter (78) have observed that although completion of Krebs's chain of reactions can take place in liver slices, the rate of formation of citrulline is considerably more rapid than the rate of conversion of citrulline to arginine. Recent work of Borsook and Dubnoff (22), confirmed by Krebs (116), indicates that the enzyme systems necessary for the conversion of citrulline to arginine are present in the kidney (79). Citrulline, which Krebs suggested (119) and

Gornall and Hunter (78) demonstrated, is formed in the liver, may be converted to arginine partly in the liver and partly in the kidney. It has not yet been determined what percentage of the conversion of citrulline to arginine normally occurs in liver and what fraction occurs in kidney. In kidney tissue suspensions either aspartic or glutamic acid or their amides could serve as the donor of $—NH_2$ to citrulline to form arginine (22). No other amino acid (except proline, hydroxyproline, lysine, and ornithine, which are believed (22) to be converted to dicarboxylic acids) can replace the dicarboxylic acids in the formation of arginine from citrulline in kidney. In the case of the animal tissues tested, the dicarboxylic acids did not appear to serve the same function in the liver.

Whether or not glutamine has a significant rôle to play in the operation of Krebs's chain is still a matter of debate. It may donate some amide nitrogen to citrulline in the liver, thus giving rise to arginine. Probably, however, most of the glutamine used for urea synthesis does not involve Krebs's ornithine mechanism.

2. Glutamine mechanism

It now appears clear that urea can be formed in the liver by a mechanism which appears to be independent of Krebs's system and that glutamine is the immediate source of nitrogen for this synthesis. Leuthardt and Glasson (130, 131, 135, 136) and Krebs (116) have reported that fasting guinea pig liver slices, and to a less extent rat liver slices, formed urea from glutamine more rapidly than from any other substrate besides ammonium chloride. Asparagine to a lesser extent (and to a still smaller extent succinamide) can replace glutamine as a substrate in the synthesis of urea by this extract. Leuthardt and Glasson report that vitamin B_1, its phosphate ester (136), and bicarbonate (135) catalyze urea synthesis in vitamin B_1 deficient rats.

We do not know as yet what fraction of urea synthesized *in vivo* is normally derived from glutamine and what portion is formed through operation of Krebs's cycle. Nor can we do little more than surmise, at present, what factors influence the relative amount of urea formed by either of these mechanisms.

3. Consideration of energy relationships in urea synthesis

As both Leuthardt (130) and Borsook (23, 24, 25) have pointed out, the process of urea formation from carbon dioxide and ammonia includes some reactions which can supply free energy to the extent of 14,000 cal. per mole of urea. Pyruvate, lactate (119), and oxalacetate (135) catalyze the reaction in slices. Leuthardt and Glasson state (134) that the presence of some autoxidizable substance, such as these substances or glucose or citric, maleic, or fumaric (not succinic) acid, is required for urea formation from ammonium chloride, but that this is not the case when glutamine, asparagine, or succinamide is the source of the nitrogen. That is, compared with the energy required for urea formation from ammonium chloride or ammonium glutamate, the energy change in the formation of urea from amide nitrogen is believed to be smaller. Nevertheless, the formation of urea from both glutamine and ammonia is prevented by cyanide

and other materials which poison oxidative, energy-giving systems. Leuthardt (130) states that the free-energy change involved in the formation of urea from glutamine and carbonic acid is less than half that involved in its formation from ammonium and bicarbonate ions. Although his calculations were based on an assumption which he would probably now change (131)—*viz.*, that pyrrolidonecarboxylic rather than glutamic acid is formed—his argument that the energy relations are such as to favor urea formation from glutamine may prove to be correct once the necessary thermodynamic data have been obtained. Leuthardt found that in the absence of glucose, 40 to 60 per cent of the amide nitrogen of glutamine was converted to urea nitrogen and he believes that, because ammonium ion does not accumulate in appreciable quantities in the liver slices, hydrolysis of glutamine to glutamic acid is not the first step in this reaction. Asparagine and the amide of succinic acid donate amide nitrogen to urea formation, but glutamic acid is only slightly active; hence it appears that the $-CONH_2$, and not the α-NH_2, nitrogen of glutamine is most easily converted to urea nitrogen. While addition of glutamic acid to liver slices taken from starved animals increased urea formation from ammonia (though not in the presence of lactate), Leuthardt believed that this was due more to the oxidation of glutamic acid as a source of energy for urea formation than to the conversion of glutamic acid nitrogen into urea nitrogen.

4. An evaluation of criticisms of Krebs's theory

A number of observations once levelled as criticisms of Krebs's theory of urea formation might better be considered as evidence indicating the existence of an alternative and independent pathway of urea synthesis. These are considered below because they offer further proof of the participation of glutamine in urea synthesis. They indicate also that the capacity for urea formation of the system utilizing glutamine is probably at least equal to that of the system postulated by Krebs.

Bach (11, 13) added ornithine to the substrate of liver slices in concentrations (600 mg. per 100 cc.) that completely inhibited and possibly reversed the enzymatic hydrolysis of arginine to ornithine and urea, and noted that after a preliminary lag, urea formation proceeded. Urea synthesis from ammonium lactate was as rapid when sufficient ornithine was present to inactivate completely the liver arginase, as when but little ornithine was present. These observations, he concluded, meant that urea could be formed by a pathway other than through arginine. He failed to prove, however, that the concentration of arginine in his system did not become so high as to overcome part of the competitive inhibition produced by ornithine (9, 11, 99, 267, 292) nor did he indicate whether or not, in the meantime, the concentration of ornithine had been decreased (116) by conversion to citrulline. Later Bach *et al.* (12) showed that the concentration of ornithine decreased during incubation with liver slices.

Trowell (263) raised another objection to Krebs's theory and questioned not only the place of arginine in the cycle but also that of citrulline. He claimed that ammonia and ornithine often yielded more urea than equivalent amounts

of arginine (especially when the concentration of ornithine was so high as to inhibit arginase) and that ammonia and citrulline yielded less urea than either. This is consistent with the statement of Gornall and Hunter (79) that the speed of conversion of citrulline to arginine in liver is considerably less than that of the conversion of ornithine to citrulline. Since liver slices contain enzymes capable of synthesizing glutamine from ammonia and glutamic acid (112), the ammonia present might form urea through the glutamine cycle. Further, the catalytic effect of ornithine observed by Trowell (263) was much more prolonged than that of citrulline. He suggested that the rôle of liver arginase was to provide free ornithine and that the main path of urea formation from ammonia is not through arginine.

Furthermore, Bach (11) and Borsook and Dubnoff (23, page 195) noted that when large concentrations of citrulline were present, the amount of nitrogen in the urea formed was equal to the amount of ammonia nitrogen used, whereas from Krebs's theory one would expect only half the urea nitrogen to come from the ammonia. This would suggest that in this case urea formation took place largely through a path such as the glutamine mechanism rather than the Krebs's cycle.

Since urea is formed rapidly from ammonia when the concentration of ornithine is so high that arginase hydrolysis of arginine is almost completely inhibited (13), and since neither Borsook and Dubnoff (22) nor Krebs (116) was able to demonstrate conversion of citrulline to arginine in liver (rat and guinea pig), it seems doubtful if more than a part of the urea formed goes through citrulline to arginine. Though Leuthardt (133) and Bach (11) suggested that glutamine played a rôle in urea formation as a nitrogen and carbon dioxide carrier, Borsook (23) has been unable to observe evidence of transport of the amide group of glutamine to ornithine. The structural similarity between ornithine and glutamine should not be overlooked, and it is to be remembered that mechanisms for the conversion of ornithine to glutamic acid and glutamine are known to be present in the kidney and liver (22, 115, 206).

The fact that glutamine is used in urea synthesis in animals reminds one of the analogy made by Boussingault (26): namely, that the detoxification of ammonia in plants by the formation of amides is the functional equivalent of urea formation in animals. It is conceivable that in animals the mechanism for ammonia detoxification by formation of amides is the same as in plants, but that in animals the process goes a step further to urea or purine formation. Björksten's evidence (18), which Chibnall considers worthless (45, page 13), that protein synthesis in wheat seedlings can occur at the expense of urea, suggests that in plants there may be an enzymatic mechanism which involves the same conversion of amide nitrogen to urea, except that in these experiments the process was observed to proceed in a direction reverse to that which we believe to be characteristic of animal metabolism. That is, in Björksten's experiments urea nitrogen appeared to be converted into a form which was useful for protein synthesis, whereas, in some animals, nitrogen in useful forms is catabolized to urea.

The observation of Bollman, Mann, and Magath (20) that hepatectomized

animals are unable to synthesize urea is not inconsistent with the idea that one step of Krebs's cycle, *viz.*, formation of arginine from citrulline, may normally occur in the kidney as well as the liver.

Formation of arginine in the kidneys could not continue unless the liver were present to replenish the supply of citrulline to the kidney.

The observation of Mann *et al.* that the concentration of blood urea rises in nephrectomized dogs is again not inconsistent with the view that the kidney may normally play an appreciable rôle in urea synthesis. In nephrectomized dogs urea may be synthesized by any or all of three mechanisms: (*1*) by hydrolysis of arginine synthesized from citrulline in the liver; (*2*) by hydrolysis of arginine liberated from proteins (either exogenous or endogenous); and (*3*) by synthesis from glutamine. The conclusion that the liver is essential for urea synthesis has not been altered by more recent advances in our knowledge of the metabolic processes involved.

Reid's observation (198) that, during the first 24 hr. after bilateral nephrectomy, rats synthesize only 66–75 per cent as much urea as control animals in which both kidneys are intact was interpreted as indicating the rôle which the kidney played in deamination of amino acids. His experiment might indicate equally well a rôle played by the kidney in synthesis of arginine from citrulline. Unfortunately, this experiment gives no clue as to the ratio of the amounts of urea formed by the two (ornithine and glutamine) cycles either under these conditions or under normal conditions. Further, if the effectiveness of one of these cycles is reduced by absence of the kidneys, it is probable that the alternative cycle will be stimulated to greater activity on this account. It is more than possible that the elevated level of blood urea in the nephrectomized animal partially inhibited urea synthesis by the glutamine cycle. It would be interesting to have for comparison the rate of urea synthesis in animals in which the ureters were tied off so that the level of blood urea would rise while the kidney tissue remained *in situ* to perform deamination and/or arginine synthesis from citrulline.

The following observation of Shiple and Sherwin (240) strongly suggests that glutamine plays a most significant rôle in urea synthesis. Feeding of phenylacetic acid (α-toluic acid) to men in nitrogen equilibrium did not increase either protein catabolism or total urinary nitrogen, but decreased urine urea to 15 per cent of its usual level. The other 85 per cent of initial urea nitrogen excretion appeared as N-α-toluylglutamine ("glutamine phenylacetic acid") (240), $C_6H_5CH_2$-$CONHCH(COOH)CH_2CH_2CONH_2$ (94, 259). While this does not prove that the nitrogen which goes to form urea passes through glutamine, it does indicate that the nitrogen, whatever its source, can be tied up in glutamine, and since urinary nitrogen was not increased by the feeding of phenylacetic acid, it suggests that both nitrogens of glutamine are ordinarily available for urea formation. Because normally only a small amount of glutamine is excreted in the urine, the large amount of glutamine which Shiple and Sherwin found to be coupled with phenylacetic acid appears to have been called forth as a response to the presence of phenylacetic acid and in a manner analogous to the way in which

glycine is made available for coupling when large doses of benzoic acid are given. Nevertheless, the fact that Shiple and Sherwin's subjects were in nitrogen equilibrium and that their total urinary nitrogen output did not increase in response to large doses of phenylacetic acid makes it appear significant that the large drop in urea nitrogen excretion was equalled by the nitrogen in the coupled glutamine excreted. This observation not only suggests a rôle for glutamine in urea formation but also indicates a rôle which glutamine plays in detoxification.

J. RÔLE OF GLUTAMINE IN DETOXIFICATION

Many early workers, chiefly Thierfelder and Sherwin (259, 260), have studied this in man. Ambrose, Power, and Sherwin (2) report that in man 95 per cent of ingested phenylacetic acid when given in small doses is excreted as a glutamine derivative (combined through the α-NH_2) and 5 per cent as a glycuronide. Power (186) showed the same to be true in the chimpanzee. Leuthardt (133) has claimed that guinea pig (but not rat) kidney and liver couple glutamine with benzoic to give hippuric acid[6] (132, 134, 135), or with phenylacetic acid, but glutamic acid will not replace glutamine in this process. If this is true, Young's suggestion (309) that the reaction is limited to man and higher apes is incorrect. Sherwin *et al.* (238) showed that on feeding 15 g. of phenylacetic acid to man half the ingested amount was excreted combined with glutamic acid and more glutamine was found in the urine combined with phenylacetic acid than was present free or combined in food (239). He concluded, therefore, fifteen years before Krebs demonstrated the enzyme glutaminase, that man is capable of synthesizing glutamine, and that glutamine is capable of acting as a detoxifying agent. Species differences, however, are fairly sharp (309). Thus dogs (212), rabbits (213), cats (217), and monkeys (237) combined most of the ingested phenylacetic acid with glycine and not with glutamine, and rat liver or kidney slices failed to use glutamine in detoxification of benzoic or phenylacetic acid; fowls (262) combine the phenylacetic acid with ornithine. The glutamine, glycine, or ornithine derivatives formed by acetylation of the α-amino groups with phenylacetic acid when fed to man or any animal are excreted unchanged (241).

K. RÔLE OF GLUTAMINE IN THE KIDNEY

1. Glutamine as precursor of urinary ammonia

The early work on this subject has been reviewed by Schneller (221). Polonovski, Boulanger, and Bizard (184, 185) and Pitts (180) have concluded that urea is not a precursor of urinary ammonia. The writer (9) was unable to demonstrate the presence of urease in dog kidney. For urinary ammonia to be derived from urea through action of urease, dog kidney would need to contain more than two hundred times the concentration of urease which could escape detection by the procedure employed.

[6] Leuthardt believes that benzoylglutamine (134) is probably the first product and that this is degraded to hippuric acid. Results of the experiments designed to elucidate the mechanism of this formation of hippuric acid are not conclusive.

Experiments by Van Slyke, Phillips, Hamilton, Archibald, Futcher, and Hiller (268), on dogs in which ammonia excretion was accelerated by administration of hydrochloric acid, yielded the following results: (*a*) The conclusion of Nash and Benedict that the ammonia concentration is greater in the renal venous blood than in arterial blood was confirmed; hence the ammonia excreted in the urine is not derived from preformed blood ammonia, but must be formed in the kidneys from some other nitrogenous material. (*b*) All the urea removed from the blood by the kidneys was excreted unchanged in the urine; hence urea could not serve as a significant source of the urinary ammonia. (*c*) The total amount of α-amino acid nitrogen extracted by the kidneys from the blood plasma was calculated from analyses of arterial and renal venous blood plasma by the ninhydrin carbon dioxide method, together with measurements of renal blood flow, and was found to be either nil or too little to provide ammonia at the rate excreted in the urine. (*d*) The amide nitrogen of glutamine, determined by Hamilton's (86, 87) application of the ninhydrin carbon dioxide method and by the author's (6, 7) enzymatic method, was much less in the plasma of renal venous blood than in the plasma of arterial blood. The difference, multiplied by the rate of plasma flow through the kidneys, indicated that the kidneys removed glutamine amide nitrogen from the blood at a rate sufficient to provide 60 per cent or more of the total ammonia passed by the kidneys into the urine and into the renal venous blood. It therefore appears that glutamine is the main precursor of urinary ammonia in the dog, although a smaller portion may be derived from deamination of α-amino acids.

In studies of the mechanism by which the ammonia is formed in the kidneys, the writer (7) examined the kidneys of dogs for glutaminase, capable of splitting ammonia from the acid amide group of glutamine, and consistently found this enzyme, as had Krebs (112) in the kidneys of other species. Glutaminase from a dog kidney extract was then added to dog plasma and ammonia was produced. That did not mean that glutaminase was necessarily the source of this ammonia. However, these findings indicated that: (*1*) blood contained something which liberated ammonia in the presence of kidney extract, and (*2*) kidney contained an enzyme capable of liberating ammonia from something present in plasma as well as from added glutamine. If seemed likely that glutamine was the substrate present in plasma. After an exhaustive study of the specificity of the glutaminase method it was concluded (6, 7, 268) that the difference in concentration of glutamine amide nitrogen in the arterial and renal venous plasma was sufficient to account for 60 per cent or more of the ammonia in urine of dogs in acidosis.

Whether or not α-amino acids, as suggested by Krebs (110), or amides of blood proteins (19), or glutamine in the red cells is the source of the remaining 40 per cent of urinary ammonia remains undetermined. There may be species differences in the proportion of urinary ammonia that is derived from glutamine. That the proportion of urinary ammonia derived from glutamine may be less in man than in the dog is suggested by the observation that normal human kidneys have a much lower concentration of glutaminase than dog kidneys (6, 9).

Wassermeyer (296) suggested erythrocyte adenylic acid as a source of urinary

ammonia. However, spectrophotometric studies of dialysates of renal venous and arterial *plasma* of dogs in acidosis (author's observations (9, 268)) failed to indicate either deamination or removal from plasma by the kidney of appreciable amounts of adenosine derivatives. The peak of the characteristic absorption curve of adenosine and of its phosphate derivatives is at a wave length of 260 mμ. The absorption of light of this wave length by the above-mentioned dialysates was found to be equivalent to an adenosine + adenosine phosphate concentration in plasma of 4×10^{-5} *M*. This is equivalent to a concentration of 2.4 mg. of adenylic acid per 100 cc. plasma. On deamination this would yield 0.1 mg. of ammonia nitrogen per 100 cc. of plasma (7). However, other constituents of plasma dialysates absorb light of this wave length; therefore the plasma concentration of adenylic acid is probably much less than is indicated by this maximal figure. For the urinary ammonia to be derived entirely from plasma adenylic acid, in the experiments quoted on acidotic dogs (268), more than twice this maximal amount of adenosine or adenylic acid would need to be present in the plasma of the renal artery and all would have to be utilized by the kidney, whereas, actually, less than 10 per cent was extracted from the plasma or deaminated as the blood passed through the kidney. It was concluded, therefore, that less than 5 per cent of the urinary ammonia could arise from deamination of plasma adenosine or its derivatives.

The value of 17.8 mg. of adenine nucleotide per 100 cc. given by Buell and Perkins (41) was for human whole blood; the nucleotide is practically all in the cells.

Whether or not adenosine and its derivatives serve as an intermediary carrier of nitrogen from glutamine to urinary ammonia or whether the relatively large reserve of adenosine in the red blood cells is used directly to form part of the urinary ammonia is still undetermined, but glutamine appears to be the plasma precursor of most of the urinary ammonia. Kleinzeller (108) has so far been unsuccessful in causing enzymatic synthesis of adenosine di- or tri-phosphate from inosine di- or tri-phosphate in the presence of adenosine triphosphate deaminase, glutamic acid, glutamine, asparagine, or ornithine. However, he assumes that the synthesis occurs *in vivo*.

2. Presence of glutaminase in human kidney

Examination of glutaminase and purine nucleoside deaminase of kidneys from fifty-two different humans showed (7, 9) that glutaminase is present also in the human. It was not found in the kidneys of cases which had had severe kidney involvement, such as chronic hemorrhagic nephritis or arteriosclerotic changes. These cases had, however, considerable purine nucleoside deaminase activity. Since it is known that the capacity to form urinary ammonia is largely lost early in kidney disease[7], the persistence of the activity of the nucleoside deaminase and the loss of glutaminase points to glutamine being the ultimate

[7] In diabetic coma, however, the urea clearance may be temporarily reduced to 5 per cent of normal, the plasma urea may rise to 150 mg. per 100 cc., and the urinary ammonia production remains unimpaired, as shown by McCance and Lawrence (150).

source of urinary ammonia rather than nucleosides or precursors thereof. While the author has had no difficulty in demonstrating the presence of glutaminase in human kidney, it is usually present in concentrations 1/10 to 1/100 those found in dog kidney (7). However, it has been found in kidneys of premature infants and of patients seventy-seven years old. Oddly enough, ten of the eighteen kidneys showing unusually high glutaminase activity have come from patients who had new growths of lung, prostate, stomach, intestine, or brain, usually without visible metastatic involvement of the kidney. The increased concentration of glutaminase in these kidneys may have been a response to increased breakdown of tissue rather than a specific response of the host to a new growth.

The fact that Marples and Lippard (145), McCance and Widdowson (151), and Branning (30) report that human premature infants are peculiarly susceptible to acidosis, indeed are constantly on the threshold of acidosis, and that the author found that kidneys of premature infants contained less glutaminase per gram than those of full-term infants, suggests that the relative lack of kidney glutaminase to provide ammonia may contribute to their acidosis. Inability of prematures to form adequate ammonia to prevent loss of fixed base in the urine may, however, not be the whole cause of their acidosis (30, 145).

3. Glutamine and citric acid metabolism

Hunter and Leloir (102) have demonstrated that citric acid formation from acetoacetic acid in the presence of oxaloacetate by kidney cortex is increased several-fold by the addition of glutamate or α-ketoglutarate. Glutamine as a precursor and reservoir of these two catalysts can be considered, in this sense, as playing a rôle in the formation of citric acid. From the work of Hamilton (87) it would appear likely that a large part of the cofactor which Hunter and Leloir found in muscle extract was free glutamine. This glutamine would be moderately stable under the heating conditions employed and would be hydrolyzed to glutamic acid by the glutaminase in the dog kidney (7) preparation of citrogenase.

L. RÔLE OF GLUTAMINE IN MISCELLANEOUS SYSTEMS

1. Relation of glutamine to reamination of nucleotides or nucleosides

There may prove to be a close tie-up between the reversible glutaminase system and nucleotide or purine nucleoside deaminase systems. Evidence at hand does not warrant a detailed discussion of this point, but suffice it to say in suggestion that (*1*) cozymase has been shown to be a necessary factor in the transamination of glutamic acid *in vitro*[8], and (*2*) Bruhl (39) observed that in convulsive states the concentration of ammonia in brain tissue increases as that of adenylic acid decreases. Riebeling (200) states that 75 per cent of the am-

[8] Glutamic acid dehydrogenase of higher plants has been said to act only in presence of coenzyme I, while that of lower plants, such as yeast or *B. coli*, acts only in the presence of coenzyme II, and that of animal tissues acts in the presence of either coenzyme I or coenzyme II (63).

monia which brain tissue can liberate comes from adenylic acid, and Weil-Malherbe (300) drew attention to the unique rôle played by glutamic acid in brain metabolism. The work of Sapirstein and the clinical work of Price, Waelsch, and Putnam would seem to favor the conclusion that ammonia formation and detoxification normally go hand in hand. That reamination of inosinic acid takes place as a result of action of enzyme systems which utilize the amide of glutamine is an attractive hypothesis, but so far the process has not been demonstrated (113). It is of interest in this connection that striated muscle (especially heart and diaphragm) is rich in both glutamine and adenosine or its products.

TABLE 1

Distribution of glutamine in animal tissues

(Milligrams of glutamine amide nitrogen per 100 g. of tissue (wet weight))

TISSUE	NINHYDRIN CARBON DIOXIDE METHOD*	ACID HYDROLYSIS METHOD†						
	Dog	Dog	Cat	Rabbit	Pigeon	Horse	Marmot	Crab
Heart	21.6	20.3	19.0	10.5	6.9	21.2	23.2	
Brain	6.1	11.0	11.0	8.5	10.0			
Liver	4.3	8.5	8.0	7.0	5.0			
Skeletal muscle	11.7	10.8	8.3	4.9				9.4
Kidney	1.1	4.5	4.3	2.5				
Spleen	5.9							
Uterus	2.3							
Lung	1.9							
Stomach	3.1							
Small intestine	5.3							
Large intestine	4.8							

* Figures reported by Hamilton (87).

† Figures reported by Ferdman *et al.* (69).

2. Presence of free glutamine in animal tissues

Ferdman *et al.* (69) studied the glutamine amide nitrogen in several organs. Because his results are not elsewhere available in English, they are reproduced in table 1. The concentration of glutamine in cats and marmots decreased in starvation and during hibernation. Hamilton (87), using the more specific ninhydrin carbon dioxide method, has obtained glutamine values for dog tissues which confirm in general those given by Ferdman. In view of the low concentration of glutamine in dog kidney it is of interest to note that Krebs (113) found that the kidneys of the dog, pig, cat, and pigeon (unlike those of the guinea pig, rabbit, sheep, and rat) do not synthesize glutamine. According to Krebs (113), certain herbivores (such as rabbits and guinea pigs, the kidneys of which synthesize glutamine rapidly) store considerable glutamine in their kidneys. Ferdman's single value for rabbit kidney does not seem to agree with Krebs's finding.

Glutamine has been isolated from three different tissues and animal species by three laboratories. McIlwain *et al.* (153) in 1939 isolated it from horse meat, following a preliminary precipitation with phosphotungstic acid which separated glutamine from 97.6 per cent of the nitrogenous impurities present in meat at the expense of a 60 per cent loss of glutamine. Örström *et al.* (170) in the same year reported the isolation of glutamine synthesized in pigeon, fowl, and duck livers, employing precipitation by mercuric nitrate and obtaining a 60 per cent yield of glutamine. Frenkel (69) isolated glutamine from horse brain after a precipitation with mercuric and silver ions by a procedure which gave a 30 per cent yield.

3. Effect of glutamine on carbohydrate metabolism

A relation of glutamine and its nitrogen metabolism to carbohydrate metabolism in animals is indicated by the observation of Örström *et al.* (170) that pigeon, fowl, and duck livers form glutamine from ammonium pyruvate at the rate of 30 mg. per hour per gram of dry tissue. It is probable that α-ketoglutarate is an intermediate when this process takes place in the kidneys of guinea pigs, since glutamine formation here is more rapid from ketoglutarate than from pyruvate. However, in liver slices of pigeon, fowl, and duck, glutamine synthesis was found to be more rapid with pyruvate than with ketoglutarate. Amide formation in rat liver was slower and was not accelerated by the addition of ammonium pyruvate. In the cases of mammalian brain and retina, and guinea pig and rabbit kidney, glutamine synthesis from ammonium glutamate was five to ten times as rapid as from ammonium pyruvate.

Further indication that glutamine is involved in carbohydrate metabolism is to be found in the observation that glutamine and glutamic acid show striking effects on carbohydrate oxidation and on lactic acid production in those tissues which contain glutaminase (298). In this connection Smythe's observation (245) that ammonia and amides of dicarboxylic acids increase the rate of fermentation by yeast should be noted. Tatum (256) noted that glutamine was necessary for the fermentation of starch by butyric acid bacteria. Harris (90) observed a marked drop in plasma and spinal fluid glutamine level following administration of insulin.

4. Effect of glutamine on purine metabolism

Örström, Örström, and Krebs (169) state that glutamine and oxalacetate stimulate the formation of hypoxanthine in pigeon liver and the formation of uric acid in fowl, duck, rat, and guinea pig livers which contain xanthine oxidase.

M. PHYSIOLOGICAL RÔLES OF GLUTAMINE IN MAMMALS

The finding that in acidotic dogs glutamine is the source of a large part of the urinary ammonia (268) provides the first demonstrated example of a physiological function for the glutamine–glutaminase system in the animal kingdom.

The earlier finding of Shiple, Sherwin, *et al.* (page 194) that phenylacetic acid is excreted combined with glutamine at the expense of urinary urea showed that glutamine was capable of acting as a detoxifying agent in certain species,

but the identity of the naturally occurring metabolites detoxified by combination with glutamine remains unknown.[9] Indeed, by stopping the normal conversion of glutamine to urea they may have done more to provide circumstantial evidence of a mechanism of urea formation than could be appreciated at the time. Nevertheless, the statement of Örström, Örström, Krebs, and Eggleston (170) is almost as true today as when it was written: "The significance of the synthesis of glutamine remains obscure as long as the rôle of glutamine in tissue metabolism is unknown. (There is) . . . no doubt that glutamine is a factor of general importance in cell metabolism but the nature of its function is not yet clear."

However, it seems legitimate to speculate that glutamine performs some of the functions in animals that it has been shown to perform in plants, such as neutral transport and storage of labile $—NH_2$ for protein synthesis. This synthesis could involve either incorporation of glutamine, as such, into the protein molecule, or of glutamic acid, or preliminary formation of other amino acids by transfer of nitrogen from glutamine to α-keto acids arising from metabolism of carbohydrate, fat, or other amino acids. The observation of Schoenheimer *et al.* (222) that isotopic nitrogen, when fed to animals as ammonia or amino acid, was recovered to a large extent as amide nitrogen of tissue proteins, supports the idea that in animals there is a rapid exchange between amide groups in proteins and ammonia or amino or amide groups of free acids. The earlier observations of Bliss (19) likewise support this view. Nitrogen stored as amide is at a higher energy level than if it were present as ammonia.

In animals (at least in omnivora and carnivora) it would at present appear that glutamine plays a more dominant rôle than asparagine, whereas in most plants asparagine is the more abundant amide.

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[9] Although phenylacetic acid is said to result from the oxidation of phenylbutyric acid by the kidneys of calf, sheep, and dog (245a), it appears unlikely that phenylbutyric acid occurs naturally in any significant concentration.

However, the odor of some specimens of old, dried, human urine cannot be distinguished from that of phenylacetic acid (α-toluic acid). It may be, therefore, that "glutamine phenylacetic acid" (N-α-toluylglutamine) or the corresponding 4-hydroxy compound occurs naturally in human urine and that phenylacetic acid or 4-hydroxyphenylacetic acid is derived from tyramine, which in turn would be derived from tyrosine by decarboxylation (94a). Amine oxidation of tyramine and β-phenylethylamine has been demonstrated by Bernheim and Bernheim (15a), using heart and liver slices. After feeding tyramine to dogs, 25 per cent was recovered from the urine as p-hydroxyphenylacetic acid (65a). A 70 per cent conversion to this acid has been observed on perfusion of cat and rabbit liver with tyramine. Action of another amine oxidase on the related compound mescaline also yields the corresponding acid (15b) in dog and rabbit but not in man (242a). The acid is then excreted in urine. It may be, therefore, that oxidation products of tyrosine provide the naturally occurring substrate for the reaction observed by Shiple and Sherwin *et al.* in humans.

REFERENCES

(1) ADLER, E., DAS, N. B., EULER, H. v., AND HEYMAN, U.: Compt. rend. trav. lab. Carlsberg **22**, 15 (1938).
(2) AMBROSE, A. M., POWER, F. W., AND SHERWIN, C. P.: J. Biol. Chem. **101**, 669 (1933).
(3) ANNAU, E., BANGA, I., GÖZSY, B., HUSZAK, ST., LAKI, K., STRAUB, B., AND SZENT-GYÖRGYI, A.: Z. physiol. Chem. **236**, 1 (1935).
(4) ANSON, M. L.: J. Gen. Physiol. **23**, 239 (1939).
(5) ARCHIBALD, R. M.: J. Biol. Chem. **151**, 141 (1943).
(6) ARCHIBALD, R. M.: J. Biol. Chem. **154**, 643 (1944).
(7) ARCHIBALD, R. M.: J. Biol. Chem. **154**, 657 (1944).
(8) ARCHIBALD, R. M.: J. Biol. Chem. **156**, 121 (1944).
(9) ARCHIBALD, R. M.: Unpublished work.
(10) ARCHIBALD, R. M., AND HAMILTON, P. B.: Unpublished work.
(11) BACH, S. J.: Biochem. J. **33**, 1833 (1939).
(12) BACH, S. J., CROOK, E. M., AND WILLIAMSON, S.: Biochem. J. **38**, 325 (1944).
(13) BACH, S. J., AND WILLIAMSON, S.: Nature **150**, 575 (1942).
(14) BERGMANN, M., AND FRUTON, J. S.: Ann. N. Y. Acad. Sci. **45**, 409 (1944).
(15) BERGMANN, M., ZERVAS, L., AND SALZMANN, L.: Ber. **66B**, 1288 (1933).
(15a) BERNHEIM, F., AND BERNHEIM, M. L. C.: (*a*) J. Biol. Chem. **158**, 425 (1945); (*b*) J. Biol. Chem. **123**, 317 (1938).
(16) BERNHEIMER, A. W., AND PAPPENHEIMER, A. M., JR.: J. Bact. **43**, 481 (1942).
(17) BERNHEIMER, A. W., GILLMAN, W., HOTTLE, G. A., AND PAPPENHEIMER, A. M., JR.: J. Bact. **43**, 495 (1942).
(18) BJÖRKSTÉN, J.: Biochem. Z. **225**, 1 (1930).
(19) BLISS, S.: J. Biol. Chem. **81**, 129 (1929).
(20) BOLLMAN, J. L., MANN, F. C., AND MAGATH, T. B.: Am. J. Physiol. **69**, 371 (1924).
(21) BORODIN, J.: Botan. Ztg. **36**, 801 (1878).
(22) BORSOOK, H., AND DUBNOFF, J. W.: J. Biol. Chem. **141**, 717 (1941).
(23) BORSOOK, H., AND DUBNOFF, J. W.: Ann. Rev. Biochem. **12**, 194 (1943).
(24) BORSOOK, H., AND JEFFREYS, C. E. P.: J. Biol. Chem. **110**, 495 (1935).
(25) BORSOOK, H., AND KEIGHLEY, G.: Proc. Natl. Acad. Sci. U. S. **19**, 626, 720 (1933).
(26) BOUSSINGAULT, J. B.: *Agronomie, chimie agricole et physiologie*, 2nd edition, Vol. 4, p. 245. Mallet-Bachelier, Paris (1868).
(27) BOVARNICK, M. R.: J. Biol. Chem. **148**, 151 (1943)
(28) BOVARNICK, M. R.: J. Biol. Chem. **149**, 301 (1943).
(29) BOVARNICK, M. R.: J. Biol. Chem. **151**, 467 (1943).
(29a) BRAND, E., SAIDEL, L. J., GOLDWATER, W. H., KASSELL, B., AND RYAN, F. J.: J. Am. Chem. Soc. **67**, 1524 (1945).
(30) BRANNING, W. S.: J. Clin. Investigation **21**, 101 (1941).
(31) BRAUNSTEIN, A. E.: Biokhimiya **4**, 667 (1939).
(32) BRAUNSTEIN, A. E., AND ASARKH, R. M.: J. Biol. Chem. **157**, 421 (1945).
(33) BRAUNSTEIN, A. E., AND BYCHKOV, S. M.: Biokhimiya **5**, 261 (1940).
(34) BRAUNSTEIN, A. E., AND KRITZMANN, M. G.: Enzymologia **2**, 129 (1937).
(35) BRAUNSTEIN, A. E., AND KRITZMANN, M. G.: Nature **140**, 503 (1937).
(36) BRAUNSTEIN, A. E., AND KRITZMANN, M. G.: Biokhimiya **3**, 590 (1938).
(37) BRAUNSTEIN, A. E., AND KRITZMANN, M. G.: Biokhimiya **4**, 303 (1939).
(38) BREUSCH, F. L.: Science **97**, 490 (1943).
(39) BRUHL, H. H.: Kinderheilk. **59**, 446 (1938).
(40) BUCHANAN, J. M., SAKAMI, W., GURIN, S., AND WILSON, D. W.: J. Biol. Chem. **157**, 747 (1945).
(41) BUELL, M. V., AND PERKINS, M. E.: J. Biol. Chem. **76**, 95 (1928).
(42) BUTKEWITSCH, F.: Tageblatt des XI Naturforscherkongresses in St. Petersburg, 1902; quoted by Prianischnikow in 1904.

(43) BYCHKOV, S. M.: Biokhimiya **4**, 189 (1939).
(44) CEDRANGOLO, F., AND CARANDANTE, G.: (*a*) Arch. sci. biol. (Italy) **28**, 1 (1942); (*b*) Chem. Abstracts **37**, 5424 (1943).
(45) CHIBNALL, A. C.: *Protein Metabolism in the Plant.* Yale University Press, New Haven, Connecticut (1939).
(46) CHIBNALL, A. C.: Private communication.
(47) CHIBNALL, A. C.: Nature **150**, 127 (1942).
(48) CHIBNALL, A. C.: Proc. Roy. Soc. (London) **131B**, 136 (1942).
(49) CHIBNALL, A. C., AND GROVER, C. E.: Ann. Botany **40**, 491 (1926).
(50) CHIBNALL, A. C., AND WESTALL, R. G.: Biochem. J. **26**, 122 (1932).
(51) COHEN, P. P.: Biochem. J. **33**, 551 (1939).
(52) COHEN, P. P.: Biochem. J. **33**, 1478 (1939).
(53) CONWAY, E. J., AND COOKE, R.: Biochem. J. **33**, 457 (1939).
(54) DAMODARAN, M.: Biochem. J. **26**, 235 (1932).
(55) DAMODARAN, M., JAABACK, G., AND CHIBNALL, A. C.: Biochem. J. **26**, 1704 (1932).
(56) DAMODARAN, M., AND NAIR, K. R.: Biochem. J. **32**, 1064 (1938).
(57) Editorial: J. Am. Med. Assoc. **124**, 577 (1944).
(58) Editorial: J. Am. Med. Assoc. **126**, 1089 (1944).
(59) EDLBACHER, S.: Z. physiol. Chem. **157**, 106 (1926).
(60) EDLBACHER, S., AND KRAUS, J.: Z. physiol. Chem. **191**, 225 (1930).
(61) EFIMENKO, O. M., AND NAUGOLNAYA, T. N.: Biokhimiya **5**, 630 (1940).
(62) ENDRES, G.: Ann. **518**, 109 (1935).
(63) EULER, H. v., ADLER, E., GUNTHER, G., AND DAS, M. B.: Z. physiol. Chem. **254**, 61 (1938).
(64) EVANS, E. A., JR., AND SLOTIN, L.: J. Biol. Chem. **136**, 805 (1940).
(65) EVERETT, M. R.: *Medical Biochemistry*, p. 349. P. B. Hoeber, Inc., New York (1942).
(65a) EWINS, A. J., AND LAIDLAW, P. P.: J. Physiol. **41**, 78 (1910).
(66) FEENEY, R. E., AND STRONG, F. M.: J. Am. Chem. Soc. **64**, 881 (1942).
(67) FERDMAN, D. L.: Uspekhi Sovremennoi Biol. **14**, 191 (1941).
(68) FERDMAN, D. L.: Biokhimiya **17**, 95, 104 (1941).
(69) FERDMAN, D. L., FRENKEL, S. R., AND SILAKOVA, L. I.: Biokhimiya **7**, 43 (1942).
(70) FILDES, P.: Rept. Proc. 3rd Intern. Congr. Microbiol. **1939**, 495.
(71) FILDES, P., AND GLADSTONE, G. P.: Brit. J. Exptl. Path. **20**, 334 (1939).
(72) FISCHER, E., AND KOENIGS, E.: Ber. **37**, 4585 (1904).
(73) FOLCH-PI, J.: Private communication.
(73a) FRAENKEL-CONRAT, H., COOPER, M., AND OLCOTT, H. S.: J. Am. Chem. Soc. **67**, 950 (1945).
(74) GEDDES, W. F., AND HUNTER, A.: J. Biol. Chem. **77**, 197 (1928).
(75) GERARD, R. W.: Ann. Rev. Biochem. **6**, 419 (1937).
(76) GIBERT, E. G.: Proc. Soc. Exptl. Biol. Med. **57**, 363 (1944).
(77) GOLUBTSOVA, A. V., AND KAHN, J. L.: Bull. biol. méd. exptl. (U.R.S.S.) **1**, 130 (1936).
(78) GORNALL, A. G., AND HUNTER, A.: Biochem. J. **35**, 650 (1941).
(79) GORNALL, A. G., AND HUNTER, A.: J. Biol. Chem. **147**, 593 (1943).
(80) GORUP-BESANEZ, v.: Ber. **7**, 1478 (1874).
(81) GORUP-BESANEZ, v.: Ber. **10**, 780 (1877).
(82) GRASSMANN, W., AND MAYR, O.: Z. physiol. Chem. **214**, 185 (1933).
(83) GREEN, D. E.: Biochem. J. **30**, 2095 (1936).
(84) GREEN, J. R.: Phil. Trans. Roy. Soc. (London) **178B**, 39 (1887).
(84a) HAC, L. R., SNELL, E. E., AND WILLIAMS, R. J.: J. Biol. Chem. **159**, 273 (1945).
(85) HAMILTON, P. B.: J. Biol. Chem. **145**, 711 (1942).
(86) HAMILTON, P. B.: J. Biol. Chem. **158**, 375 (1945).
(87) HAMILTON, P. B.: J. Biol. Chem. **158**, 397 (1945).
(87a) HAMILTON, P. B.: Unpublished work.

(88) HARINGTON, C. R., AND MEAD, T. H.: Biochem. J. **30**, 1598 (1936).
(89) HARRIS, M. N.: J. Clin. Investigation **22**, 569 (1943).
(90) HARRIS, M. M., ROTH, R. T., AND HARRIS, R. S.: J. Clin. Investigation **22**, 577 (1943).
(91) HARRIS, M. M.: Science **97**, 382 (1943).
(92) HARRIS, M. M., BLALOCK, J. R., AND HORWITZ, W. A.: Arch. Neurol. Psychiat. **40**, 116 (1938).
(93) HARRIS, J. S., AND KOHN, H. I.: J. Biol. Chem. **141**, 989 (1941).
(94) HAWK, P. B., AND BERGEIM, O.: *Practical Physiological Chemistry*, 9th edition, p. 304. P. Blakiston's Son and Co., Philadelphia (1926).
(94a) HEINSEN, H. A.: Z. physiol. Chem. **245**, 1 (1936).
(95) HERBST, R. M.: Advances in Enzymol. **4**, 75 (1944).
(96) HILL, J. H., AND MANN, E. F.: J. Urol. **47**, 522 (1942).
(97) HLASIWETZ, H., AND HABERMANN, J.: Ann. **169**, 150 (1873).
(98) HUEBER, E. F.: Wien. klin. Wochschr. **52**, 932 (1939).
(99) HUNTER, A., AND DOWNS, C. E.: J. Biol. Chem. **157**, 427 (1945).
(100) HUNTER, A., AND DAUPHINEE, J. A.: Proc. Roy. Soc. (London) **97B**, 227 (1924).
(101) HUNTER, A., AND DAUPHINEE, J. A.: J. Biol. Chem. **85**, 627 (1930).
(102) HUNTER, F. E., AND LELOIR, L. F.: J. Biol. Chem. **159**, 295 (1945).
(103) KAGANOVSKAYA, S. N., AND KAHN, J. L.: Bull. biol. méd. exptl. (U.R.S.S.) **1**, 26 (1936).
(104) KAHN, J. L., AND CHEKOUN, L.: Compt. rend. **201**, 505 (1935).
(105) KARYAGINA, M. K.: Biokhimiya **4**, 168 (1936).
(106) KEILIN, D., AND HARTREE, E. F.: Nature **145**, 934 (1940).
(107) KLEIN, G., AND TAUBÖCK, K.: Biochem. Z. **251**, 10 (1932).
(108) KLEINZELLER, A.: Biochem. J. **36**, 729 (1942).
(109) KNOOP, F., AND MARTIUS, C.: Z. physiol. Chem. **242**, 1 (1936).
(110) KREBS, H. A.: Z. physiol. Chem. **217**, 191 (1933); **218**, 157 (1933).
(111) KREBS, H. A.: Biochem. J. **29**, 1620 (1935).
(112) KREBS, H. A.: Biochem. J. **29**, 1951 (1935).
(113) KREBS, H. A.: Ann. Rev. Biochem. **5**, 247 (1936).
(114) KREBS, H. A.: Lancet **2**, 736 (1937).
(115) KREBS, H. A.: Enzymologia **7**, 53 (1939).
(116) KREBS, H. A.: Biochem. J. **36**, 758 (1942).
(117) KREBS, H. A.: Biochem. J. **36**, IX (1942).
(118) KREBS, H. A., AND COHEN, P. P.: Biochem. J. **33**, 1895 (1939).
(119) KREBS, H. A., AND HENSELEIT, K.: Z. physiol. Chem. **210**, 33 (1932).
(120) KREBS, H. A., AND JOHNSON, W. A.: Enzymologia **4**, 148 (1937).
(121) KRITZMANN, M. G.: Biokhimiya **3**, 603, 691 (1938).
(122) KRITZMANN, M. G.: Compt. rend. acad. sci. (U.R.S.S.) **21**, 42 (1938).
(123) KRITZMANN, M. G.: Biokhimiya **4**, 184 (1939).
(124) KRITZMANN, M. G.: Biokhimiya **4**, 691 (1939).
(125) KUHN, R., AND BIELIG, H. J.: Ber. **73B**, 1080 (1940).
(126) KULTZSCHER, M.: Planta **17**, 699 (1932).
(127) LANDY, M.: Nature **144**, 512 (1939).
(128) LANKFORD, C. E., SCOTT, V., COX, M. F., AND COOKE, W. R.: J. Bact. **45**, 321 (1943).
(129) LANKFORD, C. E., AND SNELL, E. F.: J. Bact. **45**, 410 (1943).
(130) LEUTHARDT, F.: Z. physiol. Chem. **252**, 238 (1938).
(131) LEUTHARDT, F.: Z. physiol. Chem. **265**, 1 (1940).
(132) LEUTHARDT, F.: Z. physiol. Chem. **270**, 113 (1941).
(133) LEUTHARDT, F.: Schweiz. med. Wochschr. **71**, 322 (1941).
(134) LEUTHARDT, F. A., AND GLASSON, B.: Helv. Chim. Acta **25**, 245 (1942).
(135) LEUTHARDT, F., AND GLASSON, B.: Helv. Chim. Acta **25**, 630 (1942).
(136) LEUTHARDT, F., AND GLASSON, B.: Helv. Physiol. Pharm. Acta **1**, 221 (1943).

(137) LEWIS, J. C., AND OLCOTT, H. S.: J. Biol. Chem. **157**, 265 (1945).
(138) LICHTENSTEIN, N.: Enzymologia **7**, 383 (1939).
(139) LICHTENSTEIN, N.: Enzymologia **9**, 185 (1941).
(140) LICHTENSTEIN, N.: J. Am. Chem. Soc. **64**, 1021 (1942).
(141) LUCK, J. M.: Biochem. J. **18**, 679 (1924).
(142) LUNDGREN, H. P.: J. Am. Chem. Soc. **63**, 2855 (1941).
(143) LUNDGREN, H. P., ELAM, D. W., AND O'CONNELL, R. A.: J. Biol. Chem. **149**, 183 (1943).
(144) LYMAN, C. M., KUIKEN, K. A., BLOTTER, L., AND HALE, F.: J. Biol. Chem. **157**, 395 (1945).
(145) MARPLES, E., AND LIPPARD, V. W.: Am. J. Diseases Children **44**, 31 (1932).
(146) MARTIUS, C.: Z. physiol. Chem. **247**, 104 (1937).
(147) MARTIUS, C., AND KNOOP, F.: Z. physiol. Chem. **246**, 1 (1937).
(148) MELVILLE, J.: Biochem. J. **29**, 179 (1935).
(149) MENDEL, L. B., AND VICKERY, H. B.: Carnegie Inst. Wash. Yearbook **34**, 298 (1935).
(150) MCCANCE, R. A., AND LAWRENCE, R. D.: Quart. J. Med. [N. S.] **4**, 53 (1935).
(151) MCCANCE, R. A., AND WIDDOWSON, E. M.: J. Physiol. **95**, 36 (1939).
(152) MCILWAIN, H.: Biochem. J. **33**, 1942 (1939).
(153) MCILWAIN, H., FILDES, P., GLADSTONE, G. P., AND KNIGHT, B. C. J. G.: Biochem. J. **33**, 223 (1939).
(154) MCMEEKIN, T. L.: Federation Proc. **1** (Part 2), 125 (1942).
(155) MILES, A. A., AND PIRIE, N. W.: Brit. J. Exptl. Path. **20**, 109 (1939); Biochem. J. **33**, 1709, 1716 (1939).
(156) MILLER, G. L., AND ANDERSSON, K. J. I.: J. Biol. Chem. **144**, 475 (1942).
(157) MOTHES, K.: Planta **7**, 585 (1929).
(158) MOTHES, K.: Planta **19**, 117 (1933).
(159) MOTHES, K.: Planta **30**, 726 (1940).
(160) NACHMANSOHN, D., JOHN, H. M., AND WAELSCH, H.: J. Biol. Chem. **150**, 485 (1943).
(161) NASSE, O.: Arch. ges. Physiol. (Pflüger's) **6**, 589 (1872).
(162) NASSE, O.: Arch. ges. Physiol. (Pflüger's) **7**, 139 (1873).
(163) NASSE, O.: Arch. ges. Physiol. (Pflüger's) **8**, 381 (1874).
(164) NEBER, M.: Z. physiol. Chem. **240**, 70 (1936).
(165) NEUBERGER, A., AND SANGER, F.: Biochem. J. **36**, 662 (1942).
(166) NEURATH, H., GREENSTEIN, J. P., PUTNAM, F. W., AND ERICKSON, J. O.: Chem. Rev. **34**, 157 (1944).
(167) NIELSEN, N.: Biochem. Z. **307**, 187 (1941).
(168) NIVEN, C. F., JR.: J. Bact. **47**, 343 (1944).
(168a) OLCOTT, H. S.: J. Biol. Chem. **153**, 71 (1944).
(169) ÖRSTRÖM, A., ÖRSTRÖM, M., AND KREBS, H. A.: Biochem. J. **33**, 990 (1939).
(170) ÖRSTRÖM, A., ÖRSTRÖM, M., KREBS, H. A., AND EGGLESTON, L. V.: Biochem. J. **33**, 995 (1939).
(171) OSBORNE, T. B., AND GILBERT, R. D.: Am. J. Physiol. **15**, 333 (1906).
(172) OSBORNE, T. B., LEAVENWORTH, C. S., AND BRAUTLECHT, C. A.: Am. J. Physiol. **23**, 180 (1908).
(173) PALLADIN, W.: Ber. deut. botan. Ges. **6**, 205, 296 (1888).
(174) PEDERSEN, S., AND LEWIS, H. B.: J. Biol. Chem. **154**, 705 (1944).
(175) PELOUZE, J.: Ann. **5**, 283 (1833).
(176) PFEFFER, W.: Jahrb. wiss. Botan. **8**, 429 (1872).
(177) PHILPOT, J. ST. L.: Biochem. J. **33**, 1725 (1939).
(178) PIRIA, R.: Compt. rend. **19**, 575 (1844).
(179) PIRIA, R.: Ann. chim. phys. [3] **22**, 160 (1848).
(180) PITTS, R. F.: J. Clin. Investigation **15**, 571 (1936).
(181) PLIMMER, R. H. A.: J. Chem. Soc. **127**, 2651 (1925).
(182) POLLACK, M. A., AND LINDNER, M.: J. Biol. Chem. **143**, 655 (1942).

(183) POLLACK, M. A., AND LINDNER, M.: J. Biol. Chem. **147**, 183 (1943).
(184) POLONOVSKI, M., BOULANGER, P., AND BIZARD, G.: Compt. rend. **198**, 1815 (1934).
(185) POLONOVSKI, M., BOULANGER, P., AND BIZARD, G.: Bull. soc. chim. biol. **15**, 863 (1933).
(186) POWER, F. W.: Proc. Soc. Exptl. Biol. Med. **33**, 598 (1936).
(187) PRIANISCHNIKOW, D. N.: Landw. Vers-Sta. **62**, 137 (1899).
(188) PRIANISCHNIKOW, D.: Ber. deut. botan. Ges. **22**, 35 (1904).
(189) PRIANISCHNIKOW, D.: Ber. deut. botan. Ges. **40**, 242 (1922).
(190) PRIANISCHNIKOW, D., AND SCHULOW, J.: Ber. deut. botan. Ges. **28**, 253 (1910).
(191) PRICE, J. C., WAELSCH, H., AND PUTNAM, T. J.: J. Am. Med. Assoc. **122**, 1153 (1943).
(192) PUCHER, G. W., AND VICKERY, H. B.: Ind. Eng. Chem., Anal. Ed. **12**, 27 (1940).
(193) PUCHER, G. W., VICKERY, H. B., AND LEAVENWORTH, C. S.: Ind. Eng. Chem., Anal. Ed. **6**, 190 (1934).
(194) PUCHER, G. W., VICKERY, H. B., AND WAKEMAN, A. J.: Ind. Eng. Chem., Anal. Ed. **6**, 140 (1934).
(195) PUCHER, G. W., VICKERY, H. B., AND WAKEMAN, A. J.: Ind. Eng. Chem., Anal. Ed. **6**, 288 (1934).
(196) PUGH, C. E. M., AND QUASTEL, J. H.: Biochem. J. **31**, 286 (1937).
(197) PUTNAM, F. W., AND NEURATH, H.: J. Biol. Chem. **150**, 263 (1943); **159**, 195 (1945).
(198) REID, C.: J. Physiol. **103**, 17P (1944).
(199) RIABINOVSKAIA, A. M.: Compt. rend. acad. sci. (U.R.S.S.) **23**, 958 (1939).
(200) RIEBELING, C.: Klin. Wochschr. **13**, 1422 (1934).
(201) RITTENBERG, D., AND WAELSCH, H.: J. Biol. Chem. **136**, 799 (1940).
(202) RITTHAUSEN, H.: J. prakt. Chem. **99**, 454 (1866).
(203) RITTHAUSEN, H.: J. prakt. Chem. **103**, 233 (1868).
(204) RITTHAUSEN, H.: J. prakt. Chem. **107**, 218 (1869).
(205) RITTHAUSEN, H.: *Die Eiweisskörper*. Max Cohen und Sohn, Bonn (1872).
(206) ROLOFF, M., RATNER, S., AND SCHOENHEIMER, R.: J. Biol. Chem. **136**, 561 (1940).
(207) RUHLAND, W., AND WETZEL, K.: Planta **1**, 558 (1926).
(208) RUHLAND, W., AND WETZEL, K.: Planta **3**, 765 (1927).
(209) RUHLAND, W., AND WETZEL, K.: Planta **7**, 503 (1929).
(210) SACHSSE, R.: Sitzber. Naturforsch.-Ges. Leipzig **3**, 26 (1876).
(211) SACHSSE, R., AND KORMANN, W.: Landw. Vers.-Sta. **17**, 321 (1874).
(212) SALKOWSKI, E., AND SALKOWSKI, H.: Ber. **12**, 653 (1879).
(213) SALKOWSKI, E., AND SALKOWSKI, H.: Z. physiol. Chem. **7**, 161 (1882).
(214) SAPIRSTEIN, M. R.: Proc. Soc. Exptl. Biol. Med. **52**, 334 (1943).
(215) SAPIRSTEIN, M. R., HERMAN, R. C., AND WALLACE, G. B.: Proc. Soc. Exptl. Biol. Med. **35**, 163 (1936).
(216) SAPIRSTEIN, M. R., HERMAN, R. C., AND WALLACE, G. B.: Am. J. Physiol. **119**, 549 (1937).
(217) SCHEMPP, E.: Z. physiol. Chem. **117**, 41 (1921).
(218) SCHLENKER, F. S.: Plant Physiol. **7**, 685 (1932).
(219) SCHLENKER, F. S.: Plant Physiol. **15**, 701 (1940).
(220) SCHMIDT, K. H.: Z. physiol. Chem. **277**, 117 (1943).
(221) SCHNELLER, H.: Ergeb. Physiol. **37**, 492 (1935).
(222) SCHOENHEIMER, R., RATNER, S., AND RITTENBERG, D.: J. Biol. Chem. **127**, 333 (1939).
(223) SCHULZE, E.: Landw. Vers.-Sta. **20**, 117 (1877).
(224) SCHULZE, E.: Landw. Jahrb. **7**, 411 (1878).
(225) SCHULZE, E.: Botan. Ztg. **37**, 209 (1879).
(226) SCHULZE, E.: Landw. Jahrb. **9**, 689 (1880).
(227) SCHULZE, E.: Landw. Jahrb. **17**, 683 (1888).
(228) SCHULZE, E.: Landw. Jahrb. **21**, 105 (1892).
(229) SCHULZE, E.: Z. physiol. Chem. **24**, 18 (1898).
(230) SCHULZE, E.: Landw. Jahrb. **35**, 621 (1906).

(231) SCHULZE, E., AND BARBIERI, J.: Ber. **10**, 199 (1877).
(232) SCHULZE, E., AND BOSSHARD, E.: Landw. Vers.-Sta. **29**, 295 (1883).
(233) SCHULZE, E., AND CASTORO, N.: Z. physiol. Chem. **38**, 199 (1903).
(234) SCHULZE, E., AND UMLAUFT, W.: Landw. Jahrb. **5**, 821 (1876).
(235) SCHWAB, G.: Planta **25**, 579 (1936).
(236) SHEMIN, D., AND RITTENBERG, D.: J. Biol. Chem. **158**, 71 (1945).
(237) SHERWIN, C. P.: J. Biol. Chem. **31**, 307 (1917).
(238) SHERWIN, C. P., WOLF, M., AND WOLF, W.: J. Biol. Chem. **37**, 113 (1919).
(239) SHERWIN, C. P., WOLF, W., AND WOLF, M.: Am. J. Physiol. **51**, 202 (1920).
(240) SHIPLE, G. J., AND SHERWIN, C. P.: J. Am. Chem. Soc. **44**, 618 (1922).
(241) SHIPLE, G. J., AND SHERWIN, C. P.: J. Biol. Chem. **53**, 463 (1922).
(242) SHORE, A., WILSON, H., AND STUECK, G.: J. Biol. Chem. **112**, 407 (1935).
(242a) SLOTTA, K. H., AND MÜLLER, J.: Z. physiol. Chem. **238**, 14 (1936).
(243) SMIRNOW, A. I.: Biochem. Z. **137**, 1 (1923).
(244) SMITH, E. L.: J. Gen. Physiol. **24**, 583 (1941).
(245) SMYTHE, C. V.: Enzymologia **6**, 9 (1939).
(245a) SNAPPER, I., AND GRÜNDBAUM, A.: Chem. Abstracts **29**, 3384 (1935).
(246) SREENIVASAYA, M., AND PIRIE, N. W.: Biochem. J. **32**, 1707 (1938).
(247) STARE, F. J., AND BAUMANN, C. A.: Proc. Roy. Soc. (London) **121B**, 338 (1936).
(248) STEINHARDT, J., AND FUGITT, C. H.: J. Res. Natl. Bur. Standards **29**, 315 (1942).
(249) STIEGER, A.: Z. physiol. Chem. **86**, 245 (1913).
(250) STRONG, F. M., FEENEY, R. E., AND EARLE, A.: Ind. Eng. Chem., Anal. Ed. **13**, 566 (1941).
(251) SULLIVAN, W. K.: Ann. Sci. Nat. (4th series) **9**, 281 (1858).
(252) SUZUKI, U.: Bull. Coll. Agr. Tokyo **2**, 409 (1897).
(253) SUZUKI, U.: Bull. Coll. Agr. Tokyo **4**, 1, 25 (1900).
(254) SYNGE, R. L. M.: Biochem. J. **33**, 671 (1939).
(255) SZENT-GYÖRGYI, A.: Z. physiol. Chem. **244**, 105 (1936).
(256) TATUM, E. L., PETERSON, W. H., AND FRED, E. B.: J. Bact. **29**, 563 (1935).
(257) TAUBÖCK, K., AND WINTERSTEIN, A.: Handb. Pflanzenanalyse **4**, 190 (1933).
(258) THIERFELDER, H.: Z. physiol. Chem. **114**, 192 (1921).
(259) THIERFELDER, H., AND SHERWIN, C. P.: Ber. **47**, 2630 (1914).
(260) THIERFELDER, H., AND SHERWIN, C. P.: Z. physiol. Chem. **94**, 1 (1915).
(261) THIERFELDER, H., AND CRAMM, E. VON: Z. physiol. Chem. **105**, 58 (1919).
(262) TOTANI, G.: Z. physiol. Chem. **68**, 75 (1910).
(263) TROWELL, O. A.: J. Physiol. **100**, 432 (1942).
(264) VALKO, E. I.: Ann. N. Y. Acad. Sci. **46**, (1945).
(265) VAN SLYKE, D. D.: J. Biol. Chem. **9**, 185 (1911); **12**, 275 (1912).
(266) VAN SLYKE, D. D.: J. Biol. Chem. **12**, 295 (1912).
(267) VAN SLYKE, D. D., ARCHIBALD, R. M., AND RIEBEN, W. R.: Unpublished work.
(268) VAN SLYKE, D. D., PHILLIPS, R. A., HAMILTON, P. B., ARCHIBALD, R. M., FUTCHER, P. H., AND HILLER, A.: J. Biol. Chem. **150**, 481 (1943).
(269) VAUQUELIN AND ROUBIQUET, P. J.: Ann. chim. **57**, 88 (1806).
(270) VERKADE, P. E.: Chemistry & Industry **57**, 704 (1938).
(271) VICKERY, H. B.: Carnegie Inst. Wash. Yearbook **36**, 325 (1937).
(272) VICKERY, H. B.: Carnegie Inst. Wash. Yearbook **37**, 334 (1938).
(273) VICKERY, H. B.: Cold Spring Harbor Symposia Quant. Biol. **6**, 67 (1938).
(274) VICKERY, H. B.: Physiol. Rev. **25**, 347 (1945).
(275) VICKERY, H. B., AND PUCHER, G. W.: Biochem. Z. **293**, 427 (1937).
(276) VICKERY, H. B., AND PUCHER, G. W.: J. Biol. Chem. **150**, 197 (1943).
(277) VICKERY, H. B., PUCHER, G. W., AND CLARK, H. E.: Science **80**, 459 (1934).
(278) VICKERY, H. B., PUCHER, G. W., AND CLARK, H. E.: J. Biol. Chem. **109**, 39 (1935).
(279) VICKERY, H. B., PUCHER, G. W., AND CLARK, H. E.: Plant Physiol. **11**, 413 (1936).
(280) VICKERY, H. B., PUCHER, G. W., CLARK, H. E., CHIBNALL, A. C., AND WESTALL, R. G.: Biochem. J. **29**, 2710 (1935).

(281) VICKERY, H. B., PUCHER, G. W., LEAVENWORTH, C. S., AND WAKEMAN, A. J.: J. Biol. Chem. **125,** 527 (1938).
(282) VICKERY, H. B., PUCHER, G. W., WAKEMAN, A. J., AND LEAVENWORTH, C. S.: Conn. Agr. Exp. Sta. (New Haven) Bull. **399,** 757 (1937); **407,** 107 (1938).
(283) VICKERY, H. B., PUCHER, G. W., WAKEMAN, A. J., AND LEAVENWORTH, C. S.: Conn. Agr. Exp. Sta. (New Haven) Bull. **424,** 7 (1939).
(284) VICKERY, H. B., AND PUCHER, G. W.: Conn. Agr. Exp. Sta. (New Haven) Bull. **424,** 107 (1939).
(285) VIRTANEN, A. I., AND LAINE, T.: Nature **136,** 756 (1935).
(286) VIRTANEN, A. I., AND LAINE, T.: Suomen Kemistilehti **9B,** 12 (1936).
(287) VIRTANEN, A. I., AND LAINE, T.: Suomen Kemistilehti **10B,** 6 (1937).
(288) VIRTANEN, A. I., AND LAINE, T.: Suomen Kemistilehti **10B,** 24 (1937).
(289) VIRTANEN, A. I., AND LAINE, T.: Nature **141,** 748 (1938).
(290) VIRTANEN, A. I., AND LAINE, T.: Nature **142,** 165 (1938).
(291) VIRTANEN, A. I., AND TARNANEN, J.: Biochem. Z. **250,** 193 (1932).
(292) VOVCHENKO, G. D.: Arch. sci. biol. (U.S.S.R.) **42,** 89 (1936).
(293) WADA, M.: Biochem. Z. **257,** 1 (1933).
(294) WAELSCH, H., AND NACHMANSOHN, D.: Proc. Soc. Exptl. Biol. Med. **54,** 336 (1943).
(295) WAELSCH, H., AND PRICE, J. C.: Arch. Neurol. Psychiat. **51,** 393 (1944).
(296) WASSERMEYER, H.: Arch. exptl. Path. Pharmakol. **165,** 420 (1932).
(297) WASSILIEFF, N. J.: Landw. Vers-Sta. **55,** 45 (1901).
(298) WEIL-MALHERBE, H.: Chemistry & Industry **54,** 1115 (1935).
(299) WEIL-MALHERBE, H.: Biochem. J. **30,** 665 (1936).
(300) WEIL-MALHERBE, H.: Biochem. J. **31,** 2080 (1937).
(301) WEIL-MALHERBE, H.: Biochem. J. **32,** 2257 (1938).
(302) WEIL-MALHERBE, H., AND KREBS, H. A.: Biochem. J. **29,** 2077 (1935).
(302a) WILSON, H., AND CANNAN, R. K.: J. Biol. Chem. **119,** 309 (1937).
(303) WOOD, J. G., CRUICKSHANK, D. H., AND KUCHEL, R. H.: Australian J. Exptl. Biol. Med. Sci. **21,** 37 (1943).
(304) WOOD, J. G., AND CRUICKSHANK, D. H.: Australian J. Exptl. Biol. Med. Sci. **22,** 111 (1944).
(305) WOOD, J. G., MERCER, F. V., AND PEDLOW, C.: Australian J. Exptl. Biol. Med. Sci. **22,** 37 (1944).
(306) WOOLF, B.: Biochem. J. **23,** 472 (1929).
(307) WORMELL, R. L., AND KAYE, M. A. G.: Nature **153,** 525 (1944).
(308) YEMM, E. W.: Proc. Roy. Soc. (London) **123B,** 243 (1937).
(309) YOUNG, L.: Physiol. Rev. **19,** 323 (1939).