DETTERMINATION OF GLUTAMINE AND GLUTAMIC ACID 57

SUMMARY

1. L-Glutamine yields one molecule each of NH₃ and of CO₂ when added to washed suspension of Clostridium welchii in acid solution. The formation of NH₃ precedes that of CO₂. The 'glutaminase' of the organism is highly specific; none of many substances tested was found to yield NH₃.

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 CO₂ evolved on decarboxylation. The NH₃ 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


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

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Some heterocyclic substances containing a trivalent nitrogen atom react with 1:2:4-chlorodinitrobenzene (Vongerichten, 1899; Reitzenstein, 1903), cyanogen bromide, phosphorus pentachloride (König, 1904) 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

and other substances to produce an unstable penta-valent nitrogen derivative which immediately changes to an open chain derivative of glutaconic aldehyde (see (I)). A similar change occurs in the 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
the development of colour is also influenced by the pH and the concentration of inorganic ions in the solution.

This reaction has been widely used for the detection and estimation of pyridine derivatives of biological importance. The procedures adopted have been reviewed by Bacharach (1941) and Waisman & Elvehjem (1941). Barta & Marschek (1937) estimated nicotine in tobacco by measuring the colour produced by the action of cyanogen bromide and \( \beta \)-naphthylamine on extracts of the plant. This procedure has now been developed for the accurate estimation of nicotine recovered from animal tissues by a distillation procedure. Quantities up to 600 \( \mu \)g of the base were recovered from 2 to 10 g. wet weight of tissues in 15 ml. of distillate. Quantities of 2 \( \mu \)g./ml. distillate and upwards were estimated with an error of \( \pm 1\% \).

**EXPERIMENTAL**

**Reagents.** (1) \( L \)-Nicotine, purified by distillation under reduced pressure, b.p. 122–123°/16 mm., \( n^\circ 1.5230 \). Standard solutions were prepared by dissolving a weighed quantity of freshly distilled nicotine in water immediately before use.

(2) Cyanogen bromide, prepared in aqueous solution by the method of Larson & Haag (1944). This was only necessary for the estimation of small quantities of nicotine with maximum accuracy, and for most purposes the solution obtained by just decolorizing saturated bromine water with 10% (w/v) KCN solution was found to be suitable. This preparation was diluted five times with distilled water and adjusted to pH 6 with KCN solution. If kept at 0° both solutions lasted for a week without deterioration.

(3) Ethanolic \( \beta \)-naphthylamine. \( \beta \)-Naphthylamine was recrystallized from ethanol immediately before use to remove the coloured substances which are rapidly formed from it on exposure to light. The purified amine was made up in 0.2% (w/v) solution in 95% ethanol before use.

(4) 0.2 m-Sorensen's phosphate buffer, pH 5.9–6.1.

(5) Acid aqueous ethanol. This consisted of a mixture containing 9 parts by volume 50% ethanol and 1 part of a mixture of equal volumes of glacial acetic acid, concentrated HCl and amyl alcohol.

The reagents were used in the following order and proportions: nicotine solution, 1.0 ml.; buffer, 0.2 ml.; \( CNBr \) solution, 0.27 ml.; ethanolic \( \beta \)-naphthylamine, 1.0 ml.

**Factors influencing the rate of colour development and intensity**

A number of workers (see Bacharach, 1941; Waisman & Elvehjem, 1941) have used this reaction for the estimation of nicotinic acid and nicotinamide. The more recent investigations have shown that the intensity of colour developed is markedly influenced by the pH in the solution and by the presence of inorganic ions. This was also found to be the case with \( L \)-nicotine. The addition of the phosphate buffer indicated above led to the development of the maximal intensity of colour. This buffer was the sole source of inorganic ions present in the first stage of the estimation.

The rate of colour development, which is slow at room temperature, was increased by heating to 37°. At 37° and the optimum pH 5.9–6.1 it was found that the colour developed was yellow which gradually increased in intensity and changed to orange red. Measurements with a spectrophotometer showed that there is a peak of extinction between the wave lengths of 490 and 550 m. In view of this, green colour filters were used in subsequent investigations. Measurements were made with the Spekker photoelectric absorptiometer, using the green filter no. 5, and in routine visual colorimetry with the Duboscq type colorimeter Chance's glass filter 0 Gr. 1 was employed. With the aid of the absorptiometer it was found that the intensity of colour reached a maximum in 2.5 hr. and remained constant up to 20 hr. A typical set of results is shown in Fig. 1. A series of tubes containing 300 \( \mu \)g of nicotine in 1 ml. of solution was treated with appropriate volumes of reagents and kept at 37°.

At intervals tubes were removed in duplicate, the contents made up to 20 ml. with 50% ethanol and the optical density read in the absorptiometer. The relation between the extinction coefficient and the time of heating is shown.

If one of the above solutions is taken after a minimum of 2.5 hr. heating, made up to 20 ml. with 50% ethanol and allowed to stand in the air, the second colour change takes place. A slow increase of optical density occurs and the tint changes towards magenta. This change is greatly accelerated by the addition of acid aqueous ethanol (reagent 5) in the place of 50% ethanol. Fig. 2 shows the relation between the extinction coefficient and the time of keeping at room temperature of solutions from the above series which had been heated for 2.5 hr. at 37° and then made up to 20 ml. with acid aqueous ethanol. The diagram shows that the change is complete within 2 hr. However, in practice it is
not necessary to wait for 2 hr. before reading, for the relative rate of increase of extinction is independent of the original concentration of nicotine. Provided, therefore, that the acidified ethanol is added to all the solutions at the same time, they may be compared with a standard, prepared simultaneously with them, at any time after the addition. Absolute readings with the absorptiometer can, of course, only be taken after the full 2 hr. has elapsed.

Fig. 2. Nicotine (300 µg.), cyanogen bromide and ethanolic β-naphthylamine. Relation between extinction coefficient and the time of keeping with acid ethanol at room temperature after the full development of the primary colour at 37° and pH 6.

Fig. 3. Relation between the extinction coefficient of the reaction mixture and the concentration of nicotine.

**Beer's law.** As is shown by Fig. 3, there is a linear relationship between colour and nicotine concentration over a wide range (in this case 1·5–20 µg./ml.).

**The estimation of micro-quantities of nicotine**

It is clear that, if it is contained in a suitably small volume, about 1 µg. of nicotine may be estimated by this method, using either a direct- vision colorimeter with small cups, capacity c. 2 ml., or a photoelectric colorimeter with 2 ml. cuvettes, with suitable colour filters. The error is less than 5%.

As will be seen in a later section, the minimal volume in which all the nicotine may be recovered from animal tissues is 15 ml., so that when very small amounts of nicotine are present it may be necessary to concentrate the solution. This may be done by reducing the pH to 4 with HCl and evaporating under reduced pressure.

**The recovery of nicotine from tissues**

Nicotine is recovered from tissues by taking advantage of its volatility in steam. The nature of the tissues under investigation and the use to be made of the distillate will determine the precise procedure employed. For the purpose of colorimetry it is desirable to have the nicotine in a minimum volume of distillate. It was found that the base could be recovered quantitatively from the tissues of the nematode *Ascaris lumbricoides* var. *suis* by a modification of the procedure of Werle & Becker (1942). The distillation apparatus, which is based on the Pregl micro-Kjeldahl apparatus, is illustrated in Fig. 4. Fresh worm tissue (2–10 g.), containing nicotine, was chopped into the detachable flask A. The following reagents were then added: (1) distilled water to a total weight of 25 g., (2) 11 g. of NaCl, and (3) 0·3 g. of MgO.

**Table 1. Recovery of nicotine from tissues of Ascaris**

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<thead>
<tr>
<th>Nicotine added (µg.)</th>
<th>Wet wt. tissues (g.)</th>
<th>Recovery of nicotine (%)</th>
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<td>30</td>
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<td>30</td>
<td>6·4</td>
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<tr>
<td>60</td>
<td>6·7</td>
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<td>60</td>
<td>5·1</td>
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<td>150</td>
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<td>600</td>
<td>5·5</td>
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<tr>
<td>600</td>
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The flask was attached to the apparatus by the ground-glass joint J and supported by a ring and gauze. The mixture was then heated by a small gas flame, and 15 ml. of distillate were collected, care being taken to avoid frothing beyond the first bulb of the still head. Some examples of the recovery of nicotine are given in Table 1. In this experiment small volumes of nicotine solution, c. 1 ml., were added to chopped *Ascaris* tissues, incubated at 37° for 6 hr., and then distilled. The final volume of solution for estimation was 50 ml.
The process of distillation was slow, taking about 30 min. for the complete operation. Recoveries adequate for exploratory and comparative work may be obtained by a much more rapid steam distillation procedure in which the flow of vapour is supplemented by the passage of steam through the tube \( B \) (broken line, Fig. 4). By this procedure about 75% of the nicotine may be recovered in 5 min. in 15 ml. of distillate.

A method is described for the recovery of nicotine from tissues and its estimation in quantities from 1 \( \mu g \).

The author is grateful to Prof. A. C. Chibnall, F.R.S., for his interest in this work, which was part of a programme of investigations into the mode of action of anthelmintics, carried out for the Agricultural Research Council.

REFERENCES


**Decomposition and Synthesis of Cozymase by Bacteria**

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(Received 5 November 1947)

Investigations concerning the metabolism of nicotinic acid and its derivatives have been surveyed recently by Schlenk (1945), and, in the case of micro-organisms, by McIlwain (1947). In the latter account, attention was directed to the synthesis, inactivation and interconversion of coenzymes I and II by micro-organisms, and particular efforts were made to obtain values for the rates at which these changes occur. Although many papers bearing on this subject were assessed, few if any were found to contain all the data required for calculating unequivocally the rates at which these important processes take place. On the other hand, such estimates of the rates as could be made by supplying likely values for missing data suggested the existence of a group of reactions with velocities of a few \( m\mumol./mg.\) dry wt. of organism/hr. The possible significance in bacteria of reactions of this magnitude has been discussed elsewhere (McIlwain, 1946b).

Studies have now been made of the behaviour of bacteria towards nicotinic acid derivatives, especially cozymase. This behaviour has been found to be dominated in many organisms by reactions which are potentially much more rapid than those inferred previously. Before studying the slower reactions (for which, also, evidence has been obtained) the course and products of the more rapid reactions have been examined, and are reported in this paper.

**SUMMARY**

A method is described for the recovery of nicotine from tissues and its estimation in quantities from 1 \( \mu g \).

**EXPERIMENTAL**

*Organisms.* A \( \beta \)-haemolytic streptococcus (R, the Richards strain; National Collection of Type Cultures no. 5631), two strains of *Streptococcus faecalis* (\( F \) 4208, N.C.T.C. no. 4208; \( FL \), a laboratory strain), a *Staphylococcus aureus* (laboratory strain) and *Proteus morganii* (N.C.T.C. no. 2818) were grown in the casein-yeast medium of McIlwain (1946a), modified as follows: in place of \( NaOH \), 0.5\% \( NaHCO_3 \) (additional 6-8 ml./100 ml.) was added; the initial pantothenate content was 10-11\%; the quantity of yeast preparation was 2.5 ml./100 ml.; and of the group \( A \) addenda only riboflavin, aneurin, \( KHPO_4 \), tryptophan, methionine, \( MgSO_4 \), \( Fe(NH_4)_2(SO_4)_2 \), 6\%H$_2$O and cystine were included. *Echerichia coli* (N.C.T.C. no. 4074) was grown in the mixture of inorganic salts and glucose of Dorfmann & Koser (1942).

*Lactobacillus arabinosus* 17-5 was grown in a medium elaborated for the use of the organism in assaying nicotinic acid (see below), and containing the quantities of nicotinic acid specified in the individual experiments described. Its pH fell from 6-8 to 5 during growth. *Haemophilus parainfluenzae* was also grown in the corresponding assay medium, with defined quantities of cozymase as V-factor.

*Reaction with cozymase.* Bacteria were normally harvested by centrifuging, washed twice with 0-9\% NaCl, suspended in that solution and a small sample taken for dry weight estimation. The suspensions were distributed between experimental vessels, which, for anaerobic experiments, were Warburg flasks with yellow \\( P \) in a centre well. Saline was that of Krebs & Henseleit (1932).

*Microbiological assays.* Nicotinic acid was determined using *Lactobacillus arabinosus* 17-5 in very nearly the way