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The effect of certain stimulants upon the growth of yeast

James B. Lesh
Iowa State College

NOTE TO USERS

THE EFFECT OF CERTAIN STIMULANTS UPON THE GROWTH OF YEAST

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James B. Lesh

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Approved:

Signature was redacted for privacy.
In charge of Major work

Signature was redacted for privacy.
Head of Major Department

Signature was redacted for privacy.
Dean of Graduate College

Iowa State College

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EFFECT OF CERTAIN STIMULANTS UPON THE GROWTH OF YEAST

I. INTRODUCTION

Interest in stimulants for the growth of yeast developed soon after the discovery of vitamins. Certain similarities of the two materials were immediately apparent. Both vitamins and growth stimulants were substances of unknown composition, whose presence in very small quantities gave rise to large effects in cell metabolism. The announcement by Williams (1919) of a method for the detection of vitamin B, based upon the supposed identity of vitamin B and bios, was responsible for many investigations in the field of yeast nutrition. The work previous to that time had consisted largely of unsuccessful attempts to replace Wildiers' bios with known compounds.

It was shown by MacDonald and McCollum (1921), Palmer, Nelson, and Sherwood (1921), Lucas (1924), and others that the growth stimulant and vitamin B are not identical, and that the yeast test for water-soluble B is invalid because the complex is synthesized by yeast. That the nature of bios is complex has been accepted. For example, the complementary action of i-inositol in the bios complex has been confirmed by several investigators since the identification, by Eastcott (1928), of that compound as Bios I. The extent of

its activity has been questioned, however, by others who were unable to obtain similar results. Other components of bios have been isolated or highly concentrated, but complete identification has not yet been accomplished.

Little attention has been paid to the effect of various inorganic constituents of synthetic media in the presence of bios. The presence of magnesium sulfate in synthetic media has been shown by Palmer, Underhofer, and Lesh (1936) to be of importance only in the media which also contain bios. The purpose of the present investigation was to make a detailed study of the effects of various salts upon the activity of several bios preparations, as determined by the growth of different strains of yeast.

II. REVIEW OF THE LITERATURE.

A. The Nature of Bios.

The first definite indication of a yeast growth stimulant appeared when Wildiers (1901) reported that he was unable to grow yeast in a solution of sugar and salts without the addition of a small amount of wort, yeast water, or beef extract. The unknown substance, or substances, to which the name "bios" was given, until its identity could be established, was organic in nature. It was not contained in yeast ash. Wildiers attempted, without success, to replace the unknown substance with such compounds as urea, alanine, tyrosine, adenine, guanine, creatine, edestin, ovalbumin, and nucleic acid.

Previously there had occurred the famous controversy between Liebig and Pasteur concerning the process of alcoholic fermentation. Pasteur had been able to grow yeast on a simple medium containing only sucrose, ammonium phosphate, and yeast ash. Liebig, using the same medium, was unable to obtain growth. The theories advanced to explain why Pasteur was able to obtain growth in the absence of bios substances are many and varied. Wildiers suggested that Pasteur used a larger inoculum than Liebig, and that the bios was added in the inoculum. These theories are reviewed by Miller (1930) who suggests that the most plausible one is that the strains of yeast used were different, and that Pasteur's yeasts were capable of growing in the

synthetic medium while those used by Liebig, Wildiers, and others were incapable of developing in the absence of the growth stimulants.

The literature on the bios problem, up to 1925, has been adequately reviewed by Tanner (1925). Later reviews have been made by Buchanan and Fulmer (1930), Miller (1930), and Fulmer and Christensen (1934). Fulmer, Ducker, and Nelson (1934) had reported on the multiple nature of bios from alfalfa, and Lucas (1934) had fractionated the stimulant from malt sprouts into two fractions, Bios I and Bios II, neither of which was very effective in the absence of the other, but the two gave large crops when combined. Eastcott (1928) had shown that one of the fractions, Bios I, from malt sprouts, was identical with i-inositol, although her findings were not accepted immediately by Williams, Warner, and Reelm (1929), and others.

Continued investigation of the fractions from malt sprouts led to discovery of several complementary components in Bios II. Miller, Eastcott, and Sparling (1932) obtained two fractions, IIA and IIB, on treatment of Bios II with charcoal. The same treatment was applied to extracts of tomato juice and of yeast, and the fractions were found by Miller, Eastcott, and Maconachie (1933) to be physiologically identical with those from malt sprouts.

Further work on the fractions of Bios II by Miller (1934) resulted in the isolation of Bios IIA as a copper salt with the composition, $Cu(C_6H_{10}O_5N)_2$, corresponding to the copper

salt of an hydroxy-amino-butyric acid. A later report by Miller (1935), states that attempts to replace IIA with various hydroxy-amino-butyric acids were unsuccessful, and that equimolecular mixtures of γ-aminobutyric acid and aspartic acid, of α-aminoisobutyric acid and aspartic acid, and of d-glutamic acid and alanine, the compositions of which approach that of hydroxy-aminobutyric acid, had no effect when substituted for Bios IIA. The effects of thirty-two amino acids on yeast crops in media containing sugar, salts, inositol, and crude Bios IIB were determined. Only l-leucine had any great effect on the crop. The increase, however, occurred when the hydroxy-aminobutyric acid as well as inositol and IIB were present. It was concluded that the activity of Bios IIA is due to the presence of both the hydroxy-aminobutyric acid and l-leucine.

Bios IIB was further purified and material was obtained which represented the most highly concentrated form of IIB yet prepared. Its identity is still unknown, however. The author, Miller (1935), states that "Eddies' bios contains at least four constituents, viz., inositol, a hydroxy-aminobutyric acid, laevo-leucine, and the purified Bios IIB".

E. J. Williams and associates have made use of electrolytic methods for the concentration of the yeast growth stimulants. The general theory of this process of fractional electrolysis, as suggested by Williams and Waterman (1929),

is that a pH gradient is established from anode to cathode, and that the ampholyte present tends to migrate to the position at which the pH approximates its iso-electric point. In carrying out such electrolyses it was found necessary to employ dilute solutions and high voltages in order to obtain relatively complete fractionations. With the use of such a method, Williams and Truesdail (1931) were able to prepare two fractions of a bios which were active for Wildiers' yeast only when both were present. One of these substances was acidic and the other basic. Williams and Bradway (1931) found that Gobrude Mayer yeast appeared to require only one nutrilite, while yeast No. 2331 of the American Type Culture Collection required four. According to the same authors, the yeast used by Narayanan (1930) and "Wildiers's yeast" responded to two nutrilites.

Further work by Williams on the nutrilite for Gobrüde Mayer yeast led to the discovery of a single widely occurring acidic substance which has definite stimulating activity. The widespread occurrence of this yeast growth stimulant was demonstrated by fractional electrolysis of extracts of rice, bran, beef livor, crab eggs, sea urchin eggs, oysters, earthworms, planarian worms, slime mold, bacteria, (B subtilis), molds (A. niger), algae, and egg white.

From the similarity of behavior of the substance in all of the extracts it was concluded that the activity is due to a single acid. It has been tentatively named by

the authors "pantothenic acid". The name is derived from the Greek meaning "from everywhere". Only moderate progress has been made toward chemical identification of the pantothenic acid. There are indications that the acid sometimes exists partly in an inactive lactone or ester form which becomes active on heating with dilute acid or alkali. The effect of pantothenic acid, inositol, and vitamin B_1, upon various yeasts has been studied by Williams and Saunders (1934) with the conclusion that "no single (unknown) substance is wholly responsible for yeast growth stimulation. The three substances studied play important roles, of which that of 'pantothenic acid' is outstanding". Recently, Williams and Rohrman (1936) reported that synthetic beta-alanine was highly potent as an agent for stimulating yeast growth in a synthetic medium containing aspartic acid and inositol. It was effective at a concentration of 0.03 micrograms per cc., and a concentration of 0.000003 micrograms per cc. is stated to be inhibitory. Pantothenic acid was necessary for maximum growth.

Miller (1936) subsequently determined the effect of beta-alanine in the presence of various bios fractions. It was found that the addition of very small amounts of beta-alanine to media containing inositol and Bios IIB greatly increases the growth of yeast. The author states, "this substance possesses the properties of Bios IIA to a much greater extent than any single chemical previously tried". It was far more effective than the unidentified hydroxy-amino-butyric acid. As with that compound, however, the

addition of levo-leucine to beta-alanine caused further increase. It was suggested that the presence of beta-alanine as an impurity in the hydroxy-amino-butyric acid to the extent of three parts per thousand was responsible for the effect of the hydroxy-amino-butyric acid. The conclusion was that the properties of Bios IIA are due to its content of beta-alanine and levo-leucine.

A highly active material has been concentrated from egg yolk and obtained in crystalline form by Kögl (1935), and Kögl and Tönnis (1936). The fraction is adsorbed by charcoal, and in chemical properties is apparently the same or similar to the Bios IIB fraction of Miller. Biotin, as it is named by Kögl, is effective alone in extremely small quantities. The effect is enhanced by the addition of inositol, and a crude fraction, Bios III, which is not adsorbed by charcoal. A yield of 1.1 mg. of crystalline material, melting at 148° C., was obtained from 250 kilograms of dried Chinese duck egg yolk. The crystals contain nitrogen, but no phosphorus or sulfur.

A summary of the bios problem shows that there are a number of different substances which stimulate yeast growth. Those which are effective alone in a solution of sugar and salts are biotin and pantothenic acid.. Those which are effective only in the presence of one or more other factors are : inositol, Bios IIA (l-leucine, and beta-alanine), Bios IIB, and Bios III. The solution to the problem of the

nature of bios lies in the identification of biotin, pantothenic acid, and Bios IIB. It is quite possible that these three substances are the same, and that the different results thus far obtained are due to differences in the composition of media and in the strains of yeast used.

In addition, it should be mentioned that a name, Bios V, has been proposed by Miller (1935) for the constituent found to be necessary for the reproduction of Saccharomyces hansenionspora valbyensis. It must be present along with inositol, crude Bios IIA and crude IIB to give growth with this yeast. It does not, however, affect the growth of Saccharomyces cerevisiae.

B. Methods of Determination of the Activity of Bios.

The progress in the study of yeast growth stimulants has been partly dependent upon developments in the methods of measuring the activity of such stimulants. The selection of cultures, test media, methods of incubation, and procedures for determining growth has had an important bearing on the problem. Many of the conflicting results which characterise the entire field of investigation have been due to lack of standardized technique.

The selection of yeast cultures has been given considerable attention in recent years. Miller (1930), referring to the Pasteur-Liebig controversy, says, "in those early

days they spoke of 'beer yeast' implying as it were that 'pigs is pigs'; the fact that Liebig used a Munich bottom-yeast seemed unimportant, at least no one for many years suggested that the race of yeast employed might make a difference". In much of the earlier work a single culture was employed, and the results thus obtained were taken as a general characteristic of all yeasts.

Wildiers (1901) used several commercial yeasts and two pure strains, and from these results assumed that bios was necessary for the normal growth of all yeasts. Later it was found that yeasts vary considerably in their bios requirements. Fulmer, Nelson, and White (1925) were able to propagate a culture, isolated from a Fleischmann cake, on a medium containing only salts and a synthetic sugar, "maltose". Fulmer and Grimes (1925) studied the growth on synthetic agar media of three types of organisms; Saccharomyces cerevisiae, Torula sphaerica, and Mycoderma. Copping (1929) compared the ability of twenty different yeasts to grow in the medium of Meader (1927), and found a wide variation among the cultures. Stantial (1932) determined the effect of inositol and Bios II on twelve different yeasts. Of these, two cultures grew rapidly in the synthetic medium alone, three were stimulated by the Bios II alone, and the others grew well only in the presence of both inositol and Bios II.

In recent years it has become customary for investigators to determine the response of several strains of yeast to the various bios fractions. Miller, et al (1935) showed

that Wildiers' yeast behaved toward inositol, Bios IIA, and Bios IIB just as the strain of yeast employed in most of the previous work. Williams and Saunders (1934) found significant differences in the behavior of Wildiers' and Miller's yeasts toward inositol and crystalline vitamin B$_1$. Both yeasts were stimulated by pantothenic acid, and by beta-alanine (Williams and Rohrman, (1936). The "Rasse P" used by Nägl (1935) was found to be similar to Miller's and Wildiers' yeasts by Miller (1935) and by Williams and Rohrman (1936).

The type of medium most suitable for bios studies is one which supplies all the nutrients necessary for the rapid growth of yeast when bios is added. The number of media which have been used is almost as large as the number of investigations. Some media have been made up empirically; in others the concentrations of salts best suited for growth have been determined. Studies by Fulmer, Nelson, and Sherwood (1931) resulted in the development of several media of the latter type. The media contain ammonium chloride, potassium phosphate, calcium chloride, and calcium carbonate, in optimal concentrations for the growth of yeast in the absence of bios preparations.

The effect of copper, zinc, and manganese salts on the growth of yeast in synthetic media was determined by McHargue and Calfee (1931). They found optimum concentrations for copper sulfate at 7.5 parts per million, manganese chloride at 10 p.p.m., and for zinc sulfate at 7.5 p.p.m. Chrzaszcz (1931) studied the effect of copper and iron salts on the

growth of yeast in the medium of Wildiers (1901) freed from copper and iron. Ferric chloride was found to stimulate growth and to increase respiration when small amounts were added to the medium. The addition of copper and iron together further increased growth. Ulvehjem suggested that availability of iron was the limiting factor in many synthetic media used in the study of growth requirements of yeast.

Copping (1929) compared the media of Reader, Wildiers, Pringsheim, Robertson, and the Medium U of Pulver, Nelson, and Sherwood. Only the first and last media were found satisfactory for yeast growth studies, and there was a much larger effect of bios in Reader's medium than in Pulver's medium.

Richards (1932), using a strain of Saccharomyces cerevisiae No. 2335 of the American Type Culture Collection, showed that thallium acetate acts as a growth stimulant in the synthetic medium of Williams. Thallium was found in the asparagine employed as a constituent of the medium. Addition of 0.001 mg. of thallium, as salt, per cc. of medium containing purified asparagine increased growth eighty per cent. A sharp optimal concentration of thallium was demonstrated, and the possibility was suggested that Wildiers' proof of the organic nature of bios is invalid. Tests by Miller, Westcott, and Maconachie (1933) show that the effect of neither Bios IIA or IIB is due to the presence of thallium. Williams and Saunders (1934) have modified the medium of Williams (1919) to include all of those elements that give a small but detectable effect upon yeast growth (Mn, Mn, Fe, Cu, Ni, Zn, and

H_3PO_3). These are included in the medium to prevent the possible addition of them as impurities from preparations of growth stimulants.

Various methods for the determination of yeast growth have been developed. Wildiers used the loss of weight in the flasks, due to evolution of carbon dioxide, as a means of comparing growth. The use of the hemocytometer is probably the most accurate method for the determination of actual proliferation, but gives no indication of the change in the size of cells which may also be considered as growth. Other methods, such as weighing the moist or dried yeast, measuring the volume of yeast in a centrifuge tube, and determining the turbidity of the yeast suspensions are also employed. Miller and co-workers incubate their cultures in L-shaped rocker tubes for twenty four hours at 25° C., and determine growth by the centrifuge tube method; the tubes are calibrated by means of a hemacytometer.

Determination of growth by means of an electrometric turbidity method calibrated in terms of the weight of dried yeast is described by Williams, McAlister, and Hoehn (1929). Incubation periods of eighteen hours at 30° C. are used. Högl and Tönnis (1936) used a test method involving a five hour growth period and electrometric turbidity measurement. Each procedure apparently has advantages, and each is sufficiently accurate for comparative purposes so that there is little danger of conflicting results arising from differences in counting methods or incubation procedures. The types of cultures and the media employed are of much greater importance.

III. EXPERIMENTAL

A. Method of Procedure.

1. Composition and preparation of media.

Several synthetic media were developed by Pulmer, Nelson and Sherwood (1921), and the optimum concentration of the components determined. Of these, the simplest, called Medium C, was selected for the following experiments. It contains, per 100 cc: 10 g. of sucrose, 0.130 g. of ammonium chloride, and 0.100 g. of dipotassium phosphate. The pH value of Medium C after the usual sterilization is 7.2. Preliminary tests showed that there was an optimum for yeast growth in the medium at pH, 6.2. In order to obtain the proper pH value, the medium was made 0.0025 N with hydrochloric acid before sterilization.

The sucrose content of the medium was reduced to 5 g. per 100 cc. in order to lessen the possibility of introducing impurities with the sugar. Variation of the concentration of sucrose between two and fifteen per cent was found to have no appreciable effect upon the growth of the yeast. Fall, Janes and Stuart (1935) found bios in many brands of commercial sugar. They determined the amount of stimulant present by the growth of yeast in a solution of the sugar alone, and showed that the stimulant was removed by recrystallization of the sugar from 80% ethyl alcohol. In Table 1 are given the

results obtained in our laboratories with various sugars before and after two recrystallizations from 80% alcohol.

TABLE 1.

Growth of yeast in Medium C containing sucrose of various sources and treatment.

(Count x 250,000 equals cells per cc.)

Sugar 5%	Count			
Beet sugar	15	19	22	22
Beet sugar, recryst.	11	11	11	15
Cane sugar	11	7	3	3
Cane sugar, recryst.	5	7	5	4
Pfanstiehl "purified"	6	7	6	6

On the basis of the above results the Pfanstiehl "purified sucrose" was selected for use in the subsequent experiments.

Mallinkrodt C. P. quality ammonium chloride was recrystallized once from distilled water, and made up in stock solution with distilled water. The dipotassium phosphate, Baker and Adamson reagent quality, was used without further purification. The stock solutions of the salts were made up in such concentration that one cc. of solution was sufficient for 100 cc. of medium. Solutions of other salts employed in the various experiments were made up in a similar manner.

In all experiments 25 cc. of medium were used in 125 cc. Erlenmeyer flasks. The basal medium was made up in concentration five-fourths of that desired. Twenty cc. of the solution were added to each flask, and made up to 25 cc. by

addition, either of solutions to be tested or of distilled water. Sterilization was carried out at 15 pounds steam pressure (121° C.) for fifteen minutes. The loss due to evaporation during the short sterilization time and during cooling was about one cubic centimeter, which was made up by the inoculum.

2. Preparation of the Inoculum.

The stock yeast cultures were preserved on malt agar slants in a refrigerator. The cultures in use were transferred daily in liquid media containing 2% glucose and 0.5% peptone. The culture which was employed in most of the experiments was No. 24 of Table 22 (p. 45), and had been isolated from a cake of Fleischmann yeast. It was grown in Medium C, and transferred every two days. There was a sufficient growth after twenty four hours to permit its use as inoculum. In using the cultures carried on glucose-peptone medium the method of preparing the inoculum described by Miller and co-workers (1933) was adopted. A twenty-four hour culture was filtered on a small filter paper which had been sterilized with alcohol. The yeast was then washed three or four times with sterile distilled water, and a small portion suspended in sterile Medium C in such concentration that one cc. of the suspension could be used to inoculate one flask.

Growth was determined by means of a Reiss-Thoma counting chamber after a twenty-four hour incubation at 30° C. A count of one is equivalent to 350,000 cells per cubic

centimeter. Inoculations were made so that the initial count was one. In all cases growth is given in terms of count. The accuracy of the counting method is greatest in the range of counts from twenty to fifty. Appropriate dilutions of heavier growth were made to bring the number of cells per cc. within the desired limits. Phenol solution (1%) was used as the dilution medium to prevent further growth during the period of counting.

B. Yeast Growth Stimulants Produced by Molds.

The fractionation of bios into Bios I and Bios II by means of alcoholic barium hydroxide has been carried out on extracts from malt, malt combings, rice polishings, tea, mushrooms, molasses, oranges, tomatoes, and yeast by Miller and co-workers. Miller, Eastcott, and Macenachie (1938) suggest that it is unnecessary to assume the existence of more than one bios, until a source of bios has been found which, with alcohol and barium hydroxide, does not behave like those already fractionated.

The production of stimulants by molds was studied by Schopmeyer and Fulmer (1951), and by Schopmeyer (1951). It was found that the stimulant produced by the growth of Aspergillus clavatus on a glycerol medium was not identical with the Bios I or II prepared from malt sprouts, as determined by their effects on the rate of growth of yeast in Medium C. The mold-produced stimulant was active when used alone, the addition of Bios I had no appreciable effect, and addition of Bios II increased growth only slightly. No attempt was made to fractionate the stimulant from A. clavatus into Bios I and II. However, the stimulant produced by A. niger appeared to be the same as that produced by the A. clavatus, and an attempt to fractionate the intracellular stimulant present in the mold felt of A. niger into Bios I and II was unsuccessful. The stimulant was produced on both glycerol and sucrose media.

In continuation of the above work, Bios II was prepared from the mold-produced stimulant and from malt sprouts. Since i-inositol was commercially available, no attempt was made to purify the Bios I fraction. The Bios II preparations, with and without the addition of inositol, were compared as to their effects upon the growth of yeast.

The mold was grown on the synthetic medium developed by Schopmeyer (1931). The medium contained, per 100 cc.: 10 g. of sucrose, 0.125 g. of dipotassium phosphate, 0.04 g. of ammonium chloride, 0.01 g. of zinc sulfate, and 0.01 g. of ferrous sulfate. Ten liters of the medium were prepared, and sterilized in 500 cc. portions in two-liter Fernbach flasks. The twenty flasks were then inoculated with a suspension of spores of Aspergillus niger, and allowed to support growth of the mold at room temperature for two weeks. The media were filtered, the mold felts washed, and the filtrate and washings were made up to the original volume of the medium. Two liters of the solution were taken for fractionation by the procedure of Lucas (1934), which is given in detail below.

The solution was concentrated in vacuo at 50° C. to one-fourth the original volume, i. e., 500 cc. One liter of 95% alcohol was then added to precipitate the inorganic salts. The mixture was allowed to settle, and was then filtered. The filtrate was concentrated to 200 cc. in vacuo, at 50° C. To this concentrate was added a slight excess of hot saturated barium hydroxide solution followed by 400 cc. of 95% alcohol. The amount of barium hydroxide necessary to cause complete precipitation in the alcoholic solution had

been determined previously with a 10 cc. sample of the concentrate. The suspension was filtered, and the precipitate, containing the Bios I, was washed with 85% alcohol and discarded. The filtrate and washings were combined and treated with carbon dioxide to precipitate the barium. The barium carbonate was removed by filtration after removal of excess carbon dioxide under reduced pressure. The filtrate was then concentrated to dryness *in vacuo*, and the residue was dissolved in 200 cc. of water. The solution was treated at 60° C. with dilute sulfuric acid to remove the last traces of barium. The barium sulfate was separated by filtration, and the filtrate was concentrated to 150 cc. The product thus obtained was called "crude Bios II solution".

The activity of the Bios II fraction, with and without inositol, was determined both in Medium C and in the medium of Clark (1923) which is used by Miller and co-workers. This medium contained, per 100 cc.: 5.0 g. of sucrose, 0.417 g. of mono-potassium phosphate, 0.054 g. of ammonium nitrate, 0.07 g. of calcium chloride, and 0.208 g. of magnesium sulfate.

Inoculations were made to a count of one in 25 cc. of medium, and the growth of yeast was determined by counting after incubation for twenty-four hours at 30° C. The results are given in Table 2.

TABLE 2.

The effect of crude Bios II solution and inositol upon yeast growth in Medium C and in Clark's medium.

(Concentration of Bios II in terms of cubic centimeters of original mold filtrate per cc. of medium. Concentration of inositol given in milligrams per cc. of medium).

Bios II	Inositol	Count	
		Medium C	Clark's Med.
-	-	14	1
-	0.008	12	2
0.12	-	54	231
0.25	-	66	197
0.25	0.008	50	359
0.25	0.016	--	362

The data given above show that the Bios II fraction is active in stimulating yeast growth in the absence of inositol. It is much more effective in the medium of Clark than in Medium C. The addition of inositol to Bios II gave no appreciable increase in Medium C, but increased growth in Clark's medium. The effect of various concentrations of inositol in the presence of Bios II was then determined. The results are shown in Table 3.

TABLE 3.

The effect of various concentrations of inositol on the bios activity of Bios II from mold filtrate in Medium C and in Clark's medium.

(Concentration of Bios II: cc. of original filtrate per cc. of medium. Concentration of inositol; mg. per cc. of medium.)

Inositol	Bios II	Count		
		Medium C		Clark's Medium
-	-	8	12	2
-	0.12	47	67	140
0.004	0.12	66	60	200
0.008	0.12	62	83	245
0.016	0.12	60	73	211
0.032	0.12	65	78	196

A concentration of 0.003 mg. per cc. of inositol appears to be optimal in Clark's medium for the amount of Bios II used. No definite effect of inositol is apparent in Medium C. The difference between Clark's medium and Medium C in regard to inositol effect and also to Bios II activity might be accounted for on the basis of the difference in composition of the two media. The initial pH of Medium C is about 6.2, and that of Clark's medium is 4.4. In Table 4 the results obtained by adjusting the pH values of both media are compared with those of the media as usually prepared.

TABLE 4.

Effect of Bios II from mold filtrate and inositol on yeast growth in Medium C and Clark's medium as a function of pH.

(Bios II: cc. of original filtrate per cc. of medium.)
(Inositol: mg. per cc. of medium.)

Bios II	Inositol	Initial pH		Count	
		Medium C	Clark's med.	Med. C	Clark's med.
0.12	-	6.2	4.4	24	154
0.12	0.003	6.3	4.4	26	285
0.12	-	5.8	5.9	24	219
0.12	0.003	5.6	5.8	23	224
0.12	0.003	5.7	6.0	28	340

The anomalous results, evidently, cannot be ascribed to differences in initial pH values of the two media.

To throw further light on the problem various combinations of the constituents of the two media were prepared, and the yeast growth observed both with and without Bios II and inositol. The results are given in Table 5.

TABLE 5.

Effect of the composition of the medium upon stimulation of yeast growth by Bios II from mold filtrate and inositol.

(Magnesium sulfate: 0.017 M.).
(Bios II: cc. of original filtrate per cc. of medium).
(Inositol: mg. per cc. of medium).

Medium	Control	With Bios II (0.12)	With Bios II (0.12) and Inositol (0.010)
Medium C	7	71	74
Medium D (1)	2	55	50
Medium C + $MgSO_4$	5	194	215
Medium D + $MgSO_4$	3	101	256
Medium C_1 (2)	3	63	65
Clark's medium	6	255	311

(1): Medium D contains, per 100 cc.: 0.138 g. of ammonium chloride, 0.100 g. of dipotassium phosphate, 0.100 g. of calcium chloride, 1.0 cc. N/10 sodium hydroxide, and 5.0 g. of sucrose.
(2): In medium C_1 ammonium nitrate was substituted for ammonium chloride in Medium C to give the same source of nitrogen as that present in Clark's medium.

The data show that magnesium sulfate is the component of Clark's medium responsible for the phenomena noted above. The effect of various concentrations of magnesium sulfate in the presence of a constant amount of the Bios II fraction were therefore determined. The results are presented in Table 6.

TABLE 6.

Effect of the concentration of magnesium sulfate on yeast growth in the presence of Bios II from mold filtrate.

(Bios II: cc. of original filtrate per cc. of medium).
(Inositol: mg. per cc. of medium).

Molarity of $MgSO_4$	Medium C + Bios II (0.12)		Medium C + Bios II (0.12) + Inositol (0.010)	
0.0	44	39	45	37
0.0003	135	147	155	153
0.0017	126	179		
0.0033	161	154		
0.000	140	178		
0.017	103	130	197	160

It may be seen that the magnesium sulfate is effective in very low concentrations. No optimal concentration for yeast growth is apparent within the range of concentrations studied. The highest concentration of the salt used was approximately that employed in Clark's medium.

It is evident from the foregoing results that the Bios II was highly stimulative in the absence of inositol, and that the presence of inositol had little additional effect. There is the possibility that the inositol had not been completely removed in the fractionation of the mold filtrate. In order to check this point the fractionation of mold filtrate was repeated. Eight liters of the mold filtrate were concentrated to 3300 cc. under reduced pressure. The concentrate was then treated according to the procedure of Lucas (1934) as previously described. The crude Bios II solution was concentrated to 250 cc., and compared with the Bios II used in the foregoing experiments. The results are shown in Table 7.

TABLE 7.

Stimulation of yeast growth by two Bios II fractions from mold filtrate.

(Bios II: concentration: 0.12 cc. of original filtrate per cc. of medium. Inositol: concentration in terms of mg. per cc. of medium).

$MgSO_4$	Inositol	Count		
		Medium C	with Bios II Frac. 1.	with Bios II Frac. 2.
-	-	16	47	35
-	0.016	14	46	35
0.017 M	-	14	130	175
0.017 M	0.016	12	224	125

The comparison shows that the two fractions differ slightly in the concentration of stimulant, but that both are active in the absence of inositol. Increasing the concentration of solids in the solution to be tested had no apparent effect upon the action of alcoholic barium hydroxide. Three explanations were considered. First, the separation of Bios I from Bios II was incomplete; second, the Bios II was active alone because of the conditions of the experiment, that is, with the strain of yeast, and the types of media employed; and third, the stimulant produced by molds was different from that obtained from various sources and fractionated into Bios I and II by Miller and co-workers. In order to find the correct explanation for the above phenomena attention was turned to studies of Bios II prepared from malt sprouts, a source used by Miller.

C. Yeast Growth Stimulant from Malt Sprouts.

The preparation of Bios II from malt sprouts was carried out exactly as described by Lucas (1934). The procedure has been given in connection with the fractionation of mold-produced stimulant. The same quantities of materials as given by Lucas were used in this preparation. Six liters of extract were obtained from 900 g. of malt sprouts. The crude Bios II solution, free from barium, was further purified by a procedure of Lucas, as described below. The crude Bios II solution (600 cc.) was diluted to five liters with distilled water, and concentrated *in vacuo* to 800 cc. To the concentrate were added five liters of acetone in 800 cc. portions. During the addition a red oily material, insoluble in acetone, separated and the acetone layer became turbid. The turbidity disappeared with further addition of acetone, but the insoluble layer remained liquid, although, according to Lucas, it should have solidified. The acetone-soluble portion was then decanted, and the acetone removed by distillation under reduced pressure. The residue from the distillation was washed eight times with 250 cc. portions of warm acetone. The washings were filtered, and the filtrate distilled. The residue from the distillation was dissolved in water to give "purified Bios II solution". The material insoluble in warm acetone was dissolved in water, freed from acetone, and concentrated under reduced pressure. This preparation was the "acetone gum" of Lucas. The red oily

layer was also freed from acetone and dissolved in water to form the "acetone precipitate". In making the tests a portion of each solution was diluted to the volume of an equivalent portion of the original extract. The solutions listed in Table 8 were then tested for bios activity.

TABLE 8.

Effect of various fractions obtained from malt sprouts.

(Concentration of bios solutions in terms of cc. of original extract per cc. of medium.)

No.	Solutions	Count	
		Medium C	Medium C plus [illegible] 0.0005%
	Control	11	9
1.	Original extract	300	352
2.	Crude Bios II solution	20	264
3.	Acetone precipitate solution	25	220
4.	Acetone gum solution	18	210
5.	Purified Bios II solution	13	60
6.	Purified Bios II inositol	15	90

It is evident that most of the stimulant of the crude Bios II fraction remained in the acetone-insoluble portions, i.e., Nos. 3 and 4.

The effect of inositol upon the crude and purified Bios II fractions is shown in Table 9. The description of the Bios fraction is given in Table 8.

TABLE 9.

The effect of inositol on crude and purified Bios II fractions.

(Concentration of Bios II fractions in terms of cc. of original extract per cc. of medium. Concentration of inositol in mg. per cc. of medium.)

Solution B	Solution S	Inositol	Medium C	Count $CuSO_4$ (0.003M)
-	-	-		10
0.1	-	-		335
0.1	-	0.000		360
0.1	-	0.016		380
0.1	-	0.032		357
0.1	-	0.064		342
-	0.1	-		35
-	0.1	0.000		92
-	0.1	0.016		128
-	0.1	0.032		110
-	0.1	0.064		102

The data show that inositol has more stimulative effect with the crude than with the purified Bios II. Both fractions, however, are active in the absence of inositol.

The fractionation of the extract of malt sprouts was repeated using a smaller amount of material (150 g.). The solutions obtained were added to Medium C as in the previous determination, so that the amount of solution present in one cubic centimeter of medium represented 0.1 cc. of the original extract. In Table 10 the acetone gum solution contains both the acetone gum and the acetone precipitate.

TABLE 10.

Effect of fractions from malt sprouts on yeast growth.

(Concentration of Bios in terms of cc. of original extract per cc. of medium. Concentration of inositol in mg. per cc. of medium.)

Bios II acetone-soluble	Acetone gum	Inositol	Count Medium C	Medium C + $MgSO_4$ 0.003 M.
--	--	--	9	9
--	--	0.010	9	9
0.1	--	--	37	316
0.1	--	0.010	51	352
--	0.1	--	42	210
--	0.1	0.010	52	271

The results given above show the acetone-soluble and acetone-insoluble fractions to be equally active. Both are highly stimulative, in the absence of inositol, in Medium C containing magnesium sulfate. The addition of inositol gives a slight increase with both fractions.

A third fractionation of the extract from malt sprouts was made. The Bios I fraction was discarded, and the Bios II freed from barium and alcohol; concentrated, and treated with acetone. The results obtained with the use of the various fractions are given in Table 11.

TABLE 11.

Bios activity of various fractions from malt sprouts.

(Concentration of bios fractions in terms of cc. of original extract per cc. of medium.)

Fractions						Count	
						Medium C plus $MgSO_4$ (0.002 M.)	
1	2	3	4	5	6	Without inositol:	With inositol (0.010 mg. per cc.)
-	-	-	-	-	-	4	6
0.1	-	-	-	-	-	217	235
-	0.1	-	-	-	-	250	207
-	-	0.1	-	-	-	222	207
-	-	-	0.1	-	-	197	231
-	-	-	-	0.1	-	20	37
-	-	-	-	-	0.1	42	42
-	-	-	0.1	0.1	0.1	206	250

1: Original extract from malt sprouts.
2: Filtrate from preliminary alcohol precipitation.
3: Crude Bios II.
4: Acetone precipitate.
5: Acetone gum.
6: Bios II.

The data of the above table are similar to those obtained in the first fractionation of malt sprouts. The addition of inositol to the Bios II fractions does not greatly increase stimulation.

In order to be certain that the Bios I was completely removed from the Bios II, half of the solutions of acetone gum and Bios II of Table 10 were combined, and again treated with barium hydroxide. The precipitate was discarded, and the filtrate, containing the Bios II, was again purified by means of acetone. The purified fractions were then compared with the solutions of the original fractionation. The results

are given in Table 12.

TABLE 12.

Effect on yeast growth of double treatment with alcoholic barium hydroxide on Bios fractions from malt sprouts.

(Concentration of Bios fractions in terms of cc. of original extract per cc. of medium. Concentration of inositol in terms of mg. per cc. of medium.)

(1) Bios II	Acetone gum	(2) Bios II	Acetone gum	Inositol	Growth Red. 0	Red. 0 M/8 (0.000 1)
-	-	-	-	-	0	0
0.1	-	-	-	-	55	-
0.1	-	-	-	0.016	51	-
-	0.1	-	-	0.016	51	-
-	-	0.1	-	-	52	191
-	-	0.1	-	0.016	64	324
-	-	-	0.1	-	41	-
-	-	-	0.1	0.016	47	-

(1) Fractions obtained from single treatment of the extract of malt sprouts with alcoholic barium hydroxide and acetone.
(2) Fractions obtained by treatment of combined fractions of (1) with alcoholic barium hydroxide and acetone.

It is evident that the second treatment of the Bios II with alcoholic barium hydroxide and with acetone did not change appreciably the activity of the fractions.

In attempts to remove the inositol from the Bios II fractions, precipitation by means of other metallic salts was attempted. Bios II fractions were treated with basic lead acetate, cupric acetate, and mercuric acetate. In each case the salt was added to the hot solution of the Bios II until no more precipitation occurred. The mixture was then

filtered; the filtrate was treated with hydrogen sulfide, filtered, and boiled to remove the excess hydrogen sulfide. The results of the comparison of the solutions, with and without inositol, are shown in Table 13.

TABLE 13.

Comparison of stimulative activity of various Dios II fractions from malt sprouts.

(Concentration of fraction in terms of cc. of the original extract per cc. of medium. Concentration of inositol: 0.010 mg. per cc. of medium.)

Dios II fractions		Count	
		Medium 6 + 50% (0.005 M.)	
No.	Concentration	Without Inositol	With Inositol
1.	0.1	125	197
2.	0.1	146	101
3.	0.1	60	76
4.	0.1	140	140
5.	0.1	175	137
Control		4	5

1: Acetone precipitate, No. 4, of Table 11; treated with basic lead acetate.
2: Dios II, No. 3, of Table 11; treated with mercuric acetate.
3: Dios II, No. 3, of Table 11; treated with cupric acetate.
4: Dios II, No. 3, of Table 11; treated with lead acetate.
5: Acetone gum, No. 4, of Table 8.

The data show that the stimulant is partly destroyed by treatment with cupric acetate. Treatment of the fractions with the other salts failed to change the activity of the solutions either in the presence or in the absence of inositol. The greatest inositol effect is shown by Solution No. 1.

From the foregoing experiments it may be seen that the Bios II from mold medium and that from malt sprouts are similar. Both stimulate the growth of the yeast employed, and this stimulation is only slightly increased by the addition of inositol. Since repeated fractionation of the stimulant failed to decrease the activity of the Bios II used alone, or to increase the stimulating effect of inositol, it was concluded that the results obtained were not due to the presence of inositol as an impurity in the Bios II fraction. An explanation for the difference between the Bios II prepared in this laboratory and that prepared by Miller and associates was sought in the conditions of the experiment. The effect of the composition of the medium upon the growth of yeast in the presence of the Bios II preparations was studied, and comparison was made of the growth of various yeast strains under different conditions.

D. The Effect of the Composition of the Medium upon the Growth of Yeast in the presence of Inositol and Bios II.

Solution No. 1 of Table 13, which had been purified by means of alcoholic barium hydroxide, acetone, and basic lead acetate, was employed in the following experiments. The effect of varying concentrations of this solution in Medium C containing inositol and magnesium sulfate is shown in Table 14.

TABLE 14.

Effect of the concentration of Bios II upon the growth of yeast in the presence of inositol and magnesium sulfate.

Concentration of inositol: 0.052 mg. per cc. of medium.
Concentration of magnesium sulfate: 0.003 M.

Bios II cc. of original ext. per cc. of medium	Count	Number of cells per unit of Bios II added
-	2	-
0.0004	5	3
0.002	34	7
0.004	71	7
0.020	138	5.8
0.040	216	2.2
0.200	344	0.7

It may be seen from the results in Table 14 that the increase in stimulation is not great at concentrations above 0.02 cc. of the Bios II per cc. of medium.

The effect of the concentration of inositol in the presence of Bios II and magnesium sulfate was then determined. Varying amounts of inositol were added to Medium C containing Bios II (0.02 cc. per cc. of medium), and $MgSO_4$ (0.017 M). The results are given in Table 15.

TABLE 15.

Effect of the Concentration of Inositol upon Growth of Yeast in the Presence of Bios II and Magnesium Sulfate.

Inositol (mg. per cc. of medium)	Count
0	170
0.002	230
0.010	340
0.050	374
0.080	519
0.100	245
0.320	338
0.800	344

The results given above indicated an optimal concentration of inositol of 0.08 mg. per cc. in the medium containing 0.08 cc. of Bios II per cc. Use of inositol at it's concentration in previous experiments would have shown a greater inositol effect than that obtained, but the large stimulation due to Bios II, alone, is still apparent.

1. Effect of magnesium salts.

Fulmer, Underkofler, and Losh (1930) have shown that the magnesium alone is not responsible for the increased activity of Bios II when magnesium sulfate is added. Thus, magnesium chloride and magnesium nitrate were effective only in the presence of sulfate added as ammonium or potassium sulfate.

Comparison is given in Table 16 of the effects of magnesium sulfate and of magnesium chloride on yeast growth in the presence of Bios II.

TABLE 16.

Effect of Magnesium Sulfate and Magnesium Chloride on Stimulation of Yeast Growth by Bios II and Inositol.

(Concentration of Bios II: 0.02 cc. per cc. of medium).
(Concentration of inositol: 0.010 mg. per cc. of medium).

	Medium C	Medium C + Bios II	
		Without Inositol	With Inositol
Control	2	25	27
$MgClO_4$ (0.01 N.)	1	121	109
$MgSO_4$ (0.10 N.)	2	149	254
$MgCl_2$ (0.01 N.)	4	29	32
$MgCl_2$ (0.10 N.)	2	30	41

When ammonium sulfate is used in place of ammonium chloride at the same normality, the addition of magnesium chloride is effective in stimulating growth. In Table 17 are given the results obtained in a medium containing ammonium sulfate and 0.02 cc. of Bios II per cc. of medium.

TABLE 17.

Effect of Concentration of Magnesium Chloride on the Growth of Yeast in the Presence of Ammonium Sulfate and Bios II.

(Concentration of inositol: 0.010 mg. per cc.)
(Concentration of Bios II: 0.02 cc. of the original extract per cc. of medium.)

Molarity of $MgCl_2$	Count	
	Without Inositol	With Inositol
0.0	58	55
0.00001	77	76
0.0001	167	159
0.001	174	165
0.01	175	159
0.1	161	201

2. The effect of calcium salts.

Calcium chloride is used in many media employed in the study of bios. In order to determine the effect of calcium chloride upon inositol and Bios II, various concentrations of the salt were used, the data being given in Table 12.

TABLE 12.

Effect of Concentrations of Calcium Chloride on Yeast Growth in the Presence of Bios II and $MgSO_4$.

(Concentration of inositol: 0.016 mg. per cc. of medium. Concentration of Bios II: 0.02 cc. of original extract per cc. of medium.)

	Medium C + Bios II			
	Without $MgSO_4$		With $MgSO_4$ (0.005 M.)	
	Without Inosit.	Inositol	Without Inosit.	Inositol
0.0	15	16	175	215
0.00001	19	16	184	251
0.0001	19	24	192	244
0.001	10	22	200	261
0.01	15	25	223	321
0.1	20	25	180	247

There is some additional growth in the presence of calcium chloride, the best results being obtained at concentration of 0.01 molar. That the effect is not due to calcium sulfate is shown in Table 13. Calcium sulfate solution was added to Medium C containing Bios II, inositol, and magnesium sulfate.

TABLE 10.

Effect of the Concentration of Calcium Sulfate on Yeast Growth in the Presence of Bios II, Inositol, and Magnesium Sulfate.

Inositol: 0.016 mg. per cc.
Bios II: Concentration in terms of cc. of the original extract per cc. of medium.
Magnesium sulfate: 0.008 M.

Molarity of $CaSO_4$	Medium C + $MgSO_4$ + Inositol	
	Bios II (0.02 cc.)	Bios II (0.04 cc.)
0.0	254	259
0.0	196	267
0.00001	193	301
0.0001	177	269
0.0002	222	313
0.0004	241	307
0.001	215	337
0.002	203	348
0.004	253	353
0.010	255	303

The results given above show that calcium sulfate has little effect on yeast growth in the presence of inositol and Bios II. Richards (1925) found an optimal concentration of calcium sulfate for growth and fermentation of yeast in William's medium at 0.0001 molar. There was no growth stimulant in the medium, however.

Comparison of the effects of calcium and magnesium chlorides was made. The medium contained ammonium sulfate (0.0176 M.) and Bios II (0.02 cc.).

TABLE 20.

Comparison of Effects of Magnesium Chloride and Calcium Chloride on Growth of Yeast in a Medium containing Ammonium Sulfate and Bios II.

	Count	
	Without Inositol	With Inositol (0.016 mg./cc.)
Control	25	39
$MgCl_2$ 0.01 M.	173	179
$CaCl_2$ 0.01 M.	45	75
$MgCl_2$ 0.005 M. plus $CaCl_2$ 0.005 M.	220	271

It is evident from the results given above that calcium salts do not replace those of magnesium in their effect upon growth of yeast in the presence of Bios II and inositol, but that it does improve the medium in the presence of magnesium salts.

3. Effect of potassium chloride.

The effect of potassium chloride on yeast growth in the presence of Bios II, inositol, and magnesium sulfate is shown in Table 21. The results indicate that slightly better growth is obtained in low concentrations of the salt. A more pronounced effect of inositol is evident in the presence of potassium chloride.

TABLE 21.

Effect of Potassium Chloride on Growth of Yeast.

Concentration of inositol: 0.010 mg. per cc.

Molarity of KCl	Medium C + $MgSO_4$ (0.000 M.) Bios II (0.02 cc. per cc.)	
	Without Inositol	With Inositol
0.0	104	181
0.0001	104	181
0.001	109	208
0.01	103	237
0.10	175	238
1.00	24	95
0.0	157	195
0.01	175	236
0.05	104	248
0.10	183	241
0.30	157	205
0.50	112	170
1.00	56	117

E. Response of Various Strains of Saccharomyces cerevisiae to Inositol, Bios II, and Magnesium Sulfate.

It seemed of interest to determine whether the effect of magnesium sulfate was the same for all yeast strains, or whether the single strain which had been used previously was alone in showing the phenomenon. Eighteen strains, all of them Saccharomyces cerevisiae, were chosen for the experiment. These were stock cultures kept in the laboratory refrigerator on malt agar slants, and maintained in good condition by periodic transfers to fresh slants.

In order to make direct comparisons of all the cultures it was necessary to standardise the method of preparing the inoculum. An attempt to carry all the cultures in Medium C was unsuccessful, because some of the cultures grew very slowly or not at all in that medium. The technique of Miller, Nesbett, and Maconachie (1955) was then adopted. The cultures were transferred daily in suitable liquid media for four days, and then a twenty-four hour culture was filtered on sterile paper; washed thoroughly with sterile distilled water, and suspended in sterile water. In the following experiments the media used consisted of 2 per cent glucose and 0.5 per cent peptone.

A key to the cultures is given in Table 22.

TABLE 22.

Key to Yeast Cultures.

Laboratory Culture No.	Name	Source
5	Saccharomyces cerevisiae, Hansen	ATCC #4300 - 1954
6	" " type Frohberg	" 2354 "
7	" " type Saaz	" 2358 "
8	" " I Hansen	" 2360 "
10	" "	" 764 "
11	" " var Berlin K 11	" 4084 "
12	" "	Canadian Distillers
14	" "	Frankfort Distillers
15	" "	G.H. Through Schopmeyer
16	" "	ATCC #764
18	" "	" 4132
19	" "	" 4100
20	" " top, bread	Callahan
21	" " Distillers	Christensen, 1933
22	" " Hansen	U. of Ill. #2255
24	" " bread	Old Fleischmann cake
26	" " Hansen	ATCC #4082 - 1954
41	" " (Fulmer)	" #4230 "
43	" " "(Gebrüde Meyer)"	Fleischmann C.14-40
45	" " "(Old Process)"	" C.15-32

Each culture was used in inoculating the series of media given in Table 23. The concentration of inositol was 0.032 mg. per cc. of medium. The Bios II had been prepared from malt sprouts by two successive treatments of the extract with alcoholic barium hydroxide (Table 12), and the amount used was equivalent to 0.04 cc. of the original extract per cc. of medium. Magnesium sulfate was employed in the concentration of 1 mg. per cc. of medium (0.008 M.).

TABLE 23.

Growth of Various Strains of Yeast in the Presence of Bios II, Inositol, and Magnesium Sulfate.

Yeast Laboratory Culture No.	Medium C								Group
	Without Magnesium Sulfate				With Magnesium Sulfate				
	1	2 Inositol	3 Bios II	4 Inositol Bios II	5	6 Inositol	7 Bios II	8 Inositol Bios II	
5	1	1	59	70	1	2	18	59	III
5	5	-	52	65	5	-	54	62	
6	3	2	4	7	2	1	1	24	III
6	1	2	8	14	2	2	1	16	
7	1	1	11	15	1	2	14	19	IV
9	2	2	-	2	1	1	10	5	I
9	1	1	3	5	1	1	3	10	
10	3	3	44	89	3	4	72	135	IV
10	6	4	96	130	11	-	120	218	
11	6	4	24	34	4	5	46	113	II
13	8	7	53	139	3	5	41	141	III
14	3	6	24	33	4	2	75	223	IV
15	13	17	42	40	11	6	127	134	IV
15	12	11	14	20	3	12	106	150	

TABLE 23.

(continued)

Yeast No.	1	2 Inositol	3 Bios II	4 Inositol Bios II	5	6 Inositol	7 Bios II	8 Inositol Bios II	Group
16	2	4	151	141	2	4	95	141	II
16	6	9	20	25	4	3	106	152	
18	12	12	15	15	2	1	117	115	I & II
18	8	8	20	16	5	4	150	150	
19	4	2	14	11	2	2	77	67	II
19	3	4	7	9	2	3	58	95	
20	8	8	13	16	5	5	66	88	I & II
20	6	10	10	14	3	4	58	70	
21	13	19	15	25	1	5	4	130	III
21	4	6	15	26	2	2	6	162	
23	1	1	21	40	2	2	46	100	IV
24	6	-	20	27	4	5	125	163	IV
24	7	7	24	26	4	5	92	105	
26	10	10	17	27	5	6	210	503	IV
26	15	15	21	24	5	7	182	540	
41	3	0	10	16	1	5	52	71	II
41	4	0	17	21	5	12	52	75	

The data of Table 23 show that in no case is the growth markedly affected by the addition of inositol, magnesium sulfate, or a combination of the two. All of the strains show greater growth in the presence of inositol and Bios II when magnesium sulfate is added. However, the strains differ in several other respects which permit their division into the following four convenient groups:

Group I: Bios II alone does not give increased growth. These include Nos. 9, 13, and 20.

Group II: Inositol does not give increased growth in the presence of Bios II when magnesium sulfate is absent. These include Nos. 11, 16, 18, 19 20 and 41.

Group III: Magnesium sulfate does not give increased growth in the presence of Bios II. These include Nos. 5, 6, 12, and 21. For the above strains the growth was actually reduced by the addition of magnesium sulfate.

Group IV: This group includes those strains which show increased growth under the conditions given for groups I, II, and III. These include Nos. 7, 10, 14, 15, 22, 24, and 26.

The groupings for the various strains of yeast are given in Table 23. The data show some reasons for discrepancies in various published results in bios studies, as related to the strain of yeast and the composition of the medium employed.

For example; if a strain of yeast belonging to Group III were grown in a medium which contained magnesium sulfate, Bios II would be found to be active only in the presence of inositol. The complementary effect of inositol and Bios II, as described by Miller and co-workers, would then be demonstrated. If, however, a strain of yeast from Group II or Group IV were employed under the same conditions, the Bios II would be active in the absence of inositol, and little complementary effect of the two stimulants would be apparent.

2. Effect of concentration of magnesium sulfate.

In the preceding experiments with the various cultures only one concentration of magnesium sulfate, which was found suitable for Yeast No. 24, was used. Another concentration of magnesium sulfate might have given an entirely different classification of the strains studied, if the toxic effect or stimulation, as the case might be, appeared only at definite concentrations of the salt. In order to test this point, series of flasks containing Medium 0 and Bios II (0.02 cc. of extract per cc. of medium) were treated with varying amounts of magnesium sulfate. A duplicate series contained inositol in concentration of 0.052 mg. per cc. of medium. The flasks were inoculated in the usual way. Table 24 gives the results with the strains of yeast selected.

TABLE 24.

Effect of the Concentration of Magnesium Sulfate upon Growth of Various Yeast Strains in the Presence of Bios II and Inositol.

Yeast No.	Inositol: mg./cc.	Control	Molarity of Magnesium Sulfate 0.000003	0.00001	0.0003	0.001	0.03	Group
24	-	34	44	150	147	173	192	IV
24	0.016	30	55	157	142	215	315	
41	-	55	50	57	86	96	112	II
41	0.016	86	83	124	167	177	205	
20	-	23	42	87	30	91	99	I & II
20	0.016	83	39	71	72	102	109	
5	-	42	66	65	49	91	50	III
5	0.016	60	60	75	91	130	157	
21	-	13	30	30	20	10	10	III
21	0.016	35	76	171	175	200	331	

Within the range of concentrations studied there appears no maximum concentration of magnesium sulfate for either the toxic effect on certain strains or the stimulating effect on the others. There is, however, a noticeable stimulation of growth at low concentrations of the salt with the strains toward which it is toxic in higher concentrations in the absence of inositol. It is evident that a higher concentration of magnesium sulfate than that used in the previous experiment would have shown more striking results in regard to the effect of inositol.

Two other cultures of yeast which had been recently received through the courtesy of the Fleischmann Yeast Co. were also grown in media with varying concentrations of magnesium sulfate. They were No. 42, Sebrifle Bayer, and No. 43, Old Process Yeast, which have been used extensively by Williams in bios studies. Magnesium sulfate was found to be toxic to both cultures in the absence of inositol. The Old Process Yeast grew in such large clumps that it was impossible to make accurate counts. The values given in the table are estimates made by comparing turbidity and sediment in each flask with that in the controls. Yeast No. 24 was also grown for comparison. The results are given in Table 35.

TABLE 35.

Effect of Concentration of Magnesium Sulfate on Growth of Various Strains of Yeast.

Yeast No.	Inositol Mg./cc.	Control	Molarity of Magnesium Sulfate 0.000008	0.00005	0.0008	0.008	0.05
24	-	55	54	159	154	105	205
24	0.016	51	73	170	179	240	239
42	-	30	55	31	32	30	30
42	0.016	46	73	156	191	274	319
43	-	X	X	2X	2X	2X	X/3
43	0.016	X	2X	5X	5X	6X	10X

The data show that cultures No. 42 and 43 may be placed in Group III. It is evident that in the absence of inositol high concentrations of magnesium sulfate are definitely toxic, although slight stimulation is apparent at low concentrations of the salt.

3. Effect of inositol upon the clumping of yeast cells.

The tendency of yeast to form clumps is affected by the presence of magnesium sulfate and of inositol. In the previous experiments it was noted that the amount of clumping increased with the concentration of magnesium sulfate when no inositol was present. In media containing inositol the number and size of clumps was reduced, or no clumps were present. Photomicrographs were made to show the effect of inositol and magnesium sulfate upon the size and shape of the cells and upon the clumping of cells of various strains. Cultures were inoculated to an initial count of one in the following four types of media:

1. Medium C plus Bios II (0.02 cc. per cc. of medium).
2. Medium C plus Bios II plus $MgSO_4$ (0.008 M.).
3. Medium C plus Bios II plus Inositol (0.032 mg. per cc. of medium).
4. Medium C plus Bios II, plus $MgSO_4$, plus inositol.

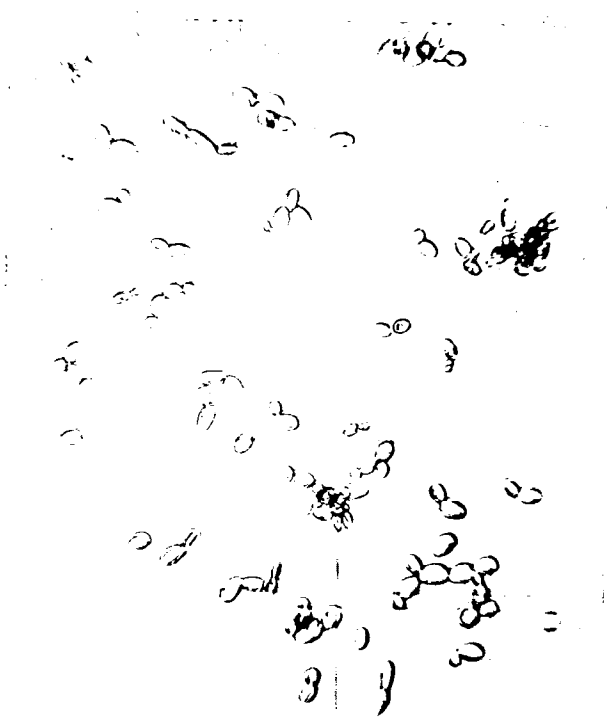
After twenty-four hour incubation, the cultures were counted, and the cells photographed. The suspensions of cells used for the photomicrographs were prepared as for counting. No dilution was made for any of the cultures, and nothing was added to prevent further growth. The cells were photographed on the rulings of a Levy hemacytometer so that comparisons might be made of the size and numbers of cells per unit area.

Figure 1.

Mica II.

Mica II and Fe_2O_3.

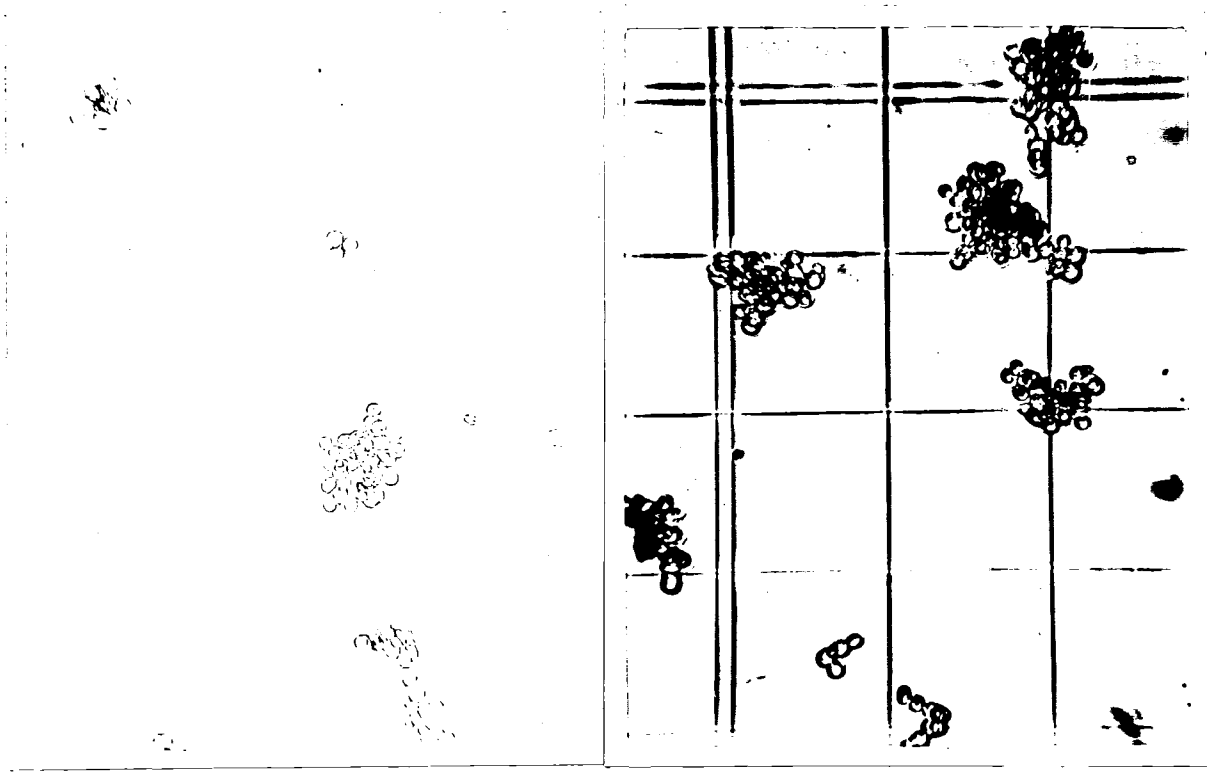
Total No. 42. Group VI.



Mica II and Ilmenite.

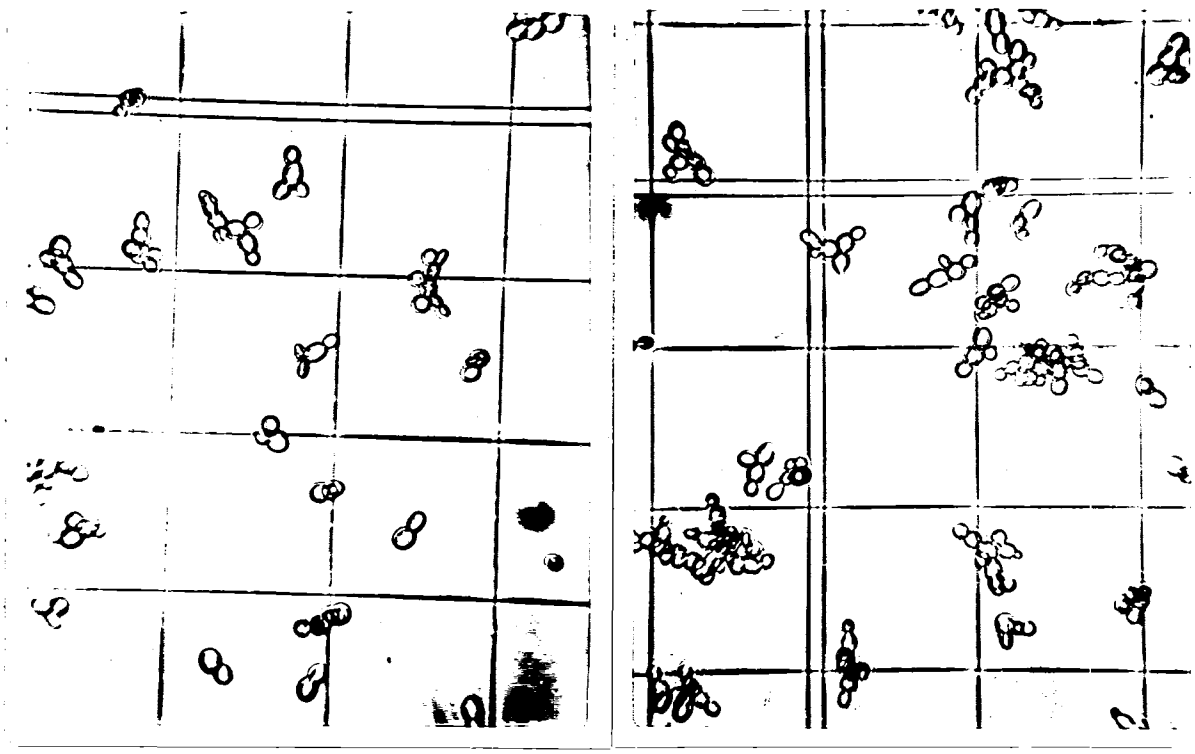
Mica II, Fe_2O_3 and Ilmenite.

Figure 2.



Bios II. Bios II and $MgSO_4$.

Yeast No. 5. Group III.



Bios II and Inositol. Bios II, $MgSO_4$, and Inositol.

Figure 5.

Disc II.

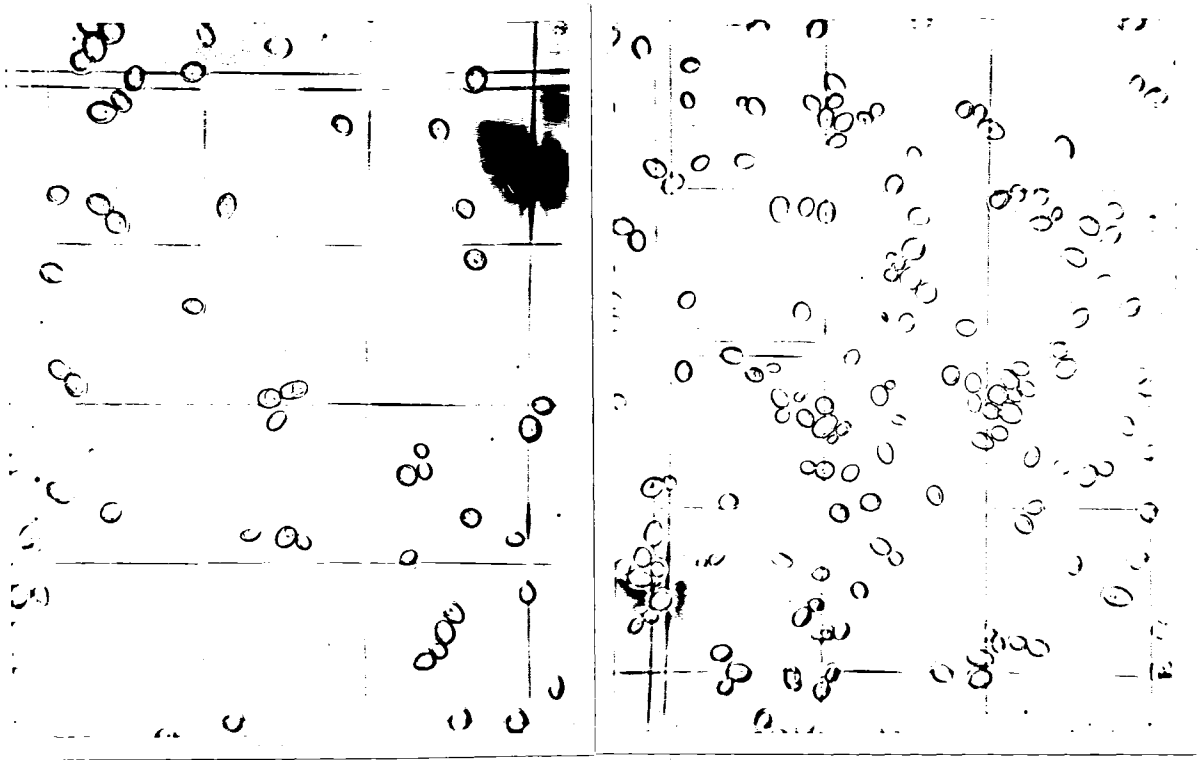
Disc IV and Light.

Table No. 62. Group III.

Disc IV and Benzol.

Disc V, Light and Benzol.

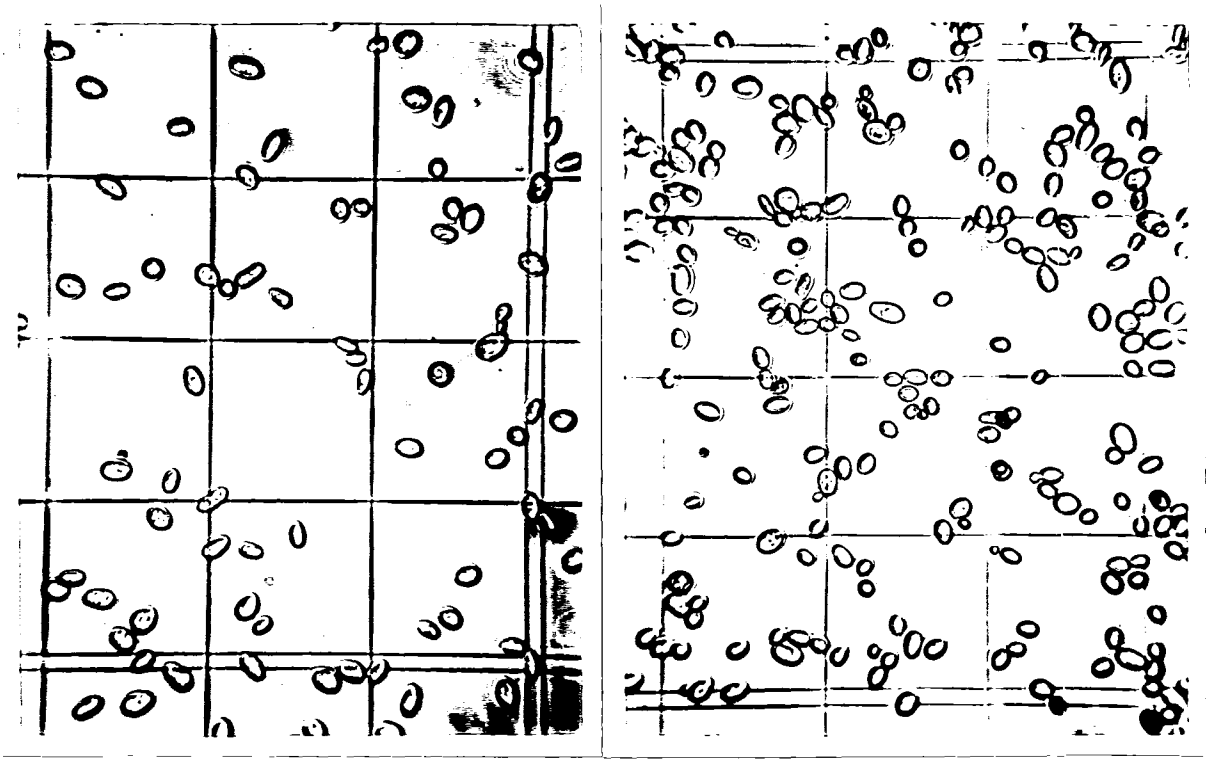
Figure 4.



Discs III.

Discs IV and $MgSO_4$.

Toast No. 24. Group IV.



Discs IV and Lactose.

Discs III, $MgSO_4$, and Lactose.

It may be seen from the photographs (Figures 1-4) that the addition of inositol to media containing magnesium sulfate and Bios II for yeasts of Group II and Group III results in marked diminution of the number and size of cell clumps. The increased growth of Group III yeasts is also apparent. Yeast No. 24 (Group IV) did not clump even in very high concentrations of magnesium sulfate, but microscopic observation during counting showed that addition of inositol brought about changes in the size and shape of the cells. Elongation of the cells was characteristic of this culture, and is apparent in Figure 4.

F. Comparison of the Growth of Yeast in Various Media.

It has been shown that the important difference between Medium C and the medium of Clark, in regard to the stimulative effect of Bios II, is the presence of magnesium sulfate in the latter medium. It became of interest to determine how the various yeast strains would grow in other media. The media selected for comparison were Clark's, Williams', Fulmer's Medium E, and Medium C to which magnesium sulfate had been added. The composition of the various media is given in Table 26.

TABLE 26.

Composition of Various Media Employed in Bios Studies.

Components	Medium			
	Clark	Williams	Medium E	Medium C
NH_4Cl	-	-	0.130	0.130
NH_4NO_3	0.33	-	-	-
$(NH_4)_2SO_4$	-	0.30	-	-
KH_2PO_4	0.42	0.20	-	-
K_2HPO_4	-	-	0.100	0.100
$CaCl_2.6H_2O$	0.07	0.04	0.200	-
$CaCO_3$	-	-	0.04	-
$MgSO_4.7H_2O$	0.21	0.05	-	0.20
Sucrose	5.0	5.0	5.0	5.0
Aspartic acid		0.15		
$KnSO_4$		0.0001		
$MnCl_2$		0.0001		
H_3BO_3		0.0001		
$FeCl_3$		0.00005		
$CuSO_4$		0.00001		
KI		0.00001		
$TlCl_3$		0.0001		

In Table 27 are given the comparative growths of four cultures in the media of Clark, Williams, and Medium C, with Bios II and with Bios II and inositol. All media were made up from the same super solution, and were sterilized together.

TABLE 27.

Comparison of the Growth of Four Yeast Strains on Various Media.

(Concentration of Bios II in terms of cc. of the original extract per cc. of medium. Concentration of inositol in mg. per cc. of medium.)

Yeast No.	Bios II	Inositol	Count Medium of Clark	Medium of Williams	Medium C
5	-	-	5	10	2
5	0.02	-	33	38	52
5	0.02	0.052	219	524	271
24	-	-	5	9	4
24	0.02	-	200	214	139
24	0.02	0.052	309	352	268
41	-	-	3	5	4
41	0.02	-	185	231	142
41	0.02	0.052	301	454	354
42	-	-	3	3	1
42	0.02	-	47	100	13
42	0.02	0.052	459	536	398

A comparison was then made of Williams' medium, Medium B and Medium C. The results are shown in Table 28.

TABLE 28.

Comparison of the Growth of Four Yeast Strains on Various Media.

(Concentration of Bios II in terms of cc. of the original extract per cc. of medium. Concentration of inositol in terms of mg. per cc. of medium.)

Yeast No.	Bios II	Inositol	Count Medium of Williams	Medium F	Medium G
5	-	-	8	1	1
5	0.02	-	55	54	75
5	0.02	0.052	180	75	250
24	-	-	4	4	2
24	0.02	-	264	102	236
24	0.02	0.052	462	150	301
41	-	-	8	5	4
41	0.02	-	251	100	128
41	0.02	0.052	402	194	270
42	-	-	5	1	1
42	0.02	-	132	55	55
42	0.02	0.052	289	158	295

It may be seen from the data of Tables 27 and 28 that no one medium is best suited for the cultures used. In regard to the inositol effect, the most striking stimulation with Yeast No. 5 is in Clark's medium, although both Williams' medium and the Medium G give good results with that yeast. Williams' medium appears to be a little better for yeasts No. 24 and 41, both of which were isolated from Fleischmann yeast. For Yeast No. 42 Medium F and Clark's medium showed the greater stimulation. Medium F, which did not contain magnesium sulfate, was in all cases the least sensitive to both Bios II and inositol.

On the basis of simplicity of composition of the medium, the Medium C containing magnesium sulfate has a slight advantage over the medium of Clark, although, as it has been previously shown, the presence of calcium chloride improves the medium to some extent. In this respect the addition of magnesium sulfate to Medium E has interesting possibilities. The disadvantage of the medium of Williams is its complexity. The addition of all possible elements affecting growth of yeast, even at optimal concentrations, does not eliminate the possibility of the effect of the same elements added as impurities in bios preparations. It has not been shown that the optimal concentration of each component as determined alone is still optimal in the presence of other components; especially when the yeast growth is rapid. The determination of the effective concentrations of copper, iron, manganese, and thallium salts by Mallargue and Calfee (1951), Nilvebjen (1951), and Richards (1933) was made in media which contained no bios. In the presence of organic growth stimulants the above salts may give entirely different results.

IV. SUMMARY.

1. The yeast growth stimulant produced during the growth of Aspergillus niger on sucrose media was found to be similar to that obtained from the extract of malt sprouts. Bios II prepared from the mold-produced stimulant by means of alcoholic barium hydroxide and Bios II from malt sprouts had similar effects upon the growth of yeast. The results obtained by Schopmeyer (1951), to the contrary, may be explained on the difference in the media and the yeast employed.

2. The importance of magnesium sulfate in media used in bios studies was emphasized. The effect of the concentration of magnesium sulfate upon the growth of various strains of Saccharomyces cerevisiae in the presence of Bios II and inositol was determined. With all the strains studied, the addition of magnesium sulfate to Medium 1 increased the stimulative effect of Bios II and inositol.

3. The effect of inositol upon the stimulation of yeast growth by Bios II was studied. The supplementary effect of inositol is dependent upon the strain of yeast employed, and upon the concentration of magnesium sulfate. In the absence of inositol the higher concentrations of magnesium sulfate are toxic toward certain strains of yeast. In such cases the addition of inositol gave large increase in growth.

4. Twenty strains of Saccharomyces cerevisiae were studied in regard to growth in the presence of inositol,

Mica II, and magnesium sulfide. The strains were divided into four groups according to their reactions to various combinations of the three substances.

5. Photomicrographs were made of four different strains to show the ability of inositol to reduce the clumping of cells.

6. Comparison was made of the growth of various strains of yeast in the media of Clark, Williams, and Fulmer in order to determine the suitability of each for use in this studies.

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