(*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.

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Relationships in Streptococci between Growth and Metabolism of Glutamine

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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-

sized by the organisms or added as such) is required by streptococci. To previous evidence suggesting this has now been added two further types of observation: one, that a number of substances related to glutamine, but which are incapable of replacing glutamine in growth, have no effect on glycolysis; and the other, that when the coupled breakdown of glutamine is inhibited, growth of the organisms is also inhibited.

Fig. 1. Points of action of inhibitors in the synthesis and breakdown of glutamine.
 $R = -CH_2.CH_2.CH(NH_2).COOH$.

The reactions concerned are summarized in Fig. 1, and evidence is given below for their differential inhibition by two compounds structurally related to glutamic acid, namely, α -amino- γ -methylsulphanylbutyric acid, and γ -glutamylhydrazine. These compounds were selected for detailed study from a series described by Roper & McIlwain (1948). It will be observed from Fig. 1 that the synthesis and breakdown of glutamine are brought about by distinct systems, each associated with energy-yielding reactions such as glycolysis. Other aspects of the association between glutamine and glucose metabolism are reported below.

EXPERIMENTAL

Growth of streptococci, the performance of metabolic experiments with streptococcal suspensions, purification of glutamine, and determination of NH_3 and of glutamine were by methods previously stated (McIlwain, 1946 *a, b*) unless others are specified. Casein hydrolysate was that of McIlwain & Hughes (1944). The streptococcal strains employed are described by McIlwain (1946 *a, b*) and by Hale & McIlwain (1947).

Disintegration of bacteria followed Wiggert, Silverman, Utter & Werkman's (1940) method, using particles of Pyrex glass about the size of the bacteria. A mass of bacteria packed by centrifuging was cooled in ice and mixed with three times its (moist) weight of the glass, transferred to a dry, ice-cooled mortar and rubbed with maximum hand pressure for 30 sec., scraped together, and the rubbing twice repeated. The resultant paste was extracted in a centrifuge tube with cold $M/18$ phosphate, pH 7, 1.5 times the moist weight of the original bacteria. Disintegration was observed microscopically and its extent determined by mounting stained films in water instead of oil. The particles of glass could then be seen and the ratio of particles to bacteria was found before and after the grinding.

Extraction by autolysis. Organisms packed by centrifuging were suspended in seven times their moist weight of 0.055 M -phosphate of pH 7, containing 0.36 M -NaF, and left with occasional shaking at 37° for periods of 3–10 days.

Experiments with streptococcal extracts. In addition to the conditions of metabolism referred to above, a medium of the following composition (cf. Utter, Reiner & Wood, 1945) was used in attempting to obtain glycolysis in streptococcal extracts: Na_2HPO_4 and KH_2PO_4 of pH 7.3, 0.01 M ; $NaHCO_3$, 0.05 M ; $MgCl_2$, 0.008 M ; glucose, 0.03 M ; hexosediphosphate,

0.0025 M ; adenosine triphosphate, 0.0006 M ; cozymase (as a specimen of about 40% purity), 0.001 M , with and without nicotinamide, 0.04 M , and in equilibrium with 5% CO_2 in N_2 .

Conditions employed in examining the actions of substances on streptococcal growth. A medium based on Bacto-peptone was used (McIlwain, Fildes, Gladstone & Knight, 1939; McIlwain, 1939), inoculated with about 500 cocci/ml. of the different streptococcal strains, and incubated at 37°. The mass of growth in control and experimental tubes was compared visually during the 7 days immediately following inoculation.

Materials. The compounds of Fig. 3 and Table 6 were either obtained commercially, or prepared according to McIlwain (1939). Hynson, Westcott and Dunning Inc., Baltimore, supplied bromsulphalein. Glutamine, glutamic acid and γ -glutamylhydrazine were the natural L-isomers. α -Amino- γ -methylsulphanylbutyric acid was the mixture of isomerides produced from DL-methionine by Roper & McIlwain (1948).

RESULTS

Effects of various circumstances on glutamine breakdown and on streptococcal glycolysis

Before comparing the behaviour of other compounds with that of glutamine, the effects of incidental circumstances on reactions between streptococci and glutamine itself were examined. The actions of glutamine which were studied were (*a*) the rate of glutamine breakdown, especially during anaerobic glycolysis, and (*b*) the associated stimulation of glycolysis.

Preparation of organisms. Results with organisms grown for different periods of time, and in different media, are given in Table 1. Though variations in the rate of NH_3 formation from glutamine occurred,

Table 1. *Variation in response to glutamine with age, medium of growth, and treatment of organisms*(The Richards strain was employed, and cultures contained 5×10^{-4} M-glutamine unless otherwise indicated.)

Culture medium	Age (hr.)	Dry wt. used/3 ml. (mg.)	Q_{NH_3} (μ mol./mg. dry wt./hr.)	Q_{CO_2} without glutamine (μ mol./mg. dry wt./hr.)	Q_{CO_2} with glutamine as % of that without	Ratio $\frac{Q_{CO_2}}{Q_{NH_3}}$ in presence of glutamine
1. Casein hydrolysate	12	2.9*	2.55	25.5	126	12.6
2. Casein hydrolysate	26	2.8†	1.07	12.7	128	15.2
3. Broth (no added glutamine)	17	2.7	0.86	11.0	129	16.5
4. Glucose-broth	17	2.7	0.87	11.1	128	16.3
5. Casein hydrolysate; glutamine, 4×10^{-3} M	18	3.2	3.1	20.7	128	8.6
6. Casein hydrolysate; glutamine, 2×10^{-6} M	18	3.8	2.7	18.3	127	8.6
7. Casein hydrolysate; organisms washed once	17	5.0	1.95	15.3	124	9.7
8. Casein hydrolysate; organisms washed thrice	17	5.0	1.90	13.6	119	8.5
9. Casein hydrolysate; organisms washed thrice and incubated in saline†	17	5.0	1.41	11.8	121	10.1
10. Casein hydrolysate; pyridoxin, 2×10^{-4} M	18	4.3	2.1	15.3	118	8.6

* Containing 2.7×10^6 viable organisms/mg. dry wt.† Containing 2.3×10^5 viable organisms/mg. dry wt.

‡ 10 mg. dry wt. of organisms in 10 ml. 0.9% (w/v) NaCl at 37° for 30 min.

these usually roughly paralleled the rates of glycolysis of the different preparations. This emphasized the dependence of glutamine breakdown on glycolysis; it was concluded that the different treatments did not have immediate effects on the reaction with glutamine. The conditions of growth included instances in which glutamine (medium 6, Table 1) or glucose (medium 3) were limiting factors in growth; organisms harvested at 12 hr. (medium 1) were at pH 7, while those harvested at 26 hr. (medium 2) had been at pH c. 5 for some hours. The effect of increased pyridoxin on growth was examined (medium 8) as a derivative of it acts as coenzyme in two reactions possibly relevant to glutamine (Gale & Epps, 1944; Gunsalus, Bellamy & Umbreit, 1944; Baddiley & Gale, 1945; Lichstein, Gunsalus & Umbreit, 1945) and the substance sometimes slightly accelerated growth of the 'Richards' streptococci in one of the experimental media, namely, that based on casein hydrolysate.

Concentration of glutamine in reaction-mixture, and time of reaction. The rate of NH_3 formation from glutamine during glycolysis by the 'Richards' streptococci (Table 2A) fell slightly during an hour's reaction, while the rate of glycolysis remained constant. In a separate experiment the streptococci were added to mixtures already containing glutamine and glucose; a relationship similar to that of Table 2A was observed, but with the superposition of short lags before glycolysis and glutamine breakdown reached their steady rates. The ratios of mol. NH_3 formed/mol. glutamine reacting did not markedly change during the experiment, but were between 0.88 and 0.95.

The fall in Q_{NH_3} observed at the end of the experiment of Table 2A was not due to the lowered concentration of glutamine which had then been reached. This is shown in Table 2B, where Q_{NH_3} is seen to be substantially unaffected by a 40-fold

variation in glutamine concentration. The range of concentrations of the table cover those of blood plasma and tissues (cf. Archibald, 1945), but do not fall to the values at which glutamine becomes a limiting factor in streptococcal growth.

Table 2. *Kinetics of ammonia production from glutamine*

A. (Glutamine (8.3 μ mol.) was added to glycolyzing streptococci (2.5 mg. dry wt.; Richards strain; final volume, 3.5 ml.) at 37° at a time taken as zero, in a series of vessels which were successively placed in ice at the times indicated. Values for Q_{NH_3} are calculated for the successive intervals.)

Time after mixing (min.)	NH_3 (μ mol.)	Q_{NH_3} (μ mol./mg. dry wt./hr.)
0	0	2.6
15	1.67	2.4
25	2.66	2.1
38.5	3.84	2.3
53.5	5.32	1.7
70	6.50	

B. (Organisms (Richards streptococci, 2.4 mg. dry wt.) were added at 37° to portions of the normal metabolic mixture containing the quantities of glutamine given below, in a final volume of 3 ml. After 34 min. the mixtures were cooled in ice and NH_3 determined. The values for mean concentrations of glutamine were calculated on the assumption that 1 mol. of glutamine disappeared for each mol. of NH_3 formed. See text, and McIlwain, 1946b.)

Initial glutamine (μ mol.)	NH_3 formed (μ mol.)	Mean glutamine concentration ($M \times 10^4$)
41.2	2.1	134
20.6	2.1	65
10.3	2.0	31.3
5.15	2.1	13.7
2.58	2.1	3.6

(Mean Q_{NH_3} 1.53 μ mol./mg. dry wt./hr.)

The effects of varying concentrations of glutamine on the duration and degree of stimulation caused in glycolysis have already been reported (McIlwain, 1946a).

Concentration of organisms in reaction mixture. This variable had a major effect on the stimulation of glycolysis. When, in the absence of added glutamine, a suspension of organisms was diluted so that the glycolyzing mixture contained c. 0.03 mg. dry weight of bacteria/ml. in place of c. 3 mg., their rate of glycolysis expressed as a metabolic quotient (i.e. after allowing for the decreased quantity of organisms) was lowered to about one-half. This occurred with organisms washed once and with others washed three times, and so was not dependent on the dilution of substances only loosely associated with the bacteria. The major effect of glutamine on glycolysis was to make good a large part of the deficiency induced by dilution (Table 3). The stimulation given by glutamine thus decreased with increasing concentrations of streptococci, as is illustrated in Fig. 2. The small stimulations in glycolysis quoted in Table 1 are associated with the relatively large concentration of organisms used in those experiments. As was shown previously (McIlwain, 1946a) the stimulation in glycolysis was not dependent on growth; optical density measurements during experiments of Table 3 were not found to change and so confirmed this.

In contrast with the results with streptococci comparable dilution of a suspension of an organism not requiring glutamine in growth did not affect its rate of glycolysis (Fig. 2).

The rates of decomposition of glutamine during glycolysis by 'Richards' streptococci in suspensions

of density 1.5 and 0.075 mg. dry wt./ml. were compared, and found to differ by < 10 %, though the stimulations of glycolysis in the two cases were markedly different (to 120 and 157 % respectively of controls without added glutamine). Similar results were obtained with 'Buxton' streptococci in suspensions of densities 2.7 and 0.27 mg. dry wt./ml.

Fig. 2. Relationship between the effect of glutamine on glycolysis, and the concentration of cocci in the glycolyzing mixture. Circles, the Richards streptococcus. Squares, a strain of *Staphylococcus aureus* which did not require glutamine in growth.

Rates of glutamine breakdown and of glycolysis. Streptococcal glycolysis continues for some hours in the absence of added glutamine, though the organisms themselves contain relatively little glutamine (see below). There is therefore no necessarily fixed ratio between rates of glycolysis and of glutamine decomposition. When both substances are

Table 3. Deficiency induced in glycolysis by dilution of bacterial suspension

(Richards (R) or Buxton (B) streptococci were added at a time taken as zero to vessels containing the normal metabolic mixture with and without glutamine (20 μ mol.) and of final volume 3 ml.)

Organisms; dry wt. (mg./3 ml.)	Change measured during period (min. after commencing reaction)	Q_{CO_2}				Ratio: $\frac{Q_{CO_2} \text{ with glutamine}}{Q_{CO_2} \text{ without glutamine}} \times 100$
		Without addition		With glutamine		
		(μ mol./mg. dry wt./hr.)	(As % of (a) in same experiment)	(μ mol./mg. dry wt./hr.)	(As % of (b) in same experiment)	
R (batch 1) 13	0-30	10.8 (a)	100	11.7 (b)	100	109
R (batch 1) 1.08	0-30	7.1	66	10.6	90	150
R (batch 1) 0.09	0-120	4.5	42	7.5	64	167
R (batch 2) 4.4	5-25	18.1 (a)	100	22.3 (b)	100	123
R (batch 2) 0.22	5-25	14.6	80	21.6	97	148
R (batch 2) 4.4	25-45	17.8 (a)	100	21.4 (b)	100	120
R (batch 2) 0.22	25-45	11.6	65	19.2	90	166
R (batch 3) 7.7	0-25	18.1 (a)	100	21.7 (b)	100	120
R (batch 3) 0.38	0-25	11.1	61	18.2	84	164
B (batch 1 washed once) 1.2	0-115	9.35	—	13.0	—	139
B (batch 1 washed thrice) 1.2	0-115	9.20 (a)	100	9.60 (b)	100	104
B (batch 1 washed thrice) 0.06	0-115	4.7	51	7.0	73	149

present in excess, a relatively stable ratio between the two rates is however observed. The ratio is not markedly dependent on the conditions of growth and treatment of the organisms. Tables 1 and 4 show that values for a given strain vary, but not greatly when it is considered that the values quoted include experiments with independently grown organisms over periods of several months. Beyond such variation, differences characteristic of the strain of organisms can be discerned.

Attempted extraction of NH₃-producing enzymes from streptococci

Though Wiggert *et al.* (1940) obtained by ground glass from *Escherichia coli* extracts which were capable of glycolyzing, such an extract was not obtained from the Richards and group G streptococci. Rubbing which disintegrated 90–99 % of the cells was carried out and extraction of the ground material was performed at pH 5, 7 and 8. Further,

Table 4. *Rates of glutamine breakdown and of glycolysis by haemolytic streptococci*

(Reactions were carried out as described in Table 1; streptococcal strains were β -haemolytic with the exception of the *faecalis* strain.)

Strain; temperature of growth	Temperature of reaction	Q_{CO_2} (μ mol./mg. dry wt./hr.)	Q_{NH_3} (μ mol./mg. dry wt./hr.)	$\frac{Q_{CO_2}}{Q_{NH_3}}$
R; 37°	30°	9.6	1.28	7.6
R; 37°	37°	17.2	2.26	7.6
R; 37°	42°	20.6	2.55	8.1
R; 25°	37°	16.0	1.92	8.3
B; 37°	37°	6.7–13.1	0.81–2.01	4–14
UC2; 37°	37°	6.8–24	0.23–1.55	13–38
<i>Strep. faecalis</i> ; 37°	37°	11.6–9.2	0.45–0.63	18–20.5
TI; 37°	37°	20.6	1.28	16.1

Tables 1 and 4 show no simple stoicheiometric relationship between the reactions in glucose and glutamine. The effect of glutamine in stimulating glycolysis may be expressed in terms of the additional CO₂ evolved while NH₃ is being formed from glutamine. No stoicheiometric relationship however exists between the NH₃ and additional CO₂ which are produced together. This reflects the observations above that (a) the percentage stimulation of glycolysis, and hence the additional CO₂, increases with decreasing concentrations of streptococci, while (b) the rate of NH₃ formation from glutamine does not markedly vary with concentration of organisms.

the initial, packed, moist organisms were prepared by centrifuging suspensions in the reaction mixture with glucose, glutamine and coenzymes in order to protect such systems as could be stabilized by these substrates.

Both the extracts and residues of ground organisms were examined for their ability to decompose glutamine, but reaction was not observed. It was absent not only in the usual media with and without glucose, but also in more concentrated solutions, buffered with bicarbonate or phosphate, in which the minimum dispersion of enzyme systems took place. In such an experiment, 1 g. moist weight of strepto-

Table 5. *Arginine dihydrolase activity of streptococcal extracts; absence of glutaminase*

(The Richards streptococci were used, ground with glass, and extracted with 0.03 M-phosphate (pH 7). Reactions were carried out as described by McIlwain (1946a) in 3 ml. of phosphate saline at 37°.)

Extract; wt. used (mg.)	Dry wt. of bacteria corresponding to portion of extract used (mg.)	Substrate (μ mol.)	Q_{NH_3} (μ mol./equiv. dry wt./hr.)
A; 142	37	Glutamine, 25	0.02
A; 99	26	Glutamine, 5	0.02 ($Q_{glut.} - 0.01$)
A; 99	26	Nil	0.02
A; 189	50	Arginine, 20	0.92
B (first extract); 91	6	Arginine, 20	0.85
B (first extract); 91	6	Nil	0.02
B (first extract); 122, heated at 100° for 3 min.	8.2	Arginine, 20	0.02
B (second extract); 282	18	Arginine, 20	0.35

cocci was ground and extracted with 1.5 ml. of a solution containing glucose, bicarbonate, adenosine triphosphate, hexosediphosphate, inorganic phosphate, cozymase and glutamine. An extract (1.1 ml.) was obtained of which 0.25 ml. was mixed with 0.1 ml. further solutions for a metabolic experiment. Glutamine breakdown and glycolysis by the extract were <5% of that of an equivalent quantity of whole organisms.

The inactivation of streptococcal enzymes by such disintegration was not a general one. The arginine dihydrolase (Hills, 1940) of such extracts was of special interest, as this enzyme leads to formation of NH_3 , but independently of glycolysis. Extracts were obtained from the streptococci which were slightly opalescent and contained no cells, and which rapidly decomposed arginine but were without effect on glutamine (Table 5). The first extract, made at pH 7, had, at pH 7, one-third of the activity of the organisms from which it was prepared. About a further one-sixth of the original activity was obtained on a second extraction of the same cells. The remainder was not necessarily lost, as the pH optimum and other properties relating to the activity of the extracted enzyme have not been determined.

Autolysis did not yield from the streptococci an enzyme decomposing glutamine.

Specificity of the glutamine structure in relation to streptococcal glycolysis

Comparison has been made of a number of substances with glutamine with respect to (1) production of NH_3 during glycolysis, and (2) stimulation of glycolysis. Results of the comparison are given in Table 6 except in the cases of a few substances whose effects have been described previously (McIlwain, 1946a). Sixteen compounds were examined, using the Richards streptococcus, and a few with a group G organism.

The rate of NH_3 production from glutamine in control experiments was about 2 $\mu\text{mol./mg. dry wt./hr.}$, and with the conditions adopted, production at a rate 1/50 of this could be detected. No material yielded NH_3 at a rate of > 0.05 $\mu\text{mol./mg. dry wt./hr.}$ with the exception of arginine and mixtures containing it; this has been reported previously and, as is noted above, occurred independently of glycolysis (Hills, 1940; McIlwain, 1946a).

Stimulation of glycolysis to the extent caused by glutamine was not produced by any compound examined. It was reported (McIlwain, 1946a) that NH_3 itself caused a lesser stimulation, and that a few compounds, known or presumed to produce NH_3 under the conditions of the present experiments, also

Table 6. *Effects on streptococcal glycolysis, and on NH_3 production, of compounds related to glutamine and to NH_3*

(Organisms (0.5–1 mg. dry wt.) were added to reaction mixtures containing glucose and bicarbonate (final vol., 3 ml.); conditions anaerobic.)

Reaction mixture		Q_{CO_2} as % of the value without addenda, for the same period		Mean Q_{NH_3} ($\mu\text{mol./mg.}$ dry wt./hr.)
Organism	Addenda ($\mu\text{mol.}$)	0-30 min.	30-60 min.	
A. Ammonia and simple amines				
R	NH_4Cl , 25	110-118	110-116	—
R	NH_4Cl , 2.5	108-116	107-115	—
R	$\text{CH}_3\text{NH}_2\text{Cl}$, 25	102	103	—
R	$\text{CH}_3\text{NH}_2\text{Cl}$, 2.5	103	104	—
R	$(\text{CH}_3)_2\text{NH}_2\text{Cl}$, 25	92	95	—
R	$(\text{CH}_3)_2\text{NH}_2\text{Cl}$, 2.5	103	97	—
R	$\text{C}_2\text{H}_5\text{NH}_2\text{Cl}$, 25.	103	97	—
B. Glutamine and related compounds				
R, G	Glutamine, 2.5-50	140-175	140-180	+1.5-2.5
R, G	L- or DL-Glutamic acid, 2.5-15	97-103	100-106	<0.05
R	Glutaric acid monoamide, 50	101	105	<0.03
R	α -Acetamidoglutaric acid amide, 10, 50	99, 102	96, 105	<0.03
R	DL-Uramidoglutaric acid, 2.5-15	97	98	<0.04
R	Pyrrolidone- α -carboxylic acid, 2.5, 15	96	100	<0.03
R	Pyrrolidone- α -carboxylic acid amide, 2.5, 15	104	102	<0.04
C. Other amino-acids				
R, G	Asparagine, 25	101	105	<0.03
R	Aspartic acid, 25	96	98	<0.04
R	Proline, 2.5, 20	103	104	<0.03
R	Citrulline, 20	101	114	c. 0.05
R	Arginine, 10	104	117	+3.9

stimulated glycolysis to values approximating to those of NH_3 . To such compounds may now be added citrulline, though the rate of NH_3 production from this compound was small (Table 6). Dimethylamine and ethylamine were without effect. The action of methylamine may be due to NH_3 in the specimen, which was not especially purified; it can be concluded that any effect of methylamine is less than that of NH_3 .

The action of inhibitors on glutamine breakdown, glycolysis and on growth

The effect of further substances on these three processes has been examined to see to what extent the response of the processes to given circumstances are correlated. Correlation would suggest their functional interrelation.

Fig. 3. Comparison of the effects of several inhibitors on streptococcal glycolysis in the presence of glutamine, and on the associated production of NH_3 from the glutamine. The Richards strain was used throughout; numbers beside the names of the inhibitors give their molarities ($\times 10^4$).

Phthaleins. Glutamine hydrolysis by streptococci has not yet been brought about in the absence of glycolysis; in previous attempts to do so by addition of substances inhibiting glycolysis, the glutamine breakdown was also prevented (McIlwain, 1946a). As bromsulphalein (a sodium salt of a disulphonic acid of tetrabromophenolphthalein) inhibits a glutaminase of animal tissues (Archibald, 1945), which is not described as being dependent on carbohydrate metabolism, its effect on glutamine metabolism in streptococci has been studied. Bromsulphalein has been found to inhibit the breakdown of glutamine by streptococci, but it inhibited also both glycolysis and

growth, often when present in relatively low concentrations. Phenolphthalein, fluorescein, eosin, and a triiodofluorescein were also potent inhibitors of both glycolysis and the breakdown of glutamine in streptococci (Fig. 3).

When the concentrations of the various substances which affect glycolysis and glutamine metabolism were compared, as in Figs. 3 and 4, glutamine breakdown was seen to be the more sensitive of the two processes. It was however the more sensitive to all reagents examined, which showed any selectivity

Fig. 4. Inhibition of growth, of the formation of NH_3 from glutamine, and of glycolysis, by different concentrations of bromsulphalein, eosin, and γ -glutamylhydrazine. Growth was studied as described in Table 7, and metabolism as described in Table 1.

between the two reactions; the reagents studied included monoiodoacetate, cyanide and propamidine. The results of Figs. 3 and 4 thus emphasize the general connexion between glutamine metabolism and glycolysis, without giving any certain evidence that the phthaleins might affect glycolysis through an action on glutamine metabolism.

The phthaleins inhibited also the growth of streptococci, and the effect of varying concentrations of eosin and bromsulphalein on growth was examined. Growth is seen (Fig. 4) to be somewhat more sensitive to these compounds than are glycolysis and glutamine decomposition. The values given in Fig. 4

for the concentrations of the substances affecting growth are approximate only, referring to those of a series of 3-fold dilutions which inhibited completely, or were without visible effect on, growth. They are however adequate to show a marked difference in character of action between the phthaleins and γ -glutamylhydrazine which is discussed below.

medium. The Richards and L strains were examined sufficiently to show that, with increasing concentrations of glutamine in growth media, increasing concentrations of the hydrazide were needed to effect a given degree of inhibition: the hydrazide competed with glutamine. The concentration of hydrazide required for a given effect did not however increase in direct proportion to the increase in

Table 7. *Action of varying concentrations of γ -glutamylhydrazine on streptococcal growth*

(Growth was described as prevented if visible opacity did not develop during the 3 days following inoculation under conditions described in the text. Little change was observed during the next 4 days.)

Strain	(a) Concentration of glutamine initially present in medium (M)	(b) Concentration (M) of glutamylhydrazine		Ratio b/a	
		Minimal to prevent growth	Maximal allowing growth	Preventing growth	Allowing growth
RNI	2×10^{-3}	10^{-2}	5×10^{-3}	5	2.5
RNI	2×10^{-4}	$2.5-5 \times 10^{-3}$	$1.25-2.5 \times 10^{-3}$	12.5-25	6.3-12.5
RNI	2×10^{-5}	$1.25-2.5 \times 10^{-3}$	$6.25-10 \times 10^{-4}$	62.5-125	31-50
L	2×10^{-3}	—	2×10^{-2}	—	10
L	2×10^{-4}	2×10^{-2}	10^{-2}	100	50
L	2×10^{-5}	5×10^{-3}	2.5×10^{-3}	250	125
<i>Strep. faecalis</i>	2×10^{-4}	4×10^{-2}	10^{-2}	200	50
<i>Strep. faecalis</i>	None added	10^{-2}	2.5×10^{-3}	—	—

The following experiments were carried out in attempting to see whether inhibition of growth by the phthaleins was related to glutamine. Inhibition of the Richards streptococci by bromsulphalein was compared in the presence of minimum glutamine for maximal growth, and in a concentration of 20 times that value. No marked difference was seen. Growth of a group G organism not requiring added glutamine was also inhibited by bromsulphalein, and the level required for inhibition was not affected by the presence of 10^{-3} M-glutamine. Tetrachlorotetraiodo-fluorescein behaved similarly to bromsulphalein in these two respects.

Effects on growth of compounds structurally related to glutamine. It was anticipated that such compounds might afford examples of inhibition connected with the metabolism of glutamine and of value in studying its effect on growth. Of the many substances examined (Roper & McIlwain, 1948) γ -glutamylhydrazine exhibited a type of action on growth which was promising from this point of view.

Results quoted in Table 7 showed several streptococcal strains to be inhibited by the hydrazide at concentrations of 6×10^{-4} to 2×10^{-2} M. The organisms of Table 7 required added glutamine to varying degrees (see Table 12) but the growth of all could be inhibited by the hydrazide; more of this was however required for inhibition of the less exacting *faecalis* or L strains. Inhibition by the hydrazide was in all cases antagonized by increasing the concentration of glutamine present in the growth

medium. The ratios of Table 7 (and also Fig. 5 which is discussed in detail below) show that a greater preponderance of the hydrazide is needed to inhibit the lower concentrations of glutamine.

The compound had no immediate bactericidal action (Table 8). As its action on growth was associated with glutamine, its effects on the metabolism of glutamine were examined.

Table 8. *Absence of immediate bactericidal action by γ -glutamylhydrazine and α -amino- γ -methylsulphinybutyric acid*

(The L strain of streptococci grown for 16 hr. was diluted in buffered casein hydrolysate.)

Substance added (M)	Colonies/ 1.5×10^{-4} ml. at times		
	0 hr.	2 hr.	6 hr.
Nil	475	317	244
γ -Glutamylhydrazine (0.02)	—	313	285
α -Amino- γ -methylsulphinybutyric acid (0.02)	—	348	255

Action of γ -glutamylhydrazine on glycolysis and on the production of NH_3 from glutamine. The type of experiment which was performed is illustrated by Table 9. From this, it can be seen that the hydrazide was effective as an agent inhibiting the formation of NH_3 from glutamine by a variety of streptococcal strains. Its action on the glycolysis with which the glutamine breakdown is associated was much less. In several instances, while NH_3 production was

Table 9. Action of γ -glutamylhydrazine on glycolysis and breakdown of glutamine by streptococci

(Warburg vessels contained the quantity of hydrazide needed to give the concentrations tested, with bacteria (c. 3 mg. dry wt.), salts and NaHCO_3 (0.06M) in 2.0 ml. of water; glutamine (in 0.5 ml. water) was added from a side arm after equilibration with 5% CO_2 in N_2 . A centre well contained yellow phosphorus.)

Strain	Reaction mixture		Ratio b/a	Products	
	(a) Glutamine (M)	(b) Hydrazide (M)		Acid formation as % of control without added glutamine and inhibitor	NH_3 formation as % of control with glutamine but without inhibitor
R	4×10^{-3}	2×10^{-2}	5	95	42
R	4×10^{-3}	6.6×10^{-3}	1.67	102	62
R	4×10^{-3}	2×10^{-3}	0.5	97	96
R	4×10^{-3}	0	0	131	100
L	4×10^{-3}	2×10^{-2}	5	99	14
L	4×10^{-3}	6.6×10^{-3}	1.67	110	50
L	4×10^{-3}	2×10^{-3}	0.5	113	100
L	4×10^{-3}	0	0	119	100
L	1.6×10^{-2}	6.6×10^{-3}	0.42	116	100
<i>Strep. faecalis</i>	3.3×10^{-4}	2.2×10^{-2}	6.7	95	33
<i>Strep. faecalis</i>	3.3×10^{-4}	{Methionine sulphoxide}	—	108	105
<i>Strep. faecalis</i>	3.3×10^{-4}	3.3×10^{-2}	—	108	100
		0			

lowered considerably, glycolysis was lowered only to a value about that found in the absence of glutamine.

Fig. 5. Comparison of the degrees of inhibition caused in growth and in the formation of NH_3 from glutamine (streptococcus R) by γ -glutamylhydrazine. Circles and shaded areas represent the effects on growth of varying concentrations of the hydrazide in the presence of varying concentrations of glutamine. The figures in squares give the percentage inhibition caused in the formation of NH_3 , at the concentration of glutamine represented by the abscissa value of the square, by the concentration of hydrazide represented by its ordinate value. C and ●, complete; P and ○, partial; N and ○, no inhibition of growth.

The process of NH_3 formation from glutamine is sensitive to about the same range of hydrazide concentrations, as is growth. Comparison of some of the values of Tables 7 and 9 shows this, and the two processes are compared directly in Fig. 4, which shows that an inhibition of 50% in the rate of NH_3

formation is associated with a marked inhibition of growth. Fig. 4, in comparing the action of γ -glutamylhydrazine on glycolysis, NH_3 formation and growth, indicates that it has a markedly different type of action from that possessed by the phthaleins and supports the suggestion that the action of the latter compounds on glutamine breakdown is a relatively indirect one.

The substance had little if any action on the assimilation of glutamic acid and glutamine (see below, and Table 11).

Comparison of the action of the hydrazide on growth and on glutamine decomposition. As the major metabolic effect which we have recognized as being brought about by γ -glutamylhydrazine is its action on the formation of NH_3 from glutamine, we have carried out a more extensive comparison between its inhibition of that process and its inhibition of growth. Both inhibitions are affected by the concentration of glutamine present, and are therefore represented in the fashion of Fig. 5. Here it is seen that in mixtures of the hydrazide and glutamine in which the decomposition is inhibited, growth is affected; 40–60% inhibition of the decomposition is associated with partial inhibition of growth, and 60–85% with complete inhibition of growth.

Glutamine synthesis by streptococci. Inhibition of the synthesis, and of growth of the organisms

Demonstration of the synthesis. It has been presumed that streptococci not needing glutamine in growth synthesize the compound, though the synthesis has not previously been demonstrated

(McIlwain *et al.* 1939; Fildes & Gladstone, 1939). Attempted determination of glutamine by NH_3 produced on hydrolysis gave negative results with culture fluids, but showed a small quantity of a glutamine-like substance to be associated with streptococcal cells. In order to study changes in the quantity of such material, the microbiological method of determining glutamine which has been described by Roper & McIlwain (1948) was developed. It was then found that a substance, possessing some chemical and microbiological properties of glutamine, could readily be obtained from streptococci grown in the absence of added glutamine. They had therefore synthesized it.

For further study it was desired to bring about the synthesis under conditions more defined than those of a growing culture. Washed streptococcal cells exposed to ammonium glutamate did not yield a glutamine-like substance; but of other materials present during the synthesis by growing cells, glucose, when added with ammonium glutamate, was found to result in the formation of a glutamine-like substance. Experimental conditions for the synthesis are given in Table 10, together with a comparison of the properties of the product with those of

glutamine. These properties, the conditions of production of the substance, and its consistent determination as glutamine by a method which itself is highly specific to glutamine make it probable that the material present in the cells is glutamine itself. Of other compounds, those known to be of the lability indicated in Table 10, and perhaps most likely to be present in the cells, are peptides of glutamine such as were studied by Melville (1935); these have however been found not to replace glutamine in growth of the assay organism (McIlwain, 1939). It can be seen from Table 10 that some 10% of glutamine is probably lost during the treatment employed for extracting it from streptococci. No correction for such loss has been applied to the results quoted in this paper, but the conditions of extraction have been rigidly adhered to and applied to washed cells; results have been found to be reproducible to within the limit of accuracy of the assay (about 4% of their value).

Kinetics. The assimilation of glutamic acid and glutamine. Gale (1947) has shown that an energy-yielding reaction such as glycolysis is required for entry of glutamic acid or glutamine into streptococcal cells, and during the present experiments ample confirmation of these observations has been found. The rate and extent of such assimilation is clearly relevant to glutamine synthesis and we have, therefore, followed assimilation while studying glutamine synthesis. Total glutamic acid and glutamine have been determined by the decarboxylase preparation from *Clostridium welchii* (Gale, 1945). Though this preparation liberates CO_2 from glutamic acid and from glutamine at different rates, we did not find the difference adequate to form the basis of a method for determining glutamine in the presence of glutamic acid in streptococcal extracts. The reagent was therefore used in quantity sufficient to determine both substances, and glutamine determined separately by the method described above. The quantity of glutamine found in cells was usually a fraction only of the quantity of glutamic acid. Both determinations were made after the extraction described in Table 10.

Several experiments on the course of entry of glutamic acid and glutamine are illustrated in Fig. 6. In these, glutamic acid was usually supplied together with 1 equiv. of NH_3 , at concentrations between 8×10^{-3} and 5×10^{-4} M. Initial concentrations of glutamine were similar. Glucose was usually present in excess of the quantity metabolized in the 20–45 min. experimental period, and phosphate buffer in quantity adequate to maintain the pH between 7.6 and 7.0. The quantity of streptococci used was between 3 and 10 mg. dry wt./ml. of reaction mixture, but in Fig. 6 results are expressed as the quantity of glutamic acid or glutamine entering unit weight of cells at various times; the

Table 10. *Properties of a glutamine-like substance produced by streptococcal cells*

(The material was obtained from 6.5 g. moist wt. of *Streptococcus faecalis*. The cells were shaken aerobically (anaerobiosis made little change in the result) with ammonium L-glutamate (0.16 mmol.), glucose (0.5 mmol.) and Na and K phosphates (1 mmol.) initially at pH 7.6, in 50 ml. water at 37° for 22 min., at which time the pH had become 6.8–7; the cells were washed and the material extracted from them by heating at 100° for 10 min. in 10 ml. 0.005 M-Na and K phosphates at pH 7. It was centrifuged free from cells and contained the equivalent of 160 μmol . glutamine/ml. Untreated cells extracted in the same way yielded a solution containing 18 μmol . /ml. of glutamine. The suspension of *Clostridium welchii* was prepared and used according to Gale's (1945) description.)

Treatment	Material undergoing treatment	Initial concentration (expressed as glutamine (M))	Material remaining (% of initial quantity)
10 min. at 100°, pH 7	Glutamine	10^{-4}	85–90
	Extract	10^{-4}	90–95
1 hr. at 100°, pH 6, in 0.2 M-phosphate	Glutamine	1.6×10^{-4}	42
	Extract	8×10^{-5}	32
	Glutamine	8×10^{-5}	30
	With extract	8×10^{-5}	
20 min. at 100° in $\text{N-H}_2\text{SO}_4$	Glutamine	8×10^{-5}	<5
	Extract	8×10^{-5}	<5
30 min. at 37° and pH 4.5 with suspension of <i>Cl. welchii</i>	Glutamine	10^{-4}	<4
	Extract	8×10^{-5}	<4

slopes of the lines joining experimental points therefore correspond to metabolic quotients.

Entry of glutamic acid and glutamine into the *faecalis* strains are compared in Fig. 6. The initial rates of entry which were observed in the two compounds were similar and corresponded to between 0.2 and 0.5 $\mu\text{mol./mg. dry wt./hr.}$ The experimental arrangements were such that these were not necessarily the maximal rates of which the organisms were capable, as the first reading was commonly taken after 8 min. exposure, and between it and the second reading a marked fall in rate of assimilation frequently occurred. A smooth curve through the experimental points of Fig. 6 suggested maximal initial rates approaching 1 $\mu\text{mol./mg. dry wt./hr.}$ in the presence of initial concentrations of glutamic acid or glutamine of $5.5\text{--}8 \times 10^{-3}\text{M}$. During assimilation, glycolysis was proceeding at the rate of about 10–15 $\mu\text{mol./mg. dry wt./hr.}$ (Table 11). Rate of entry varied with the concentration of added glutamic acid, in the manner indicated in Table 11. The initial rate of assimilation of glutamic acid from a solution $5.5 \times 10^{-3}\text{M}$ was not found to be affected by increase of the concentration of ammonium ion from 5.5 to $27.5 \times 10^{-3}\text{M}$. The final concentration of glutamine plus glutamic acid which was reached in cells was frequently greater when glutamine was supplied than when ammonium glutamate was used. This is seen in two instances in Fig. 6, and was most often found at the end of experiments in which the supply of glucose was failing.

Kinetics. The breakdown of glutamine during assimilation experiments. Streptococci rapidly break down glutamine when this is added to them in the presence of glucose. Before studying the possible

Fig. 6. The course of entry of glutamic acid and glutamine into three batches of cells of *Streptococcus faecalis*. Suspensions of bacteria were divided into portions containing c. 0.5 g. wet weight of cells, and these shaken at 37° with 15–20 ml. of 0.08M-Na and K phosphates of initial pH 7.5 and containing 0.017M-glucose and 0.008M-ammonium glutamate or glutamine. Cells were separated after the times indicated, washed, and their content of glutamic acid plus glutamine compared with that of a sample of untreated cells, after the extraction of Table 10. Full lines and black points, uptake following addition of glutamine. Broken lines and white points, uptake following addition of glutamic acid. Points of the same shape refer to experiments carried out simultaneously and with the same batch of cells.

Table 11. *Assimilation of glutamic acid from different initial concentrations; the course of assimilation and of lactic acid formation*

(The organisms and reaction mixtures (18 ml.) were as described in Fig. 6. Organisms A contained initially 1.7 $\mu\text{mol.}$, organisms B, 2.1 $\mu\text{mol.}$ and organisms C, 1.35 $\mu\text{mol.}$ of glutamic acid in the quantity of cells employed below; each contained $<0.02 \mu\text{mol.}$ of glutamine.)

Batch and dry wt. of streptococci (mg.)	Glutamic acid added		Glutamic acid assimilated ($\mu\text{mol.}$) in t min.		Glutamic acid remaining in solution (by difference)		Lactic acid formed ($\mu\text{mol.}$)
	($\mu\text{mol.}$)	($\text{M} \times 10^{-4}$)	(t)	($\mu\text{mol.}$)	($\mu\text{mol.}$)	($\text{M} \times 10^{-4}$)	
A, 120	54	30	8	3.75	50.2	28.0	294
A, 120	18	10	8	2.40	15.6	8.7	315
A, 120	9	5	8	2.00	7.0	3.9	—
A, 120	0	0	8	—	—	—	289
B, 168	100	62.5	8	1.35	99	55.0	223
B, 168	100	62.5	15	1.95	98	54.5	468
B, 168	100	62.5	30	2.30	98	54.5	502
C, 188	50	—	10	1.35	48.6	27.0	342
C, 188	50; with glutamylhydrazine, 500	30	10	1.45	48.5	26.9	322
C, 188	0	—	10	0	0	0	349
C, 188	0; with glutamine, 50	0	10	2.95	—	—	410
C, 188	0; with glutamine, 50, and glutamylhydrazine, 500	0	10	2.75	—	—	352

synthesis of glutamine, experiments were carried out to see whether any accumulation of glutamine within the cells could be demonstrated when the compound itself was added. Transitory accumulation has been found. Fig. 7 shows this. Here the concentration of glutamic acid plus glutamine rises during the first 20–40 min., and does not fall during the experimental period. Glutamine itself, however, falls in concentration after 10 min. Short experiments are therefore likely to be necessary to demonstrate its synthesis.

Fig. 7. The temporary accumulation of glutamine, and appearance of glutamic acid, in *faecalis* streptococci exposed to glutamine and glucose. Experimental arrangement, as described in legend to Fig. 6. Black points, glutamine. White points, glutamic acid plus glutamine. Points of the same shape refer to experiments carried out simultaneously and with the same batch of cells.

Kinetics. The synthesis of glutamine from ammonium glutamate. Fig. 8 shows incubation of *faecalis* streptococci with glucose and ammonium glutamate to result in a rise and then a fall of intracellular glutamine. The maximum quantity observed was only 1 mol. % of the ammonium glutamate added and 3 mol. % of the glutamic acid present in the cell at the same time. These values are to be compared with those of Fig. 6 describing the behaviour of added glutamine. Values for the same batch of organisms are available, and here the maximum accumulation observed was 8.5 % of the glutamine

added, and 16 mol. % of the intracellular glutamic acid. Synthesis is thus slower than is the entry of glutamine, but it is not known what reduction in the rate of entry would be necessary to give the observed accumulation. The maximum rate of accumulation which we have observed in synthesized glutamine is 0.02 $\mu\text{mol./mg./hr.}$, in cells capable of decomposing added glutamine at the rate of 0.6 $\mu\text{mol./mg./hr.}$

Connexion between the accumulation of synthesized glutamine in several streptococcal strains and their need for added glutamine in growth. Synthesis of glutamine has been observed in many streptococcal

Fig. 8. Accumulation of synthesized glutamine by *faecalis* streptococci. Experimental conditions, as legend to Fig. 6, employing ammonium glutamate with no added glutamine. Symbols, as legend to Fig. 7.

strains in addition to those of Fig. 8. In most cases accumulation was not followed kinetically, but the maximum values which have been observed during short experimental periods are quoted in Table 12. They are seen to vary inversely with the organisms' need for glutamine in growth. This need has been expressed as the increased lag observed in their growth, following the withdrawal of glutamine and the substitution of ammonium glutamate; details are given elsewhere (Hale & McIlwain, 1947). When so expressed organisms most exacting with respect to glutamine gave a value for increased lag (a totalled value under a set of arbitrarily chosen conditions) of about 35 days, and organisms not requiring added glutamine gave a value of zero.

Table 12 shows that the exacting organisms accumulated glutamine at a very slow rate, if at all; while non-exacting strains showed marked synthesis.

Table 12. *Variation in the accumulation of synthesized glutamine by several streptococcal strains*

(Organisms were grown in 1 or 0.5 l. of the medium based on casein hydrolysate and yeast preparation, and, in order to ensure uniformity of growth conditions, glutamine (10^{-5} M) was present in all cases although several organisms did not require its addition for their growth. At 14–24 (usually 16) hr., cells were harvested, washed twice in saline and portions representing about 0.5 g. wet wt. of organisms were used in 16 ml. of solution which contained ammonium glutamate, 0.003 M; glucose, 0.0125 M and phosphates at pH 7.6, 0.085 M. The mixtures were shaken aerobically at 37° and at 10 and 20 min. were cooled with ice and the cells separated and washed with saline. Their cell contents were freed as described in Table 10 and glutamine determined in the extracts. From values so obtained were subtracted the small quantities of glutamine initially present in the cells and determined on another portion which had been kept cold during the experiment. In instances in which it was examined, the solution after exposure of the cells to ammonium glutamate, contained no glutamine.)

Streptococcal strain	Lag on withdrawing glutamine (arbitrary total, days)	Maximum observed rate of accumulation of glutamine ($\mu\text{mol./g./hr.}$)
6176; β -haemolytic	31	<0.1
RNI; β -haemolytic	26	<0.2
6177; β -haemolytic	8	<0.1
L; β -haemolytic	3	0.3
6181; β -haemolytic	0	2.6
<i>Faecalis</i> ; laboratory strain	0	5.3
<i>Faecalis</i> ; N.C.T.C. 6796	0	2.2

Inhibition of growth by methionine sulfoxide. Of the compounds examined by Roper & McIlwain (1948), one was initially found to inhibit the growth of a *faecalis* strain of streptococci but not that of the Richards strain. This was α -amino- γ -methylsulphanylbutyric acid, the sulfoxide derived from methionine. The basis for its differential action was found to lie not in the organisms examined but in their conditions of growth; the *faecalis* strain, being non-exacting towards glutamine, was grown without added glutamine while the Richards strain was necessarily grown with glutamine. When the *faecalis* strain was grown in the presence of glutamine, α -amino- γ -methylsulphanylbutyric acid failed to inhibit it. Similar results have been reported with respect to lactobacilli by Waelsch, Owades, Miller & Borek (1946) and Borek, Miller, Scheiness & Waelsch (1946). We found the action of the sulfoxide on streptococci to be unaffected by cystine, methionine and homocystine. The relation of its action to glutamine thus showed a certain specificity. The

compound was not markedly bactericidal (Table 8); quantitative details of its action on growth are given below.

Fig. 9. Assimilation of glutamic acid and accumulation of synthesized glutamine in the presence of excess NH_3 . Conditions as legend to Fig. 6, with: (A), 100 $\mu\text{mol.}$ ammonium glutamate; and (B), 100 $\mu\text{mol.}$ ammonium glutamate plus 500 $\mu\text{mol.}$ NH_4Cl .

Inhibition of glutamine synthesis by the sulfoxide. Waelsch *et al.* (1946) suggested, on the basis of their growth experiments, that the sulfoxide affected lactobacilli through inhibiting their synthesis of glutamine. We have been able to demonstrate that the sulfoxide lowers the quantity of glutamine, formed from ammonium glutamate and glucose, and accumulating in streptococci under the experimental circumstances described in Table 13.

Many processes are occurring during such experiments, but the action of the sulfoxide was found to be a relatively specific one: the assimilation of glutamic acid is seen to be reduced to a smaller extent; in different experiments (Table 9) glycolysis was shown to be unaffected, and the breakdown of glutamine to glutamic acid not to be accelerated by the sulfoxide. It is likely, therefore, that the effect of the sulfoxide on the accumulation of glutamine is due to an inhibition of its synthesis.

Comparison of the effect of the sulfoxide on glutamine synthesis and on growth. The concentrations of methionine sulfoxide required for inhibition of growth were found to be comparable to those causing inhibition of the synthesis of glutamine. Both inhibitions depended also on the concentration of

Table 13. Action of methionine sulphoxide on the accumulation of synthesized glutamine

Organism	Reaction mixture*		Glutamic acid + glutamine of cells ($\mu\text{mol./g.}$ dry wt.)	Glutamine of cells ($\mu\text{mol./g.}$ dry wt.)	Glutamine accumulation in presence of sulphoxide as % of value in its absence $\left(\frac{c-a}{b-a}\right) \times 100$
	Ammonium glutamate (M)	Methionine sulphoxide (M)			
<i>Streptococcus faecalis</i> , laboratory strain	0	0	52	0.30 (a)	—
	2.8×10^{-3}	0	82	1.00 (b)	—
	2.8×10^{-3}	0.028	76	0.65 (c)	50
	0.55×10^{-3}	0	79	0.65 (b)	—
	0.55×10^{-3}	0.028	73	0.40 (c)	28
<i>Streptococcus faecalis</i> , N.C.T.C. 6459	0	0	15.9	0.29 (a)	—
	3×10^{-3}	0	79	13.2 (b)	—
	3×10^{-3}	0.1	43	0.29 (c)	0
	3×10^{-3}	0.033	—	0.57 (c)	2

* The reaction mixture in all cases contained approximately the quantity of organisms, glucose, and phosphate which are quoted in Fig. 6.

glutamic acid which was present at the same time, and their comparison is therefore presented in the form given in Fig. 10. This shows that decrease in the rate of accumulation of synthesized glutamine to

Fig. 10. Comparison of the degrees of inhibition caused in growth and in the accumulation of synthesized glutamine, by α -amino- γ -methylsulphophenylbutyric acid. Manner of representation: see Fig. 5.

about half its normal value results in a partial inhibition of growth; this becomes nearly complete when the accumulation is reduced to about a quarter of its normal value. It is therefore feasible that the inhibition of growth is caused by inhibition of glutamine synthesis.

DISCUSSION

The course of glutamine metabolism

It has been shown that synthesis and breakdown of glutamine occur in streptococci in accordance with the reactions of Fig. 1. These reactions are indispensable to most streptococcal strains. Both in growing and in non-proliferating organisms they are associated with other processes such as glycolysis,

and one reason for this lies in the requirement for a source of energy for the assimilation of glutamic acid and glutamine (Gale, 1947). The following considerations show, however, that this is not the only type of association between glutamine metabolism and glycolysis.

Some naturally-occurring streptococci do not synthesize glutamine, and in others the synthesis can be inhibited by α -amino- γ -methylsulphophenylbutyric acid. In these cases added glutamine allows growth, by providing the product of the system which is missing or inhibited. This confirms our previous knowledge of the importance of the first reaction of Fig. 1. But need for the second reaction is not obviated by providing glutamic acid and NH_3 . It is probable, therefore, that the second reaction is more complicated than indicated in Fig. 1 and other data support this view. First, glutamine stimulates streptococcal glycolysis. Secondly, the streptococcal system leading to production of NH_3 from glutamine may be contrasted with one of *Proteus morganii* (McIlwain, 1946c). This is easily extracted from the cells and does not require glucose for its activity. Glycolysis by *Pr. morganii* is unaffected by added glutamine.

Quantitative comparisons of processes involving glutamic acid in proliferating and non-proliferating bacteria

Four such comparisons, linking biological and chemical processes in streptococci, are made below on the basis of data from this and from other papers. The general problem which we wish to answer is whether the changes observed in non-proliferating cells are adequate in type and magnitude to form a basis for the behaviour of the organisms during growth.

Assimilation. The first problem is presented by the finding (Table 14) that streptococcal cells contain about 0.28–0.34 $\mu\text{mol./mg.}$ of glutamic acid combined in the cell structure, together with a more variable quantity of some 0.01–0.1 $\mu\text{mol./mg.}$ as free glutamic acid within the cell. The minimum rate of assimilation necessary to cause this accumulation can be calculated as follows. If $q = \mu\text{mol.}$ of substance found per mg. of cells in a culture growing exponentially, and $Q =$ the instantaneous rate of assimilation of substance in $\mu\text{mol./generation time per mg.}$, then by derivation such as that of Buchanan & Fulmer (1930), it can be shown that $q = Q \log_e 2$. The rates of assimilation necessary to account for the above cell contents of glutamic acid in cultures of a generation time of 20 min. are therefore

$$0.6 (= 0.29 \times 0.70 \times 3) \text{ to } 0.9 \mu\text{mol./mg./hr.}$$

Observed values for the rates of entry of glutamic acid and glutamine into non-proliferating streptococci have been collected in Table 14. They vary with the quantity of free glutamic acid and glutamine

already present in the cells (see below), and the rates in cells of low free glutamic acid agree reasonably well with those calculated above as necessary for growth with generation time of 20 min. The process, in the form in which it has been observed in non-proliferating suspensions, is therefore adequate to provide the quantity of glutamic acid found in cells. There is not, however, a large margin between the quantity observed to enter, and that which is required to enter for the production of cells of the observed composition at the rate of one generation in each 20 min. Also, the cells do not appear capable of assimilating and storing quantities of glutamic acid or glutamine which are large in comparison with that combined in their substance; the rate of assimilation falls as cell content increases. Figs. 7, 8 and 9 show this (see also Gale, 1947). The quantity found as free acid has rarely been one-third that existing combined, presumably as protein. These observations lead to the expectation that inhibition of reactions concerned with streptococcal glutamic acid will be reflected relatively promptly in the generation time of

Table 14. *Rates of reactions which involve glutamic acid and glutamine, in growing and in non-growing streptococci*

Process	Organism	Rate at 37° ($\mu\text{mol./mg.}$ dry wt./hr.)	Source of data; notes
Directly observed in non-proliferating suspensions, with glucose			
Assimilation of glutamic acid	<i>Faecalis</i>	0.40	Gale (1947); external glutamic acid, $9 \times 10^{-3} \text{ M}$; initial streptococcal glutamic acid, 6 $\mu\text{mol./g.}$
Assimilation of glutamic acid	<i>Faecalis</i>	0.25	Fig. 6; external glutamic acid, $8 \times 10^{-3} \text{ M}$; initial streptococcal glutamic acid, 25 $\mu\text{mol./g.}$
Assimilation of glutamine	<i>Faecalis</i>	0.72	Gale (1947); external glutamine, $9 \times 10^{-3} \text{ M}$; initial streptococcal glutamic acid, 6 $\mu\text{mol./g.}$
Assimilation of glutamine	<i>Faecalis</i>	0.40	Figs. 6 and 7; external glutamine, $8 \times 10^{-3} \text{ M}$; initial streptococcal glutamic acid, 25 $\mu\text{mol./g.}$
Conversion of glutamine to glutamic acid and NH_3	R	1.3–2.5	Table 4
Conversion of glutamine to glutamic acid and NH_3	<i>Faecalis</i>	0.5–0.6	Table 4
Accumulation of glutamine formed from ammonium glutamate	<i>Faecalis</i>	0.01	Fig. 8; organisms decomposed added glutamine at 0.6 $\mu\text{mol./mg./hr.}$
Rates calculated from content of cell or cell yield, as necessary to permit doubling of size of culture each 20 min.			
Assimilation of the glutamic acid which is found free in cells	<i>Faecalis</i>	0.02	Table 11; Gale (1947). Glutamic acid-poor cells of content 6–10 $\mu\text{mol./g.}$
Assimilation of the glutamic acid which is found free in cells	<i>Faecalis</i>	0.15	Table 13; Gale (1947). Glutamic acid-rich cells of content 50–100 $\mu\text{mol./g.}$
Assimilation of glutamyl residues of cell structure	<i>Faecalis</i>	0.60	Gale (1947); from cell content of 0.28 $\mu\text{mol./mg.}$
Assimilation of glutamyl residues of cell structure	—	0.66	Pollack & Lindner's (1942) estimate of cell content of glutamic acid (5%)
Assimilation of minimum quantity of glutamine permitting growth of ex-acting streptococci	R	0.12	Roper & McIlwain (1948)

growing cultures, and instances of this are discussed below.

Glutamine synthesis. In the growth of non-exacting streptococci, the importance of the synthesis of glutamine has previously been indicated by indirect evidence, but the process itself has not been demonstrated. The accumulation of glutamine in cells provided with ammonium glutamate and glucose has now been found to be transitory only. If the maximum observed rate is expressed as a metabolic coefficient, it corresponds to a value of $+0.01 \mu\text{mol./mg./hr.}$ As under such conditions glutamine is rapidly reconverted to ammonium glutamate, the rate of accumulation is unlikely to represent a rate of synthesis. Nevertheless, Table 12 shows that the rates of accumulation of glutamine in a series of streptococcal strains are inversely related to their need for added glutamine in growth. We may attempt to estimate the minimal needs of a streptococcus for glutamine (in distinction from glutamic acid) as follows. In an earlier paper (Roper & McIlwain, 1948) on the assay of glutamine, experimental conditions are chosen in which exacting streptococci are provided with excess ammonium glutamate but limited quantities only of glutamine, and are prevented from synthesizing glutamine from the ammonium salt. The quantity of organisms formed per unit weight of glutamine was found to be 15–20 mg. dry wt./ $\mu\text{mol.}$ of glutamine. 1 $\mu\text{mol.}$ of glutamine plus glutamic acid would be contained in 2–3 mg. organisms of the glutamine plus glutamic acid content of those of Table 14. We may conclude that the exacting organisms require at least one-fifth of their glutamyl residues as glutamine, and if non-exacting organisms make similar use of glutamine, synthesis at the rate of about $0.12 \mu\text{mol./mg./hr.}$ would be required. This is much higher than the observed rate of accumulation.

Inhibition of glutamine accumulation by α -amino- γ -methylsulphinybutyric acid. Although the observed rate of accumulation of glutamine is thus a fraction only of the probable rate of its synthesis, the sulphoxide affects the accumulation in a range of concentration closely paralleling that of its action on growth. Fig. 10 presents this well. We have also on occasions observed the sulphoxide to inhibit the assimilation of glutamic acid (Table 13), but at present express no opinion on possible causal relations between these effects.

Inhibition of glutamine breakdown. It appears from the similar comparison of Fig. 5 that inhibition of the conversion of glutamine to glutamic acid and NH_3 by the γ -hydrazide of glutamic acid also leads to a parallel inhibition of growth. This confirms the view, derived above, that this reaction in normal growth is associated with other changes, for glutamic acid and NH_3 do not prevent the action of the hydrazide on growth. Nevertheless, when the change,

which in resting cells produces these substances, is inhibited, growth is also inhibited. Both the synthesis and breakdown of glutamine are thus of functional importance to streptococci.

SUMMARY

1. (a) The rate of glycolysis (per unit weight of organisms) by non-proliferating suspensions of β -haemolytic streptococci, which required glutamine for growth, fell on reducing the quantity of organisms in suspension below values of about 3 mg. dry wt./ml.

(b) Glutamine largely made good this deficiency, but sixteen substances related to glutamine in various ways were ineffective in doing this.

(c) During its action in glycolysis, ammonia was formed from glutamine, but related compounds added in place of glutamine (e.g. asparagine, glutaric acid amide or pyrrolidone- α -carboxylic acid amide) did not lead to ammonia formation.

(d) The system responsible for glutamine breakdown was not obtained in cell-free form, though arginine dihydrolase was so obtained.

2. (a) γ -Glutamylhydrazine inhibited the production of ammonia from glutamine during streptococcal glycolysis. The concentration of the hydrazide required for this effect increased with increase in the concentration of glutamine present; when this concentration was between 0.4 and $1.6 \times 10^{-2} \text{M}$, the hydrazide at $0.2\text{--}2 \times 10^{-2} \text{M}$ gave about 50% inhibition in the rate of ammonia formation.

(b) A similar range of concentrations of the reagents gave partial inhibition of growth, and again greater concentrations of the hydrazide were required in the presence of greater concentrations of glutamine.

(c) Many substances (including a number of phthaleins), which inhibited glycolysis, inhibited also the formation of ammonia from glutamine, and inhibited growth, but the relative sensitivities of these processes were different from those found in the case of the hydrazide, and supported the suggestion that the primary action of the hydrazide was in the breakdown of glutamine, and that this led to inhibition of growth.

3. (a) In the presence of glucose, glutamic acid or glutamine entered the cells of non-proliferating streptococci at rates of about $0.5\text{--}1 \mu\text{mol./mg. dry wt./hr.}$, which is adequate to account for the production of successive generations of cells of the observed glutamic acid content of $0.3\text{--}0.5 \mu\text{mol./mg.}$ and growing with a generation time of 20 min.

(b) Streptococcal cells contained a small quantity of material with the chemical and microbiological properties of glutamine. The quantity in streptococci which did not require added glutamine in growth could be increased by brief incubation with ammonium glutamate in the presence of glucose.

(c) Glucose also led to breakdown of glutamine, and the accumulation in streptococcal cells was found to be transitory only. This applied to glutamine added as such, as well as to that produced from ammonium glutamate and glucose. The maximum observed rate of accumulation of synthesized glutamine was about $0.01 \mu\text{mol./mg./hr.}$ in cells capable of decomposing added glutamine at the rate of $0.6 \mu\text{mol./mg./hr.}$ The rate was much lower, and probably zero, in organisms requiring added glutamine in growth.

(d) α -Amino- γ -methylsulphonylbutyric acid inhibited the accumulation of synthesized glutamine in non-exacting streptococci. In one strain, $2.8 \times 10^{-2} \text{ M}$ -

solutions of the sulphoxide lowered the initial rate of accumulation from $2.8 \times 10^{-3} \text{ M}$ -ammonium glutamate by 50%. These concentrations of reagents led to partial inhibition of growth of the organism, and the action of other concentrations on growth and metabolism were similarly correlated. The compound was without action on growth in the presence of glutamine and its action on growth in the presence of glutamate was presumably due to inhibition of glutamine synthesis. Both the synthesis and breakdown of glutamine are thus of functional value to the organisms.

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The Estimation of the Leucine Isomers in Protein Hydrolysates by Infrared Analysis

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Certain of the monoamino-acid constituents of protein hydrolysates may be estimated by partition chromatography (Martin & Synge, 1941; Gordon, Martin & Synge, 1943; Tristram, 1946). This method, however, does not effect any separation of leucine $(\text{CH}_3)_2\text{CH}.\text{CH}_2.\text{CH}(\text{NH}_2)\text{COOH}$ and isoleucine $\text{CH}_3.\text{CH}_2.\text{CH}(\text{CH}_3).\text{CH}(\text{NH}_2)\text{COOH}$, the acetyl derivatives of which have identical properties under the conditions of the Martin & Synge technique. The present investigations were directed towards obtaining a rapid and accurate spectroscopic method for analyzing the isoleucine/leucine

ratio in the leucine fractions separated from protein hydrolysates by partition chromatography.

The possibility of analyzing a mixture of leucine and isoleucine by means of differences in their vibration spectra was first suggested by Edsall (1938) in work on the Raman spectra of amino-acids, but, as Edsall noted, the Raman technique presents many difficulties in such a problem. In the course of spectroscopic work on amino-acids, peptides and proteins, we recorded the infrared spectra of leucine and isoleucine and their acetyl derivatives, the latter for the first time. As might be anticipated from the