CXLV. STUDIES IN BACTERIAL NUTRITION.

III. PHOSPHATES AND THE GROWTH OF STREPTOCOCCI.

By HUGH ROBINSON WHITEHEAD.

From the Department of Bacteriology, College of Medicine, Newcastle-upon-Tyne.

(Received September 11th, 1926.)

THE analytical study of media used for growing bacteria has produced very little chemical knowledge up to the present time. Limiting oneself to the more pathogenic organisms (such as streptococci), the facts so far elucidated may be briefly summarised as follows.

(i) Amino-acids are not so important for growth as was at one time thought. Some amino-acids are favourable to growth (*e.g.* alanine, taurine and asparagine), but many are inhibitory when present in excess [McLeod and Gordon, 1926]. Tryptophan has been definitely excluded as a necessary substance for streptococci [Whitehead, 1924]. Mueller [1922] has isolated a hitherto unknown sulphur-containing amino-acid which he has found to be a growth accessory for streptococci. In general, although a certain amount of amino-nitrogen is always present in good media, the amount of growth is not dependent on the concentration of substances containing this grouping. McLeod and Gordon [1926] give a summary of the work which has been done on this point.

(ii) From another standpoint two substances both essential to streptococcal growth have been demonstrated in a protein medium; one is precipitable by phosphotungstic acid and the other is not [Whitehead, 1923].

(iii) By the use of another method of protein analysis it would appear that some substance concerned in streptococcal growth must be partially soluble in butyl alcohol, but only to a very slight extent. Extraction of a medium for 100 hours by Dakin's butyl alcohol method failed to render it useless, although growth was considerably lessened.

The methods of chemical analysis, which can be applied with any degree of success to a medium consisting mainly of protein derivatives, are very limited, on account of two experimental factors:

(i) the difficulty of avoiding changes in the proteins during manipulation, and of determining whether such changes have taken place;

(ii) the elimination of bactericidal or inhibitory substances, especially excess of neutral salts, when manipulation is finished; otherwise growth experiments on the separated fractions are negative or fallacious.

In any case the most for which we can hope at the present time is to obtain results which will eliminate certain substances as growth accessories and indicate to what class or classes of compounds the active substances belong.

A method so far not investigated in the examination of media is separation of the protein substances by using ethyl alcohol as a precipitant. This agent has the advantage of being readily removable by distillation at a low temperature which seems to render it admirably fitted for the purpose. The present paper describes results obtained by the application of this method.

In the first instance, Cole and Onslow's caseinogen broth, fresh from trypsinisation, was treated with absolute alcohol at 0° , so as to render it first 66 % and then 86 % alcoholic. Two precipitates were obtained and separated by centrifuging. Each was redissolved in water, adjusted to p_H 7.5 and made equal in volume to the original medium. The possibility of change in the protein in these precipitates due to the action of alcohol should not be overlooked, but they were at any rate readily redissolved in water giving clear solutions. The remaining liquid was freed from alcohol by distillation *in vacuo* at $45-50^{\circ}$ and similarly made up in water. Thus were obtained three fractions of the original medium:

"A" precipitated by 66 % alcohol.

"B" precipitated by 86 % alcohol.

"C" residual liquid.

Solution C had the characteristic amino-acid smell usually associated with caseinogen broth and contained about 67 % of the amino-N of the original broth. Solutions A and B were odourless and contained about equal amounts of amino-N; A was slightly opalescent.

Growth experiments were performed by distributing the liquids in small plugged tubes, sterilising, and sowing thinly with suspensions of streptococci as described in previous papers. It was found that, whilst none of the fractions singly showed any marked growth, all three together gave growth equal to that in a control tube of untreated broth. A combination of A and C in 24 hours gave almost as much growth as the control tube. A combination of B and C gave no visible growth in 24 hours, but in 48 hours the growth was almost as good as that in AC. That is to say, BC showed a lag period before growth occurred.

By chance a growth experiment was performed in which some disodium hydrogen phosphate solution was added to all the tubes, primarily with the idea of stabilising the p_H . A rather surprising result was obtained. AC now gave no growth in 24 hours and little or none in 48 hours, whilst BC in 24 hours gave growth quite as good as that in the control. A combination of A and B gave a slight growth in the presence or absence of added phosphate. It thus became evident that the phosphate was playing a much more active rôle than that of a mere buffer salt.

On examination it was found that solution A contained 70-80 % of the phosphate originally present in the medium, solution B about 15 % and solu-

tion C about 5 %. The proportion varied, of course, with different batches of medium. Moreover, the phosphate in A appeared to be mainly in the inorganic form, since a granular precipitate of ammonium phosphomolybdate could be readily obtained without previous oxidation of the organic matter. In B and C, on the other hand, treatment with ammonium molybdate without oxidation of the organic matter gave flocculent protein-like precipitates. Estimation of inorganic phosphate in the three solutions confirmed this view: no uncombined phosphate was found in B or C.

At first sight, therefore, it seemed that the above experiments might be explained on the assumption that the organism needed a certain minimum amount of inorganic phosphate or of phosphate in a readily accessible form for its growth, but that a larger amount was inhibitory. Since the phosphate in BC was all in organic combination, the organism could only grow slowly in those solutions until its enzymes had liberated phosphate from combination. So simple a hypothesis was not, however, borne out, or at any rate other factors enter into the problem, for the total concentration of phosphate in BC could be increased to nearly double the value which checked growth in AC without inhibiting the organism in the former mixture. Inhibition in AC was not, therefore, simply an osmotic effect: other substances in solution A must modify the action of the phosphate and a complete explanation of the various phenomena is lacking at present.

Experiments showed that inorganic phosphates were produced when streptococci were allowed to grow in BC for 48 hours, but whether phosphate was liberated for use by the organism, or merely as an incidental happening in its metabolism, is difficult to prove.

The effect of several other salts was tested with some interesting results which should be amplified by more thorough investigation.

Potassium dihydrogen phosphate proved incapable of activating mixture BC, and furthermore no growth occurred in 48 hours. Added to mixture AC it caused no inhibition.

Diammonium hydrogen phosphate activated mixture BC, but not to the same extent as sodium phosphate. It caused no inhibition when added to AC. This salt, however, caused slight growth to occur in the single solutions A, B and C. Its kation NH_4 may enter in some way into the growth of the organisms.

Sodium salts, such as the acetate and sulphate, gave slight growth with BC in 24 hours and good growth in 48 hours. They seemed to shorten the period of lag. No inhibition was caused in AC.

Potassium salts gave similar results to those obtained with potassium phosphate, *i.e.* inhibition in BC and no effect in AC.

It is clear from the foregoing that the nature of both anion and kation of added salts is of importance in the growth of streptococci. Potassium in particular seems to have an adverse influence unless sufficient sodium phosphate is already available in the medium. The main fact is that inorganic phosphate seems to be necessary for the production of growth in 24 hours and phosphate

in some form is probably essential. It is, however, difficult to prepare a protein medium absolutely free from phosphate, so that the latter point cannot be proved at present. Meader and Robinson [1920] maintain that phosphate is essential to the production of haemolysin and observe no effect on growth, but the horse serum which they added to their medium probably supplies the small amount of phosphate necessary for growth.

A point of practical use is that solution A can be disregarded for the present in the study of caseinogen media, since growth equal to that in whole broth is obtained in a mixture of solution B and C + phosphate. This is important as some of the more complex protein derivatives can be removed, and difficulties in experimental manipulation are overcome.

It would appear from the results obtained that three types of compound are concerned in the growth of streptococci on caseinogen broth.

(i) A complex type of protein derivative present in solutions A and B and probably identical with the active substance precipitated by phosphotungstic acid.

(ii) A simpler protein derivative, present in solution C; possibly an amino-acid.

(iii) Phosphates, preferably in inorganic form. Most probably the phosphate plays the part of a co-enzyme in one or more of the reactions by means of which the organism utilises the protein growth factors in its metabolism.

Effect of heating on the phosphate compounds in broth.

During the previous experiments it became evident that only broth which was quite fresh from digestion gave the clear-cut results which have been described. Medium which had been steamed a few times would not give B and C fractions causing a growth lag. At the same time the broth became rapidly darker in colour under the influence of heat and ultimately lost much of its growth-producing power. Also it had been noticed that broth, to which phosphate had been added, darkened and lost its growth power much more rapidly than normal broth, when steamed, so it was decided to investigate the changes which took place on heating, in the hope that light would be thrown on the nature of the protein growth factors.

In the first place the absence of growth lag in solutions B and C prepared from steamed broth was explained by the fact that traces of inorganic phosphate were detected in those solutions. Probably adsorption of phosphate on the flocculent precipitate produced by 66 % alcohol played some part in the removal of inorganic salts from the broth and, as the character of the precipitate was rather different in steamed broth (being much more granular), it seems reasonable to assume that less powerful adsorption allowed some phosphate to remain in the liquid phase.

The three fractions A, B and C, prepared from fresh broth, were steamed for about 12 hours with the addition of all the salts which had been tested

during the growth experiments. Control tubes of whole broth were similarly heated. At the end of this period it was observed that:

- (i) A did not darken with or without the addition of any salt whatever;
- (ii) B darkened a little without, and a little more with, the addition of phosphate; other salts caused no more darkening than occurred in the control;
- (iii) C darkened a little without, and markedly more with, added phosphate; other salts caused no more darkening than occurred in the control;
- (iv) tubes of whole broth darkened considerably with no addition and still more with the addition of phosphate; other salts again had no effect.

The tubes of whole broth were sown, after heating, with a streptococcus and while the broth heated alone showed some diminution of growth in the tube containing added sodium phosphate, growth was completely inhibited and greatly delayed in the tubes containing potassium and ammonium phosphates.

The broth which had been heated with additional sodium phosphate proved to have no inhibitory effect on fresh medium. It appeared, therefore, that phosphate had combined with, or changed, some growth substance present mainly in the solution C, thereby rendering it inactive as a growth factor.

The next point was to determine whether phosphate had combined with the growth factor, for this might afford valuable clues as to the nature of the protein derivative. Inorganic and organic phosphate were estimated in fresh and in heated broth, and it was found that in every instance there was an increase in inorganic phosphate after heating. When the heated broth was separated by 66 % alcohol, an increased amount of phosphate was found in the portion corresponding to fractions B and C but the increase was accounted for by inorganic phosphate, the concentration of combined phosphate being practically the same as in fresh broth. In effect there was no evidence whatever that inorganic phosphate had recombined with protein substances under the influence of heat. On the contrary, the results showed that the main change in phosphate distribution on heating was a hydrolysis of some of the organic phosphorus compounds in fraction A.

The change of the growth substance present in C must therefore be one which is catalysed by phosphates. Experiments in which broth was heated in narrow-necked flasks well stoppered, and in Erlenmeyer flasks giving a large liquid surface, showed that the reaction was probably not an oxidation, since the darkening was absolutely equal under both conditions. It can only be said, therefore, that one of the growth factors for the streptococcus, one of the simpler protein derivatives, is changed on heating in the presence of phosphates, so that it no longer supports growth of the organism.

EXPERIMENTAL.

Separation of broth by alcohol.

25 cc. of fresh caseinogen broth were poured into 50 cc. of ice-cold absolute alcohol and the mixture allowed to stand for 1 hour in the ice-chest. The flocculent white precipitate (A) was separated by centrifuging and the supernatant fluid poured into 100 cc. of cold alcohol. After another hour the second precipitate (B) was separated by centrifuging. Both precipitates were washed with the appropriate strength of alcohol and then dissolved in water to the original volume of broth, and adjusted to p_H 7.5. The alcoholic liquid (C) remaining from the separation was evaporated to dryness *in vacuo* at 45–50°, redissolved and adjusted. The following table gives the growth results obtained with a haemolytic streptococcus. Experiments with two other strains bore out the results.

Table I.

Diluent	A	B	C	AB	AC	BC	ABC	Control
Water	Trace	—	Trace	+	+++	Trace (+++)	++++	++++
Na ₂ HPO ₄	„	—	+	Trace	—	++++	.	+++ (++++)
KH ₂ PO ₄	„	—	—	„	+++	—	.	++++
(NH ₄) ₂ HPO ₄	„	Trace	Trace	+	+++	+++	.	++++
Na ₂ SO ₄	„	—	„	+	+++	+	.	++++
						(+++)		
K ₂ SO ₄	„	—	„	+	+++	—	.	++++

The results in brackets indicate changes which took place in 48 hours. Trace indicates no visible growth, but growth obtained on subculturing.

+, ++, +++, +++++ are degrees of growth estimated by opacity.

The salts used were all adjusted to p_H 7.5 and had an effective concentration of $M/45$ in the growth tubes. Dilutions were arranged so that solutions A, B and C and the broth control were one-third of their original strength in every tube.

Phosphate estimations.

Inorganic phosphate was estimated in the solutions by precipitation with magnesia mixture. Total phosphate was estimated by oxidation and precipitation as ammonium phosphomolybdate, the latter being estimated volumetrically.

Inorganic and organic phosphate in solutions A, B and C separated from fresh broth.

Exp.	A		B		C	
	Inorganic	Organic	Inorganic	Organic	Inorganic	Organic
1	0.147	0.097	0.0	0.029	0.0	0.018
2	0.191	0.106	0.0	0.049	0.0	0.019
3	0.129	—	0.0	—	0.0	—
4	0.127	0.049	0.0	0.060	0.0	0.033

Results expressed as % P₂O₅.

Change in distribution of phosphate on heating broth.

I. Three samples of broth were prepared and treated as follows.

(i) Steamed 10 minutes and adjusted to p_H 7.5.
 (ii) Steamed for 12 hours in 100 cc. graduated flask with narrow neck, tightly stoppered.

(iii) Steamed 12 hours in an Erlenmeyer flask with large liquid surface.

Samples (ii) and (iii) were subsequently adjusted in reaction (they were very slightly acid) and brought to their original volume with distilled water. When placed in similar flasks it was observed that both heated samples had darkened to the same extent, so that probably oxidation had nothing to do with the phenomenon of darkening.

Phosphate concentration (% P_2O_5).					
(i)		(ii)		(iii)	
Inorganic	Organic	Inorganic	Organic	Inorganic	Organic
0.106	0.104	0.133	0.075	0.135	0.076
Totals 0.210		0.208		0.211	

Heating therefore causes a decomposition of organic phosphorus compounds and liberation of additional inorganic phosphate.

II. Samples of fresh broth and broth steamed for varying times were treated with 66 % alcohol at 0°. After centrifuging, the distribution of phosphate was investigated in the supernatant fluid (corresponding to solutions B and C). The reaction of all the samples was p_H 7.5 at the time of separation.

Time of heating: $\frac{1}{2}$ hour		2 hours		6 hours		12 hours	
Inorganic	Organic	Inorganic	Organic	Inorganic	Organic	Inorganic	Organic
% P_2O_5 0.000	0.093	0.007	0.087	0.013	0.098	0.031	0.073
*0.003	0.089	0.007	0.087	0.017	0.082	0.037	0.062

* Solutions B and C prepared from this particular sample of broth showed a growth lag of less than 24 hours. This is accounted for by the trace of inorganic phosphate.

No great accuracy is claimed for the figures giving inorganic phosphate in the supernatant fluid, since the amounts were very small, but the results give quite definite evidence that the darkening caused by heat is not accompanied by a combination of phosphate with the substances in the solutions B and C. At the same time it is evident why B and C show no growth lag when prepared from heated broth, since inorganic phosphate is present in those solutions and not in those prepared from fresh broth.

Concentration of amino-N and total N in the three fractions of broth.

The following table gives concentrations of amino- and total N found in a typical experiment.

	A	B	C
% Amino-N	0.092	0.092	0.378
% Total N	0.224	0.227	0.648

SUMMARY.

1. A tryptic digest of caseinogen may be separated into three fractions by treatment with ethyl alcohol. All the inorganic phosphate is present in the fraction precipitated by 66 % alcohol.

2. Streptococci have a lag period of about 24 hours before growth occurs in a medium made from the two fractions containing no inorganic phosphates. Rapid growth occurs if phosphate is added to these fractions.

3. From the results obtained at least three factors appear to be concerned in the growth of streptococci in caseinogen medium; two are probably protein derivatives and the third is phosphate preferably in inorganic form.

4. The darkening and deterioration of caseinogen medium which occur on steaming are probably due to the presence of phosphate.

5. The deterioration is due to a growth factor, present among the simpler protein derivatives, being rendered inert. The change is not an oxidation nor is there a combination of the growth factor with phosphate, but phosphate appears to catalyse the reaction.

REFERENCES.

- McLeod and Gordon (1926). *J. Path. Bact.* **29**, 13.
Meador and Robinson (1920). *J. Exp. Med.* **32**, 639.
Mueller (1922). *J. Bact.* **7**, 309.
Whitehead (1923). *Biochem. J.* **17**, 742.
— (1924). *Biochem. J.* **18**, 829.