On the growth of some wine yeasts in synthetic media with special reference to vitamin requirements and amino acid utilization

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On the Growth of Some Wine Yeasts in Synthetic Media with Special Reference to Vitamin Requirements and Amino Acid Utilization

THESIS

presented to the
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for the Degree of
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by

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Citizen of Canada

Accepted on the recommendation of
Prof. T. O. Wikén and Prof. Dr. H. Deuel

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Preface

The experimental work in connection with this research was undertaken at the Department of Agricultural Bacteriology and Fermentation of the Swiss Federal Institute of Technology, Zürich, under the learned guidance of Prof. T. Wikén. For generously placing the resources of the institute at my disposal, and for valuable advice throughout the course of this work, I wish to record my sincere appreciation to my teacher.

This study was supported by an exchange fellowship granted to me by the Swiss Federal Institute of Technology, Zürich, and Macdonald College of McGill University, Montreal, Canada, as well as by funds from the "Weinbaufonds" of the "Abteilung für Landwirtschaft des Eidg. Volkswirtschaftsdepartements". To these bodies, I am deeply grateful.

The Italian wine yeasts, strain No. 67 "Saccharomyces ellipsoideus Sicilia", and strain No. 76 "Chianti Classico", were obtained from "Istituto di Microbiologia Agraria e Tecnica, Università di Perugia", Director: Prof. T. Castelli. The French wine yeasts, strain No. 14 "Entre Deux Mers", and strain No. 15 "Saint Emilion" were procured from the "Station Agronomique et Oenologique de Bordeaux", Director: Prof. J. Ribéreau-Gayon. The author wishes to express his thanks to the above mentioned for their cooperation.

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Part II

Introduction

Yeasts have long been used as favorite test objects in the fields of general and comparative biochemistry and physiology. Much of our knowledge of carbohydrate, lipid and vitamin synthesis and utilization is the result of investigations of these microorganisms. Copping (1929) established that different strains of yeast varied in their vitamin requirements when grown in synthetic substrates. Since Copping's work, many investigations concerning the vitamin requirements of culture yeasts (distilling, beer, wine and baking yeasts), and so-called wild yeasts have been made. Conclusions from these researches have included the hypothesis that the wild yeasts are able to proliferate in a synthetic medium in which water, sugar and inorganic salts are the only materials present, while the culture yeasts, which have been maintained in the laboratory in "natural" substrates for years, have lost their autotrophic characteristics, and are therefore incapable of substantial growth in synthetic substrates unless supplied with growth factors (Fries 1938, Schopfer 1949). Further, Rippel-Baldes (1952) states: "Für das Wachstum der 'Kulturhefen' ist, wie schon erwähnt, 'bios' der Hauptfaktor, ein Sammelbegriff für eine ganze Reihe wirksamer Stoffe, während 'wilde' Hefen, wie Torulopsis utilis (Eiweiss- oder Mineralhefe), diese Stoffe synthetisieren können."

Wikén and Richard (1951a, 1951b, 1952a, 1952b) and Fiechter (1953) investigated the auxo-autotrophic and auxo-heterotrophic properties of the Swiss wine yeast races "Fendant", "Herrliberg", "Salenegg", "Dézaley" and "Polymorphus II". They showed conclusively that, in so far as growth factors are concerned, "Fendant", "Salenegg" and "Herrliberg" are autotrophic, "Dézaley" is heterotrophic and "Polymorphus II" is autotrophic or heterotrophic, depending upon the type of synthetic medium employed.

The nitrogen nutrition of yeasts has been investigated from a variety of points of view by a number of workers. Nielsen (1933-41) has investigated, under conditions of minimal nitrogen content, the availability to yeasts of the nitrogen in a large number of amino acids contained in a synthetic medium utilizing a small amount of wort as growth supplement. Thorne (1945) using a chemically defined synthetic medium containing three bios constituents examined nitrogen nutrition. Wickerham (1946) conducted experiments relating to the assimilation of nitrogen from ammonium sulphate, potassium nitrate, urea, asparagine and peptone in a chemically defined medium supplemented with eight vitamins.

Presented here is a partial study of the vitamin requirements of the yeasts "Chianti Classico", "Entre Deux Mers", and "Saint Emilion", and a detailed study of the vitamin requirements of, amino acids requirements of, and amino nitrogen uptake by the yeast "Saccharomyces ellipsoideus Sicilia". Also given is a modified, simple, quantitative method of amino acid determination by means of paper chromatography.

General Methods

1. Cleaning of Glassware

Throughout this investigation all glassware coming in contact with culture material was cleaned in the following manner (Wikén and Richard 1951a) by:

1. Thoroughly washing with hot soda solution,
2. Rinsing with hot tap water followed by a distilled water rinse,
3. Autoclaving (while containing distilled water) at 120°C for 15 minutes,
4. Finally rinsing with distilled water.

2. Culture Vessels and Chemicals

Glassware used in the growing or maintenance of cultures consisted of either Jena 20 or Pyrex. Cotton plugs were of a chemically pure cotton obtained from Dr. von Buens Co., Flawil, Switzerland. All chemicals unless otherwise specified, were analytical grade. Calcium pantothenate, meso-inositol, (+)-biotin, D(-)α, γ-dihydroxy-β, β-dimethyl-γ-butyrolactone and vitamin-free glucose were supplied by Hoffman-La Roche, Basel, Switzerland.

3. Isolation of Yeast Strains

The yeasts, "Saccharomyces ellipsoideus Sicilia", "Chianti Classico", "Entre Deux Mers", and "Saint Emilion", were procured as lyophilized cultures produced in this department. The glass capsules were opened aseptically; the contents were well mixed with sterile water, and from the suspensions thus obtained, serial dilutions were prepared. One ml from each appropriate dilution was mixed with beer wort agar in Petri dishes and incubated 48 hours at 20^{o}C. The cells from four isolated colonies obtained from each yeast were suspended in sterile water and plates from these suspensions prepared and incubated as indicated above.

Similarly, four isolated colonies of strains from the latter plates were inoculated by streaking onto beer wort agar slopes, the number of strains of each yeast at this point being twenty. From these strains, one from each yeast was selected at random for preliminary study.

4. Basal Substrates

Three basal substrates were utilized. These were as follows (Wikén and Richard 1951a):

Substrate A

glucose	20.0 gm
malt extract	5.0 gm
NH_4Cl	0.5 gm
KH_2PO_4	0.5 gm
$MgSO_4 \cdot 7H_2O$	0.5 gm
$FeCl_3$ solution, 1 mg $FeCl_3$/ml	1.0 ml
distilled water to	1000.0 ml

The solution was heated 20 minutes, cooled, filtered through the same filter paper, and the pH adjusted with sodium hydroxide to 4.8-5.0 using Lyphan-type paper. Sterilization was achieved by the use of three steamings of 20 minutes duration, 24 hours apart.

Substrate A was used solely for maintenance of the yeasts, inoculations being made into 40 ml of fresh medium every other week.

Substrate B

glucose	50.0 gm
$(NH_4)_2SO_4$	6.0 gm
KH_2PO_4	2.0 gm
$MgSO_4 \cdot 7H_2O$	0.25 gm
$CaCl_2$	0.25 gm
H_3BO_3 solution, 1 mg H_3BO_3/ml	1.0 ml
$ZnSO_4$ solution, 1 mg $ZnSO_4$/ml	1.0 ml
$MnCl_2$ solution, 1 mg $MnCl_2$/ml	1.0 ml

Tl_2SO_4 solution, 1 mg Tl_2SO_4/ml	1.0 ml
$FeCl_3$ solution, 0.5 mg $FeCl_3$/ml	1.0 ml
$CuSO_4$ solution, 0.1 mg $CuSO_4$/ml	1.0 ml
KI solution, 0.1 mg KI/ml	1.0 ml
distilled water to	1000.0 ml

The glucose was dissolved separately, and the two solutions autoclaved at 120°C for 20 minutes. After being cooled and filtered, the solutions were combined, the pH was adjusted to 4.8, using Lyphan paper as indicator, the volume made to 1000 ml with distilled water, and the appropriate vitamins were added. Final sterilization was achieved as in substrate A.

Substrate C

glucose	50.0 gm
KCl	0.425 gm
KH_2PO_4	0.550 gm
$CaCl_2 \cdot 2H_2O$	0.125 gm
$MgSO_4 \cdot 7H_2O$	0.125 gm
$FeCl_3 \cdot 6H_2O$	2.5 mg
$MnSO_4 \cdot 4H_2O$	2.5 mg
citric acid	4.5 gm
casein hydrolysate, vitamin free, 10%	40.0 ml
distilled water to	1000.0 ml

The ingredients were dissolved in about 700 ml of distilled water, the pH was adjusted to 4.5 with potassium hydroxide, using Lyphan paper as indicator, and the solution autoclaved at 120°C for 20 minutes. After the solution had been cooled, it was filtered twice through the same filter paper, and the pH was again adjusted in like manner to 4.8. The volume was made to 1000 ml with distilled water.

During the preparation of the above substrates, the vitamins were added, as desired, just prior to final sterilization by steaming.

5. Preparation of the Inoculum

From the cultures, which had been maintained on substrate A, two drops of the desired yeast were inoculated into 40 ml of fresh substrate A, contained in 100 ml Erlenmeyer flasks, and incubated 48 hours at 20°C. The cells were separated from the medium by centrifuging at 3500 r.p.m., washing with sterile distilled water, followed by centrifugation. After the wash water was discarded, the cells were suspended in 20 ml of sterile distilled water, and the resulting suspension was then utilized as the final inoculation material. The above manipulations were conducted under aseptic conditions.

6. Turbidimetric Readings

Growth was determined turbidimetrically with a Beckman quartz spectrophotometer using a wave length of 660 mμ and a slit opening of 0.06 mm. The uninoculated substrate served as a blank, having been adjusted to read 100% transmittance. The growth was measured as per cent transmittance against the blank solution.

Five ml of the test substrate were removed by means of a pipette, which had been sterilized by wrapping in paper and heating in the oven for 1½ hours at 160^{o}C. Two ml of the test solution were used to rinse the cuvette, and the remaining three ml added to the cuvette for the required readings.

Part I
GROWTH EXPERIMENTS

A) The Influence of (+)-Biotin, Calcium Pantothenate and Meso-Inositol on Growth

1. Introduction

Wildiers (1901) discovered that yeast required for its nutrition a mysterious substance (or mysterious substances) which he called "bios". By introducing a very small seeding of yeast into a medium containing sugar, ammonium tartrate and the minerals in yeast ash, Wildier found that appreciable growth did not follow, but that propagation was induced when a small amount of sterile decoction of yeast was introduced into the medium. The above investigator felt justified in concluding that "bios" was indispensable for the growth of yeast. Unfortunately, the original publication on "bios" was almost completely overlooked, and between the years 1906 and 1919 "bios" all but disappeared from the biochemical literature.

Williams (1919) concluded that a substance of unknown nature, which was a constituent of yeast, was necessary for the nutrition of yeast cells, and that this substance was identical to the beri-beri-preventing vitamin. Kluyver (1913) established that crude maltose is more easily assimilated by yeast than the highly purified product, and suggested that an "oryzanin-like substance" was responsible. Abderhalden and Koehler (1919), and Bachmann (1919) showed that the growth of yeast and certain other organisms was accelerated by yeast extract. Bachmann concluded: "The ammount of organic material required is so small and the effect upon the growth and activity of the yeast is so striking that a marked similarity exists between these organic substances and the vitamins required for the normal development of animals."

It is now well known, of course, that the whole "bios" effect cannot be ascribed to any single substance, but that "vitamin B activity" is due to a considerable number of separable organic compounds, and that there is a great deal of overlapping so far as the responsible agents are concerned.

Williams (1940) established that, (a) two out of three strains of <u>Saccharomyces cerevisiae</u> were able to grow continously without inositol, (b) thiamin was important for one strain, and (c) biotin alone would not immediately stimulate the yeast growth from small seedings, but in proper combination with other vitamins, its effect was marked in very small quantities. Further, it was found that pantothenic acid (or its precursor β -alanine) was essential for the immediate growth of all yeasts tested.

Biotin was discovered and isolated in the form of the methyl ester (which is biologically active) by Kögl (1935). This growth factor, in keeping with its low concentration in tissues, possesses extraordinary activity in minute doses. However, Williams (1940) showed that three diverse strains of a yeast were able to grow continously from a small seeding in the absence of biotin, even though they responded to it strikingly under appropriate conditions. Further, Williams (1941) stated: "The stimulative effect of biotin is due, not to the fact that an exogenous source is required, but that its rate of synthesis may limit growth."

The first intimations of the existence of pantothenic acid are to be found in the publications of Williams and Bradway (1931). These were followed by an announcement by Williams et al. (1933) that a nutrilite for certain yeasts (which they named pantothenic acid) was of universal biological significance.

The discovery of the existance of β -alanine as a cleavage product of pantothenic acid was preceded by the findings that β -alanine itself was effective as a yeast nutrilite in very small amounts (Williams and Rohrmann 1935). Mitchell et al. (1940) found that the non-β -alanine portion of the molecule lactonized after cleavage, and that in this portion of the molecule a hydroxyl group was in the α -position. Williams and Major (1940), and Stiller et al. (1940) were able to condense the lactone with β -alanine to yield physiologically active pantothenic acid.

Certain molds and fungi synthesize pantothenic acid readily during growth (Williams et al. 1933), and for some it serves as a nutrilite without being an indispensable constituent (Mosher et al. 1936).

The first pure substance found to contribute definitely to "bios" activity was inositol (Eastcott 1928) which was isolated from tea. Inositol when added singly to a synthetic medium usually has little or no effect on growth, but becomes a limiting factor when certain other nutrilites are added.

The intent of this section is to determine the vitamin requirements of "Saccharomyces ellipsoideus Sicilia", "Chianti Classico", "Entre Deux Mers", and "Saint Emilion", and to select for further study the strain of yeast exhibiting the most interesting characteristics.

2. Experimental Methods and Results

a) Growth in Basal Substrates B and C with and without Vitamins

The four yeasts selected for study were inoculated into parallel series of flasks containing: (a) 40 ml of substrate B, and (b) 40 ml of substrate C.

After three days of incubation in substrate B at 20°C, "Entre Deux Mers" and "Chianti Classico" exhibited good but not abundant growth, while "Saccharomyces ellipsoideus Sicilia" and "Saint Emilion", although showing definite growth, did not attain the cell multiplication produced by "Entre Deux Mers" and "Chianti Classico".

Propagation of the four yeasts in substrate C in the absence of vitamins, was for practical purposes nil. Thus it may be concluded that these four tame wine yeasts are auxo-autotrophic in substrate B, i.e., when utilizing ammonium sulphate as a nitrogen source, and are auxo-heterotrophic in substrate C, i.e., when casein hydrolysate is utilized as a source of nitrogen.

The four yeasts were found to proliferate when inoculated into the media B or C containing vitamins (+)-biotin, calcium pantothenate, and meso-inositol in concentrations of 25 γ per liter, 2.5 mg per liter and 25 mg per liter respectively.

b) The Influence of Vitamins on Growth in Substrate B

To appropriate quantities of substrate B the following freshly prepared aqueous solutions of (+)-biotin, calcium pantothenate, and meso-inositol were added as necessary:

(+)-biotin	25.0 γ/liter,
calcium pantothenate	2.5 mg/liter,
meso-inositol	25.0 mg/liter.

Portions consisting of 250 ml of substrate B containing: (a) the three vitamins, (b) no vitamins, (c) (+)-biotin, (d) calcium pantothenate, and (e) meso-inositol were placed in 500 ml Erlenmeyer flasks. The flasks were stoppered with cotton plugs wrapped with good quality cotton cloth (in order to prevent cotton from entering), the pH adjusted to 4.0 with 2 N hydrochloric acid, and the flasks steamed 3 times for 20 minutes, allowing 24 hours between steamings.

Inoculation material was prepared from 48 hour old cultures of the four yeasts. After sterilization of the media had been completed, 0.4 ml of the inoculum were placed in each flask. The number of parallels was two.

The cultures were then incubated at $20^{o}C$. At 24 hour intervals, five ml from each culture were removed by means of a sterile pipette and the aggregate growth measured turbidimetrically by means of the Beckman spectrophotometer (Table I and Figs. 1, 2, 3, and 4).

The above experiment was repeated with the use of the yeasts "Chianti Classico" and "Saccharomyces ellipsoideus Sicilia", but with the substitution of the following mixtures of vitamins (Table II and Figs. 5 and 6):

a) (+)-biotin and meso-inositol,
b) (+)-biotin and calcium pantothenate,
c) calcium pantothenate and meso-inositol.

The inclusion of the three vitamins singly in substrate B fostered a rapid growth subsequent to a lag phase of approximately 24 hours (Table I and Figs. 1, 2, 3, and 4). With the yeasts "Entre Deux Mers" and "Saint Emilion" (Figs. 1 and 2), (+)-biotin encouraged a growth almost equal to that induced by the three vitamins. In spite of the rapid growth following the lag phase, which produced a turbidity equal to that of the combination of the three vitamins, (+)-biotin alone induced a growth during the induction and lag phases approximately equal to the growth which resulted when no vitamins were included in the medium. When offered singly to the four yeasts, meso-inositol and calcium pantothenate had less influence on the rate of growth throughout than (+)-biotin, except in the case of "Saccharomyces ellipsoideus Sicilia" where the stimulation exhibited by meso-inositol was found to parallel that of (+)-biotin. The addition of calcium pantothenate alone to each of the four yeast cultures resulted in a production of poor growth. In the case of "Saccharomyces ellipsoideus Sicilia" (Fig. 3), the growth induced by calcium pantothenate was only slightly greater than that induced by the vitamin-free substrate. This meager growth stimulation by calcium pantothenate in substrate B is interesting in view of the large stimulatory effect exhibited when later this vitamin was added in combination with either (+)-biotin or meso-inositol to substrate C, in which amino acids were utilized as a nitrogen source (Fig. 7). The yeasts "Saint Emilion" and "Saccharomyces ellipsoideus Sicilia" responded differently when combinations of (+)-biotin, calcium pantothenate and meso-inositol were offered (Table II and Figs. 5 and 6). The growth responses by "Saint Emilion" to the different vitamin combinations were similar, with the growth rates proceeding in a parallel manner. However in the case of "Saccharomyces ellipsoideus Sicilia", (+)-biotin in combination with calcium pantothenate initiated a relatively slow growth rate during the lag phase. Logarithmic growth originated after 48 hours, with the final turbidity being

Table I

The Influence of (+)-Biotin, Calcium Pantothenate and Meso-Inositol on the Growth of the Yeasts "Entre Deux Mers", "Saint Emilion", "Saccharomyces elipsoideus Sicilia" and "Chianti Classico". Expressed as % Transmittance

	Time in Hours	No Vitamins Added	(+)-Biotin 25 γ/1000 ml Calcium Pantothenate 2.5 mg/1000 ml Meso-Inositol 25 mg/1000 ml	(+)-Biotin 25 mg/1000 ml	Meso-Inositol 25 mg/1000 ml	Calcium Pantothenate 2.5 mg/1000 ml
"Entre Deux Mers"	0	98.4	98.4	98.4	98.4	98.4
	24	89.6	84.6	87.0	85.0	87.7
	48	72.2	35.5	45.1	53.6	66.3
	72	57.9	14.0	18.6	37.6	50.2
	96	-	-	-	-	-
	110	39.9	9.3	9.4	22.6	31.7
"Saint Emilion"	0	98.5	98.4	98.2	98.6	98.4
	24	91.9	87.5	89.3	87.5	90.7
	48	76.5	36.8	42.3	56.5	69.2
	72	73.7	20.6	31.9	47.5	62.4
	96	-	-	-	-	-
	110	59.9	8.8	11.7	34.2	48.3
"Sacch. ellipsoideus Sicilia"	0	98.3	98.5	98.5	98.4	98.4
	24	93.6	86.0	91.0	83.4	63.7
	48	84.2	33.7	56.2	57.5	82.9
	72	74.0	12.0	39.0	41.6	72.0
	105	66.1	8.9	28.7	30.7	62.8
	120	62.6	8.8	24.3	28.5	58.5
"Chianti Classico"	0	98.4	98.3	98.5	98.5	98.2
	24	88.5	83.4	86.4	83.4	88.0
	48	73.2	33.9	43.7	56.7	64.8
	72	61.8	15.6	34.5	49.2	55.0
	96	-	-	-	-	-
	110	50.3	10.9	26.9	38.0	40.8

Substrate: B.
Inoculation Material: From inoculated substrate A, 48 hours old.
Number of Parallels: 2.

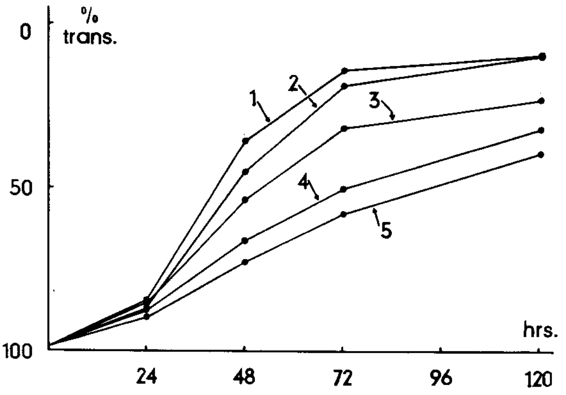


Fig. 1 The Influence of (+)-Biotin, Calcium Pantothenate and Meso-Inositol on the Growth of "Entre Deux Mers"

Substrate: B.
1. (+)-Biotin + Meso-Inositol + Calcium Pantothenate.
2. (+)-Biotin.
3. Meso-Inositol.
4. Calcium Pantothenate.
5. No Vitamins Added.

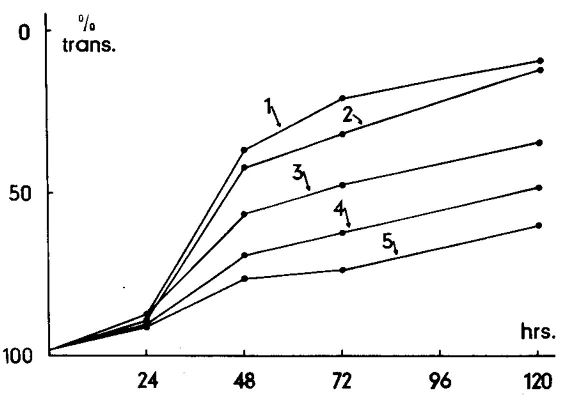


Fig. 2 The Influence of (+)-Biotin, Calcium Pantothenate and Meso-Inositol on the Growth of "Saint Emilion"

Substrate: B.
1. (+)-Biotin + Meso-Inositol + Calcium Pantothenate.
2. (+)-Biotin.
3. Meso-Inositol.
4. Calcium Pantothenate.
5. No Vitamins Added.

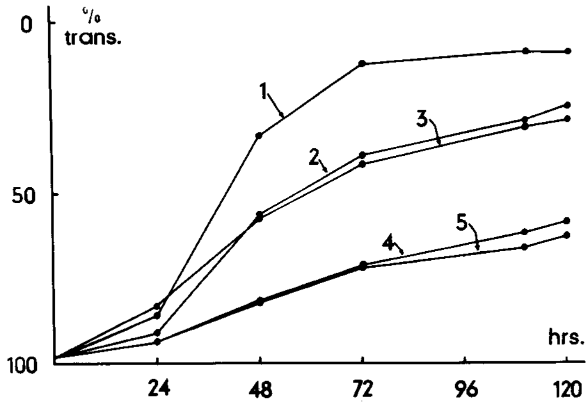


Fig. 3 The Influence of (+)-Biotin, Calcium Pantothenate and Meso-Inositol on the Growth of "Saccharomyces ellipsoideus Sicilia"

Substrate: B.
1. (+)-Biotin + Meso-Inositol + Calcium Pantothenate.
2. (+)-Biotin.
3. Meso-Inositol.
4. Calcium Pantothenate.
5. No Vitamins Added.

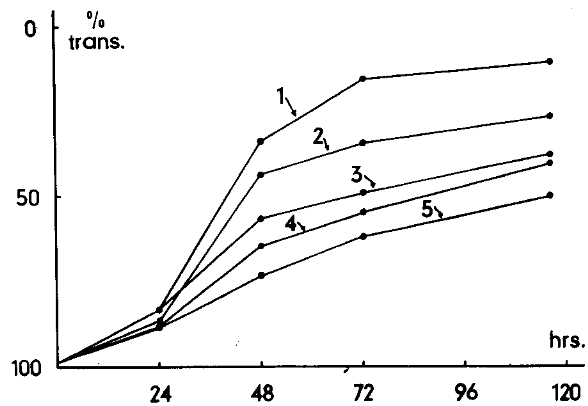


Fig. 4 The Influence of (+)-Biotin, Calcium Pantothenate and Meso-Inositol on the Growth of "Chianti Classico"

Substrate: B.
1. (+)-Biotin + Meso-Inositol + Calcium Pantothenate.
2. (+)-Biotin.
3. Meso-Inositol.
4. Calcium Pantothenate.
5. No Vitamins Added.

greater than that produced by the combinations (+)-biotin-meso-inositol and meso-inositol-calcium pantothenate. Because of the interesting growth patterns exhibited by "Saccharomyces ellipsoideus Sicilia" (Figs. 3 and 6), this yeast was selected for further study.

Table II

The Influence of Combinations of (+)-Biotin, Calcium Pantothenate and Meso-Inositol on the Growth of the Yeasts "Saint Emilion" and "Saccharomyces ellipsoideus Sicilia". Expressed as % Transmittance

	Time in Hours	No Vitamins Added	(+)-Biotin 25 γ/1000 ml Meso-Inositol 25 mg/1000 ml	(+)-Biotin 25 γ/1000 ml Calcium Pantothenate 2.5 mg/1000 ml	Meso-Inositol 25 mg/1000 ml Calcium Pantothenate 2.5 mg/1000 ml
"Saint Emilion"	24	91.9	85.4	87.7	85.4
	48	76.5	44.5	40.8	50.4
	72	73.7	25.6	23.1	29.2
	96	-	-	-	-
	120	59.9	10.3	11.3	23.7
"Sacch. ellipsoideus Sicilia"	24	93.6	82.0	94.5	84.6
	48	84.2	46.4	70.2	45.3
	72	74.0	35.2	30.7	29.7
	110	66.1	29.0	15.3	19.7
	120	62.6	24.6	12.2	14.8

Substrate: B.
Inoculation Material: From inoculated substrate A, 48 hours old.
Number of Parallels: 2.

As the amount of inoculum had been found to influence somewhat the length of the lag phase (Wikén and Richard 1952b) when the yeast "Dézaley" was incubated up to 600 hours in substrate C containing as growth factors (+)-biotin alone, meso-inositol alone, and a combination of (+)-biotin and meso-inositol, it seemed expedient to examine the influence of varying amounts of inoculum on the growth of "Saccharomyces ellipsoideus Sicilia" in a vitamin-enriched substrate B. Therefore, 0.8, 0.6, 0.4, and 0.2 ml of inoculum from a 48 hour old culture were inoculated into 250 ml quantities of substrate B contained in 500 ml Erlenmeyer flasks. The substrate included the following combinations of vitamins:

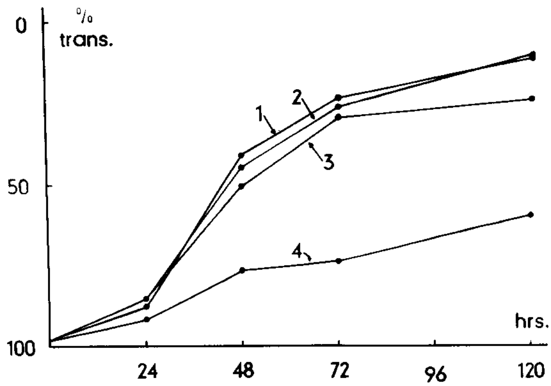


Fig. 5 The Influence of Combinations of (+)-Biotin, Calcium Pantothenate and Meso-Inositol on the Growth of "Saint Emilion"

Substrate: B.
1. (+)-Biotin + Calcium Pantothenate.
2. (+)-Biotin + Meso-Inositol.
3. Calcium Pantothenate + Meso-Inositol.
4. No Vitamins Added.

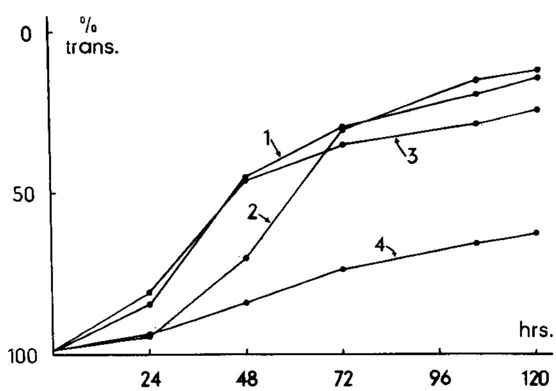


Fig. 6 The Influence of Combinations of (+)-Biotin, Calcium Pantothenate and Meso-Inositol on the Growth of "Saccharomyces ellipsoideus Sicilia"

Substrate: B.
1. Calcium Pantothenate + Meso-Inositol.
2. (+)-Biotin + Calcium Pantothenate.
3. (+)-Biotin + Meso-Inositol.
4. No Vitamins Added.

a) 25 γ/1000 ml (+)-biotin and 25 mg/1000 ml meso-inositol,

b) 25 γ/1000 ml (+)-biotin and 2.5 mg/1000 ml calcium pantothenate,

c) 2.5 mg/1000 ml calcium pantothenate plus 25 mg/1000 ml meso-inositol.

The resultant growth, expressed as per cent transmittance, during 115 hours of incubation at 20^{o}C is shown in Table III.

Table III

The Influence of the Quantity of Inoculation Material and Vitamin Combinations on the Growth of "Saccharomyces ellipsoideus Sicilia". Expressed as % Transmittance

Time in Hours	BI				BP				PI			
	Quantity of Inoculation Material per 250 ml of Substrate											
	0.8	0.6	0.4	0.2	0.8	0.6	0.4	0.2	0.8	0.6	0.4	0.2
0	99.0	99.3	99.5	99.8	99.0	99.3	99.5	99.8	99.0	99.3	99.5	99.8
24	79.8	82.3	84.6	88.0	93.1	93.4	95.5	97.6	81.0	84.8	85.5	88.2
43	31.4	35.2	35.5	39.2	76.0	79.3	84.1	91.2	51.0	55.0	55.0	57.5
52	21.5	23.9	24.5	22.0	60.0	64.9	70.7	81.6	46.8	49.0	49.2	51.3
67	13.1	14.2	15.1	16.2	30.5	33.7	37.6	48.9	36.7	38.4	38.0	37.2
75	10.6	11.1	11.1	12.5	21.9	24.8	26.6	24.3	31.2	33.2	32.9	32.7
91	10.0	10.2	10.1	10.0	14.1	17.0	17.5	20.7	25.3	27.1	26.0	26.8
115	8.9	9.0	9.2	10.0	11.7	11.6	12.1	13.1	19.3	20.7	19.8	21.2

Substrate: B.
Inoculation Material: From inoculated substrate A, 48 hours old.
Number of Parallels: 2.
BI = (+)-Biotin 25 γ/1000 ml + Meso-Inositol 25 mg/1000 ml.
BP = (+)-Biotin 25 γ/1000 ml + Calcium Pantothenate 2.5 mg/1000 ml.
PI = Calcium Pantothenate 2.5 mg/1000 ml + Meso-Inositol 25 mg/1000 ml.

From the results of this experiment, it is clear that considerable difference in growth occurs within the range of the quantities of inoculum employed. The general tendency is for a lower rate of growth to result when 0.2 ml of the inoculum are used. The utilization of inoculation quantities greater than 0.8 ml would not be feasible, as the vitamin content of the inoculated substrate would be increased somewhat due to vitamins introduced with the inoculation material. In order to keep this interference to a miniumum, and at the same time, to introduce enough of the inoculum for rapid stimulation of the yeast growth, 0.4 ml of inoculum per 250 ml of substrate was chosen as the standard to be used throughout the remainder of this work unless otherwise stipulated.

It should be noted that the differences in growth which did occur between the varied concentrations of the inoculum were most marked during the lag and the beginning of the logarithmic phases, with the induction and the latter part of the logarithmic phases showing a smaller diversity in readings.

c) The Influence of Vitamins on Growth in Substrate C

In order to observe the influence of (+)-biotin, meso-inositol and calcium pantothenate on the growth of "Saccharomyces ellipsoideus Sicilia" in a synthetic substrate, containing amino acids as the nitrogen source (substrate C), the basal substrate with the following added combinations of growth factors was prepared:

a) 25 γ/1000 ml (+)-biotin and 25 mg/1000 ml meso-inositol,
b) 25 γ/1000 ml (+)-biotin and 2.5 mg/1000 ml calcium pantothenate,
c) 2.5 mg/1000 ml calcium pantothenate and 25 mg/1000 ml meso-inositol,
d) 25 γ/1000 ml (+)-biotin and 0.935 mg/1000 ml β-alanine,
e) 25 γ/1000 ml (+)-biotin and 1.3652 mg/1000 ml D(-)-α, γ-dihydroxy-β, β-dimethyl-γ-butyrolactone,
f) 25 γ/1000 ml (+)-biotin,
g) 2.5 mg/1000 ml calcium pantothenate,
h) 25 mg/1000 ml meso-inositol.

As pantothenic acid is a condensation product of β-alanine and D (-)-α, γ-dihydroxy-β, β-dimethyl-γ-butyrolactone, these two substances were combined individually with (+)-biotin in order to determine which part of the pantothenic acid molecule is utilized by the strain of "Saccharomyces ellipsoideus Sicilia".

To 250 ml quantities of substrate C, in 500 ml Erlenmeyer flasks, the above combinations of growth factors were added prior to final sterilization by steaming. In order to prevent contamination by vitamins (traces of which might have remained on previously used cloth covered cotton plugs), new stoppers were prepared and used for this experiment. This procedure was implemented in subsequent experiments.

An inhibition by (+)-biotin upon the action of meso-inositol within certain periods of incubation (250-300 hours) has been found to exist when the Swiss wine yeast "Dézaley" was cultured in substrate C containing simultaneously (+)-biotin and meso-inositol (Wikén and Richard 1952a, Wikén and Agthe 1957). (+)-Biotin alone did not stimulate growth within 200-600 hours, while induction of growth, without lag phase, followed by a slow growth was reported when meso-inositol was offered singly. Furthermore, the simultaneous addition of (+)-biotin and pantothenic acid resulted in a growth-promoting action during the lag phase comparable with that of cultures supplied with pantothenic acid alone. However, after 96 hours, those cultures containing the mixture of (+)-biotin and pantothenic acid grew at a logarithmic rate, while those containing only pantothenic acid continued to grow slowly.

In the present work (Table IV and Fig. 7), (+)-biotin in combination with meso-inositol induced no growth after 286 hours and only a trace up to 358 hours. (+)-Biotin alone was also unable to stimulate growth. Meso-Inositol initiated slight growth, without lag phase, after 24 hours, but did not influence the turbitity to any great extent until after 190 hours, at which time growth progressed at a relatively rapid rate. An antagonistic effect of (+)-biotin upon the action of meso-inositol became evident 24 hours after the start of the incubation period. This effect continued throughout the time covered by the experiment. Pantothenic acid induced a slow but steady cell multiplication, the result of which produced a culture turbidity represented by 73.5 % transmittance after an incubation period of 262 hours. The combinations of (+)-biotin and calcium pantothenate, meso-inositol and calcium pantothenate, (+)-biotin and β -alanine, stimulated abundant growth after prolonged lag phases, the maximum growth induced by the latter combination being greater than that of the two former combinations.

In contrast to the (+)-biotin-β-alanine combination, the mixture of the pantothenic acid lactone and (+)-biotin induced negligible growth after 358 hours.

The increased growth stimulation effected by meso-inositol after an incubation period of 190 hours, may be due to one, or a combination of three factors, namely, (a) a slow synthesis of vitamins within all the yeast cells, (b) a selective process whereby a limited number of cells with autotrophic tendencies begin to multiply in the large population of heterotrophic cells, and (c) mutations from heterotrophic to autotrophic tendencies by a small segment of the cell population (Wikén and Richard 1952b).

In an attempt to explore the above possibilities, 250 ml quantities of substrate C (containing as the sole source of vitamins, 25 mg/ml of meso-inositol) were inoculated with 0.4 ml of washed inoculum. These flasks were left to incubate, without shaking, at 20°C. After approximately 300 hours it was noted that on the bottom of four out of every five flasks several small isolated colonies had developed. The appearance of these isolated colonies, although not definite proof in itself, strongly suggests that either a small number of cells are mutating to autotrophic types during the incubation period, or that a few have autotrophic tendencies.

Table IV

The Influence of Vitamins on the Growth of "Saccharomyces ellipsoideus Sicilia". Expressed as % Transmittance

Time in Hours	BI	BP	PI	BA	BL	B	P	I	BIP
0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0
24	99.0	99.4	93.3	99.2	99.6	98.8	99.3	98.2	89.2
48	98.0	97.4	53.6	90.8	97.8	98.0	98.1	96.2	43.7
70	99.0	94.2	39.4	64.9	98.4	98.0	95.9	-	26.4
94	98.6	81.3	28.0	37.3	98.5	98.1	93.0	96.2	5.4
118	98.5	65.8	20.0	16.5	98.1	98.0	97.7	-	-
166	98.3	31.3	18.8	9.7	98.5	98.0	81.6	-	-
190	98.3	16.5	18.5	-	97.9	97.7	78.7	91.5	-
238	98.2	13.3	18.5	-	98.5	98.3	75.3	-	-
262	98.4	-	-	-	98.3	98.8	73.5	62.7	-
286	98.5	-	-	-	98.5	96.3	-	-	-
310	97.3	-	-	-	96.4	98.6	-	46.0	-
334	96.2	-	-	-	96.4	98.0	-	36.8	-
358	96.5	-	-	-	96.4	96.2	-	-	-

Substrate: C.
Inoculation Material: From inoculated substrate A, 48 hours old.
Number of Parallels: 2.
A = β-Alanine 0.935 mg/1000 ml.
B = (+)-Biotin 25 γ/1000 ml.
I = Meso-Inositol 25 mg/1000 ml.
L = D(-)-α,γ-Dihydroxy-β,β-dimethyl-γ-butyrolactone.
P = Calcium Pantothenate 2.5 mg/1000 ml.

As the possibility existed that the results reported in Table IV could not be duplicated at a later date, due to the development of mutant strains in the maintenance substrate A, a similar experiment was conducted approximately one year later. In this experiment, (+)-biotin, meso-inositol, calcium pantothenate, β -alanine, and combinations thereof, were utilized as growth factors. The lactone component of pantothenic acid was not used, as it had previously produced only negligible growth. The quantity of inoculum employed was doubled (i.e., 0.4 ml of washed cell suspension to 125 ml of substrate C) in order to establish more clearly whether the same overall effect would result.

The findings of this later experiment are recorded in Table V and Fig. 9. Allowing for the heavier cell seeding, they show growth patterns similar to those previously found (Table IV and Fig. 7) with the exception of the growth induced by meso-inositol alone and (+)-biotin in combination with meso-inositol. Here, no growth occurred in the presence of meso-inositol, and slight growth was immediately initiated by (+)-biotin plus meso-inositol, followed by rapid growth after 216 hours of incubation. The different growth responses of the organism to meso-inositol alone and the mixture of (+)-biotin and meso-inositol in the later experiment, as compared with the results obtained earlier when these vitamins were employed, was not expected. The significance of these changes is not clear from the results described here, although the possibility of the development of mutant strains within the cell population cannot be entirely ignored. In the "Dézaley" yeast, (+)-biotin was found to inhibit the action of meso-inositol (Wikén and Agthe 1957).

Table V

The Influence of Vitamins on the Growth of "Saccharomyces ellipsoideus Sicilia". Expressed as % Transmittance

Time in Hours	B	I	P	A	BI	BP	BA	PI	IA	PA	BIP	BIA
24	96.7	94.5	96.1	96.4	94.3	96.8	96.3	91.5	91.7	96.5	90.6	91.8
40	95.4	92.3	94.9	91.7	92.7	95.2	91.7	48.2	51.9	94.6	42.8	50.3
96	95.0	93.2	94.4	79.9	92.2	87.1	50.5	10.0	12.0	89.1	5.5	5.9
114	95.1	93.0	81.9	76.5	92.7	33.2	14.8	10.5	13.0	81.7	-	-
216	95.2	93.2	65.7	74.1	90.0	7.7	5.8	10.3	-	62.6	-	-
264	96.0	94.3	45.8	57.3	44.4	-	-	-	-	42.5	-	-

Substrate: C.
Inoculation Material: From inoculated substrate A, 48 hours old.
Number of Parallels: 2.
Time of Inoculation: Approximately one year after the inoculation leading to the data in Table IV and Fig. 7.
B = (+)-Biotin 25 γ/1000 ml.
I = Meso-Inositol 25 mg/1000 ml.
P = Calcium Pantothenate 2.5 mg/1000 ml.
A = β-Alanine 0.935 mg/1000 ml.

In order to further demonstrate that pantothenic acid or β-alanine is an essential metabolite for the promotion of growth when utilized in combination with (+)-biotin or meso-inositol in a synthetic substrate containing casein hydrolysate as a nitrogen source, sterile calcium pantothenate was added after an elapsed incubation time of 173 hours, to cultures containing a combination of (+)-biotin and meso-inositol. The vitamin concentrations were identical with those employed in previous experiments. The amount of the inoculum utilized was 0.4 ml per 250 ml of substrate C. Combinations consisting of: (a) (+)-biotin and calcium pantothenate, (b) calcium pantothenate and meso-inositol, and (c) (+)-biotin, calcium pantothenate and meso-inositol, were inoculated simultaneously in order to act as controls.

Table VI

The Stimulatory Influence of Calcium Pantothenate on the Growth of "Saccharomyces ellipsoideus Sicilia". Expressed as % Transmittance

Time in Hours	BI	BP	PI	BIP
0	99.0	99.0	99.0	99.0
24	98.5	99.2	95.9	90.3
48	97.8	98.0	59.0	45.2
70	97.6	90.0	42.2	24.6
94	98.4	81.7	30.9	6.4
118	98.2	78.7	22.5	-
190	98.1 *)	32.2	19.9	-
238	41.9	11.4	20.2	-
262	15.0	-	-	-

Substrate: C.
Inoculation Material: From inoculated substrate A, 48 hours old.
Number of Parallels: 2.
B = (+)-Biotin 25 γ/1000 ml.
I = Meso-Inositol 25 mg/1000 ml.
P = Calcium Pantothenate 2.5 mg/1000 ml.
*) = Sterile Calcium Pantothenate Added at 173 Hours.

Table VII

The Influence of (+)-Biotin, Meso-Inositol, and Calcium Pantothenate on the Growth of "Saccharomyces ellipsoideus Sicilia", when Ammonium Sulphate is Utilized as a Source of Nitrogen. Expressed as % Transmittance

Time in Hours	B	BI	I	P	BP
24	97.9	99.1	95.1	98.2	81.5
48	92.1	93.2	84.8	96.0	21.8
72	68.1	78.1	74.9	93.5	7.9
96	37.7	46.9	68.6	91.6	-
120	18.6	31.1	62.7	89.4	-

Substrate: C, casein hydrolysate replaced by ammonium sulphate.
Inoculation Material: From inoculated substrate A, 48 hours old.
Number of Parallels: 2.
B = (+)-Biotin 25 γ/1000 ml.
I = Meso-Inositol 25 mg/1000 ml.
P = Calcium Pantothenate 2.5 mg/1000 ml.

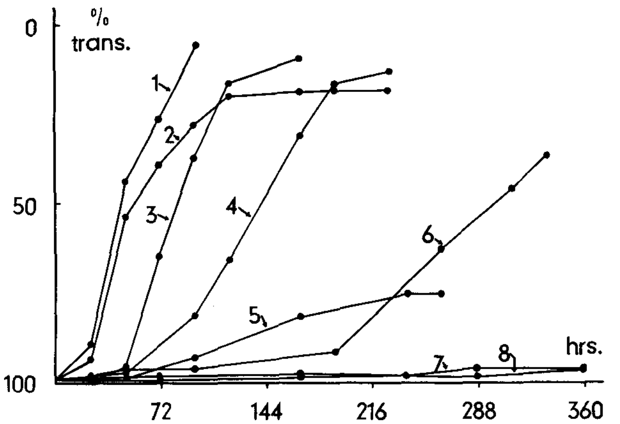


Fig. 7 The Influence of Vitamins on the Growth of "Saccharomyces ellipsoideus Sicilia"

Substrate: C.
1. (+)-Biotin + Meso-Inositol + Calcium Pantothenate.
2. Meso-Inositol + Calcium Pantothenate.
3. (+)-Biotin + β-Alanine.
4. (+)-Biotin + Calcium Pantothenate.
5. Calcium Pantothenate.
6. Meso-Inositol.
7. (+)-Biotin.
8. (+)-Biotin + Meso-Inositol.

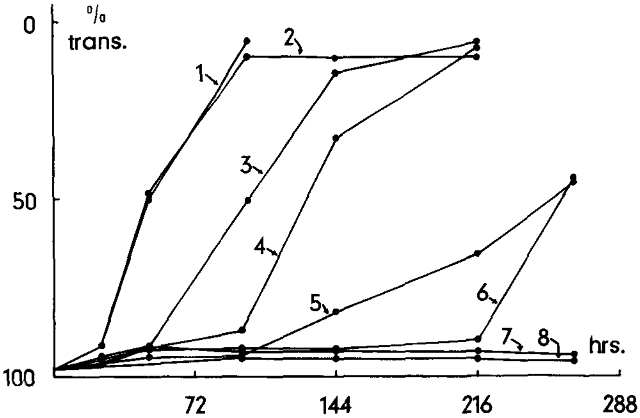


Fig. 8 The Influence of Vitamins on the Growth of "Saccharomyces ellipsoideus Sicilia"

Substrate: C.
1. (+)-Biotin + Meso-Inositol + Calcium Pantothenate.
2. Meso-Inositol + Calcium Pantothenate.
3. (+)-Biotin + β-Alanine.
4. (+)-Biotin + Calcium Pantothenate.
5. Calcium Pantothenate.
6. (+)-Biotin + Meso-Inositol.
7. Meso-Inositol.
8. (+)-Biotin.

Time of Inoculation: Approximately one year after the inoculation leading to the data in Table IV and Fig. 7.

After the addition of calcium pantothenate (Table VI), an induction period of approximately 20 hours ensued, followed by rapid growth which reached its maximum within 90 hours.

In order to uncover, if possible, a reason for the absence of growth in substrate C supplemented with (+)-biotin and meso-inositol alone, or in combination, as against good growth induced by these vitamins in substrate B, 250 ml quantities of substrate C, in which the casein hydrolysate was replaced by ammonium sulphate as a source of nitrogen, were inoculated with 0.4 ml of inoculum. The combinations of vitamins used were as follows:

a) (+)-biotin,
b) (+)-biotin and meso-inositol,
c) meso-inositol,
d) Calcium pantothenate,
e) (+)-biotin and calcium pantothenate.

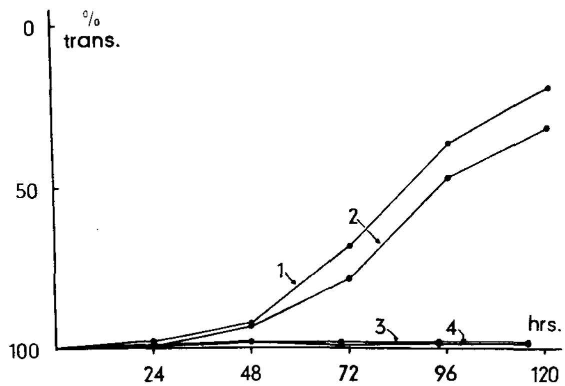


Fig. 9 The Influence of (+)-Biotin, and (+)-Biotin + Meso-Inositol, in the Presence of Different Nitrogen Sources, on the Growth of "Saccharomyces ellipsoideus Sicilia"

Ammonium Sulphate (substrate B).
1. (+)-Biotin.
2. (+)-Biotin + Meso-Inositol.

Casein Hydrolysate (substrate C).
3. (+)-Biotin.
4. (+)-Biotin + Meso-Inositol.

Concentrations of the above growth factors were identical with those which had been used in the preceding experiments.

From the results shown in Table VII, it can be observed that "Saccharomyces ellipsoideus Sicilia" proliferated in this substrate when supplemented with (+)-biotin alone. The lag phase was, however, approximately 24 hours longer than previously noted with the use of substrate B containing (+)-biotin as the lone growth factor (Table I). Meso-Inositol induced a slow but definite growth, while a mixture of (+)-biotin and meso-inositol initiated a growth almost equal to that which resulted after the addition of these vitamins to substrate B. Calcium pantothenate was found to support only slow growth, but when it was added simultaneously with (+)-biotin, growth response was rapid. Fig. 9 shows clearly the influence of (+)-biotin alone as well as a mixture of (+)-biotin and meso-inositol when ammonium sulphate or casein hydrolysate is utilized as a source of nitrogen.

3. Discussion

From the findings presented here, it is clear that "Saccharomyces ellipsoideus Sicilia" is auxo-autotrophic, or auxo-heterotrophic depending upon the nature of the medium employed, and in particular, the type of nitrogen source offered. The partial investigation as to the vitamin requirements of "Entre Deux Mers", "Saint Emilion" and "Chianti Classico" indicate auxo-autotrophic properties for these yeasts when cultured in a synthetic chemically defined medium (medium B). The growth responses found for the yeast "Saccharomyces ellipsoideus Sicilia" in the two media B and C when supplemented with various vitamins may be summarized as follows:

Substrate B without addition of vitamins: Slow steady growth without logarithmic phase. The auxo-autotrophic tendency of "Saccharomyces ellipsoideus Sicilia" in this substrate is not as evident as that exhibited by the wine yeasts "Salenegg", "Herrliberg" and "Fendant" (Wikén and Richard 1951a and b, 1952a).

Substrate B with calcium pantothenate: The same growth response as was found with the use of substrate B alone. Thus, calcium pantothenate appears to have no stimulatory influence when offered singly with this substrate.

Substrate B with (+)-biotin: Slow growth during a 24 hour lag phase, followed by rapid growth response and a high maximum yield of cells.

Substrate B with meso-inositol: Relatively rapid growth during the lag phase. Between 48 and 120 hours, growth is equal to that produced when the yeast is cultured in substrate B supplemented with (+)-biotin.

Substrate B with a mixture of calcium pantothenate and meso-inositol: Typical "S curve" type growth and a high maximum cell yield.

Substrate B with a mixture of (+)-biotin and meso-inositol: The same growth response as that which occurred with the use of substrate B supplemented with a mixture of calcium pantothenate and meso-inositol.

Substrate B with a mixture of (+)-biotin and calcium pantothenate: Relatively poor growth stimulation during the first 24 hours followed by rapid growth. The maximum yield of cells was higher than in those cultures supplied with a mixture of calcium pantothenate and meso-inositol, or (+)-biotin and meso-inositol.

Substrate B with a mixture of (+)-biotin, meso-inositol and calcium pantothenate: Excellent growth after the simultaneous addition of the three vitamins. Therefore, although the yeast "Saccharomyces ellipsoideus Sicilia" exhibits auxo-autotrophic tendencies in the vitamin deficient substrate B, it may be classed among those organisms requiring a comparatively complex mixture of vitamins for the development of normal growth.

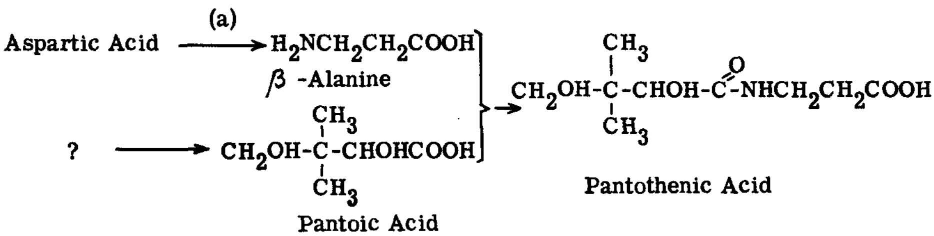
Substrate C without addition of vitamins: No growth promoting action. This is comparable to the yeast "Dézaley" which has been reported (Wikén and Richard 1952b) as unable to fully utilize this substrate. Only a slow rate of growth was reported even after an incubation period of 240 - 500 hours.

Substrate C with (+)-biotin alone, meso-inositol alone, or a mixture of (+)-biotin and meso-inositol: Little or no growth during the first 200 hours of incubation. In some experiments, substrate C when supplemented with meso-inositol alone supported growth after an elapsed incubation time of 200-300 hours. Whether this later growth stimulation was due to the action of meso-inositol or to mutations by a segment of the cell population is open to question.

Substrate C with ammonium sulphate replacing casein hydrolysate as a source of nitrogen, plus (+)-biotin alone, meso-inositol alone, or a combination of (+)-biotin and meso-inositol: A rapid stimulation of growth occurred following a 48 hour lag phase. The maximum yield of cells was high. (+)-Biotin in the presence of ammonia has been found to induce an immediate increase in the rate of both respiration and aerobic and anaerobic glycolysis (Winzler et al. 1944). From the experiments conducted here, it is clear that the action of (+)-biotin and/or meso-inositol is strongly dependent upon the presence of ammonia.

Substrate C with calcium pantothenate alone or β-alanine alone: Slow steady growth.

Substrate C with the lactone portion of the pantothenic acid molecule: No growth. As pantoic acid (the lactone portion of pantothenic acid) in substrate C will not support growth, and pantothenic acid or β -alanine, when added singly to substrate C, will do so, it is clear that, at a pH of 4.0, the yeast is unable to form β -alanine, i.e., to carry out reaction (a) in the scheme shown below.



As either pantothenic acid or β -alanine when combined with (+)-biotin and/or meso-inositol in substrate C induces excellent growth response, it may be concluded that pantothenic acid becomes a limiting factor for growth in the absence of ammonia.

B) The Influence of Certain Amino Acids both Singly and in Combination upon the Growth of "Saccharomyces ellipsoideus Sicilia"

1. Introduction

Despite the vast amount of work which has been accomplished in connection with the stimulation of yeast growth by amino acids, and assimilation by yeasts of these acids, there is by no means definite agreement as to the mechanisms involved. Ehrlich (1907, 1911 and 1912), Stickland (1934, 1935, 1935a and 1935b), and Thorne (1950) have contributed substantially to our knowledge of amino acid assimilation.

Dealt with under this section is a survey of amino acid utilization and, in addition, a study of the inhibitions caused by L-cystein hydrochloride and L-cysteic acid on the growth of the yeast "Saccharomyces ellipsoideus Sicilia".

2. Experimental Methods and Results

a) Preparation of L-Cysteic Acid

As L-cysteic acid was not available from Hoffmann La Roche, Basel, and the sample obtained from Dr. Bender & Dr. Hobein Co., Zürich, was found to be impure, as shown by the appearance of two spots when chromatographed on paper, it was necessary to prepare the monohydrate of the compound in this laboratory from L-cystine. The method described by Gortner and Hoffman (1927) was attempted, but found to give a low yield and an impure product with too low a melting point. A modification of Gortner and Hoffman's method by Clarke (1940) was used, and the product which resulted from the application of this method was found to be pure.

Essentially, the procedure was as follows: To a solution of 24 grams (0.1 mole) of L-cystine in a cold mixture consisting of 150 ml of water and 50 ml of concentrated hydrochloric acid, 80 gms (25 ml, 0.5 mole) of commercial bromine was added dropwise with occasional stirring for a duration of approximately forty minutes. The resultant solution (the temperature of which rose to about 60°C), which contained a small amount of unreduced bromine, was then evaporated under reduced pressure on the steam bath. The dark coloured, crystalline residue was then dissolved in 100 ml of distilled water and the solution filtered from a small quantity of insoluble amorphous matter. Concentration of the filtrate to 65 ml was accomplished by evaporating on the water bath. Crystallization of the filtrate was achieved by overnight refrigeration at 0°C. The crystals were filtered with suction, washed well with about 100 ml of absolute ethyl alcohol in several portions, the washings being collected separately, and dried in vacuo over phosphorous pentoxide. A second crop was obtained by diluting the washings with an equal volume of water, evaporating until free of alcohol, adding the residue to the mother liquor, and evaporating the combined solutions to dryness on the water bath. The residue was dissolved in 30-40 ml of water, decolorized with 0.5-1.0 gm of charcoal, concentrated to 15 ml, and when cold, treated with 30 ml of absolute alcohol. The crystals found were collected, washed with alcohol, and dried as before. The total yield was 29.2 gm of pure L-cysteic acid monohydrate with a melting point of 288.5°C (corrected). After chromatographing on paper, using a solvent of butanol, glacial acetic acid and water (4:1:1), drying and spraying with 0.1% ninhydrin in water-saturated butanol, one clearly defined spot developed.

b) The Influence Exerted by Individual Amino Acids on Growth

Throughout section B of Part I, unless otherwise stated, the concentrations (in grams per liter) of amino acids used, either singly or in combination with each other, were such that the nitrogen content of the amino acid or acids corresponded to the nitrogen content of 6 grams per liter of ammonium sulphate. Furthermore, the pH of the substrates containing amino acids was adjusted to 4.0.

The amino acids tested in these experiments were as follows: Glycine, DL-alanine, DL-serine, DL-valine, DL-leucine, DL-isoleucine, DL-phenylalanine, L(+)-cystein hydrochloride, DL-methionine, L(-)-tryptophane, DL-proline, L(-)-hydroxyproline, DL-aspartic acid, L(+)-glutamic acid, monohydrate of DL-histidine hydrochloride, L(+)-arginine (base), L(+)-lysine dihydrochloride, and L(+)-citrulline.

The appropriate amount of each amino acid was placed in 300 ml Erlenmeyer flasks. To these flasks 125 ml of the basal substrate B, not containing ammonium sulphate, and including a mixture of (+)-biotin, meso-inositol and calcium panthothenate in concentrations of 25 γ, 25 mg, and 2.5 mg per 1000 ml respectively, were added. With the use of a Polymetron pH meter, the pH of the media was adjusted to 4.0 by the addition of small quantities of concentrated sodium hydroxide. Sterilization was accomplished by three steamings of 15 minutes duration, 24 hours apart. The quantity of inoculum placed into each flask was 0.2 ml.

In order to compare the growth induced by the amino acids with that induced by ammonia, substrate B (prepared with ammonium sulphate) containing the three vitamins was inoculated at the same time.

After an incubation period of 24 hours at 20^{o}C, and subsequently each 24 hours thereafter up to a period of 120 hours, the turbidity of each culture was determined by means of the Beckman quartz spectrophotometer. As the colors present in the different amino acid solutions varied slightly from one another after sterilization, each series of parallels was read against an identical substrate which had not been inoculated.

It is clear from the figures shown in Table VII that "Saccharomyces ellipsoideus Sicilia" was able to utilize a majority of the amino acids when these were presented singly. Further, it will be seen from Table VII that, of the aliphatic acids containing one amino and one carboxyl group, all except glycine supported good growth. None of these acids, with the exception of DL-serine, induced a rate of growth comparable to that induced by ammonium sulphate alone.

Of the sulphur-containing acids, DL-methionine and L-cystein hydrochloride, the former supported good growth, while the latter was found to have no nutritional value.

DL-Phenylalanine, the only aromatic acid used, encouraged excellent growth.

The acids containing two carboxyl groups, DL-aspartic and L-glutamic, gave rise to growth equal to that initiated by ammonium sulphate. In contrast, the basic amino acids were utilized in a diverse manner, with the nutritional value of L-arginine and L-citrulline exceeding that of ammonium sulphate. DL-Histidine hydrochloride supported only slight growth while the nutritional value of L-lysine dihydrochloride was nil.

Two of the three heterocyclic amino acids used, L-tryptophane and DL-proline, supported limited growth, while the addition of the third acid, L-hydroxyproline, produced only a slight culture turbidity.

As the amino acids glycine, L-cystein hydrochloride, DL-proline, L-hydroxyproline, DL-histidine and L-lysine dihydrochloride, if offered individually, were not readily utilized as a source of nitrogen by the yeast, it was decided to determine what effect these acids would have on growth when combined with ammonium sulphate.

This experiment, the results of which are tabulated in Table VIII, was conducted in the same manner as was the one preceding.

Table VIII

The Influence of Individual Amino Acids, when Utilized as a Sole Nitrogen Source, on the Growth of "Saccharomyces ellipsoideus Sicilia". Expressed as % Transmittance

Time in Hours	24	48	72	96	120
Ammonium Sulphate	70.7	19.7	9.7	6.2	-
Glycine	90.2	78.8	56.8	36.2	5.0
DL-Alanine	88.6	52.0	12.1	7.1	6.0
DL-Serine	68.6	23.5	8.6	5.8	-
DL-Valine	87.5	44.8	14.7	9.9	5.5
DL-Leucine	85.4	43.1	10.6	7.7	5.3
DL-Isoleucine	90.6	74.6	31.5	14.1	9.6
DL-Methionine	87.4	53.6	16.1	10.1	6.3
L(-)-Tryptophane	84.7	68.7	45.9	27.5	20.0
DL-Proline	92.2	75.9	55.3	42.0	35.6
L(-)-Hydroxyproline	89.9	85.7	84.1	83.2	82.5
L(+)-Arginine (base)	67.6	17.3	7.6	6.4	-
L(+)-Lysine Dihydrochloride	92.1	91.0	91.0	91.0	90.8
L(+)-Citrulline	37.6	10.5	8.7	8.2	-
DL-Phenylalanine	84.1	42.2	19.1	12.3	-
L(+)-Cystein Hydrochloride	97.2	97.2	97.4	97.4	97.5
DL-Aspartic Acid	71.5	36.2	16.2	5.2	-
L(+)-Glutamic Acid	69.8	29.9	14.9	10.5	-
DL-Histidine Hydrochloride	90.9	83.9	73.8	59.0	51.2

Substrate: B, vitamin enriched, not containing ammonium sulphate.
Inoculation Material: From inoculated substrate A, 48 hours old.
Number of Parallels: 2.

It was found (Table VIII) that glycine, DL-proline, L-hydroxyproline and L-lysine dihydrochloride, when combined with ammonium sulphate, induced somewhat greater growth than that induced by ammonium sulphate alone. L-Cystein hydrochloride, however, inhibited the action of ammonium sulphate up to 120 hours, after which time rapid growth occurred. It was concluded that this later growth was caused by the oxidation of cystein into various sulfinic and sulfonic acids, and into cystine.

$$\begin{matrix} R - SH \\ R - SH \end{matrix} + A \longrightarrow \begin{matrix} R - S \\ | \\ R - S \end{matrix} + H_2A$$

A = Hydrogen acceptor

The inhibition created during the first five days of incubation was at first thought to be due to the unfavorable redox potential in the medium set up by cystein. However, later experiments proved that this was not so.

Table IX

The Influence of Single Amino Acids in Combination with Ammonium Sulphate on the Growth of "Saccharomyces ellipsoideus Sicilia". Expressed as % Transmittance

Time in Hours	24	48	72	96	120	168	192
Glycine	91.4	82.6	67.3	49.6	39.1	-	-
DL-Glycine + Ammonium Sulphate	78.1	18.8	6.4	6.0	5.7	-	-
L(-)-Cystein Hydrochloride	98.1	97.9	96.3	95.0	94.4	-	-
L(-)-Cystein hydrochloride + Ammonium Sulphate	98.5	97.1	97.2	94.5	86.5	12.6	11.4
DL-Proline	92.0	79.1	62.1	48.4	39.5	-	-
DL-Proline + Ammonium Sulphate	71.7	18.6	9.9	8.9	8.0	-	-
L(-)-Hydroxyproline	91.0	85.1	83.0	80.0	-	-	-
L(-)-Hydroxyproline + Ammonium Sulphate	79.0	16.6	7.3	7.0	-	-	-
DL-Histidine Hydrochloride	94.3	86.8	73.2	66.8	65.2	-	-
DL-Histidine Hydrochloride + Ammonium Sulphate	81.2	20.0	8.0	7.2	6.7	-	-
L(+)-Lysine Dihydrochloride	94.9	94.0	93.7	94.0	94.0	-	-
L(+)-Lysine Dihydrochloride + Ammonium Sulphate	73.0	17.7	8.8	7.6	7.0	-	-
Ammonium Sulphate	77.3	21.9	10.4	9.4	7.2	-	-

Substrate: B, vitamin enriched, not containing ammonium sulphate.
Inoculation Material: From inoculated substrate A, 48 hours old.
Number of Parallels: 2.

c) The Influence Exerted by Combinations of Amino Acids on Growth

Dyer (1938) reported from du Vigneaud's laboratory that the S-ethyl analog of methionine, called ethionine, appeared to be toxic to rats on a diet in which the cystine and methionine content was low, whereas, in the presence of sufficient methionine, neither loss of growth nor death occurred. These findings represented probably the first authentic demonstration of metabolic antagonism involving amino acids. Evidence has been reported that methionine and ethionine act as metabolic antagonists for E. coli (Harris and Kohn 1941). It appears likely that other unsuspected instances of metabolic antagonism involving amino acids may be found in the literature.

As there are some instances where two naturally occurring amino acids appear to compete with one another, e.g., arginine has been shown (Doermann 1944) to compete with lysine in the case of a mutant strain of Neurospora, it seemed expedient to explore the possibilities of such an occurrence in this work. Therefore, mixtures of those amino acids which had promoted abundant growth and those which had supported relatively less growth were combined. Furthermore, pairs of amino acids, which had induced a growth comparable to that of ammonium sulphate, were combined. The results of these two experiments are shown in Tables X and XI.

With the exception of L-hydroxyproline, inhibitions of varying degrees occurred when the amino acids supporting less growth were combined with the rapid growth supporting L-citrulline (Table X). The fact that L-hydroxyproline, when combined with citrulline, encouraged comparatively good growth is interesting in view of the almost non-existant growth produced when L-hydroxyproline was added singly. The inhibition induced by L-cystein hydrochloride, when offered with either L-citrulline or DL-methionine, although greatly in evidence, was less pronounced than was noted when this acid had been previously combined with ammonium sulphate. Glycine and DL-proline had only a slight inhibiting effect upon the action of L-citrulline, and DL-methionine. While L-lysine dihydrochloride and DL-histidine inhibited the action of L-citrulline, an inhibition did not occur when either of these acids were combined with DL-methionine. In fact, growth was enhanced. DL-Hydroxyproline inhibited slightly, both L-citrulline and DL-methionine.

L-Citrulline has been found to have a better nutritive value than ammonium sulphate (Table VIII). Certain combinations of those amino acids which had demonstrated their ability to stimulate rapid growth when presented singly, had an antagonistic effect when offered in pairs (Table XI). DL-Alanine, DL-leucine, DL-isoleucine and DL-methionine, when added singly to substrate B containing L-citrulline as the nitrogen source, tended to suppress the excellent growth supporting characteristics of L-citrulline in that the growth rates were found to be lower than those induced by ammonium sulphate alone. Of the remaining pairs of amino acids tested in this series, only the mixtures containing DL-serine with L(+)-citrulline, and DL-leucine with DL-serine promoted growth which was clearly defined as being supperior to the growth induced by ammonium sulphate.

Table X

The Inhibiting Effect of Amino Acids Supporting Poor Growth in Combination with those Supporting Rapid Growth on "Saccharomyces ellipsoideus Sicilia". Expressed as % Transmittance

Time in Hours	24	48	72	96	120	168
Glycine + L(+)-Citrulline	77.3	35.5	16.5	12.1	10.3	-
L(-)-Cystein Hydrochloride + L(+)-Citrulline	98.8	96.8	83.3	65.0	62.9	57.0
DL-Proline + L(+)-Citrulline	70.1	27.2	13.5	10.2	10.0	-
L(-)-Hydroxyproline + L(+)-Citrulline	68.8	24.2	11.2	8.0	8.1	-
DL-Histidine Hydrochloride + L(+)-Citrulline	67.7	29.5	16.4	11.1	10.7	-
L(+)-Lysine Dihydrochloride + L(+)-Citrulline	67.7	40.5	33.2	27.0	24.7	-
L(+)-Citrulline	69.1	21.1	10.3	8.9	8.5	-
Glycine + DL-Methionine	87.2	62.8	33.1	18.3	11.3	-
L(-)-Cystein Hydrochloride + DL-Methionine	98.3	98.3	96.4	89.0	88.8	79.8
DL-Proline + DL-Methionine	91.7	64.9	21.7	11.6	11.5	-
L(-)-Hydroxyproline + DL-Methionine	91.7	67.3	24.0	12.5	11.1	-
DL-Histidine Hydrochloride + DL-Methionine	89.5	57.5	36.6	9.8	9.7	-
L(+)-Lysine Dihydrochloride + DL-Methionine	90.0	51.2	11.2	7.2	7.6	-
DL-Methionine	91.5	61.1	20.0	10.7	10.4	-

Substrate: B, vitamin enriched, not containing ammonium sulphate.
Inoculation Material: From inoculated substrate A, 48 hours old.
Number of Parallels: 2.

Table XI

The Influence of Rapid Growth Promoting Amino Acids, when Offered in Pairs, on the Growth of "Saccharomyces ellipsoideus Sicilia". Expressed as % Transmittance

Time in Hours	24	48	72	96	120
DL-Alanine + L(+)-Citrulline	86.3	37.1	9.3	6.9	-
DL-Serine + L(+)-Citrulline	78.2	21.2	9.0	7.3	-
DL-Valine + L(+)-Citrulline	78.6	33.2	10.0	7.7	-
DL-Leucine + L(+)-Citrulline	88.5	42.7	15.6	9.0	-
DL-Isoleucine + L(+)-Citrulline	91.1	55.8	21.5	10.6	-
DL-Methionine + L(+)-Citrulline	89.5	51.3	18.9	9.9	-
DL-Serine + DL-Alanine	85.4	36.6	10.5	6.6	-
DL-Valine + DL-Alanine	88.7	57.5	20.0	8.9	-
DL-Isoleucine + DL-Alanine	92.0	74.5	39.5	14.9	13.2
DL-Methionine + DL-Alanine	90.5	72.0	38.5	15.5	15.6
DL-Valine + DL-Serine	84.6	31.2	11.0	6.7	-
DL-Leucine + DL-Serine	82.6	37.9	10.0	6.3	-
DL-Isoleucine + DL-Serine	87.9	32.1	16.0	6.0	-
DL-Methionine + DL-Serine	90.7	52.5	18.8	8.8	-
DL-Leucine + DL-Valine	88.4	43.6	18.3	9.6	-
DL-Isoleucine + DL-Valine	92.4	67.7	31.4	14.6	15.7
DL-Methionine + DL-Valine	89.6	56.5	27.2	13.7	13.2
DL-Isoleucine + DL-Leucine	88.8	67.2	41.3	18.7	19.0
DL-Leucine + DL-Methionine	87.7	64.9	30.4	12.9	11.5
DL-Methionine + DL-Isoleucine	92.6	56.5	46.9	19.5	20.0
Ammonium Sulphate	76.8	28.3	12.6	6.6	-

Substrate: B, vitamin enriched, without $(NH_4)_2SO_4$.
Inoculation Material: From inoculated substrate A, 48 hours old.
Number of Parallels: 2.

d) Inhibition Produced by L-Cystein Hydrochloride, and its Reversal

It has been observed in preceding experiments (Tables IX and X) that L-cystein hydrochloride inhibited the action of ammonium sulphate, L-citrulline, and DL-methionine during the first 96 hours of incubation. In order to determine the concentration of L-cystein hydrochloride at which the inhibition in the presence of ammonium sulphate ceases to function, the following quantities, in grams per liter, of the acid were added to substrate B: 7.175, 5.740, 4.305, 2.870, 1.435, 1.075, 0.718, and 0.089. The concentration of ammonium sulphate was held constant at 3 gm per liter. The vitamins (+)-biotin, meso-inositol, and calcium pantothenate were added to each culture in the concentrations of 25 γ , 25 mg, and 2.5 mg per liter respectively. The amount of inoculation material per 150 ml of substrate was 0.2 ml.

Table XII illustrates clearly, that after an incubation period of 48 hours at 20^{o}C, the critical concentration of L-cystein hydrochloride was 0.359 grams per liter which corresponds to a concentration of 3.59 mg/10 ml.

In order to determine if the above figures would be approximately the same in the presence of higher concentrations of ammonium sulphate, the following experiment was undertaken: To 100 ml quantities of the vitamin enriched substrate B, L-cystein hydrochloride was added in concentrations of 2, 4, 6, 8, and 10 mg per 10 ml of substrate. Ten ml of this substrate were then placed in 50 ml Erlenmeyer flasks in series of 8 for each concentration. The inoculum to be used was diluted 1:10, and 0.25 ml of the diluted material was inoculated into each flask. This resulted in a cell seeding equal to that of 0.2 ml of undiluted inoculum per 100 ml of substrate. The concentration of ammonium sulphate in the medium was the same as that of the basal substrate B, i.e., 6 grams per liter.

Table XIII illustrates clearly that, irrespective of the concentration of ammonium sulphate present, the inhibition created by L-cystein hydrochloride becomes apparent in the range of concentrations between 2 and 4 mg per 10 ml.

If the inhibition caused by L-cystein hydrochloride could be reversed by L-aspartic acid, L-glutamic acid, or by a product of transamination of one of these acids such as α-ketoglutaric acid, it would seem that the toxicity of L-cystein hydrochloride is not due to the low redox potential produced in the medium by the HS-group of the latter acid.

In order to explore the possibility that the above acids might possess the ability of reversing the toxicity of L-cystein hydrochloride, they were added separately in concentrations of 6, 8, and 10 mg per 10 ml to the vitamin enriched substrate B containing 6 mg of L-cystein hydrochloride per 10 ml. Ten ml of each substrate (contained in 50 ml Erlenmeyer flasks) were seeded with inoculum which had been diluted as described in the preceding experiment. The cultures were incubated 48 hours at 20^{o}C, after which time the turbidity of each culture was determined. β -Alanine was included in the experiment, in concentrations of 0.01 and 0.02 mg per 10 ml, in order to observe the manner in which growth would be affected by an increase in the concentration of this portion of the pantothenic acid molecule.

α -Ketoglutaric acid and L-glutamic acid were found to reverse the inhibition (Table XIV), the reversal being more pronounced as the concentrations of these two acids were increased. L-Aspartic acid prevented inhibition completely when added in concentrations which were slightly higher than that of L-cystein Hydrochloride. When the concentration of L-aspartic acid was increased to 10 mg/10 ml, the reversal became less marked.

Table XII

The Influence of Various Concentrations of L(-)-Cystein Hydrochloride on the Growth of "Saccharomyces ellipsoideus Sicilia". Measured as % Transmittance

Time in Hours	Concentration Grams/Liter								
	7.175	5.740	4.305	2.870	1.435	1.075	0.718	0.359	0.089
24	98.5	98.4	98.5	98.2	98.1	99.1	98.5	89.6	75.2
48	97.2	97.3	92.6	97.3	97.6	94.2	70.2	26.3	18.7
72	97.1	97.4	97.3	97.0	97.0	64.5	16.0	9.5	8.5
96	96.4	97.4	96.6	96.5	96.4	25.4	9.1	8.4	8.4
120	46.0	48.6	74.6	74.0	55.2	8.2	-	-	-

Substrate: B, vitamin enriched, containing 3 gm/1000 ml ammonium sulphate.
Inoculation Material: From inoculated substrate A, 48 hours old.
Number of Parallels: 2.

On the addition of 0.01 mg/10 ml of β -alanine, the inhibition was increased. However, by raising the concentration to 0.02 mg/10 ml, the additional inhibitory effect of β -alanine was cancelled.

Table XIII

The Influence of Various Concentrations of L(-)-Cystein Hydrochloride on the Growth of "Saccharomyces ellipsoideus Sicilia". Incubation Time: 40 Hours

L(-)-Cystein Hydrochloride mg/10 ml	% Transmittance
2	40.8
4	93.0
6	96.9
8	97.4
10	97.6

Substrate: B, vitamin enriched and containing 60 mg/10 ml ammonium sulphate.
Inoculation Material: From inoculated substrate A, 48 hours old.
Number of Parallels: 8.

Table XIV

Reversal of L(-)-Cystein Hydrochloride Inhibition. Expressed as % Transmittance

Concentration mg/10 ml	L(+)-Aspartic Acid	α-Ketoglutaric Acid	L(+)-Glutamic Acid	β-Alanine
0.01	-	-	-	95.1
0.02	-	-	-	73.5
6.00	21.6	44.6	25.5	-
8.00	10.3	42.9	22.1	-
10.00	20.4	30.2	17.2	-

Substrate: B, vitamin enriched and containing 60 mg/10 ml L(-)-cystein hydrochloride. % Transmittance = 70.1 after inoculation and incubation.
Inoculation Material: From inoculated substrate A, 48 hours old.
Number of Parallels: 4.
Incubation Time: 48 hours.

Table XV

Growth Inhibition Produced by L-Cystein Hydrochloride and its Reversal by L-Aspartic Acid

L(-)-Cystein Hydrochloride mg/10 ml	L(+)-Aspartic Acid mg/10 ml	% Transmittance
0	0	12.8
1	0	21.2
2	0	31.2
4	0	92.4
6	0	93.8
4	0.5	79.9
4	1.0	74.9
4	2.0	70.1
4	4.0	55.5
4	6.0	20.1
6	0.5	89.2
6	1.0	91.2
6	2.0	89.7
6	4.0	76.5
6	6.0	73.6
6	8.0	69.7
6	10.0	30.9

Substrate: B, vitamin enriched and containing 6 mg/ml ammonium sulphate.
Inoculation Material: From inoculated substrate A, 48 hours old.
Number of Parallels: 4.
Incubation Time: 48 hours.
Test Organism: "Saccharomyces ellipsoideus Sicilia".

A further investigation was undertaken with the intention of determining the exact concentration of L-aspartic acid that should be added in order to overcome the inhibition caused by L-cystein hydrochloride. To substrate B (containing 6 gm per liter of ammonium sulphate), L-cystein hydrochloride was offered in concentrations which varied from 0 to 6 mg/10 ml. Quantities of L-aspartic acid were united with these substrates within the range of concentrations 0 to 10 mg/10 ml. The dilution of the inoculum, quantity of medium per flask, incubation time, and temperature were the same as in the preceding experiment.

Table XVI

The Influence of L(-)-Cysteic Acid on the Growth of "Saccharomyces ellipsoideus Sicilia". Expressed as % Transmittance

Time in Hours	L(-)-Cysteic Acid	L(-)-Cysteic Acid + Ammonium Sulphate	Ammonium Sulphate
24	80.4	77.0	75.7
48	33.3	30.2	29.6
72	15.6	14.4	12.8
96	6.0	6.1	6.0

Substrate: B, vitamin enriched, not containing ammonium sulphate unless indicated.
Inoculation Material: From inoculated substrate A, 48 hours old.
Number of Parallels: 2.

Table XVII

Growth Inhibition Induced by L(-)-Cysteic Acid in the Absence of Pantothenic Acid

L(-)-Cysteic Acid mg/10 ml	% Transmittance
0	55.0
1	93.0
2	94.0
4	94.6
6	94.2
8	94.4
10	95.5

Substrate: B, vitamin enriched but without calcium pantothenate. Contains 60 mg/10 ml ammonium sulphate.
Inoculation Material: From inoculated substrate A, 48 hours old.
Number of Parallels: 4.
Incubation Time: 48 hours.
Test Organism: "Saccharomyces ellipsoideus Sicilia".

Table XV illustrates clearly at which concentration L-aspartic acid was capable of completely reversing the inhibition. When the concentration of L-aspartic acid was equal to that of L-cystein hydrochloride acid, i.e., 4 mg/10 ml, moderate growth, as represented by 55.5 % transmittance, occurred. However, as the concentration of L-aspartic acid was increased to 6 mg/10 ml, the per cent transmittance dropped to 20.1. By maintaining the concentration of L-cystein hydrochloride at 6 mg/10 ml and varying the amounts of L-aspartic acid, reversal of the inhibition occurred when 8-10 mg/10 ml of L-aspartic acid were added.

The results of a preliminary experiment, in which L-cysteic acid was utilized in combination with its analogue L-cystein hydrochloride, revealed no reversal of inhibition by L-cysteic acid. Moreover, L-cysteic acid was found to promote excellent growth, with or without the presence of ammonium sulphate, when presented singly in a vitamin enriched substrate B (Table XVI). However, L-cysteic acid completely inhibited the action of ammonium sulphate when the acid was added, in concentrations of 1 mg/10 ml and over, to a pantothenic acid-deficient substrate B (Table XVII).

3. Discussion

The importance of amino acids in yeast nutrition has initiated several investigations relating to the behavior of individual amino acids as nitrogen sources. Ehrlich (1907) demonstrated the formation of amyl alcohols during fermentation from leucines which were present in the unfermented medium. He considered that hydrolytic deamination and decarboxylation of isoleucine were involved. Similar reactions were found to occur

$$\left.\begin{array}{r}CH_3\\ CH_3CH_2\end{array}\right\rangle CH\underset{\underset{\displaystyle NH_2}{|}}{C}HCOOH + H_2O \longrightarrow \left.\begin{array}{r}CH_3\\ CH_3CH_2\end{array}\right\rangle CHCH_2OH + NH_3 + CO_2$$

with other amino acids, namely, leucine, phenylalanine, tyrosine, and tryptophane (Ehrlich 1911, 1912). Cell-free extracts are unable to carry out these reactions; in fact, not only living, but also actively growing yeast is necessary (Thorne 1950). Therefore, it would seem that the deamination of alpha-amino acids is intimately linked with the life of the yeast cell by providing the nitrogen which is indispensable for growth and fermentation. These processes can be maintained only by utilizing some of the energy liberated during fermentation.

Stickland (1934), while studying the metabolism of Cl. sporogenes, found that the organism gained energy by oxidizing some of the amino acids at the expense of others. He classified the amino acids into two groups.

1. Those which acted as hydrogen donators.
2. Those which acted as hydrogen acceptors.

The net reaction between the two groups being that one amino acid was oxidized by the other with the liberation of ammonia.

$$CH_3\underset{\displaystyle NH_2}{\underset{|}{C}}HCOOH + H_2O \longrightarrow CH_3COCOOH + NH_3 + 2H$$

Oxidative deamination
Oxidation of alanine

$$CH_3COCOOH + H_2O \longrightarrow CH_3COOH + CO_2 + 2H$$

Reductive deamination
Reduction of glycine

$$2\underset{\displaystyle NH_2}{\underset{|}{C}}H_2COOH + 4H \longrightarrow 2CH_3COOH + 2NH_3$$

$$CH_3 \cdot \underset{\displaystyle NH_2}{\underset{|}{C}}HCOOH + 2H_2O + 2\underset{\displaystyle NH_2}{\underset{|}{C}}H_2COOH \longrightarrow 3CH_3COOH + CO_2 + 3NH_3$$

In his classification of a few of the amino acids, Stickland listed as hydrogen donators, alanine, serine, valine, leucine, aspartic acid, glutamic acid, cystein, phenylalanine and histidine. Classified as hydrogen acceptors were glycine, ornithine, proline, hydroxyproline and tryptophane. Thorne (1945) found that some yeasts were also able to initiate the Stickland reaction, given suitable pairs of amino acids, not as a source of energy, but as a source of ammonia in addition to that obtained by the Ehrlich mechanism.

Thorne (1950) suggested that when yeasts are supplied with a diverse mixture of amino acids, these acids tend to be assimilated intact and at a higher rate than would be possible if they had to undergo deamination. It is suggested by the present writer, that while in many instances the Ehrlich and Stickland mechanism may play a leading role, it is possible that amino acids, such as citrulline, which promote better growth than ammonia, are assimilated intact even when presented singly. The foregoing postulations, however, do not explain the antagonisms found to exist between some amino acids, or why, for example, citrulline should be incapable of maximum growth in the presence of lysine.

Thorne (1939) suggested that residues remaining after amino acid deamination have various degrees of toxicity towards yeast. However, as lysine is not deaminated, as demonstrated by the absence of growth when this acid is offered singly, it appears unlikely that the toxicity of amino acid residues play a role in the antagonistic effect of lysine upon citrulline.

The reversal by L-aspartic acid of the inhibition caused by the addition of L-cystein hydrochloride was found to be competitive; i. e., a definite inhibition occurred at a definite ratio of the inhibitor to L-aspartic acid. The antibacterial index as defined by Shive and Macow (1946) is "the ratio of the concentration of inhibitor (c_i) to that of the essential metabolite (c_m) at which complete inhibition of the test organism results". The antibacterial index found during the reversal by L-aspartic acid of the inhibition caused by L-cystein hydrochloride was approximately 4/4 and 6/8 in the presence of 4 and 6 mg/10 ml respectively of the inhibitor used (Table XV).

Shive and Macow have suggested that the antibacterial index is a function of the inhibited enzyme system. The competitive reversal of inhibition by L-aspartic acid and the definite reversal by L-glutamic and α-ketoglutaric acid, suggests the blocking of an enzyme or enzyme system by L-cystein, the action of which occurs prior to the formation of the amino acids involved. Furthermore, as L-aspartic acid, L-glutamic acid and α-ketoglutaric acid are likely to have little effect upon the redox potential of the medium, a further possibility is indicated. The reversal agents may influence, displace, or alter the L-cystein already present in the cell in such a way that the redox potential within the cell is changed, and the normal processes of the cell are restored, followed by a resumption of growth.

As previously demonstrated (Tables II and IV), "Saccharomyces ellipsoideus Sicilia" is capable of growth in the absence of pantothenic acid when ammonia alone is present as a nitrogen source. However, growth is not possible when ammonium sulphate is replaced by casein hydrolysate as in substrate C. The inhibition of growth caused by L-cysteic acid in substrate B in the absence of pantothenic acid, indicates a blockage by L-cysteic acid of β-alanine synthesis.

Part II

ASSIMILATION OF NITROGEN BY "SACCHAROMYCES ELLIPSOIDEUS SICILIA"

A) Analytical Methods

1. Determination of Ammonia

In the present study, the estimation of ammonia nitrogen in the fermented substrates is based upon a modification (Hawk 1951) of the method of Conway and Byrne (1933). An outline of the method is as follows: In the outer compartment of a Conway unit are placed 0.2 ml of the test solution. To the inner compartment is added 1 ml of 2% boric acid solution containing bromcresol green indicator. The cover glass is smeared with a thin film of silicone grease and fixed on the unit, greased side down. The cover is slid aside sufficiently to permit the introduction of a pipette tip, and 1 ml of saturated potassium carbonate solution is added to the outer compartment after which the cover is replaced. The fluids are mixed gently by tilting and rotating the units. This is followed by incubation for one hour at 38°C. At the end of the incubation period, the cover is removed and the contents of the inner chamber are titrated with 0.0143 N sulphuric acid, using a micro-burette with a fine tip. Stirring during the titration is effected by playing a fine stream of nitrogen over the surface of the inner chamber. The titration is continued until the color matches exactly that of a control unit treated precisely as described for the analysis except that 0.2 ml of water replace the substrate.

Since one ml of 0.0143 N sulphuric acid is equivalent to 0.2 mg of nitrogen, the calculation is as follows:

$$\text{ml of 0.0143 N acid required} \times 0.2 \times 5 = \text{mg of ammonia nitrogen per ml of substrate}$$

2. Amino Acid Determination

a) Introduction

A search for a rapid routine method for the accurate estimation of several amino acids contained in a biological fluid was disappointing. The available chemical methods for the determination of individual amino acids (Block and Bolling 1945) were found wanting, as the employment of these methods involved much time for analysis, and in some instances required separation from interfering substances. The method of Van Slyke, MacFadyen and Hamilton (1941), although highly specific for alpha-amino nitrogen, was useless unless the amino acids were first separated. The separation might have been accomplished by the excellent method developed by Moore and Stein (1951, 1952) in which the acids were chromatographed on sulphonated polystyrene resins. However, a complete isolation required up to three days.

Studies have been made to determine whether a quantitative relationship might be established in the colorimetric reaction between ninhydrin and the amino acids. Harding and MacLean (1915) first developed this reaction and later condemned it as a colorimetric method, as they found a lack of specificity and a variation in the red and blue colors produced by the reaction, since ammonia and amines formed similar colors with ninhydrin. Fitzpatrick (1949), using 13 amino acids, determined by means of the ninhydrin reaction, that within certain limits of concentration, the transmission was a straight line, and concluded that it appeared feasible to adapt these studies to the quantitative estimation of amino acids separated by a partition paper chromatographic method.

A complete method, lacking in detail, was published by Landua and Awapara (1949) and Awapara (1949) in which the spots were located with 0.05 % ninhydrin solution in water-saturated butanol, cut from the chromatogram, and the color developed with 1 ml of 2 % aqueous ninhydrin solution in the presence of pyridine by heating on a boiling water bath for 20 minutes. The color intensity was found to be a straight line function of the concentration.

Moore and Stein (1948) observed that when color development was carried out in tubes exposed to the air, the unreproducible findings which had been reported by previous workers appeared to result primarily from the influence of dissolved oxygen. Improved results were obtained when the reaction was performed in tubes evacuated to 20 mm. Under these conditions, Moore and Stein found that the relationship between color yield and amino acid concentrations was more nearly linear, although deviations were still marked. By the direct addition of a strong reducing agent to the reaction medium, the oxidative side reaction was eliminated. This reagent, as used by Moore and Stein, consisted of a 2 % ninhydrin solution in methyl cellosolve mixed with a citrate buffer containing stannous chloride as the reducing agent. The development of the color was accomplished by heating at 100°C on a specially designed electrically operated water bath. The maintenance of a constant level in the reaction tubes involved the use of a constant level regulator.

Fisher et al. (1948) measured the area of the spot formed by the ninhydrin reaction and found that the size was proportional to the logarithm of the concentration of the amino acid. The concentration of an amino acid could then be determined by comparison with known standards run simultaneously. The measurement of the spot area, however, varied somewhat due to personal error.

Bull et al. (1949) determined the light transmission of the colored compound on the paper at different points along the spot in the direction of the phenol movement. By integrating the area under the curve, relating color density and distance, semi-quantitative estimation was achieved. Block (1948) used a similar method except that the amino acids were first separated into acidic, basic, and neutral amino acids on ion exchange resins before chromatography. Later, Block (1949) developed this method further in order to obtain the percentage composition of an amino acid mixture.

In the analysis of amino acids in urine by paper chromatography, Berry and Cain (1949) utilized visual comparisons of spots. Owing, however, to the effect on chromatography of water soluble materials in the urine, the standards with which comparisons were to be made were prepared by adding known amounts of amino acids to the urine. The authors claimed 10 to 15 % accuracy for their method.

Thompson et al. (1951) introduced a method which eliminated some of the variables involved during the development of the chromatogram such as temperature, access to oxygen, concentration of ninhydrin, and the solvent used to apply it to the paper. These workers developed the chromatogram by spraying the paper with a 1 or 2 % solution of ninhydrin in 95 % ethanol containing 2 % of a collidine-lutidine mixture (1:3). The papers were hung vertically in a chamber in which they were exposed for 30 minutes to an anaerobic (CO_2) atmosphere in equilibrium with free liquid ethanol at 60°C. The spots were eluted with 10 ml of 1:1 ethanol-water mixture. When heated in an anaerobic atmosphere, the chromatograms required only one spraying in order that complete development of the chromatogram be effected. This method involved the use of a complicated chamber in order to obtain anaerobic heating conditions.

b) Preliminary Experiments

During the present work, investigations were conducted in order to establish the suitability of a solvent to be used for dilution of the colored reaction mixture (resulting from the reaction between an amino acid and Moore and Stein's reagent) prior to estimation of the color density. Of the various solvents examined, water, ethanol-water (1:1), and propanol-water (1:1) brought forth a clear solution when combined with the reaction mixture. Of the above mentioned solvents, the latter two supported a color stability lasting up to four hours (Table XVIII). Ethanol-water (1:1) was selected as the diluent solvent.

In view of conflicting reports regarding the maximum absorption point of the products of the reaction between ninhydrin and amino acids, absorption curves for each amino acid to be analyzed were determined. The ninhydrin reagent used throughout the following experiments was that of Moore and Stein (1948) and was prepared as follows: 0.04 grams of reagent stannous chloride (monohydrate) was dissolved in 25 ml of citrate buffer pH 5 (see below). To this solution was added 1 gram of ninhydrin (obtained from Hoffmann-La Roche, Basel) in 25 ml of reagent grade methyl cellosolve. After the solutions were combined, a slight rose color became evident, which disappeared upon allowing to cool. The resulting ninhydrin solution was used fresh.

Citrate buffer: The buffer pH 5 (0.2 molar) was prepared from 21.008 grams of citric acid (reagent grade) and 200 ml of normal sodium hydroxide diluted to 500 ml. The pH of the buffer when diluted with an equal amount of water was 5.0 plus or minus 0.1.

Methyl cellosolve: Samples of methyl cellosolve should give a clear solution when mixed with an equal volume of water and a negative or faint peroxide test with 10 % aqueous acidic potassium iodide. The methyl cellosolve supplied by Dr. Bender and Dr. Hobein Co. Ltd., Zürich, met these requirements.

In order to determine the points of maximum absorption of the reaction products formed by the ninhydrin solution and DL-phenylalanine, L-citrulline, or L-lysine, 50 γ of each amino acid, contained in 1 ml of water, were placed into respective test tubes. Three ml of ninhydrin reagent were added, and the total volume in each tube was made to 20 ml with 95 % ethanol and water (1:1). The tubes containing the reaction mixtures were heated for 20 minutes on a steam bath, the volume again made to 20 ml, cooled under running water to 20°C, and the optical density of the reaction mixtures determined at 10 mμ intervals between 500 and 630 mμ against a standard containing 3 ml of ninhydrin reagent and 17 ml of ethanol-water solution until the inflection point was approached, when the measurements were made at 5 mμ intervals (Fig. 10). The maximum absorption point of the reaction mixtures tested was found to be 570 mμ . Throughout Part II of this dissertation, color densities were measured by means of a Beckman quartz spectrophotometer.

Preliminary work to determine the reproducibility of color densities, resulting from the use of various temperatures during the ninhydrin reaction, was performed using DL-phenylalanine. The first temperature attempted was 100°C. Ten equal quantities of DL-phenylalanine were placed in glass tubes (2 x 5 cm) to which were added 3 ml quantities of ninhydrin reagent. The tubes were placed in a steam bath for 20 minutes, after which time the volume of the solution contained in each tube was made to 20 ml with ethanol-water (1:1), and the color densities of the resulting solutions measured against a standard of 3 ml of ninhydrin reagent in 17 ml of ethanol-water solution. Deviations from the mean of the readings varied up to 15 %. This high error was due to the evaporation of the reagents during the heating period. As a constant level regulator was not available, and with a view to confining the procedures and equipment to that of simplicity, the use of steam heat during the development of color was abandoned.

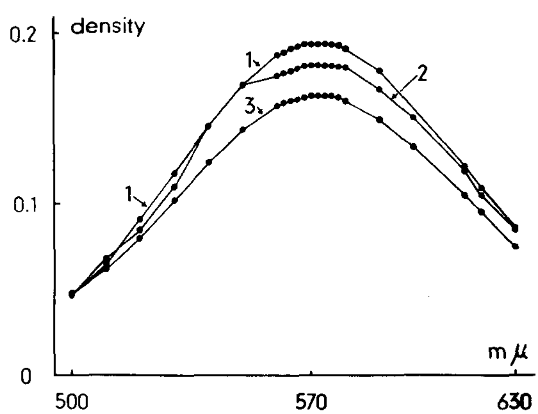


Fig. 10 Absorption Curves of the Products Resulting from the Reaction of DL-Phenylalanine, L-Citrulline or L-Lysine with Ninhydrin Reagent

1. DL-Phenylalanine.
2. L-Citrulline.
3. L-Lysine.

Table XVIII

Stability of Color Produced by the Ninhydrin Reaction Using Various Diluent Solvents. Expressed as (-) Log Transmittance

Time in Hours	Water	Ethanol-Water (1:1)	Propanol-Water (1:1)
0	0.091	0.092	0.089
0.5	0.080	0.092	0.089
1.0	0.072	0.091	0.090
2.0	0.067	0.092	0.089
3.0	0.064	0.091	0.088
4.0	0.060	0.091	0.089

Number of Parallels: 2.
Color Development: 3 ml ninhydrin reagent reacted with 10 of phenylalanine, heated 20 minutes at 100°C, and 1 ml of the reaction mixture made to 20 using the solvents indicated.

The problem relating to the method of obtaining a uniform development of color, and, at the same time, preventing a loss of solvent, was solved by the use of a lower temperature combined with the use of a longer heating time, and in addition, the employment of closed containers.

To fulfil the above conditions, exactly 50 γ quantities of DL-phenylalanine were placed into ten 20 ml volumetric glass stoppered flasks followed by the addition of 3 ml of ninhydrin reagent. The flasks were closed, using silicone grease, and heated for three hours at 60°C, cooled to 20°C, the contents made to the mark with ethanol-water solvent and the solutions read at 570 mμ . The readings of ten parallels agreed well, with a mean deviation of $\pm$ 0.0015.

Although the results of the preceding experiment indicated that, at a temperature of 60°C, color development was uniform in the reaction mixtures when each contained equal amounts of an amino acid and 3 ml of ninhydrin reagent, it was not known whether maximum color was attained. The temperature and heating times used by previous workers varied from 80°C for 3 minutes, to 100°C for 20 minutes (Moore and Stein 1948, Awapara 1949, Awapara and Landua 1949, Fitzpatrick 1949). Thompson et al. (1951) observed that the effect of time on the aerobically developed color, during the reaction between a ninhydrin solution in water-saturated butanol and an amino acid on paper, had a marked effect at 60°C in that the color density of a majority of the acids tested decreased substantially after heating for a period of 20 minutes. In order to ascertain what changes in color density might occur at the various temperatures during the development of color in closed containers using the ninhydrin reagent of Moore and Stein, 150 γ of DL-phenylalanine, L-lysine dihydrochloride, and L-citrulline were introduced into separate 15 ml brown glass, rubber stoppered bottles containing 10 ml of the ninhydrin reagent. The introduction of exact quantities of the amino acids into the bottles was accomplished with the use of an "alga" micrometer-syringe, consisting of a glass precision-made syringe attached to a micrometer screw. As the screw was revolved, the plunger was depressed: one revolution delivering 0.01 ml of solution. To the syringe was attached a 6 cm hollow needle with a beveled tip for easy application to the paper.

Quantities consisting of 150 γ were placed, by means of the micro-syringe, onto small squares of Whatman number 1 paper, allowed to dry, and the paper squares folded and placed in the bottles with the aid of tweezers. Heating at selected temperatures was achieved by placing two bottles containing parallel reaction mixtures in each of five ovens set at temperatures of 40, 60, 70, and 80°C. At chosen intervals, the bottles were withdrawn, and cooled to 20°C under running tap water. Subsequently, a 1 ml aliquot was removed from each bottle after which the bottles were immediately returned to their respective ovens. The 1 ml aliquots were made to 20 ml with ethanol-water and the color densities of the diluted solutions read at 570 mμ against a blank consisting of 3 ml of ninhydrin reagent and 17 ml of water-ethanol diluent. Readings at a temperature of 80°C could not be observed, as the higher temperature caused the stoppers to loosen or to pop from the bottles.

It is interesting to note (Table XIX and Figs. 11, 12 and 13) that the three amino acids DL-phenylalanine, L-lysine and L-citrulline, produced similar patterns in that their maximum color densities were reached after a heating time of approximately two hours at 70°C followed by a rapid drop of density with increasing time. Heating at a temperature of 60°C for two hours produced densities almost equal to those given at 70°C for the same length of time. Upon additional heating at 60°C, the drop in color density was not as pronounced as at 70°C. Color densities achieved at 40°C and 50°C were always less than those yielded at the two higher temperatures. The maximum densities attained with the use of lower temperatures became evident only after prolonged heating. By allowing the 70°C reaction mixture to be rapidly cooled after the attainment of maximum color, and set aside at room temperature, very little loss of color

Table XIX

The Influence of Time and Temperature on Color Density
(See figs. 11, 12 and 13)

	Time in Hours	Color Density ((-) Log Transmittance)				
		40°C	50°C	60°C	70°C	*)
DL-Phenylalanine	0.5	0.037	0.045	0.061	0.070	-
	1	0.047	0.057	0.075	0.088	-
	2	0.058	0.071	0.094	0.098	-
	3	0.060	0.075	0.093	0.091	-
	4	0.061	0.069	0.086	0.074	-
	5	0.060	0.0625	0.070	0.055	-
	6	0.059	0.070	0.070	0.045	0.0975
L-Lysine Dihydrochloride	0.5	0.025	0.060	0.085	0.095	-
	1	0.045	0.077	0.096	0.102	-
	2	0.070	0.092	0.108	0.112	-
	3	0.082	0.098	0.110	0.095	-
	4	0.089	0.099	0.105	0.060	-
	5	0.090	0.088	0.092	0.041	-
	6	0.087	0.082	0.080	0.039	0.109
L-Citrulline	0.5	0.029	0.065	0.115	0.118	-
	1	0.048	0.087	0.120	0.125	-
	2	0.071	0.105	0.121	0.119	-
	3	0.101	0.1095	0.122	0.087	-
	4	0.102	0.105	0.089	0.079	-
	5	0.101	0.096	0.079	0.067	-
	6	0.085	0.090	0.072	0.052	0.120

Number of Parallels: 2.
Color Development: 10 ml ninhydrin reagent reacted with 150 γ of amino acid, heated, cooled to 20°C, and one ml made to 20 with ethanol-water (1:1).

*) Represents color densities of samples withdrawn from the 70°C reaction mixture following a heating time of two hours. The samples were cooled and read after an elapsed time from the start of the experiment of six hours.

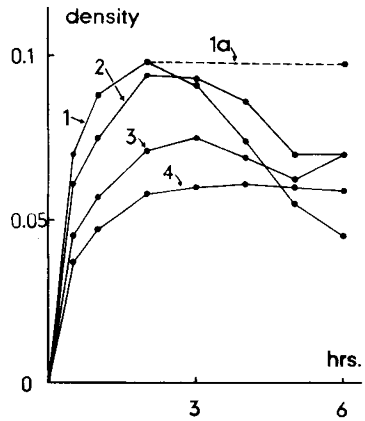


Fig. 11 The Influence of Time and Temperature on the Color Density of the Reaction Mixture Containing DL-Phenylalanine and Ninhydrin Reagent

1. 70°C.
1a. 70°C (followed by rapid cooling after two hours of heating).
2. 60°C.
3. 50°C.
4. 40°C.

Color Development: See Table XIX.

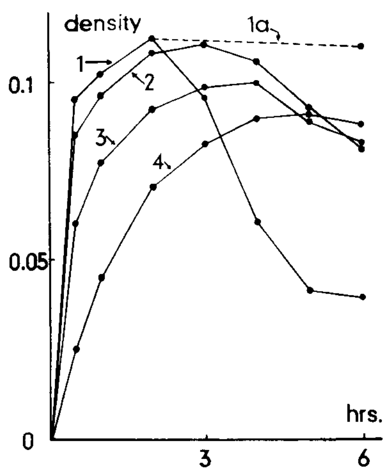


Fig. 12 The Influence of Time and Temperature on the Color Density of the Reaction Mixture Containing L-Lysine and Ninhydrin Reagent

1. 70°C.
1a. 70°C (followed by rapid cooling after two hours of heating).
2. 60°C.
3. 50°C.
4. 40°C.

Color Development: See Table XIX.

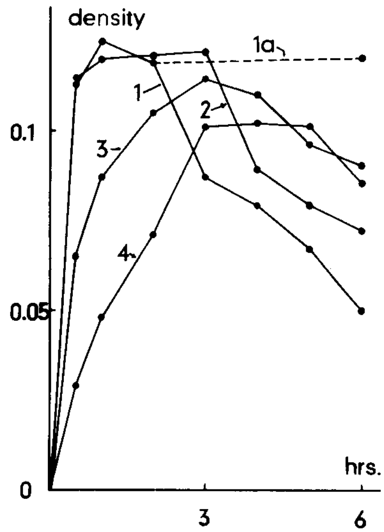


Fig. 13 The Influence of Time and Temperature on the Color Density of the Reaction Mixture Containing L-Citrulline and Ninhydrin Reagent

1. 70°C.
1a. 70°C (followed by rapid cooling after two hours of heating).
2. 60°C.
3. 50°C.
4. 40°C.

Color Development: See Table XIX.

ensued. Moreover, it has been previously shown that the color is quite stable in ethanol-water (1:1) mixture (Table XVIII). These findings indicate that once maximum color has been obtained, it can be readily stabilized during the remainder of the procedure. A heating period for the ninhydrin-amino acid mixture of 2 hours at 60°C was selected as being most suitable.

Up to this point in the present investigation, the following has been established: (a) the reagents to be used, (b) the maximum absorption point of color products, and (c) the most suitable temperature and heating time of the reaction mixture. The next step was to determine within what limits of concentration Beer's law was obeyed. Therefore, a standard curve for DL-phenylalanine was constructed from data obtained by reacting various amounts of the amino acid with the ninhydrin reagent. To this end, five ml glass-stoppered brown bottles were used. One ml quantities of distilled water containing 75, 100, 150, 200 and 250 γ of DL-phenylalanine were placed in the 5 ml glass-stoppered flasks followed by the addition of 3 ml of ninhydrin reagent. The flasks, sealed by lightly greasing the glass stoppers, were heated at a temperature of 60°C for two hours. After allowing to cool to room temperature, a 1 ml aliquot was removed from each flask, made to 20 ml with ethanol-water solution and the color density determined against a blank determination in which DL-phenylalanine was absent. By plotting (-) log transmittance against the concentration of DL-phenylalanine present in the reaction mixture, a straight line was obtained (Table XX and Fig. 14).

Subsequently, the choice of a solvent for the quantitative separation of amino acids by paper chromatography was considered. Butanol saturated with 3 % ammonia, butanol-glacial acetic acid-water (4:1:1), and freshly distilled phenol were examined. All gave excellent separation of L-lysine, L-citrulline and DL-phenylalanine. Because of the ease of preparation, the butanol-glacial acetic acid-water mixture was chosen for preliminary determinations.

Tests were conducted in order to ascertain whether equal amounts of phenylalanine, when placed on paper, chromatographed, and the color of the spots developed by the ninhydrin reagent, would yield parallel results. Several sheets of Whatman number 1 paper were placed between two pieces of white paper (to prevent contamination from the hands) and fastened together with paper clips. After marking the outside sheet with pencil, the papers were cut to the measurements of 30 cm x 40 cm. Lines were drawn widthwise 4 cm and 6 cm from the top of each sheet. At 3 cm intervals, the 6 cm line was marked for spotting, and each paper folded at the 4 cm line in order to facilitate the placing of the paper between glass plates in the tray containing the solvent. Extreme care was necessary to ensure absolute cleanliness.

A standard solution of DL-phenylalanine (3 mg/ml) was applied to the paper by means of a micro-pipette drawn from glass tubing (Brimley and Barrett 1953). The tip of the pipette was allowed to touch the surface of the solution until the ascent by capilliary action had ceased, after which time the pipette was brought against the paper until all the solution had been discharged. Drying between additional applications of the solution to the same spot on the paper was achieved by the use of warm air from a hair drier. The paper was then run in butanol-acetic acid-water solvent overnight, hung to dry at room temperature for three hours, and the spots located by spraying with 0.05 % ninhydrin in saturated butanol followed by heating at 60°C for 10 minutes.

The chromatogram was placed on a clean sheet of paper, and rectangles of equal area were drawn around the spots. The spots were cut from the paper and folded with the aid of tweezers. Each paper rectangle was placed into a 5 ml

Table XX

Color Densities Produced by the Reaction of Various Amounts of DL-Phenylalanine with Ninhydrin Reagent (See Fig. 14)

γ	Color Density ((-) Log Transmittance)
75	0.114
100	0.226
150	0.422
200	0.622
250	0.830

Number of Parallels: 4.
Color Development: Amino acid reacted with ninhydrin (3 ml), heated at 60°C for two hours, and a 1 ml aliquote made to 20 ml with ethanol-water (1 : 1).

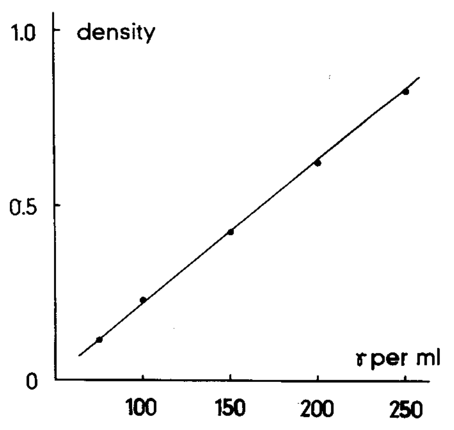


Fig. 14 Color Densities Produced by the Reaction of Various Amounts of DL-Phenylalanine with Ninhydrin Reagent

glass-stoppered brown bottle. Previous trials had indicated that the folding of the cut-out rectangles was to be preferred to the cutting of them because the paper, when cut into small pieces, shed fine particles into the solution if the bottles were shaken during the heating period.

The bottles, containing the cut out squares and ninhydrin solution, were placed in the oven and heated to 60^{o}C for a period of 2 hours. At twenty minute intervals during the heating period the bottles were gently shaken. After heating, the flasks were brought to room temperature by allowing them to stand for 20 minutes. A one ml aliquote from each bottle was made to 20 ml with ethanol-water and read at 570 mμ against a blank determination which had been subjected to identical treatment except for the addition of the amino acid. The color densities of the eight parallels did not agree within limits required for quantitative work. After calibration of the pipette, by weighing the fluid delivered onto small pieces of filter paper, it was found that the individual deliveries varied up to $\pm$ 8 % from the mean. For this reason, the micro-pipettes were discarded and the preceding experiment repeated using the micro-syringe for spot application. The color densities with the use of this instrument varied $\pm$ 0.7 % from the average of 8 readings.

Prior to this point in the present study, a standard curve of DL-phenylalanine had been determined by reacting various concentrations of the acid with the ninhydrin solution (Table XX and Fig. 14). The color density was found to be a straight line function of the concentration. In order to ascertain whether amino acid loss during the procedure of chromatographing on paper is proportional, standard curves were determined for L-lysine dihydrochloride and L-citrulline. Solutions (5 mg/ml) of the acids were prepared by weighing the appropriate amounts into volumetric flasks and filling to the mark with substrate B (not containing ammonium sulphate). Whatman number 1 paper was prepared and spotted with the use of the micro-syringe as previously described, after which duplicate spots containing 50, 100, 150, 200 and 250 γ were applied to the paper. The solution on the paper was dried thoroughly between applications to the paper. The spots were of equal size, varying only in concentration, i.e., 25 γ = 1/2 turn of the microscrew, 50 γ = 2 x 1/2 turn of the microscrew etc. The paper was run 30 hours in butanol-glacial acetic acid-water solvent, dried for 3 hours at room temperature, the spots cut from the paper, and the color developed as previously described. The color densities of the two amino acids were found to be a straight line function of their concentration (Table XXI and Figs. 15 and 16).

Table XXI

Color Densities Produced by the Reaction of Various Amounts of Amino Acids with the Ninhydrin Reagent (See Figs. 15 and 16)

γ	Optical Density	
	L-Lysine Di-hydrochloride	L-Citrulline
50	0.0465	0.042
100	0.095	0.091
150	0.138	0.139
200	0.190	0.187
250	0.230	0.232

Number of Parallels: 8.
Color Development: Amino acid placed on paper, run with Butanol-glacial acetic acid-water, developed with ninhydrin reagent 2 hours 60°C.

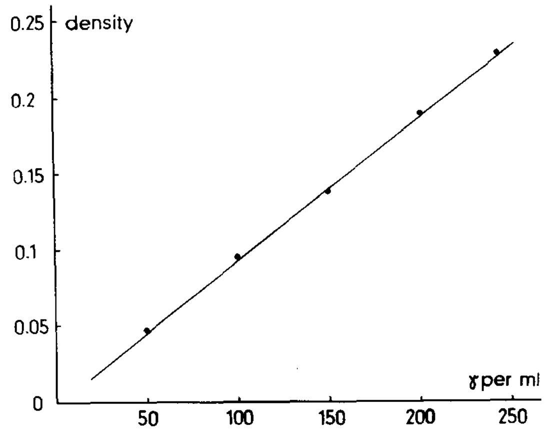


Fig. 15 The Relationship of Color Density to the Quantity of L-Lysine. The color was developed with ninhydrin reagent after the acid had been chromatographed on paper

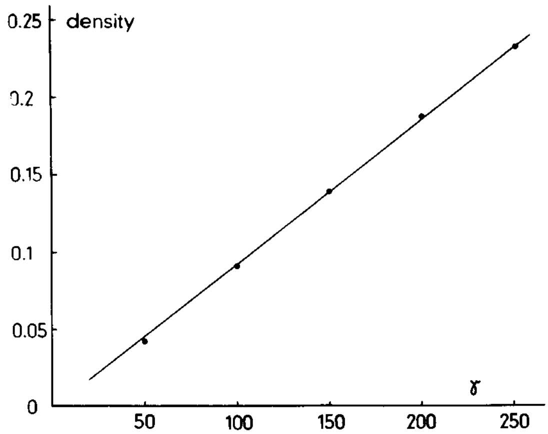


Fig. 16 The Relationship of Color Density to the Quantity of L-Citrulline. The color was developed with ninhydrin reagent after the acid had been chromatographed on paper

It is essential that all work be accomplished with the use of the same sheet of paper, as investigations have shown that the use of different sheets produce errors up to 7 % (Landua and Awapara 1949). In the present work, utilizing Whatman number 1 paper, an error of ± 4 % was indicated with the use of seven different sheets of paper. Two hundred γ of L-lysine dihydrochloride were applied to each paper in series of eight spots. The chromatograms were then run and developed as previously described, a blank determination being run with each sheet of paper. The color densities obtained from the spots of each paper were read against their respective blanks and the results averaged. Although a standard procedure was strictly adhered to in the preceding experiment as well as in the following determinations, large color variations occurred with the use of different batches of paper. These variations, however, seemed to have little or no influence on the outcome of the amino acid determinations as long as standards were utilized with each sheet of paper.

c) A Quantitative Procedure for the Ninhydrin Reaction on Paper

The procedure for the quantitative formation and measurement of the color produced by the reaction between amino acids and ninhydrin on paper is as follows:

The paper (Whatman number 1) is prepared as described previously (page 58), after which the substrate containing the amino acids is spotted in series of two, 3 cm apart and 6 cm from the top of the paper by means of a micro-syringe or other suitable device which will deliver accurately a chosen amount of the substrate. The diameter of the spots should be about 1 cm. Drying between applications of the substrate and standard to the paper is best accomplished by a warm air blower, e.g., a hair drier. The use of an infra-red lamp is not suitable, as the overheating of adjacent spots causes charring of the substrate. A standard solution containing the desired amino acids, which have been dissolved in the type of substrate used, is applied to the paper in duplicate spots. The concentration of the amino acids placed on the paper need not be known as long as established amounts of the substrate and standard solutions are applied. The paper is run for a period of 24 hours in any solvent which is suitable for the separation of the amino acids concerned, followed by drying at room temperature for not less than three hours. When the paper is dry, the spots are located by spraying lightly and evenly with 0.05 % ninhydrin in water-saturated butanol. The papers are hung for 10 minutes in an oven which has been preset to 60^{o}C. The spots are then enclosed by pencil-drawn rectangles of equal area and cut from the chromatogram. A predetermined blank section of the paper within each row of spots is also marked with an area equal to that of each cut-out spot. This serves as a blank reading of the reagents and of the paper for each amino acid. As each rectangle is cut from the chromatogram, it is folded three or four times, depending upon the size, by means of clean tweezers, and placed into a brown 5 ml glass-stoppered bottle. Care must be taken to ensure that the hands do not touch the squares at any time. To each bottle, exactly 3 ml of the freshly prepared ninhydrin reagent (page 51) is added, and the stoppered bottles are heated at 60^{o}C for a period of two hours, after which time they are allowed to cool to room temperature. During heating, the bottles are gently shaken at 20 minute intervals. On cooling, a 1 ml aliquot from each bottle is made to 20 ml with ethanol-water (1:1) mixture. The same 1 ml pipette is used throughout, care being taken to thoroughly rinse the pipette with the next solution to be used. The color may then be read at any time thereafter up to several hours, since the fading is slow. The color density is measured in a Beckman spectrophotometer with a 1-cm path length at 570 mμ for blue and purple spots, and at 330 mμ (Thompson et al. 1951) with a band width of 0.7-0.8 mμ for orange spots, against the blank determination for the particular amino acid. The duplicate readings of the standard are averaged as are those of the unknown, and the amino acid content of the un-

Table XXII

Determination of L-Lysine Dihydrochloride by Paper Chromatography

Sample	L-Lysine Dihydrochloride Added mg/ml	L-Lysine Dihydrochloride Detected mg/ml
1	1	1.030
2	1	1.016
3	1	0.923
4	1	0.945
5	1	1.033
6	1	1.001
7	1	1.010

known is calculated by:

$$\frac{\text{Reading of unknown}}{\text{Reading of standard}} \times \text{Concentration of standard} = \text{Concentration of unknown.}$$

Utilizing two standard solutions of L-lysine dihydrochloride (3 mg/ml and 1 mg/ml) dissolved in substrate B (not containing ammonium sulphate), seven determinations were made by using one of the standard solutions as the "unknown".

The mean deviation of the seven determinations was found to be $\pm$ 0.0343 (Table XXII).

B) Experiments and Results

It has been observed (Part I) that, (a) L-lysine dihydrochloride supported no growth, (b) L-citrulline supported a growth superior to that encouraged by ammonium sulphate, and (c) antagonism occurred between L-lysine dihydrochloride and L-citrulline when a mixture of these amino acids was used as the source of nitrogen.

In order to establish to what extent the nitrogen of each amino acid and that of ammonium sulphate is utilized when presented alone or in combination, the individual nitrogen determinations of L-lysine dihydrochloride, L-citrulline, and ammonium sulphate were made on the media prior to inoculation and three days after the beginning of the incubation period. Throughout these experiments, the basal substrate B (not containing ammonium sulphate unless indicated) enriched with (+)-biotin, meso-inositol, and calcium pantothenate in concentrations of 25 γ /1000 ml, 25 mg/1000 ml and 2.5 mg/1000 ml respectively, was utilized.

The amino acids and/or ammonium sulphate, each containing 25 mg of nitrogen, were weighed into shallow 100 ml glass beakers. After the addition of approximately 25 ml of the basal, vitamin enriched substrate, the pH was adjusted to 4.0 with the use of a Polymetron pH meter equipped with a glass covered magnetic stirrer. The substrate containing the nitrogen sources was then transferred quantitatively to 50 ml volumetric flasks, made to the mark with the basal substrate, placed in 100 ml Erlenmeyer flasks, and stoppered by cloth covered cotton wool plugs. Sterilization was achieved by steaming 3 times for a period of 15 minutes 24 hours apart. Ten ml samples from each flask were aseptically withdrawn and the particular amino and/or ammonia nitrogen determined by the methods previously described. As a high concentration of ammonia and sulphate ions interfere with the quantitative determination of amino acids by paper chromatography, most of the ammonium sulphate was removed from the samples containing a mixture of amino acids and ammonium sulphate. Ammonia was removed in the following way (Klein 1931/33): To 10 ml of the substrate containing 1 drop of phenolphthalein was added a 5 % water suspension of magnesium oxide until the solution was slightly alkaline. Subsequently, the solution was distilled under vacuum at 40^{o}C until the residual volume was approximately one half of the original. The contents of the distilling flask were filtered and the residue washed with small quantities of cold water. A large part of the sulphate was removed from the filtrate by precipitating as barium sulphate after the addition of concentrated barium hydroxide. Care was

Table XXIII

Assimilation of Amino and Ammonium Nitrogen by "Saccharomyces ellipsoideus Sicilia". Expressed as mg Nitrogen/ml

Substrate	% Transmittance	Ammonia Nitrogen	Lysine Nitrogen	Citrulline Nitrogen
		Before Fermentation		
$(NH_4)_2SO_4$	99.4	0.500 ± 0.008	-	-
L-Lysine	99.3	-	0.506 ± 0.016	-
L-Lysine + $(NH_4)_2SO_4$	99.4	0.500 ± 0.006	0.501 ± 0.023	-
L-Citrulline	99.4	-	-	0.499 ± 0.006
L-Citrulline + $(NH_4)_2SO_4$	99.4	0.499 ± 0.010	-	0.500 ± 0.019
L-Citrulline + L-Lysine	99.3	-	0.507 ± 0.018	0.498 ± 0.020
L-Citrulline + L-Lysine + $(NH_4)_2SO_4$	99.4	0.500 ± 0.005	0.502 ± 0.009	0.500 ± 0.008
		After Fermentation (3 days)		
$(NH_4)_2SO_4$	9.8	0.230 ± 0.004	-	-
L-Lysine	97.8	-	0.508 ± 0.010	-
L-Lysine + $(NH_4)_2SO_4$	7.0	0.255 ± 0.001	0.500 ± 0.021	-
L-Citrulline	6.2	-	-	0.207 ± 0.016
L-Citrulline + $(NH_4)_2SO_4$	7.0	0.245 ± 0.006	-	0.499 ± 0.007
L-Citrulline + L-Lysine	60.0	-	0.508 ± 0.008	0.449 ± 0.005
L-Citrulline + L-Lysine + $(NH_4)_2SO_4$	6.8	0.240 ± 0.005	0.502 ± 0.015	0.500 ± 0.013
		Amount of Nitrogen Used		
$(NH_4)_2SO_4$	-	0.270	-	-
L-Lysine	-	-	0.0	-
L-Lysine + $(NH_4)_2SO_4$	-	0.245	0.0	-
L-Citrulline	-	-	-	0.292
L-Citrulline + $(NH_4)_2SO_4$	-	0.254	-	0.001
L-Citrulline + L-Lysine	-	-	0.0	0.049
L-Citrulline + L-Lysine + $(NH_4)_2SO_4$	-	0.260	0.0	0.000

Substrate: B, vitamin enriched and without ammonium sulphate unless otherwise indicated.
Inoculation Material: From inoculated substrate A, 48 hours old.
Number of Parallels: 4.
Incubation Time: 72 hours.

taken in order to avoid the use of an excess of barium hydroxide. After separating the barium sulphate from the solution by filtration, the volume of the filtrate was reduced to about 5 ml by vacuum distillation at 40°C and the contents of the distilling flask transferred quantitatively to a 20 ml volumetric flask. The pH of the sample was adjusted to 5, the flask filled to the mark with distilled water, and the amino nitrogen of the contents determined.

From the flasks containing the remaining 40 ml of substrate 1 ml was withdrawn and ammonia nitrogen determined. The substrates were then inoculated with one drop of inoculum and incubated at 20°C for three days. At the end of the incubation period, the cells were separated from the substrate by filtering with slight suction through 1 G 4 sintered glass filters. The clear substrates were divided into four equal portions, placed into stoppered 50 ml flasks, and stored in the deep freeze cabinet (-35°C) until required for amino and ammonia nitrogen determinations.

1. Discussion

The results contained in Table XXIII confirm those previously found in Section B of Part I in that L-lysine inhibited to a considerable extent the stimulatory action of L-citrulline. Moreover, it may be observed that in the presence of L-citrulline the uptake of L-lysine nitrogen is nil, and therefore the inhibitory effect of L-lysine upon L-citrulline can not be due to the toxic effects of deaminated fragments of L-lysine as suggested by Thorne (1949), but is probably due to the toxicity of the entire L-lysine molecule in preventing the assimilation or deamination of citrulline.

When ammonium sulphate was present in the mixture of L-lysine and L-citrulline, ammonia nitrogen was assimilated rapidly accompanied by no loss of amino nitrogen from the medium. In fact, little change in ammonia nitrogen assimilation was noted when L-lysine and/or L-citrulline were present.

A slight increase in uptake of ammonia nitrogen was observed when ammonium sulphate was the only source of nitrogen present. If L-citrulline was offered singly, citrulline nitrogen was assimilated to a greater extent than was the nitrogen assimilated in the presence of ammonium sulphate alone. This lends strength to the proposal set forth earlier in this work that a possibility exists whereby a portion of the citrulline present is assimilated intact when utilized as a source of nitrogen.

Zusammenfassung

1. In der vorliegenden Arbeit wird der Einfluss von (+)-Biotin, Ca-Pantothenat und meso-Inosit auf das Wachstum der Weinhefen "Chianti Classico", "Entre Deux Mers" und "Saint Emilion" in einem synthetischen Substrat, das als N-Quelle Ammoniumsulfat enthält, dargestellt. Ferner wird eine eingehende Untersuchung des Einflusses der gleichen Vitamine (einzeln oder in Kombinationen) auf das Wachstum von "Saccharomyces ellipsoideus Sicilia" in synthetischer Nährlösung mit Ammoniumsulfat oder Caseinhydrolysat als N-Quelle beschrieben.

Fries (1938), Schopfer (1949) und Rippel-Baldes (1952) behaupteten, dass sich wilde Hefen in synthetischen Substraten ohne Vitamine vermehren, während Kulturhefen für normales Wachstum eine vielfältige Mischung von Wachstumsfaktoren benötigen.

Wikén und Richard (1951a) anderseits haben klar gezeigt, dass die schweizerische Kulturhefe "Fendant" imstande ist, in einem synthetischen Medium zu wachsen, das nur Glucose, Mineralsalze und Ammoniumsulfat enthält. Sie folgerten daraus, dass "die Theorie der auxo-heterotrophen Natur der Kulturhefen eine unzulässige Verallgemeinerung in sich schliesst" (Wikén und Richard 1951a und b).

Weitere Versuche von Wikén und Richard (1951b und 1952a) haben gezeigt, dass die Hefen "Salenegg" und "Herrliberg" in bezug auf Wachstumsfaktoren ebenfalls autotroph sind.

Es geht aus den Ergebnissen der vorliegenden Arbeit klar hervor, dass "Saccharomyces ellipsoideus Sicilia" je nach der Natur des dargebotenen Mediums und speziell der N-Quelle auxo-autotroph oder auxo-heterotroph ist. Wenn diese Hefe in einem synthetischen vitaminfreien Substrat gezüchtet wurde, das Glucose, Mineralsalze und Ammoniumsulfat (Substrat B) enthielt, ergab sich langsames, stetiges Wachstum ohne eine logarithmische Phase. Wenn jedoch die Hefe in einem synthetischen vitaminfreien Substrat mit Caseinhydrolysat (Substrat C) als N-Quelle gezüchtet wurde, war das Wachstum praktisch gleich Null.

Mit Zugabe von Ca-Pantothenat allein, (+)-Biotin allein oder meso-Inosit allein zum Substrat B (Ammoniumsulfat als N-Quelle) war das Wachstum der vier untersuchten Hefen grösser als mit Substrat B allein. Bei gleichzeitiger Zugabe der drei Vitamine wurde ein ausgezeichnetes Wachstum und eine maximale Zellausbeute erzielt. Die Kombinationen von zwei der drei Vitamine ergaben mit der Hefe "Saint Emilion" ähnliches, aber nicht so gutes Wachstum, wie bei Anwesenheit aller 3 Faktoren. Es ergaben sich grosse Zellausbeuten und Kurven vom "S-Typ".

Grosse Wachstumsunterschiede wurden von Ca-Pantothenat, (+)-Biotin und meso-Inosit hervorgerufen, wenn diese einzeln oder in Kombination dem Medium C (Caseinhydrolysat als N-Quelle) zugefügt und das Medium sodann mit "Saccharomyces ellipsoideus Sicilia" beimpft wurde. Die Zugabe von (+)-Biotin und meso-Inosit, einzeln oder zusammen, erzielte in den ersten 200 Stunden entweder kein oder nur kleines Wachstum. Immerhin, wenn Substrat C mit Ca-Pantothenat zusammen mit (+)-Biotin und/oder meso-Inosit geboten wurde, konnte bedeutend grösseres Wachstum gefunden werden als ohne Ca-Pantothenat. Auch bewirkte Ca-Pantothenat allein nur ein langsames, aber stetiges Wachstum.

Dem Substrat B zugesetztes β -Alanin konnte im Falle von "Saccharomyces ellipsoideus Sicilia" Ca-Pantothenat ersetzen, während Pantoinsäure (der Laktonteil des Pantothensäuremoleküls) dies nicht vermochte. Dies deutet darauf hin, dass die Hefe β -Alanin nicht synthetisieren kann.

2. In einem zweiten Abschnitt wird der Einfluss von 18 Aminosäuren, einzeln oder in Kombination, sowie in Verbindung mit Ammonsulfat auf das Wachstum von "Saccharomyces ellipsoideus Sicilia" beschrieben. Abgesehen von L-Cysteinhydrochlorid, L-Oxyprolin und DL-Histidin war die Hefe imstande, in verschiedenem Ausmass alle einzeln gebotenen Aminosäuren auszunützen. Die Kombinationen von DL-Glycin, DL-Prolin, DL-Oxyprolin, DL-Histidin und L-Lysindihydrochlorid, welche einzeln nur schwaches Wachstum zeitigten, mit Ammonsulfat ergaben ein wesentlich grösseres Wachstum als die Aminosäuren allein. L-Cysteinhydrochlorid andererseits hemmt das Hefewachstum bis zu 200 Stunden in Gegenwart von Ammonsulfat. Nach dieser Zeit folgt ein rasches Wachstum. Aufgehoben wurde diese Wachstumshemmung durch die Zugabe von L-Asparaginsäure, α -Ketoglutarsäure oder L-Glutaminsäure zum Substrat.

Die Aufhebung der Wachstumshemmung durch L-Asparaginsäure war kompetitiv.

Die sechs Aminosäuren, von denen oben gesagt wurde, dass sie wenig Wachstum bewirken, wenn sie einzeln zum Substrat gegeben werden, riefen einen Antagonismus verschiedener Stärke gegenüber L-Citrullin hervor. Mit Ausnahme von L-Lysindihydrochlorid und DL-Histidin zeigten sie Antagonismus auch gegenüber DL-Methionin.

L-Citrullin bewirkte ein Wachstum, das grösser war als das mit Ammonsulfat. DL-Alanin, DL-Leucin, DL-Isoleucin und DL-Methionin hatten die Tendenz, den Stimulationseffekt des L-Citrullins auf das Hefewachstum zu unterdrücken, wenn sie einzeln dem Substrat C mit L-Citrullin zugegeben wurden. Die Wachstumswerte waren niedriger als die mit Ammonsulfat allein.

Wie schon erwähnt ist "Saccharomyces ellipsoideus Sicilia" wachstumsfähig in Abwesenheit von Pantothensäure, wenn (+)-Biotin und meso-Inosit anwesend sind. Unter diesen Bedingungen und in Gegenwart von Ammonsulfat wurde eine starke Hemmung festgestellt, wenn L-Cysteinsäure zum pantothensäurefreien Medium gegeben wurde. In Gegenwart von Pantothensäure war keine Hemmung sichtbar. Die Hemmung, hervorgerufen durch L-Cysteinsäure in Abwesenheit von Pantothensäure, deutet auf eine Blockierung der β -Alanin-Synthese hin.

3. In Kulturen von "Saccharomyces ellipsoideus Sicilia" wurde die Aufnahme von L-Lysindihydrochlorid gemessen. Amino-N wurde mit einer modifizierten Methode papierchromatographisch bestimmt, die kurz in folgendem besteht: Auf Whatman-Papier No. 1 wurden die Testlösung und Standardlösung, welche die zu bestimmende Aminosäure enthielt, doppelt aufgetragen. Das Chromatogramm wurde dann ca. 30 Stunden in einem passenden Laufmittel laufen gelassen. Nach 3 Stunden Trocknen bei Zimmertemperatur wurden die Flekken durch Besprühen mit 0,05%iger Ninhydrinlösung in wassergesättigtem Butanol sichtbar gemacht. Dann erfolgte eine Erwärmung auf 60°C für 10 Min. Die Flecken wurden als Rechtecke von gleicher Grösse ausgeschnitten und in braune Glasstöpselflaschen von 5 ml Inhalt gebracht. Jeder Flasche wurden 3 ml des Ninhydrinreagens (2 % Ninhydrin in Glykolmonomethyläther ("Methylcellosolve") mit Citratpuffer und Zinnchlorür als Reduktionsmittel nach M o o r e und S t e i n 1948) zugefügt. Die Reaktionsmischung wurde 2 Stunden bei 60°C erhitzt, und die Flaschen wurden öfters geschüttelt. Aus jeder Flasche wurde 1 ml weggenommen und auf 20 ml mit Aethanol-Wasser (1:1) ergänzt. Die Farbe wurde bei 570 mμ gegen eine Nullprobe des Chromatogramms gemessen, die gleich behandelt wurde wie die Probeflecken.

Ammonium-N wurde von "Saccharomyces ellipsoideus Sicilia" rasch assimiliert. Dabei konnte kein Verlust des Substrates an Amino-N festgestellt werden, wenn gleichzeitig mit dem Ammoniumsalz eine Mischung von L-Lysin und L-Citrullin geboten wurde. Eine leichte Erhöhung der Ammonium-N-Assimilation ist beobachtet worden, wenn Ammonsulfat als einzige N-Quelle geboten wurde.

Die Aufnahme von N aus einem Substrat, das L-Citrullin allein enthielt, war grösser als wenn das Substrat nur Ammoniumsulfat enthielt.

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Academic Record

Primary School (British Columbia)	6 years
Junior High School (British Columbia)	3 years
High School (Junior Matriculation, British Columbia)	3 years
University (B.Sc. (Agr), McGill University, Montreal)	4 years
Postgraduate (Swiss Federal Institute of Technology, Zürich)	2½ years