CXXIII. THE RELATION OF THE GROWTH OF CERTAIN MICRO-ORGANISMS TO THE COMPOSITION OF THE MEDIUM.

I. THE SYNTHETIC CULTURE MEDIUM.

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(*Received June 11th, 1927.*)

This investigation had as its main object the study of the influence of the ill-defined growth-promoting factor known as bios on the development of unicellular organisms in artificial media. In the early stages it became apparent that no appreciable progress could be achieved until the other essential requirements had been more clearly defined. In particular, this referred to their needs for inorganic salts and the most suitable sources of carbon compounds from which they could derive the necessary energy for their metabolic processes.

It is a curious fact that, in spite of a very large amount of work reported in the literature, the question of the most suitable composition of the artificial medium in which a particular organism can be grown is undecided. This is particularly true of the inorganic composition of the culture fluid, and there seems to be a general impression that the requirements of yeast and bacteria for mineral salts are moderately elastic [Pasteur, 1860; Liebig, 1871; Wildiers, 1901; Windisch, 1902; Pringsheim, 1906; Osborne and Wakeman, 1919; Miller, 1921]. From the experiments reported below it must be concluded that only within a very narrow range of concentration is maximum growth obtained.

Experimental.

Preliminary experiments showed that all the organisms tried would grow on a medium consisting of pure glucose, certain inorganic salts, and water when these were added to clean flasks, sterilised on three successive days, inoculated, and incubated. Consequently it was decided to study in detail the basal requirements of three organisms and find how far (1) the inorganic salt content, and (2) the source of carbon could be varied.

The organisms used were *Sarcina aurantiaca*, *Streptothrix corallinus*, and a white *Streptothrix* as supplied by the National Collection of Type Cultures.

The strains to be used were grown on the surface of broth agar, and carefully plated to make sure that they were pure cultures. Inoculations were always made by suspending some of the dry surface growth in water, using a platinum loop. To avoid acclimatisation effects a fresh culture was used after every four or five subcultures had been made. All water used for making up the media or for the suspensions was prepared from tap water, by distilling, boiling for 3 hours with alkaline permanganate, and redistilling, only glass or silica vessels being used throughout.

The chemicals used were either Kahlbaum's chemicals "Pure for Analysis" or "Analytical Reagents" from the British Drug Houses. The NaCl and $MgSO_4$, $7H_2O$ were further recrystallised. The glucose employed was the "Puriss" variety from the British Drug Houses, but it was found to need four recrystallisations from 80, 75, 70 and 65 % of alcohol before it was rendered free from detectable traces of impurities having a definite stimulating action on growth.

Optimum hydrogen ion concentration. The limiting p_H was found for each organism by inoculating a series of plain broth media tubes and plates of different p_H before attempting to make up a suitable synthetic medium. The approximate limits were found by grading the series by means of colorimetric methods; later a more accurate adjustment was obtained by readings with the glass electrode [Kerridge, 1925]. A series of flasks for each organism was prepared, the contents of the flasks differing from each other by a p_H of 0·02 over the range 4·50 to 10·00. The results were as follows:

Organisms	Lowest limit at which appreciable growth occurred p_H	Maximum growth p_H	Highest limit p_H
1. *S. aurantiaca*	5·30	7·15	9·43
2. *Streptothrix corallinus*	6·02	7·60	9·35
3. White *Streptothrix*	6·02	7·60	9·35

Later these values were checked when a satisfactory artificial medium had been elaborated and were found to be substantially correct. Meanwhile it was decided to work at a value of $p_H = 7·4$ throughout.

The inorganic salt content. Having established the optimum hydrogen ion concentration for growth, a series of experiments was carried out in which varying quantities of inorganic salts were added to a medium containing 1 % of dextrose.

Table I.

Medium: Glucose 1 %
KH_2PO_4 0·1 %
K_2HPO_4 to $p_H = 7·4$.

Salt	Percentage concentration											
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*
$(NH_4)_2SO_4$	0·1	0·2	0·4	0·1	0·2	0·4	0·1	0·2	0·4	0·1	0·2	0·4
$MgSO_4$, $7H_2O$	0·02	0·02	0·02	0·05	0·05	0·05	0·07	0·07	0·07	0·09	0·09	0·09

(1) A series of flasks was arranged with 12 variations as seen in Table I. Six flasks were prepared for each group in an attempt to eliminate errors in inoculation, etc.

Growth at the end of 7 days was measured by opacity and found to be much better in cultures *E*, *F*, *H*, and *I* than in any of the others. Since in cultures *D* and *G* growth was less good than in *E* and *H* it was concluded that 0·1 % of $(NH_4)_2SO_4$ was not sufficient. It also seemed apparent that the optimum concentration of $MgSO_4$, $7H_2O$ lay between 0·05 and 0·09 %.

(2) All the ammonium-magnesium combinations in Table I were duplicated (*a*) with twice, and (*b*) with half the concentration of phosphates

$$(0{\cdot}1\ \%\ KH_2PO_4 + 0{\cdot}016\ \%\ K_2HPO_4),$$

but in neither case was the growth as good as in *E*, *F*, *H* and *I* above. It was therefore concluded that no advance could be made by altering the initial figure for phosphate concentration.

(3) The influence of the addition of varying concentrations of NaCl was next considered. A series of cultures similar to *D*, *E*, *F*, *G*, *H* and *I* was prepared, but containing in addition concentrations of NaCl varying from 0·01 % to 1 %. The cultures containing 0·05 % showed the best growth.

(4) A few preliminary experiments having indicated that traces of $Ca(NO_3)_2$ seemed to be beneficial, it was decided to set up a series containing $Ca(NO_3)_2$ over a range of concentrations from 0·001 % to 1 %. The improvement was very slight in the lower concentrations and growth was definitely retarded with concentrations above 0·075 %. The action seemed independent of concentration below 0·05 %.

(5) Innumerable examples occur in the literature of the last 20 years, some claiming, some disclaiming, the stimulating effect of traces of manganese and iron in the soil or culture medium on the growth of plant tissue. Experiments were designed in which traces of manganese salts, and ferrous and ferric salts were added to the glucose-water-inorganic salt medium described above. The substances used and the results obtained can be seen in Table II.

Table II.

Substance used	Concentration g. in 10 cc.	Increase in growth
MnO_2	1×10^{-4}	Nil
MnO_2	1×10^{-6}	,,
$MnSO_4$	1×10^{-4}	,,
$MnSO_4$	1×10^{-6}	,,
$FeSO_4$	1×10^{-4}	,,
$FeSO_4$	1×10^{-6}	,,
$FeCl_3$	1×10^{-4}	Slight
$FeCl_3$	1×10^{-6}	,,

The slight increase in growth with $FeCl_3$ was probably due to impurities, for after further purification (solution in ether, evaporation of the ether, and volatilisation of the salt) no sign of any action was detectable.

Conclusion from experiments with varied amounts of inorganic salts. From the experiments recorded above it was decided to use the following salt-mixture when studying (1) the effect of variation in the amount of glucose present, and (2) the most suitable source of carbon supply:

$(NH_4)_2SO_4$	0·30 g. in 100 cc. medium
$MgSO_4, 7H_2O$	0·07 ,, ,,
KH_2PO_4	0·10 ,, ,,
K_2HPO_4	0·016 g. (to bring p_H to 7·4)
NaCl	0·05 g. in 100 cc. medium
$Ca(NO_3)_2$	0·04 ,, ,,

Variation in the amount of glucose present. The arbitrary value of 1 % of glucose was used throughout the earlier experiments. It was decided to test this figure, using the salt concentrations tabulated above. Table III shows the results obtained over the range of concentration 0·01–10 %.

Table III.

Concentration glucose (%)	Growth
0·01	↑ Gradually diminishing
0·02	
0·03	
0·04	
0·06	
0·08	
0·10	
0·20	
0·30	
0·40	
0·50	No appreciable variation
0·60	
0·70	
0·80	
0·90	
1·00	
2·00	
3·00	
4·00	
5·00	
6·00	
7·00	
8·00	
9·00	
10·00	↓ Gradually diminishing

0·5 % of glucose was used for all later experiments.

Influence of source of carbon. Although the medium described above gave good results, it was thought of interest, in view of the number of publications on this subject, to study the value of sources of carbon other than glucose. The growth with glucose as the source of carbon is taken as standard and recorded by two crosses, *e.g.* ++.

Three crosses = increased amt. growth.

One cross = decreased amt. growth.

Table IV.

Source of carbon (0·5 % solution in salt-water medium)	Growth	Source of carbon (0·5 % solution in salt-water medium)	Growth
Glycerol ...	+ + +	Sodium pyruvate	+ +
Mannitol ...	+ + +	Fructose	+
Glucose ...	+ +	Rhamnose ...	+
Arabinose ...	+ +	Sodium acetate ...	+
Glycogen ...	+ +	„ propionate	+
Calcium lactate	+ +	„ butyrate	Trace
Sodium lactate...	+ +	„ oxalate ...	Trace
„ citrate...	+ +	„ succinate	0
		„ tartrate ...	0

The growth on glycerol and mannitol calls for comment. Although the suspension was more opaque, the rate of reproduction did not seem to be increased, but the growth was more filamentous, in fact almost vegetative. Whereas the segmentation of the *Streptothrix* usually begins about the third day, it could be delayed to the seventh or eighth day with mannitol or glycerol as the source of carbon. The effect of citrate is surprising, but the presence of mycelial threads in the culture fluid points to an effect similar possibly to that of glycerol or mannitol.

Amino-acids as source of carbon or nitrogen. The following experiments were designed to test the amino-acids (1) as a source of carbon, and (2) as a source of nitrogen for bacteria. Column 1 in Table V shows results obtained when the glucose was replaced by a 1 % solution of the various amino-acids tabulated. Column 2 gives results when the ammonium sulphate was replaced by the amino-acids, the glucose being added as usual.

The growth obtained with glucose (0·5 %) and ammonium sulphate (0·3 %) present was again used as standard.

Table V.

Amino-acid (0·5 % solution)	Growth: Column 1 Amino-acid as source of carbon	Growth: Column 2 Amino-acid as source of nitrogen
Tryptophan ...	−	−
Tyrosine	−	−
Alanine	+ +	Trace
Phenylalanine ...	−	„
Histidine	−	„
Cystine*	+	„
Glycine	+ +	„
Asparagine ...	+ +	„
Glutaminic acid ...	−	„
Tyrosine + alanine	+ +	„
Tyrosine + histidine	−	−
Alanine + histidine	+ +	+
Histidine + glycine	+ +	Trace

* The low solubility of cystine enabled only a 0·1 % solution to be used. The flasks were tested at the end of the experiment and cystine found to be still available.

In column 1, *i.e.* in the absence of glucose, growth was obtained with alanine, glycine, asparagine, and to a less degree with cystine.

In column 2, *i.e.* in the absence of ammonium salts, the growth was erratic and was certainly never of the same order as with a purely inorganic source of nitrogen.

Discussion.

The experiments recorded in this paper show that it is comparatively easy to obtain growth of the organisms studied on a synthetic medium, but whether the metabolism and rate of multiplication of the organisms are normal under these conditions remains to be proved. In all cases it was found possible to obtain a marked improvement of the rate of growth by adding minute quantities of broth or of any other bios-containing substance. Experiments on the effect of bacterial accessory factors will be discussed in a later paper.

The absolute qualitative requirements of the organisms studied seem to be fairly simple. It is indeed true that the formula worked out for the inorganic requirements of the culture medium differs only slightly from that of Stephenson and Whetham [1924], but what the author wishes to emphasise is the necessity for keeping within narrow limits the range of concentration of any one ingredient. The presence of Cl ions is essential and a trace of $Ca(NO_3)_2$ seems to encourage growth.

It is impossible to exaggerate the importance of the progressive purification of the glucose. The filtrate after a first recrystallisation can be shown to have a distinct stimulating effect upon the growth: the filtrate after the fourth recrystallisation has none. A similar action was pointed out by Funk and Dubin [1921] in their experiments on the stimulating effect of "vitamin B" on the growth of yeast and bacteria. They state that treatment of the sugar with an absorbent eliminates the effect. This has recently been confirmed, and it was found that after treating the glucose with charcoal only two recrystallisations were necessary.

The addition of amino-acids to the synthetic glucose-inorganic salt medium, being an addition of heavier and more complex molecules, naturally complicates the problem. In this connection the work of Aubel [1920] is interesting but his view has not been confirmed. He studied cultures in which asparagine was the only source of carbon and of nitrogen, and compared them with cultures containing also glycerol, glucose and fructose. More ammonia was used and more growth was obtained by these additions. Glycerol gave a ninefold increase; this he believed to be due to the formation of pyruvic acid, which, given directly, had nearly twice the augmenting power of glycerol. His conclusion, therefore, was that carbon as —CO is most effective, then comes —CHO and lastly —CH_2OH. On the other hand, the conclusion to be drawn from the experimental section of this paper is that pyruvate is not more efficient in promoting growth than glucose, and is obviously inferior to glycerol.

The possibility that the controlling factor in the variation of growth is a

purely physical one, such as osmotic pressure, surface tension, etc., must be considered. That the lowering of surface tension within certain limits by an addition of fatty compounds may alter the type, but not the amount, of growth is shown in the following paper [Reader, 1927].

SUMMARY.

1. Experiments have been carried out to find the concentration of salts in a synthetic medium necessary to give maximum growth of certain micro-organisms. The organisms used were *Streptothrix corallinus*, a white *Streptothrix*, and *Sarcina aurantiaca* and a definite formula was decided upon.

2. Traces of manganese or iron did not increase the rate or amount of growth.

3. The following substances may replace glucose as source of carbon without loss of efficiency in the medium: glycerol, mannitol, arabinose, lactate, citrate, pyruvate and glycogen.

4. Amino-acids, as such, do not supply an improved source of nitrogen or carbon to the medium. Glycine, asparagine, alanine, and possibly cystine may supply carbon, but ammonium salts are a more efficient source of nitrogen.

I take this opportunity of expressing my thanks to Prof. J. C. Drummond for his helpful advice and criticism throughout this work, and to Mrs P. T. Kerridge for help with the determinations of hydrogen ion concentrations.

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