II. THE CHEMICAL INVESTIGATION OF "BIOS."

PART I.

By BINDIGANAVALE TIRUMALACHAR NARAYANAN.

From the Department of Physiology and Biochemistry, University College, London.

(*Received November 27th, 1929.*)

Introduction.

Few scientific papers have given rise to more controversy than that in which Wildiers [1901] described "bios." After nearly thirty years of investigation, a review of the literature provides little or no satisfactory evidence of the essential nature of "bios" and only a series of widely conflicting statements regarding its chemical nature and properties.

Quite recently, Miss Copping [1929] has carried out in these laboratories a re-investigation of the fundamentally important question whether or not "bios" is necessary for the growth of yeasts in artificial sugar-salt media and has confirmed the observation that yeasts differ in this respect. Some species are able to reproduce indefinitely in artificial media, whilst others are unable to grow unless a factor resembling that originally described by Wildiers is supplied. *Saccharomyces cerevisiae*, the yeast most frequently employed in investigations on "bios," is definitely in the second class.

It next became of interest to re-examine the chemical nature of this substance, particularly in view of the claims that have been made regarding its isolation. The investigation reported in this paper concerns two main points, namely, the relation, if any, between "bios" and vitamin B, and the actual chemical nature of "bios" itself.

With regard to the first question, it will be recalled that Funk and Dubin [1921] were the first to suggest that the substance stimulating the growth of yeast also played an important part in the nutrition of animals. For a time active discussion took place over the possible identity of "bios" with vitamin B [see review of literature by Kruse and McCollum, 1929], with the result that they were finally regarded as distinct. The discussion has been revived, however, quite recently in the light of the discovery that the so-called vitamin B is a complex of at least two substances, of which one bears a superficial resemblance to "bios" in being stable to heat and alkali.

Regarding the nature and properties of "bios," there is, in the first place, the original description by Wildiers, who stated that it is an organic substance,

soluble in water and 80 % alcohol, stable to heat and acid, not precipitated by neutral or basic lead acetate, phosphotungstic acid, phosphomolybdic acid, or by silver nitrate in acid solution or in the presence of ammonia. Most of these statements were supported by Devloo [1906], who added the view that the active substance is related to choline and possibly associated with choline in nature.

Both Wildiers [1901] and Funk and Dubin [1920] tested a series of purines and pyrimidines, but failed to find that any of them would replace "bios." The work of Fränkel and Schwarz [1920] and of Suzuki *et al.* [1923, 1925] tended to strengthen both the growing opinion that "bios" was a nitrogenous base, and the view that it is related to choline, although Fränkel and Scharf [1921] actually found that choline itself inhibits yeast growth.

On more than one occasion the study of the chemical nature of "bios" has been complicated by the suggestion that it is not a single substance [Fulmer, Duecker and Nelson, 1924]; a view which recently seemed to be established by the reported isolation of the constituents of "bios" by Miller [1924], of which one appears to have been identified as inositol [Eastcott, 1928].

One difficulty in accepting the idea of a complex "bios" was to reconcile it with the alleged activity of a single substance isolated by Eddy, Kerr and Williams [1924, 1926].

The "bios" of this group of investigators was described as a crystalline nitrogenous base, M.P. 223°, not showing the reactions of a primary amine, not precipitated by phosphotungstic acid [cf. Williams *et al.*, 1927], but yielding a crystalline benzenesulphonamide. On slender evidence it was believed to be related to proline. Particularly striking was their claim that whereas the benzenesulphonamide was inactive, the regenerated base showed an activity equal to that of the original "bios." Eddy and his collaborators also described the isolation of a second "bios" (β), apparently also a nitrogenous base, but this has not been obtained in a pure condition.

Furthermore, Kerr [1928] has recently announced the separation of another "bios" (also called β), a very hygroscopic nitrogenous base. It is claimed that this "bios" is also capable of acting by itself.

Further discussions of these claims would, therefore, appear to be more conveniently held over until the results of this investigation have been described.

Experimental.

Technique.

Throughout this investigation a strain of *Saccharomyces cerevisiae* (Chapman) No. 2190, National Collection of Type Cultures, was employed. It was maintained by regular subculturing on wort-agar slopes; cultures of 24–30 hours' growth being employed for "bios" experiments.

As an artificial medium for the "bios" tests, that described by Reader

[1927] was employed. Copping [1929] has shown that this medium is satisfactory for the purpose. "Bios" tests were always made by inoculating 25 cc. of sterile Reader's medium in 100 cc. Erlenmeyer flasks with a suspension of yeast in the medium, so that the original count[1] in the flask was of the order of 0·01, *i.e.* 2500 cells per cc. To certain of the flasks the sterile fractions to be tested were added, care being taken that the volume was not altered by more than 2 %. If there was likelihood of greater dilution, Reader's medium was employed to dissolve the substance to be tested.

All cultures were maintained at 22°. The growth was measured by cell counts made at intervals with the Thoma haemocytometer; all 400 small squares being counted in order to reduce the error. Growth was assumed to have occurred when there was multiplication of at least a hundredfold 24 hours after incubation, and when there was visible growth after 48 hours. The activity of the substance was measured as the least amount which produced this order of growth.

In that part of the investigation concerned with the relation of "bios" to vitamin B_2, fractions were examined for the latter factor. The technique of this test is described more fully elsewhere [Narayanan and Drummond, 1930]. Briefly, it consisted in observing whether the growth of young rats fed on a basal diet adequate in all respects other than a deficiency of vitamin B_2 was stimulated by administering the fraction.

Examination of known substances for "bios" activity.

As part of the investigation, it was thought to be worth while to examine a number of substances of known composition, that might, for one reason or another, be suspected of serving as "bios."

(*a*) *Nucleic acid and related substances.* In view of the work of Wildiers, and also of Funk and Dubin, referred to earlier, the action of yeast nucleic acid was investigated, but no stimulation of growth was observed following the addition of 0·01–0·1 mg. for each cc. of Reader's medium. Tests with the same concentration of guanine as hydrochloride, adenine as sulphate, and uracil also gave negative results; the very slight stimulation of yeast growth with the highest doses of guanine can probably be disregarded. These observations are in agreement with those of Wildiers.

(*b*) *Substances possibly related to vitamin B.* In view of the stability of "bios" to heat, and the recognition of the complexity of vitamin B, it was of interest to investigate whether the substances originally described by Funk [1912–13] as vitamin B, and the products, nicotinic acid and betaine obtained from it by Drummond and Funk [1914], represented any part of the vitamin B complex or possessed "bios" action. The former tests are described elsewhere, but as regards "bios" activity, no stimulation of yeast growth was produced by concentrations of 0·01–0·1 mg. per cc. of the original

[1] Unit count = 250,000 yeast cells in 1 cc. of culture medium.

compound (M.P. 223°) of Funk, nicotinic acid, or betaine. The observations of Peters *et al.* [1928] that concentrates of vitamin B_1 may show power to stimulate the growth of yeast and micro-organisms has been confirmed, for it has been possible through the kindness of Dr Guha to test the materials obtained by him during the fractionation of the vitamin B_1 constituent of the vitamin B complex [Guha and Drummond, 1929]. Some of the earlier fractions tested showed a marked "bios" effect in concentrations of the order of 0·08 mg./cc., but, as the concentration of the vitamin progressed, the "bios" activity decreased. At the stage of the picrolonic acid fractionation no appreciable "bios" activity was shown by Dr Guha's concentrates.

(*c*) *Substances related to choline.* In view of the original statement of Devloo, an examination was undertaken to ascertain whether choline, or any of the bases derived from other phosphatides, showed "bios" action under the conditions of our tests. Tests were made on purified preparations of lecithin and kephalin, for which the writer is indebted to Dr H. J. Channon, added to the cultures in the form of highly dispersed 0·1 % emulsions, but without any increased growth of the yeast being observed. The products of hydrolysis of these preparations of lecithin and kephalin were also found to be inactive in the same concentrations, whilst, at the suggestion of Dr O. Rosenheim, aminoethyl alcohol, for a pure specimen of which the writer is again indebted to Dr Channon, was tested in concentrations of 0·02–0·07 mg./cc., without any activity being observed. No activity was shown by phrenosin, sphingomyelin and the base sphingosine, tested in concentrations of 0·005–0·10 mg. In view of these results it appears probable that the activity of Devloo's material was due to an impurity and that he was incorrect in relating "bios" to choline and lecithin.

(*d*) *Miscellaneous substances.* The following substances were tested for reasons which need not now be given, since they were all found inactive in doses ranging from 0·005 to 0·1 mg./cc.: stachydrine, lysine, spermine, guanidine, methylguanidine, putrescine, cadaverine, hexosediphosphate (fermentation) and potassium pyrophosphate. Cysteine in a concentration of 0·002–0·01 mg./cc. and the three dyes, methylene blue, potassium indigotetrasulphonate and indigo carmine in doses of 0·001–0·005 mg./cc. were also found inactive.

(*e*) *Tests on Eddy's preparations.* Through the kindness of Prof. Eddy the writer was provided with small quantities of both the α- and the β-"bios" isolated in his laboratories. They were tested in concentrations ranging from 0·025 to 0·075 mg./cc. The highest doses of both preparations produced a slight stimulation of growth of yeast, but the smaller concentrations were, according to the standards used in this investigation, inactive (see Table I). Thus, for example, after 120 hours' incubation the count in the flasks to which 0·075 mg./cc. of α-"bios" had been added was only 20, whilst that in the case of the β-"bios" was 24. These figures compare unfavourably with many of our impure concentrates, which in the same interval of time produce a count of

124 with a concentration of not more than 0·02 mg./cc. Judged by the standard adopted in this investigation the preparations of Eddy would be regarded as substances of relatively poor "bios" activity.

Table I.

Source of "bios"	Dose per cc. of medium (mg.)	Initial inoculation count/cc.	Growth in units of count after 24 hr.	48 hr.	72 hr.	120 hr.
Negative control	—	0·01	0·01	0·02	0·02	0·04
" "	—	0·10	0·10	0·40	0·90	2·40
Eddy's α-"bios"	0·025	0·01	—	—	0·50	6·80
	0·025	0·10	0·10	0·50	2·80	17·00
	0·050	0·01	0·02	0·07	1·80	14·00
	0·050	0·10	0·20	0·50	4·30	19·50
	0·075	0·01	0·03	0·06	2·00	20·00
	0·075	0·10	0·40	0·90	3·90	26·00
Eddy's β-"bios"	0·025	0·01	0·01	—	0·40	7·30
	0·025	0·10	0·10	0·30	3·20	19·00
	0·050	0·01	—	—	—	16·80
	0·050	0·10	0·20	—	—	24·00
	0·075	0·01	0·01	0·05	1·90	24·00
	0·075	0·10	0·30	0·70	3·50	28·40
Extract (*d*) Table II	0·050	0·01	2·30	—	24·00	209·00
	0·050	0·10	5·20	14·70	—	—
Extract (*e*) Table II	0·020	0·01	1·90	—	21·00	172·00
Extract (*h*) Table II	0·010	0·01	1·00	—	9·60	102·50
	0·020	0·01	1·40	—	13·70	124·00

As it was possible that these differences might be due to the fact that Eddy and his co-workers used a higher incubation temperature (31°), a few experiments were made to control this point. The following results show that the different temperature does not explain the discrepancy.

Temperature of incubation 31°.

Source of "bios"	Dose per cc. of medium (mg.)	Initial inoculation count/cc.	Growth in units of count after 24 hr.	48 hr.	72 hr.	120 hr.
Negative control	—	0·01	0·01	—	0·02	0·04
Eddy's α-"bios"	0·075	0·01	0·02	0·07	3·20	28·80
	0·075	0·10	0·20	0·60	5·30	34·60
Eddy's β-"bios"	0·075	0·01	0·02	0·09	4·10	27·60
	0·075	0·10	0·30	0·90	4·80	23·30
Extract (*d*) Table II	0·050	0·01	3·20	8·90	—	222·50

Fractionation of yeast extracts for "bios."

(*a*) *Preparation of yeast extract.* A series of experiments was made to ascertain the most suitable means of extracting "bios" from brewer's yeast. Extraction with alcohol of various strengths, the use of acid and alkaline alcohol and the effect of different temperatures were all investigated, but no outstanding advantage in any one method was discovered. The method finally adopted was as follows.

Brewer's yeast was extracted in 7 or 14 lb. batches with 2·5 or 5 litres of alcohol for 4 hours at 60 to 70°. After filtration, the pressed residue was twice re-extracted with 50 % alcohol. The united extracts were concentrated under reduced pressure.

In some cases it was found convenient to use as raw material a commercial yeast extract (marmite), although an obvious drawback to this procedure was the presence in the extract of substances other than those originally present in the yeast.

The original yeast extracts usually produced a count of 16–20 in 48–60 hours when added in concentrations of 0·3–0·4 mg./cc.

(*b*) *Alkaline hydrolysis.* The concentrates from yeast or yeast extract (marmite) were hydrolysed by heating for 2 or 3 hours in an autoclave at 15 lb. pressure with a 20 % solution of barium hydroxide. The precipitate formed during the hydrolysis was removed by filtration, and the filtrate rendered free from barium by addition of a slight excess of dilute sulphuric acid. The barium sulphate precipitate carried down a considerable quantity of organic material from the solution at this reaction (p_H 6·8), but the filtrate contained practically all the "bios."

The dose of this preparation required to produce yeast growth of 16–20 counts in 48–60 hours was usually 0·2–0·35 mg./cc. of medium.

(*c*) *Precipitation with neutral lead acetate.* The filtrate from the barium sulphate precipitate was treated with an excess of lead acetate solution, about 110 g. being usually employed to treat the material obtained from 7 lb. of yeast, and the mixture allowed to stand overnight. After filtration both filtrate and precipitate were treated with H_2S to remove lead, the lead-free liquids brought to a reaction of p_H 6·8 and concentrated.

The "bios" activity was found exclusively in the lead acetate filtrate, of which the dose was found to be 0·15–0·25 mg./cc., whereas in confirmation of Rosedale [1927] and of Chick and Roscoe [1929] it was found that the vitamin B_2 was separated almost completely in the lead acetate precipitate.

From this point of the investigation, having established that "bios" and vitamin B_2 are not identical, the two substances were studied separately.

(*d*) *Attempts to adsorb "bios" on norite charcoal.* Following the steps that have been useful in effecting a concentration of certain of the B vitamins, attempts were made to adsorb "bios" by treating the filtrate from the treatment with lead acetate (*c*) with adsorbent charcoal (norite) at different reactions. The reaction of the liquid was adjusted by units of p_H in stages from 2 to 7, at each stage being shaken with appropriate quantities of norite. The charcoal was filtered off and extracted with warm 50 % alcohol containing 1 % HCl, but no evidence of an adsorption of "bios" was obtained. After repeated trials this step was omitted from the later processes.

(*e*) *Treatment with* 80 % *alcohol.* The final filtrate from the "norite" treatment, or, in the later processes, the filtrate from the lead acetate precipitation was evaporated under reduced pressure to a thick golden brown syrup, and extracted thrice with 5 volumes of hot 80 % alcohol. This treatment removed a quantity of insoluble inorganic substances and other inactive materials, and effected a certain amount of concentration of "bios"; the dose being reduced to 0·07–0·10 mg./cc.

(*f*) *Precipitation with phosphotungstic acid.* The 80 % alcohol extract was concentrated under reduced pressure to remove the alcohol, the thick brown concentrate dissolved in 500 cc. of 5 % sulphuric acid, and treated with an excess of a saturated solution of phosphotungstic acid in 5 % sulphuric acid; a 25 % excess of the precipitant is necessary if the precipitation of "bios" is to be satisfactory. The mixture was allowed to stand overnight in the dark and filtered. Both filtrate and precipitate were treated with barium hydroxide to remove phosphotungstic and sulphuric acids. It was found that under these conditions "bios" is almost completely precipitated by phosphotungstic acid; the activity of the material present in the decomposed precipitate being 0·008–0·02 mg./cc. The filtrate showed insignificant activity.

(*g*) *Silver fractionation.* Two methods of fractionating by means of silver the materials precipitated by phosphotungstic acid were employed.

In the first place a series of fractions with silver nitrate were obtained at different reactions, baryta being employed to lower the acidity by stages. Precipitates were removed at p_H 2 (Ag I), p_H 4·6 (Ag II), p_H 6·8 (Ag III), p_H 8 (Ag IV) and finally after saturation with baryta (Ag V). Each precipitate was suspended in hot water and decomposed with hydrochloric acid gas, but no appreciable "bios" activity was traced in any one; the final filtrate from the silver fractions, rendered free from silver and barium by sulphuric acid, showed considerable potency, being active in doses of 0·01–0·02 mg./cc.

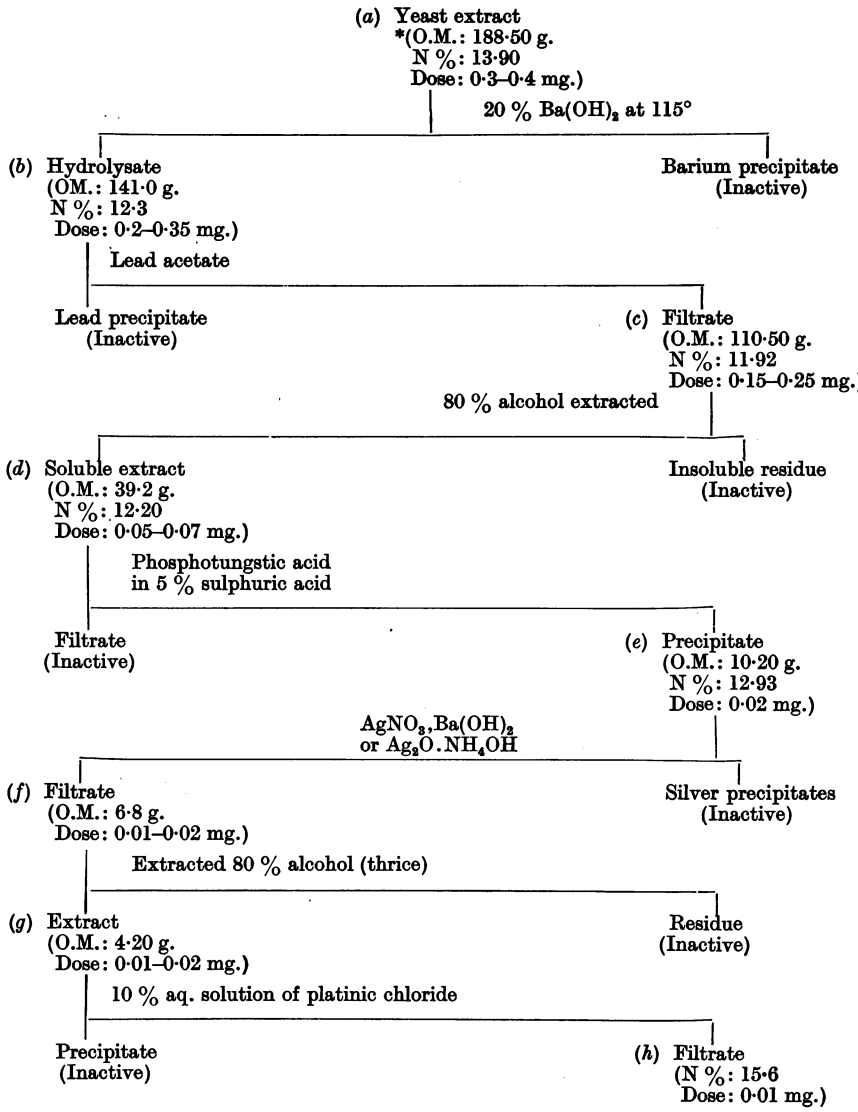
The alternative treatment with silver consisted of rendering the diluted solution from *e* (Table II) distinctly alkaline by means of ammonia and adding freshly precipitated silver oxide until maximal precipitation occurred. The "bios" activity was not removed in the heavy precipitate, but remained in the filtrate after removal of ammonia and the excess of silver. The activity of this preparation was of the same order, namely, 0·01–0·02 mg./cc.

(*h*) *Treatment with platinic chloride.* The filtrates from the silver treatments on concentration to dryness yielded semi-solid materials from which a further amount of inactive inorganic substances was removed by extraction with 80 % alcohol. The solution after removal of alcohol on the addition of a 10 % aqueous solution of platinic chloride yielded a crop of a brown crystalline solid. The crystals were removed, and well washed with water, in which they were relatively insoluble, and decomposed with H_2S. The material precipitated by platinum showed "bios" activity, but only in relatively high concentrations (0·10–0·20 mg./cc.). Most of the activity was found in the platinum filtrate of which the active concentration was about 0·01 mg./cc.

A number of attempts have been made to concentrate further the "bios" present in the final filtrate from the platinum treatment, but with no appreciable lowering of the concentration necessary to produce the required stimulation of yeast growth.

The progress of a typical fractionation is illustrated in Table II.

Table II.



* O.M. = organic matter. Dose = active dose for 1 cc. of medium.

Properties of the active concentrate.

The material obtained by the decomposition of the platinum filtrate contains 15·6 % of nitrogen (calculated on an ash-free basis) but no phosphorus. It does not give the following tests for various types of nitrogenous compounds: carbylamine reaction, nitrosamine reaction, biuret test, Folin-Denis test, Millon reaction, ninhydrin reaction and Denigè's test, but it gives a fairly strong reaction with Pauly's reagent. No precipitates were obtained

on the addition of aqueous or alcoholic picric acid, mercuric sulphate (Hopkins's reagent) or mercuric chloride. Bromine is absorbed with great avidity, yielding a gummy resinous product.

The "bios" activity is inappreciably affected by treatment with nitrous acid or dilute nitric acid, but oxidation by warming with hydrogen peroxide for 30 minutes causes inactivation. All efforts to prepare a solid benzene-sulphonamide failed, and no further evidence has yet been obtained to throw light on the chemical nature of the active substance.

Comparison of the various "bios" preparations.

The more important statements regarding "bios" are summarised in Table III; it being remembered that according to Miller [1924] the growth of yeast in a sugar-salt medium is not satisfactory unless "bios I" is added to "bios II." The former has been identified by Eastcott [1928] as inositol; furthermore, the "bios II" of these workers is described as extremely soluble in acetone, readily removed from solution by shaking with charcoal, and not precipitated by barium hydroxide [Lucas, 1924; Miller, 1924].

Table III.

	Wildiers, Devloo	Eddy and his co-workers	Author's concentrate
Solubilities:			
Water	Soluble	Soluble	Soluble
Alcohol (abs.)	—	Sol. in 95 %	Sparingly sol.
„ (80 %)	Soluble	Soluble	Soluble
Acetone	—	Soluble in aqueous acetone	Insoluble
Ether and chloroform ...	Insoluble	—	Insoluble
Stability to reagents:			
Alkalis (hot or cold baryta and NaOH)	Stable.	Stable	Stable
Acids (cold HCl, H_2SO_4, HNO_3)	Stable	Stable	Stable
Hot HCl, HNO_3 and H_2SO_4	Stable	—	Stable
Nitrous acid	—	—	No action
Hydrogen peroxide ...	—	—	Inactivated
Precipitants:			
Lead acetate (neutral) ...	Not precipitated	—	Not precipitated
„ (basic) ...	Not precipitated	—	Not precipitated
Silver nitrate (acid) ...	Not precipitated	—	Not precipitated
„ (alkali)	Not precipitated	—	Not precipitated
Phosphotungstic acid ...	Not precipitated	Not precipitated	Precipitated
Mercuric sulphate ...	Not precipitated	—	Not precipitated
Mercuric chloride ...	Not precipitated	—	Not precipitated (?)
Platinic chloride (aqueous)	Not precipitated	—	Not precipitated
„ (alcoholic)	Not precipitated	—	Not precipitated
Picric acid	Not precipitated	—	Not precipitated
Nature	Believed to be an org. N base	Nitrogenous base	Simple N compound

It will be seen that there is some disagreement regarding the properties of "bios." Actually, the disagreement is more serious than is indicated by the comparison of the properties of the various preparations, because Lucas [1924]

records a failure to observe under the conditions of his test any stimulant action with Eddy's "bios," and with our technique the preparations of Eddy were also found to be virtually inactive (see Table I).

The identification of "bios I" as inositol by Miss Eastcott [1928] has also complicated the question considerably. During the course of the investigation being reported a large number of experiments has been made to ascertain whether the activity of any of the fractions was dependent upon or was augmented by the addition of inositol. In no single case was this found to be the case; the following examples are typical (Table IV).

Table IV.

(Initial inoculation: 0·01 count/cc., incubation temperature: 22°.)

Inositol (Basle) mg./cc.	Inositol (Pryde) mg./cc.	"Bios" extract mg./cc. Table II	Growth in units of count after 24 hr.	48 hr.	72 hr.	120 hr.
Nil	Nil	Nil	0·01	0·01	0·02	0·04
0·02	,,	,,	0·01	0·01	0·02	0·04
0·04	,,	,,	0·01	0·02	0·02	0·04
0·08	,,	,,	0·01	0·02	0·04	0·04
0·10	,,	,,	0·01	0·02	0·04	—
0·20	,,	,,	0·02	0·04	0·07	0·18
Nil	0·05	,,	0·02	0·05	0·15	0·20
,,	0·10	,,	0·02	0·04	0·10	0·20
,,	0·20	,,	0·03	0·09	0·15	0·30
0·02	0·20	0·050 (*d*)	2·30	5·40	21·80	—
0·08	0·20	0·050 ,,	2·10	6·20	—	210·00
0·10	0·20	0·050 ,,	1·90	5·70	—	206·50
0·20	0·20	0·050 ,,	2·10	5·90	23·00	—
Nil	0·20	0·050 ,,	2·40	6·10	20·20	205·00
,,	0·050	0·020 (*e*)	1·80	5·00	20·50	—
,,	0·10	0·020 ,,	1·90	5·30	—	184·00
,,	0·20	0·020 ,,	—	4·70	18·30	—
,,	Nil	0·020 ,,	1·75	4·80	19·70	—
,,	0·05	0·010 ,,	0·70	2·00	—	78·00
,,	0·10	0·010 ,,	0·85	—	—	82·00
,,	0·20	0·010 ,,	0·80	2·70	—	85·50
,,	Nil	0·010 ,,	0·70	2·20	—	80·40
		(Initial inoculation: 0·10 count/cc.)				
Nil	Nil	Nil	0·10	0·20	—	0·40
0·02	,,	,,	0·10	0·20	—	0·50
0·05	,,	,,	—	0·20	—	—
0·10	,,	,,	—	0·20	—	—
0·20	,,	,,	0·20	—	—	1·50
0·02	,,	0·020 (*e*)	4·50	19·50	—	—
0·05	,,	0·020 ,,	4·90	22·30	—	—
0·10	,,	0·020 ,,	4·70	20·70	—	—
0·20	,,	0·020 ,,	4·60	20·80	—	192·00
Nil	0·10	Nil	0·40	1·20	—	—
,,	0·20	,,	0·50	1·40	—	3·00
,,	0·05	0·020 (*e*)	4·80	—	—	187·00
,,	0·10	0·020 ,,	—	—	67·90	—
,,	0·20	0·020 ,,	5·70	22·60	—	—
,,	Nil	0·020 ,,	4·70	21·50	—	186·00

A series of experiments was also conducted at 28°, the temperature of incubation employed by Miss Eastcott [1928], but the results were not essen-

tially different from those given above. Of the same character were the results of a series of experiments with varying doses of "bios" extracts (Table V).

Table V.

(Initial inoculation: 1·0 count/cc., incubation temperature: 22°.)

Inositol (Basle) mg./cc.	Inositol (Pryde) mg./cc.	"Bios" extract mg./cc. Table II	Growth in units of count after 24 hr.	Growth in units of count after 48 hr.
Nil	Nil	Nil	1·0	2·80
0·05	,,	,,	—	3·20
0·10	,,	,,	—	4·60
0·20	,,	,,	2·30	5·20
0·05	,,	0·020 (*h*)	8·70	32·00
0·10	,,	0·020 ,,	8·60	31·70
0·20	,,	0·020 ,,	9·20	33·90
Nil	0·05	0·020 ,,	7·80	29·20
,,	0·10	0·020 ,,	8·70	33·50
,,	0·20	0·020 ,,	8·50	31·00
,,	0·05	Nil	—	2·90
,,	0·10	,,	—	5·70
,,	0·20	,,	4·00	9·20 (?)
,,	Nil	0·020 (*h*)	7·90	31·00
,,	,,	0·010 ,,	4·80	19·40

One sample of inositol employed was a preparation made by the Society of Chemical Industry of Basle. It was purified by several recrystallisations from dilute alcohol and melted sharply at 215°. A few experiments were also made with a preparation isolated from mammalian muscle, for which I am indebted to Dr J. Pryde. All the experiments with inositol proved negative in the sense that no effect on the growth of yeast was observed.

The active concentrates obtained in this investigation were examined qualitatively for inositol, but no indication of its presence was obtained. The conclusion reached is, therefore, that, if the methods of studying the growth of yeast employed by the Toronto investigators differ in no fundamental manner from those used in this investigation, the activity of the inositol observed by them must have been due either to the presence of an impurity, or to some such effect as has been recently described by Miss Reader [1929] in the case of mannitol. The present study provides no explanation of the different behaviour of inositol in these experiments and those of the Toronto investigators. Until a satisfactory explanation of the discrepancies is forthcoming it might be assumed that different yeasts require different types of "bios," although the writer's experience is against making such an assumption. Miss Copping [1929] was good enough to test a number of the active concentrates and found that they stimulated the growth of a wide variety of yeasts of different types.

Discussion.

Until the investigation has been carried further, it is undesirable to attempt to reconcile the many conflicting statements regarding "bios" that are to be found in the literature.

For the present it will be sufficient to state that concentrates have been prepared from yeast which are highly active in stimulating the growth of *S. cerevisiae* in an artificial sugar-salt medium. These concentrates exhibit an activity in doses that are considerably smaller than those recorded by Eddy for his preparations, one of which is believed to be a pure substance, or those described by the group of Toronto workers. In this investigation no evidence has been obtained to support the activity of Eddy's preparations of α- and β-"bios," nor are the results described in this paper in agreement with the statements regarding the activity of the "bios I" and "bios II" of Miller, Lucas and others.

The properties of the most active concentrates prepared during the course of this work suggest that the active material is a comparatively simple nitrogenous substance[1].

SUMMARY.

(1) "Bios" and vitamin B_2 are not identical. A chemical separation of vitamin B_2 from "bios" has been effected.

(2) A series of substances of known chemical composition has been tested for "bios" activity. None of these substances has been found to exert a marked stimulating influence on the growth of the yeast.

(3) A method of fractionation of "bios" is described, which provides concentrates producing marked stimulation of yeast growth in doses of the order of 0·01 mg./cc. of an artificial sugar-salt medium. The final concentrate obtained appeared to consist largely of relatively simple nitrogenous substances and contained no phosphorus.

(4) The properties and activity of this concentrate are compared with those of Eddy's preparations, and also with those of the preparations of Miller, Lucas and their colleagues. The conclusion is reached that a more active preparation has been obtained than those described by them, and, therefore, that their claims to have isolated "bios" cannot at this stage be admitted.

(5) An extensive examination of Eastcott's statement that inositol is an essential unit of "bios" has been made, but no evidence was obtained to support her claim.

In conclusion, the writer wishes to make grateful acknowledgment to Prof. J. C. Drummond for his kind interest and advice throughout the course of this investigation.

[1] Since this typescript was prepared for publication a paper by Williams, Warner and Roehm [*J. Amer. Chem. Soc.* 1929, **51**, 2764] has appeared where the authors record results very similar to those obtained in the foregoing paper on Eddy's "bios" preparations and inositol. Williams and his co-workers claim to have obtained a very concentrated form of "bios" (their "Z" concentrate adsorbable by fuller's earth) which, however, manifests its greatest activity only in the presence of another factor present in the residue after adsorption by fuller's earth. The "Z" concentrate is claimed to produce definite stimulation of growth in as low a concentration as 0·00005 mg./cc. when duly supplemented by 0·8 mg./cc. of the unadsorbed residue. As has already been shown in the foregoing paper no evidence in support of the complex nature of "bios" has been obtained during the course of the present investigation.

REFERENCES.

Chick and Roscoe (1929). *Biochem. J.* **23**, 504.
Copping (1929). *Biochem. J.* **23**, 1050.
Devloo (1906). *La Cellule*, **23**, 361.
Drummond and Funk (1914). *Biochem. J.* **8**, 598.
Eastcott (1928). *J. Phys. Chem.* **38**, 1094.
Eddy, Kerr and Williams (1924). *J. Amer. Chem. Soc.* **46**, 2846.
—— —— —— (1926). *Proc. Soc. Exp. Biol. Med.* **23**, 416.
Fränkel and Schwarz (1920). *Biochem. Z.* **112**, 203.
—— and Scharf (1921). *Biochem. Z.* **126**, 269.
Fulmer, Duecker and Nelson (1924). *J. Amer. Chem. Soc.* **46**, 723.
Funk (1912–13). *J. Physiol.* **45**, 75.
—— and Dubin (1920). *J. Biol. Chem.* **44**, 487.
—— —— (1921). *Proc. Soc. Exp. Biol. Med.* **19**, 15.
Guha and Drummond (1929). *Biochem. J.* **23**, 880.
Kerr (1928). *Proc. Soc. Exp. Biol. Med.* **25**, 344.
Kruse and McCollum (1929). *Physiol. Rev.* **9**, 126.
Lucas (1924). *J. Phys. Chem.* **28**, 1180.
Miller (1924). *Science*, **59**, 197.
Narayanan and Drummond (1930). *Biochem. J.* **24**, 19.
Peters, Kinnersley, Orr-Ewing and Reader (1928). *Biochem. J.* **22**, 445.
Reader (1927). *Biochem. J.* **21**, 904.
—— (1929). *Biochem. J.* **23**, 61.
Rosedale (1927). *Biochem. J.* **21**, 1266.
Suzuki and Suzuki (1923). *J. Chem. Soc. Japan*, **44**, *Proc.* 228.
—— and Taira (1925). *J. Chem. Soc. Japan*, **45**, 299.
Wildiers (1901). *La Cellule*, **18**, 313.
Williams, Wilson and von der Ahe (1927). *J. Amer. Chem. Soc.* **49**, 227.