189. PROLIFERATION-PROMOTING PROPERTIES AND ULTRAVIOLET ABSORPTION SPECTRA OF FRACTIONS FROM YEAST

BY ELTON S. COOK, SISTER MARY JANE HART, O.P., AND SISTER MIRIAM MICHAEL STIMSON, O.P.

From the Research Laboratories of the Institutum Divi Thomae, Cincinnati, Ohio

(*Received 26 October 1940*)

WORK in these laboratories on proliferation-promoting factors has been concerned largely with a study of substances which appear to be produced by cells as a result of injury (intercellular wound hormones). It has also been necessary to examine factors of the bios type from "uninjured" cells. In many cases the different preparations have also been tested for their effects on the respiration of yeast and tissues and, in some instances, for their effects on glycolysis and fermentation. In most of the reported findings these various stimulating properties have been found to accumulate in different fractions.

Spectroscopic study of crude proliferation-promoting substances from ultraviolet-injured yeast cells has shown that the increase in biological potency parallels the increase in ultraviolet absorption at about 2600 Å. and this is substantiated by chemical tests [Cook *et al.* 1939; Loofbourow, Cook & Stimson, 1938]. Indications are similar with proliferation-promoting substances produced by mechanical injury [Loofbourow, Cook, Dwyer & Hart, 1939]. These facts have suggested that the growth factors from injured cells owe their activity to nucleic acid-like substances although not to nucleic acids themselves since nucleic acids and related substances possess only a very low order of activity under the conditions of assay [Loofbourow *et al.* 1940]. Similar conclusions are drawn with respect to proliferation-promoting substances prepared by the irradiation of animal tissues [Cook *et al.* 1939; Loofbourow *et al.* 1937; Loofbourow, Cueto & Lane, 1939; Loofbourow *et al.* 1940]. In this connexion reference should be made to the findings of Fischer [1939] and of Caspersson [1936; 1939] relating nucleic acids or nucleoproteins to proliferation.

Preliminary experiments showed that a bios preparation from yeast had marked absorption at 2600 Å. while bios prepared from sugar showed only end absorption [Loofbourow *et al.* 1937] as have some other bios preparations (unpublished). On the other hand, a bios sample from malt combings showed peaks at 2800 and 3000 Å. which might be due in part to cyclic amino-acids [Loofbourow *et al.* 1937]. It is of significance that Miller [1936] has found β-alanine and possibly *l*-leucine to act as components of the bios complex (Bios IIA). Williams & Rohrmann [1936] earlier pointed out the importance of β-alanine for yeast growth, and β-alanine has been recognized as a component of pantothenic acid [Weinstock *et al.* 1939] which has now been synthesized [Williams & Major, 1940; Williams, Eakin & Snell, 1940; Williams, Mitchell, Weinstock & Snell, 1940; Stiller *et al.* 1940]. The simple acyclic amino-acids show essentially end absorption in the ultraviolet [Ellinger, 1937] and pantothenic acid, as would be expected, appears to show little characteristic absorption

[Williams *et al.* 1939]. Other pure compounds having bios activity are inositol (Bios I) [Eastcott, 1928], aneurin (Bios V) [Miller, 1937; Schultz *et al.* 1938], and vitamin B_6 [Schultz *et al.* 1939; Eakin & Williams, 1939]. Inositol shows only end absorption (unpublished data). Aneurin is characterized by maxima at 2350 and 2670Å. [Wintersteiner *et al.* 1935]. The spectra of vitamin B_6 are more complex and alter with *p*H. At *p*H 2·1 there is a single peak at 2920 Å.; at *p*H 4 there are peaks at 2920 and 3260 Å.; at *p*H 5·1 there are maxima at 2550, 2920 and 3260 Å.; at *p*H 6·75 the middle peak disappears, leaving those at 2550 and 3260 Å.; at *p*H 10·2 the two peaks are shifted to 2460 and 3110 Å. [Stiller *et al.* 1939; Kuhn & Wendt, 1939].

None of the pure compounds identified as parts of the bios complex absorbs appreciably at 2600 Å. and none of them which has been tested by us (β-alanine, *l*-leucine, inositol and aneurin) shows proliferation activity on yeast under the conditions to be described [Loofbourow *et al.* 1940]. Until recently no evidence had been presented definitely to connect the biological activity of bios preparations with absorption at this wave-length, thus contrasting with the proliferation-promoting factors obtained from injured cells. Recently, however, Rainbow & Bishop [1939] carried out fractionations which gave highly purified Bios IIA and Bios IIB. The former, probably identical with pantothenic acid, exhibited only end absorption, whereas the latter, possibly equivalent to the biotin of Kögl & Tönnis [1936], showed an absorption maximum at 2620 to 2650 Å. Since many of the manifestly crude proliferation-promoting preparations obtained in our laboratories from injured cells show considerably greater extinction at 2600 Å. than Rainbow's highly purified Bios IIB, it is not yet clear to us whether the absorption of Rainbow's preparation is characteristic of his fraction or may be caused by highly absorbing impurities. Indeed, the extinctions of some of the fractions reported in the present paper equal or exceed that reported by Rainbow. Williams, Eakin & Snell [1940] have pointed out that substances other than the known bios components are important for rapid yeast growth.

Thus far it has been impossible to correlate respiration-stimulating activity of yeast fractions, either on tissues or yeast, with absorption at 2600 Å. [Cook & Walter, 1939] and this is supported by the data of the present paper; biological tests with nucleic acids and related substances have likewise been negative [Cook *et al.* 1940].

In a study of respiration [Cook *et al.* 1938], five fractions were obtained from Fleischmann's baker's yeast by a simplification of the procedure applied by Lucas [1924; also Miller *et al.* 1933] to the preparation of bios from malt combings. These fractions had already been assayed for their effects on the respiration of yeast and of rat skin and liver [Cook *et al.* 1938]. In this paper we wish to report on the ultraviolet absorption spectra of these fractions and the activities of four of them on the growth of yeast.

Experimental methods

The preparation and respiration assay of the fractions have been fully reported [Cook *et al.* 1938]. For the growth assays the following fractions were available:

Fraction N: $Ba(OH)_2$–alcohol precipitate.
Fraction C: $Ba(OH)_2$–alcohol filtrate.
Fraction O: Acetone precipitate.
Fraction E: Acetone filtrate.

Unfortunately no more of the original crude aqueous-alcoholic extract (fraction A), from which these samples were prepared, was obtainable for growth

assay although some spectra were taken of this fraction. Other crude extracts comparable with fraction A possess considerable growth activity but, of course, a significant quantitative comparison cannot be made with the fractions used in the present study.

Growth assay. These fractions were tested for their effect on the proliferation of *Saccharomyces cerevisiae* in Reader's medium [Reader, 1927] by the rocker-tube technique previously employed in these laboratories [Norris & Hart, 1937; Norris & Kreke, 1937; Loofbourow, Cook & Stimson, 1938; Loofbourow, Dwyer & Morgan, 1938; also Fraser, 1921; Lucas, 1924]. Each tube contained 24 ml. of Reader's medium and 1 ml. of added material to be assayed. The yeast used for seeding was washed with Reader's medium from 48 hr. slants on Saboraud's medium and added to the rocker-tubes to give a concentration of 0·064 mg./ml. The concentration of yeast used for seeding and the crop after 24 hr. at 30° were determined by means of a photoelectric densitometer. Since Loofbourow & Dwyer [1938] have shown that greater consistency is obtained when the yeast population increase is expressed in terms of weight rather than in terms of count, we have adopted their procedure. Haemocytometer counts reveal that the increase in weight due to the addition of the fractions studied is the result of increased cell number and not merely of increase in cell size.

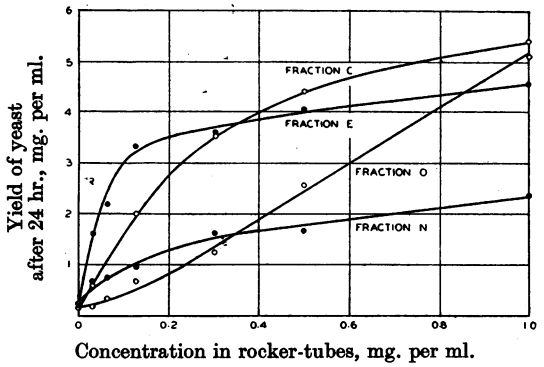

Fig. 1. Stimulating effect of yeast fractions on proliferation of yeast.

The activities of these fractions on the proliferation of yeast are shown in Fig. 1. From these curves have been calculated the potency of the samples in growth units per mg., one growth unit, as defined by Loofbourow, Dwyer & Morgan [1938], representing an increase in yeast crop of 1·6 mg./ml. greater than that in the controls. The growth curves can be regarded as approximately linear up to 0·15–0·25 mg./ml. of added material (except O, which, within experimental error, is linear over its entire length). Beyond this point there is a marked change of slope. The growth unit potency has been calculated within the early portions of the curves. It will be noted that the relative potencies obtained from these parts of the curves usually do not hold when higher concentrations of added material (0·5–1·0 mg./ml.) are considered. Therefore this means of comparing growth potencies may be misleading and, in fact, is really valid only when the growth curves are linear over their entire length. This fact must be borne in mind throughout the discussion. The total number of growth units per fraction, obtained by multiplying the weight of the fraction by the potency in growth units per mg., as well as the potency in growth units per g. of original yeast, have also been calculated. These figures are collected in Table 1. The

Table 1. *Proliferation activity of fractions from yeast*

Fraction	Total wt. of fraction, g.	Growth units per mg.	Total growth units per fraction	Growth units per g. of yeast
N	2·42	2·6	6,292	4
C	41·33	9·1	376,103	237
O	28·28	2·9	82,012	55
E	12·80	22·2	284,160	179
Irradiated yeast preparation 4/26/38*		5·0		715
Sample 14 from malt combings†		10·6		—

* Loofbourow, Dwyer & Morgan [1938]. † Norris & Kreke [1937].

weights of fractions and yields on the basis of yeast were obtained from the previously published data [Cook *et al.* 1938]. Also included for comparison are data on Norris & Kreke's [1937] most potent bios sample from malt combings and the most potent crude material prepared by irradiating yeast [Loofbourow, Dwyer & Morgan, 1938]. With respect to the latter it will be observed that, while its potency in growth units per mg. is considerably lower than some of the preparations obtained without irradiation, the yield per g. of yeast is considerably higher. Irradiated yeast preparations partially purified by chromatographic adsorption show greater potency than any of the bios samples (unpublished data).

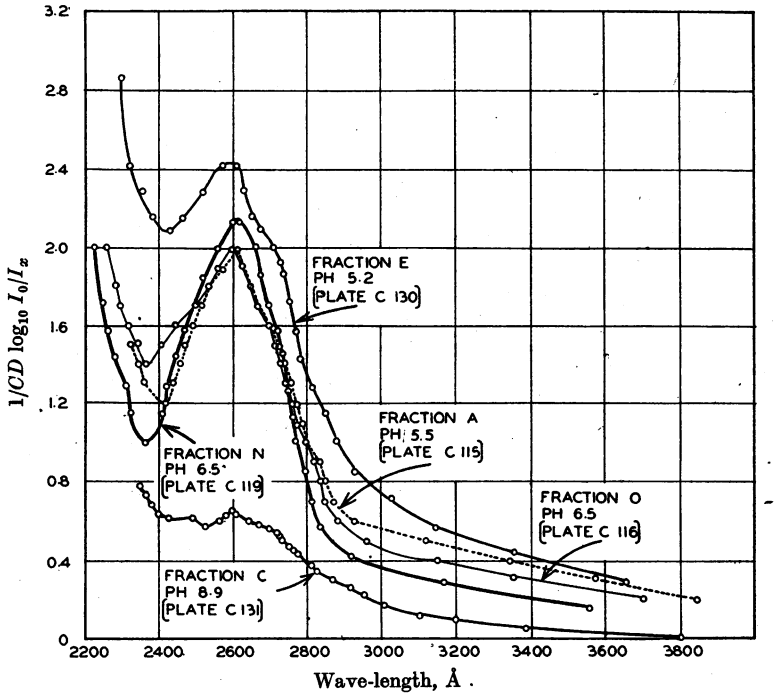

Fig. 2. Ultraviolet absorption spectra of fractions from yeast.

Ultraviolet absorption spectra. Spectra were observed at the normal *p*H of the solutions of the fractions in distilled water. As will be seen from Fig. 2, the reaction varied, being acid in all cases except fraction C. Some months later, in an

attempt to explain certain of the anomalies to be discussed, spectra were re-observed in Kolthoff buffer mixture at *p*H 7. Only slight differences were found in the extinctions with the exception of an increase for fraction N which requires further investigation. The spectra were obtained with a Hilger quartz spectrograph and Spekker photometer using a tungsten spark source and are shown in Fig. 2.

Discussion

The fractionation [Cook *et al.* 1938] employed to obtain the materials studied in this paper involved the extraction of yeast with aqueous alcohol. This crude extract, of which no sample was available for growth assay, was treated with $Ba(OH)_2$ and alcohol. A small amount of precipitate, fraction N (which corresponds to the Bios I fraction of Lucas), contained only a relatively small growth activity, most of the biological activity going into the filtrate (fraction C). This filtrate was then treated for 72 hr. with sufficient acetone to give a concentration of 95 %. This treatment precipitated a considerable quantity of material of relatively low growth potency (fraction O) and the major portion of the growth activity went into the acetone-soluble portion (fraction E), which had a potency of 22 growth units per mg. This fraction would correspond to Lucas's Bios II. There was little loss of potency during the acetone treatment, the sum of the potencies of the acetone filtrate and precipitate being nearly equal to that of the parent fraction C. It is to be presumed that an appropriate variation of conditions would allow a complete concentration of the growth activity in the acetone filtrate and thus permit the removal of a large amount of inactive acetone-insoluble material. As far as the present data go we may say, then, that the yeast growth factor is not precipitated to any great extent by $Ba(OH)_2$-alcohol and is soluble in 95 % acetone.

Reference to the paper on respiration [Cook *et al.* 1938] will show that the disposition of the yeast respiratory activity parallels that of the yeast proliferation activity. In a concentration of 1 mg./ml., fraction C caused a 21 % stimulation of yeast respiration, fraction E a 53 % increase and fraction O was inactive. The close parallelism is shown by the fact that fraction E was 2·5 times as active as C in stimulating respiration and 2·4 times as active in stimulating proliferation. The recovery of respiratory activity (i.e. the sum of E and O as compared with C) was not as complete as in the case of growth. Fraction N, which has a low growth activity, was without influence on yeast respiration. However, both fractions N and O were of considerable activity on tissue respiration (rat liver and skin).

This parallelism of growth and respiratory activity is apparently not in accord with other experiments in which the maximum growth and respiration activities have been concentrated in different fractions [Norris & Hart, 1937; Norris & Kreke, 1937; Norris & Ruddy, 1937]. It was found previously by application of the Lucas fractionation to malt combings [Norris & Kreke, 1937] that the growth activity was concentrated in the acetone precipitate (10·6 growth units per mg.) rather than in the filtrate. On examining the procedures, however, it will be seen that the acetone precipitation as applied to malt combings was a very rapid one, lasting only a few minutes, whereas a 72 hr. acetone treatment was employed with yeast. If we assume that the growth factors from the two sources are the same, which is not necessarily the case, it is readily seen that the prolonged acetone treatment might redissolve material carried down by rapid addition of acetone. In other words, the acetone treatment of Norris & Kreke was inadequate to obtain complete separation. Furthermore, it will be seen that malt combings fractions 18 and 19 [see Norris & Kreke, 1937], which were

obtained from the acetone-soluble material, both possessed high growth activity (6·3 and 5·9 units per mg., respectively) and one of these (no. 18) possessed the greatest respiratory activity of any of the malt combings samples. This incomplete fractionation is significant in view of our present findings. On account of the difference in techniques it is doubtful how far we are justified in comparing the two procedures. While the present experiments show apparent departures from some expectations based on earlier work, the bulk of the evidence at present available still seems to indicate the non-identity of the factors which increase yeast proliferation and respiration. Indeed, current experiments using electrolytic methods favour this non-identity [Cook *et al.*, unpublished]. However, further investigations are certainly required. It is apparent that the classical bios fractionations are not adequate to accomplish a good separation of growth and respiratory factors and results obtained with these methods must be interpreted with care.

All of the fractions show ultraviolet absorption peaks at 2600 Å. although the extinction is very low for fraction C. Absorption at this wave-length is characteristic of the heterocyclic rings of nucleic acids and their derivatives [Holiday, 1930; Heyroth & Loofbourow, 1934]. In keeping with this fact all of the fractions contain N, P and pentose (Bial test). Sulphur is absent, as is protein (biuret test). With the Thomas [1931] tryptophan reagent all fractions gave a light violet-brown colour quite distinct from the pale green given by yeast nucleic and adenylic acids but somewhat resembling the colours given by irradiated yeast preparations and coenzyme II [Cook *et al.* 1939; Loofbourow, Cook & Stimson, 1938]. With the Thomas [1931] β-naphthol reagent the fractions gave green rings with brown tops while yeast nucleic acid gives a blue ring with a green top. The brown coloration was absent from fraction C which thus bore a somewhat closer resemblance to yeast nucleic acid. Irradiated yeast preparations, yeast adenylic acid and coenzyme II gave blue rings with the β-naphthol reagent [Cook *et al.* 1939; Loofbourow, Cook & Stimson, 1938].

An examination of the absorption spectra and growth data appears to show no correlation between the yeast growth activity and absorption at 2600 Å. Thus, fraction N, of low yeast growth potency, has a high absorption. Fraction C has low absorption, but when it is split into its relatively inactive component (O) and its highly active component (E), both of these moieties show greatly increased absorption of the same general magnitude. It seems difficult to attribute the low absorption of C to the presence of non-absorbing materials which are not present in the component fractions O and E because recovery was practically quantitative on a weight and nearly so on a biological basis. The apparently anomalous relation between the spectra of C and its components O and E must go unexplained for the present. If we omit C as such and consider it only in terms of its components O and E, fairly good checks can be obtained between the spectra N, O, and E and the original A. This is shown in Table 2 in which the

Table 2. *Weights of samples and extinctions at 2600 Å.*

Sample	Wt. in g.	Extinction at 2600 Å.	Wt. × Extinct.	Wt. × Extinct. / Wt.
A	102·6	2·00	205·2	2·00
N	2·4	2·14	5·1	—
O	28·3	2·00	56·6	2·13
E	12·8	2·42	31·0	—

extinctions at 2600 Å. and the weights of the fractions are compared. It should also be noted that all fractions show considerable short-wave absorption, as is

true of the preparations from irradiated yeast and tissues. At this time it is not possible to say to what extent this may be connected with the biological activity but this point is under investigation.

From the facts presented it appears that the bios preparations studied may contain variable quantities of material absorbing at 2600 Å. and the biological activities of different partially purified bios fractions cannot be correlated quantitatively with the material having these absorption properties. None of the known pure bios components gives this type of absorption, with the apparent exception of Rainbow & Bishop's [1939] Bios II B. On the other hand, injury to cells seems to result in the production by the cells of proliferation-promoting substances which, at least in the crude state, have characteristic absorption at 2600 Å., the amount of this absorption paralleling the growth potency. It would thus seem possible that a more or less specific type of proliferant may be produced by cells as a result of injury. In this connexion it may be pointed out that materials from injured cells have usually given a linear relationship between growth and concentration over a wide range [Loofbourow, Dwyer & Morgan, 1938; Loofbourow *et al.* 1940]; whereas the growth curves of the bios preparations are usually not linear over their entire length, showing marked stimulation at low concentrations but little greater effect at high concentrations [cf. Norris & Kreke, 1937]. At this time it cannot be certain to what extent this distinction between "bios type" and "injury type" proliferants may exist, especially since current experiments in these laboratories [Loofbourow *et al.* unpublished] show that by chromatographic adsorption it is possible to separate from the materials produced by injured cells very potent proliferation stimulants which do not show selective absorption at 2600 Å. It is certain that injury results in the increased release of materials having these absorption characteristics, but further work alone will show to what extent injury causes cells to produce specific proliferants and to what extent the proliferants may be of the "normal" bios type.

Summary

The yeast proliferation-promoting activity of fractions obtained from yeast is concentrated in a 95% acetone-soluble fraction corresponding to Lucas's Bios II from malt combings. The greatest activity on yeast respiration is also found here. Unlike the crude cell-free preparations from ultraviolet-injured yeast cells, no direct correlation is found between ultraviolet absorption at 2600 Å. and the proliferation activity of the present fractions. This and dissimilar characteristics of the growth curves may indicate a difference in the nature of proliferants produced as a response to injury (intercellular wound hormones) and the known members of the bios complex, or may indicate that a predomination of certain proliferants is produced as a response to injury.

REFERENCES

Caspersson (1936). *Skand. Arch. Physiol.* **73**, Supplement No. 8.
—— (1939). *Nature, Lond.*, **143**, 602.
Cook, Kreke & Nutini (1938). *Studies Inst. Divi Thomae*, **2**, 23.
—— Loofbourow & Stimson (1939). *Atti X° Cong. intern. Chim.* **5**, 26.
—— & Walter (1939). *Studies Inst. Divi Thomae*, **2**, 189.
—— Walter & Eilert (1940). *Proc. Soc. exp Biol., N.Y.*, **44**, 547.
Eakin & Williams (1939). *J. Amer. chem. Soc.* **61**, 1932.
Eastcott (1928). *J. phys. Chem.* **32**, 1094.

Ellinger (1937). *Tabul. biol., Berl.*, **12**, 292 ff.
Fischer (1939). *Nature, Lond.*, **133**, 113.
Fraser (1921). *J. phys. Chem.* **25**, 4.
Heyroth & Loofbourow (1934). *J. Amer. chem. Soc.* **56**, 1728.
Holiday (1930). *Biochem. J.* **24**, 619.
Kögl & Tönnis (1936). *Hoppe-Seyl. Z.* **242**, 43.
Kuhn & Wendt (1939). *Ber. dtsch. chem. Ges.* **72**, 305.
Loofbourow, Cook, Dwyer & Hart (1939). *Nature, Lond.*, **144**, 553.
—— —— & Stimson (1938). *Nature, Lond.*, **142**, 573.
—— Cueto & Lane (1939). *Arch. exp. Zellforsch.* **22**, 607.
—— & Dwyer (1938). *Studies Inst. Divi Thomae*, **2**, 129.
—— —— & Lane (1940). *Biochem. J.* **34**, 432.
—— —— & Morgan (1938). *Studies Inst. Divi Thomae*, **2**, 137.
—— Schmieder, Stimson & Dwyer (1937). *Studies Inst. Divi Thomae*, **1**, 79.
Lucas (1924). *J. phys. Chem.* **28**, 1180.
Miller (1936). *Trans. Roy. Soc. Can.* III, **30**, 99.
—— (1937). *Trans. Roy. Soc. Can.* III, **31**, 159.
—— Eastcott & Maconachie (1933). *J. Amer. chem. Soc.* **55**, 1502.
Norris & Hart (1937). *Studies Inst. Divi Thomae*, **1**, 65.
—— & Kreke (1937). *Studies Inst. Divi Thomae*, **1**, 137.
—— & Ruddy (1937). *Studies Inst. Divi Thomae*, **1**, 53.
Rainbow & Bishop (1939). *J. Inst. Brewing*, **45**, 593.
Reader (1927). *Biochem. J.* **21**, 901.
Schultz, Atkin & Frey (1938). *J. Amer. chem. Soc.* **60**, 490.
—— —— —— (1939). *J. Amer. chem. Soc.* **61**, 1931.
Stiller, Harris, Finkelstein, Keresztesy & Folkers (1940). *J. Amer. chem. Soc.* **62**, 1785.
—— Keresztésy & Stevens (1939). *J. Amer. chem. Soc.* **61**, 1287.
Thomas (1931). *Hoppe-Seyl. Z.* **199**, 10.
Weinstock, Mitchell, Pratt & Williams (1939). *J. Amer. chem. Soc.* **61**, 1421.
Williams, Eakin & Snell (1940). *J. Amer. chem. Soc.* **62**, 1204.
—— & Major (1940). *Science*, **91**, 246.
—— Mitchell, Weinstock & Snell (1940). *J. Amer. chem. Soc.* **62**, 1748.
—— & Rohrmann (1936). *J. Amer. chem. Soc.* **58**, 695.
—— Weinstock, Rohrmann, Truesdail, Mitchell & Meyer (1939). *J. Amer. chem. Soc.* **61**, 454.
Wintersteiner, Williams & Ruehle (1935). *J. Amer chem. Soc.* **57**, 517.