XXVII. GLUTAMINE AND THE GROWTH OF *STREPTOCOCCUS HAEMOLYTICUS*

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In the course of our analysis of the nutrients of *Streptococcus haemolyticus* it was found that growth did not take place in a mixture containing peptone, cystine, glucose, phosphate and a number of other substances until an extract of meat also was added. Good growth then took place in some 16 hr. whereas in the culture without meat extract no growth was visible during the period of observation, usually 2 days but often 3 or 4 and on one occasion 9 days.

The test coccus was the well-known strain "Richards" maintained at high virulence by repeated passage through mice and kept in the intervals in rabbit blood. For the purposes of this work it was transferred from the blood to nutrient agar as required for the tests. After a variable number of subcultures on agar it was found that the coccus was of no further use as a test object in that it became able to grow on the peptone mixture without our extract of meat. This phenomenon of alteration in nutrient requirements we ascribed to the development of a power to synthesize our meat extract factor which was lacking while the organism was maintained in blood. Since the power to synthesize our factor was thus potentially present, positive results obtained by the addition of some fractions of meat, which were obtained only after 3 or 4 days' incubation, were suspect even though the control without the addition might be negative. In such cases it was possible that the fraction of meat did not contain the active substance we were seeking but did contain (*a*) a related compound from which our active substance was synthesized, or (*b*) some not necessarily specific substance which accelerated the enzymic processes utilized in the synthesis. In order therefore to ensure a test object which was incapable of growth in the absence of our meat extract we used a culture which was recovered from the blood every 7 days; and to satisfy ourselves that we were titrating our active substance itself and not something else which facilitated its synthesis we judged the potency of meat fractions by readings of growth up to 24 hr. and not 2 or 3 days.

Bacteriological technique

The general technique used on the bacteriological side of this work has followed that customary in this laboratory in nutritional studies of bacteria. The inoculum used has been relatively small. A small mass of bacteria was raked from an agar surface in such a way as to remove none of the medium and transferred to water. This was thoroughly suspended with a Pasteur pipette and diluted until the suspension was just invisible to the naked eye. One drop of this was used per tube. A precise enumeration of the number of cocci in a

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streptococcal suspension is impracticable but the number of colonies which grew on plating out the inoculum was usually about 1 million. It may, however, be stated that the strain used by us was capable of growing in the mixture to be described when the inoculum was reduced approximately to one coccus.

The inadequate basal medium used for the demonstration of the activity of the meat extracts was made as follows:

Bacto peptone (Difco)	10·0 g.
NaCl	5·0 g.
KH_2PO_4	4·5 g.
N NaOH	26 ml.
Water	to 700 ml.

Adjust to *p*H 7·4; distribute in 7 ml. lots in test tubes (6 in. × ¾ in.) and autoclave. At the time of testing add to each tube:

Sample to be tested (filtered)		1 ml.
M/18 phosphate buffer *p*H 7·4 (autoclaved)	1·05	2 ml.
M/20 cystine HCl in *N*/10 HCl (filtered)	0·2	
N/5 NaOH (autoclaved)	0·25	
Mixture *A*	0·5	
Inoculum	1 drop	

Mixture *A* consisted of:

Aneurin	0·005 g.	Thymine	0·01 g.
Nicotinamide	0·005 g.	Guanine	0·01 g.
β-Alanine	0·005 g.	KH_2PO_4	0·3 g.
Pimelic acid	0·05 g.	Glucose	5·0 g.
Uracil	0·01 g.	Riboflavin *M*/5000,	20 ml.
Cytosine	0·01 g.		

Uracil, cytosine, thymine and guanine were dissolved in 50 ml. *N*/10 H_2SO_4 and neutralized with NaOH to *p*H 6·0. The other ingredients were then added and water to 90 ml. Readjusted to *p*H 6·0 and made up to 100 ml. Filtered Seitz EK.

The tubes were incubated in an upright position at 37° in air + 5 % CO_2.

With regard to the constituents of mixture *A*, glucose is, of course, necessary as a source of energy but the part played by the others in growth has not yet been determined. In the absence of all, growth is usually but not always absent during the first day, but it may occur on the second day.

Chemical fractionation of meat

Extraction. The sodium sulphate method of Deutsch *et al.* [1938] was applied to fresh horse meat. The fat was cut from meat (20 lb.); the flesh minced (7·38 kg.; water content, 75 %), mixed with anhydrous Na_2SO_4 (2·77 kg.; ½ of the weight of water in the tissue) and the mixture twice reminced to a homogeneous stiff brown paste. This was heated with frequent stirring in a water bath at 45–50°; its temperature rose to 32° in the first 15 min. and remained so for a further 15 min. during the dissolving of the Na_2SO_4. The paste then became sloppier and its temperature rose. It was expressed at 35°; the extract cooled to room temperature; $Na_2SO_4, 10H_2O$ filtered off; the mother liquors cooled to 0° and more $Na_2SO_4, 10H_2O$ removed, leaving 1160 ml. of meat juice at about 2½ times its concentration in the tissues and containing about 4 % Na_2SO_4. In several

batches the yields ranged from 140 to 180 ml./kg. of fresh meat or about 50–60 % of the amount theoretically obtainable from the water content of the tissue. The efficacy of the various methods of separation attempted was judged by determination of the total N and growth activity of the fractions. Results obtained with the fractions described are given in Table III (p. 228).

First mercury precipitation. Mercuric acetate precipitated only part of the activity in the Na_2SO_4 extract; precipitation was complete under Neuberg's mercuric acetate-carbonate-alcohol conditions, but an equally complete precipitation was obtained with mercuric acetate in neutral 60 % alcoholic solution.

The extract (1000 ml.) was mechanically stirred and excess of finely powdered mercuric acetate (*c.* 100 g.) added, the solution neutralized with 10*N* NaOH (*c.* 60 ml.), alcohol (1500 ml.) added and the whole kept 2 hr. The precipitate was collected, freed from associated material by washing 3 times with water (500 ml.), stirred in water (500 ml.) in a closed jar and decomposed by H_2S under slight pressure. The HgS was filtered off and washed with water. The filtrate and washings from the sulphide contained almost the whole of the activity of the original extract and when evaporated in a vacuum gave a gum which retained its activity on keeping.

Phosphotungstic acid precipitation. Mercury-precipitated material equivalent to 1000 ml. of original extract was dissolved in 2*N* H_2SO_4 (300 ml.) and 20 % phosphotungstic acid in 2*N* H_2SO_4 added gradually with continuous stirring till in excess (*c.* 650 ml.). The precipitate was filtered after 4 hr. and washed 3 times with 2*N* H_2SO_4 containing a little phosphotungstic acid. H_2SO_4 and excess phosphotungstic acid were removed from the combined filtrate and washings, with baryta (*c.* 420 g.), excess Ba precipitated with H_2SO_4 and the solution filtered and evaporated under reduced pressure to 30 ml. Inorganic salts separated on keeping and were filtered off.

Properties of meat concentrates. The active material was not removed from aqueous solution by a wide variety of adsorbents and immiscible solvents at a variety of *p*H. In the Na_2SO_4 extract it was stable at 0° for several weeks. It was not very stable to heat: a little loss occurred at *p*H 5, 6 and 7 during heating for 1 hr. at 100° and $\frac{4}{5}$ of the activity was lost in 3 hr.; losses were slightly greater in all cases at *p*H 8 and 9. It was stable to mild oxidizing and reducing agents (air, silver salts; H_2S, SO_2). The following indications of its amino-acid nature were obtained: it was more completely precipitated by $Hg(CH_3CO_2)_2$-Na_2CO_3 in aqueous solution than by $Hg(CH_3CO_2)_2$ itself; it yielded a moderately insoluble copper salt which was not, however, useful in separation; it was destroyed by nitrous acid at room temperature in dilute aqueous solution and by alloxan during 10 min. at 100°.

Observations on glutamic acid and glutamine

At this point we observed the note by Rane & Subbarow [1938] in which they described the growth of *Str. haemolyticus* in a gelatin-hydrolysate medium containing, in addition to other substances, glutathione which they considered to be a "significant" constituent of the nutrients. We tested glutathione in the concentration suggested in combination with various constituents of our mixtures not referred to here and in particular as a replacement of our meat extract factor, and in the latter circumstances found that it gave a trace of activity, which trace was increased by increase in the concentration of glutathione. The activity of our meat extracts could not however be due to glutathione for the following reasons:

(1) Our active concentrates contained no demonstrable SS—SH group.

(2) Cuprous oxide, under the conditions described by Hopkins [1929] for glutathione, precipitated from our concentrates a small quantity of cuprous salts which contained only a trace of the original activity.

(3) Glutathione in concentrations equal in activity to our purer preparations, which were however still impure, contained about 5 times as much nitrogen.

(4) The stability and other properties of our material were different from those of glutathione.

The small activity of glutathione in growth tests would have suggested that this might be due to a biologically active impurity in our glutathione preparations, had not Rane & Subbarow found a synthetic preparation also active.

The somewhat trivial effects suggested that they might be non-specific in the sense already referred to and due merely to an improvement in the general metabolism brought about by increase in the amino-N content of the nutrients. In other work we had found that increases in the concentrations of particular amino-acids might have a critical effect upon growth. We therefore carried out tests in which the constituent amino-acids of glutathione, glycine and glutamic acid, were severally increased in concentration, cystine being already adequately represented.

Table I shows that high concentrations of synthetic glutamic acid were capable of inducing growth comparable in rapidity and mass with that resulting from our meat extract. It was shown that aspartic acid had no such action.

Table I. *Effect of glutamic acid as a replacement for meat extract*

A specimen of *dl*-glutamic acid synthesized by McIlwain & Richardson's [1939] method was dissolved in water, neutralized and autoclaved; concentration *M*/1·5. Dilutions were added to the inadequate mixture to give the concentrations shown.

	Growth after hr.		
	17	24	43
Inadequate medium + 0	0	0	0
+ *dl*-glutamate *M*/25	+ +	+ + + +	+ + + +
M/50	?	+	+ + + +
M/100	0	0	+ + + +
M/200	0	0	0
+ meat extract 1·0 ml.	+ + + +	+ + + +	+ + + +
0·2 ml.	+ + +	+ + +	+ + +
0·04 ml.	+	+	+ +
0·008 ml.	0	0	0

Here and elsewhere, *plus* signs are roughly proportional to the mass of growth.

Our interpretation of the results of Table I was that glutamic acid could not be the active substance in our meat preparations because there were obvious qualitative and quantitative differences between them. Glutamic acid might however be specifically associated with our active substance in the sense that the coccus could synthesize our active substance in adequate amounts in the presence of high concentrations of glutamic acid, but not in adequate amounts when the concentration of glutamic acid was low.

According to this interpretation glutamic acid might be the parent substance from which our active material was synthesized. We therefore surveyed the derivatives of glutamic acid which were known to occur in nature and which had similar properties to those of our active substance and we selected for test glutamine.

It was found that glutamine, sterilized by filtration, when added to the inadequate basal medium in a concentration as low as $M/5000$ caused full growth in as little as 16–17 hr. This growth was capable of serial subculture in the same mixture as often as desired. Asparagine tested under the same conditions was inactive.

The following specimens of glutamine from different sources had the same activity:

(1) A specimen isolated by Prof. Chibnall from natural sources, containing 98·2 % glutamine (glutamine was determined by the method of Vickery, Pucher Clark *et al.* [1935]).

(2) A specimen prepared by ourselves from beetroot by the method of Vickery, Pucher and Clark [1935], containing 97·5 % glutamine.

(3) A specimen synthesized by Prof. Harington from natural glutamic acid [Harington & Mead, 1935].

(4) A specimen synthesized by ourselves from synthetic glutamic acid.

The results with specimen 4 showed conclusively that glutamine itself and not any associated biologically active impurity was capable of replacing our active meat preparations.

A survey was then made of our various fractions of meat to estimate how far their activities could be ascribed to glutamine in them, determined as labile amide-N by the above method. The results of analysis are given in Table II on

Table II. *Glutamine content and activity of preparations*

	Total N mg./ml.	NH_3-N mg./ml.	Labile amide-N* mg./ml.	Calc. glutamine %	Activity for growth
Meat extract	27·7	0·37	0·106	0·111	+ + + +
1st Hg ppt.	4·59	0·36	0·105	0·109	+ + + +
Phosphotungstic filtrate	0·672	0·015	0·0403	0·042	+ + +

* Determined under the conditions of Vickery, Pucher, Clark *et al.* [1935] for glutamine.

solutions equivalent to the original meat extract. Negligible amounts of glutamine were found in the phosphotungstic precipitate and in the less soluble salts separated at that stage (p. 225); the loss of glutamine in the filtrate may readily be explained by the temporary alkalinity and the considerable evaporation involved in this process. It will be seen that the distribution of glutamine-N in the fractions was the same as that of growth activity.

Separation of glutamine from meat

The method used was essentially that by which Schulze & Bosshard [1883] and Vickery, Pucher & Clark [1935] isolated glutamine from plant extracts. It was not found possible to apply this to the immediate meat extract or first mercury precipitate owing to their containing much smaller amounts of glutamine than a typical beet extract (about 1/20) and larger amounts of associated materials. The method was however successfully applied to the material after phosphotungstic acid precipitation (p. 225) in which the glutamine, with 60 % loss, had been separated from 97·6 % of the associated nitrogenous impurities (Table II).

To material from 1000 ml. of meat extract at this stage, in water (50 ml.), saturated aqueous basic lead acetate solution was added till in excess (*c.* 40 ml.) while progressively neutralizing the solution with *N* acetic acid (*c.* 2 ml.). The pale cream-coloured granular precipitate settled quickly and was filtered off after 30 min. and washed 3 times with water (10 ml.). To the mother liquors

a 30 % solution of mercuric nitrate [cf. Vickery, Pucher & Clark, 1935] was added in excess (*c.* 30 ml.). The solution then had *p*H 2, and the white precipitate (Hg*P* I) was collected and washed after 2 hr. The mother liquors were neutralized with 10*N* NaOH (*c.* 6 ml.), kept overnight and the further precipitate (Hg*P* II) collected and washed with water. All fractions were decomposed with H_2S and their growth activities and glutamine contents determined. The material recovered from the lead precipitate had no activity and contained no detectable glutamine; the material from the two mercury precipitates contained the bulk of the glutamine and activity, while the mother liquors were slightly active and contained about 12 % of the total glutamine. The solutions containing the bulk of the material recovered from the two mercury precipitates were each neutralized with ammonia, evaporated under reduced pressure from a bath at 50° to about 2 ml. and the separated crystals collected and washed with 50 % aqueous alcohol. More crystals were obtained from their mother liquors by treatment with alcohol. The fractions had the properties shown in Table III. The activities of these fractions also were closely correlated with their glutamine contents, and about ½ the glutamine in the starting material had been isolated.

Table III. *Characters and growth activities of glutamine fractions isolated from meat*

	Wt. g.	M.P.	Mixed M.P. with *l*-(+) glutamine	Glutamine content %	Glutamine content g.	Activity*
Hg*PI* 1st crop	0·125	194–5°	196–7°	99	0·22	+ + +
Hg*PI* 2nd crop	0·036	194–5°	196–7°			
Hg*PI* 3rd crop	0·087	172–4°	187–9°	69		
Hg*PI* mother liq.					0·013	+
Hg*PII* 1st crop	0·076	>260°		0	0	0
Hg*PII* 2nd crop	0·027	>260°				
Hg*PII* mother liq.					0·120	+ +
Initial phosphotungstic acid precipitated meat extract					0·420	+ + + +

* Tested in concentration equivalent to that of the original meat extract.

Table IV. *The growth activity of meat in relation to its glutamine contact*

Crude meat extract estimated to contain 0·111 mg. glutamine/ml. was titrated against a solution of glutamine (specimen 3) containing 0·111 mg./ml.

Tube			Growth in hr. 24	29½	48½
1	Inadequate medium + glutamine	1·0 ml.	+ + + +	+ + + +	+ + + +
2	+ glutamine	0·2 ml.	+ + +	+ + + +	+ + + +
3	+ glutamine	0·04 ml.	+ +	+ + ⊥	+ + +
4	+ glutamine	0·008 ml.	Tr.	+	+ ⊥
5	+ glutamine	0·0016 ml.	0	Tr.	+
6	+ glutamine	0·00032 ml.	0	0	0
7	+ meat extract	1·0 ml.	+ + + +	+ + + +	+ + + +
8	+ meat extract	0·2 ml.	+ + +	+ + + +	+ + + +
9	+ meat extract	0·04 ml.	+ +	+ + ⊥	+ + +
10	+ meat extract	0·008 ml.	Tr.	+	+ ⊥
11	+ meat extract	0·0016 ml.	0	0	0
12	+ meat extract	0·00032 ml.	0	0	0
13	+ water	1·0 ml.	0	0	0

The purer glutamine fractions were recrystallized 3 times from aqueous alcohol; the product melted higher (205°) than glutamine previously isolated from natural sources, had a typical optical rotation of $[\alpha]_D^{21°} = +6·5 \pm 0·1°$ and gave the precipitation reactions described in the literature.

In a final experiment it was shown that under the conditions of our test the only contribution made by the meat to the growth of streptococcus was glutamine. A solution of glutamine was prepared at a concentration equivalent to the estimated concentration in our crude meat extract and both were titrated for growth-inducing activity. Table IV shows that the activity of each was approximately the same.

Discussion

The use of meat extract as an addition to peptone was, according to Bulloch [1930], introduced by Loeffler in 1881 and has been a routine procedure in medical bacteriology ever since. It would appear that an important function of the meat so far as *Str. haemolyticus* is concerned is to ensure the presence of glutamine in the nutrients. Arguing from the action of animal extracts and fluids (e.g. urine) upon the growth of streptococcus it may be assumed that glutamine is widely distributed in the animal body, but little reference can be found to its possible function. It is however interesting to note that Krebs [1935] has found that the brain cortex and retina of vertebrates and the kidney of the rabbit and guinea-pig can synthesize glutamine from glutamic acid and that extracts of these organs contain a glutaminase which can catalyse the reverse process. It is possible that similar processes can be carried out by streptococci and that they are a necessary feature of their growth.

The effect of glutamine described has also been demonstrated with ten other strains of haemolytic streptococci. Two others grew equally well under the conditions used without glutamine.

Summary

This work continues a study of the nutrients essential for the growth of bacteria. Previous work has shown that "simple" non-pathogenic microbes can obtain all nitrogenous compounds required in their metabolism by synthesis from ammonia. Increasing complexity of nutrient requirements, usually associated with increasing pathogenicity, calls for the provision of more complex nutrients some of which have been identified with established animal vitamins. The present paper shows that glutamine, a substance present in animal tissues but with no known function, is an "essential growth factor" for most strains of streptococci isolated from cases of disease in that it must be supplied in the nutrients.

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