CVII. STUDIES IN BACTERIAL NUTRITION. II.

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In a previous paper [1923] the separation of a tryptic digest of caseinogen into two constituents by means of phosphotungstic acid was reported. Two more experiments bearing on the same phase of the problem are now described.

Effect of autoclaving caseinogen stock broth.

Prolonged autoclaving changes some of the substances essential to streptococcal growth and it was of interest to determine whether (i) the reaction of the medium affected its heat stability, and (ii) the substances changed were those in the precipitate or filtrate portion of the broth. Three tubes were prepared, each containing 25 cc. of undiluted stock broth, adjusted to p_H 6.5, 7.5 and 8.5 respectively, and identified as *A*, *B* and *C*. The tubes were heated in steam at 117° for 20 minutes daily for 25 days, samples being taken at 5-day intervals, adjusted to p_H 7.5, diluted 1 in 3 with distilled water, and distributed in smaller tubes for testing growth. A flocculent precipitate, presumably of phosphates, appeared in tube *C* on the 9th day and in the other two tubes on the 14th day. At the end of 25 days a small tube representing each sample was sown with a suspension of the *Streptococcus*. Growth could not be judged accurately because of the flocculent precipitate in some tubes, but where a reasonable growth occurred the characteristic flakes of agglutinated streptococci appeared on the sides of the tube.

There was no apparent growth in the 20-day samples from *A* and *B* or in the 15-day sample from *C*, although all the tubes gave growth on subculturing on to agar. In the 10-day samples from *A* and *B* the growth was equal to that in the control, while the 10-day sample from *C* showed very poor growth. Thus the broth was definitely much less stable (from the point of view of streptococcal growth) in the more alkaline tube *C*.

The original samples *A*, *B* and *C* after being autoclaved for 25 days were filtered, adjusted to p_H 7.5 and tested for growth with the addition of the phosphotungstic precipitate and filtrate portions of caseinogen broth. In this case growth was fairly accurately determined by opacity since no precipitate was present.

Reaction during autoclaving	+ Precipitate	+ Filtrate	Broth alone	Control
p_H				
6.5	++++	++	++	++++
7.5	++++	++	+	
8.5	+++	+	+	

Concentration of broth 1 in 3. Concentration of precipitate or filtrate equivalent to 1 in 3.

The results indicate that the substances destroyed by heat are those contained in the portion precipitable by phosphotungstic acid, and it seems probable that the effect which prolonged autoclaving produces is hydrolysis of some of the more complex compounds in the broth. This, coupled with the fact that the total hydrolysis of caseinogen by means of acid does not produce a medium suitable for the growth of *Streptococcus*, may be taken as evidence that some substance more complex than an amino acid is essential for the growth of the organism.

Effect of adding amino acids to the precipitate portion of the broth.

It was suggested that some amino acid might be the activating principle contained in the filtrate portion and to test this the following amino acids were added to the precipitate portion: glycine, alanine, leucine, tyrosine, tryptophan, histidine, aspartic and glutamic acids. The concentration of the precipitate portion was made equivalent to 1 in 3 stock broth, and the concentration of amino acid was 0.1 % (except tyrosine which was used in saturated solution). On sowing with the *Streptococcus* the result was quite negative in every case as it was also in a solution containing the precipitate portion and all eight acids in 0.01 % concentration. This does not exclude the possibility of some other, possibly unknown acid, being the activating agent, but it is quite evident that the amino group as such is not sufficient.

Extraction by butyl alcohol.

Dakin [1920] has devised and applied a method for the analysis of the products of protein hydrolysis, using butyl alcohol as a solvent, and it was thought that this might prove of some use in elucidating the problem of the substances used by the *Streptococcus* in caseinogen broth. An apparatus similar to that of Dakin was used and various batches of caseinogen stock broth were subjected to continuous extraction by butyl alcohol *in vacuo* at a temperature of 45–50°. By this method the broth is separated into three classes of substances:

(A) Substances quite insoluble in butyl alcohol and therefore remaining in the aqueous solution. According to Dakin the diamino and the dibasic acids are included in this portion.

(B) Substances extracted by butyl alcohol but insoluble in ethyl alcohol. Most of the simple amino acids fall under this head.

(C) Substances soluble in butyl and in ethyl alcohols. Proline and hydroxyproline are the type substances in this class.

The extraction proceeds rapidly at first and a cream-coloured deposit of amino acids separates in the butyl alcohol flask; the deposit is filtered off at intervals to avoid bumping. In the case of caseinogen broth however, which, unlike the solutions used by Dakin, is not completely hydrolysed, the extraction soon slows down and it was found that continuous extraction for 100 hours was necessary before no further appreciable quantity of matter was extracted. Even then it was probable that the extraction was incomplete.

After extraction for 100 hours the whole of the butyl alcohol was separated from the aqueous solution and filtered to remove the deposit, which was then washed with fresh butyl alcohol and with ethyl alcohol. The butyl alcohol containing soluble substances was evaporated *in vacuo* to avoid decomposition and the yellowish deposit remaining was extracted with boiling ethyl alcohol, any insoluble portion being filtered off, washed and added to the solid amino acids *B*. The aqueous portion and the alcoholic solution were both evaporated and finally all three portions were separately redissolved, adjusted to p_H 7.5, and brought to the volume of the original broth. The three solutions were termed *A*, *B* and *C* according to the scheme given above. *A* and *B* were both brownish in colour and *C* was light yellow; only *B* had the sweetish amino acid smell characteristic of caseinogen stock broth. In two cases the total nitrogen and amino nitrogen of all three solutions was determined in order to get some idea of the class of nitrogenous substances present in each solution.

	<i>A</i>		<i>B</i>		<i>C</i>	
	% N	% Amino N	% N	% Amino N	% N	% Amino N
Broth (Nov. 1923) digested 10 days	0.603	0.369	0.338	0.281	0.105	0.038
„ (Jan. 1924) „ „	0.623	0.317	0.309	0.246	0.077	0.044

Amino nitrogen was determined by Foreman's [1920] method.

The following table gives a summary of the protein reactions by stock broth and by solutions *A*, *B* and *C*.

	Broth	<i>A</i>	<i>B</i>	<i>C</i>
Presence of phosphorus	+++	+++	+	-
Biuret	+++	+++	-	-
Xanthoproteic	No precipitate with HNO ₃ . making alkaline	+++	Yellow colour → orange on	-
Millon's	+++	+++	+++	-
Glyoxylic	+++	-	+++	+++
Molisch's	-	-	-	-
Saturated with ammonium sulphate	Precipitate	Precipitate	Slight precipitate	Slight precipitate

All three solutions gave a precipitate with phosphotungstic acid though the bulk was small in *B* and *C*; this shows, however, that some peptide bodies must be extracted by the butyl alcohol since in Dakin's experiments the diamino acids lysine, arginine and histidine were shown to be left unextracted in the aqueous solution. The presence of phosphorus in *B* seems to indicate that some of the phosphates in the broth are still in organic combination.

Growth of the Streptococcus. The three solutions were prepared in small tubes as usual, being diluted so that the concentrations were proportional to stock broth 1 in 3, and sown with equal amounts of the *Streptococcus*. The results in general were remarkably constant.

<i>A</i>	<i>B</i>	<i>C</i>	<i>AB</i>	<i>AC</i>	<i>BC</i>	<i>ABC</i>	Control
+	±	-	+++	+++	++	++++	++++

The incompleteness of the separation was shown by the fact that doubling the concentration of *A* caused a growth of + + +, while doubling *B* caused very little difference in growth. Therefore, the essentials for growth of the

organism are all present in *A* though with some insufficient concentration as is shown by the increase in growth obtained when the other solutions are added.

Tryptophan is definitely excluded as an essential growth factor for the *Streptococcus* since solution *A* gives a negative glyoxylic test. Also the results afford further evidence that concentration of amino N is not the deciding factor in the growth of the *Streptococcus* and that specific substances are essential. On the other hand, *B. coli* grew in all three solutions, approximately according to the nitrogen content.

Several attempts were made to separate each of the solutions *A*, *B* and *C* into two fractions by means of precipitation with phosphotungstic acid, but unfortunately without success so far as streptococcal growth was concerned.

The division into six solutions could be carried out so that very little growth-producing power was lost but the separation was not sufficiently clear-cut to enable any useful information to be gained. The separation was particularly incomplete in solutions *B* and *C*, since the phosphotungstic precipitate and filtrate portions of these all seemed capable of giving increased growth when combined with *A*. As mentioned before, the amounts of precipitable substance in *B* and *C* were small, and this may account for the incomplete precipitation. The filtrate portion of solution *A* appeared to be the more important fraction.

Undoubtedly other methods of protein analysis will have to be applied before any clear light is thrown on the subject of bacterial growth substances. The main difficulty from the chemical point of view is the extremely small amount of matter which is sufficient to cause growth of an organism. It has been found, for instance, that the *Streptococcus* will grow in an emulsion of caseinogen unless the protein has been purified by repeated precipitation and that trypsin acting on purified caseinogen for a very short time liberates sufficient substance to cause growth of an organism. Experiments bearing on this point will probably be reported later.

Under these circumstances it is certain that no method, which does not give the accurate separation characteristic of inorganic chemistry, will give absolutely clear-cut results when tested from the growth-producing standpoint.

REFERENCES.

- Dakin (1920). *J. Biol. Chem.* **44**, 499.
Foreman (1920). *Biochem. J.* **14**, 451.
Whitehead (1923). *Biochem. J.* **17**, 742.