REFERENCES

Abel, J. J. & Geiling, E. M. K. (1925). *J. Pharmacol.* **25**, 423.
Bergmann, M., Fruton, J. S. & Pollok, H. (1939). *J. biol. Chem.* **127**, 643.
Blatherwick, N. R., Bischoff, F., Maxwell, L. C., Berger, J. & Sahyun, M. (1927). *J. biol. Chem.* **72**, 57.
Brand, E. & Kassell, B. (1941–2). *J. gen. Physiol.* **25**, 167.
du Vigneaud, V. (1927). *J. biol. Chem.* **75**, 393.
Fruton, J. S. (1946). *J. biol. Chem.* **166**, 721.
Grassmann, W., Dyckerhoff, H. & Schoenebeck, O. V. (1930). *Hoppe-Seyl. Z.* **186**, 183.
Greenstein, J. P. (1938). *J. biol. Chem.* **125**, 501..
Grob, D. (1945–6). *J. gen. Physiol.* **29**, 219.
Hopkins, F. G. & Morgan, E. J. (1938). *Biochem. J.* **32**, 611.
Kuhn, R. & Desnuelle, P. (1938). *Hoppe-Seyl. Z.* **251**, 14.
Kunitz, M. (1938–9). *J. gen. Physiol.* **22**, 207.
Kunitz, M. & Northrop, J. H. (1934–5). *J. gen. Physiol.* **18**, 433.
Kunitz, M. & Northrop, J. H. (1935–6). *J. gen. Physiol.* **19**, 991.
Mirsky, A. E. & Anson, M. L. (1934–5). *J. gen. Physiol.* **18**, 307.
Neville-Jones, D. & Peters, R. A. (1948). *Biochem. J.* (in the Press).
Northrop, J. H. & Kunitz, M. (1932–3). *J. gen. Physiol.* **16**, 313.
Philpot, J. St L. (1938). *Nature, Lond.*, **141**, 283.
Stern, K. G. & White, A. (1937). *J. biol. Chem.* **117**, 95.
Walker, E. (1925). *Biochem. J.* **19**, 1082.
Webb, E. C. & van Heyningen, R. (1947). *Biochem. J.* **41**, 74.

Quantitative Determination of Glutamine and Glutamic Acid

By H. A. KREBS, *Unit for Research in Cell Metabolism (Medical Research Council), Department of Biochemistry, The University, Sheffield*

(*Received* 23 *October* 1947)

Washed suspensions of *Clostridium welchii*, strain S.R. 12, as shown by Gale (1945, 1947), specifically decarboxylate L-glutamic acid and L-glutamine and are thus a specific reagent for the determination of these two substances. It is shown in this paper that the decarboxylation of glutamine is accompanied by the formation of an equivalent amount of ammonia, and that the glutaminase responsible for this reaction is highly specific. No other compound has so far been found to yield ammonia when incubated with washed suspensions of the organism at pH 4·9. This glutaminase preparation is more convenient to make than Archibald's (1944) glutaminase preparation from kidney and is more specific.

The following method for the quantitative determination of glutamic acid and glutamine rests on these facts, and combines the principles used by Gale (1945) and by Archibald (1944). The sum of the substances is determined according to Gale's procedure, slightly modified, by manometric determination of the carbon dioxide evolved on decarboxylation. The ammonia is then estimated in the solution treated with *Cl. welchii*, and in a sample not so treated. The difference represents the glutamine.

Preliminary experiments indicated that the rate of CO_2 evolution from glutamine is under some conditions considerably slower than the CO_2 evolution from glutamic acid, and a detailed study of the conditions favourable for the quantitative decarboxylation of glutamine was therefore required.

EXPERIMENTAL

Bacteria. The strain S.R. 12 of *Cl. welchii* as recommended by Gale (1945) was used for most experiments. In a few cases, a strain of the same species supplied by the Department of Bacteriology of this University and containing a histidine- and a glutamic-decarboxylase was used. The organisms were grown at 38° in the medium suggested by Gale except that 50 ml. of yeast extract were added to 1 l. medium (prepared by autoclaving baker's yeast with an equal volume of water and filtering). The washed cells from about 1 l. medium were suspended in about 15 ml. of 0·45% (w/v) NaCl. The dry weight of the bacteria in the suspension was determined in a 0·2 ml. sample. The yield from 1 l. of medium was 1·1–1·5 g. of dry bacteria. All data on bacterial weights in this paper refer to dry weight. The suspension was kept in a refrigerator where its activity did not appreciably deteriorate within a month.

Chemical determinations. CO_2 was measured manometrically with air in the gas space of the vessels, NH_3 according to the method of Parnas & Heller (see Parnas, 1934).

Chemicals. The L-glutamine used was prepared from mangel-wurzels. In spite of frequent recrystallizations from ethanol-water and water, its purity was only 93% as judged by amide-N content and CO_2 evolution. Among the impurities were about 1·3% arginine (see Archibald, 1945). The L-glutamic acid preparation was 96% pure. The impurity was probably its optical enantiomorph, as the amino-N content agreed with the theoretical value whilst Gale's method yielded 96%.

Table 1. *Rate of* CO_2 *evolution from glutamine and glutamic acid by* Clostridium welchii *at varying substrate concentrations*

(Each cup contained 2 ml. substrate solution, 1 ml. 0·2 M-acetate buffer (pH 4·9), 0·05 ml. bacterial suspension (≡ 1·1 mg.); 30°.)

Substrate ...	Glutamine				Glutamic acid			
Final substrate concentration (M) ...	0·013	0·0065	0·00325	0·001625	0·013	0·0065	0·0034	0·001625
Total amount of substrate/cup (μl.) ...	448	224	112	56	448	224	112	56
CO_2 (μl.) evolved after: 10 min.	19	12	6·5	1	34	30	17·5	13
20 min.	39	28	14	3	65	55	36	23·5
40 min.	91	58	29·5	7	122	120	65	46

Table 2. *Effect of* pH *on the rate of* CO_2 *evolution from glutamine and glutamic acid by* Clostridium welchii

(Each cup contained 3 ml. 0·1 M-acetate or lactate buffer, 1 ml. 0·01 M-substrate, 0·5 ml. bacterial suspension (11 mg.); 30°.)

Substrate ...	Glutamic acid							Glutamine						
Buffer ...	Acetate					Lactate		Acetate					Lactate	
pH ...	5·0	4·7	4·4	4·1	3·7	3·4	3·0	5·0	4·7	4·4	4·1	3·7	3·4	3·0
CO_2 (μl.) evolved after:														
5 min.	60	64	74	74	75	54	34	26·5	21·5	15	10·5	8·5	2·1	1
10 min.	154	160	168	166	143	115	74	72	57	35	22	18	5	2
15 min.	189	198	203	203	179	170	110	110	109	52	30	25·5	9	3
50 min.	211	211	216	218	208	218	203	190	184	132	82	64·5	26	12

Metabolism of glutamine in Clostridium welchii

Rate of CO_2 *evolution from glutamine.* Data comparing the rates of CO_2 evolution from glutamine, and from glutamic acid under the same conditions, are recorded in Table 1. Differences in the rates are especially marked at low concentrations of the substrates. As will be shown below, these results, obtained on pure solutions of the substrates, can be modified by the presence of other substances.

Effect of pH. Glutamic acid decarboxylase shows only small changes in activity between pH 3·4 and 5·0 (Table 2; see also Gale, 1941). The optimum is fairly broad and extends from about pH 3·7 to 4·4. At pH 3·0 (lactate buffer) the activity is still about 50% of the optimal activity. In contrast, CO_2 evolution from glutamine falls fairly rapidly with pH between 5·0 and 3·0; at pH 3·0, the rate is only 3% of that observed at pH 5·0.

Production of ammonia. Fig. 1 shows that the evolution of CO_2 from glutamine is accompanied by the production of ammonia. During the early stages of the reaction more ammonia than CO_2 is formed, but later the yields of both substances become identical. This indicates that the formation of ammonia precedes decarboxylation, as is borne out by the observation that the CO_2 evolution from glutamine, unlike that from glutamic acid, has a short lag period during which the rate of decarboxylation gradually rises (Table 2, Fig. 1). This is to be expected if the substrate of decarboxylation, glutamic acid, is not present at the start, but is gradually formed under the influence of glutaminase. Further evidence of the primary hydrolysis of the amide group is afforded by inhibitor experiments. Cyanide (3×10^{-3} M), which completely inhibits decarboxylation (Gale, 1945), but has no effect on the evolution of ammonia, separates the action of glutaminase and glutamic decarboxylase, whilst $HgCl_2$ inhibits both reactions (Table 3).

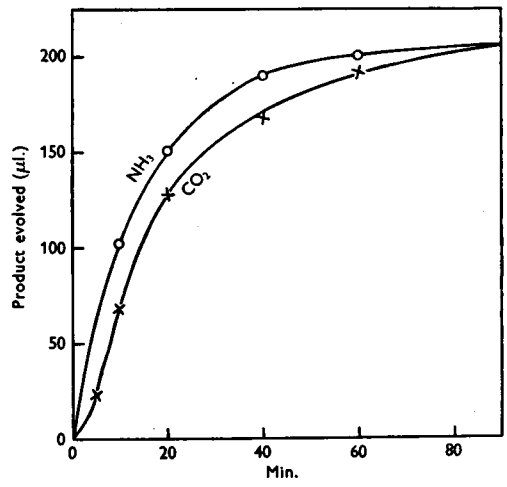


Fig. 1. Formation of NH_3 and CO_2 from glutamine in the presence of *Clostridium welchii*. 3 ml. 0·2 M-acetate buffer, pH 4·9; 1 ml. 0·01 M-glutamine; 0·5 ml. bacterial suspension (≡ 11 mg.); 30°. The reaction was interrupted by addition of 1 ml. 2 N-HCl at the specified time.

Table 3. *Effect of* HCN *and* $HgCl_2$ *on the action of* Clostridium welchii *on glutamine*

(pH 4·5, 1·46 mg. glutamine, 30°.)

	Inhibitor		
	None	3×10^{-3}M-$HgCl_2$	3×10^{-3}M-HCN
CO_2 after 60 min. (μl.)	171	2	3
NH_3 after 60 min. (μl.)	183	~Nil	190

Temperature coefficient of decarboxylation. The rate of decarboxylation of glutamine and of glutamic acid at 40° was about 60 % faster than at 30°. There was no indication that the bacterial cells suffered damage through prolonged incubation at 40°.

Effect of narcotics. Gale (1946) has already noted that ethylurethane does not inhibit bacterial decarboxylases. Other unspecific 'narcotic' inhibitors like octanol or phenylurethane (both in saturated solutions) were found not to affect the decarboxylation of glutamine.

Salts. At pH 4·9 no difference in the rates of decarboxylation was found when the concentration of the acetate buffer was varied between 0·05 and 0·2M. Higher buffer concentrations caused inhibitions, *c.* 40 % by 0·4M-buffer and *c.* 60 % by 0·8M-buffer. NaCl, in presence of 0·2M-acetate buffer, had no appreciable effect as long as its concentration did not exceed 1M (see also Taylor & Gale, 1945).

Effect of detergents. In the course of experiments on inhibitors it was observed that addition of cetyltrimethylammonium bromide ('cetavlon') accelerated the decarboxylation of glutamine. Examples are shown in Table 4. The magnitude of the effect depends, among other factors, on pH. Under the conditions shown in Table 4, the increase of the initial rate was *c.* 2·5-fold at pH 4·9, *c.* 10-fold at pH 4·1 and *c.* 20-fold at pH 3·8. Thus the shape of the pH-activity curve of the decarboxylation of glutamine is changed by the detergent, and the curve obtained with washed cell suspensions is therefore not the true pH-activity curve. Other factors, presumably the permeability of the cells, affect the rate of interaction between enzyme and substrate.

Table 5, giving the effects of varying concentrations of cetavlon, shows that 0·48 mg. detergent can appreciably increase the activity of 6 mg. bacteria and that the maximum effect is produced by about 1 mg. detergent.

The decarboxylation of glutamic acid was, under the same conditions, also acclerated by cetavlon, but owing to the relatively high rate of the decarboxylation of glutamic acid without detergent the percentage increase produced by cetavlon was smaller than in the case of glutamine. The effect of adding the detergent to the bacteria before the substrate, e.g. incubation with cetavlon for 20 min. before the addition of the substrate, was to produce inactivation (see also Baker, Harrison & Miller, 1941).

An anionic detergent, sodium dodecyl sulphate, completely inhibited the decarboxylation of glutamine under the conditions stated in Table 4, when 0·05 % detergent was added to the substrate solution.

In experiments with another strain of *Cl. welchii*, which decarboxylated histidine in addition to glutamic acid and glutamine, cetavlon likewise accelerated the glutamic decarboxylase but inhibited the histidine decarboxylase, whilst sodium dodecyl sulphate inhibited the decarboxylation of glutamic

Table 4. *Effect of cetavlon on the decarboxylation of glutamine at different* pH

(1·46 mg. glutamine in 3 ml. 0·2M-buffer; 40°; 0·1 ml. bacterial suspension (3 mg. bacteria); cetavlon added to substrate solution.)

Buffer		Acetate	Acetate	Lactate	Acetate	Acetate	Lactate
pH		4·9	4·1	3·8	4·9	4·1	3·8
Cetavlon (%)		0	0	0	0·13	0·13	0·13
CO_2 (μl.) evolved after:	5 min.	26	6	2	68	55	39
	10 min.	52	11	4	137	111	86
	15 min.	77	13	6	174	172	125
	20 min.	96	16	7	187	192	161
	30 min.	126	21	8	195	204	186

Table 5. *Effect of varying cetavlon concentrations on the decarboxylation of glutamine*

(1·46 mg. glutamine in 4 ml. 0·1M-acetate buffer, pH 4·1; 0·2 ml. bacterial suspension (6 mg.); cetavlon added to substrate solution.)

Cetavlon concentration (final) (%)		0	0·012	0·024	0·048	0·096	2·0
Total amount of cetavlon added (mg.)		0	0·48	0·96	1·92	3·84	80
CO_2 (μl.) evolved after:	5 min.	2	17	89	78	87	85
	10 min.	8	44	176	182	181	179
	15 min.	13	65	201	205	202	205

acid and glutamine and had no effect on the histidine decarboxylase. These experiments will be reported in full elsewhere.

Effect of serum. Blood serum (Table 6) also accelerated the decarboxylation of glutamine by *Cl. welchii*, the effect depending on pH. At pH 4·1, larger (*c.* 10-fold) amounts of serum were required than of cetavlon (on a w/w basis) and the maximum effects of serum were smaller. Gelatin (0·6 %) had no effect; 'Bacto' peptone (0·6 %) caused a small increase (about 30 %) of the initial rate. When both cetavlon and serum were added, the effects observed were somewhat smaller than those of cetavlon alone (Table 6). At pH 4·9, the maximum effects produced by cetavlon and by serum were about equal, and smaller quantities, about one half, were required for the maximum acceleration. Aqueous extracts of animal tissues had effects similar to those of serum.

Cetyltrimethylammonium bromide. Commercial cetavlon in water (2 %) served as a stock solution. According to the makers, cetavlon contains about 70 % of cetyltrimethylammonium bromide. A purer preparation (about 97 %), kindly supplied by Mr S. Ellingworth of Imperial Chemical (Pharmaceuticals) Ltd., did not appreciably differ from commercial cetavlon in its action on the decarboxylation of glutamine.

Manometric arrangement. Conical flasks provided with a side arm are used. The solution to be examined is placed in the main compartment. If very acid or very alkaline it is first adjusted to *c.* pH 4·9 by the addition of HCl or NaOH, and finally by 3 M-acetate buffer, usually $\frac{1}{15}$ vol. The total volume of the fluid in the main compartment should not exceed 4 ml. The side arm contains 0·5 ml. of bacterial suspension. Two types of control are required, one to measure the gas exchange of the bacteria alone, the other to measure the gas exchange of the solution to be examined. The first contains the bacterial suspension in the side arm and 4 ml. 0·2 M-acetate buffer in the main compartment. The second contains a sample of the unknown solution, treated in the same way as in the test (no bacterial suspension in the side arm). The manometers are shaken at 40°; the contents of the side arm are mixed when equilibrium is established, and readings are taken until evolution of CO_2 ceases. Glutamic acid usually reacts completely within 15 min., glutamine within 20–30 min.

Table 6. *Effect of serum on the decarboxylation of glutamine at* pH 4·1

(1·46 mg. glutamine in 4 ml. total volume; 0·1 M-acetate buffer, pH 4·1; 40°; 0·1 ml. bacterial suspension (3 mg.). The serum contained 7·9 % protein.)

Sheep serum added (ml.)	...	0	0·2	0·4	0·8	1·6	0	0·8
Cetavlon added (mg.)	...	—	—	—	—	—	1·6	1·6
CO_2 (μl.) evolved after: 5 min.		3	11	23	37	52	66	52
10 min.		6	20	50	86	117	149	133
15 min.		7	32	79	120	166	186	176

Procedure for the determination of glutamine and glutamic acid

Data presented in the previous section indicate that Gale's conditions (pH 4·5; 30°), though satisfactory for the determination of glutamic acid, are not optimal for the decomposition of glutamine. A less acid medium of pH 4·9 and a temperature of 40° is therefore proposed. A pH higher than 5·0 would be disadvantageous with regard to the manometric determination of CO_2, because at such a pH the medium would retain CO_2. Furthermore, addition of cetavlon is recommended if the material to be examined does not contain some protein. Extracts of animal tissues usually accelerate the decarboxylation of glutamine sufficiently to make the addition of cetavlon unnecessary.

Reagents

Bacterial suspension. The stock suspension of *Cl. welchii* is diluted with 0·2 M-acetate buffer (pH 4·9) to contain *c.* 25 mg. dry bacteria/ml. If kept in a refrigerator the suspension may be used for about a month.

Stock acetate buffer. 160 ml. 3 M-Na acetate are mixed with 100 ml. 3 M-acetic acid. This buffer (pH 4·9; 3 M) is diluted to a final concentration not exceeding 0·2 M, since higher concentrations inhibit the enzyme.

The control containing bacteria alone usually gave a small negative pressure, e.g. 4 μl. in the first 30 min. and 6 μl. in the second 30 min., and it contained no more than a trace of NH_3 (4 μl.). The gas exchange in the second control, also, is often negligible. An O_2 uptake, however, may occur when tissues are examined in which the enzymes have not been inactivated (see below).

Determination of NH_3. On completion of the CO_2 evolution the cups are detached from the manometers, and NH_3 is determined in the solutions by steam distillation *in vacuo* according to Parnas (1934). A modified apparatus which allows the collection of 20 ml. of distillate was used.

Calculation. For convenience the amounts of CO_2 and NH_3 are both expressed in μl. After correction for blanks, the NH_3 produced in the unknown solution is equivalent to the glutamine, and the CO_2 to glutamine + glutamic acid.

Recovery of glutamine

Recovery of glutamine from pure solution was satisfactory as shown in Table 7. The errors in eight determinations did not exceed 4 % in the yield of CO_2 and 6 % in the yield of NH_3.

Table 7. *Recovery of glutamine from pure solutions*

(Procedure as described in text.)

Amount of glutamine analyzed (ml. 0·01 M-solution) ...	2	1	0·5	0·25
Glutamine expected (μl.) (allowing for 93% purity)	416	208	104	52
CO_2 (μl.) evolved: Duplicates	416	212	105	56
	419	212	103	50
Average	418	212	104	53
NH_3 (μl.) found: Duplicates	420	210	106	54
	400	216	110	50
Average	410	213	108	52

Table 8. *Changes in the concentration of glutamic acid and glutamine in animal tissues on storage*

(Procedure as described on p. 56 except that the tissue was not placed in liquid air; 'fresh' tissue was placed in iced water within a few minutes of death of the animal, and analyzed after being kept in the ice for about 50 min. 'Stored at 25°' refers to a sample of the same liver kept in ice for the same time and then left in a covered vessel at 25° for 24 hr.)

		Substance (μl./g. wet liver)		
		Glutamic acid	Glutamine	Preformed NH_3
Liver I:	Fresh	131	35	59
	Stored at 25°	225	14	386
Liver II:	Fresh	146	24	97
	Stored at 25°	305	15	305
Liver III:	Fresh	84	29	36
	Stored at 25°	152	5	278
Spleen:	Fresh	137	15	34
	Stored at 25°	179	~0	492

Specificity

The following substances, in quantities of 5 mg. each, did not produce NH_3 or CO_2 under the conditions of the glutamine determination:

Amino-acids: glycine, DL-alanine, DL-citrulline, L-arginine, L-phenylalanine, L-tyrosine, L-tryptophan, L-cysteine, L-cystine, DL-methionine, L-histidine, L-aspartic acid.

Acid amides: L-asparagine, urea, nicotinamide.

Purine derivatives: adenine, guanine, uric acid, allantoin, adenylic acid (muscle), adenylic acid (yeast), adenosinetriphosphate.

Miscellaneous: glucosamine, nicotinic acid, creatine, creatinine, succinimide.

Preparation of animal tissues for the determination of glutamic acid and glutamine

Preliminary experiments indicated that special precautions have to be taken when glutamic acid and glutamine are to be determined in animal tissues. The concentration of these two substances can undergo rapid changes, owing to the presence of proteolytic enzymes and glutaminase in these materials. Inactivation of the enzymes is not always practicable because operations commonly employed for this purpose are liable to destroy glutamine or to generate glutamic acid from protein or peptides.

When intact fresh tissue was kept at room temperature the glutamic acid increased and the glutamine fell (Table 8). In minced tissue, mixed with 2 parts of 0·1 N-HCl (resulting pH 3·7), both glutamic acid and glutamine rapidly increased even if the suspension was kept in the refrigerator (Table 9). The parallel increase in amino N indicates that the increase is due to proteolysis.

Table 9. *Changes in the concentration of glutamic acid and glutamine in acidified liver suspensions*

(Chilled sheep liver ground with 2 parts 0·1 N-HCl (pH of suspension 3·71 electrometrically). Amino N determined with nitrous acid method after deproteinization with trichloroacetic acid.)

	Substance (μl./g. wet wt.)			
Treatment of liver suspension	Glutamic acid	Glutamine	Preformed NH_3	Amino N
Fresh suspension	110	29	69	920
Stored 24 hr. near 0°	216	57	195	5,040
Stored 24 hr. at 25°	442	148	250	13,900

When 0·1 N-HCl was replaced by 0·3 N-HCl, the pH of the resulting tissue suspension being 2·2, an increase, though smaller, of both glutamic acid and glutamine was observed on 24 hr. storage in the refrigerator. On the other hand, the concentrations of glutamic acid and glutamine in a suspension containing 1 part of tissue and 2 parts of 0·5 N-HCl (pH about 1·0) remained almost constant in the refrigerator for 24 hr. After 48 hr., however, some of the glutamine had been converted into ammonium glutamate. The higher amount of

acid (0·5N) also inhibited the rise in 'preformed' NH_3, i.e. the NH_3 present in the tissue before the addition of *Cl. welchii* (Table 10).

Table 10. *Changes in the concentration of glutamic acid and glutamine in acidified liver suspensions*

(Rabbit liver frozen in liquid air for 20 min., thawed, minced and mixed with 2 vol. 0·5N-HCl.)

	Substance (μl./g. wet wt.)		
	Sum of glutamic acid and glutamine	Glutamine	Preformed NH_3
Examined immediately	138	27	50
Stored 24 hr. near 0°	138	28	48
Stored 48 hr. near 0°	139	12	61
Stored 24 hr. at 20°	152	—	—

A further error to be avoided is a loss of glutamic acid and glutamine, which occurs when tissue is packed in ice. The water from the melting ice penetrates into the tissue and causes it to swell. At the same time, amino-acids (and other substances) diffuse into the hypotonic medium. The result is that the concentrations of the amino-acids/unit weight of tissue fall. The magnitude of the changes is illustrated by the following example: 69 g. of liver, placed in 200 ml. water and 200 g. ice in a Dewar vessel, weighed 99 g. after 24 hr., whilst 52 mg. of glutamic acid had diffused into the water.

A complication interfering with the manometric CO_2 determination is the O_2 absorption which extracts or suspensions of liver, and to a less extent of testis, exhibit when shaken in air at pH 4–5. Most tissues tested (kidney, cortex and medulla, brain, spleen, heart, skeletal muscle, pancreas, thyroid gland) have no appreciable O_2 uptake under the experimental conditions. Sheep liver absorbed about 0·7–1·25 μl./mg. dry wt./hr. at 40°, without yielding CO_2. Often the rate increased with time. The substrate of this oxidation has not been identified. In some experiments the rate of the O_2 uptake increased on addition of the bacteria, and the tissue suspension alone is therefore not a satisfactory control to measure the O_2 uptake. The difficulties are overcome by filling the gas space of the manometers with N_2.

Procedure for the preparation of animal tissues. On the basis of the above observations the following procedure was adopted. The material, cut into 5–10 g. pieces, was thrown into liquid air as quickly as possible after death, and left there for at least 15 min. After removal from the Dewar vessel it was minced in a cooled tissue grinder as soon as the consistency permitted, transferred to a tared beaker, weighed, and 2 ml. of cooled 0·5N-HCl/g. tissue were added with thorough mixing. If the suspension could not be analyzed immediately it was stored in a refrigerator. For the determination of glutamic acid and glutamine, 2 ml. of suspension were pipetted into the main compartment of a conical Warburg vessel, followed by 0·1 ml. of 0·1% aqueous methyl orange and just sufficient N-NaOH to change the indicator to yellow (usually about 0·6 ml.). Next, 0·2 ml. 3M-acetate buffer (pH 4·9) was added to the main compartment. The further procedure was as already described, except that the gas space of the vessels was filled with N_2 and the centre contained a stick of yellow phosphorus.

Recovery of glutamine added to tissue suspensions. When known amounts of glutamine were added to acidified liver suspensions recovery was satisfactory: for example, 73 μl. added, 74 μl. recovered; 36·5 μl. added, 41 μl. recovered.

Presence of inhibitors. Like other analytical methods based on the use of enzymes, the present procedure cannot be applied to solutions containing inhibitors of the enzymes concerned. If the presence of inhibitors in the unknown material cannot be excluded, glutamine should be added and its recovery tested. As the decarboxylation of glutamine is more sensitive towards some inhibitors than that of glutamic acid, the latter is not always a suitable test substrate. In pure solutions various indicators (0·01%), such as phenolphthalein, bromocresol green, bromocresol purple and anionic detergents such as sodium dodecyl sulphate (0·05%), were found to inhibit the decarboxylation of glutamine. These inhibitions were largely abolished by the addition of cetavlon (0·05%) or serum (1 ml. to 3–4 ml. solutions), and the addition of one of these reagents may therefore be advisable if inhibitors are present. If serum is used, a blank determination is required; human serum contains about 8 mg. of glutamine and 2 mg. of glutamic acid/100 ml.

DISCUSSION

The main advantage of the present method over some previous techniques is the higher degree of specificity, coupled with relative simplicity. Vickery, Pucher, Clark, Chibnall & Westall (1935) deprecated the general application of their procedure (heating for 2 hr. at 100° at pH 6·5 and estimating the increase in NH_3-N) to unknown material because of lack of specificity. They reported that urea and allantoin yielded NH_3. Bartley & Krebs in unpublished experiments found that nicotinamide methochloride formed about 0·25 mol. of NH_3 when subjected to the procedure of Vickery *et al.* As nicotinamide methochloride is analogous to the pyridine nucleotides it is possible that these coenzymes also form NH_3. The specificity of Archibald's glutaminase from kidney is likewise imperfect, in that it is contaminated with deaminases liberating NH_3 from amino-purine nucleotides.

It cannot be claimed that the specificity of the glutaminase of *Cl. welchii* (strain S.R. 12) is absolute. However, it seems to be very high, since so far no substance apart from glutamine has been found to form NH_3 under the experimental conditions. Moreover, in a large number of analyses of animal and plant tissues, no material was encountered where the yield of NH_3 exceeded that of CO_2. If substances other than glutamine can produce NH_3 with the bacterial enzyme, an excess of NH_3 over CO_2 might have been expected.

SUMMARY

1. L-Glutamine yields one molecule each of NH_3 and of CO_2 when added to washed suspension of *Clostridium welchii* in acid solution. The formation of NH_3 precedes that of CO_2. The 'glutaminase' of the organism is highly specific; none of many substances tested was found to yield NH_3.

2. The method for the determination of glutamine and glutamic acid combines the principles used by Gale and by Archibald. The sum of the substances is determined according to Gale by manometric determination of the CO_2 evolved on decarboxylation. The NH_3 is then estimated in the solution treated with *Cl. welchii* and in a sample not treated with the bacteria. The difference represents the glutamine.

3. The conditions affecting the quantitative reaction of glutamine were examined. In pure solutions glutamine reacted much more slowly than glutamic acid, and the rate of reaction of glutamine, and to a smaller extent that of glutamic acid, was much (in some cases more than tenfold) accelerated by cetyltrimethylammonium bromide, serum or tissue extracts. In the presence of these substances the maximum rates of the decarboxylation of glutamine and glutamic acid were approximately equal.

4. The slow reaction of glutamine seems to be due, under some conditions, not to the low activity of the glutaminase or glutamic decarboxylase, but to permeability barriers. Removal of these barriers would account for the accelerating effect of cetyltrimethylammonium bromide.

5. The glutamine and glutamic acid content of animal tissues rapidly increases after death owing to autolysis. A procedure has been elaborated which minimizes the effects of autolytic enzymes.

REFERENCES

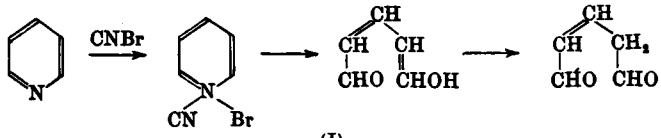
Archibald, R. M. (1944). *J. biol. Chem.* **154**, 643.
Archibald, R. M. (1945). *J. biol. Chem.* **159**, 693.
Baker, Z., Harrison, R. W. & Miller, B. F. (1941). *J. exp. Med.* **73**, 249.
Gale, E. F. (1941). *Biochem. J.* **35**, 66.
Gale, E. F. (1945). *Biochem. J.* **39**, 46.
Gale, E. F. (1946). *Adv. Enzymol.* **6**, 1.
Gale, E. F. (1947). *J. gen. Microbiol.* **1**, 53.
Parnas, J. K. (1934). *Biochem. Z.* **274**, 158.
Taylor, E. S. & Gale, E. F. (1945). *Biochem. J.* **39**, 52.
Vickery, H. B., Pucher, G. R., Clark, H. E., Chibnall, A. C. & Westall, R. G. (1935). *Biochem. J.* **29**, 2710.

The Recovery of *l*-Nicotine from Animal Tissues and its Colorimetric Micro-estimation

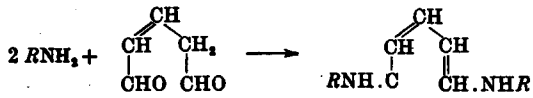
By A. R. TRIM, *Biochemical Laboratory, University of Cambridge*

(*Received* 3 *November* 1947)

Some heterocyclic substances containing a trivalent nitrogen atom react with 1:2:4-chlorodinitrobenzene (Vongerichten, 1899; Reitzenstein, 1903), cyanogen bromide, phosphorus pentachloride (König, 1904) and other substances to produce an unstable pentavalent nitrogen derivative which immediately changes to an open chain derivative of glutaconic aldehyde (see (I)). A similar change occurs in the action of cyanogen bromide on furfural, when the ring opens at the cyclic oxygen atom. The glutaconic derivative obtained in this way will condense with certain aromatic amines to form stable coloured compounds (see (II)). The tint and intensity of colour produced depend primarily upon the structure of the heterocyclic base and the amine. In the cases which have been investigated



(I)



(II)