same samples by the yeast method. As expected, the chemical values are lower and may give a rough indication of the proportion of the total vitamin B_6 present as pyridoxin.

SUMMARY

1. The microbiological method for the determination of vitamin B_6 with *Saccharomyces carlsbergensis* (Atkin *et al.* 1943) was satisfactorily applied, with slight modifications, to a number of foodstuffs.

2. Two chemical methods were also used and criticisms of them are presented.

3. Data obtained upon the effectiveness of different methods of extraction of vitamin B_6 from natural materials can be explained without assuming the existence of unknown compounds possessing vitamin activity.

4. Indications were obtained that the particular strain of *S. carlsbergensis* used might also be used for the estimation of biotin and inositol.

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Intercellular Hormones

6. RELEASE AND SYNTHESIS OF FACTORS OF THE VITAMIN B COMPLEX BY DAMAGED LIVING CELLS

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When cells are damaged under conditions which leave them grossly intact, proliferation-promoting factors ('wound hormones') are released into the intercellular suspension medium (see Cook & Cronin, 1941; Loofbourow, 1942*b* for earlier references). The mechanism of the effect appears to involve (*a*) increase in cell-membrane permeability, (*b*) diffusion of non-protein substances through the more permeable membranes, and (*c*) partial replacement within the injured cells of some of the substances lost through diffusion (Loofbourow, 1942*b*).

It has been suggested that the substances responsible for the proliferation-promoting activity might be amino-acids or vitamin B complex factors (Davidson, 1940), purines or pyrimidines (Loofbourow, Cook & Stimson, 1938) or combinations of nucleotides with vitamin B complex factors (Loofbourow, 1942*c*). Duplication of the proliferation-promoting effect of damaged-cell products by combinations of B complex factors, amino-acids, and adenine nucleotides has afforded presumptive evidence that all of these substances are involved in the effect (Loofbourow, 1942*b*).

To determine the role of any substance in the 'wound hormone' phenomenon it is desirable to establish the presence and relative concentration of the substance in products from damaged cells, and then to demonstrate its proliferation-promoting activity under like conditions of concentration and combination with other substances. The present paper presents quantitative assays of damaged-cell and undamaged-cell preparations with regard to factors of the vitamin B complex, together with growth tests employing B complex factors in comparable concentrations. Subsequent communications will deal with the role of amino-acids, nucleotides and nucleosides in the 'wound hormone' phenomenon.

METHODS

Preparation of products from damaged and undamaged cells. After thrice washing, by emulsification in volumes of 400 ml. of water and centrifugation, 400 g. wet weight (about 112 g. dry weight) of Fleischmann starch-free baker's yeast was suspended in distilled water to make a total volume of 4 l. Equal 2 l. amounts were placed in Pyrex glass containers. The two suspensions were stirred constantly by motor-

driven glass paddles. One suspension was irradiated for 8 hr. with lethal ultra-violet radiation from a Hanovia air-cooled d.c. quartz mercury arc operated at 65 V., 5·2 amp. and placed 24·5 cm. from the surface of the suspension. The non-irradiated suspension served as an undamaged-cell control. The irradiated suspension was maintained at approximately the same temperature as the non-irradiated suspension (22–23°) by means of a glass cooling coil. The depth of the suspensions in the Pyrex glass vessels was 6·3 cm. The lethal ultra-violet energy (wave-length shorter than 2950 A.) absorbed by the irradiated suspension during the 8 hr. irradiation period was approximately $4{\cdot}2 \times 10^8$ ergs/ml. of suspension. The cell volume before irradiation was 12·5% of the total volume of the suspension; after irradiation, it was 12% for the non-irradiated and 9% for the irradiated suspension. Details of the energy and cell-volume measurements are given in an earlier communication (Loofbourow, 1942*a*).

Following irradiation, both suspensions were centrifuged. The supernatant fluids were decanted, filtered through sterile Seitz G.P. pads, and autoclaved (15 min. at a pressure of 15 lb./sq.in.), the yield of intercellular fluids being 1842 ml. from the irradiated cells and 1765 ml. from the non-irradiated cells.

After centrifugation, the cell residues were washed thrice in 200 ml. volumes of water. The wash water from each residue was pooled, made up to 700 ml. with distilled water, and autoclaved.

To each washed sludge residue, an equal volume of distilled water and 5 ml. of toluene were added. To permit autolysis of the cell residues, the washed sludge residues were stored in the dark for 8 days at room temperature in loosely corked flasks, during which period they were shaken frequently. The acidity of autolysates was then adjusted to pH 2·7–2·8 with HCl, steamed for 30 min. at 100°, to afford further extraction of the cellular residue for B factors, and centrifuged. The supernatants were decanted, filtered through Seitz G.P. pads, and autoclaved.

Assays for B complex factors were carried out on the intercellular suspension fluids, the sludge-residue wash waters, and the autolyzed-cell extracts, in the case of both undamaged (control) cells and damaged (irradiated) cells.

Vitamin assay methods. Aneurin was assayed by the thiochrome method (Hennessy, 1941; Hennessy & Cerecedo, 1939). Nicotinic acid was assayed colorimetrically (Melnick & Field, 1940*a*, *b*; Melnick, 1941). A fluoriphotometric method was employed for riboflavin, following the technique of Hodson & Norris (1939) as modified in the laboratories of Prof. Robert S. Harris at the Massachusetts Institute of Technology. Biotin was assayed by the microbiological method of Williams (1941); 10 ml. cultures of yeast (*S. cerevisiae*, F.B. strain) were used. Pantothenic acid was assayed by Williams's (1941) yeast-growth method (using *S. cerevisiae*, G.M. strain) with the following modifications: (1) the cultures were grown in rotor tubes (Loofbourow, Webb, Loofbourow & Abramowitz, 1942), (2) an innoculum of 0·001 mg./ml. was used instead of 0·004 as used by Williams, (3) the medium supplements included a folic acid preparation, obtained from R. R. Williams, and casein in addition to the supplements employed by Williams, and (4) the pH was adjusted to 4·7. Folic acid was assayed by the microbiological growth method of Landy & Dicken (1941), using *L. casei* as the organism and the previously mentioned preparation of folic acid as the reference standard. The reference standard was an impure sample; the percentage of folic acid it was assumed to contain was based on data supplied by R. R. Williams. Both the Landy & Dicken (1941) *L. casei* growth method and the Williams (1941) yeast-growth method, with *S. cerevisiae* G.M. strain, were used for pyridoxin; the results reported here were obtained by Williams's method. Inositol was assayed by Williams's (1941) yeast-growth method, with *S. cerevisiae* G.M. strain.

The approximate errors of the assays (Tables 1 and 2) were determined by repeated assays of the same preparations in these and other experiments.

Determination of proliferation-promoting effects. The proliferation-promoting effects of the various preparations were determined by yeast-growth assays, with *S. cerevisiae*, F.B. strain, grown in rotor tubes (Loofbourow *et al.* 1942).

RESULTS

Vitamin assays. The results of the assays for vitamin B complex factors are shown in Table 1. In the case both of the intercellular fluids and of the sludge washings, the damaged cells gave greater yields of all factors assayed than did the undamaged cells. In the case of autolyzed sludge extracts, however, only biotin showed a greater yield from the damaged than from the undamaged cell sludge residue; the folic acid yield was the same, within experimental error, from both residues, while all of the remaining factors showed less yield from the damaged-cell sludge residue than from the undamaged-cell residue. The total yields in the intercellular fluids, wash waters, and sludge residue autolysates were significantly greater for damaged-cell preparations than for undamaged-cell preparations in the case of biotin, folic acid, and pantothenic acid. The total yields of inositol and nicotinic acid were nearly the same for damaged-cell and undamaged-cell products. In the case of pyridoxin, riboflavin, and aneurin, however, the total yields were significantly less from damaged than from undamaged cells. These lesser total yields were accounted for entirely by the lesser amounts in the sludge-residue autolysates.

Proliferation-promoting effects. It was reported previously (Loofbourow, 1942*b*) that the total proliferation-promoting potency of intercellular fluids and residue autolysates from slowly damaged cells is greater than that from undamaged cells. Such results suggest a replacement within the living, injured cells of proliferation-promoting factors lost to the intercellular fluids. However, in the previously reported experiments, the proliferation-promoting potencies of the intercellular fluids and of the residue autolysates were assayed separately and the numerical results were added. More conclusive data should result if the intercellular fluids and residue autolysates were combined and assayed together. Accordingly, in the present experiments, like portions of the intercellular fluids, wash waters, and residue autolysates were combined and assayed in the case

Table 1. *Assay of products from damaged and undamaged cells for B complex factors*

Factor	Source	Yield of factors (μg./g. of moist yeast in original suspension) Intercellular fluid	Sludge washings	Sludge autolysate extract	Total	Approximate error of assay (%)
Biotin	Irrad. susp.	0·120	0·0415	0·695	0·857	± 3
	NI* susp.	0·0095	0·0035	0·508	0·52	
	Ratio I*/NI	12·5	11·9	1·4	1·6	
Folic acid	Irrad. susp.	0·19	0·069	0·27	0·53	±10
	NI susp.	0·0025	0·005	0·27	0·28	
	Ratio I/NI	76·0	13·8	1·0	1·9	
Inositol	Irrad. susp.	124·0	105·0	625·0	854·0	± 5
	NI susp.	53·0	18·5	860·0	931·5	
	Ratio I/NI	2·36	5·7	0·76	0·9	
Nicotinic acid	Irrad. susp.	53·5	17·0	87·0	157·5	± 5
	NI susp.	6·18	2·1	132·0	140·3	
	Ratio I/NI	8·7	8·1	0·7	1·1	
Pantothenic acid	Irrad. susp.	36·0	9·9	13·4	59·3	± 5
	NI susp.	0·393	0·079	30·7	31·2	
	Ratio I/NI	91·5	125·0	0·44	1·9	
Pyridoxin	Irrad. susp.	3·3	0·81	23·0	27·1	± 7
	NI susp.	0·96	0·39	64·0	65·4	
	Ratio I/NI	3·4	2·1	0·36	0·41	
Riboflavin	Irrad. susp.	1·13	0·024	4·33	5·48	± 4
	NI susp.	0·068	0·012	9·15	9·23	
	Ratio I/NI	16·7	2·0	0·47	0·61	
Aneurin	Irrad. susp.	0·355	0·158	1·35	1·86	± 2
	NI susp.	0·079	0·035	3·55	3·66	
	Ratio I/NI	4·5	4·6	0·38	0·57	

* NI = non-irradiated suspension of yeast. I = irradiated suspension of yeast.

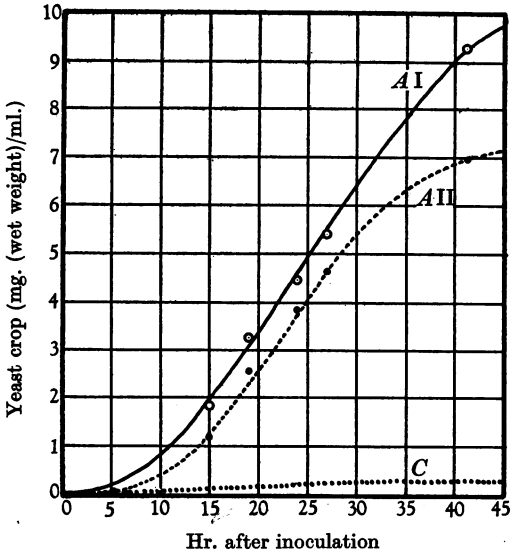

Fig. 1.

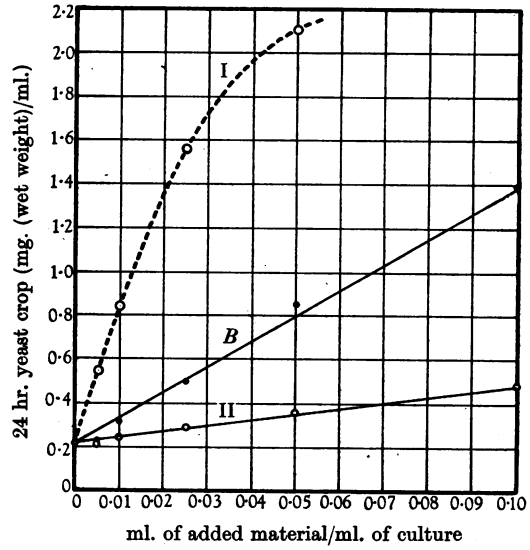

Fig. 2.

Fig. 1. Comparative yeast-growth curves obtained with combined samples of supernatant fluids, wash waters, and autolysates of cell residues from damaged cells (*A* I) and undamaged cells (*A* II). Each ml. of culture contained 0·2 ml. of the sample assayed. Growth in control cultures (Reader's medium only) is shown by curve *C*.

Fig. 2. Proliferation-promoting effect versus concentration for intercellular fluids from damaged cells (I) and from undamaged cells (II) in comparison with a solution of B complex factors (*B*) containing biotin, folic acid, inositol, nicotinic acid, pantothenic acid, pyrodoxin, riboflavin and aneurin in concentrations equivalent to those in (I).

both of products from damaged and from undamaged cells. The comparative proliferation-promoting effect of the combined samples is shown in Fig. 1. As in earlier experiments (Loofbourow, 1942*b*), the combined proliferation-promoting effect of products from damaged cells was greater than that of products from undamaged cells.

The 24 hr. growth obtained with various added quantities of intercellular fluids from damaged and undamaged cells is shown in Fig. 2, where also is shown, for comparison, the growth resulting from the addition of a solution containing B complex factors in concentrations corresponding to those found in the intercellular fluids from damaged cells (Table 2). The solution of vitamin B factors stimulated growth, but to an extent less than that of the intercellular fluids from damaged cells.

Table 2. *Content of B complex factors in intercellular fluids from damaged and undamaged cells*

	μg. of B factor/ml. of suspension fluid	
Factor	From undamaged cells	From damaged cells
Biotin	0·00108 ± 0·00003	0·013 ± 0·004
Folic acid	0·00028 ± 0·00003	0·020 ± 0·0002
Inositol	6·0 ± 0·3	13·5 ± 0·7
Nicotinic acid	0·70 ± 0·04	5·8 ± 0·3
Pantothenic acid	0·045 ± 0·002	3·9 ± 0·2
Pyridoxin	0·109 ± 0·008	0·35 ± 0·02
Riboflavin	0·0077 ± 0·00031	0·123 ± 0·005
Aneurin	0·009 ± 0·00018	0·039 ± 0·00077

The intercellular fluid volume obtained from the 2000 ml. of suspension of irradiated cells was 1842 ml. That from the 2000 ml. suspension of control cells was 1765 ml.

The relative growth-stimulating potencies of the intercellular fluids and of the solution containing B complex factors in concentrations corresponding to those found with intercellular fluids from damaged cells are shown in Table 3 in terms of PP units.* Approximately 18% of the potency of the intercellular fluids from damaged cells was obtained with the solution of B factors present in such fluids.

Table 3. *Proliferation-promoting potencies of intercellular fluids and of B factor equivalent of damaged-cell fluid*

One proliferation-promoting (PP) unit represents a 24 hr. yeast crop twice that of the control in 1 ml. of culture.

Preparation	ml. of prep./PP unit	PP units/ml.	Relative potency
Intercellular fluids from undamaged cells	0·083	12·2	1·0
Intercellular fluids from damaged cells	0·0032	312·8	25·9
Solution containing B complex factors in concentrations corresponding to those found in the intercellular fluids from damaged cells	0·018	55·6	4·5

DISCUSSION

The Seitz filter pads were examined for possible retention of fluorescing materials; no such retention was evident. Assays for riboflavin, aneurin and nicotinic acid at various stages of the procedure indicated that loss or destruction of these factors as a result of autoclaving and Seitz filtration was negligible. In the case of the other B factors, the extent of loss or destruction as a result of such treatment is not known, nor are data available as to the possible loss or destruction of B factors in the sludge autolysates as a result of standing at room temperature. However, the preparations from damaged cells were treated precisely the same as those from undamaged cells with regard to filtration, autoclaving, and temperature during autolysis. It would seem, therefore, that destruction or loss as a result of such treatments, if proportionate to the amounts of the substances present, would not influence the relative quantities of such substances in the final preparations from damaged cells as compared with those from undamaged cells.

With respect to irradiation, the conditions were not the same in the control and damaged-cell preparations. It is perhaps significant, therefore, that the instances in which appreciably lower total yields were obtained from damaged than from undamaged cells were those in which the factors concerned are known to be photochemically unstable, i.e. riboflavin, aneurin and pyridoxin.

Irradiation was carried out at about pH 5·5; hence conversion of riboflavin to lumichrome was to be expected. We attempted to assay lumichrome as well as riboflavin in the damaged-cell preparations by a fluorimetric method based on the difference in the fluorescent spectrum of lumichrome and riboflavin (Karrer, Salomon, Schöpp, Schlittler & Fritsche, 1934). This method was unsatisfactory, in part because lumichrome itself appears to be photochemically degraded into non-fluorescent products.

* The potencies of preparations assayed in previous investigations have been expressed in terms of 'growth units', one growth unit representing a 24 hr. yeast crop in 1 ml. of culture approximately 1000% greater than the control crop in a like volume of culture (i.e. a test-culture growth approximately 11 × the control-culture growth). It is preferable to adopt a smaller biological effect as the standard in order to avoid the necessity of extrapolations from the data obtained with undamaged-cell products and other low-potency materials. The proliferation-promoting unit (PP unit) employed in the present experiments represents a 24 hr. yeast crop 100% greater than that of the control in 1 ml. of culture. This unit corresponds to the 'relative growth unit' used by Cook & Cronin (1941).

The method did indicate, however, that the total amount of riboflavin and photochemical destruction products thereof in the damaged-cell preparations may have equalled or exceeded the total riboflavin content of the undamaged-cell preparations.

In the case of aneurin, computations were made of the photochemical destruction to be expected on the basis of a quantum efficiency for inactivation of the pyrimidine component of 0·0184 at $\lambda = 2537$ A. (Uber & Verbrugge, 1940*a*). Uber used a biological test for inactivation. His data indicate, however, that the initial photochemical change was a splitting off of the amino group in the 6-position. This would be expected to block the formation of thiochrome, and hence should lead to 'inactivation' in so far as the thiochrome assay, used in our experiments, is concerned. Uber found the thiazole component to be more sensitive photochemically (quantum efficiency for biological inactivation 0·347 at $\lambda = 2537$ A.), but the photochemical changes to which he attributed inactivation in this instance were not such as would be expected to interfere with thiochrome formation (Uber & Verbrugge, 1940*b*). From the energies in the 2500–2650 A. region employed in irradiation, the estimated absorption by interfering substances in the cells and suspension fluids, the above quantum efficiency for pyrimidine inactivation, and the average of the concentration of aneurin in damaged- and undamaged-cell preparations, it was computed that about 2·6 μg. of aneurin/g. of moist yeast may have been degraded as a result of photochemical action. The total yield for the damaged-cell preparations was 1·86 μg./g. of moist yeast (Table 1). This, together with 2·6 μg. assumed to have been photochemically degraded, leads to an hypothetical total of 4·46 μg. of aneurin/g. of moist yeast in the damaged-cell preparations as compared with 3·66 for the undamaged-cell preparations. Hence it seems possible that photochemical destruction may have accounted for the lesser total yield of aneurin from damaged as compared with undamaged cells (Table 1).

The results of these experiments are of primary interest in relation to the following three questions: (1) What factors may account for the increased proliferation-promoting effect of products from damaged cells as compared with those from undamaged cells? (2) Is there evidence for resynthesis of any of these factors within the living, damaged cells accompanying loss of these factors to the intercellular fluids? (3) Do the factors assayed in these experiments account entirely for the increased proliferation-promoting effect of products from damaged cells as compared with those from undamaged cells?

With regard to the first question, the data of Table 1 indicate that all of the factors determined may contribute to the increased proliferation-promoting effects of damaged-cell intercellular fluids (I) as compared with undamaged-cell intercellular fluids (II), since the quantities of the various factors were greater in all instances in I than in II. The assays establish only the possibility that each of these factors is important in the effect; the greater yields in I than in II are a necessary but not a sufficient condition for the contribution of the various factors to the proliferation-promoting effect. In the case of the combined samples of intercellular fluids, wash waters, and residue autolysates from damaged cells (*A* I) and from undamaged cells (*A* II), biotin, pantothenic acid, and folic acid were apparently the only ones of the factors assayed which could have contributed materially to the greater proliferation-promoting potency of *A* I, since only these were present in *A* I in significantly greater quantity than in *A* II, as indicated by the total yields in Table 1 (last column).

With regard to the second question, that of resynthesis, the data of Fig. 1 on growth assays of the combined samples *A* I and *A* II support the hypothesis that proliferation-promoting factors of some kind are produced, as well as released, during the course of cell damage. The assays in Table 1 show quantitatively a greater total yield of biotin, pantothenic acid, and folic acid from damaged cells and their suspension fluids than from undamaged cells and their suspension fluids, indicating elaboration of these substances by the damaged cells. Rogosa (1944) has found that, for normal yeast of the strain employed by us, both biotin and pantothenic acid (or its component β-alanine) are critical exogenous growth factors, indicating that the normal cells are incapable of elaborating such substances (at least in sufficient quantity to support optimum growth).

In considering the possibility of resynthesis of the various factors in the damaged cells, it should be borne in mind that the irradiated cells were not killed quickly but were damaged slowly. No appreciable cytolysis took place. The majority of the damaged cells were alive and metabolically active during most of the 8 hr. irradiation period. During this time, B factors and other proliferation-promoting substances were being released from the cells into the intercellular fluids, and it seems not unreasonable that replacement within the cells might have accompanied depletion resulting from diffusion into the suspending medium.

With regard to the third question, it appears that the factors assayed in the present experiments do not account entirely for the proliferation-promoting effects observed, as shown by the data of Fig. 2 and Table 3. This substantiates similar conclusions obtained by other methods (Cook & Cronin, 1941, 1942; Loofbourow, 1942*b*, *c*).

SUMMARY

1. Assays of the content of factors of the vitamin B complex of suspension fluids from ultra-violet damaged cells (I) and from undamaged cells (II)

showed greater amounts of biotin, folic acid, inositol, nicotinic acid, pantothenic acid, pyridoxin, riboflavin and aneurin in the former. The ratio of such factors in I as compared to II varied from a maximum of 91·5 × for pantothenic acid to a minimum of 2·3 × for inositol.

2. A solution of factors of the B complex corresponding to the quantities found in I stimulated the growth of yeast more than did II but not as much as did I, the relative proliferation-promoting potencies as compared with II being 25·9 for I and 4·5 for the B factor solution. It is concluded that factors of the vitamin B complex account for a part, but not all, of the proliferation-promoting effect in yeast-growth assays of products from damaged yeast cells.

3. Combined equal samples of the intercellular fluids, cell-residue wash waters, and residue autolysates from damaged cells (*A* I) and from undamaged cells (*A* II) were assayed for proliferation-promoting effect on yeast. Greater growth stimulation was obtained with *A* I than with *A* II. This confirms previously reported evidence that certain proliferation-promoting factors are replaced within the damaged cells as they are lost to the intercellular fluids by diffusion from the cells throughout the course of cell damage.

4. The total yield of factors of the B complex from intercellular fluids, residue wash waters, and residue autolysates was considerably greater from damaged than from undamaged cells in the case of biotin (1·6 ×), folic acid (1·9 ×) and pantothenic acid (1·9 ×) indicating that replacement within the living injured cells accompanies loss of these factors to the intercellular fluids. It is suggested that the instances in which lesser yields were obtained may possibly be explained by photochemical destruction in the irradiated (damaged-cell) preparations.

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Intercellular Hormones

7. RELEASE OF AMINO-ACIDS BY DAMAGED LIVING YEAST CELLS

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It has been demonstrated that factors of the vitamin B complex account in part for the proliferation-promoting activity ('wound hormone' effect) of products from yeast cells slowly damaged by sublethal ultra-violet radiation, and that the living, damaged cells appear to elaborate considerable quantities of certain of these factors (notably biotin and pantothenic acid) during the course of injury (Webb & Loofbourow, 1947).

Davidson (1940) found the amino-N content of suspension fluids from yeast cells damaged by ultra-violet radiation to be approximately 7 times that of suspension fluids from non-irradiated cells. This suggested that amino-acids may be involved in the