of the various fractions of the combined cystine of wool are directly due to the heterogeneous nature of wool. It was also considered possible that the ($A + B$) fraction of the non-keratinous proteins might be identical with the maximum titratable thiol groups found for these proteins in solution, but it cannot be claimed that the results lend much support to this suggestion. On this point the results are in conflict with those of Hess & Sullivan (1943) who found the cysteine content of the hydrolysates of a number of proteins to be equivalent to the titratable thiol groups of the intact protein. Unfortunately few independent estimations are available, but it would seem that in the case of myosin the present results are in agreement with the findings of Bailey (1937 *a, b*).

SUMMARY

1. It has been shown that the combined cystine of a number of keratins can be subdivided into fractions of differing reactivity towards NaHSO_3 solutions in a manner analogous to that previously found for the combined cystine of wool.

2. Evidence of a similar subdivision of the combined cystine S has been found for other non-fibrous proteins.

The author would like to express his thanks to Dr H. Phillips for his interest and advice in this work, to Dr N. Palmer for her assistance in the preparation of the rabbit myosin and to Miss I. Rennie for technical assistance. Thanks are also due to the Council of the Wool Industries Research Association for permission to publish this paper.

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Preparation and Antibacterial Action of some Compounds Structurally Related to Glutamic Acid. Their Application in Microbiological Determination of Small Quantities of Glutamine

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(Received 10 July 1947)

In studying the metabolism of glutamine (McIlwain, Roper & Hughes, 1948) use was made of compounds structurally related to glutamic acid which differentially inhibited certain of the reactions concerned. These inhibitors were selected from a larger group of analogues and derivatives of glutamic acid, whose preparation and properties are described in the present paper.

The substances concerned are listed in Table 1. Expectation that γ -glutamylhydrazine might be inhibitory was based on analogous findings with

respect to pantoylhydrazine (Madinaveitia, Martin, Rose & Swain, 1945; McIlwain & Hughes, 1945). Inhibition by α -amino- γ -methylsulphonylbutyric acid or 'methionine sulphoxide' was reported while the present investigations were in progress (Borek, Miller, Scheiness & Waelsch, 1946; Waelsch, Owades, Miller & Borek, 1946). Several examples are known of inhibition by homologues of biologically active compounds and this led to the examination of the α -amino dicarboxylic acids of Table 1.

Table 1. *Effects on streptococcal growth, glycolysis and glutamine hydrolysis, of compounds structurally related to glutamine*

((a) The growth response of *Streptococcus faecalis* (laboratory strain, *F*) was examined in the medium employed in assay, but lacking the yeast preparation and methionine sulphoxide; for the Richards strain (*R*), glutamine (final concentration, 5×10^{-5} M) was added. Substances were examined at the following concentrations: 2×10^{-2} M, 4×10^{-3} M and 8×10^{-4} M, except in the cases of compounds (1), (3) and (6) when the highest concentration was 10^{-2} M. No compound replaced glutamine in permitting growth of the glutamine-requiring streptococci nor in stimulating streptococcal glycolysis. γ -Ethyl glutamate behaved towards the exacting streptococci in a fashion similar to glutamic acid, in that high concentrations permitted a delayed growth in absence of glutamine. This did not occur with the other compounds examined, and was presumably due to glutamic acid formed from the ester.

((b) Glycolysis was carried out anaerobically in NaHCO_3 -NaCl solution (cf. McIlwain, 1946). Compounds were examined at the following concentrations: (2), (4), (5), (7), (9) and (10): 0.033, 0.0067, and in some cases 0.0013 M; of (1), (3), (6), (8) 0.016 and 0.0016 M.)

Compound	(a) Effect on growth		(b) Effect on glycolysis or NH_3 formation from glutamine	
	Inhibition (+) or absence of inhibition (0)	Organisms examined	Inhibition (+) or absence of inhibition (0) not tested (-)	Organisms examined
I. α -Amino dicarboxylic acids				
(1) α -Amino- α -propylsuccinic acid	0	<i>R</i>	0	<i>R</i>
(2) α -Amino- β -butylsuccinic acid	0	<i>R</i>	0	<i>R</i>
(3) α -Amino- β -octylsuccinic acid	0	<i>R</i>	0	<i>R</i>
(4) α -Aminoadipic acid	0	<i>R</i>	0	<i>R</i>
(5) α -Aminopimelic acid	0	<i>R</i>	0	<i>R</i>
(6) α -Aminoundecane- α -dicarboxylic acid	0	<i>R</i>	0	<i>R</i>
II. Other amino carboxylic acids				
(7) α -Amino- α -methylvaleric acid	0	<i>R</i>	0	<i>R</i>
(8) α -Amino- β -furylpropionic acid	0	<i>R</i>	0	<i>R</i>
III. Methionine derivatives				
(9) α -Amino- γ -methylsulphonylbutyric acid	0	<i>R</i>	0	<i>R, F</i>
(10) α -Amino- γ -methylsulphonylbutyric acid	+	<i>F</i>	-	-
IV. Glutamic acid derivatives				
(11) <i>N</i> -(γ -Glutamyl)hydroxylamine	+	<i>R, F</i>	-	-
(12) γ -Glutamylhydrazine	+	<i>R, F</i>	+	<i>R</i>
(13) Acetone derivative of (12)	+	<i>R, F</i>	-	-
(14) γ -Ethyl glutamate	0	<i>R, F</i>	-	-

Knowledge that one of the analogues, namely methionine sulphoxide, inhibited the synthesis of glutamine from glutamic acid suggested its application in the microbiological determination of glutamine. A method for determining 10–20 μmol . of glutamine was required in studying the metabolism of the compound. The existing methods, either chemical or enzymic (cf. Archibald, 1945) depend finally on determination of NH_3 produced from glutamine under conditions more or less specific for glutamine. If the NH_3 was estimated by photometric measurement of the colour yielded with Nessler's reagent, quantities of about 500 μmol . of NH_3 could be determined in this way. Such methods were, however, not necessarily sensitive to 500 μmol . of glutamine. Thus it was necessary that any NH_3 initially present, or produced non-specifically under the conditions of hydrolysis of glutamine, should be removed or determined separately by blank estimations. In many cases we desired to

determine glutamine in the presence of up to 100 times its (molar) quantity of ammonium glutamate. This demanded a very complete removal or accurate estimation of the NH_3 , and in practice the determination of glutamine by the NH_3 formed from it did not give satisfactory results.

The possible use of microbiological methods for determining glutamine presents problems of a different type. The growth response of virulent group A β -haemolytic streptococci was applied in detecting and roughly estimating glutamine in natural materials in 1939 (McIlwain, Fildes, Gladstone & Knight, 1939). Since that time glutamine has been described as stimulating the growth of other micro-organisms (cf. Knight, 1945) and in some cases an organism's response to glutamine has been studied in detail as a possible basis for assay of glutamic acid or glutamine. In all such cases, difficulty has been encountered in discriminating between the effects of glutamic acid, ammonium salts,

and glutamine. Response to glutamic acid has been found slower than response to glutamine with both streptococci (McIlwain *et al.* 1939) and *Lactobacillus arabinosus* (Lewis & Olcott, 1944; Lyman, Kuiken, Blotter & Hale, 1945; Hac, Snell & Williams, 1945); and equimolar concentrations of the substances may finally give the same response, or glutamine may give the greater response. Hac *et al.* (1945) applied the different chemical properties of glutamic acid and glutamine, together with their effects on the growth of *L. arabinosus*, to the determination of glutamine.

In the present study it was preferred to try to obtain conditions of growth which more sharply discriminated between glutamic acid and glutamine. This was especially necessary in determining glutamine in the presence of a large excess of glutamic acid. Conditions were therefore chosen in which ammonium and glutamic acid salts were throughout present in a deficient basal medium consisting of glucose-peptone with various addenda. Recently passaged β -haemolytic streptococci did not grow in this medium within 5 days or so unless glutamine was added. With excess glutamine, maximum growth was usually reached in 18–24 hr. Sub-optimal concentrations of glutamine resulted in less growth, but although the graded response of Fig. 1A was obtained, this was not a suitable basis for the assay of glutamine. The reason for this can be seen by considering the wide differences between the curves obtained from readings at different periods of growth. With quantities of glutamine between 5 and 25 $\mu\text{mol.}$, readings increase 2- to 3-fold between 18 and 25 hr.; at 5 or 10 $\mu\text{mol.}$ readings increase 2- to 6-fold between 25 and 41 hr. Any substance, therefore, which was present in a material being assayed for glutamine, and which accelerated or retarded growth of the streptococci during assay, would cause large variations in the organism's response to a given quantity of glutamine.

Growth of the type shown in Fig. 1A appeared likely to be due to synthesis of glutamine by the organisms from ammonium glutamate. We therefore tried to obtain a more stable response to glutamine by adding α -amino- γ -methylsulphinylbutyric acid to the deficient basal medium. The glutamic acid content of this medium was about 10^{-3}M , and it was anticipated from the results of McIlwain *et al.* (1948) that, with the specimen of sulphoxide employed (a mixture of isomerides), a concentration of about $2 \times 10^{-2}\text{M}$ would be required. Trial showed that this concentration offered no advantage over one of $1.1 \times 10^{-2}\text{M}$, which was that adopted in the method as described below. Media containing the sulphoxide afforded a much more stable response to glutamine. This is illustrated in Fig. 1B, which shows the increase in readings between 16 and 24 hr. to be no more than 25 % of their

values at the earlier time. Three strains of β -haemolytic streptococci gave results similar to that of Fig. 1B, and of them the Richards strain was adopted for use in assay, as its response to glutamine is known to be highly specific (McIlwain, 1939).

Fig. 1. Growth of streptococcal cultures in media initially containing varying quantities of glutamine, measured by their optical densities at different times. B, using the medium described in the text and normally employed in assay; A, using a medium without methionine sulphoxide, but otherwise identical with that of B.

EXPERIMENTAL AND RESULTS

Preparation of compounds related to glutamic acid

γ -Ethyl glutamate was prepared by the method of Bergmann & Zervas (1933) modified as follows. After esterification of glutamic acid (5 g.) in anhydrous ethanol (35 ml.) the volume was made up to 150 ml. with ethanol and British Drug Houses Ltd. Universal Indicator added. Aqueous NH_3 (sp.gr. 0.88) was added dropwise until the solution was orange-red, when the precipitated NH_4Cl was removed at the pump. The filtrate was cautiously made more alkaline with NH_3 , any immediate amorphous precipitate being removed by filtration. The solution was finally brought to an orange-yellow colour, when the ester separated as plates. The whole was kept several hours at 0° and filtered, the filtrate being tested with NH_3 for a further precipitation of ester. After recrystallization from aqueous ethanol yields of more than 90% were obtained.

γ -Glutamylhydrazine. γ -Ethyl glutamate (5 g.) with 50% (w/w) hydrazine hydrate (10 ml.) and water (5 ml.) were warmed at 70° for 15 min. Absolute ethanol (200 ml.) at 70° was added slowly and the mixture kept at 0° for several hours. The fine crystalline precipitate was filtered off and recrystallized from aqueous ethanol. A white crystalline solid (3.9 g., yield 72%; m.p. 165°) was obtained. (Melting points here and elsewhere are uncorrected.) (Found: C, 32.2; H, 7.38; N, 22.2%; loss in weight at 105°, 16.3%. $C_5H_{11}O_3N_3 \cdot 1.5H_2O$ requires C, 31.9; H, 7.44; N, 22.3; H_2O , 14.6%.) To minimize the possibility of glutamine production from γ -ethyl glutamate and NH_3 present as impurity in the hydrazine hydrate, the purest specimen of hydrazine hydrate available was used. This was estimated to contain 0.14 mol. % of NH_3 .

Picrate. γ -Glutamylhydrazine (260 mg.) with picric acid (360 mg.) and water (30 ml.) was heated until complete solution was obtained. A yellow solid separated on cooling. This was filtered off and crystallized three times from water containing 5% (v/v) of ethanol. A fine yellow crystalline solid (445 mg., yield 78%; m.p. 190° (decomposition)) was obtained. (Found: C, 33.9; H, 3.48; N, 21.5%. $C_5H_{11}O_3N_3 \cdot C_6H_3O_7N_3$ requires C, 33.9; H, 3.59; N, 21.5%.)

Acetone γ -glutamylhydrazone. γ -Glutamylhydrazine (0.29 g.) with acetone (2 ml.), ethanol (4 ml.) and water (1 ml.) was warmed on a water bath for 10 min. The clear solution obtained was kept at 0° for several hours. Acetone (50 ml.) was added. A microcrystalline solid separated and was filtered, washed with small portions of ice-cold ethanol and recrystallized from aqueous acetone, giving a white microcrystalline solid (150 mg.; yield, 70%; m.p. 171°). (Found: H, 7.58; N, 21.2%. $C_5H_{11}O_3N_3$ requires H, 7.46; N, 20.9%.)

Benzaldehyde γ -glutamylhydrazone. γ -Glutamylhydrazine (1 g.) with benzaldehyde (2 ml.) and ethanol (25 ml.) was heated on a water bath for 10 min. Water (25 ml.) was added gradually and a clear solution was obtained on continued heating. The solution was cooled slowly to 0° and held there for several hours. The white solid which slowly precipitated was filtered off and recrystallized from aqueous ethanol to give an odourless crystalline solid (1.06 g., yield 80%; m.p. 192° with decomposition). (Found: C, 57.5; H, 6.08%. $C_{12}H_{15}O_3N_3$ requires C, 57.8; H, 6.02%.)

β -Aspartylhydrazine. Asparagine monohydrate (10 g.) was refluxed with 50% (w/w) hydrazine hydrate (30 ml.) for 20 min. The solution was evaporated *in vacuo* to a gum which was taken up in water (15 ml.). Ethanol (70 ml.) was added gradually, the precipitated amorphous white solid filtered off, washed well with ethanol and crystallized from aqueous ethanol. It gave fine white crystals (9.0 g.; yield 92%; m.p. 194–195° with decomposition). (Found: C, 32.8; H, 6.26; N, 28.7%. $C_4H_9O_3N_3$ requires C, 32.7; H, 6.12; N, 28.8%.)

N-(γ -Glutamyl)hydroxylamine. To hydroxylamine hydrochloride (5 g.) suspended in ethanol (20 ml.), 10N-NaOH was added slowly and with shaking until the pH became 8 to 8.5. The mixture was filtered and the filtrate (20 ml.) with γ -ethyl glutamate (1.2 g.) was kept at room temperature in a closed vessel for 16 hr. and then at 0° for 48 hr., with shaking at intervals. The clear solution was then evaporated in a vacuum desiccator over P_2O_5 for 4 days, solid matter being removed by filtration each day. On the fifth day the volume of liquid was c. 3 ml. The pH was adjusted with concentrated HCl to 3.2 and the solution evaporated to

a gum. The gum was rubbed with ethanol (40 ml.) and left at 0° overnight. The ethanol was decanted and the semi-solid mass taken up in water (10 ml.) and slowly precipitated as a pale-brown solid by the gradual addition of ethanol (50 ml.). Three recrystallizations from aqueous ethanol yielded pale buff-coloured needles (0.64 g., yield 57%; m.p. 155°), which gave a cherry-red colour with $FeCl_3$. (Found: C, 36.6; H, 6.11; N, 17.2%. $C_5H_{10}O_4N_2$ requires C, 37.0; H, 6.17; N, 17.3%.)

N-(β -Aspartyl)hydroxylamine. Asparagine monohydrate (5 g.) and hydroxylamine sulphate (5 g.) were boiled for 20 min. in water (25 ml.). The solution was cooled and a few drops of 10% (v/v) acetic acid added. Excess of saturated aqueous copper acetate was added gradually and the dark green precipitate filtered off and washed with water (200 ml.). The yield of copper salt was almost theoretical. The salt was suspended in 50% (v/v) aqueous ethanol and 1 drop of 0.1N- H_2SO_4 added. H_2S was passed in and the mixture filtered at 60°. The pale-blue filtrate was repeatedly treated with H_2S until a clear colourless filtrate was obtained. The filtrate was evaporated to c. 5 ml. *in vacuo* at less than 50°. Ethanol (20 ml.) was added and the pale-brown precipitate was recrystallized three times from aqueous ethanol to yield off-white crystals (2.2 g., yield 45%; m.p. 179–180° with decomposition) which gave a cherry-red colour with $FeCl_3$. (Found: C, 32.1; H, 5.4; N, 19.2%. $C_4H_8O_4N_2$ requires C, 32.4; H, 5.2; N, 18.9%.) Estimation of the hydroxamic acid grouping by the method described below gave 97.5 ± 3% of the theoretical value.

Determination of hydroxamic acid grouping by $KBrO_3$. The method is based on that of Junell (1934) and consists of the oxidation of the hydroxamic acid grouping with $KBrO_3$, excess bromate being estimated by its oxidation of KI with subsequent estimation of liberated I_2 . Solutions are required of $KBrO_3$ (0.04M), $Na_2S_2O_3$ (0.025M), HCl (N), KI (saturated aqueous), and of the hydroxamic acid (about 0.01N). To the solution of hydroxamic acid (5 ml.) was added $KBrO_3$ (3 ml.) and N-HCl (2 ml.). Within 2 min. of the acidification excess KI solution was added and the liberated I_2 was estimated immediately with $Na_2S_2O_3$. The method gave consistent results to within 3% of the theoretical values. The time required for oxidation varied according to the hydroxamic acid being estimated. In the case of the two aminohydroxamic acids described above the oxidation was found (by spot testing with $FeCl_3$) to be complete within 1 min. under the conditions cited. For consistent results it was necessary to complete the estimation in less than 5 min. after acidification. Other hydroxamic acids (e.g. acetylhydroxamic acid) require up to 30 min. for complete oxidation.

α -Amino- γ -methylsulphonylbutyric acid. The method described is a modification of that used by Toennies & Kolb (1939).

DL-Methionine (10 g.) with glacial acetic acid (40 ml.) was warmed to 60° and then cooled to room temperature. Hydrogen peroxide (11 ml. of 100 vol.) was added slowly, the vessel being shaken and cooled between additions. The clear solution obtained was cooled to 0° and absolute ethanol (350 ml.) added. The mixture was cooled at 0° for several hours and the solid filtered off. The white amorphous powder (yield, 98%; m.p. 237° with decomposition) was washed with ethanol and dried *in vacuo*. Recrystallization from aqueous ethanol gave a white odourless crystalline solid (yield, 95%; m.p. 240° with decomposition). (Found:

C, 36.4; H, 6.63; N, 8.30; S, 19.4%. Calc. for $C_6H_{11}O_4NS$: C, 36.5; H, 6.68; N, 8.48; S, 19.4%.)

No attempt was made to separate the isomerides which are presumably present in this product, and which have been examined by Waelsch & Borek (1947).

α -Amino- γ -methylsulphonylbutyric acid. Preparation of this compound has been described by Toennies & Kolb (1941). The method below gives considerably poorer yield but was employed because of its simplicity.

DL-Methionine (4 g.) was suspended in 50% (v/v) acetic acid (20 ml.). $KMnO_4$ (45 ml. of 0.67 M in 50% (v/v) acetic acid) was added slowly and the solution was allowed to stand 15 min. at room temperature. H_2O_2 (100 vol.) was added dropwise until a clear solution was obtained. The straw-coloured solution was treated with charcoal, filtered, and ethanol (150 ml.) was added. The white precipitate was filtered off and recrystallized from aqueous ethanol. A white odourless crystalline solid (2.4 g., yield 53%; m.p. 254° with decomposition) was obtained. (Found: C, 33.3; H, 6.23; N, 7.6; S, 18.1, 17.5%. Calc. for $C_6H_{11}O_4NS$: C, 33.2; H, 6.08; N, 7.7; S, 17.7%.)

Compounds 1 to 8 of Table 1. These were all DL mixtures for which we are indebted to Dr J. Elks of Glaxo Laboratories Research Division.

Some microbiological properties of the compounds related to glutamic acid

The properties of the compounds related to glutamic acid have been investigated primarily in relation to haemolytic streptococci with results which are summarized in Table 1. Homologues of glutamic acid (compounds 1-6) and some other amino carboxylic acids (compounds 7 and 8) gave no instances of inhibition, of growth, of glutamine decomposition, or of glycolysis.

The two methionine derivatives proved inhibitory (see above) and the action of the sulphoxide (compound 9) on streptococcal growth is described by McIlwain *et al.* (1948). The sulphone (compound 10) had an entirely different action. Though without action on three streptococcal strains of varying glutamine requirement and also without action on a strain of *Staphylococcus aureus*, it inhibited growth of *Escherichia coli* in the medium based on ammonium lactate and described by Fildes (1938). The sulphone (0.02 M) prevented visible growth from an inoculum of $c. 5 \times 10^4$ cells/5 ml. during the first 2 days and resulted in less growth than in controls during the following 2 days. These effects were prevented by the presence of 0.01 M-methionine and diminished by 0.002 M-methionine.

Hydrazine derivatives. The effect on streptococcal growth of the following compounds has been examined: γ -glutamylhydrazine and its acetone derivative; β -aspartylhydrazine, and hydrazine itself. Of these, the most inhibitory were hydrazine and γ -glutamylhydrazine. Hydrazine itself prevented growth of the Richards strain at 4×10^{-3} M and of the *faecalis* strain at 2×10^{-2} M, the concentrations in

each case being independent of added glutamine. Inhibition by the glutamyl derivatives was, on the other hand, markedly influenced by the concentration of glutamine also present in growth media; the action of γ -glutamylhydrazine is described fully by McIlwain *et al.* (1948). Its acetone derivative acted similarly, but higher concentrations of it were required. Of the acetone derivative, 0.013 M (but not 0.01 M), prevented growth of the Richards strain for 3 days in the presence of 2×10^{-4} M-glutamine; with 2×10^{-5} M-glutamine, 5×10^{-3} M (not 3×10^{-3} M) was adequate. The benzaldehyde derivative was only slightly soluble, and at the concentrations which could be employed it was without action on growth. β -Aspartylhydrazine was required in concentrations of 4×10^{-2} M for inhibition of either the Richards or *faecalis* strains for more than 1 day, and in some cases this inhibition was diminished by either asparagine (0.01 M) or glutamine (2×10^{-4} M). Although the compound was not sufficiently active to investigate in detail, it indicates that the substitution of hydrazine by a residue similar to the glutamyl residue (as well as by the glutamyl residue itself) can produce a compound disturbing the action of glutamine. A less closely related compound, pantoylhydrazine (McIlwain & Hughes, 1945), has, it will be noted, a distinct type of action.

Hydroxylamine and hydroxamic acids. Growth of the Richards organism was partly inhibited by 4×10^{-5} M-N-(γ -glutamyl)hydroxylamine, and completely by concentrations of 2×10^{-4} M or greater, in the presence of either 2×10^{-6} or 2×10^{-4} M-glutamine. A *faecalis* and a group G strain were less sensitive to the compound, of which 2×10^{-3} M gave only a slight action, but this effect also was not altered by addition of glutamine. Hydroxylamine itself was inhibitory to streptococcal growth under the present conditions in concentrations of 5×10^{-5} M or more. To explain inhibition by the glutamyl derivative as due to hydroxylamine would require a considerable degree of breakdown, which model experiments showed should be detectable by reduction of Fehling's solution at room temperature. Such reduction was not found to be brought about by solutions of the glutamyl derivative; any hydroxylamine produced under the conditions of testing was found to represent only 0.01 mol. % of the glutamyl derivative used.

N-(β -Aspartyl)hydroxylamine was required in concentrations of 5×10^{-3} to 2×10^{-2} M, and N-acetylhydroxylamine in concentrations of 2×10^{-2} M or higher, for any inhibition of streptococcal growth. It was observed that the hydroxylamine derivatives reacted with iron salts of the growth medium to yield a yellow coloration (red in more concentrated solutions). Increase in iron content of the medium during growth did not however prevent the action of the inhibitors.

The determination of glutamine

Organisms. The Richards strain of β -haemolytic streptococci (National Collection of Type Cultures, no. 5631), passaged frequently through mice and of M.L.D. (in mice) of less than 100 cocci, was initially employed. Face masks were worn during the pipetting involved in photometric measurement of the growth of this organism. For the assay using methionine sulphoxide, a highly virulent organism was not required and this precaution was not taken. The same strain, after maintenance in laboratory media, and having a M.L.D. in mice of some 10^8 organisms, was then used. It was preserved by growing in serum broth for 7 hr., and drying *in vacuo* in a number of ampoules (cf. Hale & McIlwain, 1947). For use, a dried inoculum was grown in serum broth and from this, serum-broth-agar slopes were prepared daily for inoculation of the assay medium. Slopes were grown for 16 hr. and used only on the day following their inoculation; serum-broth cultures were kept in a refrigerator after growth and used only during the next 14 days. Incubation throughout was aerobic and at 37° . During preliminary studies, three other strains of β -haemolytic streptococci were examined as potential test organisms, but these offered no advantages over the Richards strain. As the specificity of this organism's response to glutamine in growth is already established (McIlwain, 1939), it has been used in the present study.

Medium and inoculation. A sterile solution of peptone, NaCl, KH_2PO_4 and NaOH was prepared according to McIlwain *et al.* (1939) and kept in a dark cupboard for periods of up to 1 month. For use, the following sterile solutions were added to each 100 ml. of peptone solution: the group A addenda of McIlwain (1944), 10 ml.; 0.2 M-methionine sulphoxide, 10 ml.; the yeast preparation of McIlwain (1944), 2 ml.; and N-NaOH, 2 ml. The medium was then inoculated with cocci taken from a serum-broth-agar slope; these were suspended in about 1 ml. of the peptone solution and the density of the suspension adjusted so that (by comparison with opacity standards) it contained approximately 10^6 organisms/ml. It was then diluted 1000-fold in the peptone solution and 2.5 ml. of the dilution added as inoculum. The inoculated medium was distributed in 5 ml. portions in a number of $6 \times \frac{3}{4}$ in. test tubes.

The inoculum chosen was sufficiently small not to require washing to free it from glutamine; a 5-fold increase or decrease in its size was tolerated without much effect on the assay.

Solutions containing glutamine, and incubation. 0.01 M-Glutamine was prepared at weekly intervals from the solid, sterilized by filtration through Ford's

Sterimats in an apparatus of the type described by Knight & Fildes (1936) and kept cold. On the day of its use in assay, solutions were prepared from it which were 5×10^{-6} , 10^{-5} , 1.5×10^{-5} , 2×10^{-5} and 2.5×10^{-5} M with respect to glutamine. Solutions to be assayed for glutamine were also filtered to sterilize them and diluted so that two or three dilutions could be expected to be within that range of concentrations. Known and unknown solutions, with the necessary control solutions (2 ml.) were then added to tubes of inoculated medium and incubated at 37° in 5% CO_2 -air. An ordinary bacteriological incubator was used; shaking the tubes in a thermostat at 37° did not give a more regular response.

Measurement of streptococcal growth. This was carried out photoelectrically, using a Hilger absorptiometer to obtain the optical densities of the cultures after growth. While the conditions of assay were being chosen (i.e. in producing curves such as those of Fig. 1), portions of about 1.5 ml. of the cultures were taken at intervals, aseptically, with Pasteur pipettes. Cells were used which held c. 0.5 ml. of solution but gave an absorbing layer 1 cm. in depth. Densities were normally read after 16–24 hr. incubation, but sometimes also at 40 hr.; in the first case it was not necessary to take specimens aseptically.

Reference curves such as those of Fig. 1B were constructed from the readings obtained from the known quantities of glutamine. The glutamine content of each of the tubes containing solutions being assayed was computed by reference to the standard curve. Some results are quoted in Table 2. The standard curves often contained an almost linear portion as illustrated by the full lines in Fig. 1B, but sometimes were sigmoid, as shown by the broken line.

It will be observed that the assay could be made sensitive to 1/5 of the quantity of glutamine employed in the present account by using the same method of measurement but 1/5 of the quantity of media.

Specificity of assay. The effect of a number of compounds related to glutamine on growth of the present strain of streptococci has been examined (McIlwain, 1939), without finding any which replaced glutamine. The present medium already contains ammonium glutamate, and the addition of a further 2000 m μ mol. of the substance per tube was found to have no effect on the assay of 20 m μ mol. of glutamine. Glutamine added to bacterial extracts of the type assayed frequently by McIlwain *et al.* (1948) was satisfactorily recovered, indicating the absence of antagonistic or potentiating substances, but such experiments have not been performed with a wider range of materials.

Table 2 also gives results which show the value of the sulphoxide in assay. Values in the presence of the compound are seen to be much more consistent.

Table 2. Results of glutamine assay, with and without sulphoxide and added glutamine

Quantity taken of 1/50 dilution of horse plasma (ml.)		Glutamine found, calculated to nearest 10 μ mol./ml. plasma			
		By assay without sulphoxide		By assay with sulphoxide	
		Reading after 17 hr. growth	Reading after 25 hr. growth	Reading after 17 hr. growth	Reading after 25 hr. growth
2.0		830	710	530	490
1.6		840	830	520	500
1.2		730	770	520	520
0.8		690	750	510	510
Averages: 770			760	520	510

Quantity taken of		Glutamine found, calculated to nearest 0.1 μ mol./ml. extract			
		Reading after 18 hr. growth		Reading after 26 hr. growth	
		Directly	After subtracting quantity added	Directly	After subtracting quantity added
Bacterial extract* (ml.)	Glutamine (μ mol.)				
2.0	0	11.0	—	10.9	—
1.6	0	10.8	—	10.3	—
1.2	0	10.1	—	11.2	—
0.8	0	11.3	—	11.2	—
Averages: 10.8			—	10.9	—
1.0	25	35.0	10.0	35.0	10.0
0.8	25	35.7	10.7	36.2	11.2
0.6	25	35.9	10.9	34.8	9.8
0.4	25	35.5	10.5	35.0	10.0
Averages: 35.5			10.5	35.3	10.3

* Prepared by extracting *Staphylococcus aureus* (after treatment similar to that described by McIlwain *et al.* 1948; Table 10) with phosphate buffer pH 7 (4 ml./1.6 g. moist wt. of cells) at 100° for 15 min.; sulphoxide present during assay.

The reason for their absolute values being about 2/3 of those in the absence of the sulphoxide is not known. Acceleration of growth of the assay organism, by compounds unrelated to glutamine, might be expected to have a greater effect on the earlier than on the later readings, but the average values at the two times are found to be approximately equal. It is also possible that there exist in plasma substances which simulate glutamine in the absence of the sulphoxide, or promote its synthesis.

Measurement of response by acid formation. The assay organism converts glucose to lactic acid in a yield of c. 95% (McIlwain, 1946). When this was measured by titration, a period of growth of some 3 days gave the most regular response to added glutamine (Fig. 2). As this period is longer than that required for measurement of growth by photometric readings, we have not attempted to base an assay on acid formation, although the type of response is adequate for such an assay.

Weight of streptococci produced by limiting quantities of glutamine. Assay curves such as those of Fig. 1B afford data which can be used in calculating the minimum quantity of glutamine required to produce a given quantity of streptococci. Comparison of the optical densities (α) and dry weights

Fig. 2. Acid formed in streptococcal cultures initially containing varying quantities of glutamine, and measured by the alkali required at different times to take 2 ml. portions to a standard pH of about 11. Medium and conditions of growth: as described for photometric assay. Indicator: alizarin yellow G.

(*b*; mg./ml.) of different batches of the present organisms gave values for *b/a* of 0.368, 0.345, 0.377, 0.390 and 0.353 (mean: 0.367). The slopes of the initial portions of the lines of Fig. 1*B* show suspensions of optical densities 0.18 (in 16 hr.) to 0.23 (in 24 hr.) to be produced in the first day from 30 mμmol. of glutamine in 7 ml.; 0.015–0.02 mg. of organisms are thus formed per mμmol. of added glutamine. Eight other standard curves have given values differing from this by less than 25 %.

SUMMARY

1. A number of substances structurally related to glutamic acid has been prepared, including the hydrazide, *R*.CO.NH.NH₂; its acetone derivative, *R*.CO.NH.N:C(CH₃)₂; its benzaldehyde derivative, *R*.CO.NH.N:CH.C₆H₅; the hydroxamic acid, *R*.CO.NHOH; and an analogous sulphoxide, *R*.SO.CH₃ and sulphone, *R*.SO₂.CH₃

(*R* = —CH₂.CH₂.CH(NH₂).COOH).

The hydrazide and hydroxamic acid similarly related to aspartic acid have also been prepared.

2. Some of these compounds, and also other substances related to glutamic acid (especially homologues of glutamic acid), had no effect on growth and glycolysis of streptococci. Those formulated above, except the benzaldehyde derivative and the sulphone (which however inhibited *Escherichia coli*), inhibited streptococcal growth. The basis for the action of the hydroxamic acid is unknown, but action of the other compounds on streptococci is related to the metabolism of glutamine or of glutamic acid.

3. Inhibition by the sulphoxide has been applied in the microbiological determination of glutamine. Glutamic acid can to some extent replace glutamine in growth of streptococci in ordinary media, but the sulphoxide (0.01M) prevents this and results in a prompt and adequately stable growth of the organisms in the presence of 5–40 mμmol. of glutamine in 7 ml. of medium. Photometric measurement of the resulting growth has been used to determine glutamine in natural materials.

We are greatly indebted to Mr D. E. Hughes and Miss E. Ellis for assistance during these investigations.

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Relationships in Streptococci between Growth and Metabolism of Glutamine

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(Received 25 August 1947)

Previous investigation of the behaviour of glutamine as a growth essential for streptococci has suggested its action to have a metabolic basis in two senses. First, requirement for glutamine in growth appeared to depend on the inability of those streptococci which needed added glutamine to synthesize it at an adequate rate. This has now been confirmed by demonstrating the synthesis in organisms not re-

quiring added glutamine, and further by inhibiting such synthesis when growth also has been found to be inhibited. Secondly, glutamine when added to streptococci has metabolic effects, among which has been recognized (McIlwain, 1946 *a, b*) a stimulation in glycolysis coupled with the conversion of glutamine to glutamic acid and ammonia. This group of reactions might be that for which glutamine (synthe-