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The fixation of atmospheric nitrogen by yeast

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Mechanic Arts

THE FIXATION OF ATMOSPHERIC NITROGEN BY YEAST

A Dissertation

Submitted to the Graduate
Faculty in Candidacy for the
Degree of

Doctor of Philosophy

by

Leo M. Christensen

Approved:

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INTRODUCTION

In studying the effect of ammonium chloride on the growth of yeast, it was found by Dr. E. I. Fulmer that a good growth of yeast could be obtained in a medium containing no added nitrogen compounds. This suggested that the yeast must be able to synthesize its protein from the nitrogen of the atmosphere. Growth was slow in this nitrogen free medium not only because of the lack of nitrogen compounds but also because the favorable effect of the ammonium ion was lacking. It was thought possible, however, that this favorable physico-chemical effect might be shown to some degree by some other ion. And in such a medium it should be possible to demonstrate nitrogen fixation by yeast if it were able to utilize atmospheric nitrogen.

Both positive and negative results have been reported in the literature on nitrogen fixation by yeast. Whether or not yeast has the ability to build up its protein from the nitrogen of the atmosphere is important, not only from the taxonomic standpoint but also from the industrial, because aeration of the medium is commonly practiced in the propagation of yeast. And studies on nitrogen fixation by yeast may be a convenient method for getting at the mechanism of biologic nitrogen fixation in general, because yeast is more easily grown and studied than are the organisms commonly used for such investigations.

HISTORICAL

It was known as early as the time of the ancient Greeks that there was an increase in fertility in ground that was allowed to remain fallow, and that some crops had the ability to increase the fertility of the soil upon which they were grown. These facts and the grave discrepancy in the balance sheet for soil under cultivation early led to many speculations by the first agricultural chemists. The problem that attracted the most attention was that of the nitrogen supply. Liebig held that the nitrogen content was maintained by addition thru rainfall as ammonia and nitrogen oxides coming from the atmosphere. But his views were not accepted by Boussingault nor by Lawes and Gilbert. Boussingault had evidently arrived at the idea of nitrogen fixation by micro-organisms. (18,40)

Bertholet (3) added confirmation to this conclusion by his studies on sterilized soil. Greaves (18) says that, with Guignard, he later isolated from the soil organisms capable of fixing atmospheric nitrogen. Greaves also states that a number of chemists, König and Kiesow, Armsby, Birner, Kellner, Dehérain and Avery, had found that when organic matter decomposed there was a gain in nitrogen. Some of these thot that this was due to a reaction between nitrogen of the air and nascent hydrogen which was formed during the decomposition. An alkaline reaction and the presence of iron, manganese or lime were thot to be essential in order to observe this phenomenon.

Hellriegel and Wilfarth (25) and Hellriegel (24) demonstrated the role of microorganisms in the fixation of nitrogen by legumes. This important contribution stimulated research to determine whether other organisms present in the soil might also be able to fix atmospheric nitrogen. And in 1893 Winogradsky (43) showed that an organism which he isolated from the soil and named Clostridium pasteurianum had this ability when grown in pure culture. These studies were the beginning of work on symbiotic and non-symbiotic nitrogen fixation in the soil. Almost immediately studies were made to determine whether or not other organisms had the ability to utilize atmospheric nitrogen or whether, as Winogradsky thot, this power was characteristic of a limited group of organisms. A great deal has been done with organisms found in the soil and with many fungi and bacteria more or less closely related to soil forms, with algae and higher green plants and with protozoa and some insects, to determine their ability or inability to fix atmospheric nitrogen.

Along with other fungi, yeasts were early studied with regard to this nitrogen fixing ability. Jodin (28) and Hallier (19) thot that yeasts and other fungi were able to utilize atmospheric nitrogen, but their results were not confirmed by Woff and Zimmerman (46). Heinze (21,22) noted gains in nitrogen in cultures of yeast during their spore forming stages, but later (23) considered that yeasts, fungi and algae were important in nitrogen fixation in the soil

only because they could furnish food to azotobacter. Gerlach and Vogel (17) reported negative results with yeasts, while Zikes (47) isolated a pseudo-yeast, Torula weisneri, which he showed to be able to fix atmospheric nitrogen. Lohnis and Pillae (37) also noted fixation by a Torula but found greater fixation by Dematium pullulans, and de Kruijff (7) also claimed that certain Torula had this ability.

This problem was taken up by Kossowics (31) in 1913 and he first reported that yeasts were able to fix from 1.0 to 1.4 mgms of nitrogen per 100 cc of culture media. But later (32,33) he reversed this opinion and stated that yeasts and molds could live on small traces of nitrogen compounds which they could get from the air, but that they were unable to utilize elementary nitrogen. Lindner and Naumann (35) did not find any fixation by yeasts when the air was free from ammonia and nitrogen oxides. C. B. Lipman (36) found nitrogen fixation by 18 yeasts, pseudo-yeasts and fungi. These were grown in a medium containing practically no nitrogen, incubated four weeks and analyzed by the Kjeldahl method. No mention was made of precautions to prevent absorption of ammonia from the air. Mulvania (39) thot that yeast should be able to fix nitrogen from its similarity to azotobacter, but had no quantitative evidence. Fulmer (12) found that the yeast, Saccharomyces cerevisiae, Race F, would grow in a medium containing only potassium chloride, dipotassium phosphate and sucrose in water, using atmospheric nitrogen

as the sole source of that element. Fulmer and Christensen (15) showed that a Mycoderma isolated by them from a commercial yeast cake could fix atmospheric nitrogen when grown in a molasses medium, and that the fixation was a function of the hydrogen ion concentration. Two optima were found, one at pH 6.00 and the other at pH 7.90, the latter being the more effective.

There has been some speculation as to the mechanism of nitrogen fixation by microorganisms, but work has not progressed sufficiently to give support to any particular theory. It has long been thot that the reaction

$$NH_4NO_2 \longrightarrow N_2 + 2H_2O$$

should be reversible and this seems to have occurred to many investigators, but apparently there was little encouragement from experimental results. Lewis (34) has calculated from free energy data that a pressure of 10^{51} atmospheres would be necessary before any determinable amount of ammonium nitrite could be formed. But somewhat later Falk and McKee (11) showed that there was a fixation of 6 parts of nitrogen per million of water when the reaction was carried out in an iron tube at a pressure of 300 pounds per square inch and at room temperature. One half of the nitrogen was in the form of ammonia and the other half nitrite or nitrate. Some oxygen was present in the water and in the nitrogen gas. Increased fixation was found when higher pressures were used and when substances increasing the solubility of nitrogen in

water were present. Thus 10% alcohol or acetone caused an appreciable increase. Fixation was found in glass or quartz vessels only when a piece of iron was added.

Falk and McKee suppose that this reaction proceeds by virtue of the opposite signs of the two nitrogens in the nitrogen molecule. Thus they write the reaction,

$$\overset{---}{N}\ \overset{+++}{N} + H_2O \rightarrow NH_4NO_2.$$

They point out that since it has been shown by Stieglitz and by themselves that the nitrogens in diazo and diazonium compounds are charged differently, such compounds might be expected to be readily formed from atmospheric nitrogen and suitable benzene and napthalene derivatives.

This would suggest two mechanisms for fixation of nitrogen by biologic forms. Nitrogen may dissolve and hydrolyze and become available to the organism as ammonium nitrite, and this is suggested by Greaves (18) as a possibility. If this is true it should be possible to demonstrate nitrogen fixation by any organism which can build up its protein from ammonia, nitrite or nitrate nitrogen. And the number of organisms found to be capable of "fixing" nitrogen is increasing. But this is not the only possibility. At some stage in the growth or decomposition of the organism compounds may be formed which would lend themselves to the reaction with atmospheric nitrogen to form diazo, diazonium or similar compounds containing differently charged nitrogens. It will be seen later how this last suggestion may

be applicable in the case of yeast.

The great influence of the hydrogen ion concentration on nitrogen fixation by azotobacter has been shown by Gainey and Batchelor (16), who found that there was practically no fixation when the hydrogen ion concentration was greater than pH 5.9-6.0. Vigorous growth and nitrogen fixation were noted at pH 6.1-6.5, the optimum pH for fixation being somewhat higher than the optimum for growth. It would be expected that fixation by other organisms should be likewise affected by the hydrogen ion concentration.

STATEMENT OF THE PROBLEM

1. To develop a nitrogen-free synthetic medium in which yeast will grow and in which fixation of atmospheric nitrogen can be demonstrated.

2. To devise a satisfactory analytical procedure for following changes in nitrogen content in a medium in which yeast is growing.

3. To study the effect of the hydrogen ion concentration on fixation of nitrogen in a suitable medium.

METHODS AND MATERIALS

Isolation of Cultures.

The work in this laboratory has been almost entirely with Saccharomyces cerevisiae, Race F, as isolated from a

Fleischmann yeast cake. During the early part of the work on nitrogen fixation it was found that two yeasts were present in the pressed yeast as manufactured by this company, and these were isolated by plating on 2% glucose 0.5% peptone agar.. These are identical with those described by Eddy, Kerr and Williams (9), isolated by them from the same source at about the same time, cultures of which Dr. Eddy kindly sent us for comparison. It also seems probable that they are identical with those mentioned by Miss MacDonald (38), which she obtained from a commercial yeast cake.

These two yeasts we have designated as Nos. 11 and 12. No. 11 is the yeast used in this laboratory and designated as Saccharomyces cerevisiae, Race F, but No. 12 has not been identified. Miss MacDonald referred to it as a Mycoderma. Whether or not these yeasts are always present in the pressed yeast is not definitely known. They were found on three different occasions within the course of a year, and every time in the same ratio, 2% of No. 12 and 98% of No. 11, by count. But a year later No. 12 was not found, altho plates were made from several cakes of yeast. Characteristics of the two organisms are given in TABLE I.

TABLE I

	Yeast No. 11	Yeast No. 12
Colonies on 2% glucose, 0.5% peptone agar.		
Growth	Slow	Rapid
Form of Colony	Circular	Circular
Surface	Smooth, moist	Rough, dry
Elevation	Convex	Convex
Edge	Entire	Filamentous
Internal Structure	Amorphous	Granular
Branching	Absent	Predominant
Shape and Size of Cells	Spherical or ellipsoidal. 6-8μ diam.	Elongated 8-20μ x 2-4μ
Size of Colony 48 hours	1 mm diameter	4 mm diameter
Growth in Medium E. Inoculated from colony on glucose-peptone-agar.		
48 hours	Slight growth	Visible growth
72 hours	Count 89.5 Cells spherical, little branching	Count 1,0 Cells spherical, little branching.
Growth in Medium K. Inoculated from colony on glucose-peptone-agar.		
48 hours	No growth	Visible growth
72 hours	No growth	Count 23.0
Fermentation of carbohydrates. (Peptone 0.5%, carbohydrate 2.0%)		
Glucose	Gas and alcohol	Gas
Levulose	Gas and alcohol	Gas
Sucrose	Gas and alcohol	Small amount of gas.
Maltose	Gas and alcohol	Gas Esters rather than alcohol are formed
Growth in Wort.		
24 hours	Foam	Heavy froth
48 hours	Count 620 Bottom growth Cells spherical 6-8μ diameter, no branching.	Count 3380 Dry, wrinkled surface growth. Cells elongated, 5μx7μ, branching predominant.
Spore formation. 10 day growth on carrot infusion-$CaSO_4$-agar.	Typical ascospores of S cerevisiae	No spores

Yeast No. 11 is evidently Saccharomyces cerevisiae while No. 12 may perhaps best be classified as a Mycoderma, altho it causes gas and alcohol and ester production in suitable media. But it is almost impossible to classify a yeast which does not form spores and shows as variable morphologic characteristics as does this yeast. In fact there is almost no means for classifying yeasts growing on the surface as a scum and forming no spores. Altho Mycoderma generally implies that the organism utilizes alcohol as a food, oxidizing it to acetic acid or to carbon dioxide and water, this characteristic is by no means shown by all the organisms commonly included in the Genus Mycoderma. Yeast No. 12 does not grow on the surface in all media. In all the synthetic media used it showed only slight and generally transient surface growth and the cells resembled those of Yeast No. 11. But in wort or molasses media there was the typical wrinkled, dry surface scum, which was present from 12 hours to about 2 weeks of incubation, when most of this scum settled as a slimy mass. But growth seemed to continue below the surface and in acid solutions was often found to form clusters that resembled mold growth. Long mycelial threads radiating from a clump were found on several occasions in flasks which had not been disturbed. But these mycelia readily broke up, on shaking the flask, into the elongated irregular cells typical of this yeast growing in such media.

Yeast No 12 did not grow well in Medium E (13) when first taken from the colony, but better growth was found after several transferals in this medium. But it never grew as well as did Saccharomyces cerevisiae. To what extent this acclimatization may be carried is not known. It seemed, however, to become better suited for growth in this medium thru about three transferals, after which there was little improvement. But No. 12 grew in a low nitrogen medium, Medium K, very much better than did No. 11, which did not seem to become very well acclimatized to this medium. Yeast No. 12 was therefore regarded as being the better suited for studies on nitrogen fixation.

Since surface growth seemed a desirable characteristic, several surface growing yeasts, furnished by Dr. F. W. Tanner, were investigated. These all showed the tendency to creep up the side of the flask exhibited by No. 12. Results of this investigation are given in Table II, showing the relative growth of these yeasts in Medium K, which had the composition,

Sucrose	2.00
K_2HPO_4	0.10
NH_4Cl	0.002

Made up to 100 cc with conductivity water.

This amount of ammonium chloride represents the lowest optimal concentration for Yeast No. 12.

TABLE II

Yeast	Growth
No. 12	++++
Willia saturnus	++++
Torula humicola	+++
Willia anomala	+++
Saccharomyces chevalieri	++
Mycoderma vini	++
Pichia membranaefaciens	+
Saccharomyces anomalus	+
Saccharomyces neoformans	+
Saccharomyces hominis	+
No. 11	+

Yeast No. 12 gave as large growth as any, and because of its source was used in preference to the others. Saccharomyces cerevisiae was used in some of the last experiments in order to see whether or not this yeast also was capable of fixing nitrogen.

Materials Used in Media.

In all investigations of salt effects, the salts were purified by recrystallization from water wherever possible. And in these investigations conductivity water was used and the experiments carried out in glassware that had been thoroly cleaned in hot sulphuric acid and rinsed finally with conductivity water.

The third fraction of molasses was a material obtained by fractional precipitation of molasses with alcohol after the method given by Fulmer, Duecker and Nelson (14). It was prepared by Mr. H. A. Hill and shown by him to be rich in the growth stimulant, bios. It contained 0.3667% nitrogen. The molasses used in these experiments was chosen

particularly with regard to the nitrogen content and the growth stimulant it contained. Richelieu brand of refined cane molasses was found to give very good growth and was low in protein content. Two samples varied in their nitrogen content, as is indicated in the experiments in which they were used.

In some of the earlier work in which it was desired to obtain a medium absolutely free from nitrogen, the sugar used was recrystallized from 90% ethyl alcohol. But we were never able to demonstrate any nitrogen compound, nor to find any evidence of bios in the sugar before recrystallization.

In the experiments with molasses, where it was not necessary to use such precautions for purity, ordinary distilled water was used, but it was almost entirely free from ammonia, as shown by testing with Nessler's reagent. The acid and alkali used for pH regulation were made from nitrogen free materials. The hydrochloric acid was made up with constant boiling hydrochloric acid and the sodium hydroxide made up from a saturated solution which had been allowed to settle and was known to be free from nitrogen compounds.

Adjustment of Hydrogen Ion Concentration of Culture Media.

In the study of the effect of the hydrogen ion concentration it was found necessary to make the adjustment of the medium after sterilization. Otherwise there would

be physical and chemical changes in the medium as a result of the variation of the hydrogen ion concentration which would have an effect upon the growth of the yeast. In addition the concentration of the hydrogen ion would be changed as a result of these reactions. The method and apparatus finally developed are described by Christensen and Fulmer (4).

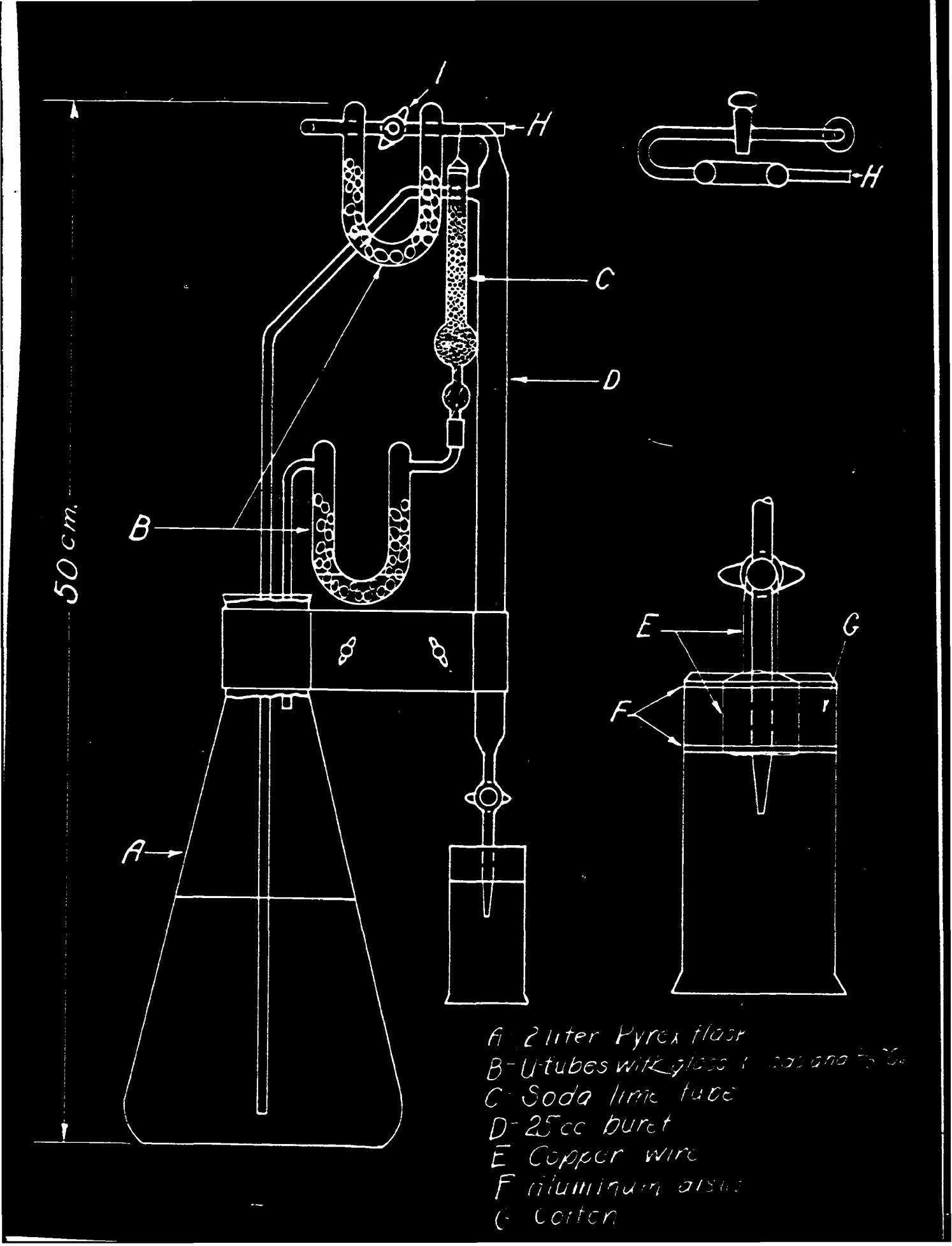
A sketch of the apparatus is shown and the various parts indicated in the legend. Three such apparatus should be constructed. One is used for alkali, one for water and one for acid. One liter of the acid, alkali or water is placed in the flask, the cotton plug inserted in the neck and the whole apparatus sterilized in the autoclave. The shield should be in place and enough acid in the U-tubes to just fill the bottom of the tube, but the soda lime tube is not sterilized. During sterilization all stopcocks should be closed and caps should be placed over the openings of the U-tubes in order to prevent too great increase in the volume of acid in the U-tubes due to condensation of water.

After sterilization the caps are removed from the U-tube openings, the soda lime tube is put in place and the cotton plug pushed down into the neck of the flask so that it may be closed off with sealing wax. The buret is filled by applying suction at H and the stopcock I is used to prevent too rapid filling of the buret and con-

sequent too rapid passage of air thru the U-tubes, which might result in splashing the acid out into the side arms.

To determine how much acid or alkali must be added in order to produce a given pH, a set of flasks, usually 10 or 12, is made up and sterilized just as those to be used for the experiment. The medium is made up of such a concentration as to allow for addition of sufficient acid or alkali to produce the extremes of the series desired. Ordinarily this series of flasks is made up and sterilized along with the ones to be used for the growth of the organism. Of course the medium must be carefully measured into each flask and care must be exercised in sterilization not to allow very great evaporation, so that there will be no large error due to different rates of evaporation in the different flasks. To part of the flasks thus prepared add varying amounts of acid and sufficient water to bring the volume to that which will give the desired concentration of materials, and to the rest add varying amounts of alkali and the difference in water. Now measure the pH of the contents of each flask and plot against the amount of acid and alkali added. From this curve it is possible to read directly the amount of acid or alkali and water necessary to produce any desired hydrogen ion concentration at the desired concentration of materials.

In adding this acid, alkali and water, the neck of the flask should be flamed as usual and the inside of the shield


I
H
C
D
B
A
50 cm.
H
E
G
F
A 2 liter Pyrex flask
B- U-tubes with glass
C- Soda lime tube
D- 25cc buret
E Copper wire
F Aluminum strip
G Cotton

flamed. A micro burner is very convenient. The flask is then inserted under the shield, the acid, alkali or water added, the flask withdrawn and the plug inserted. This operation can be carried out very rapidly and there is little chance for contamination if ordinary care be used.

All hydrogen ion measurements were made by the potentiometric method, using a Leeds and Northrup Students Potentiometer, Bailey electrodes (1) and a saturated KCl calomel electrode. Corrections for temperature and pressure are made in the formula

$$pH = \frac{E_{cell} - E_{calomel} + E_{bar}}{R\,T\,/\,N\,F} = \frac{E_{cell} - E_{calomel} + E_{bar}}{0.000198\,T}$$

No correction was made for liquid potentials, which for saturated KCl are small (10,34). The emf of the saturated type calomel half cell is given by Clark (5) and is due to Michaelis.

20^{o}	0.2488	volts	Interpolation over this range gives
25^{o}	0.2458	volts	0.0006 volts per degree, decreasing
30^{o}	0.2438	volts	as the temperature increases.

The E_{bar} of the above formula is the correction in the total emf of the cell to a pressure of one atmosphere of hydrogen. Clark (5) gives the value of E_{bar} for various barometric pressures and temperatures. Or the value may be readily calculated by the following formula,

$$E_{bar} = \frac{0.000198\,T}{2} \log \frac{1}{x}$$

where x is the barometric pressure minus the vapor tension of the solution being measured.

Equipment for Aeration.

A number of experiments were carried out with aeration of the medium. Since volatile nitrogen compounds are formed during the growth of the yeast, it was necessary to scrub the gases coming from the flasks. Dilute HCl was found satisfactory in holding these compounds. The aeration device was made to allow for this and to make it possible to sterilize the entire apparatus and to allow for easy handling during inoculation. Contamination was rarely found to occur during reasonable lenghts of time of aeration, but apparently the cotton filter used was not altogether satisfactory, for contamination was apt to develop when aeration was continued for a longer period. Aeration was not used when incubation was for more than one week.

In this work the air used was freed from ammonia and oxides of nitrogen by passing it thru 50% sulphuric acid and 10% KOH-permanganate solution and finally thru water at the temperature of the incubator. The air was distributed to the flasks by a header system provided with capillary valves which insured even distribution of air to the flasks regardless of how many were being aerated. By regulating the pressure on the header the same aeration could be secured at any time.

Regulation of Temperature and Purification of Air in Incubator.

In some of the earlier work on salt effects it became

apparent that careful temperature regulation in the incubator was necessary. By using a fan for circulation of the air within the incubator and an improved thermo-regulator, the temperature was constant to within ±C.2°C and the same in all parts of the incubator. The temperature variation in the flasks was approximately 1/10 that of the air.

The air in the incubator was kept practically free from ammonia or oxides of nitrogen by passing it over large surfaces of 10% KOH and 50% sulphuric acid. It was kept saturated with water vapor in order to prevent evaporation from the flasks.

Kjeldahl Method Used and Calculation of Results.

The contents of one flask, usually 50 cc or less, were rinsed with a minimum amount of water into a 500 cc Kjeldahl flask. 25 cc of concentrated sulphuric acid(free from ammonia) 10 gms of K_2SO_4 and 0.10 gm of copper wire were added and the flask heated gently over a free flame in the digestion rack. Generally one hour was necessary before foaming had entirely ceased, when the flame was increased and the digestion continued until the contents of the flask were a clear green. The heating was continued at such a rate that the acid boiled gently for three hours longer. When the flask had cooled, 150 cc of ammonia free water were added and the flask again allowed to cool. Then 80 cc of saturated NaOH solution were added and 100 cc

were distilled into a 360 cc E flask containing 25 cc of 0.2 n HCl, also ammonia free. Ammonia was determined by back titration with 0.1 n NaOH or by Nesslerization. Nesslerization was used during most of the experiments on nitrogen fixation, using a Dubosc colorimeter and a standard containing a known amount of ammonium chloride.

In all the experiments, differences in nitrogen found in inoculated flasks and in identical flasks not inoculated were recorded as nitrogen fixed. Positive fixation means that the inoculated flasks were higher in nitrogen, indicating fixation. Negative fixation indicates that the inoculated flasks were lower in nitrogen than the uninoculated, meaning that they must have lost nitrogen as a result of the growth of the organism. The only change in this procedure in the modification was the method of digestion, which is described later.

RESULTS

Development of the Media and Preliminary Quantitative Experiments.

It was reported by Fulmer (12) that yeast would grow in a good state of nutrition in a medium consisting of

Cane sugar	10.00 gms
K_2HPO_4	0.45 gms
Water	to 100 cc

This amount of dipotassium phosphate was found optimal for Saccharomyces cerevisiae, Race F. The amount of dipotassium phosphate was not found to be very important with Yeast 12, and several concentrations were used at various times. In studying the effect of salts the following basic medium was used.

Cane sugar	2.00 gms
K_2HPO_4	.02 gms
Water	to 100 cc

Varying amounts of several salts were added to this medium, and results of these investigations are given in the following tables. The effect of the third fraction of molasses is given, also. Incubation was at 30°C and for 4 days. The initial count* was approximately 0.01.

*When the count is 1 there are 250,000 cells per cc.

TABLE IV

Sodium Chloride

NaCl Gms/100 cc	Count
0.00	6.5
0.20	5.5
0.40	10.5
0.60	5.3
0.80	3.2
1.00	2.7
1.20	2.4
1.40	5.7
1.60	4.2
1.80	4.4
2.00	3.8

TABLE V

Potassium Chloride

KCl Gms/100 cc	Count
0.00	6.50
0.20	6.8
0.40	5.9
0.60	5.1
0.80	4.8
1.00	5.3
1.20	8.0
1.40	5.5
1.60	4.7
1.80	5.5
2.00	3.5
2.20	11.3
2.40	23.0
2.60	3.3
2.80	3.6

TABLE VI

Lithium Chloride

LiCl Gms/100 cc	Count
0.000	3.7
0.005	3.0
0.010	2.4
0.025	3.2
0.050	1.9
0.075	2.5
0.100	2.2
0.120	2.4
0.140	2.0
0.160	1.6
0.180	1.5
0.200	1.1
0.250	3.6
0.350	2.3
0.500	1.4

TABLE VII

Ferric Chloride

Adjusted to pH 7.00

$FeCl_3$ Gms/100 cc	Count
0.0000	5.1
0.0005	5.6
0.0010	3.7
0.0015	4.3
0.0020	3.4
0.0025	3.8
0.0030	3.4
0.0035	3.6
0.0040	5.8
0.0045	7.4
0.0050	9.0
0.0060	5.2
0.0070	4.6
0.0080	5.9
0.0100	4.4

TABLE VIII

Manganese Sulphate

Adjusted to p_H 7.00

$MnSO_4$ Gms/100 cc	Count
0.0000	2.6
0.0005	0.3
0.0010	3.4
0.0015	4.8
0.0020	0.7
0.0025	1.0
0.0030	0.0
0.0035	0.4
0.0040	0.9
0.0045	2.4
0.0050	1.5
0.0060	2.0
0.0070	1.5
0.0080	2.0
0.0090	0.9
0.0100	0.3

TABLE IX

Magnesium Sulphate

Adjusted to p_H 7.00

$MgSO_4$ Gms/100 cc	Count
0.000	3.3
0.001	2.5
0.002	2.6
0.003	2.7
0.004	2.8
0.005	2.3
0.006	2.1
0.007	2.6
0.008	4.0
0.009	2.2
0.010	2.3
0.015	3.0
0.020	4.2
0.025	3.0
0.030	4.0
0.040	2.2
0.050	3.6

TABLE X

Calcium Chloride

Adjusted to p_H 7.00

$CaCl_2$ Gms/100 cc	Count
0.000	3.9
0.001	5.4
0.002	5.3
0.003	4.2
0.004	4.4
0.005	5.8
0.006	4.5
0.008	4.9
0.010	4.8
0.020	5.5
0.030	6.4
0.040	7.4
0.050	6.6
0.060	7.1
0.070	7.8
0.080	8.3
0.090	6.9
0.100	6.4

TABLE XI

Third Fraction of Molasses

Gms/100 cc	Count
0.00	7.7
0.12	7.7
0.16	7.1
0.20	8.4
0.24	8.4
0.28	8.9
0.32	6.8
0.36	7.1
0.40	7.7
0.44	7.3

TABLE XII

Potassium Chloride

With optimum 3rd Fraction of Molasses (0.28 gms/100 cc)

KCl Gms/100 cc	Count
0.20	16.5
0.40	13.0
0.60	13.1
0.80	16.2
1.00	14.8
1.20	14.3
1.40	13.5
1.60	19.9
1.80	22.2
2.00	Heavy ppt.
2.20	Count was
2.40	estimated
2.60	at 30-40
2.80	22.0
3.00	21.6

TABLE XIII

Ammonium Chloride

NH_4Cl Mgms/100 cc	Count
0.001	2.4
0.002	2.5
0.003	2.2
0.004	2.0
0.005	1.7
0.006	2.1
0.007	1.5
0.008	1.3
0.010	1.5
0.020	7.2
0.030	3.0
0.040	1.6
0.050	4.7
0.060	6.6
0.070	2.8
0.080	5.6
0.100	3.3
0.200	4.9
0.300	5.2
0.400	5.6
0.500	8.4
0.600	10.3
0.700	10.9
0.800	12.1
1.000	15.5
2.000	26.0
3.000	19.7
4.000	18.1
5.000	10.5
6.000	7.5

Potassium chloride has the most pronounced effect, with a very sharp optimum at 2.40 gms/100 cc. The third fraction of molasses is also effective in stimulating growth and the same optimum of potassium chloride is found when the optimum concentration of the third fraction of molasses is present. Evidently the effects are additive. This was the only attempt to combine the effects of two materials, altho there are several possibilities indicated by the data in the tables.

From these data the following synthetic medium was chosen as being suitable for studies of nitrogen fixation by yeast:

Cane sugar	2.00 gms
K_2HPO_4	0.10 gms
KCl	2.40 gms
Water	to 100 cc

And the following medium containing the third fraction of molasses:

Cane sugar	2.00 gms
K_2HPO_4	0.10 gms
KCl	2.40 gms
Molasses 3rd	0.28 gms
Water	to 100 cc

Using the synthetic medium developed, and incubating four days at 30°C, the fixation of 0.16 mgms/100 cc was determined. In a second experiment with the same medium but with the hydrogen ion concentration adjusted to pH 7.00 and incubation for four days at 30°C, with aeration, there was a fixation of 0.20 mgms/100 cc. In another experiment

with this same medium it was found that whenever the count was 20 or above, a fixation of 2.0 mgms or more per 100 cc could be demonstrated when incubation was for about five weeks. But this medium was never very reliable, sometimes not giving counts higher than 6.0 - 8.0. It was observed that the optimum of potassium chloride was very sharp and only slight changes would be necessary to change favorable conditions to unfavorable ones.

Using Lipman's medium (36),

Glucose	2.00 gms
K_2HPO_4	0.02 gms
$CaCl_2$	0.02 gms
$MgSO_4$	0.02 gms
1% $FeCl_3$	3 drops
Tap water	to 100 cc

and incubating for seven days with aeration, there was a loss of 0.17 mgms of nitrogen per 100 cc. The tap water and glucose contained nitrogen. Incubating two weeks without aeration, a gain of 0.07 mgms per 100 cc was noted.

With Gainey's medium (16),

Dextrose	2.00 gms
K_2HPO_4	0.02 gms
$MgSO_4$	0.02 gms
NaCl	0.05 gms
1% $FeCl_3$	2 drops
Water	to 100 cc

and regulating the hydrogen ion concentration to pH 7.00 with NaOH after sterilization, and adding 0.5 gms of $CaCO_3$ per 100 cc, there was a fixation of 2.5 mgms per 100 cc in five weeks at 30°C, without aeration. After six weeks incubation without aeration at 30°C, a fixation of 4.6 mgms

per 100 cc was found.

Fixation was found using the medium containing the optimum of the third fraction of molasses and optimum of potassium chloride. In this medium, with four days incubation at 30°C, with aeration, a gain of 0.83 mgms per 100 cc was noted. In another experiment which was not aerated, a loss of 0.24 mgms per 100 cc was found.

Nitrogen fixation has been demonstrated in several synthetic media and in the medium containing the optimal concentration of the third fraction of molasses. It appears that the hydrogen ion concentration is important, for no fixation was found with Lipman's medium, which was acid, while fixation was demonstrated with Gainey's medium, which was alkaline. This would seem to account for the failure to observe fixation of nitrogen in several cases and also for the fact that many times there was no apparent relation between count and nitrogen found. It would appear also, that the period of incubation should be at least one month.

Since it was found that the third fraction of molasses greatly stimulated yeast growth, it was thot that this same stimulation might be obtained with molasses. Using a refined molasses containing 0.21% nitrogen, a study was made to determine the optimal concentration as measured by the amount of yeast growth per unit of molasses added. It was desirable to obtain the maximum growth

per unit of nitrogen. And since this medium was to be used for a study of the effect of the hydrogen ion concentration, it was also desirable to have a considerable quantity of buffer present. Using the following medium the results given in TABLE XIV were obtained.

K_2HPO_4	0.50 gms
Molasses	varied
Water	to 100 cc

Incubation was for 48 hours at 30°C.

TABLE XIV

Molasses Gms/100cc	Count	Count / Gms Molasses
0.10	2.5	25
0.20	9.8	47
0.25	14.7	59
0.30	13.2	44
0.35	12.8	37
0.40	30.5	76
0.45	34.4	74
0.50	40.7	81
0.60	44.1	74
0.80	62.4	78
1.00	100	100
2.00	146	73
3.00	239	79
4.00	252	63
5.00	381	76
6.00	484	81
7.00	522	75
8.00	548	68
9.00	600	66
10.00	665	66

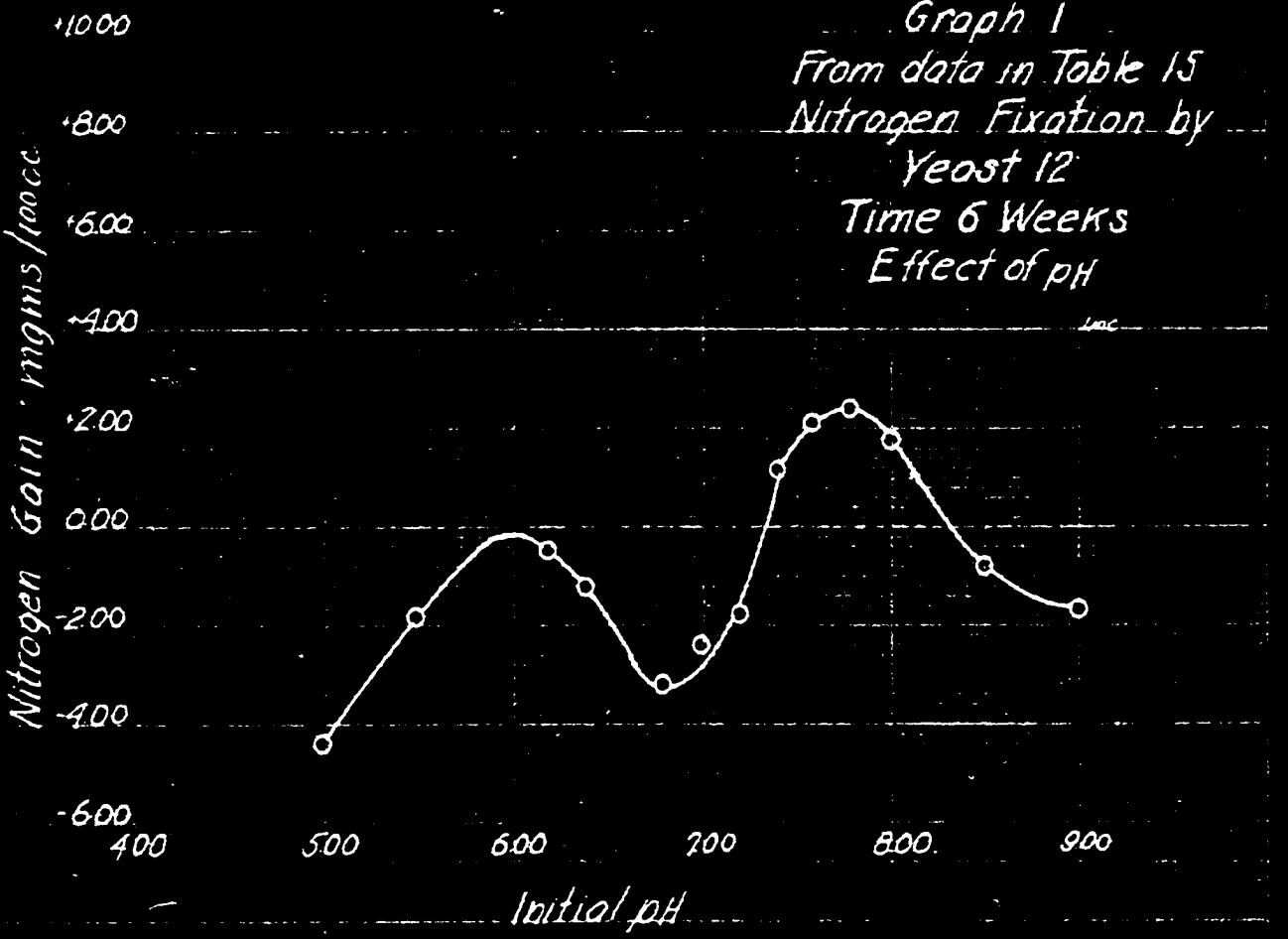
Above 0.40 gms per 100 cc the count was directly proportional to the amount of molasses, so arbitrarily, 6.00 gms per 100 cc was used. 50 cc of this medium, adjusted to the desired hydrogen ion concentration as previously

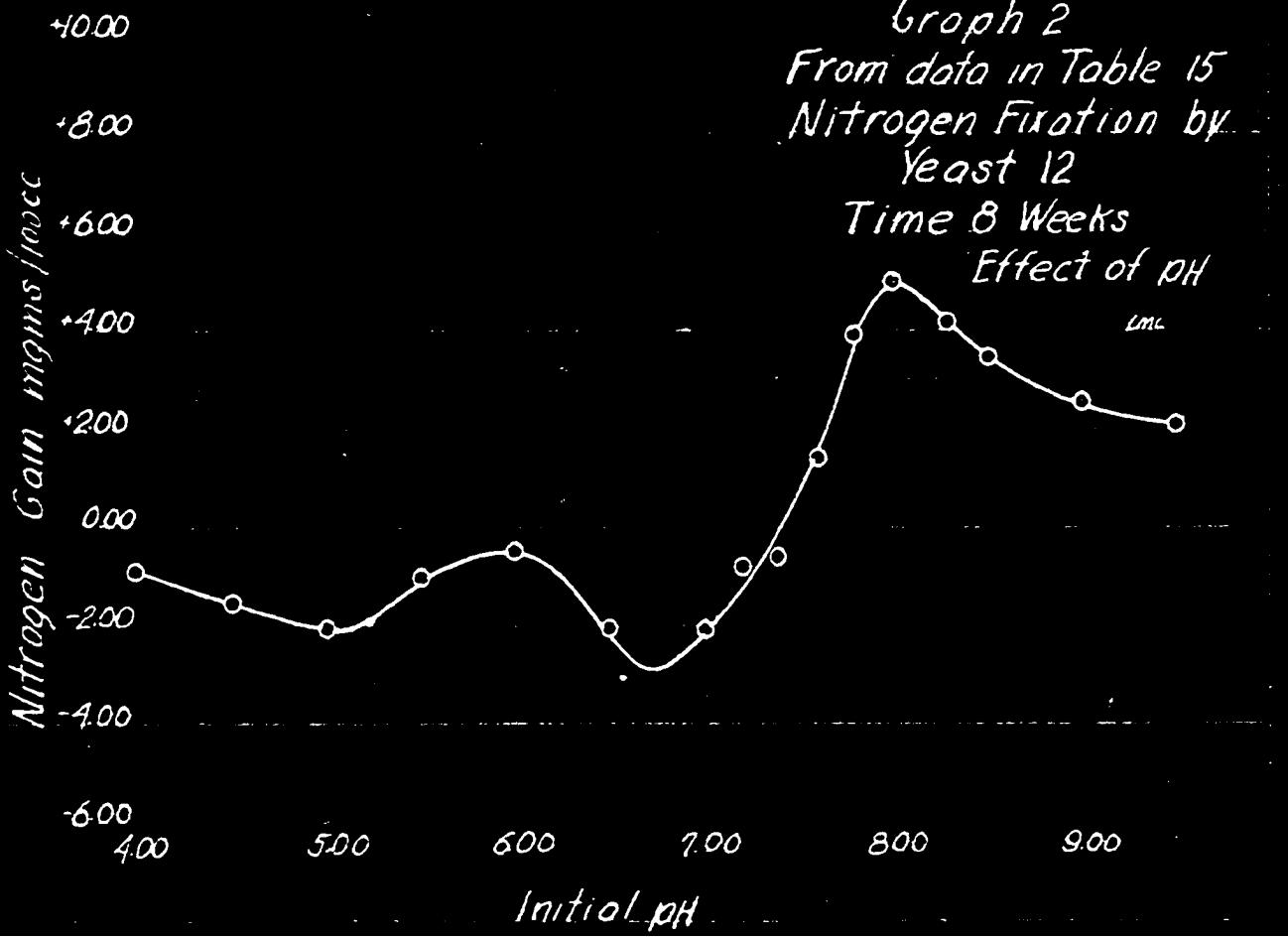
described, were used in a 150 cc Erlenmeyer flask. These were inoculated with 0.01 cc of a 3 day culture of Yeast 12 in the same medium. After inoculation the cotton plugs were trimmed level with the necks of the flasks and sealed with paraffin, leaving a 1 mm vent for pressure equalization. These were incubated at 30°C ± 0.02° for varying lengths of time and the nitrogen determined by the Kjeldahl method. The results are given in TABLE XV and shown graphically on pages 30 and 31.

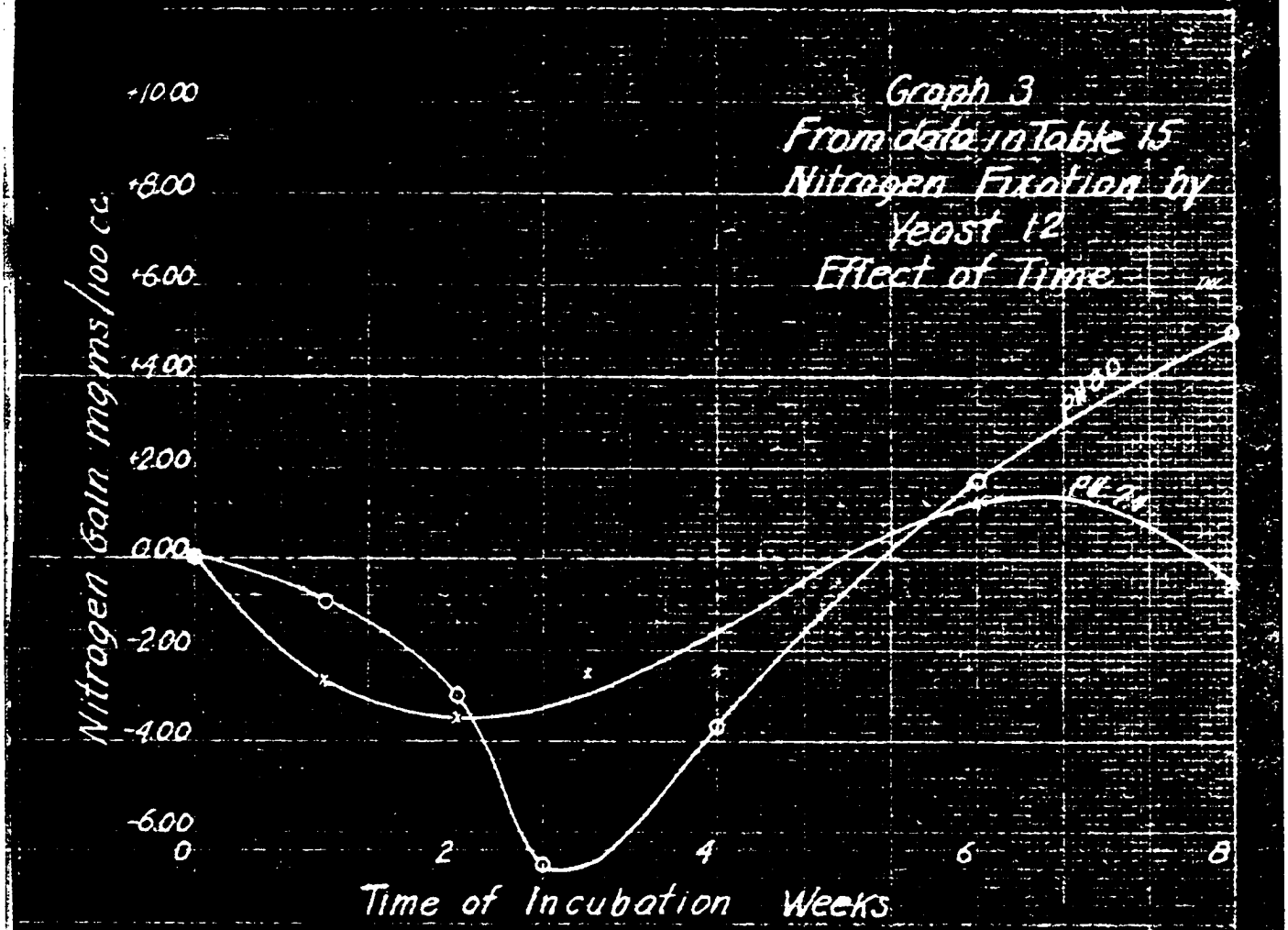
TABLE XV

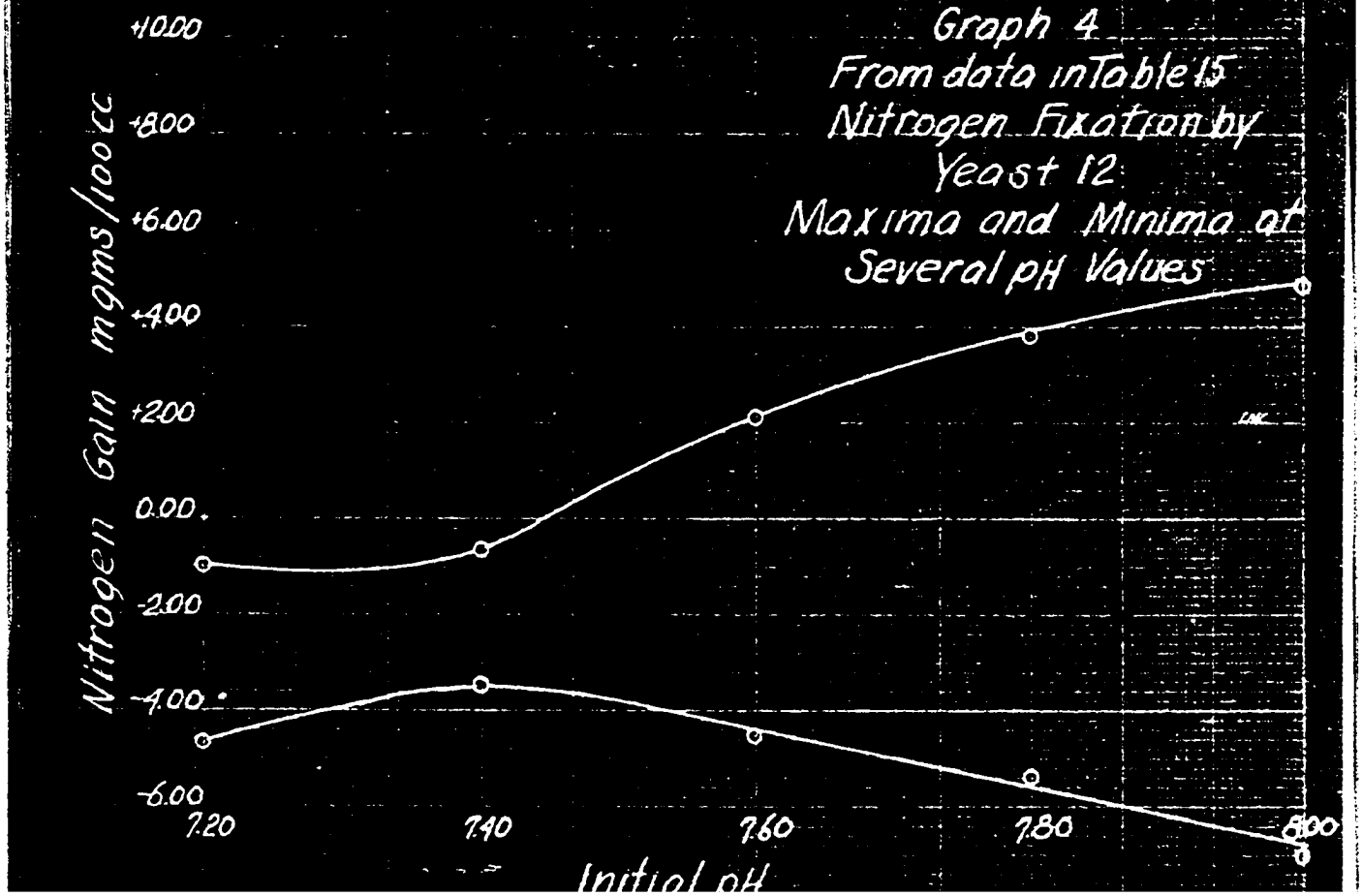
pH	Gain in nitrogen in mgms/100 cc of culture					
	Time in weeks					
	1	2	3	4	6	8
4.0						-0.92
4.5						-1.52
5.0					-4.46	-2.10
5.5					-1.91	-1.19
6.0						-0.51
6.2					-0.50	
6.4					-1.25	
6.5						-2.10
6.8					-3.22	
7.0					-2.48	-2.10
7.2	-3.60	-3.40	-4.72	-4.19	-1.85	-0.92
7.4	-2.60	-3.50	-2.56	-2.46	+1.19	-0.65
7.6	-3.20	-4.50	-3.63	-3.36	(+2.01)	(+1.35)
7.8	-1.00	-1.20	-5.35	-3.63	+2.31	+3.82
8.0	-0.90	-3.20	-7.00	-3.71	(+1.64)	+4.90
8.5					-0.84	+3.32
9.0					-1.80	+2.41
9.5						+1.99

Fixation was greatly affected by the initial hydrogen ion concentration. The final pH value was not determined in this series, but in a separate experiment it was found


Graph 1
From data in Table 15
Nitrogen Fixation by
Yeast 12
Time 6 Weeks
Effect of pH
Nitrogen Gain · mgms/100cc.
+10.00
+8.00
+6.00
+4.00
+2.00
0.00
-2.00
-4.00
-6.00
4.00
5.00
6.00
7.00
8.00
9.00
Initial pH


Graph 2
From data in Table 15
Nitrogen Fixation by
Yeast 12
Time 8 Weeks
Effect of pH
Nitrogen Gain mgms/100cc
+10.00
+8.00
+6.00
+4.00
+2.00
0.00
-2.00
-4.00
-6.00
4.00
5.00
6.00
7.00
8.00
9.00
Initial pH


Graph 3
From data in Table 15
Nitrogen Fixation by
Yeast 12
Effect of Time
Nitrogen Gain mgms/100 cc.
+10.00
+8.00
+6.00
+4.00
+2.00
0.00
-2.00
-4.00
-6.00
0
2
4
6
8
Time of Incubation Weeks


Graph 4
From data in Table 15
Nitrogen Fixation by
Yeast 12
Maxima and Minima at
Several pH Values
Nitrogen Gain mgms/100 cc
+10.00
+8.00
+6.00
+4.00
+2.00
0.00
-2.00
-4.00
-6.00
7.20
7.40
7.60
7.80
8.00
Initial pH

that there was only a small change during the first two weeks, and then only at the extremes. Due to the great difficulties in the method, no attempt was made to maintain a constant hydrogen ion concentration. It was thot advisable to allow it to change as it would, and in later experiments to measure the magnitude of this change.

Two optimal values were found, one at pH 6.00 and the other at pH 7.9, the latter being the more effective. This optimum at pH 6.00 corresponds with the value found by Dernby (8) to be optimal for the autolysis of yeast. It was observed that at the hydrogen ion concentration at which nitrogen fixation was found, the yeast had undergone disintegration and the remaining cells were Gram negative, whereas at other hydrogen ion concentrations the yeast was Gram positive. de Bord (6) has found that thermophilic bacteria also change in a similar way in time, but in this case the change took place when the culture was a few hours old. Such a change undoubtedly results from some chemical change in the cell material, and it is believed that it marks some step in the development of resting cells or in autolysis. Perhaps it may be said to indicate some stage of death, or at least an advanced phase in the life cycle.

It was thot that there might, perhaps, be an inadequate supply of carbohydrate present to observe maximum fixation, so a duplicate of the above experiment was

carried out using the following medium.

Molasses	6.00 gms
Sucrose	10.00 gms
K_2HPO_4	0.50 gms
Water	to 100 cc

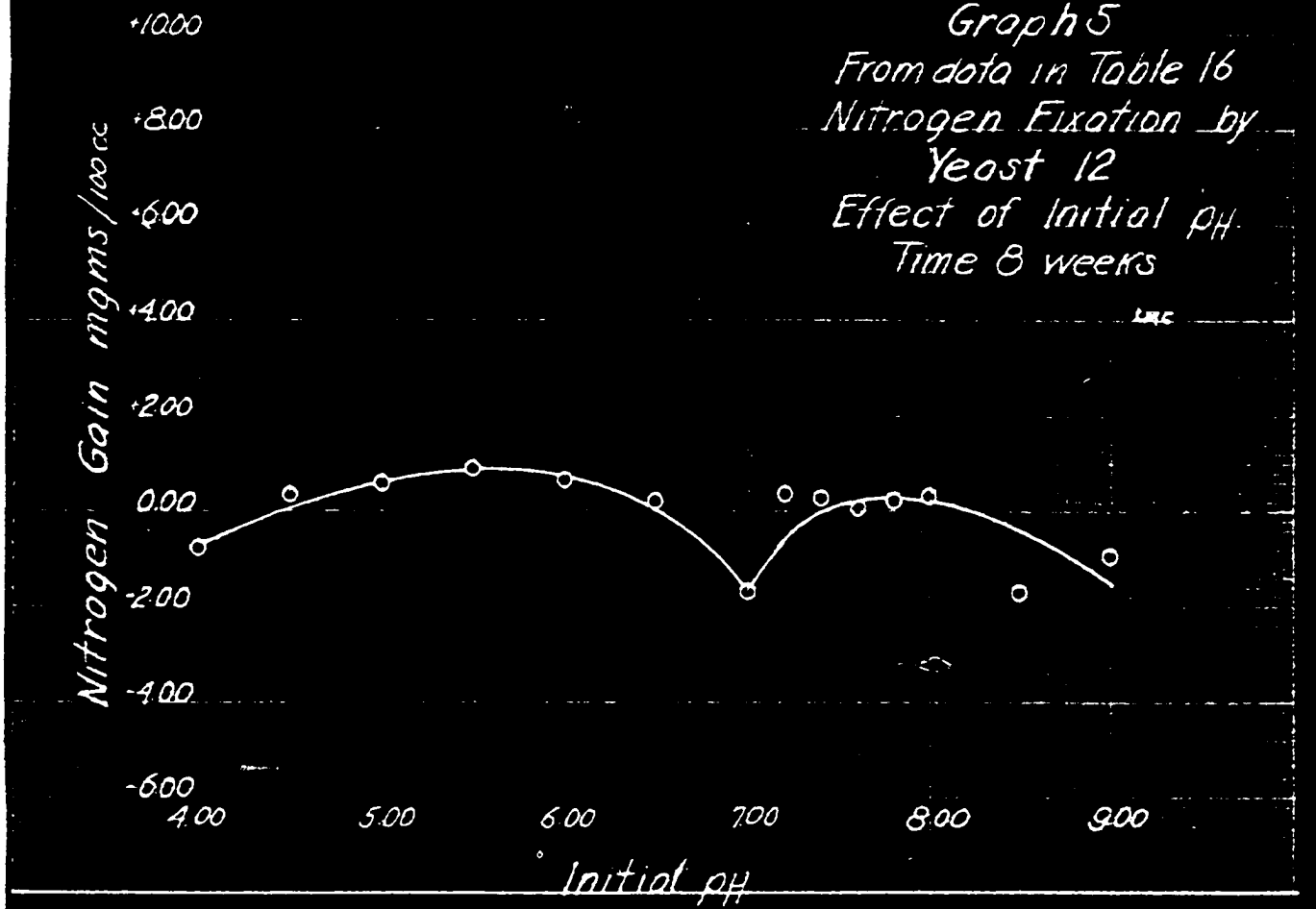
The results are given in TABLE XVI and shown graphically on page 34.

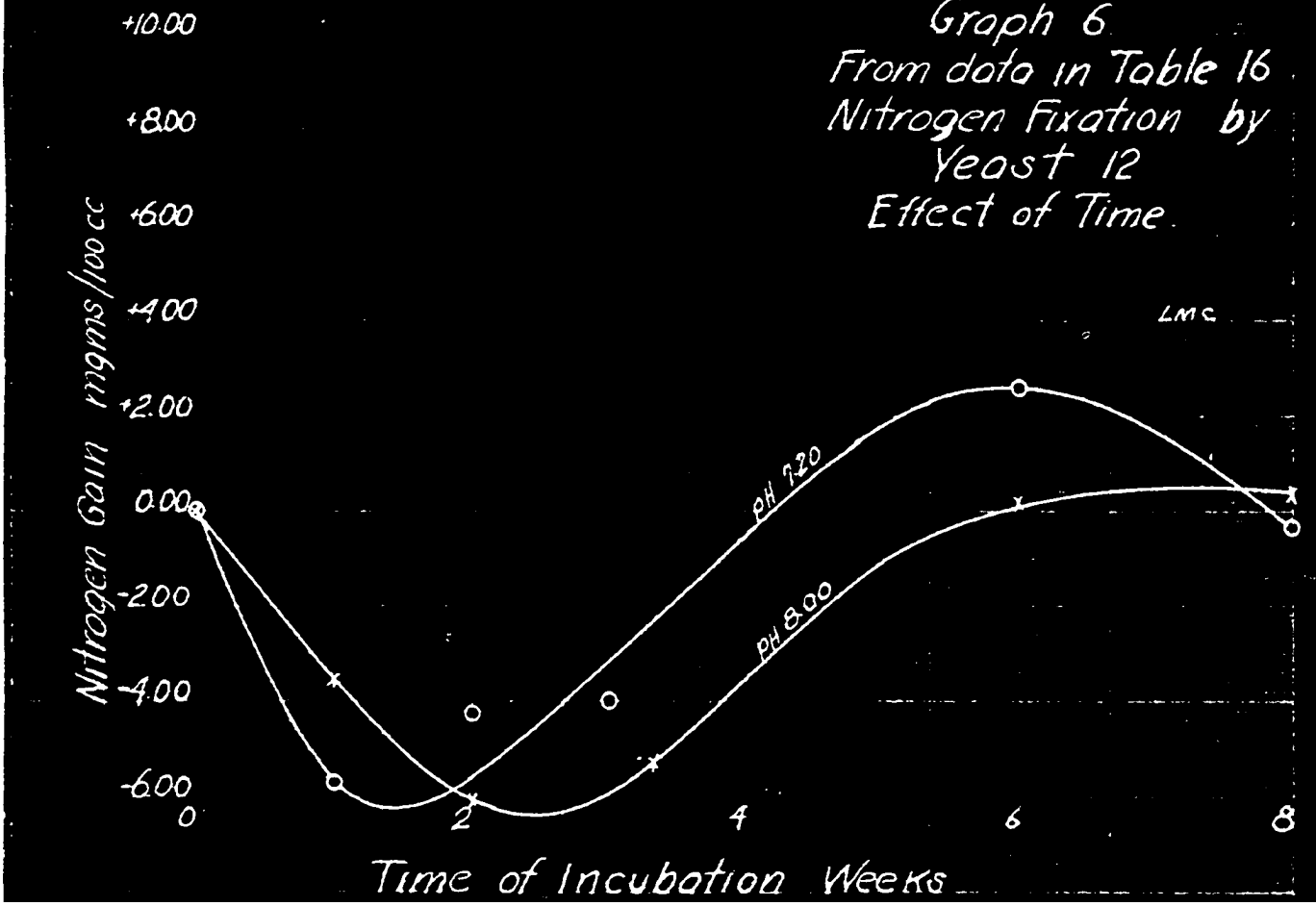
TABLE XVI

pH	Gain in nitrogen in mgms/100 cc of culture				
	Time in weeks				
	1	2	3	6	8
4.0					-0.79
4.5					+0.38
5.0					+0.57
5.5					+0.88
6.0					+0.52
6.5					+0.20
7.0					-1.68
7.2	-5.75	-4.21	-4.01	+2.60	+0.38
7.4	-5.12	-2.93	-2.68	+1.20	+0.23
7.6	-4.69	-3.80	-4.63		+0.02
7.8	-4.37	-6.88	-4.47	+0.20	+0.20
8.0	-3.49	-6.10	-4.10	+0.20	+0.38
8.5					+0.20
9.0					-1.68
9.5					-0.91

It would seem that the large amount of sugar acted as a preservative. There was no indication of cell disintegration and the cells were Gram positive in all flasks. The same two optima were in evidence, but there was no large fixation of nitrogen.

The first interpretation of these results was that the Kjeldahl method failed to obtain all of the nitrogen


Graph 5
From data in Table 16
Nitrogen Fixation by
Yeast 12
Effect of Initial pH
Time 8 weeks
LMC
Nitrogen Gain mgms/100 cc
+10.00
+8.00
+6.00
+4.00
+2.00
0.00
-2.00
-4.00
-6.00
4.00
5.00
6.00
7.00
8.00
9.00
Initial pH


Graph 6
From data in Table 16
Nitrogen Fixation by
Yeast 12
Effect of Time.
LMC
Nitrogen Gain mgms/100 cc
+10.00
+8.00
+6.00
+4.00
+2.00
0.00
-2.00
-4.00
-6.00
pH 7.20
pH 8.00
0
2
4
6
8
Time of Incubation Weeks

of the yeast until some change in the yeast protein had occurred, a change which was indicated by a change in the Gram stain. And the hypothesis was advanced (15) that this change was mainly concerned with the heterocyclic ring compounds in the yeast protein. It therefore seemed necessary to find some other analytical method which would obtain this nitrogen if the change in nitrogen content of the medium was to be followed.

Development of the Analytical Method.

The Kjeldahl method for nitrogen in one of its many modified forms has been exclusively used in studies on nitrogen fixation by biologic forms. So much has been written of the modifications of the Kjeldahl method that no attempt will be made to even mention them. Discussions of these modifications will be found in such books as Allen's "Commercial Organic Analyses", Lassar-Cohn "Arbeits Methoden fur Organisch-chemische Laboratorien", Wiley's "Principles and Practise of Agricultural Analyses" and in most analytical texts. They are described in detail by Hepburn (26). The more recent modification, the use of hydrogen peroxide, seems to be due to Kleeman (29).

There are three general methods for the determination of nitrogen in organic compounds:

1. Dry combustion and oxidation to elementary nitrogen (Dumas),
2. Wet combustion and reduction of all nitrogen to ammonia (Kjeldahl),
3. Liberation of the nitrogen as NH_3 by heating

with an alkali.

The Dumas method is probably the most universally applicable. It is apt to yield high results because of the formation of methane and because all errors are in the same direction, that is, toward high results. But the results obtained by this method will be considered as standard, and the yield of nitrogen from yeast by other methods will be compared with that obtained by the Dumas method.

As a preliminary experiment to determine whether or not the Kjeldahl method gave the same amount of nitrogen as did the Dumas, a sample of dried yeast was analyzed by both methods. A large supply of this yeast was obtained and stored in a tightly stoppered bottle and the same yeast was available for all the investigations on the analytical method. At the same time a sample of fresh yeast was analyzed by both methods. Fresh yeast was supplied each week by the Fleischmann Company so that it was available at all times for analytical experiments.

Dried yeast		
	Dumas	9.00%
	Kjeldahl	7.96%
Fresh yeast		
	Dumas	2.24%
	Kjeldahl	2.02%

The Kjeldahl method yielded 88.5% of the nitrogen of the dried yeast and 91.0% of the nitrogen of the wet yeast, taking the Dumas results as standard. These results are

the average of four of more determinations checking closely.

From these results it is apparent that some other method of analysis is essential in order to follow the change in nitrogen content of a medium in which yeast is growing. The Dumas is not applicable because of the time required for a determination and because of the difficulty in handling as large amounts of material with so little nitrogen. While it is reasonably accurate for large amounts of nitrogen, it certainly is not when there is a very low percentage of nitrogen present. And it is next to impossible to handle solutions of the volume used in this work. The alkali distillation methods seem never to have been very generally applicable and only one was tried. Some modification of the Kjeldahl method would seem most desirable. The following experiments were carried out in an attempt to devise a more suitable Kjeldahl method.

1. Catalyst.

A large number of modifications are based on the use of some ;articular catalyst. The Gunning modification made use of potassium sulfate, which acts by raising the temperature of the digestion and therefore is not strictly a catalyst, and this more than any other factor affects the rate of digestion. Without K_2SO_4 (H_2SO_4alone) it required 6 hours to digest 0.5 gms of yeast, while with potassium sulfate present the digestion was shortened to 2 hours and 30 minutes. The maximum amount of potassium sulfate that can be used without solidification of the digestion mass on cooling is 10 gms per 25 cc of sulfuric acid.

Many metals or their salts have been used. Copper and mercury are the most common ones, and mercury has been regarded as particularly effective. Both shortened the time of digestion approximately the same amount. Thus in the above experiment the time of digestion was 40 minutes with either one present or with both, and the results were the same. But when potassium sulphate was not present lower results were obtained. When it was present the results checked those obtained when Cu or Hg or both were also added. Other metals have been used as catalysts. Vanadium, chromium, manganese were all used and found to be as effective as Cu or Hg, but no more so. Copper was therefore used in most of the work because the copper ammonium complex is broken up on heating and no sulphide need be added, and the Cu is very readily measured by using a definite length of standard wire. The use of mercury is to be discouraged because sulphides must be added and the results are sometimes not reliable because the mercury has not been completely precipitated free from ammonia, and block tin condensers last much longer with copper as the catalyst than with mercury.

2. Length of Time of Digestion.

Using 25 cc of H_2SO_4, 10 gms of K_2SO_4 and 0.1 gm of Cu wire and 0.5 gm of yeast, it was found that there was a quite definite optimal length of time of digestion after the contents of the flask had become clear. There was a

very definite optimum at three hours.

3. Use of Modification for Nitrates.

There are a number of modifications to include the nitrogen of nitroso, nitro, azoxy, nitrate and nitrite nitrogen. The salicylic acid method gave no higher result than did the ordinary Kjeldahl method.

4. Addition of Oxidizing Agents to the Sulphuric Acid, During or at the End of Digestion.

A large number of modifications are based on the use of oxidizing agents during or at the end of digestion. $KMnO_4$ was commonly used very early. Directions generally are to add one or two crystals at the end of the digestion, and to avoid an excess, as indicated by a blue or purple color. Persulfates and hydrogen peroxide have been used by a number of investigators. They are generally added early in the digestion.

It has been found that permanganate does not give higher yields of nitrogen from yeast when added in varying amounts either during or at the end of the digestion; but if added in excessive amounts (sufficient to cause purple color) there is a decided lowering of the nitrogen obtained, especially if the solution is heated after the addition of $KMnO_4$.

H_2O_2 in the form of a 30% solution known as perhydrol or superoxol has been added during digestion, in quantities of 5 to 15 cc. Altho the digestion was materially hastened

by the addition of such amounts, there was no increase in the yield of nitrogen, altho like $KMnO_4$ it was apt to cause lowered yields when added in too large quantities. When it is used a blank must always be run to take care of the nitrogen in the superoxol, which may amount to 15 mgs per 100 cc. Several other oxidizing agents were used. V_2O_5, CrO_3, MnO_2 did not appear to have any beneficial effect but were apt to be detrimental when added in large amounts.

5. Preliminary Hydrolysis and Oxidation.

The hypothesis was advanced that certain ring nitrogen compounds in the yeast did not yield all of their nitrogen in the usual Kjeldahl method. If this is true it should be possible to render these compounds more amenable to analysis by an acid hydrolysis and oxidation preliminary to the digestion with sulfuric acid.

Refluxing 0.5 gms of yeast with 30 cc of water to which was added 1 cc of concentrated sulfuric acid and then digesting in the usual way gave slightly higher results than were obtained by the usual Kjeldahl procedure. Thus yeast which yielded 7.80% nitrogen by the Kjeldahl method gave 7.90% by this method. But when 30% H_2O_2 was added in this acid hydrolysis there was a very marked improvement. The preliminary experiments were carried out with 0.5 gms of yeast which was placed in a 500 cc Kjeldahl flask and treated with 30 cc of 20% H_2O_2 in water, 1 cc of concentrated sulfuric acid, 10 gms of K_2SO_4 and 0.4 gms of $CuSO_4.5H_2O$ and heated over a low

flame in the digestion rack. When the contents of the flask were almost to dryness the flame was removed and the flask allowed to cool and 24 cc of concentrated sulfuric acid were added and the digestion carried out in the usual way. It was found that the potassium sulfate was without effect but that copper sulfate was beneficial. It was more convenient to add both at one time so this was continued. The amount of hydrogen peroxide and of sulfuric acid were found to be important. The optimal results were obtained by the use of 15% H_2O_2 by weight (Superoxol diluted one-half nearly), to which *were* added the potassium sulfate, copper sulfate and sulfuric acid. With more than 1 cc of sulfuric acid lower results were obtained while less acid gave occasional higher results but also had a tendency to give very low results. This method gave results considerably higher than the Kjeldahl method, yielding about 95% of the nitrogen obtained by the Dumas method.

6. Effect of Autolysis Preceding Analysis.

Results of the first experiments on nitrogen fixation in a molasses medium showed that there was some relation between autolysis and fixation and it was thot that possibly autolysis rendered yeast more amenable to the Kjeldahl method. But when yeast was autolyzed in tubes closed off from the air there was no apparent change in the nitrogen content as indicated by the Kjeldahl method.

7. TER Meulen Method.

H. TER Meulen (41,42) devised a modification of the old soda lime process with some real improvements. In the last paper he describes what is the more applicable method. The sample is well mixed with anhydrous sodium carbonate and a little reduced nickel and placed in a quartz combustion tube. The rest of the tube is filled with asbestos coated with reduced nickel. Hydrogen, freed from ammonia and saturated with water vapor, is passed over the sample and then over the reduced nickel, both at 350° C. The gases issuing from the combustion tube are passed thru a condenser and thru a dilute acid. Ammonia may be determined in any of several ways. He obtained very good results with this method in the analysis of several kinds of flour, coal, oil cake, casein, gelatin, glue and other materials and stated that the analysis required only a few minutes. In general higher results were obtained than by the Kjeldahl method. This seemed to offer possibilities in the analysis of yeast but even with several hours of heating and with temperatures of 800-900° C, the best result obtained was 6.05% nitrogen, which is but 75% of the Dumas result and considerably lower than the Kjeldahl.

The method involving preliminary acid hydrolysis and oxidation with H_2O_2 seemed by far the best possibility and it was decided to use this method to see whether or not the Kjeldahl method had failed to indicate the progress of the fixation of nitrogen in the first experiments.

Since the hypothesis had been advanced that some compound or compounds associated with the nucleoprotein in yeast were responsible for the difference between the nitrogen as determined by the Kjeldahl and the Dumas methods, some pure nucleoprotein and nucleic acid were prepared from yeast, following the method as described by Hawk (20). Several samples of yeast, several molds and a few pure compounds were analyzed by the three methods and the results are given in TABLE XVII.

TABLE XVII

Compound or material	Percent of Nitrogen Found			
	Calc.	Dumas	Kjeld.	M.Kjeld.
Caffeine	28.9	29.77	28.65	27.04
Uric acid	33.3	33.24	31.36	31.58
Theobromine	31.1	31.79	30.56	29.62
Diphenyl amine	8.3	8.62	8.18	4.40*
Casein		14.89	13.66	13.88
Penicillium expansum, powdered air dried. Mycelium and spores		5.23	4.66	5.00
Aspergillus niger, powdered air dried. Mycelium and spores		4.49	4.42	3.98*
Yeast nucleic acid		7.78	6.72	7.58
Yeast nucleoprotein		14.85	12.14	13.59
Yeast, Fleischmann, dried at factory		9.00	7.95	8.60
Same yeast one year later		8.28	7.88	7.93
Yeast, Fleischmann, fresh, air dried		8.46	7.17	7.45
Yeast, Fleischmann, fresh, vac.dried		7.54	7.58	7.63
Yeast, Fleischmann, fresh, wet		2.19	2.16	2.18
Yeast, Fleischmann, fresh, wet		2.24	2.01	2.28

*These are the highest results obtained by several determinations.

The modified Kjeldahl method gave results on yeast nucleic acid and yeast nucleoprotein as well as on dried and fresh yeast nearly as high as those obtained by the

the Dumas method, and considerably higher than those obtained by the Kjeldahl method. When Fleischmann's yeast was dried in a vacuum at 40°C there was practically no difference in the nitrogen content as determined by the three methods, whereas there was a great difference when the same yeast was dried at atmospheric pressure and room temperature. Dried yeast seemed to lose nitrogen on storage, as indicated by the Dumas method, but there was a much lower loss as measured by the Kjeldahl method. These phenomena would indicate that the compound not amenable to the Kjeldahl method was volatile.

The heterocyclic ring compounds analyzed gave very nearly the same results by all three methods. It will be noted that diphenylamine gave very much lower results by the modified method, due to volatilization during the preliminary treatment. Of the two molds analyzed, one gave a higher result by the modified method and the other a very much lower result. Casein contained more nitrogen as determined by the modified than by the regular Kjeldahl method.

This modification is one which is well suited to the analysis of yeast and culture medium and should give quite different results than those obtained by the usual Kjeldahl procedure. But it is not perfectly reliable, for it does give low results in some cases, but never with yeast nor with yeast protein.

The Effect of the Hydrogen Ion Concentration on Fixation of Atmospheric Nitrogen by S Cerevisiae and Mycoderma, as Indicated by the Kjeldahl and the Modified Kjeldahl Methods.

In this experiment the same molasses medium developed previously was used. But this molasses contained 0.41% of nitrogen as compared to the 0.21% in the earlier experiments. The nitrogen content was the same as measured by both methods. The medium used had the composition:

Molasses	6.00 gms
K_2HPO_4	0.50 gms
Water	to 100 cc

The hydrogen ion concentration was regulated as previously described. 10 cc of this medium in a 50 cc Erlenmeyer flask were inoculated with one loop, 0.01 cc, of a three day culture of the yeast indicated at the head of the tables grown in this same medium. Incubation was at 30° C ± 0.02° C in a saturated atmosphere free from ammonia and oxides of nitrogen.

At the end of the time indicated in the tables the flasks were removed and analyzed by the regular and modified Kjeldahl methos. pH, count, and Gram stain were taken from a similar series of flasks inoculated in the same way at the same time. The flasks were plugged with cotton, but not otherwise closed.

Blanks were run at the following pH values: 4.00, 5.00, 6.00, 7.00, 8.00 and 9.00 and were made up at the same time and in the same way as were the other two series of flasks, and

were incubated with them, but were not inoculated. They were analyzed by both methods also. Nitrogen gain is equal to the nitrogen in the inoculated flask minus the nitrogen in the blank, calculated to 100 cc.

The results of this experiment are given in TABLES XVIII - XXIII and shown graphically on pages 50 - 52. Fixation has proceeded much more rapidly in this medium than in that used previously. This must be due to the molasses, and probably to its higher nitrogen content. It has proceeded so rapidly that rate curves are not given, due to the lack of necessary data. Again it was found that nitrogen fixation was a function of the hydrogen ion concentration and of time. But it is difficult to make any generalizations from the data obtained. Quite different results were given by the two methods of analysis. Again it was noted that there was some connection between nitrogen fixation and the Gram stain, altho this was not nearly so clear cut as in the first experiments.

TABLE XVIII

Saccharomyces cerevisiae

Time - one week

Initial pH	Final pH	Count	Gram Stain	N Gain mgms/100 cc Kjeldahl	Modified
4.00	4.00	310	+	-0.33	-5.55
4.50	4.60	380	+	-0.49	-5.94
5.00	5.00	345	+	-0.33	-6.05
5.25		325	+	-0.49	-5.55
5.50		360	+	-0.33	-5.79
5.75		355	+(few -)	-0.49	-6.05
6.00	5.20	415	+	-1.26	-5.79
6.25		390	+	-1.26	-6.05
6.50		400	+	-1.26	-5.27
6.75		370	+	-1.49	-5.55
7.00	6.40	334	+	-2.16	-5.55
7.25		360	+(few -)	-2.60	-6.39
7.50		370	+(few -)	-3.06	-6.05
7.75		445	+	-2.60	-6.53
8.00				-2.29	-6.14
8.25	6.60	350	+		-5.55
8.50		360	+(few -)	-2.29	-5.47
9.00	6.80	285	+	-0.86	-5.55

TABLE XIX

Saccharomyces cerevisiae

Time - two weeks

Initial pH	Final pH	Count	Gram Stain	N Gain mgms/100 cc Kjeldahl	Modified
4.00	4.45	294	+	-2.06	-0.25
4.50	4.68	303	+	-1.37	-1.57
5.00	4.85	304	+(few -)	+2.52	-2.47
5.25	4.96	304	+	+0.17	-2.83
5.50	5.06	348	+	+1.94	
5.75	5.15	348	+(few -)	+0.04	
6.00	5.74	348	+	-3.28	-2.83
6.25	5.80	386	+	+0.44	
6.50	6.08	382	+(few -)	+1.18	
6.75	6.11	408	+	-1.18	
7.00	6.14	394	+	-0.68	-1.25
7.25	6.20	376	+	-0.28	-0.85
7.50			+	-0.68	-1.25
7.75			+	-2.08	-1.64
8.00	6.24	372	+	-1.27	
8.25	6.28	393		-0.68	-0.80
8.50	6.43	342	+	-1.17	
9.00	6.62	301		+0.80	

TABLE XX

Saccharomyces cerevisiae

Time - four weeks

Initial pH	N Gain mgms/100 cc Kjeldahl	Modified
4.00	+0.72	-1.12
4.50	-0.30	+3.76
5.00	+0.52	+1.48
5.25	-1.28	+4.70
5.50	+1.46	+3.71
5.75	+0.42	+0.18
6.00	-2.68	+0.88
6.25	+0.22	+1.48
6.50	+1.54	+5.20
6.75		-3.52
7.00	+2.14	-4.67
7.25	+1.12	+0.98
7.50	-0.48	-3.52
7.75	+0.50	
8.00	+0.04	+0.24
8.25	+1.17	-0.52
8.50	+1.98	+0.78
9.00	+0.22	+0.18

Saccharomyces cerevisiae

Time - six weeks

Initial pH	N Gain mgms/100cc Kjeldahl
4.00	
4.50	-3.06
5.00	-3.80
5.25	+0.84
5.50	-2.70
5.75	-1.49
6.00	
6.25	-1.49
6.50	
6.75	
7.00	-1.49
7.25	
7.50	
7.75	-5.89
8.00	-6.02
8.25	-0.17
8.50	
9.00	

TABLE XXI

Mycoderma

Time - one week

Initial pH	Final pH	Count	Gram Stain	N Gain mgms/100 cc Kjeldahl	Modified
4.00	5.02	260	+	-1.03	-3.05
4.50	5.71	220	±	-1.84	-3.05
5.00	6.38	130	±	-3.46	
5.25	6.05	322	±	-0.58	-2.78
5.50	6.00	290	±	+0.70	-2.05
5.75	6.28	255	±	+0.98	+6.85
6.00	6.30	292	±	-0.24	+6.95
6.25	6.85	130	±	-0.67	+8.75
6.50			±	+0.16	+5.46
6.75	7.22	195	±		
7.00	7.31	193	+	-0.48	+4.35
7.25	7.35	122	+	+0.03	-7.47
7.50	7.48	182	+	-0.48	-9.00
7.75	7.67	194	±	-3.48	-2.70
8.00	7.77	149	±		-2.35
8.25	7.83	102	±	-3.43	-1.21
8.50	7.91		+	-2.26	-1.19
9.00	8.54		+	-1.00	-15.60

TABLE XXII

Mycoderma

Time - two weeks

Initial pH	Final pH	Count	Gram Stain	N Gain mgms/100 cc Kjeldahl	N Gain mgms/100 cc Modified
4.00	5.36	1081	+	-0.26	+3.25
4.50	6.11	945	+	-0.78	+8.95
5.00	6.34	1022	+(few -)	+1.12	+12.17
5.25	6.39	1230	+(few -)	+3.30	+1.95
5.50	6.34	1088	+(few -)	+1.30	+0.70
5.75	6.54	1350	+(few -)	+1.12	+6.64
6.00	6.52	1037	-		+6.64
6.25	7.03	942	-(few -)	+3.07	+6.10
6.50	7.27	876	+(few -)	+1.00	+8.75
6.75	7.69	580	-	+1.34	+0.56
7.00	7.69	730	+(few -)	-1.48	+9.25
7.25	7.75	816	+	-1.36	+1.48
7.50	8.10	775	+	-1.36	
7.75	8.12	725	+	+0.30	+6.37
8.00	8.31	867	±	+1.24	+1.12
8.25	8.37	862	-(few +)	+3.27	+0.98
8.50	8.55	819	±	-1.12	+4.96
9.00	8.74	610	+	-2.28	+6.10

TABLE XXIII

Mycoderma

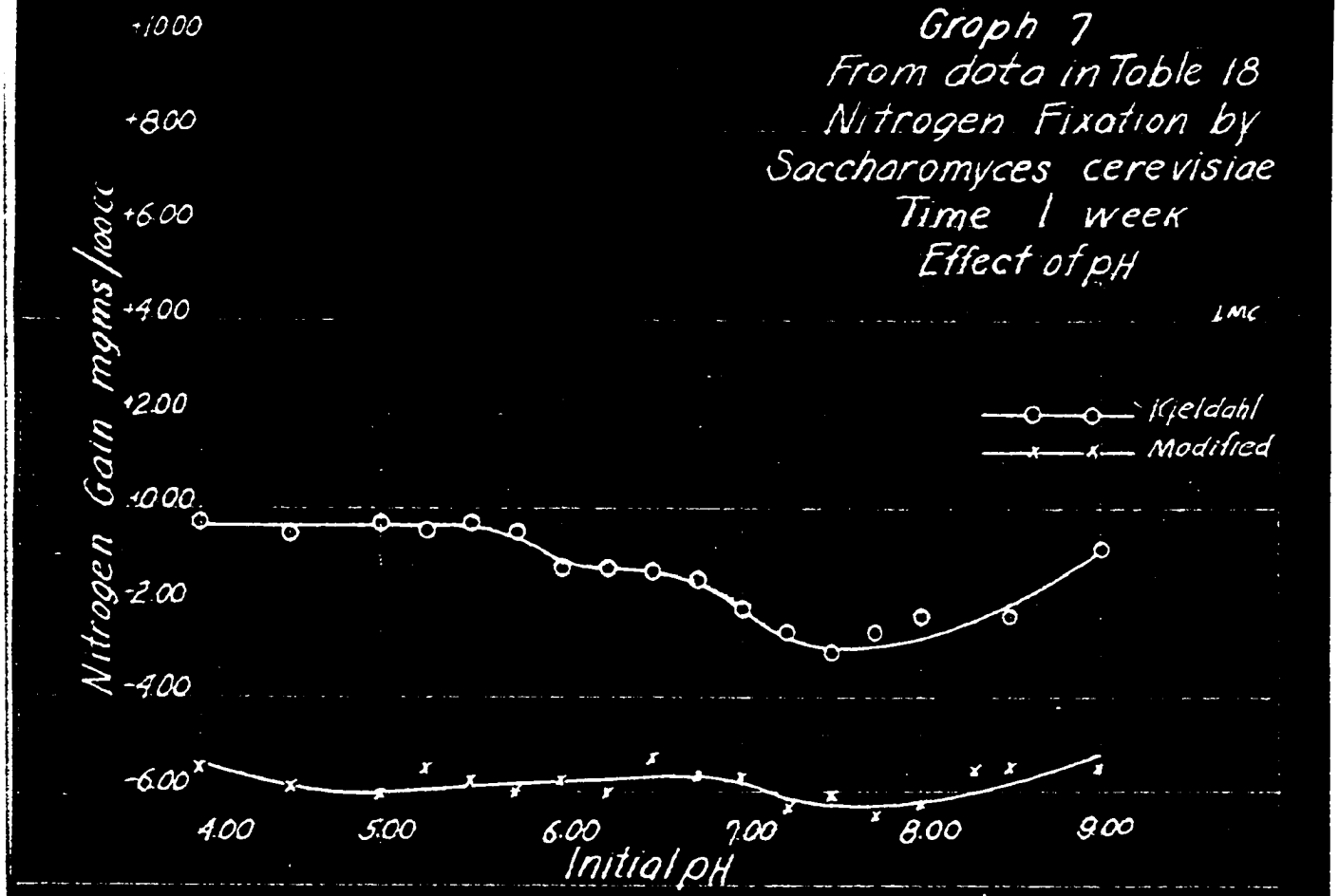
Time - four weeks

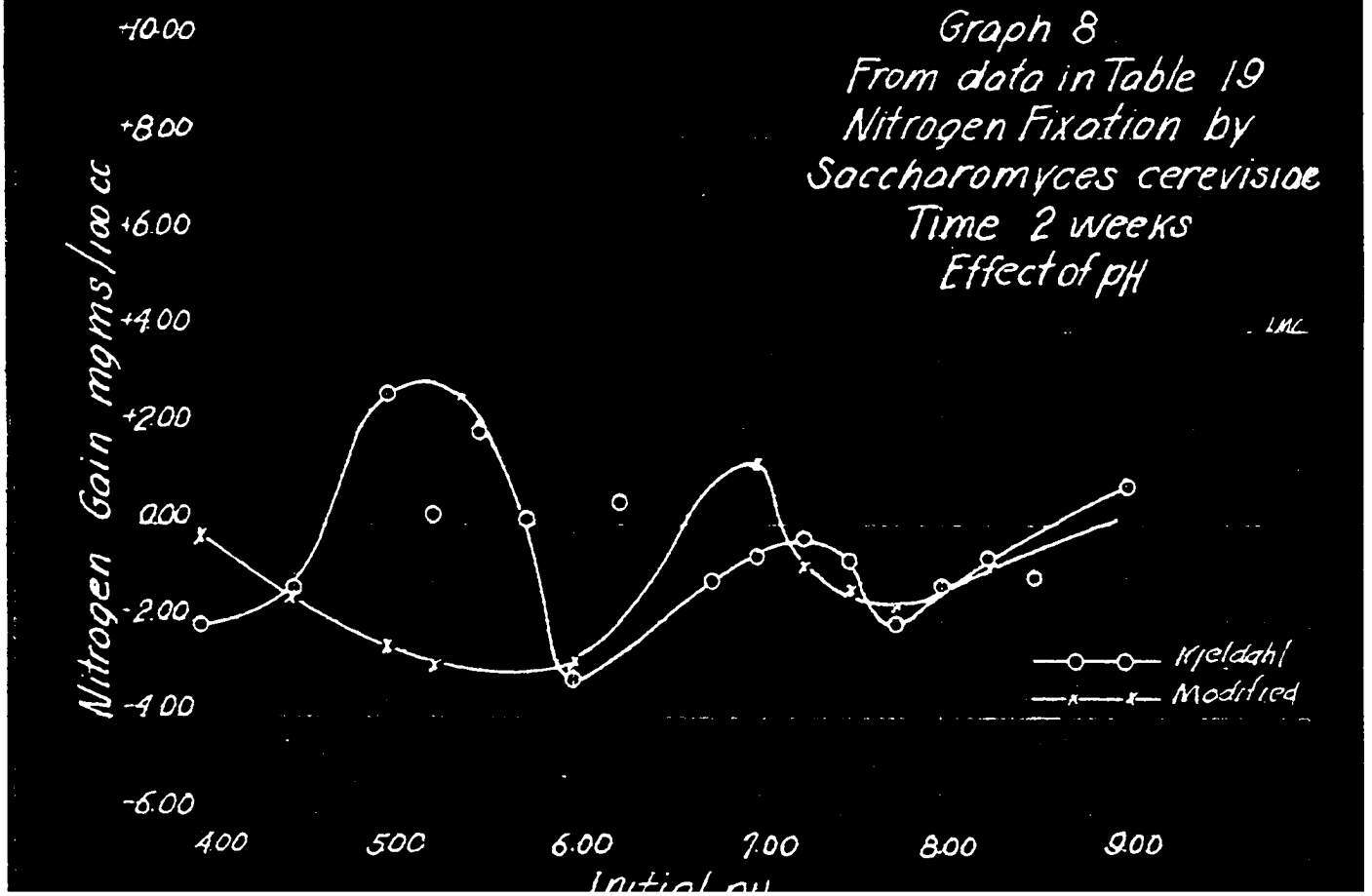
Initial pH	N Gain mgms/100 cc Kjeldahl	N Gain mgms/100 cc Modified
4.00	+3.22	-2.18
4.50	+1.14	+1.15
5.00	+0.01	+2.25
5.25	+1.24	+3.51
5.50	+1.63	+1.25
5.75	+2.37	+3.40
6.00	+0.72	+3.55
6.25	+1.97	+0.55
6.50	-0.58	-0.25
6.75	+0.41	-1.18
7.00	+0.97	+0.15
7.25	+3.24	+1.49
7.50		+0.40
7.75		-3.06
8.00	-0.58	-2.78
8.25	+0.32	-0.95
8.50	+1.62	+2.67
9.00	+1.57	

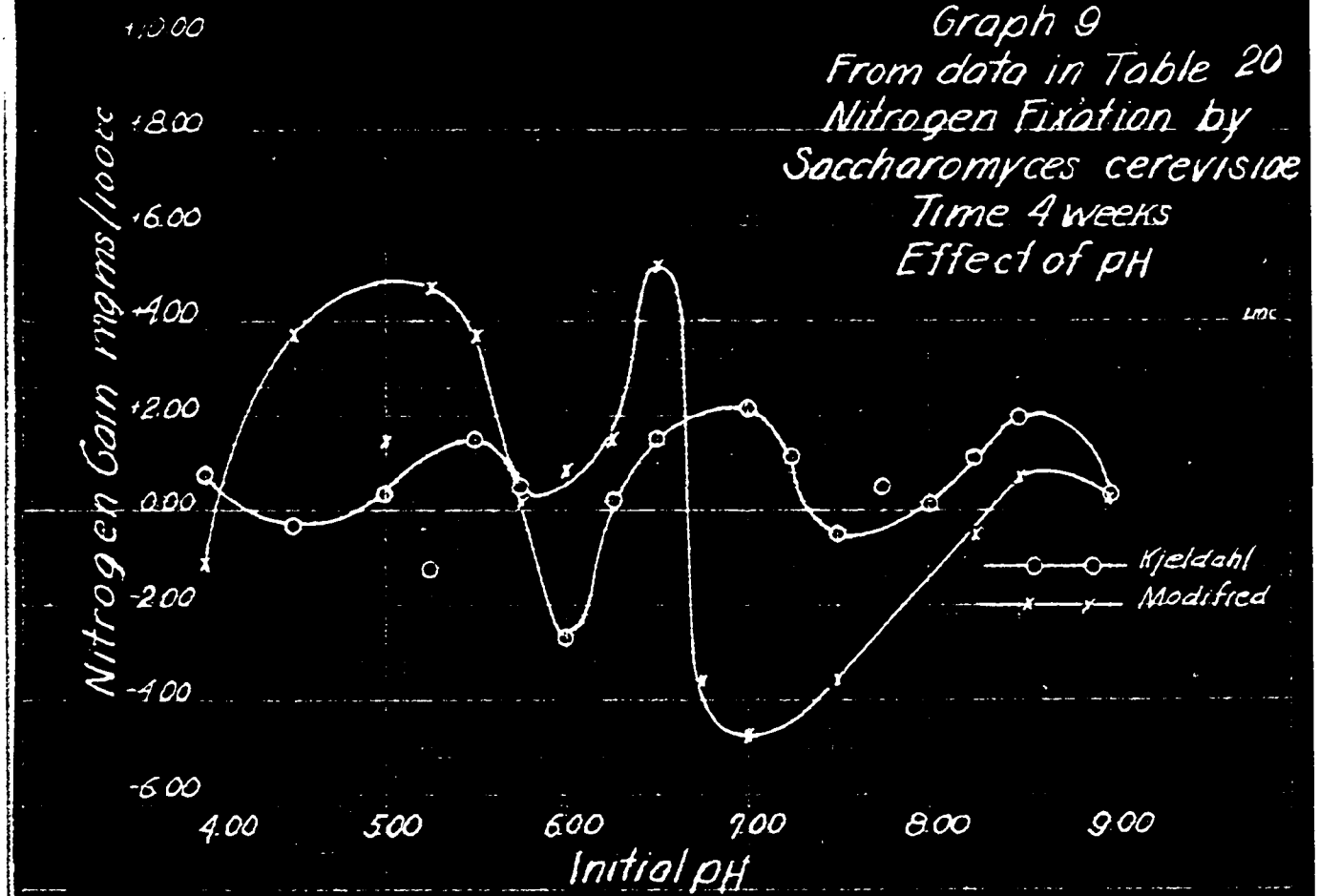
Mycoderma

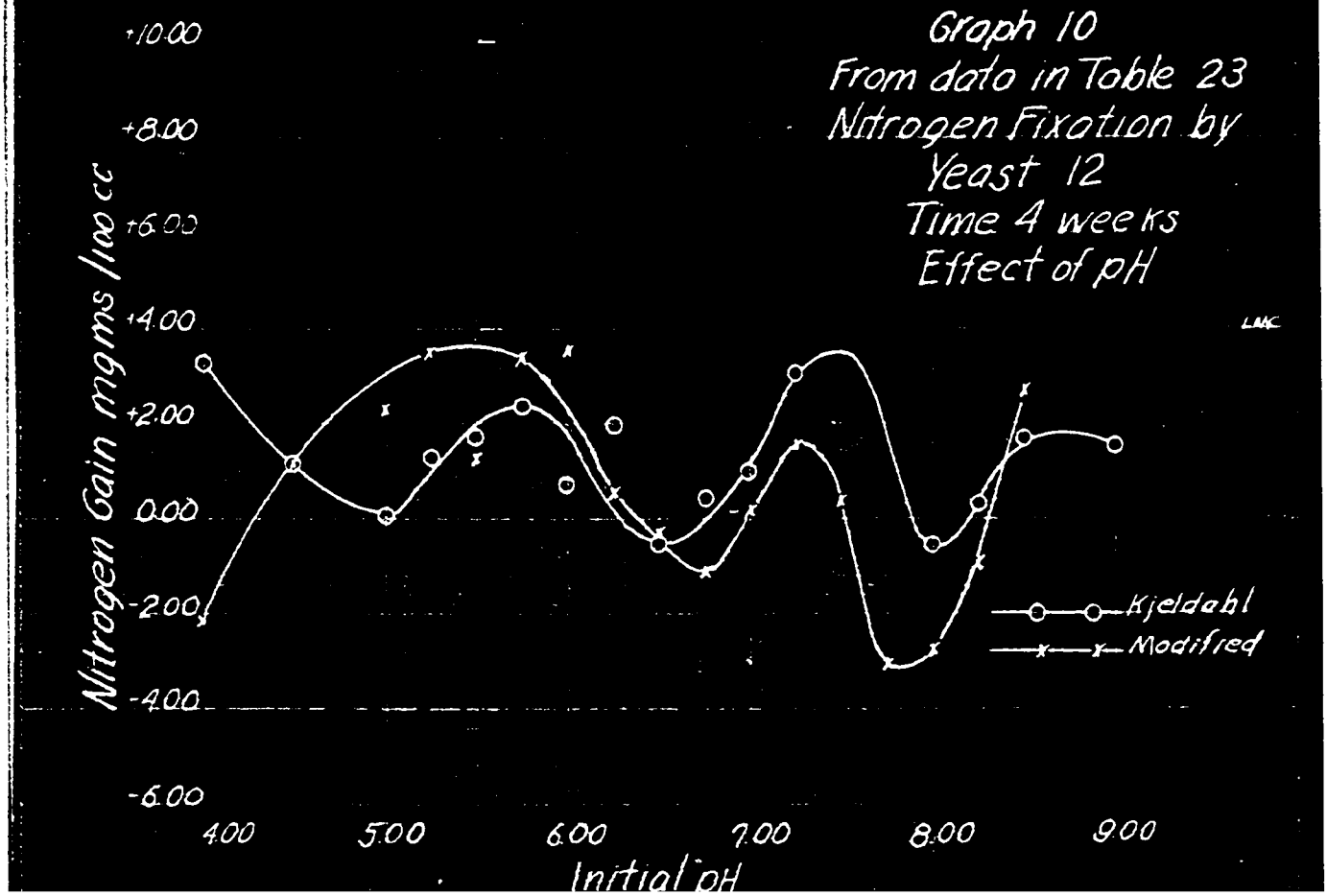
Time - six weeks

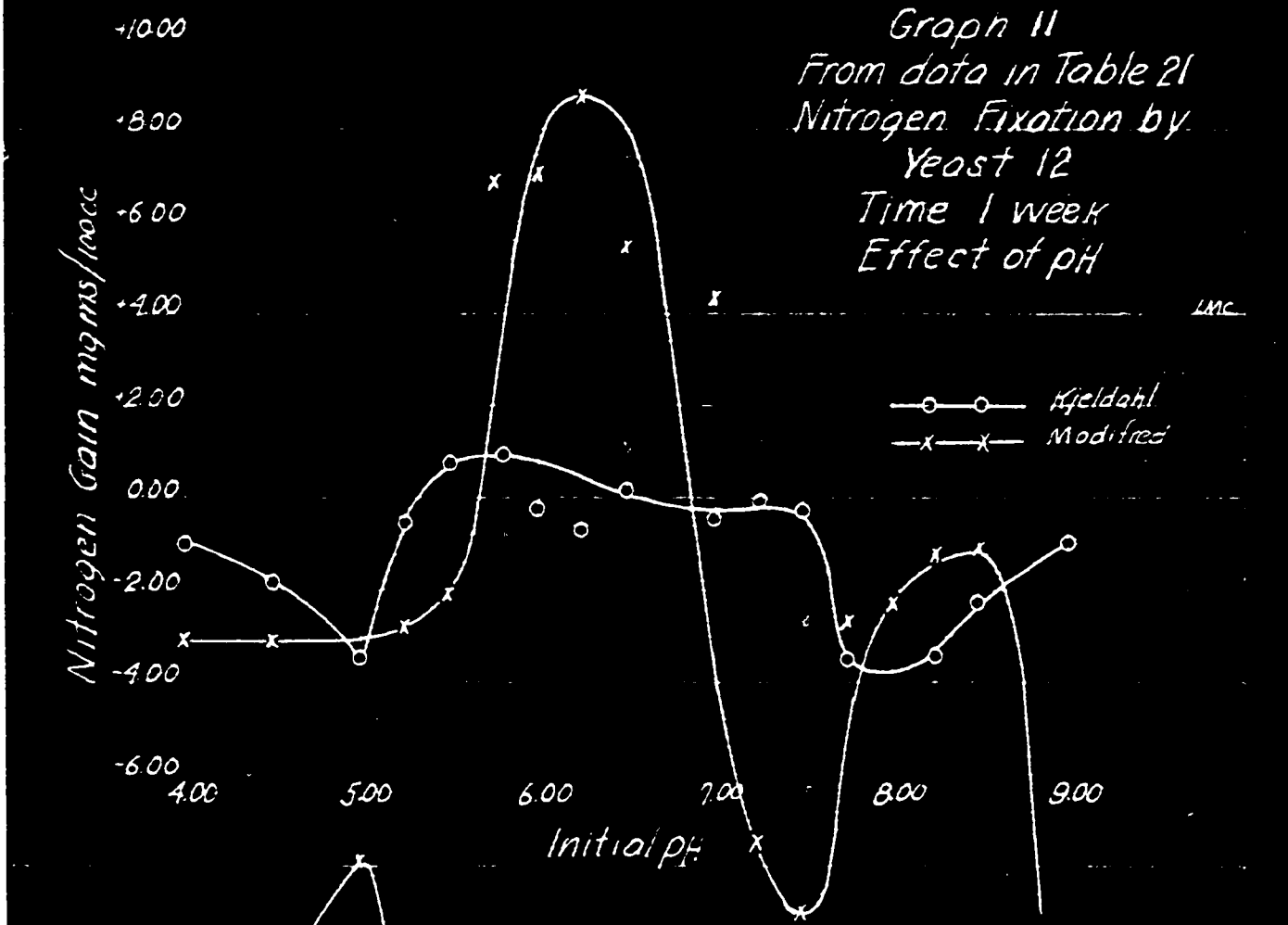
Initial pH	N Gain mgms/100cc Kjeldahl
4.00	-5.08
4.50	-1.36
5.00	
5.25	+0.42
5.50	-3.18
5.75	
6.00	
6.25	
6.50	-2.28
6.75	-0.48
7.00	-4.25
7.25	
7.50	-2.60
7.75	
8.00	-2.37
8.25	-3.46
8.50	+1.64
9.00	

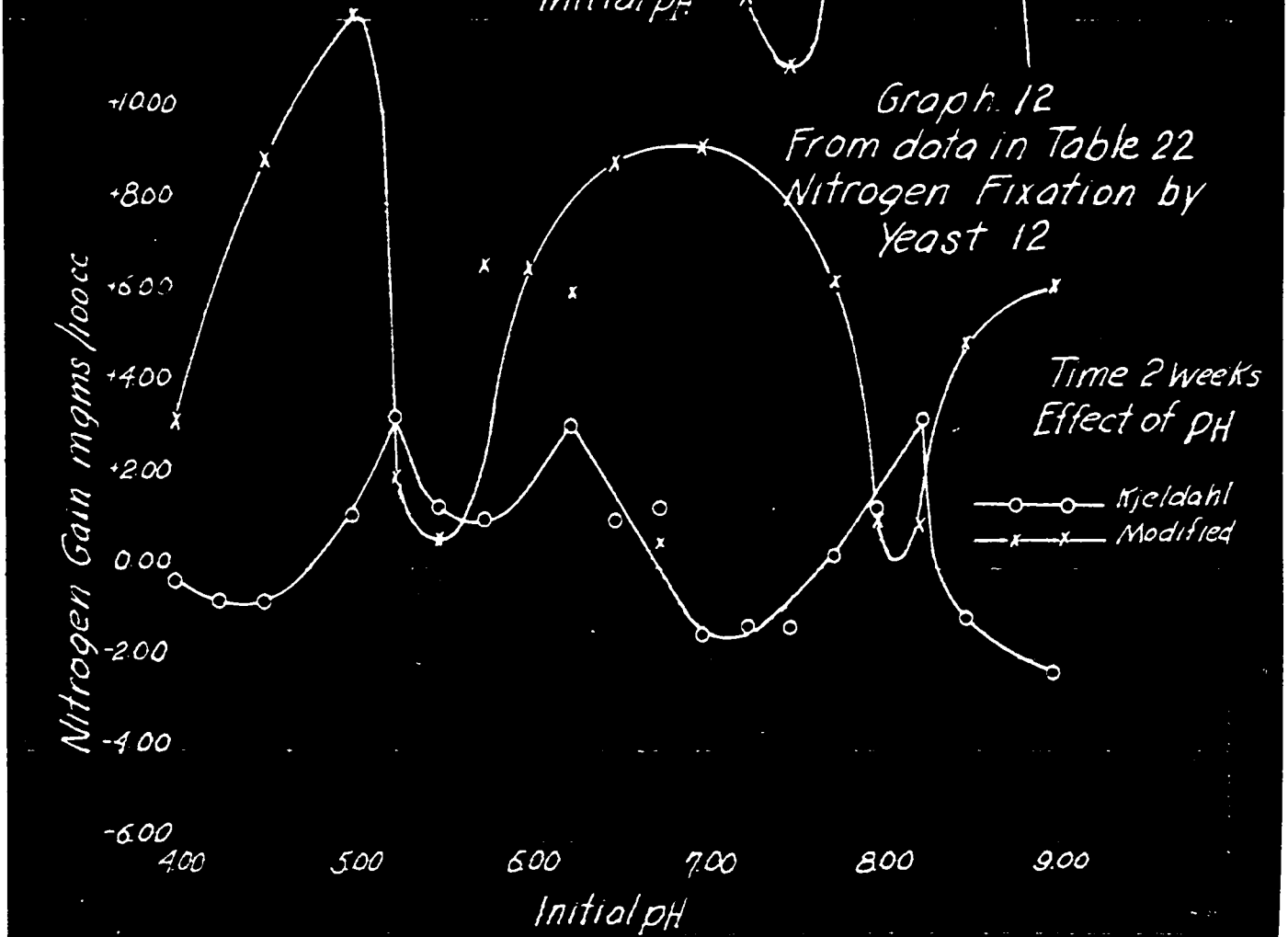

Graph 7
From data in Table 18
Nitrogen Fixation by
Saccharomyces cerevisiae
Time 1 week
Effect of pH
LMC
Kjeldahl
Modified
Nitrogen Gain mgms/100cc
+10.00
+8.00
+6.00
+4.00
+2.00
-2.00
-4.00
-6.00
4.00
5.00
6.00
7.00
8.00
9.00
Initial pH


Graph 8
From data in Table 19
Nitrogen Fixation by
Saccharomyces cerevisiae
Time 2 weeks
Effect of pH
LMC
Kjeldahl
Modified
Nitrogen Gain mgms/100 cc
+10.00
+8.00
+6.00
+4.00
+2.00
0.00
-2.00
-4.00
-6.00
4.00
5.00
6.00
7.00
8.00
9.00


Graph 9
From data in Table 20
Nitrogen Fixation by
Saccharomyces cerevisiae
Time 4 weeks
Effect of pH
LMC
Nitrogen Gain mgms/100cc
+10.00
+8.00
+6.00
+4.00
+2.00
0.00
-2.00
-4.00
-6.00
4.00
5.00
6.00
7.00
8.00
9.00
Initial pH
Kjeldahl
Modified


Graph 10
From data in Table 23
Nitrogen Fixation by
Yeast 12
Time 4 weeks
Effect of pH
LMC
Nitrogen Gain mgms/100cc
+10.00
+8.00
+6.00
+4.00
+2.00
0.00
-2.00
-4.00
-6.00
4.00
5.00
6.00
7.00
8.00
9.00
Initial pH
Kjeldahl
Modified


Graph 11
From data in Table 21
Nitrogen Fixation by
Yeast 12
Time 1 week
Effect of pH
Kjeldahl
Modified
Nitrogen Gain mgms/100cc
+10.00
+8.00
+6.00
+4.00
+2.00
0.00
-2.00
-4.00
-6.00
4.00
5.00
6.00
7.00
8.00
9.00
Initial pH


Graph 12
From data in Table 22
Nitrogen Fixation by
Yeast 12
Time 2 weeks
Effect of pH
Kjeldahl
Modified
Nitrogen Gain mgms/100cc
+10.00
+8.00
+6.00
+4.00
+2.00
0.00
-2.00
-4.00
-6.00
4.00
5.00
6.00
7.00
8.00
9.00
Initial pH

In general it may be said that two optimal hydrogen ion concentrations were found. For Mycoderma these were approximately pH 5.00 and pH 8.00. For S cerevisiae two optima were noted also, at approximately pH 5.00 and pH 7.00. But the optima found seem to depend upon the time of incubation, and there was such a great change of hydrogen ion concentration that it is difficult to say what are the optimal values. It is thot that more easily interpreted results would obtain with molasses of lower nitrogen content and with flasks provided with only a small capillary vent or preferably with a trap to catch nitrogen compounds evolved.

It is significant, however, that nitrogen fixation can be shown for Mycoderma and Saccharomyces cerevisiae in a molasses medium containing 0.0246 gms of nitrogen per 100 cc. This is about one half that contained in Medium E. The maximum fixation found for Mycoderma was at an initial hydrogen ion concentration of pH 5.00 and final hydrogen ion concentration of pH 6.34 with two weeks incubation at 30°C, as determined by the modified method, amounting to 12.17 mgms per 100 cc. For Saccharomyces cerevisiae the maximum fixation was 5.20 mgms per 100 cc, at an initial hydrogen ion concentration of 6.50 and a final of 6.00 (ca), with four weeks incubation at 30°C, as determined by the modified method.

But it cannot be said that either method of analysis

has determined what has actually taken place. Probably both tend to follow changes in nitrogen compounds rather than in total nitrogen content.

In general the modified method gives higher maxima and lower minima. Until some entirely reliable analytical method is available, no real knowledge of the change in the nitrogen content of a medium in which yeast is growing can be obtained.

SUMMARY

1. A synthetic medium containing only potassium chloride, dipotassium phosphate and sucrose in water has been developed in which yeast will grow and utilize atmospheric nitrogen in building up protein. A fixation of 2.0 mgms per 100 cc of medium can be demonstrated with five weeks incubation at 30°C.

2. It has been shown that yeast will grow in Lipman's medium and in Gainey's medium. While little fixation was noted in Lipman's medium, a fixation of 4.6 mgms per 100 cc in six weeks was found in Gainey's medium.

3. Yeast will grow very well in a 6.0% molasses, 0.50% dipotassium phosphate medium, and fixation of atmospheric nitrogen has been shown to be a function of the hydrogen ion concentration, two optima being found, one at pH 6.0 and the other at pH 7.9. Apparent losses of nitrogen were noted for three weeks, when those flasks which apparently had lost the most began to gain in nitro-

gen until in 6-8 weeks they had shown the most gain. Nitrogen was determined by the Kjeldahl method.

4. It has been found that yeast does not yield all its nitrogen in the Kjeldahl analysis. The difference in results by the Kjeldahl and Dumas methods seems to be due to compounds associated with the nucleoproteins and with the nucleic acid, and to be volatile.

5. Autolysis does not make yeast more amenable to the usual Kjeldahl method.

6. A modification of the Kjeldahl method has been developed which will give more nearly the values given by the Dumas method. It has been found superior to the Kjeldahl method for yeast, yeast nucleoprotein and yeast nucleic acid and for casein and one mold, but with another mold it gave very poor results, considerably lower than either the Dumas or Kjeldahl results. It seems to be of rather limited value, but in many cases preferable to the usual Kjeldahl method.

7. Nitrogen fixation as determined by the modified method and by the usual Kjeldahl method, has been shown to be a function of the hydrogen ion concentration. Somewhat different results are given by the two methods. Fixation was determined for Mycoderma and for Saccharomyces cerevisiae in a 6% molasses, 0.5% dipotassium phosphate medium.

8. The maximum fixation found for Mycoderma was

12.17 mgms per 100 cc of medium, as determined by the modified method, in two weeks at 30°C with an initial hydrogen ion concentration of pH 5.00 and a final hydrogen ion concentration of pH 6.34. Saccharomyces cerevisiae showed the maximum fixation in four weeks with the same medium, temperature and method of analysis, at at an initial hydrogen ion concentration of pH 6.50 and a final hydrogen ion concentration of pH 6.00, amounting to 5.20 mgms per 100 cc.

CONCLUSIONS

Two yeasts, Saccharomyces cerevisiae, Race F, and a Mycoderma which is described, have been found to fix atmospheric nitrogen when grown in a molasses medium. This fixation is a function of the hydrogen ion concentration and of time. Fixation by Mycoderma has been demonstrated in three nitrogen-free synthetic media. Fixation has been shown to be in some way associated with autolysis. It does not appear to be simultaneous with growth nor proportional to growth. Two hypotheses are advanced.

1. Fixation does not begin until autolysis has started.
2. The analytical method has failed to indicate what has taken place before autolysis.

Results by two analytical methods are similar but far from identical. Maxima and minima occur at nearly the same points as determined by both methods, and neither

indicates any fixation until some of the yeast cells have changed from Gram positive to Gram negative, indicating beginning of autolysis. The fact that the two methods agree even fairly closely and that autolysis out of contact with air seems not to make yeast more amenable to the ordinary Kjeldahl method, would indicate that fixation of nitrogen occurs during autolysis. But it is also evident that both analytical methods tend to follow changes in nitrogen compounds, to some extent, rather than changes in total nitrogen only. The phenomenon is further complicated by the losses in nitrogen occurring during incubation.

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