Thus the curves of response to doses of vitamin B_1 are not of significantly different slope when the doses of riboflavin are different and also the curves of response to doses of riboflavin are not of significantly different slope when the doses of vitamin B_1 are different. That is, with the rather small doses of vitamins employed in this experiment, there was no evidence of interaction of vitamin B_1 and riboflavin as judged by the increase in weight of the rat.

SUMMARY

1. The design is described of an experiment for testing (*a*) the interdependence of two vitamins (B_1 and riboflavin) in their influence on the increase in weight of the rat and (*b*) their possible interaction.

2. It was found that doubling the dose of either vitamin while keeping the dose of the other constant resulted in a significantly greater increase in weight of the rats. Doubling the dose of both vitamins resulted in a still greater increase in weight. Thus the response to one vitamin may be limited by the amount of another one given at the same time.

3. By an analysis of variance and by comparing the slopes of the response/log dose curves, it was found that the slopes of the vitamin B_1 curves were not significantly different when different amounts of riboflavin were given. The same was true of riboflavin in the presence of different amounts of vitamin B_1. Thus it was shown that with the small doses of vitamins used in this experiment there was no interaction of vitamin B_1 and riboflavin in their influence on the increase in weight of rats.

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The Metabolism and Functioning of Vitamin-like Compounds

3. PRODUCTS OF THE DECOMPOSITION OF GLUTAMINE DURING STREPTOCOCCAL GLYCOLYSIS

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Interest in the present reaction lies in the connexion which it may afford between a biological response of haemolytic streptococci to glutamine, and a metabolic response. The biological effect is a stimulation of growth (McIlwain, Fildes, Gladstone & Knight, 1939) and the metabolic one an increase in rate of glycolysis. While glycolyzing, the streptococci form a volatile base from added glutamine; but if glucose is not present—or if, while present, its breakdown is prevented—the reaction with glutamine does not occur (McIlwain, 1946). The products of the reaction have now been found to be glutamic acid and ammonia. As the reaction differs from that reported for some glutaminases in its dependence on glycolysis, it has been examined quantitatively, by several methods, for evidence of any chemical connexion with glucose or products of glycolysis. Also, attempts have been made to modify or couple the reaction by providing additional substances which might take part in further reactions with NH_3.

EXPERIMENTAL

Organisms. Certain of the strains of haemolytic streptococci used in Part 1 (McIlwain, 1946) were employed in the present work; they were grown as there described, but were maintained by drying newly passaged cultures *in vacuo*.

After recovery from mice, organisms were grown in blood-broth for 5 hr. and a drop of the culture pipetted to each of a number of ampoules. These were dried at room

temperature over P_2O_5 at 0·5 mm. Hg and sealed at that pressure. A fresh ampoule was opened each 10–14 days, its organisms grown for 5 hr. in blood-broth and the culture afterwards kept at 0°. Experimental material was grown from an inoculum of one loop of such a culture.

Metabolism. Organisms were collected by centrifuging and material from a culture of 100 ml. washed twice with saline. The material was then suspended in saline and measured samples normally placed in the sidearms of Warburg vessels of 15–20 ml. or of 100 ml. if several determinations were being made. The main compartments of the vessels contained glucose, 0·067 M; $NaHCO_3$, 0·05 M; glutamine, 0·0083 M, and the inorganic salts of Krebs & Eggleston (1940), with, however, only 0·001-M phosphate. Centre pots contained yellow phosphorus and the gas space, 5% CO_2 in N_2. Organisms were added to the metabolites at 37°, and the glycolysis followed manometrically. Reaction products were determined without separating the bacteria, which contributed relatively little of the substances concerned. Suspensions of the 'Richards' *Streptococcus* as used for the metabolic experiments were found to contain (μmol./mg. dry wt.): NH_3, 0·05; glutamine, 0·05, 0·09; 'amino-N' after 5 min. reaction with nitrite, 0·60, 0·71; after 20 min. reaction, 0·81.

Large-scale experiments from which glutamic acid was isolated were carried out with sterile precautions in a 250 ml. flask at 37° in a slow stream of N_2 with 5% CO_2, but without other attempt at anaerobiosis. Bicarbonate (M/2) was added during fermentation, to maintain the pH at 7–7·5.

l-(+)-Glutamine was purified as previously described (McIlwain, 1946). As arginine has been recorded as an unnoticed impurity in specimens of natural glutamine and as the present organisms readily liberate NH_3 from arginine, any of this substance present in a specimen of glutamine was separated by Archibald's (1945*a*) method; but no change was observed in the streptococcal reaction to the material. Glutamine was determined as amide-NH_3, labile under the conditions of Vickery, Pucher, Clark, Chibnall & Westall (1935) (cf. McIlwain, 1946).

Ammonia was determined after distillation *in vacuo* in a Parnas apparatus, usually acidimetrically. When estimated by Nessler's reagent, hypochlorite (Barrett, 1935) was added to the reagent described by Peters & Van Slyke (1932), and colour intensities were determined with a photoelectric absorptiometer.

'Amino-N.' Peters & Van Slyke's (1932) method and apparatus were used. Before carrying out determinations in solutions containing NH_3, this was removed by adding a borate buffer of pH 9 and evaporating twice *in vacuo* at 40°. This was shown not to cause decomposition of glutamine and to have little effect on the 'amino-N' of the bacteria. Glutamine yielded, in the concentrations in which it was being determined in mixtures from bacterial metabolism, 1·9 mol. of 'amino-N' per mol.

Substances yielding succinic acid after treatment with chloramine-T, and acid hydrolysis. Cohen's (1939) method for determination of glutamic acid was applied. In the present reaction mixtures, the yield of succinic acid from glutamic acid was 95–100%, and from glutamine 55–65% (cf. Cohen, 1939; Örström, Örström, Krebs & Eggleston, 1939). Chloramine-T did not liberate CO_2 from pyrrolidone-α-carboxylic acid, and no succinic acid was yielded by the subsequent treatment.

Separation of glutamic acid and glutamine. Repetition of Foreman's (1914) precipitation gave the necessary separation; the following experiment was designed to simulate circumstances anticipated after a streptococcal reaction with glutamine.

To 300 μmol. ammonium *l-(+)*-glutamate and 100 μmol. *l-(+)*-glutamine in 10 ml. water cooled by ice, were added 0·5 ml. 10% $CaCl_2$ and excess of a 20% suspension of $Ca(OH)_2$. The mixture was rubbed occasionally during 20 min., filtered, washed, and 60 ml. ethanol added to the filtrate and washings. A precipitate formed and was separated after 2 hr. at 0° and washed with 80% ethanol, dissolved in water, made to 10 ml., an aliquot kept for determination of glutamine and NH_3, and the bulk re-precipitated as above.

Both precipitates contained negligible NH_3; the first precipitate had 11·7 μ mol. of glutamine and the second, 1·9. Calcium was removed from the remainder of the second precipitate with oxalic acid, the solution evaporated to crystallization and ethanol added to 75% v/v. The yield of glutamic acid corresponded to 207 μmol. in the whole (70% of that added).

Recovery of glutamic acid from mixtures after streptococcal glycolysis. When glutamine was known to be absent, Neuberg & Kerb's (1912) precipitation as the Hg salt of the carbamido derivative was employed; the following experiment showed its applicability.

Streptococci ('Richards' strain; 30 mg. dry weight), in the metabolic mixture above (80 ml., but containing no glutamine), were allowed to glycolyze for 11 hr.; this required the addition of a further 8 ml. M/2$NaHCO_3$. The mixture was halved and *l(+)*-glutamic acid (578 μmol.) added to one half. Na_2CO_3 (0·5 g.) was added to each half, the mixtures separated from bacteria by centrifugation, and mercuric acetate added until a yellow precipitate formed. This happened almost immediately in the control, but in that containing glutamic acid, only after a considerable white precipitate had separated. Ethanol (250 ml.) was added to each mixture and after 2 hr. the precipitates were collected, washed with 80% alcohol, suspended in water, and decomposed with H_2S. The solutions after removal of HgS were evaporated *in vacuo* to *c.* 1 ml., taken to pH 3 by NaOH, and ethanol added to 60% (v/v). Glutamic acid separated from the solution to which it had been added: yield, 503 μmol. (87%). None was obtained from the other.

RESULTS

(1) *Characterization of volatile base.* The base, produced in a ratio of just less than 1 mol. per mol. of glutamine decomposed (McIlwain, 1946) could be determined consistently by Parnas' method for NH_3. It has been shown to be NH_3, rather than other simple amines, by comparison of the apparent quantities of substances when estimated by acidimetry and by Nessler's reagent (cf. François, 1907) (Table 1). The quantities were in close agreement, in the case of the base derived from glutamine

either during glycolysis or by acid hydrolysis; they differed markedly in the case of substituted ammonias.

Table 1. *Characterization of the volatile base from glutamine*

Metabolism (occupying about 1 hr.) was carried out anaerobically in Warburg vessels of 15–20 ml., with the reaction mixture described in the text. Material neutralizing acid after distillation in steam at 30° in a Parnas apparatus is described as a volatile base.

Origin of base	(*a*) Volatile base determined by acidimetric titration (μmol.)	(*b*) NH_3 determined by Nessler's reagent (μmol.)	$b/a \times 100$
Aliquots ($\frac{3}{20}$) of mixture from glycolysis by 6·9 mg. 'Richards' strain streptococci for 45 min. in the presence of 50 μmol. glutamine	2·28, 2·28	2·22, 2·38	98, 104
Aliquots ($\frac{1}{2}$) of mixture from glycolysis by 4·3 mg. Group G streptococci for 100 min. in the presence of 30 μmol. glutamine	5·80	5·78	100
10^{-3}M glutamine heated for 2 hr. at 100° in phosphate buffer, pH 6	7·30	7·48	102
Methylamine hydrochloride	2·50	0·56	22
Methylamine hydrochloride	1·25	0·21	17
Methylamine hydrochloride	0·63	0·09	14
Ethylamine hydrochloride	20	0·05	0·3
Dimethylamine hydrochloride	20	0·05	0·3

(2) '*Amino*-N' *change during reaction.* The N_2 produced by HNO_2 from the reaction mixture (after removal of NH_3) fell during glycolysis to the extent shown in Table 2 (A, B). The loss amounts to 92% of the loss in labile amide-NH_3, and 107% of the gain in NH_3. Such values are consistent with the main products from glutamine being glutamic acid and NH_3. As, however, carbohydrate metabolism is proceeding much more quickly than glutamine breakdown (Table 2 indicates the ratio Q_{CO_2}/Q_{NH_3} to be about 10), and the two processes are interdependent, many other products are possible.

(3) *Change in yield of succinic acid after treatment with chloramine-T and acid hydrolysis.* Direct production of succinic acid by the present streptococci during glycolysis in the presence or absence of glutamine, was found to be very small (McIlwain, 1946). Succinic acid as determined in reaction mixtures after Cohen's (1939) treatment, increased during glycolysis in the presence of glutamine. The only interpretation of this result, which is consistent with the known behaviour of substances as recorded by Cohen (1939), Örström, Örström, Krebs & Eggleston (1939), and in the experimental part of the present paper, is the production of a large proportion of glutamic acid. The increase in succinic acid formation then follows from its production in greater yield from glutamic acid than from glutamine. Assuming (*a*) that the yield of succinic acid from glutamic acid is 100% and from glutamine after metabolism is the same as that before metabolism; and (*b*) that glutamic acid and NH_3 are the only products from glutamine, then the quantity of glutamic acid apparently formed can be calculated from the data of Table 3. The accuracy of the result is limited by the variable yield of succinic acid from glutamine, which has been noted above. In A, Table 3, this yield is 56%; of the final succinic acid, 56% of 36 μmol., or 20·1 μmol., is then contributed by glutamine leaving 53·2–20·1, or 33 μmol., to be derived from glutamic acid. This constitutes 1·14 mol. per mol. of glutamine decomposed. In B, Table 3, the corresponding ratio is 0·85.

Table 2. *Loss of 'amino-N' during glutamine metabolism*

Experiments were carried out as described in Table 1, but in vessels of about 100 ml. capacity. Reaction mixtures contained glutamine (4×10^{-3}M) except the final one, which contained no added glutamine but 72 μmol. of ammonium glutamate. The CO_2 recorded is that from bicarbonate, mainly by displacement by the lactic acid formed in glycolysis (McIlwain, 1946) and the values are given to indicate the extent of glycolysis during the glutamine metabolism.

Reaction mixture	Substance determined	μmol. Originally	μmol. Finally	μmol. Change	Metabolic quotients (μmol./mg. dry wt./hr.)
A	Labile amide-N	47·7	11·1	−36·6	−3·17
	NH_3	0	29·8	+29·8	+2·58
	'Amino-N'	103	72	−31	−2·70
	CO_2 from bicarbonate	—	—	263	+22·8
B	Labile amide-N	60·5	19·5	−41	−1·61
	NH_3	0	36·2	+36·2	+1·42
	'Amino-N'	129	89	−40	−1·57
	CO_2 from bicarbonate	—	—	449	+17·1
C	Labile amide-N	0	0	0	0
	NH_3	74·7	71·3	−3·4	−0·24
	'Amino-N'	74·3	70·0	−4·3	−0·31
	CO_2 from bicarbonate	—	—	289	+21·0

Table 3. *Increase in succinic acid obtainable by Cohen's (1939) procedure, following streptococcal metabolism of glutamine*

Reaction mixtures (containing 4×10^{-3}M glutamine) and metabolic methods were those described in Table 2. Reaction B refers to the same batch of organisms as Table 2, B.

Reaction mixture	Substance determined	μmol. Originally	μmol. Finally	μmol. Change	Metabolic quotients (μmol./mg. dry wt./hr.)
A	Labile amide-N	65	36	−29	−2·4
	NH_3	0·5	26	+25·5	+2·1
	Succinic acid	36·3	53·2	+16·9	—
	CO_2 from bicarbonate	—	—	269	+22·3
B	Labile amide-N	60·5	19·5	−41	−1·61
	NH_3	0	36·2	+36·2	+1·42
	Succinic acid	37·2	46·8	+9·6	—
	CO_2 from bicarbonate	—	—	449	+17·1

(4) *Isolation of glutamic acid.* Foreman's method was applied, as described in the experimental part, to a mixture resulting from the action of the 'Richards' strain streptococci (27 mg. dry wt.) on the normal metabolic mixture containing glutamine (250 μmol.). Of this, 195 μmol. were decomposed and 85 μmol. of glutamic acid was isolated; it had m.p. 206° and mixed m.p., with an authentic specimen of *l*(+)-glutamic acid (m.p. 211°), 210°.

A higher yield was obtained by taking the reaction with glutamine to completion and separating the product by Neuberg & Kerb's (1912) method. This was applied to a mixture resulting from the action of 'Richards' strain streptococci (51 mg. dry wt.) in the normal metabolic mixture containing 878 μmol. of glutamine. After 10 hr., analysis of aliquots showed 35 μmol. of glutamine to remain; after 11 hr. the product was separated as described above and yielded 735 μmol. of glutamic acid, m.p. 203°; mixed m.p. with an authentic specimen of the *l*(+) acid (m.p. 211°), 209°. It was once recrystallized from aqueous ethanol, and dried *in vacuo* over P_2O_5 at 100°; it then melted at 211° and yielded (Kjeldahl) NH_3, 6·6 μmol./mg. (calc. for $C_5H_9O_4N$, 6·8). The optical rotation in 3N-HCl was

$$[\alpha]_D^{22°} = +31° (\pm 0·5°),$$

which is that of the 'natural' isomeride (cf. Huffman & Borsook, 1932).

(5) *Secondary changes in glutamic acid and* NH_3 *after their formation.* The changes which have been recorded in glutamine, NH_3, and 'amino-N' are only roughly in stoichiometric proportions. To see to what extent this could be attributed to changes in glutamic acid and NH_3 after their formation, streptococci were caused to glycolyze in the presence of added ammonium glutamate (C, Table 2). The disappearance of a small proportion of both NH_3 and 'amino-N' was observed, but without the formation of glutamine. The changes are of such a magnitude as may explain the greater change which has been observed to take place, during the main reaction, in glutamine than in NH_3 and 'amino-N'.

(6) *Influence of possible* NH_3 *acceptors on* NH_3 *formation from glutamine.* Ammonia formation by streptococci from glutamine during glycolysis, was followed in the presence and absence of substances which might combine with the NH_3 (Table 4).

Table 4. *Influence of added substances on glutamine breakdown*

Reactions between glutamine, glucose, the 'Richards' strain streptococci and added substances were carried out anaerobically in the bicarbonate-saline; controls lacked the added substance.*

Added substance (molarity)		NH_3 produced (% of control)	Glutamine reacting (% of control)	CO_2 evolution (% of control)
Pyruvate	$(1·4 \times 10^{-2})$	109	103	100
Pyruvate	$(3·5 \times 10^{-3})$	105	103	102
α-Keto-glutarate	$(1·4 \times 10^{-2})$	106	100	97
α-Keto-glutarate	$(3·5 \times 10^{-3})$	103	100	97
α-Keto-glutarate	$(1·4 \times 10^{-2})$*	98	—	100
α-Keto-glutarate	$(1·4 \times 10^{-3})$*	96	—	102
Oxaloacetate	$(1·4 \times 10^{-2})$*	105	—	102
Oxaloacetate	$(1·4 \times 10^{-3})$*	100	—	102
Inosine triphosphate	$(6·7 \times 10^{-3})$	100	100	104
Inosine triphosphate	$(1·4 \times 10^{-4})$	103	100	110
Hypoxanthine	(5×10^{-3})	100	—	105
Uracil	$(7·5 \times 10^{-3})$	51	45	86
Uracil	(5×10^{-3})	74	75	93

* The streptococci used were grown with added pyridoxin (see text).

Pyruvate, α-ketoglutarate, oxaloacetate, inosine triphosphate (Kleinzeller, 1942) and hypoxanthine were without marked effect. Uracil decreased NH_3 formation but also decreased glutamine breakdown and glycolysis. The growth of the streptococci in the present medium was sometimes slightly accelerated by pyridoxin, derivatives of which act as co-enzymes in decarboxylation of (Gale & Epps, 1944; Gunsalus, Bellamy & Umbreit, 1944; Baddily & Gale, 1945) and transamination (Lichstein, Gunsalus & Umbreit, 1945) between amino-acids. A batch of streptococci grown with 2×10^{-4}M pyridoxin reacted with glutamine at the usual rate and did not behave differently towards the keto-acids (Table 4).

DISCUSSION

The isolation of glutamic acid and characterization of NH_3 in the products from the action of non-proliferating streptococci on glutamine leave no doubt that the overall reaction is, to the extent of at least *c.* 85%, a simple hydrolysis:

$$l(+)C_5H_{10}O_3N_2 + H_2O \rightarrow l(+)C_5H_9O_4N + NH_3.$$

The various determinations which were carried out during the course of the reaction did not indicate the temporary accumulation of other substances. Ammonia as produced from glutamine did not appear more capable of undergoing further changes in streptococci than did NH_3 added as such.

The following aspects of the relationship between glutamine and streptococci must be considered in assessing the present results.

(1) *Glutamine and glutamic acid as cell constituents.* A major need in growing organisms is the production of new bacterial substance. Pollack & Lindner (1942) calculated that 1 mg. of several organisms which needed added glutamine or glutamic acid, both contained and required in growth about 0·05 mg. (or 0·3 μmol.) of the substances. Taking 15 min. as a minimum mean generation time for the present streptococci, in this time 1 mg. of organisms of $Q_{glut.} - 1$ to -3 will have decomposed 0·25 to 0·75 μmol. of glutamine. They thus perform the reaction at about the rate at which they would be incorporating glutamine or glutamic acid into new cells, were they growing. This may be a coincidence or may indicate an association of the present reaction with growth. Gale (1946) observed that neither glutamic acid nor glutamine entered the cells of certain streptococci in the absence of substances which could act as energy-sources; this is relevant also to (3), below.

(2) *The stimulation of glycolysis by glutamine* nevertheless is not secondary to growth in the ordinary sense, as it occurs within two minutes of the addition of glutamine to glycolyzing streptococci. A function of glutamine in intermediary metabolism, such as that discussed earlier (McIlwain, 1946) remains likely, especially in view of many of the widespread biological activities of glutamine referred to by Archibald (1945*b*).

(3) *Dependence of glutamine breakdown on glycolysis.* If the computations of (1), above, reflect a functional connexion, it is one between glutamine breakdown and synthetic processes, which in very general terms might be expected to require the energy and materials made available by glycolysis. The means by which the connexion is normally effected, and by which a certain connexion is maintained in non-proliferating suspensions, remain unknown.

SUMMARY

1. Non-proliferating suspensions of haemolytic streptococci can convert $l(+)$-glutamine to $l(+)$-glutamic acid and NH_3.

2. The action differs from that of other 'glutaminases' in that it has so far been observed only during glycolysis; but intermediate compounds which might link glutamine decomposition with glycolysis have not been detected, nor have conditions been discovered under which the NH_3 takes part in secondary reactions at a comparable speed.

3. The rate of glutamine decomposition (1–3 μmol./mg. dry wt./hr.) is of the same order of magnitude as that at which glutamine or glutamic acid would be assimilated by growing organisms.

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