separates in flat needles and is easily purified by recrystallization from methyl alcohol; yield, 24 g.

Anal. Calcd. for $C_{15}H_{11}O_2N$: C, 77.1; H, 4.4. Found: C, 77.2; H, 4.5.

3,5-Diphenyl-6-oxo-orthoxazine, XV, is sparingly soluble in ether, moderately soluble in alcohol, readily soluble in acetone. It melts at 153°. It does not combine with bromine but its solution in acetone readily reduces permanganate.

Ozonization.—The oxazine was ozonized in chloroform and the ozonide decomposed with water in the customary manner. The water solution, when heated with phenylhydrazine hydrochloride, gave the phenylhydrazone of benzoylformic acid.

Change to 3,5-Diphenyl-isoxazole.—Silver nitrate was added in excess to the strongly alkaline solution of the oxazine in potassium hydroxide. The resulting mixture, when warmed on a steam-bath, almost immediately began to deposit a new substance in glistening plates. After recrystallization this melted at 142°. It was identified as diphenyl-isoxazole by comparing its melting point with that of a mixture of the substance and the isoxazole. The same isoxazole was obtained when a solution of the oxazine in acetyl chloride was boiled for several hours.

Summary

1. The principal products of the reaction between methyl alcoholic potassium acetate and α-bromo-β-phenyl-γ-nitropropiophenone are a nitrocyclopropane derivative and an hydroxy-oximido ester and not, as stated in an earlier paper, a nitrocyclopropane derivative and an hydroxy-isoxazolidine.

2. One of the by-products of the reaction is an oxazine derivative. This, like the oximido ester, has been synthesized by methods that leave no doubt as to its structure.

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[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT OF THE UNIVERSITY OF OREGON]

THE CONTROL OF "BIOS" TESTING AND THE CONCENTRATION OF A "BIOS"

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I. Introduction

The stimulating effect produced by a given extract upon the growth of yeast cannot, as originally suggested by one of us,[1,2] be taken as a satisfactory measure of the amount of vitamin B in the extract. This fact has been proved by our own experiments[3] as well as those of others. It remains an interesting question whether or not the substances which are responsible for the stimulation of yeast growth are among those which also affect the growth of animals. Little doubt remains that what has been for convenience called "vitamin B" is a group of substances. Evi-

[1] Williams, *J. Biol. Chem.*, **38**, 465 (1919).

[2] Williams, *ibid.*, **42**, 259 (1920).

[3] Williams, *ibid.*, **46**, 113 (1921).

dence has been presented by others,[4] for which we find confirmation, that what has been called "bios" is also more than one substance. Whether there is an overlapping of these substances is not settled. It is possible, and from the standpoint of their occurrence in milk and in animal tissues it is probable, that some of the substances which have the properties of bios may affect animal growth, and that some or all of the substances constituting vitamin B may also affect yeast growth. These questions can hardly be answered satisfactorily, however, until actual chemical isolation of the substances involved is accomplished. On account of the possible importance in animal as well as yeast nutrition we are studying the properties of bios with the end in view of the possible isolation of some of the active substances.

II. Development of Testing Method

A most important phase in bios investigations is the application of tests. Various methods have been suggested, and in some cases the differences in procedure involve very essential details. We have reinvestigated the problem of a suitable test in the light of recent work and the results are given below.

a. Choice of a Duplicable Strain.—The conflicting results obtained by different workers in studies concerned with bios have no doubt been due in part to the failure of different workers to use the same strain of yeast for their experimentation. We were led to see the tremendous importance of this factor in experiments which were preliminary to the main portion of the work here presented. In an attempt to repeat the work of Eddy, Kerr and Williams[5] on the isolation of an active bios in supposedly pure form we obtained a product which seemed to have the characteristics described by these authors, but failed to show any marked stimulating effect upon either of the strains which we then had on hand. On the strain of baker's yeast the stimulating effect was not detectable, while on the sample of brewer's bottom yeast which we had isolated, the crystalline bios had a definite though very small effect. We could explain the discrepancy between their results and ours only on the basis that we worked with different strains of yeast. (Experiments reported in Section III below seem to bear out the reasonableness of this explanation. Different strains of *Saccharomyces cerevisiae* seem to give diametrically opposite results.)

In order to work with a strain which could be duplicated by other workers we obtained three cultures of *Saccharomyces cerevisiae* from the American Type Culture Collection, 637 South Wood Street, Chicago, Illinois. These pure cultures were investigated in preliminary experiments, and

[4] Lucas, *J. Phys. Chem.*, **28**, 1180 (1924).

[5] Eddy, Kerr and Williams, THIS JOURNAL, **46**, 2846 (1924).

No. 578 was chosen for experimentation because it gives very little growth in the synthetic medium used, and is very responsive to small additions of bios-containing material. Later experiments have shown that if we had chosen a strain other than No. 578 some of our results would have been quite different. In order to maintain the yeast as constant as possible it is grown at 30° on slants made up from the following formula: Blackstrap molasses (Hawaiian cane) 16.5 g., ammonium dihydrogen phosphate 0.6 g., agar 6.5 g., water 500 cc. This medium is reasonably easy to reproduce, and aside from the agar used to solidify it, approximates a medium used commercially for growing yeast.

b. **Choice of Synthetic Medium.**—Fulmer's Medium F[6] was first considered as a blank medium, but found to be highly unsuitable, especially for a gravimetric method, due to the repeated formation of a precipitate on resterilization. It may be pointed out also that this medium was devised for a given seeding of a particular strain of yeast and for a long growth period (see Section II c). If we grant that it is the best synthetic medium for the particular strain of yeast and the seeding and growth period for which it was designed, it by no means follows that it is the best medium for all strains of *Saccharomyces cerevisiae*, or for the smaller seeding and shorter growth period discussed in Section II c. Incidentally, it may be remarked that the sugar content of Fulmer's medium is much higher than would seem to be necessary, and we have found no record justifying such a large excess of this material. It is liable to be a source of contamination and irregularity, due to the impurities which it may contain.

Finding Fulmer's medium unsuitable for our purpose, we tentatively adopted for trial the medium which was previously used by one of us.[2] To test the suitability of this medium for the strain of yeast under investigation we tried the addition of various materials to the medium to see whether it was materially improved. Fulmer's medium, which is undoubtedly favorable for some yeasts, contains more calcium and magnesium than the medium under trial. We therefore added enough calcium chloride and magnesium sulfate to bring the content up to that of Fulmer's medium. The effect of these additions on the growth of the yeast as compared with the growth in the original medium was entirely negligible. The addition of dextrin as suggested by Fulmer also had very little effect, if any, on the growth produced under the conditions present. The concentration of ammonium ion in our medium is about one-third greater than in Fulmer's medium, so from the standpoint of his work our medium will not be improved by a further addition.

Some writers[7] have given the impression that various known substances, probably including protein decomposition products, are capable of acting

[6] Fulmer, Nelson and Sherwood, THIS JOURNAL, **43**, 191 (1921).

[7] See, for instance, Tanner, *Chem. Rev.*, **1**, 461 (1925).

as yeast-growth stimulants in the same sense that bios does. To test out the possibility of our medium being improved by such additions and hence unsuitable as a blank medium, we carried out a number of experiments which are reported only briefly. Casein was coagulated from skim milk with pepsin and digested for varying lengths of time with pancreatin in the presence of toluene and thymol. After being freed from toluene and thymol by steam distillation, these digests in various amounts were tested as to their effect on yeast growth. We were unable to prepare any digest which had an appreciable stimulating effect. The pancreatin used contained some bios, but the amount which it was necessary to add to carry on the digestion was too small to contribute materially to the final dosages used. We next tried the effect of the addition of sodium bromate, which is a constituent of certain "yeast foods," and has been said to be a yeast-growth stimulant. When 1 and 10 mg. were added to 55 cc. of the blank medium, the growths produced were practically identical with that obtained in the blanks.

These experiments lead us to the tentative conclusion that any appreciable stimulating effect produced by any extract should be ascribed to the unknown factor which for convenience has been called bios. We are ignorant of the existence of any pure, identified substance, which is likely to be present in any extracts used, which is capable of producing any appreciable stimulation when added to the synthetic medium used and tested according to our method. We cannot claim that the medium which we have chosen is the ideal one for the strain of yeast under consideration or for the seeding and growth period used. However, our experiments indicate that it may be safely used as a basal medium in testing for bios activity, and this is all we desire of it.

c. **Initial Growth Rate Measured.**—Before discussing our work further it is necessary to make clear the reason why we have chosen, as in earlier work, to study the growth of yeast during its initial period of growth. Biological experiments at best are complicated by various factors which are beyond the control of the investigator. It is none the less desirable to control those factors which can be controlled, and to reduce the number of variable conditions to as few as possible. In the extensive experiments of Fulmer and his co-workers[6,8] in which they arrive at definite conclusions as to "the composition of the optimum medium for the growth of yeast," nothing is said about the length of the growth period used. In a later work[9] from the same laboratory is the statement "the numbers [in the table] represent the yeast count after 48–72 hours of growth." It is not indicated which numbers refer to each growth period. Presumably, the yeast is allowed to grow until no further growth is observed or expected. A funda-

[8] Fulmer, Nelson and Sherwood, THIS JOURNAL, **43**, 186 (1921).

[9] Fulmer, Duecker and Nelson, *ibid.*, **46**, 723 (1924).

mental objection to this method of experimentation, from our point of view, lies in the fact that the growth of yeast is tested in a medium which *during the experiment is allowed to change radically.* At the start of the experiment the medium is one in which yeast can grow rapidly, while at the end of the experiment the medium is one in which yeast will not grow at all, or very little, presumably due to the formation of toxic products of metabolism. Though it seems impossible to keep the medium from changing to some extent, it is possible, as in our experiments, to conclude the experiment while the medium is still capable of promoting growth. This is accomplished by using a small seeding (one-sixth of that used by Fulmer and his co-workers) and by stopping growth at the end of 18 hours. If the longer growth period is used, it is obviously erroneous to compare the results so obtained with those obtained using an 18-hour growth period and a smaller seeding, unless it is proved that the two methods yield parallel results.

Comparison of media with varying bios content has shown that when the dosage is increased above a certain point, the yeast crop is diminished, provided the yeast is allowed to grow until the medium can no longer support growth. A similar result is obtained no matter what growth period is used, if the bios containing material contains in addition substances which are poisonous to yeast. The existence of this optimum is stressed by Eddy, Kerr and Williams[5] and has been observed by other investigators.

Our results, in which the growth rate is measured as far as feasible in the medium as originally made up, are quite different from those in which a distinct maximum appears. This is shown in the experiment reported below.

A series of media was prepared, each member containing 50 cc. of the blank medium and in addition the indicated amounts of yeast extract plus enough water to make the solution up to 55 cc. These were seeded with yeast in the manner indicated below (Section II d) and the yeast crops obtained at the end of 18 hours at 30° were weighed in Gooch crucibles. The yeast extract was prepared as indicated in Section III a.

TABLE I

EFFECT OF VARYING CONCENTRATION

	I	II	III	IV	V	VI	VII	VIII	IX	X
Yeast extract added, cc.	0	0	0.039	0.078	0.156	0.313	0.625	1.25	2.50	5.00
Yeast crop, mg.	0.8	0.7	2.8	4.9	10.1	19.4	29.8	60.5	97.9	112.4

The results show that within wide limits the growth rate in the initial medium is greater, the greater the concentration of bios, and that in the lower concentrations the yeast crop is directly proportional to the yeast extract added. We attribute the lack of an optimum bios concentration

to the fact that the growth is not allowed to proceed until the medium is no longer suitable, and also to the fact that yeast extract prepared in the manner described seems to lack the inhibiting substances which are present in yeast autolysate, as well as in some other extracts. It is to be noted that the stimulation which is shown in this experiment is extraordinary; the yeast crop in one case is 150 times as great as in the blank. This must be due to the introduction of some specific growth stimulant and not to improvement of the medium by accidental proper balancing of the ingredients or to the introduction of some ordinary nutrient.

d. **Procedure Adopted for Testing.**—Solutions are made up in 250cc. Erlenmeyer flasks containing 50 cc. of the synthetic medium, to which are added 5 cc. portions of solutions containing the material to be tested for bios. The solutions are sterilized for ten minutes at $^2/_3$ atmosphere gage pressure and allowed to come to the temperature of the incubator (30°) previous to seeding. To each solution is added 1 cc. of a suspension of yeast in sterile water, of such a concentration that approximately 15 cells are present in each large square (25 small squares) on a Levy counting chamber. The solutions are then kept in an incubator at 30 ± 0.3° without agitation for 18 hours, at the end of which time the yeast is filtered into weighed Gooch crucibles fitted with asbestos on top of filter paper. The yeast is washed twice with distilled water, placed in an oven at 105° for at least an hour, cooled in a desiccator for at least another hour and weighed.

This method is a modification of the one previously suggested,[2] inasmuch as a pure culture of yeast is used, the seeding is controlled by actual count and the quantity of medium used in each test is one-half of that used formerly. The method has advantages over some others suggested because it does not involve the use of any equipment which must be made especially for the purpose, and hence can be readily followed in any laboratory with a promise of uniform results.

III. Concentration and Fractionation

a. **Preparation of Yeast Extract.**—For the concentration of bios we have chosen as a starting material an extract of baker's yeast, which was prepared as follows.

Starch-free baker's yeast (1600 g.) and 3.2 liters of distilled water were heated together in a copper vessel nearly to boiling for an hour. During the heating the mixture was stirred frequently and then allowed to stand overnight. The following day the mixture was heated for two hours longer. The mixture was then brought back to its original volume and filtered with a laboratory filter press, using diatomaceous earth to help in the filtration. On heating the filtrate for five minutes to $^1/_3$ atmosphere gage pressure, considerable precipitation took place, so the extract was filtered clear.

The extract obtained in this manner is, according to indirect comparison, almost as rich in bios as is yeast autolysate from brewer's yeast. It con-

tains, however, only about one-tenth as much solid material (about 0.038 g. per cc.). One disadvantage of using yeast autolysate is that during autolysis the complex compounds of which the yeast is composed are broken down into simpler compounds. These simpler compounds, especially those containing nitrogen, are particularly troublesome since their general behavior resembles that of the substances which go to make up bios. Our experience has also indicated that yeast autolysate invariably contains materials which are toxic to yeast.

b. Precipitation with Alcohol.—In accordance with the results of Eddy, Kerr and Williams[5] on yeast autolysate, we have found that the addition of sufficient alcohol to the yeast extract to bring the alcoholic content up to 70% removes a considerable amount of solid without materially reducing the bios content. After treatment with alcohol, the alcohol was removed by evaporation and the solution brought back to the volume of the original extract. The total solids of the extract was then 0.028 g. per cc. as compared with 0.148 g. per cc. in the yeast autolysate after similar treatment. In some of our experiments the alcohol treatment seemed to increase definitely the stimulating action of the extract.

c. Treatment with Iron Hydrosol.—Precipitation of the bios by means of iron hydrosol as carried out by Eddy, Kerr and Williams[5] was attempted. In the earlier experiments mentioned in Section II a, using a different yeast culture, we found that the hydrosol removed the activity practically quantitatively when the solution treated had the P_H value of 5.3. This is in accord with those results obtained by the originators of the method though the final crystals which we obtained had very little stimulating action on the yeast which we used at that time. In the case of the yeast *Saccharomyces cerevisiae* No. 578, the hydrosol removed part of the activity both at P_H 4.7 and 5.3, but did not remove much over half of the activity at either acidity. The use of iron hydrosol does not at present give particular promise as a procedure in the isolation of the substances which stimulate the growth of this strain of yeast.

d. Phosphotungstic Acid Precipitation.—Phosphotungstic acid alone causes little precipitation when added to the alcohol-treated extract, but in the presence of sulfuric acid the bios activity is practically completely removed. Since only about one-half of the total solids is removed by the precipitation, the procedure may be of advantage in further concentration.

e. Fuller's-Earth Treatment.—In the preliminary experiments using another yeast culture we confirmed the conclusions of Funk and Dubin[10] and of Eddy, Kerr and Williams,[5] that an extract can be treated with fuller's earth without removing appreciable amounts of the growth-stimulating substances. In earlier work one of us[1] had found the stimulating activity

[10] Funk and Dubin, *Proc. Soc. Exptl. Biol. Med.*, **17**, 175 (1919–1920).

to be removed by fuller's earth and to be recoverable from the earth by extraction with alkali. When the alcohol-treated extract described in Section III c is treated with fuller's earth at various acidities, it loses its bios activity toward *Saccharomyces cerevisiae* No. 578. However, it is necessary to combine both the material extracted from the fuller's earth and the extract which has been treated with fuller's earth before the bios activity is restored. This is shown in the results given below. The hydrogen-ion concentrations in this experiment were determined colorimetrically, using Bromocresol green as the indicator. Although we use a hydrogen electrode for standardizing buffer solutions, we have found it difficult to keep the electrode in condition when it is used directly in the extracts, so where possible we have used a colorimetric method. The fuller's-earth treatment consisted in shaking the extract with 5% of its weight of fuller's earth (Eimer and Amend) for one-half hour and filtering. The extract used in Sections VII and VIII was obtained by shaking with barium hydroxide and removing the excess of barium with sulfuric acid. The extraction was probably not complete.

TABLE II

RESULTS OF FULLER'S EARTH TREATMENT

	Addition to blank medium	Yeast crop, mg. I	II
I	5 cc. of H_2O	0.5	0.4
II	5 cc. of soln. containing 7.5 mg. of solids of alcohol-treated extract	15.7	16.5
III	5 cc. of soln. containing same as II except treated with fuller's earth at P_H 4.7	1.3	1.2
IV	5 cc. of soln. containing same as II except treated with fuller's earth at P_H 5.0	1.1	0.9
V	5 cc. of soln. containing same as II except treated with fuller's earth at P_H 5.3	1.1	0.9
VI	5 cc. of soln. containing same as II except treated with fuller's earth at P_H 5.6	1.6	1.7
VII	5 cc. of soln. containing same as V plus 0.43 mg. of solids extracted from the equivalent quantity of fuller's earth	11.0	11.1
VIII	5 cc. of soln. containing only 0.43 mg. of solids extracted from fuller's earth	2.6	2.4

Further experiments, which are not given in detail in order to save space showed that the material which is adsorbed by fuller's earth is also adsorbed by charcoal, and therefore may correspond to Lucas' "bios II,"[3] though this is not certain, since we may not be working with the same strain of yeast.

We wish to thank the Research Committee of the University of Oregon for grants to carry on the investigation, and the Fleischmann Company of Sumner, Washington, for generous supply of yeast and other materials.

Summary

1. A repetition of the isolation of a bios carried out by Eddy, Kerr and Williams led us to the conclusion, which has also been recently recognized by others, that the particular strain of yeast chosen is an all-important factor in bios investigations. We therefore chose a strain of *Saccharomyces cerevisiae* from the American Type Culture Collection which can be duplicated at will. Further experiments on the concentration of a bios strongly emphasize the importance of having a duplicable strain.

2. Evidence is given which indicates that the medium previously suggested by one of us can safely be used with this strain of yeast as a basal medium for bios tests.

3. When the yeast is allowed to grow until the medium becomes unsuitable, the results are complicated by the fact that the medium has radically changed during the experiment. If the seeding is small and the growth period short, and the extracts are free from inhibiting substances, there appears to be no "optimum" concentration of bios. The higher concentrations produce a larger crop.

4. A yeast extract such as is described is thought to be a more suitable starting material for concentrating bios than yeast autolysate. The means so far utilized to concentrate the bios are described.

5. By treatment with fuller's earth an extract yields two fractions, either of which by itself shows very little bios activity toward the yeast chosen for experimentation. A mixture of these two fractions produces a growth approximating that produced by the original extract. The fraction adsorbed on fuller's earth is concentrated to such an extent that a concentration of 0.008 mg. per cc. produces a stimulation perhaps ten times as great as the least that can be detected, when tested by the method outlined.

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