353. *The Growth of an Aneurin-requiring Yeast Strain in Various Substrates related to the Tricarboxylic Acid Cycle.*

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A pure strain isolated from brewery yeast proved to be unusual in being independent of growth factors other than aneurin.

In a glucose–ammonium sulphate medium, the total cell population was proportional to the aneurin concentration up to 10^{-8} M., whereas down to 10^{-9} M., and in much higher aneurin concentrations, the growth rate was constant. The aneurin is thus either a necessary structural unit or, if a catalyst, one which is consumable.

With ammonium sulphate as the nitrogen source, acetate was the only carbon source which supported growth in the absence of aneurin. Even in its presence, certain substances which are intermediates in the tricarboxylic acid cycle (Krebs) did not support growth.

With glucose as the carbon source, amino-acids supported growth only in the presence of aneurin. The growth rate was not increased by serial sub-culture in these media. The highest rate was attained with ammonium sulphate or asparagine as the nitrogen source; with other amino-acids the rate was lower, probably because it was limited by the rate of de-amination.

Origin of the Strain.—A brewery yeast was grown at 25° in an aerated synthetic medium containing glucose, ammonium sulphate, certain growth factors, and small amounts of inorganic salts, buffered to pH 5 with sodium dihydrogen phosphate. Growth was vigorous under these conditions and after six sub-cultures in the synthetic medium, the yeast was plated out on wort agar and individual colonies were selected.

The single colony strains were serially sub-cultured in the synthetic medium incorporating six growth factors. From these series, test transfers were made to similar media, each lacking one of the original six growth factors. The strains were found to be of two types. One was able to grow well in all media containing biotin, but only very slowly, at least initially, in its absence. The other was able to grow vigorously in all the media which contained aneurin, but in its absence only to slight extent in the first sub-culture and not at all in the second. Such a strain is unusual; there appears to be no report in the literature of a strain solely dependent on aneurin (cf. Atkin, *Wallerstein Lab. Comm.*, 1949, xii, **37**, 141).

A single cell was isolated from one of the aneurin-dependent strains, and the experiments to be described were all carried out with cultures originating from this.

Growth of the Strain in Glucose–Ammonium Sulphate Medium containing an Ample Supply of Aneurin.—A detailed examination was made of the growth characteristics of the strain in media containing glucose as the carbon source and ammonium sulphate as the nitrogen source.

The media (Dr. Barton Wright) were made by mixing solutions as follows, under sterile conditions: 10 ml. of glucose solution (50 g./l.); 10 ml. of NaH_2PO_4 solution (3·9 g./l.; pH 5); 5 ml. of a solution containing $(NH_4)_2SO_4$ (10 g./l.), $MgSO_4,7H_2O$ (2·5 g./l.), $CaCl_2$ (1·3 g./l.), KI (0·5 mg./l.), boric acid (0·5 mg./l.), $MnSO_4,4H_2O$ (0·2 mg./l.), $ZnSO_4$ (0·1 mg./l.), $CaSO_4$ (0·1 mg./l.), ammonium molybdate (0·1 mg./l.), and $FeSO_4,7H_2O$ (0·1 mg./l.).

To this medium, growth factor solutions were added as required. Thus, to complete the standard aneurin medium, 0·1 ml. of a solution containing 0·5 mg. of aneurin hydrochloride per l. was added; to complete the fully enriched medium, the other growth factors were added as follows: 0·1 ml. each of calcium D-pantothenate solution (0·5 mg./l.), nicotinic acid solution (0·5 mg./l.), pyridoxine hydrochloride solution (0·5 mg./l.), inositol solution (2·5 mg./l.), and biotin solution (0·5 mg./l.).

The cultures were kept at 25° ± 0·05° in a water-bath and were aerated with filtered air.

In all glucose media there was only a very short lag, followed by a phase of rapid, logarithmic growth which ended when the yeast population reached 50—200 million cells/ml.; thereafter, growth became slow and was not very reproducible in rate or extent. The rate of logarithmic growth, however, was satisfactorily reproducible.

Growth rates were obtained by determining the yeast populations of samples taken at suitable intervals. A Spekker absorptiometer was used to measure the light transmission; it was calibrated against counts of cell population made with microscope and hœmocytometer. The mean generation time, *i.e.*, the time taken for the cell population to double, is the most convenient expression of growth rates.

The mean generation time in the standard aneurin medium was 120 minutes and the final population was about 500 million cells/ml. These remained unchanged when the other growth factors were added and when the aneurin concentration was increased by factors of 10, 100, 1000, or 10,000. The behaviour was the same whether the growth factors were sterilized by heat or by filtration through a bacterial filter. It is clear that the growth factor requirements of the strain are entirely satisfied by the standard aneurin concentration.

In a medium made up without growth factors, growth was limited by exhaustion of the small amount of aneurin carried over with the inoculum from the parent culture. Growth only occurred to this slight extent with uracil in place of aneurin.

Growth of the Strain in Glucose–Ammonium Sulphate Medium containing only Small Amounts of Aneurin.—In Fig. 1, the final populations attained in media containing low aneurin concentrations are plotted against the aneurin concentration. The final population is directly proportional to the aneurin concentration below about 10^{-8} M. The standard concentration was $6{\cdot}7 \times 10^{-7}$

Dependence of final population on:

FIG. 1.

Aneurin concentration.

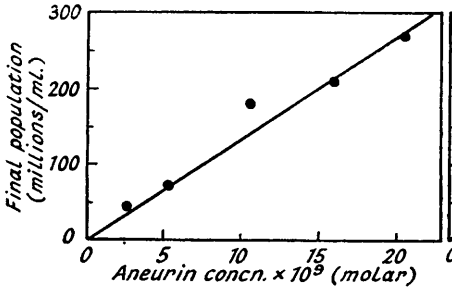


FIG. 2.

Ammonium sulphate concentration.

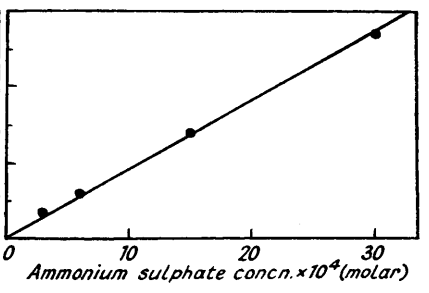
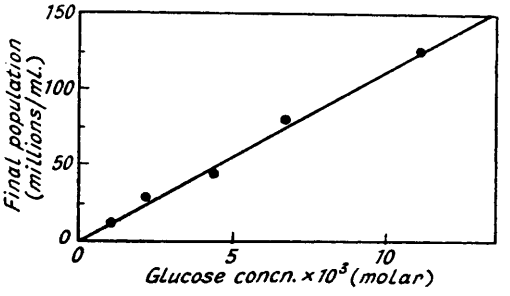


FIG. 3.

Glucose concentration.



M.; in the standard aneurin medium the population must be limited by factors other than the exhaustion of aneurin.

From the slope of the line in Fig. 1, it is possible to calculate the number of aneurin molecules required for the synthesis of each yeast cell. Each cell requires $\frac{22{\cdot}4 \times 10^{-9} \times 6 \times 10^{23}}{300 \times 10^{9}} = 4{\cdot}5 \times 10^{4}$ molecules of aneurin.

It is interesting to compare this figure with the corresponding requirements of glucose and ammonium sulphate. Plots of final population against nutrient concentration over the limiting ranges are shown in Figs. 2 and 3. From the slopes, similar calculations can be made. Each cell requires $\frac{13{\cdot}3 \times 10^{-3} \times 6 \times 10^{23}}{150 \times 10^{9}} = 5 \times 10^{10}$ molecules of glucose and $\frac{32{\cdot}8 \times 10^{-4} \times 6 \times 10^{23}}{300 \times 10^{9}} = 6{\cdot}5 \times 10^{9}$ molecules of ammonium sulphate.

Evidently each molecule of aneurin catalyses the metabolism of about one million molecules of one of the substantial nutrients.

Since the final population is proportional to the aneurin concentration over the appropriate

range, it is evident that aneurin does not function as a simple catalyst. Probably it is incorporated in the structure of the yeast cell or consumed at a constant rate in some other way.

If the aneurin functions as a co-enzyme (cocarboxylase has been identified as aneurin pyrophosphate) it should be diffusible and the growth rate should decrease as the aneurin concentration is lowered.

There is no sign that this occurs. Table I contains the results of an experiment designed to examine the growth rate of the strain in solutions of low aneurin content. To provide a direct comparison, Spekker instrument readings are listed. The method of measurement is not applicable to yeast populations of less than one million cells/ml., so that it was not convenient to make measurements at aneurin concentrations below 10^{-9} M.

TABLE I.

The Growth Rate of the Strain in Solutions of Low Aneurin Content.

Samples were taken from cultures grown in different aneurin concentrations, at the time intervals shown in the first column.

The Spekker absorptiometer readings of the samples are listed under the headings giving the aneurin concentration.

Time intervals (mins.).	Aneurin concentration (moles per l.).					
	$6{\cdot}7 \times 10^{-7}$.	$3{\cdot}3 \times 10^{-7}$.	$1{\cdot}5 \times 10^{-7}$.	$2{\cdot}1 \times 10^{-8}$.	$1{\cdot}1 \times 10^{-8}$.	$2{\cdot}7 \times 10^{-9}$.
0	0·50	0·50	0·50	0·52	0·51	0·52
90	0·46	0·46	0·47	0·46	0·47	0·51
180	0·47	0·41	0·44	0·36	—	0·44
300	0·31	0·33	0·34	0·16	0·37	0·28
390	1·23	1·20	1·22	1·15	1·21	1·22
Final population	390	570	420	270	180	44

The range of population covered in the table is from 2 to 20 million cells per ml. The discontinuous change between the fourth and fifth readings is due to a new standardisation of the Spekker absorptiometer.

Growth of the Strain in Alternative Sources of Carbon or Nitrogen.—The rôle of aneurin in the metabolism of our yeast strain was further investigated by examining the characteristics of growth with different sources of carbon or nitrogen.

Tests were made in media containing the standard aneurin concentration and in aneurin-free media. 10 Ml. of a solution of the new carbon source, at a concentration of 15, 20, or 25 g./l., adjusted to pH 5 with sodium hydroxide, was used in place of 10 ml. of glucose solution. For tests of new sources of nitrogen, ammonium sulphate in the " inorganic solution " was replaced by an amino-acid at a concentration of 6 or 10 g./l. 5 Ml. of this solution were used in making up each tube of medium.

Cultures in which growth was feeble were aerated by shaking. Special tubes, which could be mounted in the Spekker absorptiometer, were used. The light transmission of the whole culture could thus be measured, eliminating the need for sampling.

The results of these experiments are collected in Table II. The most striking features are the unique property of acetate in supporting growth in the absence of aneurin, and the failure of aconitate, citrate, α-ketoglutarate, and oxaloacetate to support growth even in its presence.

The final population in a glucose medium is much greater than that in carboxylic acid media. This is probably because growth is limited by different factors. During the growth of a culture in glucose, the medium becomes more acid, but this does not have a serious effect until the final pH of 2 units is reached; however, in carboxylic acid cultures the medium becomes more alkaline as the acid is consumed, and yeast does not flourish in these conditions.

Amino-acids support a relatively higher final population than ammonium sulphate, probably because the pH scarcely changes at all during growth in amino-acid media.

Although serial sub-culture leads to an increase in the growth rate in most of the carboxylic acid media, this is not the case in amino-acid media. Ammonium sulphate, and asparagine from which ammonia may be readily obtained, supports the highest growth rate; it is not increased when 26 amino-acids are supplied, in contrast to the behaviour of *Bact. lactis aerogenes* (Stephens and Hinshelwood, *J.*, 1949, 2516). According to Thorne (*J. Inst. Brewing*, 1945, **51**, 114), the growth rate of yeast in amino-acid media may be limited by the rate of de-amination. This appears to be the case with our yeast strain.

Metabolism of Carbon by the Yeast Strain.—Acetate is unique among the carbon sources in supporting growth in the absence of aneurin. It might, therefore, be supposed that aneurin can be synthesised from acetate, but this seems unlikely, because acetate is more efficiently utilised when aneurin is present. Growth is slow and erratic and the final population is only

TABLE II.

The Behaviour of the Strain with Alternative Sources of Carbon or Nitrogen.

Medium :	Mean generation time at standard aneurin concn. (mins.).	Stationary population at standard aneurin concn. (millions per ml.).	Stationary population when tested in aneurin-free medium (millions per ml.).
(*A*) $(NH_4)_2SO_4$ *plus* :			
Glucose	120	500	2·7
Glycerol	120	300	3·9
Lactate	300 (140)	100	3·6
Pyruvate	>500 (210)	30	4·3
Acetate	300 (150)	100	23·6
Citrate		Does not support growth	
Aconitate		Does not support growth	
α-Ketoglutarate		Does not support growth	
Succinate	220 (140)	130	3·7
Fumarate	190 (140)	50	3·1
Malate	220	40	1·7
Oxaloacetate		Does not support growth	
(*B*) *Glucose plus* :			
β-Alanine	270	400	3·2
Glutamate	120	550	4·1
Aspartate	150	480	4·7
Asparagine	110	1,300	2·6
Glycine	300	250	2·0
L-Leucine	170	580	2·5
Ammonium sulphate	120	500	2·7
26 Amino-acids	110	450	3·3

In the case of nutrients which support a faster growth rate after serial sub-culture than that attained when cells are first tested in the new medium, the initial mean generation time is listed, followed by the minimum generation time in parentheses.

25 million cells per ml. in the absence of aneurin, whereas in its presence the initial generation time is 300 minutes and the final population is 100 million cells per ml. It seems more probable that there are alternative routes for the oxidation of acetate, one of which is independent of aneurin. In the presence of aneurin, a more efficient route can be brought into action.

Although it is unlikely that the complex of metabolic processes occuring in living organisms can be precisely described in terms of a single scheme of reactions, there is no doubt that reaction cycles play an important part in such processes (cf. Krebs, *Enzymologia*, 1947, **12**, 88). The well-known tricarboxylic acid cycle (cf. Baldwin, " Dynamic Aspects of Biochemistry," Cambridge, 1947, Chapters XII and XIII; Ochoa, *Physiol. Rev.*, 1951, **31**, 56) may be taken as a guide to the mode of metabolism of carbon compounds and to the interpretation of the behaviour of our yeast strain in different carbon sources.

When our results are considered in this light, it seems reasonable to suppose that most substrates support growth only in the presence of aneurin because the latter is required as a catalyst in the oxidative decarboxylation of pyruvate (cf. Peters, *Nature*, 1940, **146**, 387). Acetate may be able to support growth in the absence of aneurin because it enters the scheme after this stage.

It is noticeable that citrate, aconitate, and α-ketoglutarate do not support growth, while succinate does. The proposal of a condensed cycle, in which acetate is directly oxidised to succinate, has received some support (cf. Weinhouse *et al.*, *J. Amer. Chem. Soc.*, 1948, **70**, 3680. It is conceivable that, in our strain, reactions of this type could occur instead of those involving tricarboxylic acids, which would thus fail to support growth.

The explanation of the comparative behaviour observed in succinate, fumarate, malate, oxaloacetate, acetate, and pyruvate cannot, however, be so simple. After training of the yeast, succinate, fumarate and acetate can support rapid growth, but growth in malate or pyruvate is much slower, and oxaloacetate does not support it at all.

The interpretation of these results in terms of reaction schemes cannot seriously be attempted until more evidence is available. The possible effects of different rates of penetration into the cell by different substrates have not been investigated. However, apart from them, the behaviour of our yeast strain in different substrates must be dependent on their relation to the possible modes of carbon metabolism.

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