84. Intercellular Hormones

5. Evidence that the Proliferation Promoting Effect of Damaged-Cell Products is Attributable to Adenine Nucleotides and known Growth Factors

By **John R. Loofbourow**, *Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass.*

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Evidence was presented [Loofbourow, 1942*a*] that the mechanism whereby proliferation-promoting (PP) factors appear in the intercellular fluids when cells are slightly damaged involved three steps: (1) an increase in cell-membrane permeability, (2) a diffusion of non-protein substances through the more permeable membranes into the intercellular fluids, and (3) a synthesis within the living, injured cells of substances whose concentration therein is disturbed by diffusion. Such a mechanism might be expected to result in the presence in the intercellular fluids of a variety of non-protein growth-stimulating materials rather than of a single active substance. The investigations reported here indicate that the release of PP factors by damaged cells does involve a multiplicity of active substances, among which are adenine nucleotides and certain known growth factors.

Experimental

The preparation of intercellular fluids has been previously described in detail [Loofbourow, Webb, Loofbourow & Abramowitz, 1942]. As in the earlier experiments, the organism employed was yeast (*S. Cerevisiae*, F.B. strain) suspended at 100 g./l. in distilled H_2O, the injuring agent was lethal ultraviolet radiation, and the suspensions were irradiated for 8 hr. under such conditions that the cells were exposed repeatedly to sublethal doses of radiant energy and were damaged slowly. The PP activity of the preparations was assayed on yeast cultured in roller tubes [Loofbourow *et al.* 1942]. Characteristic properties of a typical preparation are listed in Table 1.

Table 1. *Characteristics of intercellular fluids from damaged and normal yeast cells*

(Cell-free suspension fluids from 100 g. (wet weight) yeast per l. distilled H_2O.)

	I. Suspension fluid from damaged cells	II. Suspension fluid from normal cells
Colour	Clear light straw	Water clear
Fluorescence excited by 3650 Å. radiation:		
Colour	Yellow-green	Blue
Intensity*	42·8	9·5
Ultraviolet absorption spectrum†	Marked maximum at 2600 Å., $D=7{\cdot}0$. No maxima characteristic of proteins, tyrosine, or tryptophane	Maximum at 2600 Å., $D=1{\cdot}4$. No maxima characteristic of proteins, tyrosine, or tryptophane
Solid content, mg./ml.	3·17	0·245
Biuret and phosphotungstic acid tests for protein	Negative	Negative
Potency,‡ growth units per ml.	98·0	3·3
Growth units per mg.	30·9	13·5

* Referred to quinine sulphate, 0·27 mg./l. in 0·1 *N* H_2SO_4, as 48·0 (all readings net, after subtraction of blanks).

† $D=1/x \log_{10} I_0/I_x$.

‡ One growth unit represents an increased 24 hr. yeast crop of approximately 1000% in comparison with the controls [Loofbourow, Dwyer & Cronin, 1941].

Presence in the preparations of heat-labile as well as heat-stable PP factors. Autoclaving for 20 min. at 15 lb. at *p*H 5·4–6·0 caused a loss in activity with regard to the later periods (30–70 hr.) of the growth tests of about 10 % in damaged-cell preparations and about 25 % in normal-cell extracts (Table 2). This effect was overlooked in earlier assays

Table 2. *Effect of autoclaving for 20 min. at 15 lb. on PP activity of intercellular fluids from injured and normal cells*

Material tested for proliferation promoting effect (at 0·1 ml./ml. of culture)	Yeast crop, mg. (wet weight) per ml. after hr.					
	0	16	24	30	45	60
I. Injured-cell extracts:						
Unautoclaved	0·064	1·55	4·05	4·95	5·53	5·55
Autoclaved	0·064	1·55	4·05	4·76	4·95	4·95
II. Normal-cell extracts:						
Unautoclaved	0·064	0·47	0·84	1·00	1·30	1·39
Autoclaved	0·064	0·42	0·70	0·82	0·97	1·06
Controls	0·064	0·07	0·12	0·12	0·12	0·12

[Loofbourow, Dwyer & Morgan, 1938] because they were based entirely on 24 hr. yeast crops. No further loss in activity could be detected on repeatedly re-autoclaving; the tests for further loss in activity involved as many as nine successive assays extending over periods up to 3 months. Exposure of the autoclaved preparations to diffuse daylight during storage caused no measurable change in activity. After storage for approximately 3 months, an autoclaved damaged-cell preparation was re-autoclaved for 65 min. at 15 lb. in 0·05 *M* H_3PO_4, 0·05 *M* NaOH, and at *p*H 5·5, 6·8, and 9·6 without further loss in activity as exhibited in growth periods up to 72 hr.

These results indicated that the preparations contained heat-labile factors responsible in part for the PP effect of non-autoclaved preparations in the growth period after 24 hr., as well as heat-stable factors responsible for the early PP effect and for most of the later growth response.

Presence in the preparations of heat-stable factors other than nucleotides. Close correlation was found between the ultraviolet absorption at 2600 Å., characteristic of nucleotides, and the PP potency of intercellular fluids from damaged rat-embryo tissues [Loofbourow, Dwyer & Lane, 1940] and from damaged yeast cells suspended in isotonic saline [Cook, Loofbourow & Stimson, 1939]. In more recent experiments, suspension fluids from damaged yeast cells in distilled H_2O did not show such correlation (Fig. 1). It appeared, therefore, that the PP activity of damaged-yeast preparations could not be accounted for by the ultraviolet absorbing moiety alone. Against the possibility that the absorbing moiety played no part in the PP effect was the fact that crude preparations, and fractions thereof, having $E^{1\%}_{1\,cm.} = 10$ to 15 at λ 2600 Å., generally had almost inappreciable activity, especially in the early growth periods. A more likely interpretation seemed to be that the crude preparations contained a multiplicity of active factors

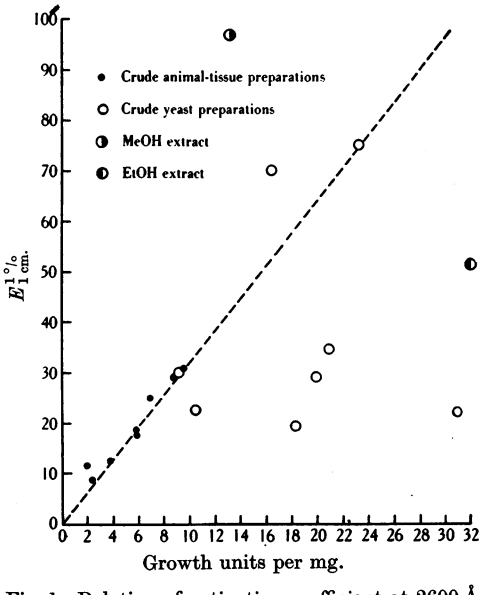

Fig. 1. Relation of extinction coefficient at 2600 Å. to activity of various preparations.

among which the absorbing moiety ceased to be limiting for yeast growth when it reached a level corresponding approximately to $E^{1\%}_{1\,cm.} = 20$ at λ 2600 Å.

Various fractionation procedures failed to result in proportionate concentration of PP factors and ultraviolet absorbing substances in the same fractions. In early experiments [Stimson, Aquinas & Loofbourow, 1940], crude damaged-yeast preparations, both before and after reduction in volume by vacuum distillation and precipitation of inactive material with hot 95 % ethyl alcohol, were passed through adsorption columns made up of equal parts of magnesium oxide and Filtercell. In 3650 Å. radiation, a brilliant fluorescing band regularly appeared at the top of the column. This could be developed into three bands, the top one fluorescing yellow, the middle one white, and the lower one blue. The filtrates fluoresced blue. Various portions of the columns were eluted with boiling H_2O, and the PP activity and ultraviolet absorption of the eluted fractions and filtrates were determined. It was thought at first that the major portion of the PP activity and ultraviolet absorbing moiety accumulated together in the yellow-fluorescing band. Further experiments indicated, however, that the activity, but not the ultraviolet absorption of fluorescence, was rather uniformly distributed among the fractions. Interpretation of these results is difficult because of the possibility that the presence of oxidation products may have complicated the observations. Thus, it was noted that when white or blue fluorescing eluates were dried on a water bath, the portion which dried most rapidly often exhibited a change in fluorescent colour to yellow, corresponding to the top bands of the columns. Similar changes from blue to yellow fluorescence were noted in solutions of barbituric acid dried rapidly and redissolved [Joyce, 1940], and a somewhat analogous development of new fluorescent properties occurs when thiamine is oxidized to thiochrome.

In recent experiments, damaged-cell products prepared from yeast in distilled H_2O were fractionated by various solvents. A preparation taken to dryness on a water bath was successively extracted, in a Soxhlet apparatus, with hot ethyl ether, methyl alcohol, and 95 % ethyl alcohol, with the results shown in Table 3. Ether removed approximately 7 % of the material but only a negligible proportion of the PP activity or ultraviolet absorption. The PP factors and ultraviolet absorbing materials were readily soluble in hot methyl or ethyl alcohol, the final residue of insoluble material, which represented about 40 % of the original material, retaining but a small proportion of the activity or absorption. The extinction coefficients of these fractions showed even less correlation with their PP activities than that found for crude preparations (Table 3 and Fig. 1).

Table 3. *Fractionation of injured yeast preparations by successive extractions with hot solvents*

	Fraction by weight, W	$E^{1\%}_{1\,cm.}$ at λ 2650 Å.	$E \times W$	Growth units per mg., G.U.	G.U. × W
I. Crude material	1·00	29·0	29·0	20·0	20·0
II. Ether extract of I	0·072	0·021	0·00	—	—
III. MeOH extract of I	0·514	51·2	26·3	32·0	16·5
IV. EtOH extract of I	0·016	97·0	1·6	13·3	0·2
V. Residue from IV	0·400	3·0	1·2	8·8	3·5
Totals, II–V	1·00	—	29·1 (+0·3 %)	—	20·2 (+1·0 %)

An aliquot of the methyl alcohol extract was dried and extracted successively with hot acetone and 95 % ethyl alcohol. The material was insoluble in acetone. The ultraviolet absorbing moiety divided approximately equally between the ethyl alcohol extract and the residue. The sums of the activities of the fractions did not equal the activity of the original material, however, so that comparison of the extinction coefficients and activities of the fractions was not possible. The apparent loss in activity in the fractions may have

been due to separation of factors which show maximum effectiveness only in combination, or to chemical breakdown. The solubility behaviours of the PP factors was similar to that of bios preparations [Bishop & Rainbow, 1939] and of various known water-soluble growth factors. The solubility behaviour of the ultraviolet absorbing moiety was suggestive of various nucleotides, rather than of nucleic acid or its constituent purines and pyrimidines [Levene & Bass, 1931]. The observed lack of correlation of extinction coefficients and PP activity corresponded to similar results obtained by Cook, Hart & Stimson [1940] with bios fractions from yeast.

From these experiments, it appears that the ultraviolet absorbing moiety of damaged yeast cell products cannot account for the entire PP effect of such preparations.

Presence in the preparations of heat-stable PP factors other than certain known growth factors. To determine whether the PP activity could be attributed entirely to certain known growth factors, two supplements, A and B, were made up in Reader's medium [Reader, 1927]. Supplement A contained the following substances (concentrations in μg. per ml.): thiamine hydrochloride, 2·5; riboflavin, 5·0; pyridoxine, 5·0; calcium pantothenate, 1·25; biotin (free base), 0·00125; choline, 7·5; and nicotinamide, 25·0. Supplement B contained the following amino-acids, each at a concentration of 6·25 mg./ml.: glutamic acid, asparagine, aspartic acid, leucine, and arginine. These concentrations were selected on the basis of data of Williams, Eakin & Snell [1940] to provide an excess of each of the factors when the supplements were added to Reader's medium in the proportion of 1 ml. of supplement in 10 ml. of supplemented medium. The PP effects of intercellular fluids from damaged and undamaged yeast cells were then tested in Reader's medium with and without these supplements.

Cook [1942] had found previously that the addition of preparations from damaged yeast cells gave increased growth in either Reader's or Williams' medium supplemented with thiamine, pyridoxine, pantothenic acid, biotin, inositol, and twenty amino-acids. Preliminary data substantially confirming Cook's results have been reported elsewhere [Loofbourow, 1942*b*]. These data have been extended by further studies with regard to (*a*) concentration effects, and (*b*) growth kinetics. At concentrations of 0·1 ml. each per ml. of culture for supplements A and B and of 0·05 ml./ml. of culture for preparations from damaged and undamaged yeast cells, the increased growth resulting from the addition of damaged-cell products to the supplemented media was clearly apparent (Table 4). When, however, the concentration level of the supplements was increased, the

Table 4. *Effect of adding suspension fluids from damaged and undamaged cells to cultures containing various growth factors and amino-acids*

Added material per ml. of final culture	Yeast crop, mg. (wet weight) per ml. (initial seeding, 0·064 mg./ml.) after hr.					
	9	20	24	33	50	75
None (controls)	0·115	0·147	0·152	0·160	0·166	0·160
0·1 ml. supplement A	0·349	2·57	3·08	3·48	3·85	3·73
0·1 ml. each supplements A and B	0·348	3·57	4·43	5·29	6·07	6·02
0·05 ml. damaged-cell preparation	0·434	3·32	3·70	4·38	4·57	4·43
0·05 ml. damaged-cell preparation plus 0·1 ml. supplement A	0·452	3·57	4·03	4·53	4·90	4·80
0·05 ml. damaged-cell preparation plus 0·1 ml. each supplements A and B	0·367	4·35	5·40	6·77	6·77	6·30
0·05 ml. undamaged-cell preparation	0·309	0·771	0·803	0·891	0·983	1·03
0·05 ml. undamaged-cell preparation plus 0·1 ml. supplement A	0·359	2·63	3·00	3·70	4·07	—
0·05 ml. undamaged-cell preparation plus 0·1 ml. each supplements A and B	0·359	3·44	4·31	5·19	5·89	5·66

effect of adding damaged-cell preparations became less apparent, until at a level of 0·2 ml./ml. of culture for each of the two supplements, almost negligible increased growth resulted from the addition of damaged-cell preparations. This is illustrated in Fig. 2, which, although plotted for 24 hr. growth, is substantially representative of the growth-concentration relationships observed throughout the growth periods from 20 to 70 hr.

The suppression of the PP effect of damaged-cell preparations by high concentrations of the two supplements suggested the presence in the damaged-cell products of at least some of the factors in the supplements. That the damaged-cell products also contained other PP factors was indicated by two results of these experiments. First, greater growth

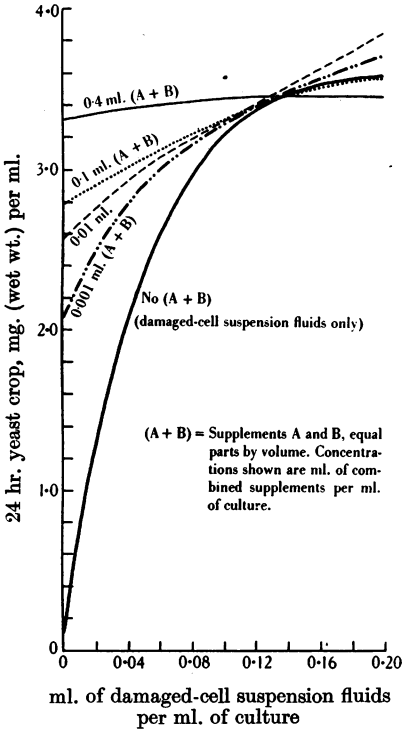

Fig. 2.

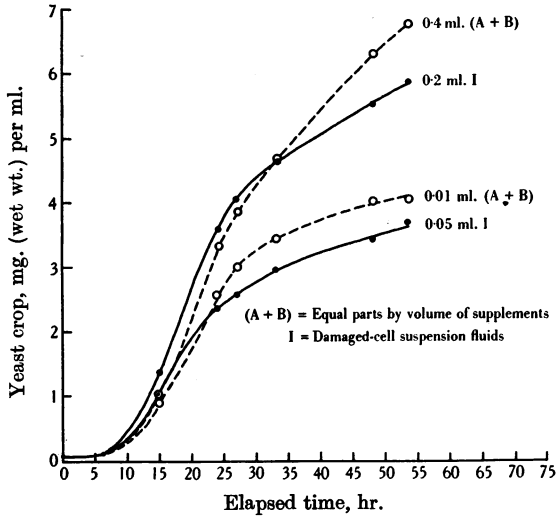

Fig. 3.

Fig. 2. Effect on 24-hour yeast crop of adding damaged-cell products to media containing various concentrations of supplements A and B. (Supplement A contained known growth factors and supplement B contained amino-acids. See text for details.)

Fig. 3. Comparison of growth kinetics obtained with damaged-cell products and with supplements A and B.

was obtained with the higher concentrations of damaged-cell preparations and 0·005 ml. of each supplement per ml. of culture than with even the highest concentration of the combined supplements alone. Secondly, the growth kinetics with the combined supplements alone differed from those with the damaged-cell products alone, the latter being characterized by greater initial PP stimulation and less final crop than the former at comparable concentration levels (Fig. 3). These data indicated a qualitative as well as a quantitative difference between the damaged-cell preparations and the supplements. The possibility has to be considered that the qualitative difference might be attributable to variation in relative proportions of the same factors, present in both the supplements and the damaged-cell products, as well as to the presence of additional factors in the damaged-cell products.

Fig. 4 shows 24 hr. growth *v.* concentration-by-weight relationships for the combined supplements and for damaged-cell preparations. Again, a qualitative difference is evident. It should be noted that at the higher concentration levels the crude damaged-cell preparations were more potent, weight for weight, than the combined supplements. This was true despite the fact that the damaged-cell suspension fluids diluted the Reader's medium in proportion to the amount of fluid added, whereas the supplements did not, since the latter were made up in Reader's medium.

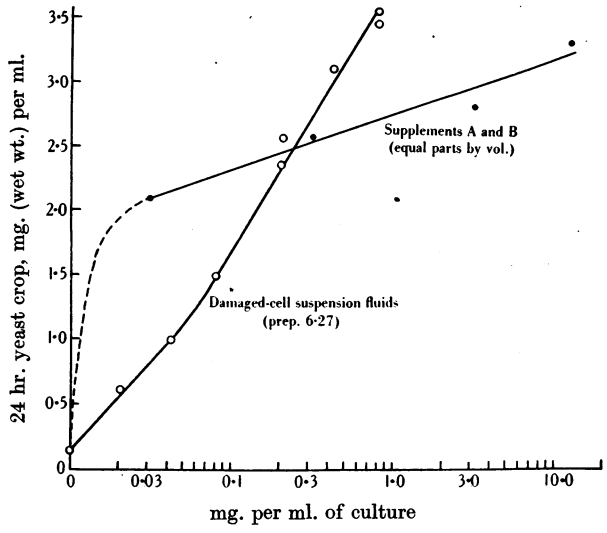

Fig. 4. Comparison of the PP potencies of damaged-cell products and of the combined supplements A and B at various concentration levels.

Concurrently with these investigations, Mr Webb of our laboratory undertook the chemical and microbiological assay of various preparations from damaged and undamaged cells for the presence of certain known growth factors. His results will be published in detail as a subsequent paper in this series; it is necessary to refer to them here in connexion with the following experiments. According to his assays, a typical damaged-yeast preparation contained (in μg./ml.) biotin 0·0125, pyridoxine 1·28, pantothenic acid 2·86, thiamin 0·01, nicotinic acid 5·40, and folic acid 0·0136. A water solution of these factors, in corresponding concentrations, was made up and designated 'supplement C'. A comparison was then made in Reader's medium of the PP effect of C, of a typical damaged-cell preparation (I), and of C and (I) in equal proportions by volume. Supplement C failed to reproduce the PP effect of (I), hence factors other than those in C apparently accounted in part for the PP activity of (I). The mixture of C and (I) exhibited, however, approximately the same PP activity as that of (I), indicating that the additional factors in (I), other than those in C, were present in excess of limiting quantities.

Simulation of the PP effect of damaged-cell products by combinations of known growth factors and adenine nucleotides. Since neither the ultraviolet absorbing moiety alone nor certain known growth factors appeared to account entirely for the PP activity of damaged-cell products, an investigation was undertaken of the combined PP effects of nucleotides and growth factors. Adenine complexes were employed because, as previously reported [Loofbourow *et al.* 1942; Loofbourow, 1942*b*], spectroscopic and chemical evidence indicated that the nucleotide moiety contained adenine. Because of the marked effect of the damaged-cell preparations in stimulating cell respiration, and because of the markedly greater PP effect of damaged-cell preparations when the cultures were well aerated, it had been thought that the nucleotide moiety might be diphospho- or triphosphopyridine nucleotide [Loofbourow *et al.* 1942]. Preliminary evidence (biological tests and effects of hyposulphite reduction on the spectra) indicated that this was not the case, but it is felt that the possibility that a part of the nucleotide absorption is attributable to these coenzymes has not been excluded entirely.

The effects of adenosine, yeast adenylic acid, muscle adenylic acid, and adenosine triphosphate on the growth of yeast in Reader's medium were investigated. The concentrations employed ranged from 5×10^{-6} to $2{\cdot}5 \times 10^{-4} M$, after dilution by the Reader's

medium. The extinction coefficients at 2600 Å. of typical damaged-cell preparations were equivalent to adenine molarities of about 5×10^{-4} in the crude preparations. Hence, on this basis, the concentrations of the nucleotides employed corresponded to the equivalent of 0·01–0·5 ml. of damaged-cell preparations per ml. of culture.

None of the nucleotides alone duplicated the activity of damaged-cell preparations in Reader's medium. Yeast adenylic acid appeared to have negligible effect. Adenosine triphosphate, muscle adenylic acid, and adenosine all stimulated proliferation, the effect decreasing in the order listed and being in all instances considerably lower in magnitude that the PP effect of equivalent concentrations of damaged-cell preparations. In the period less than 24 hr., the growth kinetics obtained with the damaged-cell preparations appeared to be most closely simulated by adenosine triphosphate.

Adenosine triphosphate, muscle adenylic acid, and adenosine were then tested in Reader's medium in combination with supplements A and B. Each ml. of culture contained either 0·1 ml. of A or 0·1 ml. each of A and B. At a concentration in the cultures of $5 \times 10^{-5} M$ the three adenyl compounds each caused increased growth response when added to media supplemented with A alone or supplemented with both A and B. At $2{\cdot}5 \times 10^{-4} M$ adenosine triphosphate added to media supplemented with A resulted in essentially the same growth as did a damaged-cell preparation alone at 0·05 ml./ml. of culture (Fig. 5). Furthermore, adenosine triphosphate at this concentration caused essentially the same increase in growth above that obtained with the combined supplements A and B as did the addition to the supplemented media of 0·05 ml. of damaged-cell products per ml. of culture. It was possible, therefore, (*a*) to duplicate the effect of damaged-cell products alone by a combination of known growth factors and adenosine triphosphate, and (*b*) to duplicate the effect of adding damaged-cell products to well-supplemented media by the addition of this nucleotide to similarly supplemented media. It should be pointed out that the concentration of the nucleotide necessary to produce these effects corresponded to 10 times the nucleotide equivalent of the damaged-cell products as computed from their ultraviolet absorption at 2600 Å.

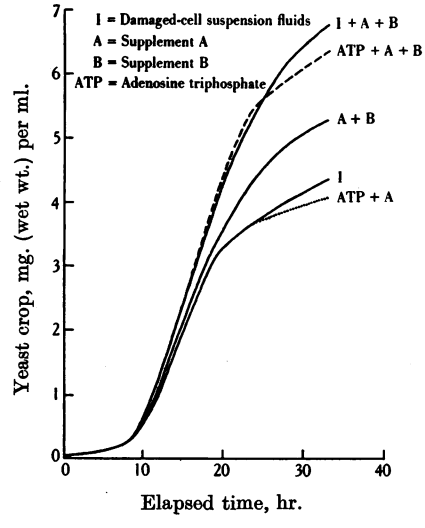

Fig. 5. Duplication of the proliferation-promoting effect of damaged-cell products by adenosine triphosphate and known growth factors.

Discussion

The interrelationships of cells through the intercellular *milieu* appears to be more complex than has previously been realized. When cells are damaged by such widely different agents as radiant energy, mechanical injury, and chemical injury [Loofbourow & Dwyer, 1938; 1939 *a*, *b*; Loofbourow, Cook, Dwyer & Hart, 1939], proliferation-promoting factors and substances capable of markedly stimulating cell respiration [Fardon, Carrol & Ruddy, 1938; Sizer & Loofbourow, 1942] are released into the intercellular fluids. This general type of phenomenon is observed either with animal-tissue cells [Menkin, 1941; Loofbourow, Cook *et al.* 1939; Loofbourow, Cueto & Lane, 1939; Loofbourow *et al.* 1940] or yeast. The phenomenon occurs not only when cells are sufficiently damaged to cause their breakdown and disintegration, but when the cellular injury is insufficient to cause cytolysis. In the latter case, the release of active factors by the living, injured cells appears to be due to increased membrane permeability and to be accompanied by

resynthesis of some of the materials lost from the cells [Loofbourow, 1942*a*]. Subjecting cells to anaerobiosis leads to much the same type of phenomenon [Loofbourow & Dwyer, 1939*a*, *b*], perhaps because of the effects of accumulated CO_2 on membrane permeability.

The present studies suggest that among the substances released by damaged cells into the intercellular *milieu*, various known growth factors and adenine nucleotides may account, in large part, for the effects of the suspension fluids on the metabolism of normal cells. The effects appear to be due to a variety of substances. This may explain the difficulties encountered in the application of chemical purification procedures alone to the elucidation of the problem—hence the emphasis now being placed on microbiological and chemical methods of analysis for known growth factors. From the standpoint of the possible relationship of the general phenomenon to wound healing and overgrowth, it might appear to be more profitable to concentrate upon studies of animal tissues. We believe, however, that a thorough study of the phenomenon with yeast, as a convenient and easily utilized organism, should point the way to the most direct approach to the problem of tissue repair in animals following cell damage.

The fact that, of the adenine complexes tested, adenosine triphosphate appeared more effective than the others in simulating the effects of damaged-cell products should not be interpreted as too strong an indication of the identity of this substance with the nucleotide moiety of the damaged-cell products. The stability of the damaged-cell preparations was greater than might be expected were adenosine triphosphate an essential constituent. Furthermore, the adenosine triphosphate preparation very likely contained an appreciable proportion of the di- and monophosphates. The question of the identity of the nucleotide moiety is being investigated in greater detail.

Summary

The proliferation promoting effect of products from damaged cells appears to be due to a variety of substances, among which are known growth factors and adenine nucleotides. Damaged-cell products caused increased growth of yeast when added to media well supplemented with biotin, pantothenic acid, folic acid, thiamine, riboflavin, nicotinic acid, pyridoxine, choline, and amino-acids. Addition of adenosine triphosphate produced the same effect as addition of the damaged cell products. Adenosine triphosphate, muscle adenylic acid, and adenosine, but not yeast adenylic acid or yeast nucleic acid, increased the growth rate of yeast in synthetic media, with or without the addition of growth factor and amino-acid supplements. It appears, therefore, that certain adenine nucleotides act as growth factors. The metabolic effects of damaged-cell products were reproduced by a combination of known growth factors and adenosine triphosphate.

I wish to express my thanks to Dr Fritz Lippmann of the Harvard Medical School for supplying the preparations of adenosine triphosphate, muscle adenylic acid, and adenosine, to Mr Robert Sinsheimer for obtaining the data on solvent fractionations and to Mrs R. K. Abramowitz for assistance in the growth assays.

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