g. per kg. killed a dog almost instantaneously; 0.075 g. produced almost instant anesthesia, lasting about an hour, after which a rapid recovery occurred, while 0.0375 g. caused only a temporary muscular incoördination. It is interesting to note that the convulsive symptoms observed previously with ethylbenzyl-barbituric acid were absent. Administered orally, the substance is much less active, probably because of its insolubility and slow rate of absorption. One gram given orally to a 12.5-kg. dog produced only a slight drowsiness and muscular incoördination after about an hour, with complete recovery by the end of the second hour.

Summary

Ethylphenethyl-barbituric acid and several related derivatives and intermediate products are described.

The hypnotic action of this substance was demonstrated and appears to be of the same general type as that of numerous other dialkylbarbituric acids. If we regard luminal, ethylbenzyl-barbituric acid and ethylphenyl-barbituric acid as homologs, differing not merely in the number of carbons in the side chain but also in the location of the phenyl group, the evidence thus far is in support of the recurrence of physiological characteristics with substitution of a given group on alternate carbon atoms.

DETROIT, MICHIGAN

[CONTRIBUTION FROM THE LABORATORY OF PHYSIOLOGICAL CHEMISTRY, TEACHERS COLLEGE, COLUMBIA UNIVERSITY]

THE ISOLATION FROM AUTOLYZED YEAST OF A CRYSTALLINE SUBSTANCE MELTING AT 223°, HAVING THE PROPERTIES OF A BIOS

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For convenience of presentation this report is classified under four divisions: (I) the method employed in isolating the product; (II) the characteristics of the product as shown by chemical and physical tests; (III) the evidence as to its "bios" nature; (IV) a discussion of its relation to vitamin B and the work of other investigators in the field.

I. The Method of Isolation for Autolyzed Yeast

a. Brewer's yeast[2] was allowed to autolyze under toluene until an autolyzate with a dry weight of 0.418 g. per cc. of filtered material was obtained. This filtrate had a Sörensen (P_H) value of 5.93; 3 kg. of yeast was used to obtain 500 cc. of autolyzate.

[1] Presented before the Biological Chemistry Division of the American Chemical Society at the Washington Meeting, April, 1924.

[2] The yeast for the purpose was generously donated by the Jacob Ruppert Co. of New York.

b. To 500 cc. of this product ethyl alcohol was added to make the solution 70% in alcohol. Proteins, yeast gum and other colloidal impurities were thus precipitated. With these impurities was lost about 20% of the bios activity of the original autolyzate. When the precipitate was filtered off and the alcohol removed from the filtrate by distillation, a water solution was obtained which when made up to 500 cc. had a dry weight of 0.148 g. per cc. and a P_H of 4.95.

c. Preliminary tests confirmed Funk and Dubin's[3] contention that fuller's earth would remove vitamin B from autolyzed yeast with little effect upon its bios activity. To the water solution obtained after removal of alcohol as described in Paragraph b, fuller's earth (50 g. per liter of solution) was added and the mixture stirred thoroughly for 30 minutes with a mechanical stirrer. When the fuller's earth was then filtered off a clear solution remained that showed little loss in "bios" activity. The dry weight of this filtrate was then reduced from 0.148 g. per cc. to 0.140 g. per cc. and the Sörensen value was then 5.06.

d. The next step in purification was Dr. Kerr's suggestion, based on his use[4] of ferric oxide hydrosol in the purification of invertase preparations. Ferric oxide hydrosol, first described by Arnold Maus[5] was prepared. For details as to method of preparation and properties the reader is referred to the paper of Nelson and Kerr[4] and papers by Thomas and co-workers.[6,7] For our purpose this iron sol constituted a selective adsorbent whose selectivity could be controlled by varying the hydrogen-ion concentration (P_H) of the solution to which it was added. Experiments demonstrated that at P_H 4.7 the iron sol precipitated from the filtrate left after treatment with fuller's earth a considerable amount of material, but with little diminution of the "bios" activity; that when the filtrate from this precipitation was made exactly P_H 5.3 and treated with an excess of iron sol (four times its volume) a second precipitate was formed and that after this precipitate was formed the remaining filtrate was devoid of "bios" activity. Our procedure was as follows.

To the filtrate from the treatment with fuller's earth (Step c) hydrochloric acid was added until the Sörensen value became 4.7. An equal volume of iron sol was then added and the resultant precipitate discarded. The filtrate was then made exactly P_H 5.3 with sodium hydroxide solution. Four times its volume of iron sol was then added and the precipitate formed filtered off. It carried the bios.

e. The separation of the bios from the iron sol precipitate was then accomplished as follows. The moist precipitate was quickly dissolved in 30% sulfuric acid and as soon as solution was completed diluted with water to 5% acid strength. Barium hydroxide was then added until the solution became alkaline to litmus, care being taken during this operation to keep the solution at about 5°. By this treatment the iron was precipitated as hydroxide and the barium as sulfate. Excess of barium hydroxide was quantitatively removed with sulfuric acid. The residual solution was then clear and slightly yellow; P_H about 6. It carried the bios in solution but not in pure form.

f. By evaporation on the steam-bath the yellow solution yielded a complex which was soluble in 50% alcohol. Our procedure for separating this complex was as follows. It was dissolved in 50% alcohol, the alcohol removed by evaporation and the resultant water solution concentrated by evaporation to a honey-yellow sirup. The addition of absolute alcohol to this sirup precipitated a hygroscopic product that was filtered off, washed with ether and dried in a vacuum desiccator over sulfuric acid. The product

[3] Funk and Dubin, *Proc. Soc. Exptl. Biol. Med.*, **17**, 175 (1919–20).

[4] Nelson and Kerr, *J. Biol. Chem.*, **59**, 495 (1924).

[5] Maus, *Ann. Phys. Chem.*, **11**, 75 (1827).

[6] Thomas and Frieden, THIS JOURNAL, **45**, 2522 (1923).

[7] Thomas and Johnson, *ibid.*, **45**, 2532 (1923).

was difficult to dry and readily absorbed water on exposure to air. It was apparently unstable and when allowed to stand for some time gave off an amine-like odor. In solution it gave a red ninhydrin reaction and was precipitable by phosphotungstic acid. It was readily soluble in 50% alcohol but less so in higher concentrations. Its taste was distinctly bitter. Our next step was the fractionation of this complex.

g. The complex was warmed in 95% alcohol when it first softened to a wax and then after prolonged heating separated into two fractions. One fraction was gummy and settled to the bottom of the containing vessel. This fraction showed no bios activity. The other fraction was soluble in the *hot* 95% alcohol and could be precipitated in two ways. This fraction is at least *a* bios.

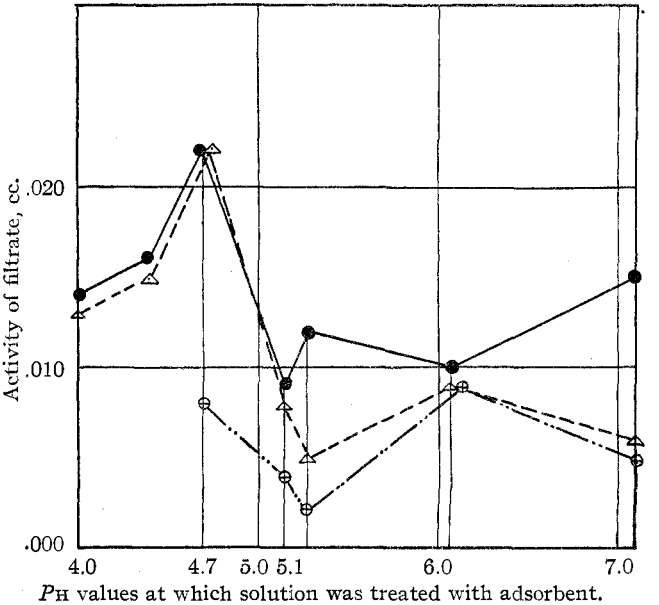

Fig. 1.—The effect of P_H values upon the adsorbent power of ferric oxide hydrosol for bios. Ordinates give the activity of the filtrates upon yeast growth after treatment with the iron sol at the P_H expressed in the abscissas.

h. The bios was separated by one of two methods. (1) The hot alcohol solution was slowly cooled, when a fine cloud of spherulytes of different sizes was formed. (2) The hot alcohol solution was cooled and the product permitted to crystallize in the alcohol concentrate, when it formed orthorhombic crystals. Since, however, the gummy fraction of the complex is insoluble in all concentrations of alcohol, the complex itself more soluble in cold 95% alcohol than in hot, and the crystalline product is almost insoluble in cold 95% alcohol, the separation of the complex fractions can be very effectively accomplished by one treatment with alcohol. After three successive recrystallizations from alcohol the properties of the crystalline fraction were unaltered. It was by far the most active fraction physiologically and this activity did not diminish per unit of weight on successive recrystallizations. The bios activity of the complex was less than half that of the crystalline fraction and the gummy fraction was practically inert—although it is obvious that no claim for even approximate purity of the gum can be made.

Our yield of crystalline bios by this method has been consistently about 70 mg. for each 3 kg. lot of brewer's yeast or, in terms of autolyzate, about 0.03% of the dry weight of the autolyzate (five separate fractionations). A proportionate yield was obtained from yeast boiled with 0.01% acetic acid according to the Osborne-Wakeman[8] method and we have isolated the same product from Fleischmann yeast, boiled alfalfa-meal extract and from a water extract of corn.

II. Chemical and Physical Properties of the Crystalline Product

Our first studies were made with the spherulytes obtained by evaporating the hot alcoholic solution. These showed a constant melting point, sharp at 223°. Later, the orthorhombic crystals were shown to possess an identical melting point. Under the petrographic microscope the spherulytes exhibit diffraction with characteristic maltese cross effects and uniform color with every evidence of homogeneity. The indices of refraction were established to lie between 1.52 and 1.53. Chemical analysis for carbon and hydrogen and for nitrogen by the Dumas method gave the following results.

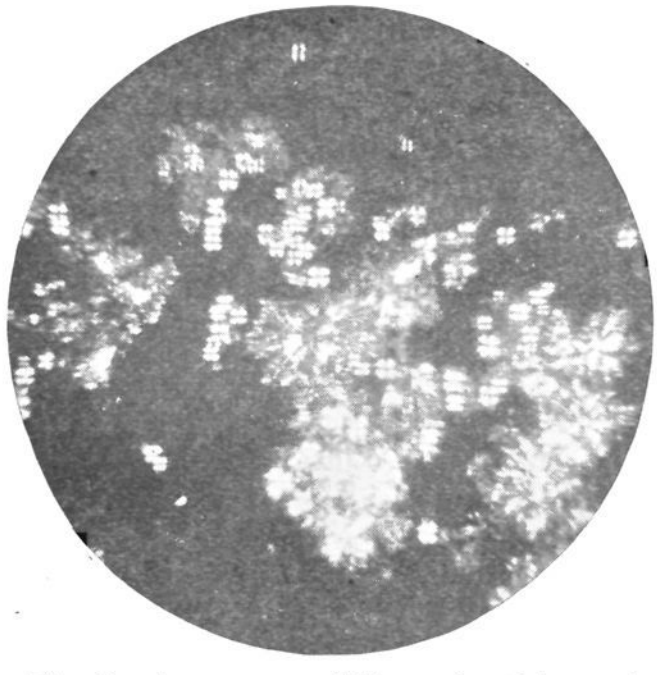

Fig. 2.—Appearance of bios spherulytes under petrographic microscope. Note the homogeneity of the diffraction picture.

Anal. Found: C, 43.29; H, 8.31; N, 10.7.

These are equivalent to the theoretical formula, $C_5H_{11}NO_3$, admitting possible variation in the hydrogen values. The substance was shown by further tests to be free from sulfur, phosphorus, halogens and ash.

The above formula calls for a molecular weight of 133. Actual determinations based upon the elevation of the boiling point of alcohol (McCoy method) gave the following results: 132, 115, 128.

The crystals are slightly bitter. They are soluble in water, acid and alkaline solutions. In cold alcohol they are soluble so long as the concentration is less than 80%, becoming almost insoluble in cold 95% alcohol. These solubilities explain in part the controversy between Fulmer[9] and

[8] Osborne and Wakeman, *J. Biol. Chem.*, **40**, 383 (1919).

[9] Fulmer and Nelson, *ibid.*, **51**, 77 (1922).

ourselves[10] and also justify Willaman and Olsen's[11] use of 80% alcohol instead of 95% to free the sugar of their culture medium from contaminating bios. The crystals are insoluble in ether and 100% acetone but a trace of water added to the latter reagent makes it an excellent solvent.

By heating the substance in a vacuum tube (showing a pressure of 1×10^{-6} mm.) a sublimate was obtained. This sublimate, scraped from the sides of the tube, melted at 223° and had bios activity.

We are not prepared as yet to speak definitely of its chemical constitution. The water solution of the crystals is not precipitable by phosphotungstic acid. It gives no ninhydrin reaction and fails to give the mustard-oil reaction; hence it is not a primary amine. The crystals when warmed in a tube give a red pine splinter test and the distillate obtained by treatment with lime also gives this test. The crystals are optically inactive and show no reducing action upon Fehling solution, nor does prolonged boiling with hydrochloric acid convert them into a reducing substance.

The pine splinter test suggests a nitrogen ring, possibly pyrrole. The lime distillate, however, exhibited a constant boiling point of 96°. This suggests the presence of the pyrroline ring (pyrrole boils at 131°, pyrroline at 91° and pyrrolidine at 88°) but to determine whether such a ring exists in the compound or is produced by the lime distillation requires further study. The pyrroline ring would indicate a secondary amine. An addition product was obtained with benzyl-sulfo-chloride. This product was precipitable in acid solution and soluble in alkaline solution. It also showed liquid crystals at 91° which melted at 119°. Since the mustard-oil reaction for a primary amine was negative we have interpreted this result as possible breaking of the pyrroline ring to form a primary amine addition product, but as the substance probably carried a carboxyl group we cannot be sure as yet that the above reaction and the behavior of the addition product toward acid and alkali is not due to the replacement of the carboxyl hydrogen with base, instead of the amine hydrogen.

The similarity of the constitutional formula and the properties indicated above led us to consider its relation to Fischer's oxyproline ($C_5H_9NO_3$). We therefore repeated his work on the isolation of this substance from gelatin. The oxyproline obtained began to decompose at about the same temperature as the melting point of our bios, but was both optically active and physiologically inactive. On the other hand, reduction of our product with hydriodic acid produces a substance similar to proline in melting point and in percentage of nitrogen content. We are following this lead in our present studies.

Another striking coincidence which may or may not be significant lies in the similarity of our formula to that of the new amino acid described

[10] Eddy, Heft and Stevenson, *J. Biol. Chem.*, **47**, 249 (1921).

[11] Willaman and Olsen, *ibid.*, **55**, 815 (1923).

by J. H. Mueller.[12] His formula was $C_5H_{11}NSO_2$, ours is $C_5H_{11}NO_3$. Through the kindness of Dr. Mueller we are now studying the relation of his product to ours.

While the above data help to delimit the chemical composition they do not establish any structural positions or groups and such information must follow further study. The amphoteric character has apparently been established by its behavior toward adsorbents and by electrolytic experiments.

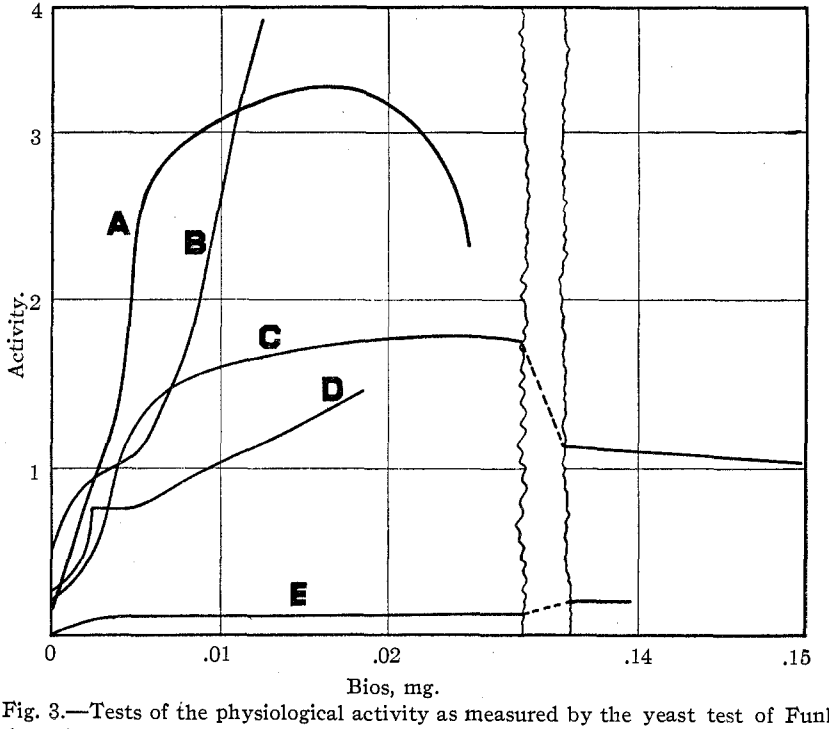

Fig. 3.—Tests of the physiological activity as measured by the yeast test of Funk and Dubin. A, Fulmer's medium, F, plus various amounts of autolyzed yeast; B, Fulmer's medium, F, 72-hour incubation using bottom yeast and bios; C, 24-hour incubation using bios, medium F and top yeast; D, Nageli's medium, 72-hour incubation using bios and bottom yeast; E, contrast between crystalline bios and residue of mother liquor, showing that activity is in the crystals. Note that A and C show an optimum concentration of bios.

III. Evidence of its "Bios" Character

In testing the physiological activity of the product we have made use of a procedure worked out by Funk and Dubin.[13] For culture medium we have used Fulmer, Nelson and Sherwood's Medium F[14] and also the

[12] Mueller, *J. Biol. Chem.*, **56**, 157 (1923).

[13] Funk and Dubin, Ref. 3, and *J. Biol. Chem.*, **44**, 487 (1920).

[14] Fulmer, Nelson and Sherwood, THIS JOURNAL, **43**, 186, 191 (1921).

Nageli solution employed by us in earlier work.[10] The sugar for our media was purified by recrystallization from 80% alcohol.

For yeast cells we have used two types, one of which persistently grows at the bottom of the tube of medium and the other equally persistently at the top of the medium. The needs of these two types for bios are markedly different, the bottom-growing variety being much the more responsive to additions of our product.

Charts are presented showing that in minute doses (0.005–0.025 mg. per cc. of culture medium) marked increase in growth of cells is attained as compared with growth on the medium alone. These stimulations have been obtained with both types of yeast and with both media. In our experience, however, these tests have not been absolutely uniform. The variations do not affect the general conclusion, namely, that our product is a stimulus in minute amounts, but they do suggest the need of further study of the mechanism of yeast testing before it can be considered satisfactory as a quantitative tool. Subliming the product did not lessen its growth-stimulating properties. Preparations from brewer's yeast, baker's yeast and alfalfa meal, all produced similar physiological activity. There is, however, some evidence that the product slowly decomposes on standing and thereby loses some activity.

IV. Relation to Vitamin B and to Bios Reported in Other Studies

Wildier's[15] paper in 1901 first suggested the term "bios" to designate the unknown constituent of plant extracts which stimulates the growth of yeast. In 1914 Bottomley[16] coined the term "auximones" to describe plant growth factors. Roger Williams[16] was, so far as we know, the first to suggest that bios was a vitamin and was also the first to introduce Wildier's term to vitamin workers in this country. In Williams' 1919 paper[16] he showed that selected extracts known to be rich in vitamin B invariably gave the yeast growth stimulation and conversely that extracts selected because of absence of vitamin B failed to show this property. He therefore suggested the use of yeast as a measure of vitamin B content on the assumption that bios and vitamin B are identical and outlined a test method.

Williams' work has been extensively checked by several groups of workers. Our own conclusions were expressed in 1921[10] as follows: "The

[15] Wildier, *La Cellule*, **18**, 313 (1901).

[16] Bottomley, *Proc. Roy. Soc.*, (London) **88**, 237 (1914); **89**, 102, 481 (1915–17); Williams, *J. Biol. Chem.*, **38**, 465 (1919); **42**, 259 (1920); **46**, 113 (1921). Fulmer, Nelson and co-workers, Ref. 14; THIS JOURNAL, **46**, 723 (1924); *J. Biol. Chem.*, **46**, 77 (1921); **57**, 397 (1923). McCollum, MacDonald and co-workers, *ibid.*, **44**, 113 (1920); **45**, 307 (1920–21); **54**, 243 (1922); **56**, 489 (1923). Bachman, *ibid.*, **39**, 235 (1919). Emmett and co-workers, *ibid.*, **43**, 265, 287 (1920). Funk and Dubin, *ibid.*, **48**, 437 (1921). Robertson and Davis, *J. Infect. Dis.*, **32**, 153 (1923). Clark, N. A., *Soil Science*, **17**, No. 3, 193 (1924).

cumulative effect of the data obtained is to suggest that in its present state the test is distinctly unreliable as a quantitative measure of vitamin (B) content.... Until a basal medium is worked out that provides an optimum of all factors except vitamin B the test must be considered of little value in the estimation of true vitamin content."

The necessity of bios for the growth of yeast was first seriously questioned by Fulmer and co-workers[16] and by MacDonald and McCollum.[16] Of this work Fulmer says: "These investigators came to the conclusion that bios is not a necessary component of media for the growth of yeast, but place it in the class of growth stimulants. If this contention be true, bios cannot be considered as analogous to a vitamin, since vitamins are absolutely necessary for the growth of animals and do not merely act as growth accelerators." McCollum's view is expressed in the following passage from a letter discussing nomenclature: "Should we restrict the system of nomenclature now generally accepted for a series of vitamins necessary for mammalian nutrition, *i. e.*, A, B, or C, or should we admit from time to time other vitamins or growth-stimulating substances such as Funk and Dubin suggest for yeast, and such as Wildier believed in when he coined the work 'bios.'"

The issue raised by McCollum refers to a viewpoint first suggested by Funk and Dubin. These investigators first showed that it is possible to free an extract of the anti-neuritic factor by use of fuller's earth without removing bios and therefore suggested that we call the bios "vitamin D."

The above is a very brief outline of the controversy that has arisen over the place of bios in or out of the vitamin series. For details the reader is referred to the bibliography.[10,11,16]

None of the above controversial points can be considered as settled. Much of the evidence that bios is non-essential to yeast growth has been vitiated by the discovery that cane sugar carries an appreciable amount of bios. By using a synthetic sugar (methose) Fulmer believes he has met the matter of sugar contamination, but the question is still open. It is not our purpose here to review the evidence. We have been concerned solely with seeking experimental evidence as to whether our product had, in addition to its yeast stimulating properties, any value in mammalian growth.

In April, through the kindness of Dr. Atherton Seidell, we were supplied with a sample of his highly concentrated picrate of the anti-neuritic factor. Using this product and our own we carried out the tests reported graphically in Fig. 4. We believe they demonstrate conclusively that our product possesses no antineuritic power, while doses of 1 mg. per day per rat of the Seidell product is effective. In most of our experiments bios does produce an increase in rat growth after a period of vitamin B-free diet, but this recovery is slight and temporary; furthermore, addition

of bios to the Seidell product does not improve the growth curve. On the other hand, the Seidell product is admittedly a mixture and it does give a bios test. These tests, therefore, leave entirely unsettled the question of whether vitamin B can produce bios effects or whether bios can affect mammalian growth if polyneuritis is prevented.

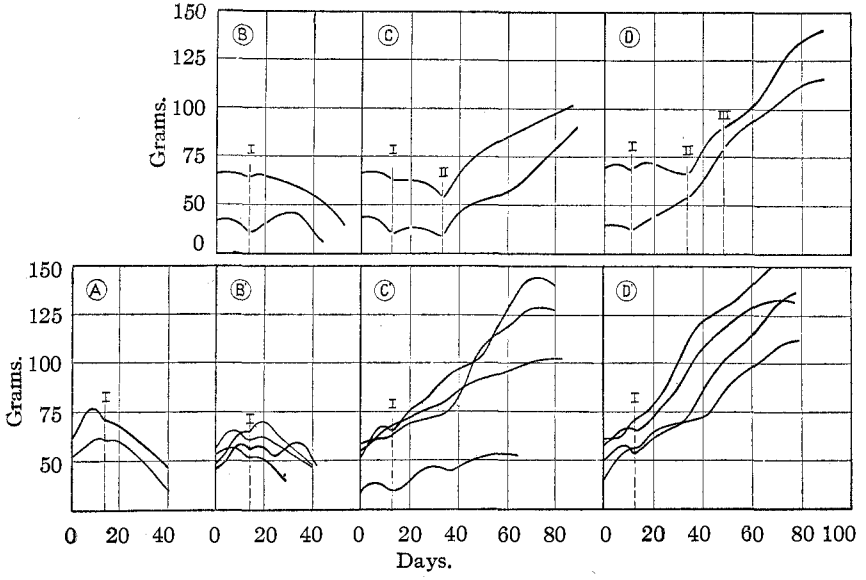

Fig. 4.—The effect of bios on rat growth—A, Controls, no bios; B, Vitamin B-free diet followed by 0.116 mg. of bios daily; C, Vitamin B-free diet followed by 0.116 mg. of bios and then 1.0 mg. of Seidell's picrate daily; D, Vitamin B-free diet followed by 0.116 mg. of bios and then by bios and Seidell's picrate, finally by Seidell's picrate alone; B′, Vitamin B-free diet followed by 0.116 mg. of bios; C′, Vitamin B-free diet followed first by 0.116 mg. of bios and then by 1.0 mg. of Seidell's picrate in addition; D′, Vitamin B-free diet followed by 1.0 mg. of Seidell's picrate and no bios. Note that there is a slight recovery when rats are shifted from a vitamin B-free diet to bios but that this recovery is temporary and that bios shows no anti-neuritic value.

Is our product the sole bios? Recently Fulmer and co-workers[17] cited evidence for believing in the multiple nature of bios. Much more extensive experiments suggesting that there are at least two bioses have been made the subject of a preliminary report by Miller.[18] Neither of these investigators claims chemical purity for these factors.

Nothing in our method of isolation negates the possibility of more than one bios. In fact, the relative stimulation producible by the addition of our pure product and by the use of autolyzate lends strong probability to the suggestion that the latter contains more than one growth stimulant.

[17] Fulmer, Duecker and Nelson, THIS JOURNAL, **46**, 723 (1924).

[18] Miller, *Science*, **59**, 197 (1924).

We have recently been privileged through the courtesy of Dr. Miller and Dr. Lucas to make tests with their material and these tests will be the subject of a later communication. For the present we submit the preceding data as evidence: (1) that our product meets all the established criteria for a pure crystalline substance and is of relatively simple composition (mol. wt., 133); (2) that the identical substance can be removed by our process from brewer's yeast, baker's yeast and alfalfa meal and that the extracts after precipitation of the product by iron sol are no longer physiologically active; (3) that it can form compounds possessing physiological activity (such as the bios complex).

Summary

We report herewith the isolation of a crystalline substance meeting the established criteria for chemical purity and possessing the functions of Wildier's bios. In minute doses (0.005 mg. per cc.) it is markedly stimulating to yeast growth. Its effect varies with the type of yeast used, being less stimulating to top-growing yeast cultures than to bottom-growing forms. A process of separation is described in detail involving selective adsorption or precipitation by ferric oxide hydrosol and recovery by removal of the iron with barium hydroxide. The physical and chemical properties of the substance are outlined so far as experiments have progressed. These apparently establish the melting point as 223°, the crystalline system as orthorhombic, the formula as $C_5H_{11}NO_3$, the molecular weight as approximately 133, the index of refraction as between 1.52 and 1.53. The chemical structure is still undetermined, but the experiments completed suggest a heterocyclic nitrogen-carbon ring with a carboxyl group attached. We have positive data demonstrating its lack of anti-neuritic power, showing that it is not vitamin **B**, but are as yet unable to conclude that it is entirely without effect on mammalian growth.

NEW YORK, N. Y.