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Production of yeast growth stimulants by molds on various media

H. H. Schopmeyer
Iowa State College

NOTE TO USERS

PRODUCTION OF YEAST GROWTH STIMULANTS BY MOLDS ON VARIOUS MEDIA

BY

H. H. Schopmeyer

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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.

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HISTORICAL

Pasteur (1860) was one of the first to propagate yeast in nutrient solutions containing mineral salts and sugar. He used most successfully a medium consisting of 10 grams of sucrose, 0.1 gram of ammonium tartrate, one gram of yeast ash, and 100 c.c. of water, and inoculated this with a small bit of yeast the size of a pin-head. A small inoculation was followed by a slow fermentation which extended over a long period of time. A heavier inoculation gave a more rapid rate of evolution of gas bubbles, Pasteur's criterion for judging the rate of fermentation. In describing his experiments, he says in part: (translation)

"After 24 - 36 hours microscopic bubbles of carbon dioxide appeared, and on the following days foam filled the flask and a deposit covered its bottom. Under the microscope a drop of this deposit revealed beautiful yeast-cells, much bunched and extremely young in looks, that is, the globules were turbid-translucent and non-granular; among these could be distinguished with surprising ease each globule of the small quantity of yeast used as seed; the young cells were infinitely more numerous than the old ----------. It must not be thought that in this solution fermentation ever became so active as it did when in place of using ammonia as the source of nitrogen, a suitable albuminoid substance was employed, such as that of grapes, beet juice, or yeast water. In sugar solutions containing one of these albuminoids fermentation was much more active; instead of after 36 - 48 hours, the first small bubbles appeared in 12 - 24, and much more yeast was formed in any given time. Nothing is more curious than this influence of the composition of the medium on the activity of the fermentation, and I have made a number of experiments on the matter. I was much surprised to find that in a medium formed by dissolving sugar in a filtered solution of albumen from the whites of eggs, yeast cells will not bud at all, and there is no trace of fermentation ----- when blood-serum is used, or juices squeezed from muscles, the cells

develop with marvelous ease and the sugar ferments almost as well as in a natural saccharine juice or clear yeast-water; this is not because the albumen of eggs differs from that of serum but because blood contains other albuminoid constituents which by their special nature are suitable for the nourishment of yeast; for if the blood be coagulated, boiled with water and then filtered to remove the coagulated albumen, sugar dissolved in the limpid filtrate forms a medium in which yeast multiplies and gives rise to well-characterized alcoholic fermentation; while if white-of-egg be treated in the same way, no fermentation at all occurs. These experiments were repeated many times, and always gave the same results -----. It is obvious that there may be very great differences between members of the group called albuminoids or proteids. I have also observed that certain proteids are much more favorable than others to the development of the lactic acid ferment; for example, the soluble part of gluten or casein, the nitrogenous residue of liquids in which alcoholic fermentation has taken place, etc."

The scientific questions with which we are concerned in this thesis one can promptly see originated with these experiments. It is clear that Pasteur recognized the importance of some "albuminoid materials" in the nutrition of yeast and other microbes. Liebig went further, and questioned the possibility of cultivating yeast at all without them, and when his own experiments with sugar, yeast ash and ammonium tartrate gave negative results and those of Naegeli the same, he promptly stated that Pasteur had deceived himself and what he took for yeast must be something else. However, he failed to follow Pasteur's suggestion to, "Come to Paris and see for yourself," (L. Pasteur, 1872) and apparently Pasteur had won his point.

Nildiers (1901) while working in Ide's laboratory at Louvain, found that he could not raise a healthy crop of yeast in salts and sugar solutions unless a little wort, yeast-water, peptone, or beef extract were

added. The unknown substance -- perhaps a whole category of substances -- which so improves the medium, he called provisionally "Bios", and thus the name is defined by him; also, he must be credited with the first attempts to prove the chemical identity of this newly discovered substance. Although he did not isolate and identify a substance of Bios properties, he did show that Bios is not contained in yeast ash, nor is it urea, alanine, tyrosine, adenine, guanine, creatine, cuistin, ovalbumen, or nucleic acid, for none of these substances can take the place of wort, yeast-water, peptone, or extract of beef.

Wildiers described his Bios as follows:

1. Soluble in water.
2. Insoluble in absolute alcohol and ether. 80% alcohol, however, allows a good extraction of Bios.
3. Not present in yeast ash. Therefore, is not an inorganic substance.
4. Not destroyed by one-half hour boiling with 5% sulfuric acid.
5. It seems to be changed by 30 minutes boiling with 1% solution of sodium hydroxide.
6. Not precipitated by lead acetate.
7. Diffuses readily through parchment.
8. Contained in Liebig's meat extract, commercial peptone, and beer wort.
9. Bios is not present in such substances as urea, asparagin, alanine, tyrosine, nuclein bases, adenine, guanine, thymus nucleic acid, creatine, peptic and

tryptic digestion products of albumin.

If Bios is necessary for yeast growth it is very difficult to explain Pasteur's success and Liebig's apparent failure to get yeast growth in synthetic media. Naturally, there have been many theories advanced to explain this very apparent discrepancy. Wildiers explained it on the basis that Pasteur used a large inoculum (a portion the size of a pin-head), while Liebig used a smaller bit of yeast. Wildiers contended that the Bios was introduced with the yeast in Pasteur's case, while this was not true with Liebig, for he used a much smaller quantity of yeast. These explanations have been very well summarized by Miller (1930):

"Liebig's suggestion that Pasteur could not distinguish yeast from lactic acid microbe may be dismissed at once; the microscope was Pasteur's favorite tool. Wildiers' explanation that Pasteur added Bios with his yeast requires that Pasteur's pins have heads of more than 0.1 c.c. in volume, for two drops of Wildiers' own "strong wort" were insufficient to cause healthy growth. That Liebig failed because he used too little yeast for seed can hardly be maintained; to him a pin-head meant 2 mm., he tells us so, and also that he used one-tenth of this amount ---about a million cells--- for seed. Lindner's suggestion that perhaps Liebig's solutions were better supplied with air than were Pasteur's and that this might have caused fatty degeneration of the yeast he used for seed, is of course no better than a guess. Kossowicz's discovery, on the other hand, that bacteria in a yeast culture may generate Bios then, offers a plausible explanation of Pasteur's famous crop; for it was only after 1880 that the technic of pure cultures was developed. The explanation that to me seems likeliest, however, is founded on a difference long ignored: 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. Wildiers, who worked with "various commercial yeasts" and two pure races, thought he had proved the need of Bios to be "a general characteristic of all beer yeasts;" and this conclusion was supported by the work, though not by the words, of Hunneberg. Fulmer, however, found a yeast in Fleischmann's yeast cake which he kept alive and well

more than a year, by constantly transplanting in his Medium F made up of inorganic salts and sugar, although the crops were always less than those in wort; to make quite sure that Bios was excluded, cane sugar was replaced by methose (a synthetic sugar) and the yeast flourished as before. This shows there is at least one yeast that gives fair crops in media free from Bios; might not Pasteur's have been another?"

The subject of yeast growth stimulants known under the name of Bios has been adequately reviewed up to 1925 by Tanner (1925) and later developments by Buchanan and Fulmer (1930).

It was first shown by Fulmer and Nelson (1924) and by Fulmer, Nelson, and Dueker (1924) that Bios was of a multiple nature. They separated the water-soluble extracts of alfalfa into four fractions with alcohol and found that the optimum concentration of these fractions when used in pairs materially affected the total yeast count. This should not have been true if but one substance were present.

Miller and co-workers (1924) (Lucas, C. H. J. 1924) have been able to fractionate the water-soluble extract of malt sprouts and from this water-soluble extract precipitate an inactive substance, Bios I, with barium hydroxide. The material not precipitated is called Bios II and is only slightly active. Great stimulation occurred when the two fractions were reunited in the proper proportions. Bios I was identified as an inositol (Eastcott, J. V. 1928). Bios II has been further fractionated by the use of acetic acid, acetic anhydride, or acetyl chloride, (Sparling, J.H. 1928) into two fractions which can be activated with NaOH.

In an attempt to determine whether the organic compounds essential to yeast growth could be produced by other fungi, Kossowicz (Tanner, F. S. 1925) inoculated nutrient solutions simultaneously with small amounts of yeast and Penicillium glaucum. Yeast cultures inoculated with mold showed an active fermentation. This stimulation according to Kossowicz was not a mere symbiosis because dead mold mycelium would also give the same stimulation and growth, it was due to substances which were water-soluble.

It is contended by Suzuki and co-workers (1920) that yeasts are divided into three classes based on whether or not they need Bios:

1. Those that must have Bios for growth.

2. Those that are greatly accelerated by Bios.

3. Those that are not stimulated by Bios.

They state Saccharomyces cerevisiae must have Bios for growth, which is in direct discord with the findings of Fulmer, Nelson, and White (1928) and considerable strain must be placed on Suzuki's theory in attempting a satisfactory explanation of Fulmer, Nelson, and Whites' work in which they grew yeast in a salt and synthetic methose medium. Suzuki does show, however, that Aspergillus oryzae when cultured on Bios free media does produce Bios in appreciable quantities.

It has been previously reported by Fulmer and Schopmeyer (1931) that during the growth of the molds Aspergillus niger, Aspergillus clavatus, or Trichoderma

lignorum, on either glycerol or sucrose, there is produced in the medium a growth stimulant for yeast Saccharomyces cerevisiae. It is the object of this work to further study the production of the stimulant in the following respects:

1. To study various molds to determine whether or not the production of Bios is a common property of them.
2. To study the effect of various kinds and concentrations of media on the growth of molds and the production of stimulants.
3. To study the nature of the stimulant from some specific mold:
    a. The extra-cellular stimulant.
    b. The intra-cellular stimulant.
4. To compare the stimulant with other varieties of Bios, or Bios from other sources.

PRODUCTION OF YEAST GROWTH STIMULANTS BY MOLDS

During the investigation of the growth of yeast in synthetic media, it has been observed that occasionally a greatly enhanced growth was shown in media which had been accidentally contaminated by molds. From this bit of information the idea was conceived that it might be possible that the mold was synthesising or at any rate was liberating a substance or substances in the media which might act as a yeast growth stimulant. Therefore, a systematic study of this phenomenon was outlined to attempt to find out definitely whether or not the mold was actually producing a Bios, and if in appreciable quantities, and also to find out if this was a general characteristic of molds, and also to study the Bios produced by some particular molds.

Rather than to attempt to isolate at first the molds contaminating yeast flasks and identify them, it was decided to start with pure cultures of these various fungi. The following molds were obtained:

1. Aspergillus niger
2. Aspergillus clavatus
3. Trichoderma lignorum
4. Rhizopus nigricans (plus and minus) (male and female)
5. Penicillium roqueforti.

Molds numbers 1, 2, 3, and 4 were furnished by Dr. Gilman of the Botany Department while number 5 was obtained from Dr. Taylor of the Chemistry Department. (This culture

having previously been furnished by Dr. Gilman.)

In attempting this study two kinds of media were used, Medium Q of Fulmer, Nelson, and Sherwood (1931) which contained the following constituents per 100 cc:

0.188 gms. of NH_4Cl
0.100 gms. of K_2HPO_4
10.00 gms. of sucrose

The pH of this medium as prepared was about 6.9. The other medium was a modification of Czapek according to Naylor, Weisbrodt-Smith, and Collins (1930) with glycerine substituted for sucrose, its composition being as follows:

$MgSO_4$	---	0.5 gm.
K_2HPO_4	---	~~0.1~~ 1.0 gm.
KCl	---	0.5 gm.
$FeSO_4$	---	0.01 gm.
NH_4Cl	---	3.5 gms.
Glycerine	---	10.0 gms.

Water to one liter.

The reasons for the glycerine were as follows:

1. To obtain an entirely different type of substrate;
2. To so far as possible eliminate anything of a directly biological origin;
3. To eliminate the possibility of the production of stimulative caramels through the high temperature sterilization that might result with sucrose. (Fulmer and Nuesselman, (1927), Fulmer, G.I. Williams, A. L. and Workman, C.H. (1931).

The procedure generally used was as follows: The medium was prepared as indicated and placed in 500 c.c. quantities in two-liter Erlenmeyer flasks and sterilized at 15 pounds for twenty minutes and then cooled and inoculated from stock spores of the respective molds kept on agar slants in the ice box and set aside at room temperature, which remained rather constant at 25 degrees, and allowed to grow. The more rapidly growing molds had a luxuriant mycelium in two weeks or less while the slow growers seemed to be making so little progress that the time of two weeks was arbitrarily used for the period of mold growth. The mold pads were then removed and the remaining media were sterilized, part of them by steam and part by filtration. Before sterilizing, however, the glycerine medium was enriched by adding 100 grams of sucrose per liter, while the sucrose media were used as obtained. For the steam sterilization the medium was placed in 50 c.c. quantities in 125 c.c. Erlenmeyer flasks and sterilized with steam at 15 pounds pressure for 20 minutes. The filtration sterilization was effected by filtering the material through a sterile Berkefeld filter, and then transferring 50 c.c. quantities into sterile 125 c.c. Erlenmeyer flasks. The flasks of media were then inoculated at the various indicated pH values (see Tables I and II) with a stock culture of Saccharomyces cerevisiae to a count of one. (Palmer, Nelson, and Sherwood, (1921)). This culture was number 4226 of the American Type Culture Collection and

was carried in stock culture in Medium C. After inoculation the flasks were incubated at 30 degrees Centigrade for 48 hours and the yeast count taken at that time. The reason for preparing the two series of flasks was to check the heat stability of the suspected stimulant, and also to avoid the excessive caramelization that would result from heating the inverted sugar. The data for the 5 molds both on glycerine and sucrose media are given in Tables I and II. The data show quite definitely that there is a stimulant produced by each of the several molds both on glycerin and sucrose substrates. This material withstands considerable heat since there is no appreciable decrease in the stimulative properties of the media sterilized by steam over that sterilized by filtration.

There is not a very great difference apparently in the relative amounts of stimulants produced by the various molds. Since the molds Aspergillus niger and Aspergillus clavatus are the more rapid growers, they will lend themselves most readily to further study.

It is interesting to note that the Penicillium roqueforti has none of the toxic properties described by Fleming (1929) as characteristic of certain of the Penicillia. This point was checked with much care with media from cultures of various ages. . It was found in one instance that a six weeks old culture had developed a very decided toxicity toward the yeast when grown on sucrose, but repeated attempts to check this point failed.

TABLE I

Counts Indicating Promotion of Stimulants by Various Molds Grown on Sucrose Media

Mold	Aspergillus niger				Aspergillus clavatus				Trichoderma lignorum			
Method of Sterilization	Steam		Filt.		Steam		Filt.		Steam		Filt.	
pH of Media	3.2	6.9	3.2	6.9	2.7	6.9	2.7	6.9	5.5	6.9	5.5	6.9
Yeast Count	73	93	88	86	45	102	32	88	109	89	67	80
	81	101	91	97	43	131	33	83	105	93	90	83
	75	111	97	89	48	115	30	98	87	92	85	93
	71	107	85	93	43	112	32	110	99	97	89	97
					47	151	36	93	103	85	79	88
					53	138	35	86	93	90	82	92
Final pH	2.2	2.4	2.3	3.7	2.6	3.0	2.8	3.1	2.5	2.7	3.6	3.1

Mold	Penicillium roqueforti				Rhizopus nigricans (plus)				Rhizopus nigricans (minus)				Medium C for check	
Method of Sterilization	Steam		Filt.		Steam		Filt.		Steam		Filt.		Steam	Filt.
pH of Media	4.0	6.9	4.0	6.9	3.7	6.9	2.7	6.9	3.7	6.9	2.7	6.9	6.9	6.9
Yeast Count	49	23	45	79	89	12	134	10	8	11	94	69	27	21
	22	22	50	72	94	12	126	7	5	10	97	75	26	22
	28	19	48	66	99	15	123	8	8	8	103	73	30	23
	31	24	53	71	105	13	138	3	3	3	99	80	33	20
					95	18	119	12	5	7	110	66	24	18
					90	14	127	6	9	9	93	73	28	22
Final pH	3.0	5.6	3.0	5.0	3.8	3.8	3.2	6.5	2.7	5.8	2.8	3.8	5.9	5.9

TABLE II

Counts Indicating Production of Stimulants by Various M

Grown on Glycerine Media

Mold	Aspergillus niger				Aspergillus clavatus				Trichoderma lignorum			
Method of Sterilization	Steam		Filt.		Steam		Filt.		Steam		Filt.	
pH of Media	2.3	6.9	2.3	6.9	3.6	6.9	3.7	6.9	3.1	6.9	3.1	6.
Yeast Count	8	55	7	66	50	94	45	74	6	131	2	
	3	54	11	66	58	93	49	77	7	116	4	
	8	51	5	76	46	89	58	83	4	116	3	
	7	49	5	69	48	97	53	73	5	133	7	
	7	53	6	57	53	103	47	63	2	106	4	
	9	50	8	68	49	89	53	69	3	137	4	
Final pH	2.2	2.7	2.3	2.9	3.3	3.6	2.4	3.4	2.1	2.6	3.1	5

Mold	Penicillium roqueforti				Rhizophus nigricans (plus)				Rhizophus nigricans (minus)			
Method of Sterilization	Steam		Filt.		Steam		Filt.		Steam		Fil	
pH of Media	2.7	6.9	2.7	6.9	3.1	6.9	3.1	6.9	3.2	6.9	3.2	6
Yeast Count	34	54	35	35	72	79	77	76	74	86	78	
	33	48	36	30	76	65	76	68	84	81	69	
	39	65	42	38	69	85	82	73	81	88	73	
	36	63	38	32	81	89	87	63	86	73	80	
	38	62	41	30	83	90	83	66	79	81	72	
	40	55	33	37	79	78	84	59	81	83	84	
Final pH	2.4	3.7	3.4	5.1	2.3	3.5	2.3	3.0	2.2	2.4	2.4	

From the data in Tables I and II it is evident that a substance has been produced by the various molds which acts distinctly as a yeast growth stimulant. This material apparently is of an extra-cellular nature and seems to be quite stable to heat. It is shown quite definitely that it is not a product of the caramelization of the hydrolyzed sugars of the media by the fact that it is present in the glycerine as well as in the sucrose media.

However, since there had been much charring in the sterilization of the sucrose media after the molds had grown and since, according to Fulmer and Nuscelman (1927), this caramelization is capable of producing marked stimulation, the following experiments were carried out to prove that this was not a significant source of the stimulant. A 100 gram portion of sucrose was dissolved in about 400 c.c. of water and to this about 25 c.c. of 1 to 4 HCl were added. The solution was allowed to stand for two days for inversion. It was then neutralized by adding NaOH to pH 6.9. The solution was made up to one liter, and one half of this sterilized by filtration, and one half by steam. A similar medium was made up using the same amount of HCl and NaOH, but adding the NaOH to neutralize the HCl before adding the sugar. This was sterilized in the same way. In addition to this Medium U was prepared and similarly sterilized. The data are given in Table III. These data show that there is a slight stimulation in the filtration sterilized inverted sugar, but not nearly enough stimulation to warrant saying that this was the reason for the enhanced growth when using the mold filtrates.

TABLE III

Studies on Invert Sugar

	Inverted Sugar		Sugar not Inverted containing NaCl		Medium C for check	
Method of Sterilization	Steam	Filt.	Steam	Filt.	Steam	Filt
pH before Growth	6.9	6.9	6.9	6.9	6.9	6.9
Yeast Counts:	39	57	46	45	46	36
	44	65	38	47	34	36
	41	58	37	37	44	32
	47	62	46	45	45	34
	46	59	38	42	40	38
	43	61	36	40	39	41
Average Count	43	60	40	41	42	35
pH after Growth	3.4	3.2	3.2	2.9	3.8	4.6

COMPOSITION OF MEDIA AS THEY AFFECT GROWTH AND PRODUCTION OF ACIDS BY MOLDS

In order to study the "Bios" content of molds as well as their "Bios" producing properties, it seemed desirable to attempt to get as rapid and luxuriant a growth of mycelium as possible and at the same time to attempt to increase the yield of products formed by them, acids, etc.

Since Aspergillus niger produced "Bios" in what appeared to be as large a quantity as any mold studied and seemed to be a mold that grew well and with considerable rapidity, it was chosen as the one to do further work on, and a medium highly suited for its growth was sought. Raulin (1863) was probably the first to substitute synthetic media for natural substrata, such as potato, carrots, meat, and their extracts. He sought to replace the ash of yeast used by Pasteur with salts essential to growth, and through this formulated the well known Raulin solution. This medium had the following composition: Water -- 1500 grams, can sugar -- 70 grams, tartaric acid -- 4 grams, ammonium tartrate -- 4 grams, ammonium phosphate -- 0.6 gram, potassium carbonate -- 0.6 gram, magnesium carbonate -- 0.4 gram, ammonium sulfate -- 0.25 gram, zinc sulfate -- 0.07 gram, ferrous sulfate -- 0.07 gram, potassium silicate -- 0.07 gram. Because this medium was so very complex, it has been considerably modified by various workers. Currie (1917) in his work on production of citric acid by Aspergillus niger proposed the following medium: Sucrose -- 125-150 grams, ammonium nitrate - 2-2½ grams, potassium dihydrogen phosphate -- 0.75 gram to 1 gram, magnesium sulfate -- 0.25 gram, hydrochloric acid to a pH to 3.4 to 3.5 in enough water to make a liter. Iron and zinc are not, he contends,

essential to maximum production of citric acid, but are essential to the normal functioning of the fungi. This medium is to be contrasted with the much more dilute medium originally used by Czapek which was revised by Naylor, Smith, and Collins (1930) in their work on Penicillium roqueforti. Duclaux (Jorgensen, (1925)) developed the mold on Raulin's medium and he showed that oxalic acid occurred as an intermediate product in the complete combustion of sucrose to carbon dioxide. Wehmer (Jorgensen, (1925)) showed that oxalic acid is sometimes absent during the growth of the fungus, whereas, it is invariably present when potassium nitrate is used as the source of nitrogen. If ammonium chloride is used no oxalic acid is found.

Elfing (Jorgensen, (1925)) found that there exist races of Aspergillus niger which tend to generate citric acid under circumstances when otherwise oxalic acid is produced.

Pruess, Peterson, Steenbock, and Fred (1931) give the following medium as one which produces very good yield of Aspergillus niger mycelium: Ammonium nitrate -- 10 grams, potassium dihydrogen phosphate -- 6.9 grams, magnesium sulfate -- 5 grams, ferric chloride -- 0.16 gram, zinc sulfate -- 0.05 gram, glucose -- 40 grams, water -- 1000 cc. With this medium the authors were able to produce 25.6 grams of Aspergillus niger mycelium per 100 grams of glucose.

Naylor, Smith, and Collins (1930) maintain that the source of the nitrogen is a very important factor in the growth of molds and give the following medium as producing the best growth of (largest felts) Pencillium roqueforti: Magnesium sulfate -- 0.5 gram, dipotassium phosphate -- 1.0 gram, potassium chloride - 0.5 gram,

ferrous sulfate -- 0.01 gram, ammonium chloride -- 5.3 grams, sucrose -- 2.5 grams, enough water to make a liter of solution.

In the mold study to be carried out, it was desired to attempt to determine whether the molds produced a stimulant of an extra-cellular nature or an intra-cellular nature or both. Consequently, Medium Q (Fulmer, Nelson, and Sherwood, (1921)) was inoculated in 500 cc quantities with Aspergillus niger, the medium having been previously sterilized at 15 pounds pressure for 30 minutes in an autoclave. The growth of the mold was slow but after considerable time had elapsed (about 6 to 8 weeks) very good growth resulted. Addition of magnesium sulfate in half gram quantities per 500 cc of medium greatly speeded up the growth. The Aspergillus niger grew reasonably well in Naylor's medium and also in Naylor's medium modified by substituting 10 grams of glycerine for the $2\frac{1}{2}$ grams of sucrose, But Aspergillus niger refused to grow at all in medium containing only the following: Glycerine -- 10 grams, ammonium chloride -- 1.88 grams, di-potassium phosphate -- 1.0 grams. This medium is essentially Fulmer, Nelson, and Sherwood's Medium Q with glycerine substituted for sucrose. The reason for the failure to obtain growth in this medium might lie in the fact that the glycerine was of a high degree of purity, while the sucrose was of a commercial grade and consequently traces of mineral impurities might be in the sucrose which would not be present in the glycerine.

Herrick (1931) in his work on the production of kojic acid by Aspergillus flavus gives the following medium as the one he found to produce maximum yields of that compound: Magnesium

sulfate -- 0.5 gram, potassium chloride -- 0.1 gram, phosphoric acid -- 0.054 gram, ammonium nitrate -- 3.5 grams, dextrose -- 30 grams, water to a liter.

The previously discussed media were prepared in accordance with the directions of the authors and each was inoculated with a small quantity of spores of Aspergillus niger. The original source of this organism was unknown, it had been obtained from the Botany Department through the kindness of Dr. Gilman. The results of these experiments are given in Table IV.

TABLE IV

Data Comparing the Growth of Aspergillus niger on Various Media

Worker	Time Grown	cc N/10 base per 10 cc of media	Wt. of mycelium per 500 cc of media
Fred, Peterson, Steenbock & Pruess	14 days	11.7	6.17
Currie	14 days	32.4	6.18
Herrick	14 days	18.18	6.53
Naylor, Smith, Collins, (modified Czapek)	14 days	3.3	2.035
Fulmer, Nelson, & Sherwood Medium C	14 days	5.5	3.63

In obtaining these data the following procedure was carried out; to obtain the cc of tenth normal base equivalent to 10 cc of the media that had supported mold growth a 10 cc sample of

this material was diluted with distilled water, boiled to expel CO_2, and titrated to neutrality with tenth normal base in the presence of phenolphthalein. To obtain the weight of the mycelium the medium was filtered and the residue which consisted of mycelium washed with distilled water and dried at 60 degrees C.

Since these data are not conclusive, it was considered desirable to attempt to find as nearly as possible the optimum conditions of growth for Aspergillus niger. To do this, the following media were prepared:

1. Glycerine -- 10 grams, K_2HPO_4 -- 1 gram, NH_4Cl -- 5.3 grams, water to one liter.
2. Glycerine -- 10 grams, K_2HPO_4 -- 1 gram, NH_4Cl -- 5.3 grams, $FeSO_4$ -- 0.01 gram, water to one liter.
3. Glycerine -- 10 grams, K_2HPO_4 -- 1 gram, NH_4Cl -- 5.3 grams, $MgSO_4$ -- 0.5 gram, water to one liter.
4. Glycerine -- 10 grams, K_2HPO_4 -- 1 gram, NH_4Cl -- 5.3 grams, $FeSO_4$ -- 0.05 gram, $MgSO_4$ -- 0.5 gram, water to one liter.
5. Glycerine -- 10 grams, K_2HPO_4 -- 1 gram, NH_4Cl -- 5.3 grams, $FeSO_4$ -- 0.01 gram, $MgSO_4$ -- 0.5 gram, water to one liter.
6. Sucrose -- 10 grams, K_2HPO_4 -- 1 gram, NH_4Cl -- 5.3 grams, water to one liter.

These media were placed in 100 cc quantities in 300 cc Erlenmeyer flasks, sterilized with steam at 15 pounds for 15 to 30 minutes and inoculated with Aspergillus niger at the pH of the medium

itself. The results are given in Table V.

TABLE V

Study of the Composition of the Media as Effecting Mold Growth

Media Number	Relative growth of mycelium	Sporulation	Wt. of mold per flask
1	Very slight	Some	0.035 grams
2	Very slight	Some	0.040 grams
3	Fair	Much	0.37 grams
4	No growth	0	0
5	Fair	None	0.30 grams
6	Good	Much	0.48 grams

From the preceding experiment the following conclusions may be drawn for the concentrations used:

1. The presence of iron retards sporulation.
2. The presence of iron is not essential.
3. Magnesium is essential in glycerine media.
4. Magnesium is not essential in a medium containing commercial sucrose.

Much work has been done studying the effect of the reaction of the medium in its relation to mold growth. Webb (1919) contends that Aspergillus niger gives increasing percentage of germination as the acidity rises from pH 7.0 toward a maximum which is about 3.0. Currie (1917) claims that a pH of 3.4 to

3.5 is optimal for the growth of Aspergillus niger in a production of citric acid. Other workers have found that a growing range for this fungus varies from a pH of 2.8 to 6.6 showing that there is much less tolerance for alkaline than for acid conditions. To check this, Currie's medium was prepared, placed in 500 cc quantities in two liter Erlenmeyer flasks, sterilized with steam, inoculated with Aspergillus niger spores, hydrochloric acid being added to the medium in varying quantities to produce media of different pH. In addition to this, 0.01 gram of zinc sulfate was added to another. The mold was allowed to grow for fourteen days. At the end of that time, 10 cc samples of the solution under the mold were diluted with water, boiled to expel carbon dioxide and titrated with sodium hydroxide in the presence of phenolphthalein, and the mycelium from the various flasks was removed, washed by pouring in a Buchner funnel and pouring water over it, then dried at 60 degrees C. and weighed. From the data one might conclude that there is even better growth at a pH of 3.0 than at 3.5 as recommended by Currie. It was quite evident that sporulation was much retarded as the acidity increased. Further, the addition of small quantities of iron and zinc salts produced greatly enhanced growth with very little sporulation. The data for the preceding experiment are given in Table VI.

TABLE VI

The Effect of pH on the Growth of

Aspergillus niger in Currie's Medium

pH	cc N/10 base after deduction mg. of HCl added.	Wt. of mycelium from 500 cc.
4.7	20.0	4.72
4.0	18.0	0.10
3.3	16.1	0.87
3.0	15.0	11.42
2.5	10.1	0.11
2.0	8.0	0.27
1.5	4.1	0.58
1.1	no very slight growth	
1.0	no growth	

	pH	cc N/10 NaOH for 10 cc	
0.01 gm. $FeSO_4$	5.5	20.7	11.85
0.01 gm. $MnSO_4$	5.5	30.5	10.45

Literature studies indicate that Aspergillus niger is decidedly omnivorous with respect to its nitrogen requirements. Czapek used sodium nitrate as a source of nitrogen in his synthetic media. Hellard (1914) in studying the alcoholic fermentation by Saccharomyces niger reports greater activity from the addition of potassium nitrate than from the use of ammonium chloride in the same quantity. Others contend that in general ammonium salts tend to increase the production of mycelium and that for mold growth, nitrogen is more readily available in this form than in the nitrate form. In order to study the effect of various sources of nitrogen, the

following media were devised. At the same time, it was deemed advisable to check the effect of iron salts in these various media. It was not considered necessary, however, to repeat the same experiment using zinc salts because of the similarity of the action of the two substances. The media prepared were as follows:

1. Sucrose - 135 grams, ammonium nitrate - 2 grams, potassium dihydrogen phosphate - 0.75 grams, magnesium sulfate - 0.2 grams, water to one liter.
2. Same as number one but containing in addition 0.01 gram of ferrous sulfate per liter.
3. Sucrose - 135 grams, potassium nitrate 5.59 grams, potassium dihydrogen phosphate - 0.75 grams, magnesium sulfate - 0.2 grams, water to one liter.
4. Same as number three but containing in addition 0.01 gram of ferrous sulfate per liter.
5. Sucrose - 135 grams, ammonium chloride - 3.55 grams, potassium dihydrogen phosphate - 0.75 gram, magnesium sulfate - 0.2 gram, water to one liter.
6. Same as number five but containing in addition 0.01 gram of ferrous sulfate per liter.

The pH of each of these media was adjusted to 5.0 by adding hydrochloric acid after sterilization. The nitrogen present in each was in equimolar proportions. Five 300 cc Erlenmeyer flasks each containing 100 cc of the medium were run for each kind of medium. The data are given in Table VII.

Table VII

Data Showing Effect of Varied Nitrogen Sources and Also Showing the Stimulating Effect of $FeSO_4$

Medium No.	pH	cc N/10 NaOH per 100 cc of media	Relative Sporulation	Wt. of mycelium per 500 cc
1	3.0	9.12	Much	10.45
2	3.0	19.63	Moderate	16.16
3	3.0	19.45	Very much	7.15
4	3.0	19.50	Much	15.30
5	3.0	20.40	Much	7.45
6	3.0	20.80	Slight	20.90

Medium No.	Source of Nitrogen	Remarks
1	NH_4NO_3	No Fe
2	NH_4NO_3	$FeSO_4$ added
3	KNO_3	
4	KNO_3	$FeSO_4$ added
5	NH_4Cl	
6	NH_4Cl	$FeSO_4$ added

From the data of Table VII it is evident that the ammonium ion as a source of nitrogen gives both a higher total titratable acidity and a larger quantity of mycelium per unit quantity of nutrient solution. In addition to this the iron

added tended to repress sporulation and caused a marked increase in mycelium.

It was now considered advisable to study the effect of both iron and zinc salts on the growth of Aspergillus niger over a rather wide range of concentrations. In order to do this a medium was prepared containing per liter: Sucrose - 125 grams, potassium dihydrogen phosphate - 0.75 gram, ammonium chloride - 3.55 grams, magnesium sulfate - 0.2 gram. 100 cc portions were placed in 300 cc Erlenmeyer flasks and the pH adjusted to 3.0 with hydrochloric acid after sterilization. To this medium zinc sulfate and ferrous sulfate were added in varying amounts from a stock solution. In order to more accurately study the effect of these salts on the growth of the fungus, the acidity of the various flasks was titrated in the usual manner at intervals throughout a fourteen day growing period. The results obtained are given in Tables VIII and IX.

TABLE VIII

The Effect of Ferrous Sulfate and Zinc Sulfate

On The Growth of Aspergillus niger

$ZnSO_4$

Grams of $ZnSO_4$ per 100 cc	cc N/10 base for 10 cc		Wt. of mycelium per 100 cc
0.005	11.4		4.37
0.01	12.4		4.34
0.05	14.55		3.92
0.1	18.15	Not reliable	3.845
0.5	23.5	because of	3.66
1.0	24.2	$ZnSO_4$ titration	1.65
2.0	20.3		0.11

TABLE VIII (Continued)

$FeSO_4$

Grams of $FeSO_4$ per 100 cc	cc N/10 base for 10 cc	Wt. of mycelium per 100 cc
0.005	14.3	3.23
0.01	11.7	3.28
0.05	11.7	3.10
0.1	11.3	3.28
0.5		2.08
1.0		None
2.0		None

TABLE IX

The Effect of Ferrous Sulfate and Zinc Sulfate on the Production of Acid and Mycelium by Aspergillus niger

Grams of $FeSO_4$ per 100 cc	Grams of $ZnSO_4$ per 100 cc	cc N/10 base for 10 cc	Wt. of mycelium per 100 cc
0.005	0.005	18.3	3.47
0.01	0.01	15.8	3.56
0.05	0.05	11.7	3.80
0.1	0.1	9.6	3.97
0.5	0.5		2.96
1.0	1.0		0.15
2.0	2.0	No growth	0.0
Check 0	0	7.13	3.45

The experiment pertaining to $ZnSO_4$ and $FeSO_4$ was repeated using a narrow range of concentrations. In addition, the acidity was titrated at frequent intervals to determine the rate of its formation.

TABLE X

Data Showing the Effect of $FeSO_4$ and of $ZnSO_4$ on Aspergillus niger over a Range of Concentrations

Gms. of $FeSO_4$ per 100 cc	cc of N/10 base for 10 cc 3rd day	5th day	7th day	10th	14th	Wt. of mycelium
0.0001	3.6	5.33	6.0	6.4	6.8	1.015
0.0005	4.7	6.6	6.4	6.4	7.5	1.047
0.001	3.42	5.3	6.4	8.6	9.0	0.997
0.005	3.84	6.4	6.6	7.2	7.5	0.917
0.01	4.6	6.6	6.6	8.1	7.5	1.077
0.05	5.75	9.9	11.3	14.5	15.6	1.543
0.1	4.7	10.4	14.3	17.1	19.2	1.546

$ZnSO_4$

Gms. of $ZnSO_4$ per 100 cc	cc of N/10 base for 10 cc 3rd day	5th day	7th day	10th	14th	Wt. of mycelium
0.0001	5.4	10.7	13.0	13.7	14.7	1.501
0.0005	5.6	18.1	28.2	36.3	43.5	2.023
0.001	6.4	29.2	38.0	42.5	51.0	2.379
0.005	4.7	23.6	33.0	41.0	47.8	2.549
0.01	9.0	30.2	34.2	39.6	47.0	2.432
0.05	6.8	8.5	20.3	32.4	36.1	2.039
0.1	6.0	18.3	23.7	26.1	30.2	1.938

It is evident from the data in Tables VIII, IX, X, and XI that the addition of $ZnSO_4$ and $FeSO_4$ has a very marked effect on the production of both mycelium and acid. There is quite definite stimulation by $FeSO_4$ alone, much more stimulation with $ZnSO_4$ alone, and a very marked increase of stimulation by combinations of $ZnSO_4$ and $FeSO_4$. The optimum condition of these salts is not very definite but lies somewhere around 0.01 to 0.1 gram of each salt per liter of media.

TABLE XI

The Effect of Mixtures of Ferrous Sulfate and Zinc Sulfate on Production of Acid and Mycelium by Molds

Gms. $FeSO_4$ per 100 cc	Gms. $ZnSO_4$ per 100 cc	Titratable acidity N/10 3rd day	5th day	7th day	10th day	14th	Wt. of mycelium per 100 cc
0.001	0.001	6.4	28.9	39.5	46.0	48.0	1.913
0.001	0.005	11.7	38.9	49.5	54.5	56.2	2.005
0.001	0.01	8.85	36.2	49.1	55.4	57.3	2.068
0.001	0.05	6.85	29.9	36.6	43.7	49.5	2.414
0.005	0.001	11.7	34.2	44.7	49.4	53.2	1.908
0.01	0.001	6.85	28.8	40.1	44.6	48.6	1.902
0	0	3.85	7.7	5.35	6.0	6.1	1.045

In a manner similar to that used in finding the optimum concentration of zinc and iron salts, magnesium sulfate was added to the following medium in varying amounts: Sucrose - 135 grams, potassium dihydrogen phosphate - 0.75 gram, ferrous sulfate - 0.1 gram, zinc sulfate - 0.1 gram, ammonium chloride - 3.55 grams. This medium was placed in 500 cc quantities in two liter Erlenmeyer flasks and the acidity and weight of mycelium tested after two weeks growth. The acidity is distinctly low. The reason for this probably lying in the fact that with the larger quantity of medium the surface volume ratio was upset and was not so near the ideal surface volume ratio as one would obtain using 100 cc of media in a 300 cc flask. The data are given in Table XII. It will be noted that the optimum concentration of magnesium sulfate lies somewhere in the vicinity of 0.8 gram per liter, but as in the case of the zinc and iron salts this optimum is not extremely sharp.

TABLE XII

Effect of $MgSO_4$ on the Growth of Aspergillus niger over a Range of Concentrations

Grams of $MgSO_4$ per 500 cc of media	cc of N/10 base for 10 cc of media	Wt. of mycelium per 500 cc of medium
0.0	5.8	4.15
0.001	7.3	9.66
0.01	7.3	11.45
0.1	18.8	12.03
0.2	14.9	14.13
0.4	15.4	14.23
0.8	14.9	14.100
1.6	14.9	14.08
2.0	19.2	14.95
3.0	18.2	13.02
4.0	13.8	13.11

Since nitrogen is said to exert a very marked effect on the growth of the fungus, the effect of varying the concentration of ammonium chloride was studied using the following medium: Sucrose - 125 grams, magnesium sulfate - 0.2 gram, ferrous sulfate - 0.1 gram, zinc sulfate - 0.1 gram, potassium dihydrogen phosphate - 0.75 gram. The above medium was placed in 100 cc quantities in 300 cc Erlenmeyer flasks, and the acidity titrated at intervals throughout the fourteen day growing period. It is extremely interesting to note that the production both of acids and of mycelium is very markedly affected by varying concentrations of ammonium chloride. It is also noted that the optimum concentration for the production of acids is quite distinct and lies around 1.875 grams of ammonium chloride per liter of solution. This is particularly interesting because this was the same optimum found by Fulmer,

Nelson, and Sherwood, (1921) in their studies on the nutritional requirements of yeast. The optimum for production of mycelium is considerably higher than this lying around 3.2 to 3.5 grams per liter and is not nearly so sharply defined. The data for this experiment are in Table XIII.

TABLE XIII

Data Showing the Effect of NH_4Cl over a Range of Concentrations

Grams NH_4Cl per 100 cc	3rd day	5th day	7th day	9th day	11th	14th	Wt. of mycelium per 100 cc
0	1.07	1.60	1.55	1.60	1.60	1.60	0.083
0.005	1.28	1.48	2.52	2.63	2.60	3.60	0.179
0.05	5.34	5.03	5.48	5.40	5.50	5.50	0.665
0.1	14.95	16.00	17.50	17.75	16.85	21.30	1.273
0.125	18.20	20.50	21.70	22.40	20.56	23.65	1.629
0.1375	22.45	23.83	25.20	26.50	26.55	27.00	1.71
0.150	29.95	38.90	40.02	40.05	38.40	43.00	1.95
0.1625	27.90	36.30	37.90	37.95	38.00	39.50	2.20
0.175	28.30	46.90	55.00	55.00	56.30	56.30	2.13
0.1875	26.30	35.63	74.50	73.50	67.85	64.33	2.66
0.200	22.83	61.10	73.25	72.40	66.60	63.20	2.67
0.2125	20.05	55.50	71.50	70.00	63.20	56.10	2.38
0.225	19.30	54.30	60.06	45.00	16.65	5.83	3.91
0.2375	17.65	46.20	60.03	55.00	28.07	9.00	3.08
0.250	21.51	54.30	60.10	46.70	14.30	6.58	3.02
0.275	16.30	43.75	47.30	30.80	7.35	6.05	3.37
0.300	12.78	33.30	36.63	20.81	7.45	6.83	3.36
0.325	13.45	30.40	31.20	9.50	7.05	7.05	3.44
0.350	13.85	29.30	28.00	8.50	6.60	7.80	3.41
0.400	14.15	26.95	21.90	9.33	7.20	7.67	3.36
0.500	11.73	16.23	17.32	13.15	11.30	10.51	3.44
0.750	10.66	13.34	16.45	17.75	17.20	18.40	3.49
1.00	11.73	14.53	17.32	20.00	18.10	19.36	3.83

In a similar manner the optimum concentration of potassium dihydrogen phosphate was found using the following

medium : Sucrose - 135 grams, ferrous sulfate - 0.1 gram, zinc sulfate - 0.1 gram, ammonium chloride - 1.875 mg., magnesium sulfate - 0.8 gram, per liter of solution, potassium dihydrogen phosphate being added in varying amounts from a stock solution. (The procedure in varying the concentrations of these various salts was exactly the same. The medium was prepared in such a concentration that 90 cc contained nutrients equivalent to 100 cc. This allowed 10 cc for dilution. The required amount of salt solution being studied was added, then sufficient distilled water added to make up the total volume to 100 cc per flask.) In the case of potassium dihydrogen phosphate series of flasks, the pH was not adjusted but was left that of the medium. This was to evade the difficulty arising from the buffer action of the potassium dihydrogen phosphate. The optimum concentration for the potassium dihydrogen phosphate, it will be noted, lies around 1.25 grams per liter and is rather sharply defined. This optimum holds rather closely both for acid production and for production of mycelium. The data are given in Table XIV.

In an effort to determine the optimum concentration of sucrose the following medium was devised: Potassium dihydrogen phosphate - 1.38 grams, ferrous sulfate - 0.1 gram, zinc sulfate - 0.1 gram, magnesium sulfate - 0.8 gram, ammonium chloride - 1.875 grams. Sugar was added to this in varying amounts and the acidity adjusted to a pH of 3.0

with the aid of hydrochloric acid. Fermentation was carried out in 100 cc quantities of medium in 300 cc Erlenmeyer flasks. The yield of acid was extremely low as compared to previous yields and gave the maximum yields in concentrations of 12% to 18% sucrose. The data are given in Table XV.

TABLE XV

Data Showing Effect of KH_2PO_4 on the Growth of Aspergillus niger over a Range of Concentrations

Grams KH_2PO_4 per 100 cc of media	cc of N/10 base for 10 cc*					
	4th day	6th day	8th day	11th day	14th day	Weight of mycelium
0.001	0.86	1.71	2.3	2.2	2.35	0.16
0.005	1.49	3.00	3.4	4.0	4.0	0.24
0.01	2.35	4.7	6.4	6.4	6.4	1.18
0.03	10.3	17.1	19.6	20.6	21.1	1.86
0.05	14.5	32.5	38.8	32.6	43.2	3.04
0.07	18.3	35.9	41.9	45.4	48.2	3.23
0.08	11.1	37.2	45.6	50.3	54.6	2.26
0.09	23.4	48.2	59.3	64.6	68.5	2.40
0.1	28.6	52.1	60.2	65.8	67.5	2.39
0.135	32.0	60.0	70.1	73.6	74.0	2.36
0.150	22.6	47.0	56.0	62.9	58.5	2.13
0.175	24.3	47.0	53.6	53.5	52.0	2.15
0.200	21.8	43.6	49.6	49.6	51.3	2.14
0.250	24.3	39.9	42.8	44.0	45.3	2.36
0.300	27.8	39.8	40.1	39.9	40.6	2.13
0.40	33.5	34.2	36.3	36.2	36.2	2.22
0.45	21.4	29.1	31.2	30.8	35.4	2.15
0.50	32.6	31.1	31.6	31.6	32.0	2.08
0	0.64	0.64	1.07	1.2	1.3	0.15

* Not corrected for titration of phosphate (PO_4) in media.

Table XV

Yields of Acid and Mycelium Produced with Varying Quantities of Sucrose

Gms. of Sucrose per 100 cc of media	cc of N/10 base per 10 cc of acid						Wt. of mycelium per 100 cc
	3rd day	5th day	7th day	9th day	11th	14th	
1	1.71	1.7	1.7	1.5	1.5	1.5	0.40
2	2.13	2.2	2.0	1.7	1.7	1.8	0.71
5	2.8	4.9	2.6	2.6	2.4	2.4	1.39
6	4.5	7.5	8.8	8.8	7.5	4.5	1.67
7	4.1	6.7	9.9	10.9	10.7	10.6	1.82
8	5.8	11.9	15.9	14.9	14.7	13.9	1.91
9	7.8	12.8	14.1	18.1	17.7	17.1	2.08
10	8.5	13.1	16.0	18.3	18.3	19.1	2.33
11	11.1	19.3	21.1	23.1	23.1	22.4	2.45
12	10.6	17.1	19.8	20.3	21.4	22.0	2.23
13	10.3	19.0	21.6	23.2	23.6	24.8	2.33
14	10.5	17.2	19.0	20.0	20.7	20.9	2.51
15	11.3	20.5	23.1	23.5	24.1	24.2	2.89
17	11.6	22.9	25.9	27.5	25.6	26.5	2.56
18	12.4	20.5	22.9	25.2	24.4	24.2	2.53
20	10.7	19.0	21.4	21.1	23.3	20.0	2.17
22	8.6	15.6	17.5	18.8	18.8	19.1	1.95
24	9.4	17.1	18.4	19.0	19.0	19.4	2.05
25	6.7	10.5	13.6	14.3	14.9	15.8	2.04

Summary of the Studies on the Effect of the Composition of the Media on the Growth of Aspergillus niger

From the preceding data one may readily see that the composition of media may be varied somewhat and still produce extremely good growth along with high yields of acid. However, the medium which would probably give the highest yields of acid would have approximately the following composition per liter:- Sucrose - 100 to 125 grams, potassium

dihydrogen phosphate - 1 to 1.5grams, ferrous sulfate - 0.05 to 0.15 gram, zinc sulfate 0.05 to 0.15 gram, magnesium sulfate - 0.7 to 1 gram, ammonium chloride - 1.75 to 2.25 grams. In order to get slightly higher yields of mycelium one can increase just a little the zinc and iron and the ammonium chloride.

It must be borne in mind too that the surface volume ratio is of much importance. Much better results were obtained using 100 cc of medium in 500 cc flasks than in using 500 cc of media in two liter Erlenmeyer flasks. In the first case, one has a total surface area of about 44.8 square cm. In the latter case, about 178 sq. cm. of surface area. If one divides each surface by the volume of the liquid under it, one has the surface volume ratio. One has then, 44.8/100 or 0.448 as the surface volume ratio for the small flask and 176/500 or 0.352 for the two liter flasks. However, 0.448 may not be the best surface volume ratio for there is not sufficient data to determine that definitely.

THE NATURE OF THE MATERIALS PRODUCED BY MOLDS THAT STIMULATE YEAST GROWTH

It has been previously shown that a stimulative material has been produced by each of the several molds studied. Of these molds, however, the two, Aspergillus niger and Aspergillus clavatus grew the most rapidly and produced a comparatively very high yield of the "Bios". Therefore, these two molds were chosen to do further work with; namely, to attempt to study the material or materials produced by them that seem to stimulate yeast. The object being to grow these molds in sufficient quantities that both the composition of the media on which they had grown and the composition of their mycelia could be studied.

For a study of the stimulant remaining in the media or the extra-cellular stimulant it was found to be more desirable to use the glycerine medium because the sucrose remaining behind after the mold had grown made further fractionation and purification very difficult. In each instance the composition of the media was that previously indicated by the Medium G of Fulmer, Nelson, and Sherwood, or the modified media of Czapek used by Naylor, Weisbrodt-Smith, and Collins (1940) and will for simplicity be merely designated by the sucrose medium and the glycerine medium respectively.

The procedure in general was as follows: The medium was prepared as indicated, sterilized in 500 c.c. quantities

in two liter Erlenmeyer flasks and cooled and inoculated with the desired cultures and incubated at room temperature for fourteen days. The mycelium was then removed by filtration, washed with distilled water and dried, and the filtrate used for further fractionation and stuey. In this way a large volume of filtrate was obtained as well as a considerable quantity of mycelium.

A STUDY OF THE STIMULANT FROM ASPERGILLUS CLAVATUS ON GLYCERINE

The Volatility of the Stimulant

One liter of the Aspergillus clavatus filtrate from glycerine medium was distilled until 100 c.c. of solution remained. The distillate was saved as well as the residue. Now, if the stimulant is volatile, it should be found in the distillate, if non-volatile, it should be found in the residue for it has been previously shown that the material is quite stable to heat. Therefore, the following series of media were prepared using the distillate and the residue:

A. Using nutrient salts in the proper proportions to prepare the original glycerine medium plus 50 grams of sucrose, 500 c.c. of medium were prepared using the distillate as a solvent.

B. The 100 c.c. of residue contain sufficient nutrient salts for one liter of the glycerine medium if they have not been used up by the molds. Therefore, 50 c.c. of this residue were

enriched with 50 grams of sucrose and made up to one-half liter with distilled water.

C. It might be possible that there is more than one substance responsible for the stimulation, and that part of the stimulant might be volatile and part non-volatile, so using 25 c.c. of the residue plus 27½ grams of sucrose, plus enough distillate to make 250 c.c., one has then a combination of all the possible stimulants.

D. The original mold filtrate made up to a 10% concentration of sucrose. This is to check if there is actually a stimulant present.

E. The glycerine medium made up to 10% sucrose to serve as a check on yeast growth.

Summarized, the purpose of the five media are as follows:

A. Checks the stimulant in the distillate.

B. Checks the stimulant in the residue.

C. Detects whether or not the stimulant is a combination of volatile and non-volatile substances.

D. Checks if there is a stimulant.

E. Checks the yeast growth.

The above media were prepared and placed in 50 c.c. quantities in 125 c.c. Erlenmeyer flasks and sterilized with steam and inoculated with Saccharomyces cerevisiae to a count of one, and incubated for 43 hours at 30 degrees and the count taken. This is the usual procedure.

The data are given in Table XVI

TABLE XVI

The Relative Stimulative Properties of the Residue and Distillate from *Aspergillus clavatus* on Glycerine

Fraction being tested	A	B	C	D	E
Original pH	6.55	2.55	2.55	2.2	6.9
Adjusted to	6.9	6.9	6.9	6.9	6.9
Yeast Counts	48	89	108	98	16
	45	95	100	106	18
	47	104	99	113	15
	50	98	99	95	19
	43	110	95	93	17
	48	93	89	99	19
Average Count	47	98	98	98	17
Final pH	3.1	3.25	3.25	3.55	5.1

From the preceding data it is evident that the stimulant is non-volatile and is not destroyed by boiling, therefore, it should be possible to concentrate it by evaporation of its solution and thus obtain it in a form for further study and possible identification.

Concentration of the Stimulant

Eight liters of *Aspergillus clavatus* filtrate from the glycerine medium were evaporated to a small volume on a steam plate and then to almost complete dryness on a water

bath. About 50 grams of a dark somewhat gummy mass remained. This was washed thoroughly with 95% alcohol and about 35 grams of crystalline material remained behind in the alcohol. The original residue was designated as "A". The insoluble material as "B", and the alcohol soluble material as "C". A small quantity of the alcoholic solution was evaporated to dryness, the residue so obtained tested along with fractions "A" and "B" for their Bios activities.

In testing the activity of the various solid fractions the following procedure was used: The material to be tested was weighed and dissolved or suspended in water generally to 1% concentration. Then medium Q which was used as the standard medium for most of the yeast work, was prepared in such concentration that 45 c.c. of this solution contained sufficient nutrient salts for 50 c.c. of medium Q. This medium was then measured out in 49 c.c. quantities in 125 c.c. Erlenmeyer flasks and sterilized. After sterilization, the sterilized material to be tested was added in varying amounts to these flasks and the total volume made up with distilled water to 50 c.c. The media were then inoculated with yeast in the usual way. The data for the three fractions are given in Table XVII. It is clearly indicated from these data that the stimulant is concentrated in the alcohol soluble fraction. However, very likely there would be present some inorganic salts from the media as well. To remove as much of these as possible this alcoholic solution was concentrated to about 500 c.c., and an equal volume of ether added. A copious whitish precipitate formed which weighed about 6 or

7 grams. The liquid was evaporated to dryness on the water bath and a residue of about 6 grams of tarry material obtained. The material precipitated by ether will be indicated by "B", while the material soluble in the ether alcohol mixture will be designated by "C". The potency of these two fractions was tested as before and the data are given in Table XVIII.

Table XVII

Fractionation of Stimulant by Means of Alcohol

A		B		C		D	
Mg.St.*	Count	Mg.St.*	Count	Mg.St.*	Count	Check Mg.St.*	Count
2	14	2	14	2	21	0	21
10	28	10	17	10	47		15
20	48	20	19	20	80		14
40	92	40	23	40	105		15
60	91	60	32	60	150		19
80	90	80	42	80	196		20

A is original residue after evaporation.

B is alcohol insoluble material.

C is alcohol soluble material.

D is original media that has not supported mold growth plus 10% sucrose for check.

* Mg.St. is milligrams of solid stimulant concentrate per 100 c.c. of media.

TABLE XVIII

Further Fractionation of Alcohol Soluble Material with Ether

Material precipitated by ether		Material not precipitated by ether		
Fraction D Mg.St.*	Count	Fraction E Mg.St.*	Count	Checks
2	33	2	140	29
10	21	10	197	28
20	29	20	220	28
40	34	40	179	29
60	23	60	164	24
80	26	80	154	

* Mg.St. is milligrams of stimulant per 100 c.c. of media.

It has been shown by the data in Table XVIII that the stimulant is present in its highest concentration in the residue from the ether alcohol solution. Consequently, this fraction was used for further purification. A 2 gram sample of this fraction was boiled with norite, then filtered and evaporated very carefully to dryness. A clear sirupy material considerably more viscous than glycerine remained. This residue weighed about 1½ grams. This material retained almost the full potency of the original material as is shown by the data in Table XIX.

TABLE XIX

Effect of Norite on Alcohol Ether Soluble Fraction

Before decoloring Mg.St.*	Count	After decoloring Mg.St.*	Count	Checks
2	70	2	63	20
10	144	10	139	24
20	176	20	150	28
40	173	40	164	25
60	185	60	189	22
80	183	80	195	

It has been contended by other workers (Wildiers, (1901)) that Bios is not soluble in strong alcohol, but is soluble in dilute alcohol. Addition of absolute alcohol had little effect on the decolorized material. Consequently, some of this material which had been purified by norite, was dissolved in just a very little water (it is very soluble) and about 200 c.c. of absolute alcohol added and shaken thoroughly. A white somewhat granular material separated which weighed about 0.7 of a gram (one gram of material was started with), and on evaporating the alcohol solution to dryness, about 0.25 gram of the whitish crystal material remained. The activity of the two fractions was tested in the usual way and it was found that the stimulant could be prepared in a considerably purer form by the use of the very strong alcoholic solutions. This is shown by the data in Table XX.

TABLE XX

Fractionation of Stimulant by Absolute Alcohol

Mg. per 100 c.c. of media	A*	B*	Checks
2	132	38	28
10	186	31	27
20	195	35	21
40	205	30	
60	195	42	
80	167	48	

* A = yeast counts for the fraction insoluble in absolute alcohol.

B = yeast counts for the fraction soluble in absolute alcohol.

Stability of the Stimulant

A one gram sample of the material soluble in the ether alcohol solution was boiled with 10% KOH for 15 minutes then neutralized with HCl and diluted to 100 c.c. In a similar way a one gram sample was boiled with HCl solution made by adding 5 c.c. of concentrated HCl to 45 c.c. of water, for 15 minutes, then neutralized with KOH. The potency of these two preparations was then tested in the usual way. The results are given in table XXI.

TABLE XXI

Stability of Stimulant

Mg. of substance per 100 c.c.	A	B	C	D
2	19	31	29	
10	34	56	48	
20	42	65	65	
40	49	83	99	
60	61	136	152	
80	104	149	143	
0				17
0				19
0				16

A represents yeast counts for the material which was boiled with KOH

B represents the yeast counts for the material which was boiled with HCl when used in the various indicated concentrations.

C represents the yeast counts in medium C under the same conditions, which serves as a check.

From the data in the preceding table it is evident that the material which is active in the stimulant is quite stable to acid and to alkali. This does not seem to be

entirely in accord with Wildiers who described (1901) Bios as being stable to dilute acid but changed by boiling in one percent NaOH. Therefore, it might be reasonable to assume that the material with which we are working is not the same as that with which Wildiers worked.

The Optimum Concentration of the Stimulant

In an effort to determine the optimum concentration of the material, a quantity of the material soluble in the ether alcohol mixture and decolorized by norite was added to Medium G in varying concentrations to form a series of flasks. These were inoculated in the usual way and the counts taken in 48 hours. The data are indicated in table XXII.

TABLE XXII

Optimum Concentration of Stimulant Present In Alcohol-Ether Fraction Decolorized by Norite

Mg. of Material	Count	Count per Mg. of Stimulant	Checks
2	77	38.5	
20	107	5.35	
40	184	4.6	
60	218	3.66	
80	212	2.65	
100	220	2.20	
200	238	1.19	
400	228	0.57	
600	237	0.398	
800	221	0.276	
0			20
0			22
0			22
0			23

From the preceding table it may be seen that the optimum concentration of the stimulant in this degree of purification is about 30 milligrams per 100 c.c.

Study of the Filtrate from Aspergillus niger Grown on Glycerine Media

Twenty liters of filtrate of Aspergillus niger grown on glycerine media were evaporated to dryness on a steam bath. About 150 grams of a somewhat dirty brown mass were obtained. This was fractionated in the same way that the material from the Aspergillus clavatus filtrate had been fractionated. The various fractions were designated as follows:

Fraction A. The original residue. Yield 150 grams.

Fraction B. Material insoluble in alcohol. Yield 80 grams.

Fraction C. Alcohol soluble material. Yield 70 grams.

Fraction D. Thrown out of the alcohol solution by ether. Yield 25 grams.

Fraction E. Alcohol ether soluble material. Yield 45 grams.

Fraction F. Fraction E decolorized with norite.

Fraction G. Fraction of F, insoluble in absolute alcohol.

Fraction H. Fraction of F, soluble in absolute alcohol.

The stimulative properties of these various fractions were tested the usual way and the data are given in Table XXIII.

TABLE XXIII

Yeast Count for the Various Fractions Obtained from *Aspergillus niger* Filtrate Grown on Glycerine

Mg. of Stimulant per 100 c.c.	A	B	C	D	E	F	G	H	Checks
	Yeast Counts								
0.5	16	-	-	-	-	-	-	-	-
1.0	16	-	-	-	-	-	-	-	-
2.0	-	17	59	14	65	87	88	21	
10.0	18	14	164	13	87	102	99	53	
20.0	21	21	165	14	135	152	115	35	
25.0	26	-	-	-	-	-	-	-	
30.0	26	-	-	-	-	-	-	-	
40.0	39	18	183	13	165	138	165	42	
50.0	45	-	-	-	-	-	-	-	
60.0	-	16	178	17	178	167	195	43	
80.0	-	16	197	15	157	157	187	47	
100.0	74								
125.0	64								
150.0	101								
175.0	94								
200.0	115								
225.0	102								
0									14
0									17
0									14

The data in the preceding table are quite similar to the data obtained with *Aspergillus clavatus* and it is possible that the material or materials producing stimulation are the same in both cases.

Further Fractionation of the Stimulant

The filtrate from two 500 c.c. quantities of glycerine media that had supported the growth of *Aspergillus niger* for two weeks was made just alkaline with ammonium hydroxide, then

lead acetate added as long as a precipitate formed. The copious yellowish precipitate thus obtained was removed and suspended in water, acidified with HCl and the lead removed with hydrogen sulfide. The lead was removed from the filtrate in the same way. The two fractions thus obtained were saved for further work.

Another one liter portion of the same filtrate was treated with barium hydroxide as long as a precipitate would form, the material was filtered and the barium removed from both the filtrate and precipitate with sulfuric acid.

It was assumed then that sufficient nutrient salts were still present in the solutions of the filtrate to permit the growth of yeast. But in order to get growth in solution of the precipitate it was deemed necessary to enrich these solutions with the proper concentrations of salts to make these media the same as the original medium. These four fractions were then inoculated, after sterilization, with yeast in the usual manner. It was noticed that apparently the stimulant has been completely destroyed by the lead precipitation. The material, however, was not precipitated or destroyed by barium hydroxide. The data are indicated in Table XXIV.

Table XLIV

Yeast Counts for the Various Fractions Obtained by Barium Hydroxide and Lead Acetate

A	B	C	D	E	F	G	H
Yeast Counts:							
98	15	12	15	71	14	81	21
102	14	13	14	75	19	83	19
100	11	14	10	70	12	84	24
85	12	13	10	82	15	88	

Yeast Counts represent quadruplicate runs on each substance tested.

Fraction A is original filtrate plus sucrose.

" B is the lead acetate precipitate.

" C is the lead acetate filtrate.

" D is barium hydroxide precipitate.

" E is barium hydroxide filtrate.

" F is mixture of filtrate and precipitate from lead acetate.

" G is a mixture of filtrate and precipitate from barium hydroxide.

" H is check.

The Production of Stimulants by Aspergillus niger as a Function of the Glycerine Concentration

In order to determine the optimum conditions for the mold growth and also to determine the optimum conditions for production of growth stimulants the following basal medium was used. $MgSO_4$ -- 0.4 grams, $FeSO_4$ -- 0.1 gram,

$ZnSO_4$ -- 0.1 gram, KH_2PO_4 - 1.5 grams, NH_4Cl -- 2 grams. To this medium, glycerine was added to the various flasks in varying amounts. It was then sterilized and inoculated with Aspergillus niger and allowed to grow for fourteen days. The mold was removed and the filtrate enriched by adding 100 grams of sucrose per liter of solution and the pH adjusted to 6.9. It was then added to the original basic media, enriched by adding 100 grams sucrose per liter, in varying amounts. This basal or control medium was the same as the original plus 100 grams of sugar per liter. One has then the following variable factors:

1. The varying concentration of glycerine from which one hopes to be able to establish an optimum for the production of stimulant relative to the concentration of glycerine.
2. The varying concentration of filtrate in the media should enable one to determine an optimun concentration of filtrate. By plotting the concentration of glycerine against average count of yeast per c.c. of filtrate used, one gets the optimum concentration of glycerine for filtrate produced. (Graph No. I.) By plotting the concentration of glycerine against weight of mycelium, one gets the optimum for the production of mycelium from glycerine. (Graph No. II.) By dividing the values obtained from the average count per c.c. of glycerine filtrate by the grams of glycerine per 1000 c.c., one gets the average count per gram of glycerine used.

TABLE XXV

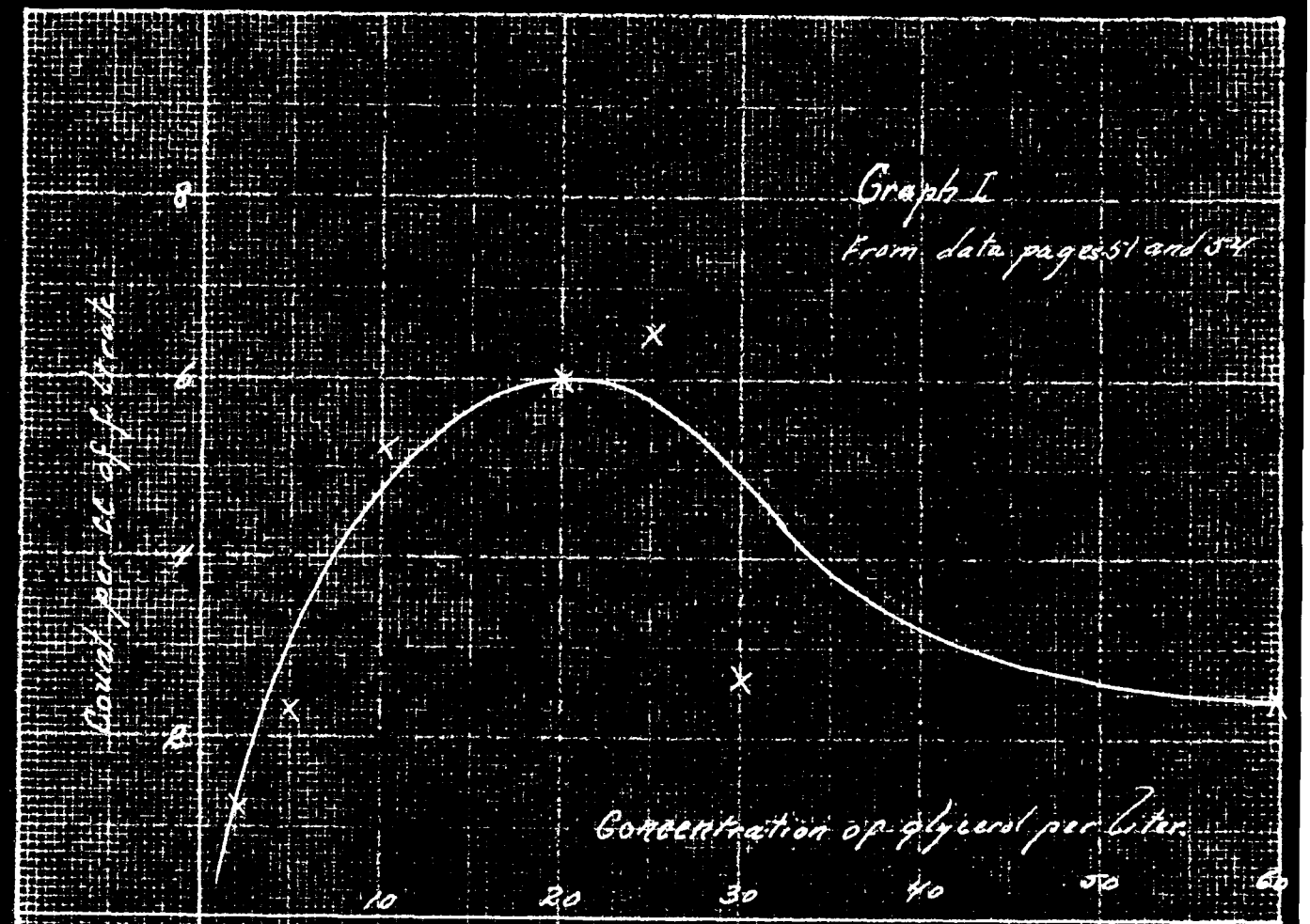
The Effect of Varying the Concentrations of Nutrients in the Production of Stimulant

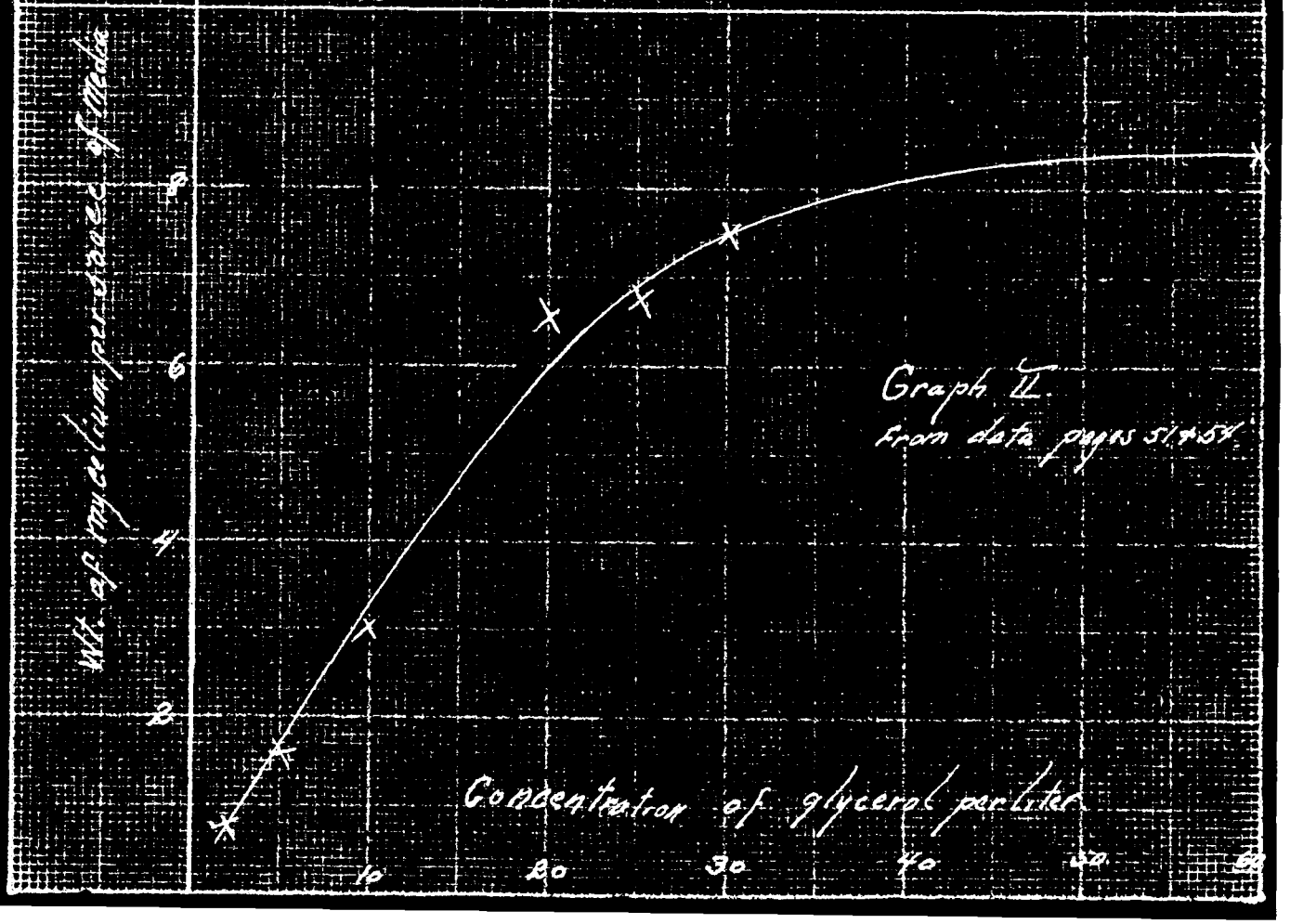
Media Number		I		II		III		IV	
c.c. of control media	c.c. of mold filtrate	A*	B**	A	B	A	B	A	B
45	5	10	2	13	2.6	32	6.4	86	17.2
40	10	15	1.5	24	2.4	97	9.7	100	10.0
30	20	19	0.95	33	1.65	105	5.25	97	4.85
25	25	28	1.12	55	2.2	125	5.0	89	3.67
20	30	30	1.0	62	2.06	122	4.66	68	2.93
10	40	42	1.05	81	2.02	120	3.0	90	2.25
0	50	39	0.78	101	2.02	122	2.4	72	1.44
50	0	7	-	6	-	7	-	11	-
Average count per c.c. of filtrate			1.20		2.13		5.20		6.05

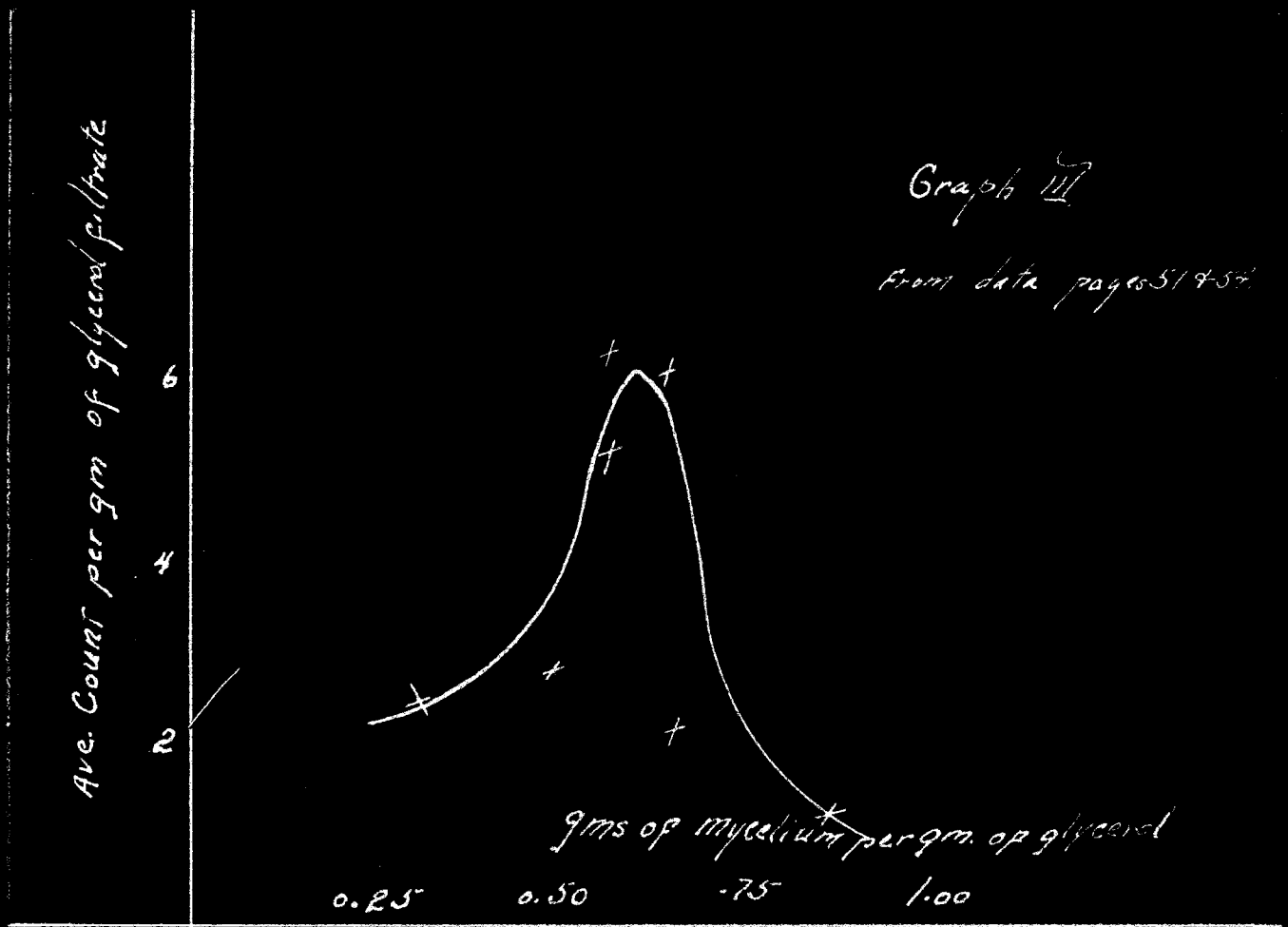
Media Number		V		VI		VII	
c.c. of Control media	c.c. of mold filtrate	A	B	A	B	A	B
45	5	97	19.4	26	5.2	27	5.4
40	10	105	10.5	36	3.6	30	3.0
30	20	110	5.05	57	2.85	44	2.2
25	25	103	4.10	70	2.80	53	2.1
20	30	99	3.30	64	2.13	63	2.1
10	40	85	2.07	52	1.3	60	1.5
0	50	51	1.02	48	0.94	51	1.02
50	0	9	-	9	-	7	-
Average count per c.c. of filtrate			6.46		2.69		2.48

* A = average count.

**B = count per c.c. of filtrate.


Graph I
from data pages 51 and 54
Concentration of glycerol per liter
10
20
30
40
50
60


Graph II
from data pages 51 & 54
Concentration of glycerol per liter
10
20
30
40
50
60



Graph VI

From data pages 51 & 52

The data are given in Tables XXV and XXVI

TABLE XXVI

Table Showing Yields of Acid and Mycelium by Aspergillus niger on Varying Amounts of Glycerine

Series Number	Gms. of Glycerine per 1000 c.c.	c.c. of N/10*	Wt. of mye.**	Gms. of mye. per gm. of glycerin
1	2	2.5	0.857	0.257
2	5	3.0	1.622	0.649
3	10	3.3	3.038	0.605
4	20	3.9	6.604	0.661
5	25	3.8	6.887	0.555
6	50	4.3	7.591	0.506
7	60	4.0	3.416	0.231

* c.c. of N/10 base per 10 c.c. of acid at the end of a fermentation.

** Weight of mycelium dried from 500 c.c. of medium.

The Production of Stimulant by Aspergillus niger on Glycerine as a Function of Time.

In order to more definitely follow the production of stimulantsby Aspergillus niger, it was deemed necessary to study the production of stimulants as a function of time. It had been previously shown that the following medium, magnesium sulfate - 0.4 gram, ferrous sulfate - 0.1 gram, zinc sulfate - 0.1 gram, potassium dihydrogen phosphate -1.55 grams, ammonium chloride - 2.00 grams, glycerine - 20 grams, produced the maximum yield of stimulant per gram of glycerine used. This medium was sterilized in 500 c.c. quantities in two-liter Erlenmeyer flasks and inoculated with Aspergillus niger. At varying intervals, the stimulative properties of the filtrate were determined by using a flask of the medium

TABLE XXVII

Production of Stimulant by Aspergillus niger as a Function of Time

Length of Mold Fermentation in days	5	7	9	11	14	18	24	30
Wt. of mycelium per 500 c.c.	0.65	1.71	3.69	5.09	5.14	4.84	3.84	3.84
c.c. N/10 base per 10 c.c.	2.2	2.8	3.5	4.3	4.7	3.2	3.2	3.2

c.c. of control media	c.c. of mold filtrate	Yeast Count							
45	5	12	11	15	35	46	60	52	44
40	10	15	16	30	70	87	92	65	69
30	20	20	30	51	81	88	118	79	70
25	25	27	58	59	95	106	112	88	55
20	30	32	57	62	85	122	112	88	61
10	40	41	47	89	83	115	107	100	62
0	50	46	59	77	89	109	109	117	67
50		6	7	10	7	7	9	11	8
50		8	9	10	10	8	11	14	8

Length of Mold Fermentation in days	36	48	50	56
Wt. of mycelium per 500 c.c.	3.32	2.82	2.90	2.80
c.c. N/10 base per 10 c.c.	3.4	3.2	3.2	3.0

c.c. of control media	c.c. of mold filtrate	Yeast Count			
45	5	54	28	35	37
40	10	65	44	41	56
30	20	72	57	45	43
25	25	79	45	67	64
20	30	88	44	76	83
10	40	90	60	93	87
0	50	91	85	72	90
50	0	11	13	12	12
50	0	10	14	11	11

enriched with sucrose to a concentration of 100 grams per liter. The pH was also adjusted to 6.9 with sodium hydroxide, then this medium was added in varying amounts to the original medium which also contained 100 grams of sucrose per liter. This enabled one to have a medium of somewhat definite composition and yet to vary the concentration of the stimulant. At the same time, a 10 c.c. sample was titrated with sodium hydroxide, and the mold mycelium dried and weighed. It was noticed that the concentration of stimulant appeared to be at a maximum in about 14 to 18 days, and after that time it seemed to lose some of its potency. The data are given in Table XXVII.

The Production of Stimulants by Aspergillus niger on Sucrose as Correlated with the Production of acid.

Previous studies have shown the following medium to be optimal in the production of acid by Aspergillus niger on sucrose: Sucrose - 100 grams, magnesium sulfate - 0.4 gram, ferrous sulfate and zinc sulfate each - 0.1 gram, ammonium chloride - 2.00 grams, and water to one liter, pH - 3.0. This medium was prepared and placed in 100 c.c. quantities in 300 c.c. flasks, sterilized, and inoculated with Aspergillus niger. It has been previously shown that using a larger volume surface ration than the one obtained in this instance gives poorer yields of acids. At varying intervals of time throughout the 14 day growing period of the mold, flasks of the material were used to study their zinc content.

TABLE XXVIII

Production of Stimulants by Aspergillus niger on Sucrose as a Function of Time and Correlated with Acid Production

Days of Mold Fermentation	2	3	4	5	7	9
Wt. of mycelium per 500 c.c.	0.868	1.61	1.85	1.74	1.98	2.25
c.c. N/10 base per 10 c.c.	4.1	24.8	38.0	47.3	61.0	64.0

c.c. of control media	c.c. of mold filtrate	Yeast Count A*	A*	A*	A*	A*	B*	A	B
45	5	35	44	60	53	53	53	82	94
40	10	49	52	62	66	56	71	80	87
30	20	65	75	92	98	75	88	84	91
25	25	86	116	159	128	107	121	114	89
20	30	97	122	94	114	103	122	87	90
10	40	93	136	71	90	83	100	43	82
0	50	105	115	58	62	58	90	33	78
50	0	16	10	17	13	11	-	10	-

Days of Mold Fermentation	11	13	14
Wt. of mycelium per 500 c.c.	2.53	2.65	2.74
c.c. N/10 base per 10 c.c.	65.5	59.5	66.0

c.c. of control media	c.c. of mold filtrate	Yeast Count A	B	A	B	A	B
45	5	81	79	93	88	88	79
40	10	104	98	88	97	93	98
30	20	101	106	97	108	99	117
25	25	99	117	99	113	108	103
20	30	64	96	86	88	61	92
10	40	30	54	54	81	51	75
0	50	10	12	39	32	29	43
50	0	11	-	11	-	14	-

A* Media sterilized by steam.
B* Media sterilized by filtration.

To do this the filtrate from the mold was obtained in the usual way and enriched with 5 grams of sucrose per 100 c.c. to compensate for that used up by the mold. This new filtrate was then added in varying amounts to the original medium in which no moldhad grown. These flasks of media were then sterilized by steam at first. However, this was not so successful because the invert sugar from the mold filtrate charred badly. Consequently, the material was sterilized through a Berkefeld filterer before mixing. The data indicate that the concentration of stimulants reaches a maximum within 3 to 5 days after inoculation with the mold. After that time there appears to be a gradual decrease in the concentration of the stimulants. Also, there is some evidence that the filtrate becomes toxic or at any rate much less stimulative in higher concentrations in the older flasks of medium. The data are given in table XXVIII.

The Production of a Stimulant of an Intra-Cellular Nature by Aspergillus niger.

The previous data indicate and describe an extra-cellular product. It seemed of interest to test the mold itself. A 200 gram sample of dried Aspergillus niger mycelium that had been grown on sucrose medium, was extracted for 10 hours with 3 liters of water at 50 to 60 degrees. The suspension was filtered and evaporated to a thick syrupy mass in vacuum at 50 degrees and dried in a desiccator over calcium chloride. The resulting material was quite black

and dough-like in consistency and amounted to about 30 grams. This was designated as Fraction I. A 25 gram sample of this fraction was continuously extracted for four days with 95% alcohol. The extract in contact with the mold was greenish in color while that in the reservoir was reddish brown. On concentrating the alcoholic extract and cooling it to minus 10 degrees a considerable quantity of needle-like crystals separated, together with some tarry material. The alcohol insoluble material was designated as Fraction II. The alcohol soluble material not removed by cooling was Fraction III. The material separated by cooling is Fraction IV. The effects of these four fractions on the growth of yeast at 30 degrees in Medium C are shown in Table XXIX. It will be noticed from the data in Table XXIX that Fractions I, II, III, and IV are very rich in the stimulant.

Table XXIX

Yeast-growth Stimulating properties of extracts of Aspergillus ni___ mycelium. Basal Medium C for Yeast. Count taken after 48 hours. Concentration of Extract in Milligrams Dry Material per 100 cc.

I		II		III		IV	
Concentration	Count*	Concentration	Count	Concentration	Count	Concentration	Count
0	41	0	60	0	57	0	58
0	35	0	48	0	38	0	38
0	37	0	54	0	38	0	37
5	57	2	50	2	67	2	49
10	74	4	49	10	112	10	77
20	119	10	52	20	106	20	74
30	116	20	56	40	148	40	120
50	162	40	56	60	114	60	106
60	141	60	55	80	99	80	115
80	159	80	55	-	-	-	-

$$*\text{Count} = \frac{\text{cells per cubic centimeter}}{250{,}000}$$

I. Water-soluble fraction, pH of 1% solution = 4.4
II. Alcohol-insoluble fraction, pH of 1% solution =4.7
III. Alcohol-soluble fraction, pH of 1% solution = 3.3
IV. Material removed from alcohol-soluble fraction by cooling to minus 10 degrees C. pH of 1% solution = 3.7

Relationship between Bios I and Bios II of Miller, and the Material of Bios Activity Produced by Molds.

According to Bush Miller and co-workers (Lucas, C.H.W., (1934)) the water soluble extract of malt sprouts can be divided into two separate constituents by barium hydroxide. The one, Bios I, is precipitated by barium hydroxide and is non-stimulative, and the other, Bios II, which is not precipitated by barium hydroxide, is only slightly stimulative alone, but combinations of I and II are highly stimulative. Bios I has been identified as inositol by Eastcott (1928).

Bios I and Bios II were prepared according to Miller's directions and made up in concentration five times that described by him. In so doing, it was possible to use 1 c.c. in 50 c.c. of media and have the same concentration as Miller did with 1 c.c. in 10 c.c. in his rocker tube. The first several attempts to prepare Bios I and II did not yield satisfactory results. Generally both the separate fractions, Bios I and II, were quite highly stimulative even though Miller's directions were followed with great pains. The most satisfactory preparation according to results obtained gave the following counts. The stimulative properties of inositol were tested along with Bios I and Bios II. See Table XXX.

* The malt sprouts were kindly furnished through Mr. G. S. Miner, of the Miner Laboratories.

TABLE XXX

The Relationship between Bios I and II of Miller and the Stimulant Obtained from Aspergillus clavatus Filtrate

Stimulant from Mold*	Bios I	Bios II	Inositol**	Count
10				114
20				161
40				131
60				147
80				148
20	1 c.c.			117
20	1 c.c.			105
40	1 c.c.			136
40	2 c.c.			127
20		1 c.c.		157
20		1 c.c.		182
20		1 c.c.		187
40		2 c.c.		198
40		2 c.c.		192
20			20	136
20			40	132
20			40	142
40			60	121
			5	32
			10	42
			20	31
			60	34
			40	38
			60	34
			80	37
	1 c.c.			103, 101, 100
		1 c.c.		117, 103, 98
	1 c.c.	1 c.c.		212
	1 c.c.	1 c.c.		353
	1 c.c.	1 c.c.		223
		0.5 c.c.	10	159
		1.0 c.c.	10	190
		1.0 c.c.	20	211
		1.0 c.c.	20	204
		2.0 c.c.	20	193
		3.0 c.c.	20	201
		2.0 c.c.	40	219
		2.0 c.c.	40	221
Check Medium 0				33, 28, 35, 2

* Stimulant from Aspergillus clavatus on glycerine after decolorizing with norite. Concentration in milligrams per 100 c.c.

** Inositol from Eastman Kodak Company. Concentration mg. per 100 c.c.

It was considered of interest to see whether or not the Bios obtained from Aspergillus clavatus which was soluble in alcohol-ether mixture bore any relationship to the Bios I and II of Miller. Consequently, a series of flasks was prepared using Medium J. To these flasks a solution of the mold filtrate was added along with Bios I or Bios II in varying concentrations. The relationship to inositol was tested also. The mold fraction itself is quite potent and its stimulative qualities are increased by addition of Bios II. Inositol and Bios I have no appreciable effect. The data are incorporated in Table XXXI.

The Alfalfa Extracts of Palmer, Nelson, and Visscher, and their Relationship to Bios I and Bios II of Miller.

Palmer, Nelson, and Visscher (1924) separated the water soluble extract of alfalfa into four fractions by fractional precipitation with alcohol, and with these four fractions proved the multiple nature of Bios.

Williams (1931) gives Hilditch credit for the original discovery of Bios, but goes further and gives Nash Miller credit for having discovered an entirely new system of Bioses from malt sprouts, namely, Bios I and Bios II. It seems absurd that each yeast or each variety of yeast should have a specific Bios or Bioses. It would seem much more logical to believe that there are many things that might function as a Bios. Perhaps too, Bios might really be a "set of conditions". In other words, when one produces optimum conditions with respect to nutrients salts, pH,

oxidation-reduction potential, one then has optimum conditions and, consequently, maximal growth, and that such a thing as Bios or Elixir of Life might be shelved in the same category as Philosopher's Stone as a thing beautiful to think about but absurd to practical minds.

If the four fractions from alfalfa as obtained by Fulmer and co-workers cannot be separated into Bios I and II by Miller's method, and if addition of Bios I and II separately to each of these four fractions does not produce enhanced growth, it would be logical to conclude that the stimulative material was something other than Bios I and Bios II of Miller, and that four new substances had been added to the already large number of materials with Bios properties.

Starting with 1600 grams of alfalfa and following carefully the method of Fulmer, Nelson, and Sucker the four fractions were obtained as designated by them. The yields were as follows:

Fraction I	47.9 grams
Fraction II	84 grams
Fraction III	215 grams
Fraction IV	152 grams

These were used in yeast nutrition studies in the usual manner. The optimum concentration of each was found. Bios I and Bios II and inositol added to each at the optimum concentration, and Fractions III and IV subjected to Miller's method of fractionation for Bios I and II.

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The Alfalfa Fractions of Fulmer, Nelson, and Snecker

	Yeast Counts			
Mg. per 100 c.c.	Fraction I	Fraction II	Fraction III	Fraction IV
0	20	11	11	11
0	19	12	12	11
0				
.5	20	18	34	34
2.5	19	24	50	45
5.0	21	29	55	76
10.0	28	35	84	85
15.0	20	47	102	95
20.0	36	55	120	114
25.0	36	46	153	130
30.0	17	61	152	124
35.0	32	71	151	138
40.0	24	80	161	140
45.0	36	94	168	138
50.0	31	107	162	156
55.0	28	115	179	146
60.0	31	117	172	162
70.0	21	127	163	172
80.0	19			

Taking the optimum concentration of these four fractions (Tables XXII and XXIII) and combining the various fractions using the optimum conditions, one gets increased stimulation over the original optimum for the separate fractions. This gives one evidence again of the multiple nature of the stimulant present.

The data obtained by adding Miller's Bios I and II to the four alfalfa fractions (Table XXXIII) are somewhat confusing. It will benoticed that there is increased stimulation when Bios I is added to fractions I, III, and IV of the alfalfa extract while there is not so much stimulation noticed with Bios I and Fraction IV. It would be logical to assume that Fractions I, III, and IV were deficient in Bios I. However,

a considerable stimulation is also obtained with combinations of fractions III and IV and Mice II which would indicate that these are fractions more deficient in Mice II which is unlikely. It seems more logical to believe that the essential or substance present in Mice I and IV and the four vitally fractions may be of an entirely different nature, and the increased stimulation is merely due to the addition of larger quantities of a variety of stimulants to the tissue. It is further noticed that the attempted fractionation of vitally fractions III and IV into Mice I and II, according to Miller's directions, was unsuccessful. In this fractionation a precipitate was obtained in both cases with barium hydroxide, but apparently it was more ether substance than Mice I. (Table XXXVI)

An Attempted Fractionation of the Meta of pancreatic ducts Into Mice I and II.

One hundred grams of thoroughly washed and dried mycelia from Aspergillus niger grown on manure were extracted with warm water and fractionated by means of barium hydroxide according to Miller's directions, (Amer. J.P.S. (1914)). Only a slight precipitate formed with the barium hydroxide. However, the two fractions so obtained were worked through with the usual procedure and made up in concentrations five times the concentration specified by Auer (29 c.c.). This enabled one to add 1 c.c. of this material to 10 c.c. of media and have the same concentration as injected with his 1 c.c. in 10 c.c. of his Mice preparations from A. niger. It will be seen that this attempted fractionation was

unsuccessful. Fraction I proves to be definitely toxic in higher concentrations. Fraction II is somewhat stimulative but the combination of I and II does not produce great stimulation. Further, the addition of Miller's fractions from malt sprouts to these fractions from the molds does not give greatly enhanced growth showing that apparently they are not rich in the respective Bions indicated. See Table XXXV.

TABLE XXXII

Yeast Counts for the Optimum Concentrations of the Four Alfalfa Fractions and for the Various Combinations of Them.

Fractions				Yeast Count***			Ave.	Check*
I	II	III	IV					
20*				73	77	81	77	29
30				117	145	130	131	30
40				134	117	136	129	28
50				93	104	99	98	25
	20			86	93	89	89	29
	30			113	104	103	107	36
	40			173	165	160	166	33
	50			167	159	162	163	28
		30		130	134	136	130	26
		40		134	134	132	130	28
		50		138	157	135	141	22
		60		130	153	144	142	28
			20	179	169	163	170	26
			30	165	173	161	166	28
			40	153	165	159	159	32
			50	130	136	143	133	-
30	30			162	149	162	134	26
40	20			135	115	127	126	28
30	30			293	343	318	327	33
40	40			214	188	237	210	-
20		30		84	94	108	95	24
30		30		108	107	106	107	25
40		40		154	162	164	160	25
50		10		132	140	156	143	24

TABLE XXXII (Continued)

Fractions I	II	III	IV	Yeast Count***			Ave.	Check*
30			30	202	198	188	196	23
30			30	346	358	332	358	21
40			40	256	182	185	191	22
50			50	355	350	342	340	24
	30	30		315	342	345	334	23
	30	30		206	238	223	221	21
	40	40		230	275	245	250	22
	50	30		185	192	160	179	24
	30		30	272	326	360	386	24
	30		30	290	208	252	246	25
	40		40	270	266	240	259	25
	50		30	226	190	190	202	24
		30	30	277	277	265	273	25
		30	30	243	250	258	250	21
		40	40	270	252	259	267	23
		50	30	242	230	265	246	24

* Yeast count in basal Medium G for check

** Milligrams of dried alfalfa fraction per 100 c.c. of medic.

*** Yeast counts in flasks containing stimulant, run in triplicate.

TABLE XXXIII

Yeast Counts for Optimal Concentrations of the Four Alfalfa Fractions plus Bios I and II of Miller.

Fractions from Alfalfa I	II	III	IV	Bios I*	Bios II**	Yeast Counts***			Ave.	Check****
30*****				1 c.c.		190	176	170	178	24
40				2 c.c.		224	210	202	212	23
40				1 c.c.		230	250	212	230	25
40				2 c.c.		216	238	220	225	24
	30			1 c.c.		58	64	58	59	17
	30			2 c.c.		78	74	80	78	19
	40			1 c.c.		77	98	91	89	22
	40			2 c.c.		76	82	78	79	-
		40		1 c.c.		176	190	164	176	17
		40		2 c.c.		179	167	185	177	19
		50		2 c.c.		175	180	191	182	22
		60		1 c.c.		187	193	211	197	-

TABLE XXXIII (Continued)

Fractions from Alfalfa I	II	III	IV	Bios I	Bios II	Yeast Counts			Ave.	Check
			20	1 c.c.		141	180	167	169	23
			30	2 c.c.		178	158	180	162	22
			40	1 c.c.		209	207	217	210	28
			40	2 c.c.		253	222	237	237	-
20					1 c.c.	120	91	100	104	18
30					1 c.c.	103	112	129	116	21
40					2 c.c.	131	143	156	143	24
40					2 c.c.	159	160	153	157	25
	20				1 c.c.	116	97	98	104	18
	30				1 c.c.	127	135	149	137	21
	40				2 c.c.	161	157	176	168	24
	40				2 c.c.	160	189	178	175	25
		20			1 c.c.	198	233	195	205	18
		30			1 c.c.	196	214	187	200	21
		40			2 c.c.	184	182	178	186	24
		40			2 c.c.	238	208	218	219	25
			20		1 c.c.	199	192	187	193	18
			30		1 c.c.	206	207	227	213	21
			40		2 c.c.	210	220	197	209	24
			40		2 c.c.	211	226	206	218	25

* Bios I of Miller. Concentration five times that used by him. This gives the same concentration in 50 c.c. as he had in 10 c.c. in his rocker tube method.

** Bios II of Miller. Same concentration as Bios I.

*** Yeast counts on triplicate flasks containing the stimulants.

**** Medium 2 for check.

***** Milligrams of dried alfalfa extract per 100 c.c. of media.

TABLE XXXIV

Yeast Counts for Bios I and II Prepared from Alfalfa Fraction III and IV. Concentration Equals Five Times Miller's Preparation. Concentration in 50 c.c. of Yeast Media Same as Miller's.

Bios I and II from Alfalfa Fraction III

Alfalfa Bios		Miller's Bios		Yeast Counts
Bios I	Bios II	Bios I	Bios II	
1 c.c.				28
1 c.c.				29
2 c.c.				37
2 c.c.				41
3 c.c.				33
3 c.c.				39
	1 c.c.			58
	1 c.c.			63
	2 c.c.			77
	2 c.c.			79
	3 c.c.			99
	3 c.c.			103
1 c.c.	1 c.c.			57
1 c.c.	1 c.c.			53
2 c.c.	2 c.c.			95
2 c.c.	2 c.c.			93
1 c.c.		1 c.c.		135
1 c.c.		2 c.c.		186
2 c.c.		2 c.c.		193
2 c.c.		2 c.c.		183
	1 c.c.	1 c.c.		78
	1 c.c.	1 c.c.		75
	2 c.c.	2 c.c.		93
	2 c.c.	2 c.c.		86

Bios I and II Prepared from Alfalfa Fraction IV (same concentration as for previous fraction)

1 c.c.	3	0
2 c.c.	6	4
3 c.c.	7	5
4 c.c.	6	8

TABLE XXXIV (Continued)

Alfalfa Bios		Miller's Bios		Yeast Counts.	
Bios I	Bios II	Bios I	Bios II		
	1 c.c.			33	35
	2 c.c.			36	33
	3 c.c.			38	41
	4 c.c.			43	39
1 c.c.	1 c.c.			46	43
2 c.c.	2 c.c.			51	46
3 c.c.	1 c.c.			66	63
1 c.c.	3 c.c.			40	39
1 c.c.	2 c.c.			29	28
2 c.c.	1 c.c.			43	45
Check Counts on Medium 3:				19	
				23	
				28	
				34	

TABLE XXXV

An Attempted Fractionation of the Pelts of Aspergillus niger into Bios I and II

Bios from Mold		Miller's Bios		Yeast Count
Bios I	Bios II	Bios I	Bios II	
0.5 c.c.				45
0.5 c.c.				39
1.0 c.c.				37
1.0 c.c.				24
2.0 c.c.				10
2.0 c.c.				15
3.0 c.c.				8
3.0 c.c.				5
4.0 c.c.				0
4.0 c.c.				8
	0.5 c.c.			134
	0.5 c.c.			135
	1.0 c.c.			139
	1.0 c.c.			128
	2.0 c.c.			136
	2.0 c.c.			142
	3.0 c.c.			70
	3.0 c.c.			87
0.5 c.c.	0.5 c.c.			140
0.5 c.c.	0.5 c.c.			107
1.0 c.c.	1.0 c.c.			91
1.0 c.c.	1.0 c.c.			87
2.0 c.c.	2.0 c.c.			47
2.0 c.c.	2.0 c.c.			89
1.0 c.c.			1.0 c.c.	117
1.0 c.c.			1.0 c.c.	104
2.0 c.c.			2.0 c.c.	78
2.0 c.c.			2.0 c.c.	67
	1.0 c.c.	1.0 c.c.		145
	1.0 c.c.	1.0 c.c.		158
	2.0 c.c.	2.0 c.c.		122
	2.0 c.c.	2.0 c.c.		149
Check Medium 3				96 - 85 - 32

A Comparison of the Growth Curves of the Various Stimulants

According to Buchanan (1925) and Buchanan and Fulmer (1928) the life history of a typical yeast begins with a period of quiescence, budding then sets in slowly at first but gradually more rapidly until "the stage of logarithmic growth" is reached. After this period, the rate of reproduction decreases and in the last stage there is negative growth acceleration, or more cells die than are formed. Miller (1930) lays great stress on the value of growth curves in estimating and studying Bios preparations. He says in part:

> "The slope of the logarithmic curve and the crop obtained when logarithmic production ceases are the important matters; the former is characteristic for a given preparation, the latter measures the amount of active substance present, or if there be more than one that of the active substance present in (physiologically) the least amount ---. If a suitable race of yeast be used the crop increases so slowly when the logarithmic stage is passed that a few hours delay in measuring this is of no practical importance."

Comparative growth curves were run on the various fractions of Bios obtained. The material being tested was added to Medium Q in the desired ratio and the flasks after inoculation placed in a rocker in an incubator, (Clark, H.A., (1928)). This furnished sufficient agitation to enable one to obtain accurate and consistent results. These flasks were removed from time to time in order that counts might be taken.

It is interesting to note how well the growth curves (Graph No. III) carry out Miller's predictions. Curves I and II are for the same material but in different amounts, thus the curves are nearly parallel showing the common properties of the stimulant while the crop obtained when logarithmic growth ceases

is larger in the case of the larger quantity of stimulant. The curves for Bios I and II are of considerably different slope than for the sold stimulant indicating a different preparation.

TABLE XXXVI

Yeast Counts Throughout 48 Hour Growing Period for the Various Bios Fractions

	A		B		C		D	
Time	Count	Log Count	Count	Log Count	Count	Log Count	Count	Log Count
0	1	0	1	0	1	0	1	0
2	3	0.4771	2	0.3010	1	0	1	0
4	1	0	2	0.3010	2	0.3010	2	0.3010
6	3	0.4771	3	0.4771	3	0.4771	1	0
8	3	0.4771	6	0.7782	7	0.8451	2	0.3010
10	6	0.7782	14	1.1461	12	1.0792	5	0.6990
12	30	1.4771	20	1.3010	19	1.2788	6	0.7781
14	41	1.6128	47	1.6721	38	1.5798	7	0.8451
16	65	1.8129	65	1.8129	64	1.8062	5	0.6990
18	83	1.9191	101	2.0043	100	2.0000	5	0.6990
20	105	2.0212	239	2.3784	110	2.0414	–	–
22	113	2.0531	339	2.5302	102	2.0086	–	0.6990
24	99	1.9956	390	2.5910	188	2.2742	5	0.6989
26	95	1.9777	400	2.6021	156	2.1931	8	0.9031
28	103	2.0128	422	2.6253	195	2.2900	9	0.9542
30	97	1.9868	372	2.5705	198	2.2967	11	1.0414
32	110	2.0414	488	2.6884	192	2.2833	40	1.6021
36	114	2.0569	465	2.6675	200	2.3010	82	1.9138
48	108	2.0334	475	2.6767	212	2.3263	179	2.2529

	E		F		G	
Time	Count	Log Count	Count	Log Count	Count	Log Count
0	1	0	1	0	1	0
2	2	0.3010	1	0	2	0.3010
4	3	0.4771	2	0.3010	1	0
6	2	0.3010	1	0	1	0
8	6	0.7782	5	0.6990	2	0.3010
10	16	1.2041	12	1.0792	3	0.4771
12	18	1.2553	16	1.2041	4	0.6021
14	33	1.5185	38	1.5797	7	0.8451
16	72	1.8573	72	1.8573	9	0.9542
18	130	2.1139	206	2.3139	12	1.0792
20	164	2.2148	285	2.4548	16	1.2041
22	165	2.2175	340	2.5315	15	1.1761
24	165	2.2175	352	2.5465	16	1.2041
26	164	2.2148	342	2.5340	18	1.2553

Table XXVII (continued)

* Four digits taken at random.

A = Alcohol ether soluble fraction from *Aspergillus clavatus* on glycerine, 50 mg. per 100 cc.

B = Alcohol ether soluble fraction from *Aspergillus oryzae* on glycerine, 100 mg. per 100 cc.

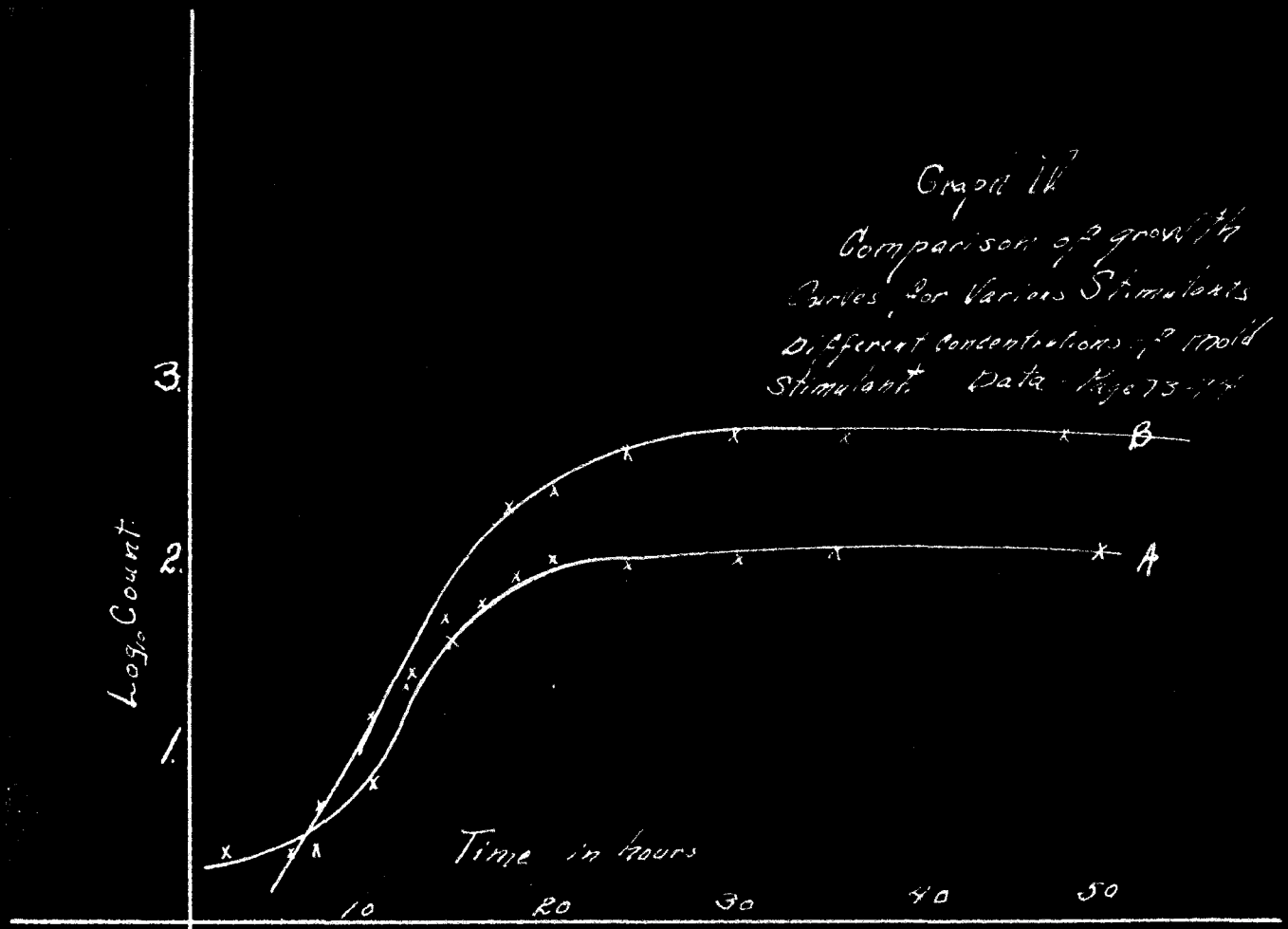
C = Plus Na. Desoxycholate - 20 mg. per 100 cc.

D = Plus H clinic = 3 cc. per 100 cc. of Medium 2.

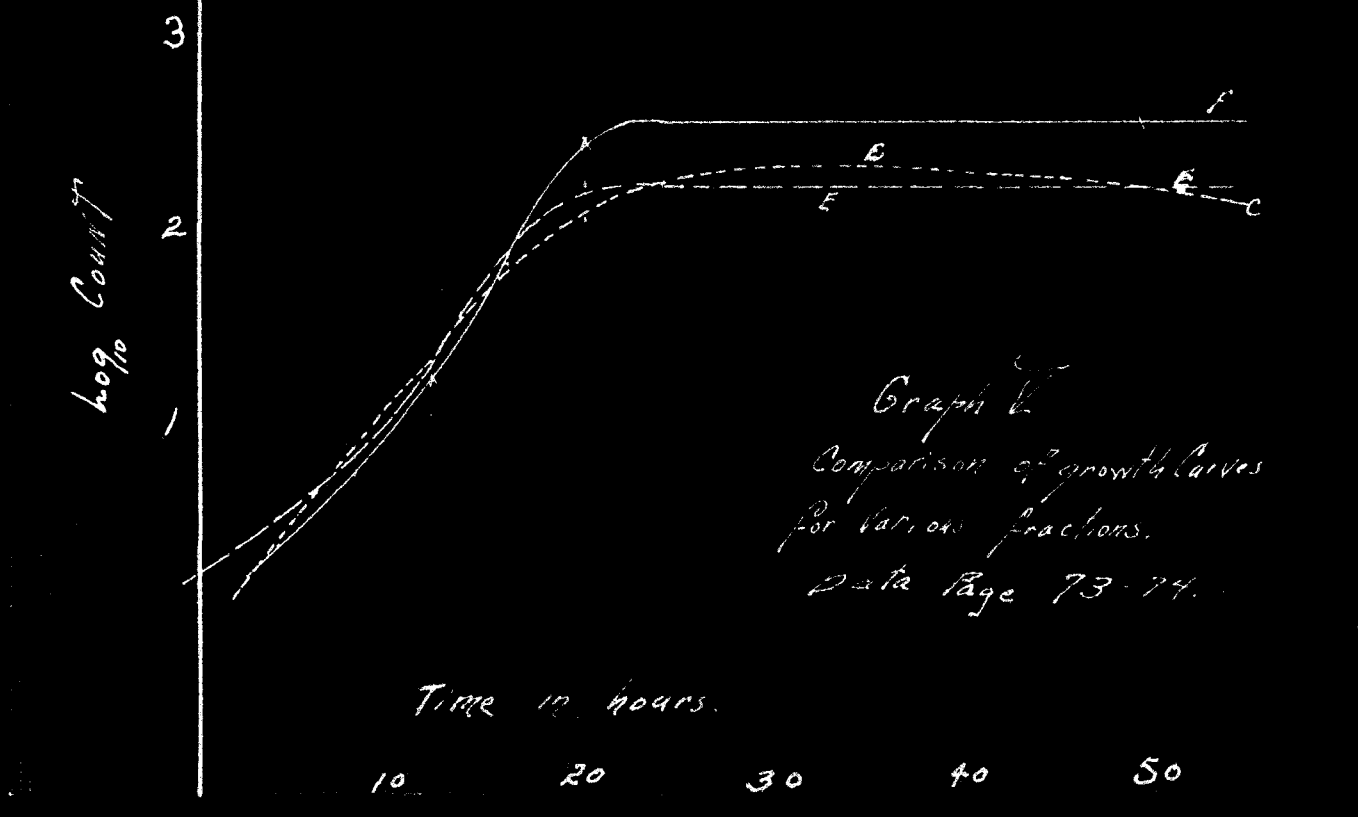
E = Plus I = 3 cc., Plus II = 3 cc. per 100 cc.

F = Plus II = 6 cc., Plus I = 6 cc. per 100 cc.

G = Medium 2 = Sur Check



Graph IV
Comparison of growth Curves for Various Stimulants Different Concentrations of mold Stimulant. Data Page 73-74



Graph V
Comparison of growth Curves for various fractions.
Data Page 73-74.

Summary

1. It has been shown that the molds *Aspergillus niger*, *Aspergillus clavatus*, *Trichoderma lignorum*, *Rhizopus nigricans*, and *Penicillium roqueforti* are capable of producing a substance or substances stimulating yeast growth.

2. It has been shown that this material of a Bios nature is produced from both glycerine and sucrose substrates.

3. The bios producing properties of *Aspergillus niger* have been studied with respect to time, the composition of the media, production of acids, and of mycelium.

4. A study of the nature of the stimulant has been made.

5. It has been shown that the Bios produced by mold is apparently not the same as Miller's bios fractions.

6. A synthetic medium has been devised which gives high yields of acid and mycelium with *Aspergillus niger*.

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