tri-*p*-tolylselenonium chloride which has been isolated in 77% yield as the zinc chloride addition compound.

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Wildiers' Bios: The Fractionation of Bios from Yeast[1]

BY W. LASH MILLER, EDNA V. EASTCOTT AND J. E. MACONACHIE

The fractionation of the bios contained in an aqueous infusion of malt-combings was described by Dr. G. H. W. Lucas[1a] in 1924. After preliminary purification of the infusion, baryta and alcohol were added and the precipitate so formed was filtered off; to the filtrate, freed from alcohol and barium, he gave the name "crude Bios II solution." The precipitate was extracted with hot water, and the solution freed from barium; this "crude Bios I solution" was then precipitated by lead acetate and ammonia, the precipitate suspended in water and treated with carbon dioxide and sulfuric acid; the solution so obtained, after removing the last traces of lead with hydrogen sulfide, he called "Bios I solution." The physiologically active constituent of this Bios I solution was identified with *i*-inositol by Miss E. V. Eastcott[2] in 1928.

The yeast crop resulting under standardized conditions when a solution of salts and sugar is seeded with certain kinds of yeast[3] is not much increased by adding either Bios I solution (*i. e.*, a solution of *i*-inositol) or Bios II solution; but if both are added together, the crop is large—Lucas[1a] obtained counts of 15 to 25 from his Bios I solutions, 35 to 50 from his Bios II solutions purified by acetone, and 165 to 240 from the mixtures. In Wildiers' language, neither Bios I (*i*-inositol) nor Bios II contains much bios, but their mixture contains a considerable quantity of that substance; thus precipitation by alcohol and baryta "fractionated" the bios of the combings, and has subsequently been used to fractionate the bios from malt, rice polishings, tea, mushrooms (*agaricus campestris*), molasses, tomatoes, oranges and yeast. It is obvious that such a fractionation of the bios is very different from the mere removal of inactive materials,

(1) Financial assistance from the National Research Council of Canada is gratefully acknowledged.

(1a) G. H. W. Lucas, *J. Phys. Chem.*, **28**, 1180 (1924).

(2) E. V. Eastcott, *ibid.*, **32**, 1094 (1928); THIS JOURNAL, **51**, 2773 (1929).

(3) Dr. Helen Stantial finds that *Wildiers' yeast* and the following yeasts from the American Type Culture Collection behave with inositol and Bios II like the race with which most of our own experiments have been carried out: 2361 *Sacch. validus*, 4206 *Schizosacch. octosporus*. Mr. Maconachie found the same for 2331 (*brewers' yeast*) and for 2335 (*bakers' yeast*) and Dr. Lucas (Ref. 1a, p. 1190) for one of the yeasts used by Prof. W. H. Eddy. Miss Stantial also found that 2361 *Sacch. pastorianus* gives a large crop in media containing only salts and sugar [see Lash Miller, *J. Chem. Ed.*, **7**, 263 (1930)] like the top yeast used by Prof. Eddy (Lucas, Ref. 1a); and that the crops of 2333 *Sacch. mandschuricus* and of 2602 *Zygosacch. mandschuricus* are much increased by Bios II, but are not further increased by adding inositol to media containing Bios II. (Helen Stantial, *Trans. Roy. Soc. Canada*, Sec. III, p. 163 (1932).

(*e. g.*, by precipitation with alcohol, etc.); it would prevent confusion if the word "purification" were used when processes of the latter type are meant.

Experiments with Bios II solutions prepared from malt combings, from tomatoes, and from yeast, show that these solutions also can be fractionated;[4] the provisional names "Bios IIA" and "Bios IIB" are proposed for their constituents—the former for that which is left behind, and the latter for that which is adsorbed when, after preliminary purification if necessary, the Bios II solution is shaken with charcoal. Thus, up to the present, three separable constituents of Wildiers' bios have been recognized, *viz.*, Bios I (*i. e.*, inactive inositol), Bios IIA and Bios IIB. Media containing salts, sugar, and only one of these constituents give a small yeast crop; in media containing any two of them the crop is also fairly low; but if the medium contains all three constituents the crop is large, as shown in Table XI, which gives the crops obtained with Wildiers' yeast. This method of fractionating Bios II was described at the May meeting of the Royal Society of Canada; the present paper gives some details of the work with yeast.

I. Apparatus and Procedure

The technique followed in these experiments is substantially that described in detail in Miss Eastcott's paper,[2] except that dextrose[5] was used instead of cane sugar in the culture media, and that the seed yeast was cultivated in a tomato medium[6] instead of in wort.

Culture media, seeded to a count of 1 or 2 with a race of yeast whose isolation is there described, were rocked for twenty-four hours in a thermostat at 25° after which a measured volume was pipetted into a centrifuge tube containing chloroacetic acid, and the crop determined from the height of the yeast column after centrifuging. By the "count" is meant the number of cells in 4×10^{-6} cc.; 250,000 times the count gives the number of cells in one cc. of the culture medium. Except where otherwise stated, the inositol used was Eastman's ash-free inositol, and the Bios II was from a stock prepared on the large scale from malt combings.

Rocker-tubes

These are L-shaped glass tubes, closed at one end, which are rocked (in the plane of the L) thirty times a minute through an angle of 60° in the water of a thermostat. A size convenient for 10 cc. of culture medium is 27 cm. long and 14 mm. inside diameter; tubes of about the same length, but 27 mm. in diameter, are used for 50 cc. of solution.

(4) Miller, Eastcott and Sparling, *Trans. Roy. Soc. Canada*, Sec. III, p. 165 (1932).

(5) It is only when the medium contains nothing but sugar and salts that the trace of bios contained in a good brand of cane sugar seriously affects the crop. Eastman's "practical dextrose" gives the same low crops as the more expensive "dextrose" if it has been cleaned by shaking 100 g. with 100 cc. of a mixture of 10 volumes of acetone with one volume of water. A second washing is hardly necessary; not much dextrose is dissolved.

(6) 4 g. KH_2PO_4, 2 g. $MgSO_4 \cdot 7H_2O$, 8 g. NH_4NO_3, 0.7 g. $CaCl_2 \cdot 6H_2O$, 2 g. Rochelle salts, 100 g. cane sugar, water to one liter; to this is added 300 cc. of tomato juice, obtained by heating the contents of a can of tomatoes to 100°, filtering through cloth and paper, and neutralizing half of it to litmus with about 5 cc. of 2 *N* sodium hydroxide. This is easier to make than wort, and yeast cultivated in it is white, not dark colored.

If the culture medium, seeded with yeast, be left in a stationary flask instead of being rocked in a rocker-tube, the cells form a layer at the bottom of the flask; as this layer grows thicker, the upper cells more and more shut off the supply of sugar, salts, oxygen and bios from those below, and hinder the escape of alcohol and acid formed by them. Even the topmost cells are supplied only by diffusion and by accidental convection currents; and because of the small concentration of bios in media used for crop determinations, the diffusion of that component is particularly slow. That under such conditions the twenty-four-hour count may depend more on the shape and size of the vessel and the volume of the culture medium than on the quantity it is intended to measure (*viz.*, the amount of bios present), is shown by the results of Table I.

In these experiments the rocker-tube and the flasks were immersed in the water of the same thermostat; with one exception each contained 50 cc. of a medium made of 25 cc. of S. & S. (p. 1504, note 7) 23 cc. of water and 2 cc. of wort, seeded with Yeast 2331 of the Am. Type Cult. Coll'n whose rate of reproduction is practically the same as that of the yeast commonly used here; the smallest flask was supplied with only 40 cc. of the same medium; if it had been filled to the neck the count would doubtless have been lower still.

Stopping the rocker lowers the count, but doubling the usual rate of rocking leaves the count unchanged; 10 cc. in the small rocker-tube gives the same count as 50 cc. of the same medium in the larger tube. Thus there is ample evidence that results obtained in rocker-tubes are independent of diffusion, while those obtained in flasks may be much influenced by that factor.

TABLE I

Rocker-tube	$C = 270$	125-cc. Erlenmeyer	$C = 93$
250-cc. Erlenmeyer	169	50-cc. Erlenmeyer	60
200-cc. Erlenmeyer	112		

With our yeast the twenty-four-hour count in the 250-cc. Erlenmeyer agrees with that in the rocker-tube if the latter does not exceed 100; when the count in the rocker-tube is larger, that in the flask may catch up if it be left standing long enough, as shown in Table II.

In the experiments of Table II, the 50 cc. of medium in each vessel contained 5 cc. of wort; only one rocker-tube was used, but seven flasks, as it would obviously have been impossible to take samples from a single flask without shaking it.

TABLE II

Hours after seeding	14.3	16.2	17.2	19.8	24.6	38.4	48.5
Rocker-tube $C =$	170	232	288	345	430	444	446
250-cc. Erlenmeyer $C =$	138	165	193	242	300	427	445

Sugar and Salts.—Clark's sugar and salts solution[7] was used; when bios is present, moderate changes in the proportions and absolute amounts

(7) 15 g. KH_2PO_4, 7.5 g. $MgSO_4 \cdot 7H_2O$, 30 g. NH_4NO_3, 2.5 g. $CaCl_2 \cdot 6H_2O$, and 360 g. dextrose, in 1800 cc.; 5 cc. of this "S. & S." (2.5 cc. in the half-strength medium of Tables III and IV) was used in each rocker-tube, and made up to 10 cc. by the other ingredients of the culture medium.

of the salts do not matter very much; although, as Fulmer[8] has shown, in absence of bios they may be of the first importance. Cutting down Clark's quantities of salts and sugar to half does not affect counts below 250–300; but Miss Reader's[9] *streptothrix* medium (with one per cent. of dextrose) is too dilute for yeast counts as high as 100.

The results of Table III were obtained with different quantities of the same bios (p. 1509) in the half-strength Clark's medium and in Miss Reader's medium; those of Table IV with a constant amount of bios in half-strength Clark's medium and in media containing up to five times the salts and sugar of Miss Reader's.

TABLE III

Proportionate amount of bios	1	2	3	4
Clark's medium, half strength	C = 125	175	225	283
Miss Reader's medium	C = 83	110	123	125

TABLE IV

Miss Reader's medium, C =	125	Miss Reader's medium × 4, C =	221
Miss Reader's medium × 2	170	Miss Reader's medium × 5	271
Miss Reader's medium × 3	200	Clark's half strength	283

Acidity of the Culture Medium.—The P_H of Clark's medium (equal volumes of S. & S. and water) is 4.6, about the same as that of wort; while addition of 4 cc. of water and one cc. of $N/8$ sodium hydroxide to 5 cc. of S. & S. gives a nearly neutral medium, P_H = 6.8. In the experiments described below, counts were determined both with and without the addition of this much alkali to the medium before seeding; those to which alkali has been added are the "neutral media," the others "Clark's media."

It is clear from the results of Tables VI to XII that there is no "best" initial P_H for media containing bios; high twenty-four-hour counts are often higher in the initially neutral medium, low counts often lower. This behavior is no doubt due to the formation of acid in the medium while the crop is growing; carbon dioxide does not lower the P_H of Clark's medium, but even so low a count as 27 may be accompanied by a change of P_H from 4.6 to 3.6 or from 6.8 to 5.8, and higher counts increase the acidity much more. Addition of sodium bicarbonate or replacement of part of the acid phosphate by dibasic phosphate has, of course, the same effect as addition of sodium hydroxide; high counts can also be improved by adding certain salts, which probably act as buffers.

For example, a count of 200 in Clark's medium with wort as source of bios was raised to 325 by adding sodium hydroxide in the proportion given above, ammonium mucate raised it to 329, sodium lactate to 330, sodium potassium tartrate to 360, sodium acetate to 291, sodium chloride had no effect; the amount of salt used in each case con-

(8) Fulmer, Nelson and Sherwood, THIS JOURNAL, **43**, 191 (1921).

(9) 0.10 g. KH_2PO_4, 0.016 g. K_2HPO_4 (to bring P_H to 7.4), 0.07 g. $MgSO_4 \cdot 7H_2O$, 0.30 g. $(NH_4)_2SO_4$, 0.05 g. NaCl, 0.04 g. $Ca(NO_3)_2$ and 0.5 g. dextrose, in 100 cc. This medium was worked out by Miss V. Reader [*Biochem. J.*, **21**, 904 (1927)] for *streptothrix corallinus*, not for yeast; she found that the dextrose might be varied from 0.6 to 2.0%; one per cent. was used in the experiments of Tables III and IV above.

tained metal or ammonium equivalent to that in the hydroxide; the medium to which sodium hydroxide had been added was not further improved by adding acetate, lactate or tartrate.

This whole matter is being further investigated.

II. Crops from Yeast Infusions, with and without Inositol

Workers in other laboratories have confirmed Miss Eastcott's observation that adding inositol does not much improve the yeast crop obtained from sugar and salts solutions; and Williams[10] with his co-workers, and Narayanan,[11] report little or no improvement when inositol is added to media containing sugar, salts and yeast-infusion or certain other yeast preparations. These preparations (made from Fleischmann's starch-free yeast, and from brewers' yeast, respectively) thus resembled the juice of oranges or grapes, the infusions of bran, dried catnip, cottonseed meal or saffron, and the solutions of meat extract (lemco) or dried milk (klim), studied by Miss Eastcott; for she found[2] that any one of the latter added to salts and sugar solution gave a good yeast crop which was not appreciably improved by adding "Bios I" as well. In view of these results we were quite unprepared for Mr. Maconachie's observation that an aqueous infusion[12] made from a pure culture of Yeast No. 2335 of the Am. Type Cult. Coll'n gave in Clark's medium a twenty-four-hour count of 125, but on addition of 0.02 mg./cc. of Eastman's ash-free inositol a count of 234; the yeast whose crop was measured was the same as that from which the infusion had been made, *viz.*, No. 2335.

Mr. Maconachie then prepared an extract[13] from brewers' yeast (for which we are indebted to the O'Keefe Brewing Company of Toronto), following the procedure of Narayanan.

One cc. of this extract in 10 cc. of culture medium seeded with a brewers' yeast (Am. Type Cult. Collection No. 2331) gave a twenty-four hour count of 440, which was increased to 500 by adding 0.02 mg./cc. of Eastman's inositol.

On centrifuging in a Sharples laboratory centrifuge, 680 g. of this same yeast yielded 350 cc. of beer (*i. e.*, culture liquid); thus rather less than half of this sample of "brewers' yeast" consisted of yeast cells, and a considerable part of the "yeast extract" made from it was really beer extract.

One cc. of the beer in 10 cc. of culture medium gave a count of 248, which was not increased by adding inositol but was raised to 474 by adding an amount of Bios II from

(10) Williams, Warner and Roehm, THIS JOURNAL, **51**, 2764 (1929).

(11) Narayanan, *Biochem. J.*, **24**, 6 (1930).

(12) 2.5 liters of sugar and salts solution (p. 1504, note 7) mixed with 2 liters of water and 500 cc. of a strong wort (Eastcott, Ref. 2, p. 1097), seeded with 50 cc. of a suspension of Yeast 2335 (count 150), and stirred for twenty-four hours at room temperature, gave a crop of count 450. The yeast was filtered out, heated with 150 cc. of water to 100° for three hours, and filtered. The cloudy filtrate was autoclaved for a few minutes and the resultant precipitate removed by centrifuging.

(13) 3.7 kg. of the yeast as received was heated to 65° for four hours with 2.9 liters of 93% alcohol, and the residue after filtration was similarly extracted first with 3 liters and then with 2 liters of 50% alcohol. The combined filtrates were evaporated *in vacuo* to 1350 cc.

combings which by itself (in the 10 cc.) gave a count of 50; this shows that the beer contained a large excess of inositol. On the other hand, one cc. of an extract made (as described above) from the thoroughly washed yeast cells gave a count of 488, which was raised to 660 by adding 0.02 mg./cc. of inositol, but was left unchanged by Bios II.

Thus the extract made from the cells was deficient in inositol, but in the original "yeast extract" this deficiency was largely made up by the excess of inositol introduced with the beer.

These observations suggested an explanation for Narayanan's negative results, *viz.*, that his brewers' yeast may have contained a little more beer than ours, or beer a little richer in inositol. If this were the case, adding inositol to his extract would not have improved his yeast crops; for as Miss Eastcott has shown, addition of inositol beyond a certain amount (which depends on the quantity of Bios II in the medium) does not go on increasing the crop.

That this is not the only possible explanation, however, was shown by the behavior of the next batch of yeast obtained from the same brewery. This yielded only 16% of beer, as compared with over 50% from the first batch; and neither the beer, nor the extract from the yeast as received, nor the extract from the washed yeast-cells was improved as a culture medium by adding inositol. Neither was the extract prepared from a pure culture of the race of yeast used in most of the experimental work of this laboratory; but whereas for Maconachie's preparation Yeast 2335 was freshly cultured, that of our own race was taken from the bottom of a culture-flask two days after seeding, when (as shown by crop determinations) most of the Bios II had been removed from the wort. That the amount and quality of the bios in a yeast extract depends upon the condition of the cells used to make it, is evident from the results with Fleischmann's yeast described below; it follows even more directly from an observation of Maconachie, who found that the extract prepared from cells of No. 2335 which had been stirred for twenty-four hours with salts and sugar gave a count of only 35 instead of 125.

Two half-pound cakes of Fleischmann's yeast were then purchased, one (I) from the Canada Bread Co., and the other (II) from Fleischmann's agency in Toronto; neither gave the iodine test for starch. An aqueous extract and a 50% alcoholic extract was prepared from each; and the twenty-four-hour counts given in Table V were obtained in Clark's medium containing the extract from 0.05 g. of yeast cake per cc., either with or without 0.02 mg. of Eastman's inositol per cc.

TABLE V

0.05 g. Cake I	Alcoholic extr., alone	$C = 250$	With inositol $C = 275$
.05 g. Cake II	Alcoholic extr., alone	$C = 155$	With inositol $C = 175$
.05 g. Cake I	Aqueous extr., alone	$C = 217$	With inositol $C = 367$
.05 g. Cake II	Aqueous extr., alone	$C = 167$	With inositol $C = 209$
.01 g. Cake III	Extract alone	$C = 100$	With inositol $C = 320$

The Fleischmann Company then presented us with a fresh one-pound cake of starch-free yeast, Cake III, sent from their factory in Montreal in a refrigerator car.

This was chopped up into 2 liters of boiling water, left in a steam-box for two hours with occasional shaking, autoclaved for an hour, and filtered. The filtrate was evaporated *in vacuo* to 175 cc., 350 cc. of alcohol added, the precipitate filtered off, and the solution (mixed with 50 cc. of washings) evaporated to dryness in a vacuum. The residue, dissolved in water, neutralized with a few drops of normal sodium hydroxide, and made up to 180 cc., is the extract whose fractionation is described below; one cc. corresponds to 2.5 g. of yeast cake.

The extract made from this cake contained much more bios per gram of yeast, and a much larger excess of Bios II, than the preparations from either of the other yeast cakes; the counts given in Table V were obtained with only one-fifth the weight of yeast used in the other cases.

Table VI gives the counts obtained when each cc. of the culture medium contained the extract from 0.01 g. of yeast cake III together with from $^1/_{800}$ to $^1/_{25}$ milligram of the Eastman Company's "ash-free inositol."

TABLE VI

Extract from 0.01 g. of yeast per cc. of medium

Inositol	Mg./cc. =	0.00125	0.0025	0.0050	0.010	0.020	0.040
Clark's medium	C =	170	200	260	294	320	316
Neutral medium	C =	163	217	300	370	410	410

Miss Eastcott has already shown that there is such a thing as "enough" inositol for a given amount of Bios II, *i. e.*, more does not increase the yeast crop. The experiments of Table VI show that 0.02 mg./cc. together with that in the extract used, is enough inositol for the Bios II contained in that extract; the reason why inositol did not improve the crops given by the extracts of Williams and of Narayanan is simply that these extracts themselves contained enough inositol for their Bios II.

As the suggestion has been made (though not supported experimentally) that the results obtained by Miss Eastcott with Kahlbaum inositol and with her own preparations from tea might be due to some unspecified impurity (which if it exists must have been present in equal quantity in all of them), we have subjected both Kahlbaum (pre-war) inositol and Eastman ash-free inositol to the process used in purifying Miss Eastcott's inositol from tea: that is, each of them was heated for twenty hours to 110° with 22% hydrochloric acid, and after evaporating to remove the acid, was fractionally crystallized and recrystallized from mixtures of methyl alcohol and water. The counts obtained with these purified inositols, both alone and with Bios II from various sources, agreed within the experimental error with those given by the commercial preparations.

Table VII gives the counts obtained with Kahlbaum inositol, Eastman ash-free inositol (both as purchased) and Miss Eastcott's original preparation from tea, when 0.02 mg./cc. of each in turn was added to media containing per cc. the extract from ten

milligrams of yeast cake III (*i. e.*, *Y*/cc. = 0.01).[14] The counts agree with each other and with the two corresponding determinations of Table VI (Eastman inositol) as closely as can be measured with the technique employed.

TABLE VII

Yeast extract *Y*/cc. = 0.01, with inositol 0.02 mg./cc.

Clark's med.: Eastman's, *C* = 324; Kahlbaum's, *C* = 320; from tea, *C* = 326
Neutral med.: Eastman's, *C* = 416; Kahlbaum's, *C* = 400; from tea, *C* = 410

III. Fractionation of the Bios from Brewers' Yeast by Baryta and Alcohol and by Lead Acetate and Ammonia

Both extracts from the first batch of brewers' yeast (*viz.*, that from the yeast as it was received, and that after the beer had been removed) on treatment with baryta and alcohol as described by Lucas, yielded "crude Bios I" and "crude Bios II" solutions just like those from the other eight sources of bios enumerated above. It seemed worth while, however, to repeat these experiments with the extract from the second batch, which like Narayanan's extract gave as large a crop without inositol as with it. Following Narayanan's procedure, the extract was autoclaved with baryta solution and the filtrate after removing the barium was precipitated with acetate of lead. Like the extract itself, neither the baryta-filtrate nor the lead-acetate-filtrate[15] (after removing metals and acetic acid, neutralizing, adding water salts and sugar as usual, and seeding with yeast) gave a larger yeast crop when inositol was added than without it. But when, after autoclaving the extract with baryta, *two volumes of alcohol were added before filtration*, the result was very different: 0.02 mg. of Eastman inositol added to each cc. of the culture medium in which this filtrate was used, raised the count from 140 to 340. The reagent generally used for precipitating inositol[16] is basic lead acetate with ammonia; and when the filtrate from the neutral lead acetate precipitation was precipitated with this reagent, the count obtained from this basic-lead-acetate-filtrate was raised from 110 to 325 by adding 0.02 mg./cc. of inositol.

Neither of these reagents for precipitating inositol was used by Narayanan in preparing his "concentrates;" and so, naturally enough, he "obtained no evidence to support Eastcott's claim that inositol is an essential constituent of bios."

IV. Details of the Fractionation of Bios from Fleischmann's Yeast by Baryta and Alcohol, and by Charcoal

Because of its large content of Bios II, the extract from Cake III was chosen for these experiments; the preparation of the extract is described on p. 1508.

(14) The symbol *Y*/cc. means the number of grams of yeast cake used to make the preparation contained in one cc. of culture medium.

(15) Used in the experiments of Tables III and IV.

(16) Needham, *Biochem. J.*, **17**, 422 and 431 (1923).

Table VIII gives the counts obtained with the extract from 0.0025–0.040 g. of yeast per cc. of culture medium; the counts from media containing the extract from 0.01 g. of yeast per cc. together with various amounts of inositol have been recorded in Table VI.

TABLE VIII

	Y/cc. = 0.0025	0.005	0.010	0.020	0.040
Clark's med.	C = 62	70	108	160	260
Neutral med.	C = 114	124	120	142	274

The Baryta-Filtrate

In each of several 50-cc. Erlenmeyer flasks, 2 g. of powdered crystallized barium hydroxide and 10 cc. of extract (from 25 g. of yeast) were heated to 100° for three hours with occasional shaking. After cooling, the contents of one flask were filtered and the precipitate washed with 5 cc. of hot water; filtrate and washings were heated and 6.25 cc. of 2 N sulfuric acid added (just enough to change the color of Congo paper), the barium sulfate was removed by filtration and volatile acids by repeated evaporation *in vacuo*, the residue was neutralized by sodium hydroxide, and made up to 25 cc. Thus one cc. of this "baryta-filtrate" comes from one gram of yeast.

For equal values of Y/cc., the counts obtained with baryta-filtrate are much lower than those with extract; this can hardly be due to the presence of a poison in the former, because when added to a medium containing wort it increased the count (wort alone, C = 350; wort with bar.-filt. Y/cc. = 0.0375, C = 434). Adding Bios II does not increase the Count (Y/cc. = 0.0125, neut. med., C = 142; with Bios II, 138), adding inositol, however, does (see Table IX); thus in the baryta-filtrate as in the extract there is excess of Bios II, but some of the latter has been destroyed by the baryta treatment. Table IX gives the counts obtained in neutral media containing 0.02 mg./cc. of inositol and various amounts of the baryta-filtrate.

TABLE IX

	Y/cc. = 0.0025	0.005	0.010	0.0125
Bar.-filt.				
Neutral med.	C = 36	54	130	225

There is much more difference between the forty-eight-hour counts and the twenty-four-hour counts when the media contain baryta-filtrate than when they contain yeast extract; moreover, after suitable treatment with acid the baryta-filtrate gives higher twenty-four-hour counts. These experiments, which suggest that the Bios II of the extract is not irrecoverably destroyed by the baryta treatment, are being extended; they may throw light on changes which some of the solutions undergo when kept.

Fractionation by Precipitation with Baryta and Alcohol

To the contents of another of the 50-cc. Erlenmeyer flasks (see *baryta-filtrate*, above) as soon as it was taken from the steam-bath, 20 cc. of 95% alcohol was added, with shaking; after standing for some hours the contents were filtered, and the precipitate washed with 15 cc. of a mixture of two volumes of alcohol and one of water. filtrate precipitate and washings being kept separate. A "crude Bios II" solution was prepared from the filtrate, by removing barium and volatile acids (as described above) and neutralizing;

the washings were treated in the same manner; and a "crude Bios I" solution was prepared from the precipitate by extracting with 15 cc. of hot water and treating this filtrate like the other; each of the three was brought to a volume of 25 cc., *viz.*, one cc. per gram of yeast used in making them. A mixture of equal volumes of these three solutions gave practically the same count as the baryta-filtrate from the same amount of yeast (neutral medium. Y/cc. = 0.0125. bar.-filt., C = 142, mixture, C = 136); with 0.02 mg./cc. inositol, the mixture gave a somewhat lower count (bar.-filt., C = 225, mix., C = 178). suggesting loss of Bios II.

Counts from the "crude Bios I solution" were not increased by adding inositol, but were increased by adding Bios II from combings, and the opposite is true for counts from the "crude Bios II solution" (see Table X, 0.02 mg./cc. inositol was used). Thus bios from Fleischmann's yeast is fractionated by baryta and alcohol just like bios from brewers' yeast and from the other eight sources enumerated on p. 1502.

TABLE X

Crude Bios I, Y/cc. = 0.02	Clark's med.: C = 20	With Bios II	C = 143
Crude Bios I, Y/cc. = 0.02	Neutral med.: C = 24	With Bios II	C = 158
Crude Bios II, Y/cc. = 0.02	Clark's med.: C = 60	With Inositol	C = 264
Crude Bios II, Y/cc. = 0.02	Neutral med.: C = 120	With Inositol	C = 263

Fractionation of the yeast extract itself, omitting the baryta treatment, gives a better yield of Bios II.

The crude Bios II solution obtained by adding 2 g. of baryta (dissolved in 3 cc. of hot water) and 26 cc. of alcohol to 10 cc. of yeast extract (from 25 g. of yeast), gave a count of 340 instead of the 263 of Table X (Y/cc. = 0.02, inositol 0.02 mg./cc., neutral medium).

The preparation of crude Bios II solution can be still further simplified, by extracting the yeast (6 lb. Fleischmann's starch-free) with 80% alcohol (6 liters) and adding baryta (360 g. dissolved in 360 cc. of water) and alcohol (750 cc. 95%) to the filtrate, which of course contains water taken from the yeast cake.

The counts from a crude Bios II solution prepared in this way by Mr. A. J. Mueller were: Y/cc. = 0.014, C = 350; Y/cc. = 0.028, C = 444 (both with 0.02 mg./cc. of inositol in Clark's medium). This preparation therefore was better than that from the baryta-filtrate, though not so good as that obtained by directly fractionating the extract from Cake III.

Fractionation of the Bios II by Charcoal

One gram of Darco charcoal was shaken for fifteen minutes with 30 cc. of a solution containing one cc. of 2 N sulfuric acid and the crude Bios II solution (that of Table X) from 20 g. of yeast, filtered off, and washed with 15 cc. of water; the filtrate was shaken with another half gram of charcoal, which was filtered off and washed as before. The combined filtrates and washings were freed from sulfuric acid by baryta, and made up to 80 cc.; each cc. of this neutral "crude Bios IIA solution"[17] came from 0.25 g. of the

(17) This is the correct designation according to the nomenclature of Lucas' paper; but in the present paper (except where otherwise stated), this was the solution used in the experiments for which quantities of Bios IIA or IIA are given. Similarly Bios IIB or IIB are printed when the crude Bios IIB solution was used.

yeast cake. The charcoal from the two operations was shaken for half an hour with 40 cc. of freshly prepared "acetone–ammonia reagent,"[18] filtered off, washed with 10 cc. of the reagent, and then shaken again with 25 cc. of the reagent and washed as before. The united extracts and washings were evaporated *in vacuo* and the residue dissolved in water to a volume of 25 cc.; this "crude Bios IIB solution" was neutral to litmus, each cc. came from 0.80 g. of yeast.

With 0.02 mg./cc. or 0.04 mg./cc. of inositol, whether in Clark's medium or in the neutral medium, and whether Y/cc. = 0.01, 0.04 or 0.05, the crude Bios IIA solution gave counts of 20 or lower, and the crude IIB solution gave counts of 50 to 60 or lower. But when IIA (Y/cc. = 0.01) and IIB (Y/cc. = 0.01) and inositol (0.02 mg./cc.) were present in the same (neutral) medium, the count was 127. *Thus charcoal fractionates Bios II into Bios IIA and Bios IIB, just as baryta and alcohol fractionates bios into Bios I and Bios II.* Mr. Mueller's crude Bios II solution (p. 1511) was fractionated in the same way; the counts (with 0.04 mg./cc. of inositol in neutral media) obtained from his IIA and IIB separately and together, are given in Table XVI.

To make quite sure that it was "Wildiers' bios" that had been fractionated, and not some other "nutrilite,"[19] the media of Table XI were seeded with Wildiers' own yeast, a culture of which was courteously furnished us by Professor R. J. Williams of the University of Oregon.

The inositol used in these experiments (0.04 mg./cc.) was Eastman's: the Bios IIA (Y/cc. = 0.04) and the Bios IIB (Y/cc. = 0.02) were the crude solutions whose preparation from the baryta-filtrate has just been described in detail.

TABLE XI

(Wildiers' yeast)	Clark's medium	Neutral medium
Inositol	C = 20	C = 30
Bios IIA	13	20
Bios IIA + inositol	25	50
Bios IIB	42	38
Bios IIB + inositol	92	62
Bios IIA + Bios IIB	30	98
Bios IIA + Bios IIB + inositol	262	225

A series of measurements was carried out in which increasing amounts of Bios IIB were added to media containing excess of inositol and a fixed amount of Bios IIA, and another in which the amount of Bios IIB was kept constant and that of Bios IIA was varied; the results are given in Table XII. In Clark's medium (but not in the neutral medium) the counts first rise and then fall again as the amount of Bios IIB is increased; it is proposed to determine the nature and the amount of the acid to whose formation this behavior is probably due.

When a considerable amount of the variable constituent has been added, the effect of further additions is small (Table XII); just as when inositol is

(18) Acetone–ammonia reagent: 5 cc. of conc. ammonia, 35 cc. of water, acetone to 200 cc.

(19) Williams and Bradway, THIS JOURNAL, 53, 783 (1931).

TABLE XII

Inositol 0.04 mg./cc.

Bios IIA. Y/cc. =	0.0025	0.005	0.01	0.02	0.04	0.08	
Bios IIB Y/cc. = 0.01. C =	54	74	178	230	270	....	Clark's
Bios IIB Y/cc. = 0.01. C =	54	60	127	179	220	....	Neutral
Bios IIB Y/cc. = 0.02. C =	.....	65	140	280	306	340	Clark's
Bios IIB Y/cc. = 0.02. C =	.....	54	118	278	360	418	Neutral
Bios IIB. Y/cc. =	0.0025	0.005	0.01	0.02	0.04	0.08	
Bios IIA Y/cc. = 0.01. C =	100	156	178	140	150	....	Clark's
Bios IIA Y/cc. = 0.01. C =	92	116	127	118	187	....	Neutral
Bios IIA Y/cc. = 0.02. C =	.....	150	230	280	228	217	Clark's
Bios IIA Y/cc. = 0.02. C =	.....	113	179	278	396	390	Neutral

added to the yeast extract (Table VI) or to Bios II solutions (Table V of Miss Eastcott's paper)[2] or when Bios II is added to a solution of inositol. On this behavior we hope to base a method for determining Bios IIA and Bios IIB in arbitrary units, which will enable their amounts in Bios II solutions from different sources to be compared, even before these constituents have been obtained in the pure state—although, of course, attempts to isolate and identify them are being made.

The interesting discovery of Professor O. R. Richards,[20] that one part of thallium per million of Williams' culture medium[21] increases the count of Yeast 2335, led to the experiments with Kahlbaum thallium–potassium alum whose results are given in Table XIII. For, although the twenty-four-hour counts obtained by Richards with thallium were small, so are those with inositol under similar circumstances; and just as inositol exerts its full effect only in the presence of Bios II, so might the counts with thallium be heightened by addition of inositol and one of the constituents of Bios II. The results, however, show that this is not the case, and that neither Bios IIA nor Bios IIB owes its potency to thallium.

TABLE XIII

Thallium 0.001 mg./cc., Inositol 0.04 mg./cc.

Bios IIA, Y/cc. = 0.02: Clark's medium C = 15, Neutral medium C = 15
Bios IIB, Y/cc. = 0.02: Clark's medium C = 40, Neutral medium C = 40

V. Identity of Constituents from Different Sources

Miss Eastcott's work with bios from sixty different sources, the fractionation by baryta and alcohol of bios from nine sources, and the fractionation by charcoal of Bios II from three sources,[4] make it probable that there is only one bios and not "a whole category" of them, as Wildiers thought possible. This probability is increased by the results of Table XIV which show that mixtures of inositol (in excess) with Bios IIA from any one of the three sources and Bios IIB from either of the other two, give large counts; so, whether chemically identical or not, the constituents from the

(20) O. R. Richards, *J. Biol. Chem.*, **96**, 405 (1932).

(21) 2 g. KH_2PO_4. 0.25 g. $MgSO_4$. 3 g. $(NH_4)_2SO_4$. 0.25 g. $CaCl_2$. 1.5 g. asparagine (Eimer and Amend). 20 g. sucrose, one liter of water.

three sources are at least physiologically interchangeable. Work now in progress on the behavior of these constituents with acids and bases, with organic solvents, and on acetylation,[22] will no double reveal any differences that may exist; although the isolation and identification of the active principles of these obviously impure solutions is, of course, the main object of the investigation.

TABLE XIV

Inositol 0.04 mg./cc.

	IIB (*Y*/cc. 0.02)		IIB (tomato)		IIB (combings)		No Bios IIB	
	Clark	Neut.	Clark	Neut.	Clark	Neut.	Clark	Neut.
IIA (*Y*/cc. 0.04)	304	396	284	286	286	237	20	20
IIA (tomato)	300	324	284	230	280	214	37	35
IIA (combings)	220	212	187	162	233	150	15	19
No Bios IIA	50	60	54	60	57	52	..	..

Closer examination of Table XIV shows that the counts[23] in neutral media (where relations between count and amount of constituent in the medium are simpler than they are in Clark's, see Table XIII) increase toward the left of each line and toward the top of each column; *i. e.*, the three lines agree in saying that most IIB was added with the yeast preparation and least with that from combings, and the three columns agree that the order of the amounts of IIA is the same.

With the data at present available it is not possible to make an accurate computation, but as a rough approximation the amounts of Bios IIA from tomato and from combings, respectively, used in these experiments, correspond to *Y*/cc. = 0.025 and *Y*/cc. = 0.016; and the amounts of Bios IIB to *Y*/cc. = 0.014 and *Y*/cc. = 0.010.

VI. Presence of Bios IIA in Crude Bios I Solutions

That the Crude Bios I solutions from combings and from tomatoes must contain some active constituent other than Bios I (*i. e.*, inositol) forced itself on our attention long ago; the same is true of the Crude I from yeast; for as is shown by the results of Table XV, higher counts can be obtained from media containing Bios II by adding Crude Bios I solution than by adding inositol; the preparations used are those of Table X. The experiments of Table XVI show that this substance is Bios IIA; for adding IIA to the Crude I leaves the count unchanged, while adding IIB greatly increases it.

The Crude I of these experiments is that of Tables X and XV; the IIA and IIB used as reagents were prepared from Mr. Muellers' Bios II (p. 1512 above), the quantities used per cc. of medium came from 0.03 g. of his yeast cakes.

As previous experiments had shown that Lucas' "Bios I solution" does not contain Bios IIA, it seemed probable that that substance could be found in the filtrate after precipitating "Crude Bios I solutions" with lead acetate and ammonia. Experiments with the Crude Bios I solution from tomatoes show that this is the case; the IIA and IIB of Table XVII are those of Table XVI, details of the treatment of the tomato juice are given in

(22) E. M. Sparling, *Trans. Roy. Soc. Canada*, **22**, Sec. III, p. 271 (1928).

(23) Those in media containing all three constituents are meant.

TABLE XV

Neutral Medium

Crude Bios I (Y/cc. = 0.10), with 0.02 mg./cc. inositol	C = 50
Crude Bios II (Y/cc. = 0.02), with 0.02 mg./cc. inositol	263
Crude Bios II (Y/cc. = 0.02), with 0.04 mg./cc. inositol	280
Crude Bios II with the Crude I and 0.02 mg./cc. inositol	394

TABLE XVI

Inositol 0.04 mg./cc., Neutral Medium

Bios IIA	C = 12	Crude Bios I	C = 50
Bios IIB	37	Crude I and Bios IIA	54
Bios IIA and Bios IIB	334	Crude I and Bios IIB	304

TABLE XVII

Inositol 0.02 mg./cc., Clark's medium

Tomato preparation	C = 60
Tomato preparation with Bios IIA	64
Tomato preparation with Bios IIB	470

the footnote.[24] Unfortunately the time at our disposal did not permit the repetition of these experiments with the Crude Bios I solution of Table XV; that of Table XVIII had been prepared, with another object in view, by precipitating Mr. Mueller's Crude Bios I solution (see p. 1511) with lead acetate and ammonia, but as his baryta–alcohol precipitate had been washed with 50% alcohol instead of 65%, and as the barium compound of Bios IIA is more soluble than that of inositol, most of the former had been carried into the crude Bios II solution; enough remained, however, to leave no doubt as to its presence. The IIA and IIB of Table XVIII are those of Tables XVI and XVII.

TABLE XVIII

Inositol 0.02 mg./cc., Clark's medium

Yeast preparation	C = 24
Yeast preparation with Bios IIA	32
Yeast preparation with Bios IIB	92

Summary

1. Addition of inositol to media containing aqueous or alcoholic extracts of yeast may or may not increase the twenty-four-hour yeast crop, depending on the condition of the cells from which the extracts were made.

(24) Evaporated *in vacuo* 3730 cc. of filtrate from canned tomatoes to 1910 cc., added 3820 cc. alcohol; evaporated filtrate to 350 cc., added 400 g. of baryta dissolved in 425 cc. of water, and 1600 cc. of alcohol; filtered, washed precipitate with 150 cc. of dilute alcohol (2 volumes of alcohol, one volume of water) and extracted with 1500 cc. of water. Removed barium from this Crude Bios I liquor by carbon dioxide and sulfuric acid, neutralized with ammonia, and precipitated with 100 g. of crystallized lead acetate dissolved in 100 cc. of water, and 45 cc. of conc. ammonia (11.8 N) diluted with 17 cc. of water; filtered, did not wash precipitate. Removed lead from filtrate by sulfuric acid and hydrogen sulfide, and acetic acid by repeated evaporation *in vacuo*. Diluted a portion (corresponding to 60 cc. of tomato juice) to 10 cc., shook with 0.5 g. of Darco charcoal, neutralized the filtrate with sodium hydroxide and made up to 20 cc.; thus 1 cc. came from 3 cc. of juice, this amount was present in the 10 cc. of culture medium of each experiment of Table XVII.

Before using brewers' yeast to make a yeast extract, the liquid culture medium in which the cells are suspended ought to be removed: as this liquid may contain enough inositol to render further addition of that substance to the "yeast" extract unnecessary.

2. By treating yeast extracts (from brewers' yeast and from Fleischmann's yeast) with alcohol and baryta as Lucas treated his infusion of malt combings, we have fractionated the bios contained in them; just as the bios from combings, malt, rice polishings, tea, mushrooms, molasses, oranges and tomatoes has already been fractionated.

Neither of the two fractions so obtained, added to salts and sugar medium, gives a large yeast crop; but the two together do. The crop obtained with one of them (*viz.*, the crude Bios I solution) is not increased by adding inositol, but is much increased by adding Bios II, whether the latter be derived from yeast or from malt combings. The crop obtained with the other (*viz.*, the crude Bios II solution) is much increased by adding inositol.

A similar fractionation has been effected with lead acetate and ammonia, which like baryta and alcohol precipitates inositol.

3. By shaking the crude Bios II solution from Fleischmann's yeast with charcoal, it has been fractionated into two constituents for which the provisional names "Bios IIA" (not adsorbed) and "Bios IIB" (adsorbed) are proposed. Thus the bios from yeast has been fractionated into three constituents. With salts and sugar, each of these constituents gives a small yeast crop: mixtures of any two of them give fairly small crops; but all three together give a large crop.

Bios II from malt combings, and Bios II from tomatoes have been fractionated in the same way.

4 Crude Bios I solutions, as Lucas called them, whether prepared from malt combings from tomatoes or from yeast, contain Bios IIA; but his Bios I. solutions do not, for the Bios IIA remains in solution when the Crude Bios I solution is precipitated with lead acetate and ammonia.

5. The Bios IIA from yeast is physiologically equivalent to that from combings and to that from tomatoes; *i. e.*, either of the latter with inositol and Bios IIB from yeast gives a large crop. In the same sense, Bios IIA from combings is equivalent to Bios IIA from tomatoes; and Bios IIB from any of the three sources is equivalent to Bios IIB from either of the others.

As the crude Bios IIA solutions from the three sources were obtained by the same chemical procedure, the chemical properties of their active constituents are to that extent the same; and as their effects on the yeast crop are also the same, there is at present no reason to regard them as separate species. For the same reasons there is no basis for assuming more than one Bios IIB.

Until a source of bios has been found which with alcohol and baryta does not behave like the nine already studied, or which with charcoal does not behave like the three whose behavior is described in this paper, it is unnecessary to assume the existence of more than one bios.

6. Measurements with Wildiers' yeast in media containing one of the three constituents, or two, or all three of them, show that it behaves just like Toronto yeast; there is therefore no necessity to assume the existence of a class of "nutrilites" distinct from Wildiers' bios.

The names are given of four species of yeast from the American Type Culture Collection which with inositol and Bios II from combings behave the same as Wildiers' yeast and as the Toronto yeast; and the names of three species which behave otherwise.

7. To check the spread of misapprehensions, experiments are described which show that the twenty-four-hour crop obtained with yeast extract is just as much increased by inositol bought from Kahlbaum or from Eastman as it is by that prepared by Miss Eastcott from the Bios I soln. of tea.

8. Experiments are described which show to what a great extent yeast crops obtained in stationary vessels may depend on factors other than the amount of bios in the culture medium; and some which show that the agitation and aeration afforded by Fraser's rocker-tubes are sufficient.

9. The effect of the initial P_H of the culture medium on the yeast crop is discussed. In media containing bios, there is no one initial P_H that in every case gives a larger crop than any other; the nature and amount of the acid or acids formed during crop determinations is being studied.

10. Professor O. R. Richards' discovery of thallium in asparagine led to experiments which show that the effects obtained with the crude solutions of Bios IIA and Bios IIB are not due to thallium; work on the isolation and identification of the active principles of these solutions is in progress.

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