TABLE II
DERIVATIVES OF 2-KETOPIPERAZINE

	M. p., °C. cor.	N, % Calcd.	Kjeld.[a]
Picrate	180	21.28	21.19
Hydrochloride	208	20.51	20.65
Phenylurea	171	19.18	19.18
Phenylthiourea	199	17.87	17.85

[a] Reported analytical results are the averages of two determinations, neither of which differs from the calculated by more than 0.15% absolute error.

Summary

1. A practical synthesis for the hitherto unknown 2-ketopiperazine and its 3-alkyl and 3,3-dialkyl derivatives has been developed.

2. A study of the reaction between ethylenediamine and α-halogen esters under different conditions has been carried out.

3. The piperazines prepared in this study have been fully characterized by reaction with such typical amine reagents as benzenesulfonyl chloride, picric acid, hydrogen chloride, phenyl isocyanate and phenyl isothiocyanate.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND CHEMICAL ENGINEERING, THE UNIVERSITY OF TEXAS]

The Relationship of Inositol, Thiamin, Biotin, Pantothenic Acid and Vitamin B_6 to the Growth of Yeasts

BY ROGER J. WILLIAMS, ROBERT E. EAKIN AND ESMOND E. SNELL

The specific chemical substances found to be effective constituents of culture media for promoting the growth of yeasts are of general biological interest. Inositol, found to be a growth factor for yeast in the laboratory of W. Lash Miller,[1] is of widespread occurrence in the tissues of plants and animals. Thiamin (vitamin B_1), first found to be effective for yeast in the author's laboratory,[2] was a recognized vitamin long before and has since proved to be of widespread significance in cellular physiology, functioning as the organic moiety of co-carboxylase (and perhaps in other ways). Biotin, discovered in the laboratories of F. Kögl,[3] is widely distributed and is physiologically effective for a number of organisms.[4,5,6] Pantothenic acid, discovered and concentrated in the author's laboratory,[7,8] is now recognized as identical to the filtrate factor or the chick antidermatitis vitamin,[9,10] and probably stimulates the growth of rats.[11] It is of ubiquitous occurrence and has been found essential for the growth of many bacteria[12,13] and to stimulate the growth of green plants[14] and the respiration of widely different tissues. It appears to be an essential constituent of some important enzyme systems.[15] β-Alanine is a component part of pantothenic acid[16] and may itself serve as a precursor of pantothenic acid both for yeasts and for certain bacteria.[13,16,17] Vitamin B_6 (adermin), recently found to be a growth factor for yeast,[18,19] bacteria,[20] and higher plants,[21] has for some time been recognized as a vitamin essential for rats.

Through the kindness of Professor Kögl, who has placed a sample of biotin at our disposal, we are now in a position to study the interaction of all of the factors enumerated above in promoting the growth of yeasts. The results of such studies may prove of great interest both from the standpoint of yeast physiology and because of the light which they may throw on the interplay of these

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physiological principles in biological phenomena in general.

While aspartic acid[22] (placed in our basal medium), leucine,[23] ethanolamine, riboflavin, nicotinamide, carotene and doubtless other recognized compounds may under suitable conditions increase growth or respiration of yeast, the effects are relatively small and these effects are not included in the present study.

The "optimal" concentration of any specific substance in a nutrient medium depends upon the strain of yeast, the cultural conditions, and particularly upon the other constituents of the medium. For practical reasons, however, we have fixed the concentration of each nutrilite at a level somewhat above that required to produce a "maximum" response when tested alone and have studied the growth curves of three distinct (and highly different) strains of "*Saccharomyces cerevisiae*" under the influence of the five more significant substances mentioned above.

The basal medium used throughout was similar to that used in other studies from the author's laboratory[16,24] and contained as the source of organic nitrogen 0.1 g. of *l*-aspartic acid per liter. This has the general effect of increasing yeast crops, though experiments have shown that it can be omitted (in some cases at least) without changing the general trend of the results in any major way.

Experimental

The carefully sterilized media (50 ml. batches in 250 ml. Erlenmeyer flasks) were each seeded with 0.2 mg. of the yeast under investigation and allowed to grow for one hundred to one hundred and sixty hours at 30°. At intervals the cultures were shaken and samples aseptically withdrawn and their yeast content determined with a thermocouple turbidimeter.[25,26]

The media used were composed as indicated below. An addendum when used was always in the same concentration, as follows: inositol 5 γ per ml. of the final medium; thiamin 0.04 γ per ml.; biotin 0.0001 γ per ml.; pantothenic acid 0.006 γ per ml.; β-alanine 1 γ per ml.; vitamin B_6 0.04 γ per ml. Since previous experiments had shown that β-alanine could serve as a precursor of pantothenic acid, this substance was substituted for pantothenic acid in some of the media as indicated. The amount of β-alanine used is much more than equivalent on a stoichiometric basis to the pantothenic acid addendum.

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(1) Basal alone.
(2) Basal + pantothenic acid (p. a.).
(3) Basal + β-alanine.
(4) Basal + biotin.
(5) Basal + inositol, thiamin, biotin and B_6 (no p. a.).
(6) Basal + inositol, thiamin, biotin and β-alanine (no B_6).
(7) Basal + inositol, biotin, β-alanine and B_6 (no thiamin).
(8) Basal + thiamin, biotin, β-alanine, and B_6 (no inositol).
(9) Basal + inositol, thiamin, β-alanine and B_6 (no biotin).
(10) Basal + inositol, thiamin, biotin, p. a. and B_6 (complete p. a.).
(11) Basal + inositol, thiamin, biotin, β-alanine and B_6 (complete β-alanine).
(12) Same as 11 above + 0.2 mg./ml. crude liver extract.

The strains of yeast used were (1) Gebrüder Mayer (G. M.), (2) "Old Process" (O. P.), and (3) yeast isolated (repeatedly) from Fleischmann baker's yeast (pound goods) (F. B.).[27]

The results of the tests are presented in Figs. 1, 2 and 3.

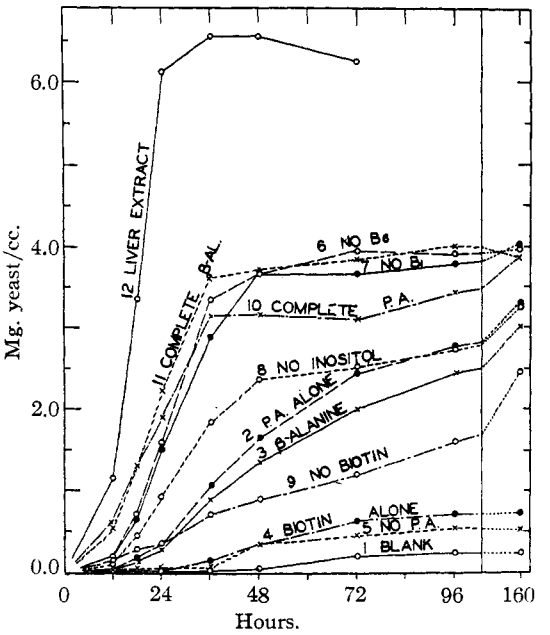

Fig. 1.—Growth curves of "Gebrüder Mayer" yeast.

Discussion of Results

In order to conserve space, separate curves are not given showing in more detail the growth during the first twenty-four hours. From many standpoints this is the most interesting period, since in the practical utilization of yeasts in the fermentation industries, the immediate response to the medium is often of much greater importance than what may happen after a long time interval. The great majority of studies of yeast growth have

(27) Cultures of these yeasts, all of which are or have been grown commercially for baking purposes, are being sent to the American Type Culture Collection, Georgetown Univ. Med. School, Washington, D. C., where duplicates may be obtained.

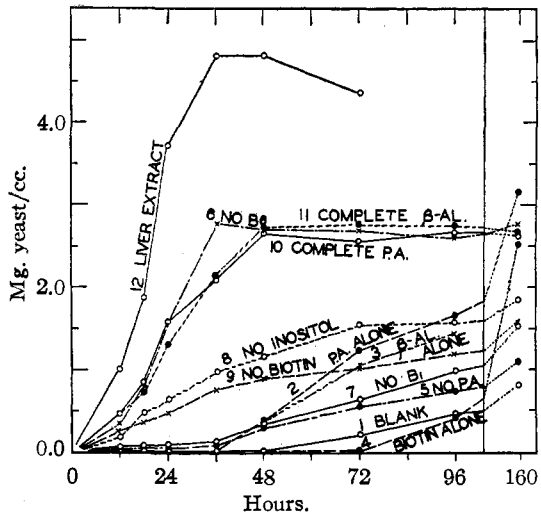

Fig. 2.—Growth curves of "Old Process" yeast.

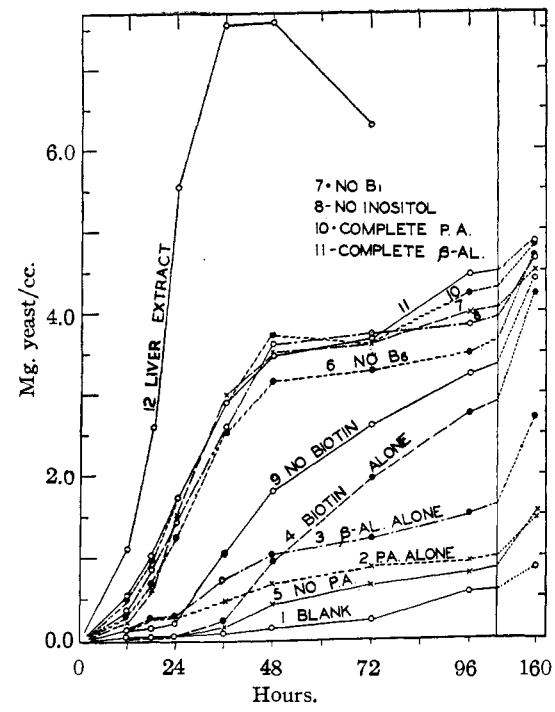

Fig. 3.—Growth curves of Fleischmann baker's yeast.

also involved a comparatively short period of incubation. The problem of whether and to what extent yeast is able to grow in a medium after many days of incubation is of scientific and academic, rather than practical interest.

It is obvious that when continued growth takes place in a medium devoid of a particular substance the substance is either dispensable or can be synthesized by the yeast from other constituents of the medium. On the basis of available evidence we shall assume tentatively in such cases that synthesis is taking place.

Inositol.—F. B. yeast grows at only a slightly diminished rate, and up to high levels in the absence of inositol. G. M. yeast is more dependent upon an exogenous source of inositol, and O. P. yeast most dependent. There is a question in view of the fact that the growth curve of the O. P. yeast flattens after seventy-two hours, whether it is capable of indefinite growth in the absence of inositol.

Thiamin.—Both F. B. and G. M. yeasts are able to grow to high levels in the absence of thiamin, but O. P. yeast is dependent on an external source for rapid growth. It appears that O. P. yeast may develop an ability to synthesize thiamin, because after thirty-six hours the growth curve rises more rapidly; growth continued throughout the period of observation.

Biotin.—In no case does biotin alone induce an appreciable increase in growth during the first twenty-four hours (under our conditions, light seeding etc.). F. B. yeast is markedly affected by biotin (alone) after this period; other yeasts are less affected. When biotin is left out and the other nutrilites are put in, each yeast grows less than the maximum (with all five nutrilites), but F. B. yeast curiously yields most nearly maximum growth.

Pantothenic Acid (and β-Alanine).—The close correspondence between curves 2 and 3, Figs. 1, 2 and 3, and curves 10 and 11, Figs. 1, 2, and 3, in which pantothenic acid and β-alanine are interchanged, shows that for the purpose of these experiments pantothenic acid and β-alanine are practically equivalent physiologically. This is in line with the known fact that β-alanine is a precursor of pantothenic acid.[16]

None of the yeasts grow, except very slowly, during the first twenty-four hours in the absence of an exogenous source of pantothenic acid. Each yeast appears to be able to grow continuously in the synthetic medium when pantothenic acid or β-alanine is present. When the other four nutrilites are present and pantothenic acid is absent, growth continues at a low rate with F. B. and O. P. yeast, but with G. M. yeast growth ceases after a few days. This is in line with the previous observation that this yeast is apparently unable to bring about the synthesis of pantothenic acid.[17]

Vitamin B_6.—This vitamin appears dispensable for all three yeasts, yet its physiological effects

are noticeable in each case. Its absence from the medium is most noticeable in the case of G. M. yeast at the end of a twelve-hour growth period. In this case the "complete" medium yielded about three times the amount of yeast as the same medium lacking B_6.

Unknown Factors.—That unknown factors play an important part in stimulating the rapid growth of yeasts is evidenced by the fact that each yeast is strikingly stimulated when a liver extract is added to the medium containing all five of the nutrilites. Extracts of yeast also contain these additional stimulatory substances, which cannot be replaced by a higher concentration of the five nutrilites under consideration. For some yeasts at least biotic acid[28] is an important nutrilite.

Growth over Long Periods.—Although biotin and pantothenic acid are unlike chemically and are tested for under entirely different conditions, it does appear that when growth over a long period is concerned, they are in a measure interchangeable. Thus in the case of the F. B. and O. P. yeasts either biotin alone or pantothenic acid (or β-alanine) alone causes a substantial increase (100% or more) over the blank. For G. M. yeast biotin alone was relatively ineffective even after one hundred and sixty hours (*cf.* curves 1–4, Figs. 1, 2, 3). The same apparent replaceability of one nutrilite by others is observed in some cases where only one nutrilite is omitted from the medium. Similar phenomena have been observed in the senior author's laboratory when the mold *Trichophyton interdigitale* failed to grow on an amino acid medium but did grow appreciably when *any one* of three nutrilites (inositol, thiamin or pantothenic acid) was furnished.[29]

These results suggest that when one nutrilite alone is furnished it leads to the more ready synthesis of those which are lacking in the medium. It may be that the one nutrilite by furnishing the means of completing one of several enzyme systems enables the organism to derive energy for metabolic and synthetic activity.

Ability to Grow in a Synthetic Medium.—From the growth curves it appears that F. B. and O. P. yeasts are eventually able to grow continuously, though very slowly, in a medium containing none of the nutrilites. The growth of G. M. yeast, however, ceases after about three days, at which time about a 60-fold increase has taken place. The original yeast seeding of course has in it all of the requisites for growth. The extent to which growth can take place in a synthetic medium seems to depend on how far these original substances can be "diluted" by passage to the daughter cells and to what extent the yeast is able to synthesize the substances. G. M. yeast seems to be limited in its ability to grow in a synthetic medium by the fact that synthesis of pantothenic acid cannot take place. Whether G. M. yeast can through long "training" be induced to synthesize pantothenic acid is, again, unknown.

This work has been generously supported by grants from the Rockefeller Foundation and from Standard Brands, Inc., of New York.

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Summary

The growth curves of three distinct (and highly different) strains of "*Saccharomyces cerevisiae*" were studied in media containing different combinations of inositol, thiamin, biotin, pantothenic acid (or β-alanine) and vitamin B_6. Among the conclusions drawn are the following. At least two of the yeasts can grow continuously without inositol. Thiamin is especially important for one yeast only. Biotin alone will not immediately stimulate the yeast growth from small seedings but in proper combination its effect is striking and evident at very minute doses. Pantothenic acid (or its precursor β-alanine) is highly essential for the immediate growth of all of the yeasts and when added alone is able to induce continued growth of all the yeasts tested. B_6 is relatively unimportant as a constituent of the culture media. Substances of unknown nature present in liver and yeast extracts play an important role in promoting rapid growth. Yeasts during a long incubation period show considerable ability to grow in the absence of various nutrilites, and under such conditions one nutrilite alone may serve whereas for rapid growth several are required.

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