529. *The Conditions for Optimum Growth Rate of* Bact. lactis aerogenes.

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In synthetic media containing carbohydrate, phosphate buffer, the necessary inorganic salts, and ammonium sulphate or individual amino-acids, *Bact. lactis ærogenes* grows with a mean generation time of not less than about 30 minutes; in meat extracts of unknown composition this falls to about 20 minutes.

The requirements for this rapid growth have been shown to be the presence not of trace elements, ready-made nucleic acids, nucleotides or their constituents, or other compounds of high molecular weight, but a complete series of amino-acids. None of these occupies a key position, but the addition of each new member of a list of about 20 causes a stepwise shortening of the mean generation time. The kinetic reason for this is discussed.

Introduction.—*Bact. lactis aerogenes* grows readily in simple artificial media containing glucose, ammonium sulphate, magnesium sulphate, and potassium phosphate. In the logarithmic growth phase, during which the number of cells varies with time according to the law $1/n(\mathrm{d}n/\mathrm{d}t) = k$, the time taken for the bacterial population to double is approximately 33 minutes at 40·0°. This value for what is termed the mean generation time (M.G.T.) is not lowered by substitution of any other carbon source for glucose, though replacement of ammonium salts by certain compounds such as asparagine shortens the M.G.T. to about 30 minutes. On the other hand, meat extracts of unknown composition, such as that sold under the name of " Lemco broth " permit M.G.T.s as short as 20 minutes.

Considerable interest attaches to the question of the compounds which must be provided ready-made in the solution to enable the cells to multiply at the maximum rate of which they are inherently capable, and the experiments to be described were designed to yield systematic information on this matter.

It will be convenient first to classify the experiments and to outline the main results, and then to describe the details in the appropriate sub-sections.

The first question is whether for optimum growth rate the cells require compounds of complex structure and high molecular weight, such as polypeptides and nucleic acids. The answer is negative, since a solution formed by hydrolysis of the bacteria themselves with sulphuric acid at 100°, a process which none of these complex compounds would survive, supports growth with an M.G.T. of 20—24 minutes (section 1).

The next question is whether the meat preparations contain traces of metallic elements, which are absent from the synthetic media, and are essential for the highest rates of growth. This possibility is also disproved by the use of bacterial hydrolysates. Bacteria are grown for many generations in the normal synthetic media, and can not be richer in the trace elements than the content of these media permit. Yet the hydrolysates derived from them support growth at rates comparable with those in the broth. The virtue of these solutions must thus depend upon the organic compounds which the bacteria have synthesised (section 1).

Much work has already been done in this laboratory on the utilisation of various carbon sources and it seemed unlikely that there would be a significant improvement in growth rate on

the replacement of glucose by any other sugar with the possible exception of ribose. This compound, in view of its importance in nucleoproteins, might have had special virtues, which in fact it proved on trial not to have (section 2).

A group of substances related to nucleic acids and nucleotides was then investigated. This consisted of yeast and thymus nucleic acids themselves, the nucleotides adenylic acid and guanylic acid and the compounds guanine, thymine, uracil, guanosine, and adenosine. None of these proved capable of inducing growth at the optimum rate though certain combinations, mainly those containing guanylic acid, gave a transitory enhancement of the rate of division without correspondingly increased rate of synthesis, the result being a decrease in the size of the cells present (section 2).

Finally, numerous combinations of amino-acids were tested. A mixture of all those normally contained in protein hydrolysates, added to a glucose–magnesium sulphate–phosphate medium, gave a solution capable of supporting growth with M.G.T. in the neighbourhood of 21 minutes which closely approaches that observed in the " natural " medium. Investigation of various combinations showed that the optimum rate is only attainable when the acids are all present together, and that, although some are more efficacious than others, the addition of each new one to a basic medium gives an extra shortening of the M.G.T. (sections 3 and 4). The significance of this step-wise shortening of the M.G.T. as fresh amino-acids are added and the non-existence of any individual key-compounds is considered in the Discussion.

It is also of interest to note that the solution formed by complete lysis of a bacterial suspension with ultra-violet light is much less effective in supporting growth than that formed by hydrolysis with sulphuric acid. Certain important structures are evidently destroyed by the ultra-violet light. Indeed it is not impossible that this destruction may be the cause initiating the lysis itself (section 5).

The general experimental methods are similar to those described in previous papers from this laboratory. What will be referred to as the normal medium is a sterile solution containing magnesium sulphate, ammonium sulphate, glucose, and phosphate buffer. Usually it is made up in boiling tubes in 26 ml. volume, consisting of 1 ml. of 1 g./l. magnesium sulphate solution, 5 ml. of 5 g./l. ammonium sulphate solution, 10 ml. of M/15-potassium dihydrogen phosphate which has been adjusted to pH 7·12 with sodium hydroxide, and 10 ml. of 50 g./l. glucose solution. All these solutions are kept in flasks plugged with sterile cotton wool and are sterilised daily by boiling. A " normal culture " refers to a culture of *Bact. lactis aerogenes* in the normal medium.

Growth rates were determined either (*a*) by turbidimetric examination of samples with a photoelectric " Spekker " instrument or (*b*) by direct counting in a hæmocytometer chamber under the microscope. Except when otherwise stated the curves of log (count) plotted against time were of the standard linear form, from which the M.G.T. could be read off.

Section 1. *Growth of* Bact. lactis aerogenes *in Media which contain the Product of Acid Hydrolysis of the Bacteria.*—The cells from about 500 ml. of a fully-grown normal culture were centrifuged free from the mother-liquor and suspended in 20 ml. of glass-distilled water. This suspension was brought to normal acid strength by addition of sulphuric acid and was then kept at 100° in an oil-bath for 24 hours. The resulting solution was decanted or filtered, neutralised with pure sodium hydroxide dissolved in glass-distilled water, and sterilised by boiling. Sufficient of the neutral and sterile solution was added to a normal medium to support growth up to high cell counts. The amount was estimated in terms of the number of cells from which the lysate had originally come. The M.G.T. of *Bact. lactis aerogenes* in this medium was measured by either turbidimetric or direct-counting methods. Results are given in Table I.

It is concluded that neither trace elements nor very complex compounds are essential for fast growth : the first because the trace-element content of the hydrolysate could not exceed that of the artificial medium from which it had been grown; the second because the complex compounds would not be stable under the drastic conditions of the hydrolysis.

Section 2. *Effect on the Mean Generation Time of* Bact. lactis aerogenes *of Nucleic Acid Constituents added to the Medium.*—Each medium was prepared by weighing out the compounds with a glass spatula into a normal medium less glucose and sometimes less ammonium sulphate, warming to dissolve the compounds, filtering off any undissolved guanine if present and boiling to sterilise, and then cooling and adding the other constituents of the normal medium. Growth was measured by counting the cells in a sample with a microscope and hæmocytometer.

The plots of log (count) against time were often not of the standard linear form but were curves concave towards the time axis. In such cases the initial (and steepest) slope was used to calculate the M.G.T., so that the results given in Table II correspond to the periods of fastest growth and are thus biassed in favour of short M.G.T.s. The Table shows that in

TABLE I.

Mean generation times in media containing acid lysate.

Treatments other than neutralisation accorded to lysate.	Amount of lysate added.*	Method of measuring growth.	Mean generation time in mins.
Decanted from ppt.	approx. 0·75	Counted by microscope	24
,, ,,	approx. 2	,, ,,	20
,, ,,	approx. 1·5	,, ,,	24
,, ,,	approx. 3	,, ,,	23·5
Filtered, then ppt. separately dissolved in conc. HNO_3, neutralised, and added to lysate	approx. 1·5	,, ,,	24
Filtered	approx. 0·75	Counted by Spekker absorptiometer	23·5
,,	approx. 1	,, ,,	21

* Amount of lysate measured in equivalents of a normal fully-grown culture as explained in the text.

TABLE II.

Ammonium sulphate.	Asparagine.	Aspartic acid.	Glutamic acid.	Guanine.	Adenine.	Uracil.	Thymine.	Ribose.	Guanosine.	Adenosine.	Guanylic acid.	Adenylic acid.	Thymus nucleic acid.	Yeast nucleic acid.	Range of count in millions/ml. over which M.G.T. applies.	M.G.T. in mins.
—	25	—	—	—	—	—	—	—	—	—	—	—	—	—	20—1000	32·5, 32
—	15	5	5	—	—	—	—	—	—	—	—	—	—	—	,,	32·5, 30
25	15	5	5	S	—	—	—	—	—	—	—	—	—	—	15— 30	32
25	15	5	5	—	25	—	—	—	—	—	—	—	—	—	15—200	40
25	15	5	5	—	—	25	—	—	—	—	—	—	—	—	150—300	46
25	—	—	—	—	—	—	10	—	—	—	—	—	—	—	50—250	30 *
25	—	—	—	—	—	—	—	200	—	—	—	—	—	—	40—200	31
25	15	5	5	—	—	—	—	—	15	—	—	—	—	—	30—250	32, 36
25	15	5	5	—	—	—	—	—	—	15	—	—	—	—	30— 80	50
25	15	5	5	—	—	—	—	—	—	—	10	—	—	—	20—100	21, 23, 30, 33
25	15	5	—	—	—	—	—	—	—	—	5—30	—	—	—	15— 30	32, 32, 32
25	15	5	5	—	—	—	—	—	—	—	—	10	—	—	20— 80	27
25	15	5	5	—	—	—	—	—	—	—	—	—	100	—	20—250	31·5
—	15	5	5	—	—	—	—	—	—	—	—	—	—	100	20—1000	33·5
25	15	5	5	S	25	25	—	—	—	—	—	—	—	—	50—250	46
25	15	5	5	—	—	—	—	—	—	—	10	10	—	—	15— 50	30 *
25	15	5	5	S	—	—	—	—	15	—	10	—	25	—	10— 40	25
25	15	5	5	S	10	10	—	—	10	10	10	—	20	20	40—100	38
25	15	5	5	S	10	10	—	100	10	10	10	20	20	10—100	24, 24

Figures show mg. of the compound added to normal medium (26 ml.) less ammonium sulphate.
S = saturated solution of the compound in the medium.
* = measured by absorptiometer only.

spite of this no combination of these compounds supported fast growth over an appreciable range, although there seems to be a short initial stage of rapid cell division in some media containing the nucleotides adenylic and, especially, guanylic acid. This short period of rapid division is evidently not accompanied by a commensurate rapid growth of cell material, for turbidity measurements (which essentially indicate the latter) do not yield such short values for the M.G.T., and observation shows that the cells become smaller as multiplication proceeds. In all cases, above counts of about 100 million/ml. growth and multiplication rates are slow.

The conclusion is that absence of these compounds from the normal medium is not the cause of growth rates less than the optimum.

Section 3. *Growth Rates of* Bact. lactis aerogenes *in Media containing Mixtures of "Essential" Amino-acids.*—The first few experiments showed that variation from 2 to 10 mg. in the amount of an amino-acid added to a normal medium did not alter the M.G.T. Consequently the later media were prepared by addition of approx. 5 mg. of each amino-acid. Otherwise the media were prepared by the same technique as that described in Section 2. Growth was measured by turbidity since this method is much quicker than counting, and the values obtained yielded straight-line plots of log (count) against time. The M.G.T.s measured for these media are given in Table III.

TABLE III.

More important amino-acids.																Less important amino-acids.			
Serine.	Leucine.	Alanine.	Methionine.	Valine.	*iso*Leucine.	Phenylalanine.	Tryptophan.	Threonine.	Cystine.	Aspartic acid.	Proline.	Cysteine.	Glutamic acid.	Tyrosine.	Hydroxyproline.	Arginine.	Glycine.	Lysine.	Histidine.	Mean generation time in mins.
P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	21, 22, 22, 23, 23, 23, 23, 24
P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	—	21
P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	—	—	—	23, 24, 27, 25
P	P	P	P	P	P	P	P	—	P	P	—	—	P	P	P	—	—	—	—	24, 25, 25, 31
P	P	P	P	P	P	P	P	—	P	P	—	—	P	P	—	—	—	—	—	24
P	P	P	P	P	P	P	P	P	P	P	P	—	—	P	P	—	—	—	—	25
P	P	P	P	P	P	P	P	P	—	—	—	—	—	—	—	—	—	—	—	25
P	P	P	P	P	P	P	P	—	—	P	P	—	P	—	P	—	—	P	—	25·5
P	P	P	P	P	P	—	—	—	—	—	—	—	P	—	—	—	—	—	—	26·5
—	P	—	P	P	P	P	P	P	P	P	—	P	P	P	—	P	—	—	—	26, 28
—	P	—	P	P	—	—	P	—	P	P	—	—	P	P	—	—	—	—	—	26, 30
P	P	P	P	P	—	—	P	P	—	P	P	—	P	P	P	—	P	P	P	27·5
P	P	P	P	P	P	P	—	—	—	—	—	—	—	—	—	—	—	—	—	28
P	P	P	—	P	—	—	—	—	—	P	—	—	—	—	—	—	P	P	—	28
P	P	P	P	P	—	—	P	—	P	P	P	—	P	P	P	—	—	—	—	28·5
P	P	P	P	P	P	—	P	—	P	—	—	—	—	—	—	—	—	—	—	28, 30
P	P	P	P	P	P	P	P	—	P	P	—	—	—	P	—	—	—	—	—	29
—	P	—	P	P	P	P	P	P	P	—	—	P	—	P	—	P	—	P	P	29
P	P	P	P	—	—	—	P	—	—	—	—	—	—	—	—	—	—	—	—	29, 30
P	P	P	P	P	P	—	—	—	—	P	P	—	P	P	P	P	P	—	—	30
P	—	P	—	—	P	P	—	P	—	—	P	P	—	—	P	P	—	—	—	30
P	P	P	P	P	P	P	P	—	P	—	—	—	—	P	—	—	—	—	—	31
P	P	P	P	P	P	—	P	—	P	—	—	—	—	P	—	—	—	—	—	32
P	—	P	—	—	—	P	P	P	P	P	P	P	P	—	P	—	P	P	P	34

P = compound present in the medium.

The results show that addition of amino-acids to the medium helps growth and that as more acids are added so the M.G.T. is progressively shortened until, when all 20 acids are present, the optimum value of 21—23 minutes is attained. In general, attempts to eliminate certain amino-acids as ineffective were unsuccessful, although glycine, lysine, and histidine seemed to contribute comparatively little. It was, however, possible to grade the remaining 17 acids in a rough order of importance by measurement of the M.G.T.s in media containing all these acids less each member in turn. The results of the experiments are shown in Table IV.

TABLE IV.

Mean generation times in media containing all amino-acids less one.

Normal medium + all amino-acids except	M.G.T. in mins.	
Serine	31·5, 35	Most effective acids
Leucine	31, 35	
Alanine	30, 30	
Methionine	26·5, 30	
Valine	27·5, 25	
*iso*Leucine	27, 24	
Phenylalanine	27	
Tryptophan	28, 23	
Threonine	26	
Cystine	26·5, 23·5	
Aspartic acid	25·5	
Proline	25	
Cysteine	25	
Glutamic acid	25	
Tyrosine	24, 23·5	
Hydroxyproline	25, 22	
Arginine	23	Least effective acids

Section 4. Growth Rates of Bact. lactis aerogenes *in Media containing Nucleic Acid Constituents and Amino-acids together.*—Results of these experiments, which were carried out in the usual way, are shown in Table V. It appears that adding nucleic acid constitutents to a medium containing all the amino-acids causes no further shortening of the M.G.T.

TABLE V.

Mean generation times in media containing mixtures of amino-acids and nucleic acid constituents.

Amino-acid mixture.	Guanine.	Adenine.	Thymine.	Ribose.	Adenylic acid.	Guanylic acid.	Yeast nucleic acid.	Thymus nucleic acid.	Nicotinic acid.	Riboflavin.	M.G.T. in mins.
A									P		27
A										P	30
A				P	P	P	P	P			33, 33
A				P	P		P	P			27, 31
A											27·5
B									P		24·5
B											21, 23
B				*							33
B			P								22, 23, 25
B	P										23
B		P									22·5
B	P	P									22·5

Amino-acid mixtures.

A: Serine, leucine, alanine, methionine, tryptophan, valine, *iso*leucine, tyrosine, phenylalanine, aspartic acid, glutamic acid, and cystine.

B: All 20 amino-acids.

P = compound present in the medium.

* = Ribose present but no glucose in medium.

TABLE VI.

Mean generation time in media containing lysates.

Ml. of 24-hour acid lysate.	Ml. of unboiled u.-v. lysate.	Ml. of boiled u.-v. lysate.	Method of measurement.	M.G.T. in mins.
	2		Turbidity + counting	27, 31
	4		,,	30, 27
	8		,,	35
1			,,	25, 24, 21
3			Counting	20
2			,,	24, 24
5			,,	23·5
		2	Turbidity	27, 30, 28
		4	Counting	32, 32
2		2	Turbidity + counting	23, 21, 23·5
2		4	Counting	22
With 9 ml. of u.-v. lysate which had been kept at 100° for 24 hours with N-H_2SO_4			Counting	35
Ditto with 2 ml. of above solution			Turbidity	34
With 3 ml. of acid lysate which had been irradiated with u.-v. light for 5—7 days			Turbidity	29, 26·5
Acid lysate + all amino-acids except cystine			Turbidity	23
Irradiated acid lysate + all amino-acids except cystine			Turbidity	25

Section 5. The Difference between the Effects of Ultra-violet and Acid Lysates on the Growth Rate.—The effect of the acid lysate in speeding up growth of *Bact. lactis aerogenes* has already been described in Section 1. A solution was obtained by irradiating a suspension of bacteria in glass-distilled water with ultra-violet light for several days until almost complete lysis had occurred. This when it was added to the normal medium supported an appreciably slower growth than the acid hydrolysate. The M.G.T.s for these media are given in Table VI.

There seem to be three possible reasons why the ultra-violet lysate might support slower

growth than the acid lysate : (i) ultra-violet lysate contains an inhibitor, (ii) ultra-violet light breaks down cell material less completely than does acid, and the cells prefer the more completely broken down products as food, (iii) ultra-violet light breaks down cell material more completely than does acid, and cells prefer the less broken down (*i.e.*, more complex) compounds as food. To decide between these alternatives the following experiments were made : (i) A mixture of ultra-violet and acid lysates was added to normal medium, and it was found that the cells grew at the same speed as they did in the acid-lysate medium alone. This showed that the ultra-violet lysate contained no inhibitor, or possibly that an inhibitor is present but its action is reversed by presence of compounds contained in the acid lysate. (ii) Ultra-violet lysate was heated with normal acid for 24 hours at 100°, and then neutralised and added to the normal growth medium. The M.G.T. in this solution equalled that of the untreated ultra-violet lysate, so that the superiority of the acid lysate is not merely due to more complete decomposition of the cell material. (iii) Acid lysate was irradiated with ultra-violet light for 7 days, and then neutralised and boiled, and its effect on growth compared with that of a similar sample of acid lysate which was not irradiated. In the first experiment it was found that both the irradiated and non-irradiated lysates yielded the same slow growth with M.G.T. of 29 minutes. In a second experiment in which ultra-violet treatment lasted for only 5 days, the irradiated lysate gave growth with an M.G.T. of 26·5 minutes and the non-irradiated lysate faster growth with M.G.T. 23·5 minutes.

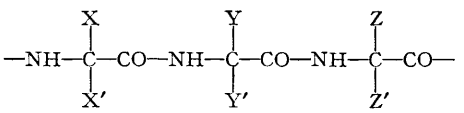
It thus seems likely, but not certain, that ultra-violet light decomposes compounds which assist rapid growth. Further, some amino-acids (*e.g.*, phenylalanine and tryptophan) have strong absorption bands in the ultra-violet, and if a clear solution of 20 essential amino-acids is irradiated it darkens. This suggests that perhaps the difference between the ultra-violet and acid lysates is that the former is without certain essential amino-acids which are decomposed by ultra-violet light. To test this, the M.G.T. was measured, by absorptiometer, for media containing all the amino-acids and a sample of irradiated acid lysate, as well as the normal compounds. The value of 25 minutes which was found is faster than that for the irradiated lysate alone but slower than that for the amino-acid mixture alone.

The tentative conclusion drawn from these experiments is that the ultra-violet lysate is without some important amino-acid constituents of the acid lysate and may contain slightly inhibitory compounds. Further support for this view was later obtained by chromatographic analysis of the acid and ultra-violet lysates, the technique described by Dent (*Biochem. J.*, 1948, **43**, 169) being used. Two independent experiments failed to reveal any amino-acids in the ultra-violet lysate, whilst serine, valine, leucine, alanine, glycine, lysine, and aspartic acid were identified in the acid lysate.

Discussion.

The Effect of Amino-acids on Growth Rate.—The experiments have not indicated any inhibitory action of amino-acids. Nor has it been possible to find a small group of acids which gives as fast a growth as a mixture of them all. There seems rather to be a stepwise shortening of the M.G.T. with each addition of a fresh amino-acid, and the following simple scheme shows how this phenomenon might arise.

It is assumed that the bacterium builds up its proteins by forming amino-acids into peptide chains



which can be written shortly as

—A—B—C—

where A, B, and C are amino-acid radicals.

On the assumption that in an experiment all conditions are constant except the amino-acid concentration, the rate of arrival of any one constituent at the growing end of the peptide chain will be proportional to a function of its concentration, *i.e.*,

$$\text{Rate of arrival of A} = f_A([A]).$$

where [A] is the concentration of A in the medium.

Therefore the average time interval between the attachment of the preceding constituent and the arrival of A is given by

$$t_A = 1/f_A([A])$$

and the time taken to build up the unit A—B—C by

$$T = t_A + t_B + t_C = \frac{1}{f_A([A])} + \frac{1}{f_B([B])} + \frac{1}{f_C([C])}.$$

The M.G.T. during the period of steady growth will be the sum of such times, so that we can write

$$\text{M.G.T.} = 1/\Sigma f_A([A])$$

As $[A] \longrightarrow \infty$, so we should expect $1/f_A([A]) \longrightarrow 0$ or some finite limiting value. But however low the value of $1/f_A([A])$ the M.G.T. will be kept up by the other terms $1/f_B([B])$ etc. Only when each of these other terms has successively been brought to its lower limit (either finite or zero) does the M.G.T. reach the lowest limit possible.

In this way it can be explained how omission of amino-acids from the medium causes stepwise increments in the M.G.T. of the cell and why a large number of acids must be present to give the fastest growth.

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