CCXLI. THE SPECIFICITY OF GLUTAMINE FOR GROWTH OF *STREPTOCOCCUS HAEMOLYTICUS*

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GLUTAMINE was established as an essential nutrient for *Streptococcus haemolyticus* by McIlwain *et al.* [1939] and its importance in growth of other bacteria, notably pneumococcus, was shown by Fildes & Gladstone [1939]. It is involved in many other biological materials and processes, which makes desirable the determination of the specificity of the glutamine structure in producing these effects. The present results of attempted growth of streptococcus in the presence of compounds related to glutamine show that glutamine is extremely specific in this respect.

Conditions of testing

The bacteriological technique was that described by McIlwain *et al.* using, however, a small inoculum of approximately 1000 organisms. The compounds were tested as neutral solutions sterilized by filtration and added to the glutamine-free medium in maximum concentrations of 2×10^{-3} *M* and minimum concentrations of 8×10^{-5} *M* except when otherwise indicated. Incubation was continued for at least 7 days. Control tests with glutamine itself consistently attained maximum growth in 1 day with 4×10^{-4} *M* solutions and in 5–7 days with 6×10^{-7} *M* solutions.

Preparation and growth activity of glutamine analogues

Compounds tested are listed below in order of their deviation from the glutamine structure. When activity was observed, results of growth are given; all other specimens were inactive.

A. *Compounds differing from glutamine at its amide grouping*

Glutamic acid is inactive under the present conditions. Its effect under different conditions has been described by McIlwain *et al.* [1939] and by Fildes & Gladstone [1939].

α-Aminobutyric acid. A commercial specimen was used.

α-γ-Diaminobutyric acid was given by Dr R. L. M. Synge [Synge, 1939].

Glutathione. A commercial specimen was inactive under the present conditions [cf. McIlwain *et al.* 1939].

B. *Compounds differing from glutamine at its amine grouping*

Glutaric acid monoamide. Glutaric acid was converted to the anhydride which was distilled (B.P. 151–152°/20 mm.), crystallized (M.P. 51°) and converted to the amide (M.P. 94°, from acetone-ether). (Found: N, 10·8%. Calc. for $C_5H_9O_3N$: N, 10·7%.)

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l-*Leucyl*-d-*glutamine* was given by Prof. Chibnall.

Cysteylglutamine was given by Prof. Harington [*v.* Harington & Mead, 1936].

N-*Acetyl*-l-*glutamine*. Glutamine (see note *a*, p. 1946) was acetylated according to Synge [1939] in 80 % yield. The immediate product, and a specimen given by Dr R. L. M. Synge, caused slight growth of streptococcus in 1 day at a concentration of 2×10^{-3} *M* and in 3 days at 4×10^{-4} *M*. In view of the negative results with glutamine peptides, this was suspected to be due to contamination with glutamine, of which about 0·01 % would give the observed effect. Such was found to be the case, for on repeated crystallization from alcohol, 4–1 acetone-water and 4–1 alcohol-water, a specimen was obtained (M.P. 208°; N, 14·8 %. Calc. for $C_7H_{12}O_4N_2$, 14·9 %) which was without growth-promoting activity.

C. *Compounds altered at the carboxylic acid grouping of glutamine*

d-*Glutaminylglycine* and d-*glutaminyl*-d-*glutamic acid* were given by Prof. Chibnall [*v.* Melville, 1935].

Glutaminylcysteine was given by Prof. Harington [*v.* Harington & Mead, 1936].

D. *Compounds altered at the amide and amine groupings of glutamine*

Pyrrolidone-α-carboxylic acid. Specimens were obtained from glutamine by the method of Vickery *et al.* [1935] and from glutamic acid according to Foreman [1914], but the following, based on a method outlined by Haitinger [1882], was found best. *dl*-Glutamic acid (20 g.) was heated in a slow stream of N_2 in a metal-bath at 190–200°. Water was evolved vigorously after about 10 min.; the temperature remained at 160° during this, but after 5 min. rose and was kept at 185–190° for 2 hr. The resulting light brown liquid was allowed to cool to 100°, water (18 ml.) and HCl (2 ml. conc.) added and the mixture filtered. Pyrrolidone-α-carboxylic acid separated on cooling; more was obtained from its mother liquors and the whole recrystallized from dilute HCl (11 g., M.P. 183°. Found: N, 10·8 %. Calc. for $C_5H_7O_3N$: N, 10·8 %).

This compound is of interest as ammonium pyrrolidone-α-carboxylate is the product of breakdown of glutamine in aqueous solution [Vickery *et al.* 1935]. It was attempted to reverse this reaction by treating the inactive ammonium salt with NH_4OH and ammonium salts under various conditions; no appreciable amount of active material was found in the products.

Glutaric acid. A commercial specimen was used.

α-Oximinoglutaric acid. Diethyl-α-oximinoglutarate [McIlwain & Richardson, 1939] was very easily hydrolysed by NaOH according to Wislicenus & Grützner [1909] to the acid, which was recrystallized from water with the minimum of heating; M.P. 154–155° with gas evolution in a sealed capillary. Found: N, 8·6 %. Calc. for $C_5H_7O_5N$: N, 8·7 %.

α-Uramidoglutaric acid. The methods of Lippich [1908] and Dakin [1919] were found to yield mixtures of the acid with unchanged glutamic acid, from which it was very difficult to separate by crystallization. The solubilities of the heavy metal salts of the uramido-acid were investigated in order to discriminate between it and pyrrolidone-α-carboxylic acid and glutamic acid. The uramido-acid only was found to be precipitated by Pb salts at *p*H 5·5–6. The following method takes advantage of this.

dl-Glutamic acid (10 g.) urea (25 g.) and $Ba(OH)_2, 8H_2O$ (40 g.) in water (1000 ml.) were boiled in an open flask and 2 % $Ba(OH)_2$ added to restore the volume each time the *p*H reached 8. $BaCO_3$ was precipitated and NH_3 evolved. When the solution remained strongly alkaline after long boiling (after the

addition of about 3 l. of solution in 60 hr.), CO_2 was passed to remove excess $Ba(OH)_2$, the solution filtered and evaporated in vacuum to about 300 ml. Saturated aqueous basic Pb acetate solution was added till in excess, maintaining the solution at *p*H 5·5–6 by the addition of glacial acetic acid. The crystalline Pb salt was collected, suspended in warm water and H_2S passed. After removal of PbS the solution was concentrated to crystallization. Uramidoglutaric acid separated and was washed with water and alcohol: 5 g., M.P. 166° with gas evolution. Found: N, 14·8 %. Calc. for $C_6H_{10}O_5N_2$: N, 14·7 %. Its M.P. was not depressed by admixture with glutamic acid, but varied between 180 and 190°. *l*-Uramidoglutaric acid, similarly prepared, melted at 174°.

E. *Compounds altered at the amide and carboxylic acid groupings of glutamine*

dl-*Glutamic acid imide.* α-Oximinoglutaric acid imide (see under: 2 g.) in water (20 ml.) and HCl (1·5 ml. conc.) was shaken with palladium charcoal (0·5 g.) in H_2 at slightly above atmospheric pressure. Gas was rapidly absorbed until 580 ml. had reacted (6 hr.) when the solution was filtered from catalyst and evaporated in vacuum at 45° to crystallization. Further material was obtained by the addition of alcohol and ether to the mother liquors. The combined products (M.P. 204–206°) were recrystallized from aqueous alcohol containing a little HCl, giving colourless prisms, M.P. 206°, of glutamic acid imide hydrochloride. Found: N, 17·15 %. Calc. for $C_5H_9O_2N_2Cl$: N, 17·1 %. The crude material exhibited small activity, which was lost on repeated crystallization.

dl-*Isoglutamine* was prepared from *dl*-glutamic acid according to Bergmann & Zervas' [1932] preparation of the *l*-acid. This is known to yield a mixture containing about 14 % of glutamine [Melville, 1935]. When purified by heating the aqueous solution under conditions in which glutamine decomposes [Melville, 1935] it still contained labile amide (estimated by the procedure of Vickery *et al.* [1935]) corresponding to 1 % of glutamine. Repetition of the process, and various crystallizations, reduced the glutamine to an amount not chemically detectable and at the same time gave greatly reduced growth activity. Though this was not completely removed the evidence obtained indicated it to be due only to traces of glutamine.

F. *Compounds altered at the amide, amine and carboxylic acid groupings of glutamine*

α-*Oximinoglutaric acid imide.* Diethyl-α-oximinoglutarate [McIlwain & Richardson, 1939] (8·8 g.) in a glass-stoppered flask, and saturated NH_4OH (20 ml.) were cooled in ice and mixed. The solid softened and two layers formed; it was shaken at room temperature until homogeneous (about 1 hr.) and kept for 6 hr. in all at room temperature. Periodical titrations of excess NH_3 in aliquot portions indicated the reaction to be complete at that time, when 0·205 mol. of NH_3 had reacted with 0·2 mol. of ester. The solution was evaporated in vacuum from a bath at 45° to about half its bulk, when crystals had begun to appear; it was cooled to 0°, the solid (5 g.) collected and recrystallized from water to colourless long or short prisms. The pure imide melted with decomposition to a green-blue liquid at temperatures between 155 and 165° according to the conditions of heating. Found: total N, 20·5 %; amide N, 10·1 %. Calc. for $C_5H_6O_3N_2$: 19·8 and 9·9 %. The imide was soluble in water to a solution of *p*H 4–5. NH_3 was liberated by the action of cold conc. NaOH but the compound recrystallized unchanged from dilute HCl and was thus not an ammonium salt. It formed a precipitate with Hg^{++} salts in neutral, acid and alkaline solutions, but not with Pb or Ag salts.

dl-*Hydantoin-5-β-propionic acid* was prepared by the action of HCl on *dl*-uramidoglutaric acid and formed colourless crystals M.P. 164–165°.

dl-*Pyrrolidone-α-carboxylic acid amide* was prepared from the acid according to Abderhalden & Kautzsch [1912] and repeatedly crystallized from aqueous acetone, yielding colourless prisms of the amide, M.P. 217°. Found: N, 21·5%. Calc. for $C_5H_8O_2N_2$: 21·85%.

G. *Miscellaneous*

Asparagine and *insulin* were commercial specimens.

Discussion

Thus no growth occurred when practically any of the above compounds was substituted for glutamine in conditions under which glutamine itself caused growth. The immediate interpretation of these results is that the above compounds cannot perform the functions of glutamine, which are thus seen to be extremely specific.

There is also the further implication that none of the above compounds can be converted into glutamine by the organism at a rate sufficient to meet its demands in even extremely slow growth. Thus streptococcus is seen to be incapable of such relatively simple operations as forming the γ-amide of glutamic acid, hydrolysing an *N*-acetyl, *N*-leucyl or *N*-cysteyl grouping; hydrolysing peptide linkages between the carboxylic acid grouping of glutamine and the amino groupings of glycine, glutamic acid or cysteine. It also cannot open the rings of pyrrolidone-α-carboxylic acid or glutamic acid imide by addition of NH_3 or water in the necessary positions.

These results can be considered in relation to the mode of action of glutamine. Particularly significant in this connexion is the inactivity of the glutamine peptides. It may be argued that if *Streptococcus haemolyticus* has no enzyme mechanisms capable of breaking down simple peptides to release glutamine, then, from the normal reversibility of enzyme systems, it is also incapable of building up glutamine into normal peptides. Thus the purpose for which glutamine is used by the organism is not peptide formation unless enzymes specific to a particular peptide are involved. The inability of *Streptococcus haemolyticus* to form unusual amide linkages has already been commented on [McIlwain, 1939]. With this reservation, it would appear that glutamine is used by the organism as such rather than for building up a larger molecule, as the compounds tested present examples of every likely type of combination with glutamine.

A suggestion as to the use of glutamine obvious from its peculiar properties is that of NH_3 transference. If glutamine is involved in such a cyclic process, the compounds into which it is converted would be expected to show glutamine activity. Nevertheless, ammonium pyrrolidone-α-carboxylate, the product of breakdown of glutamine in aqueous solution, is inactive, as also is glutamic acid, the product of action of glutaminase on glutamine.

Summary

A large variety of glutamine analogues and derivatives have been found incapable of replacing glutamine in supporting growth of *Streptococcus haemolyticus*. The mode of action of glutamine is discussed.

I have pleasure in thanking Prof. A. C. Chibnall, Prof. C. R. Harington and Dr R. L. M. Synge for gifts of glutamine derivatives.

ADDENDUM

Note on the stability of glutamine. Data on the stability of glutamine in simple aqueous solutions have been recorded by Melville [1935] and by Vickery *et al.* [1935]. The following are points of practical importance which may be taken as supplementing those observations. (*a*) Solid glutamine prepared from beet and containing a little moisture was found to have decomposed to the extent of 66 % (as judged by labile amide-N) in the course of keeping for 3 months at room temperature. Later specimens have been kept over $CaCl_2$ at 0°. (*b*) In certain bacteriological culture media containing agar, glutamine was stable to autoclaving at 120° and *p*H 7·2 for 20 min.; $1{\cdot}5 \times 10^{-5}$ *M* glutamine in one medium supported growth equally before and after such autoclaving. In peptone infusion not containing agar, some destruction of glutamine occurred (P. Fildes, G. P. Gladstone and G. M. Hills).

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