72. Intercellular Hormones

4. Further Observations Regarding the Mechanism of the Production and Release of Proliferation-promoting Factors by Injured Cells

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

(Received 1 July 1942)

When cells of yeast or animal tissues are injured by agents such as lethal ultra-violet which do not lead directly to rupture of the membrane or to rapid death, proliferation-promoting factors other than dead-cell disintegration products or autolysis products appear in the intercellular fluids [Loofbourow, Dwyer *et al.* 1938; 1940; 1941; Davidson, 1940]. Evidence that such proliferation-promoting factors (intercellular wound hormones) are released by living injured cells rests primarily on the following observations [Loofbourow, Dwyer & Cronin, 1941]: (1) slow injury leads to higher potencies of intercellular fluids than rapid killing, (2) the potency of the fluids increases rapidly before appreciable mortality is noted, (3) the potency is greater when cells are injured in a physiologically favourable suspension medium, (4) active factors appear in suspensions of cells subjected to anaerobiosis without killing the cells, and (5) cells killed quickly by grinding or boiling and then subjected to injurious ultra-violet yield less potent preparations than cells injured slowly by ultra-violet prior to quick killing by grinding or boiling. It has been concluded from such data that the proliferation-promoting activity of intercellular fluids from slowly-injured cells is attributable to either (a) release of materials already present in the cell as a result of increased membrane permeability, (b) release of materials synthesized in the living cell as a response to injury, or (c) both of these processes. The further studies presented here were directed at a more complete elucidation of the mechanism involved in this phenomenon.

EXPERIMENTAL METHODS AND RESULTS

S. cerevisiae Fleischmann, bakers' strain, was washed repeatedly and suspended in distilled H₂O at a concentration of 100 g. wet weight per litre of suspension. The suspension was divided into two equal portions, one of which was irradiated with lethal ultra-violet while the other served as a control. Following irradiation, the intercellular fluids were separated off by centrifuging and filtration through Seitz filters. Assays for proliferation-promoting activity were made on yeast cultured in Reader's medium at 30° in a roller tube device. Details of the irradiation and growth techniques have been reported [Loofbourow, Webb, Loofbourow & Abramowitz, 1942].

1. *Cell counts.* There was no significant change in haemocytometer counts throughout the course of the injury period, either in controls or irradiated suspensions as illustrated by the two examples in Table 1. This confirms similar observations by Davidson [1940], who used isotonic saline as the suspension medium. In experiments of longer duration (up to 22 hr.) at lower ultra-violet intensity levels, and employing Reader's medium, increase in cell number was noted in both controls and irradiated suspensions, but in no instance was a significant decrease in cell number, indicative of loss of cells by cytolysis, observed.

2. *Results of microscopic examinations.* No evidence of cytolysis was found on examination of the suspensions at intervals throughout the course of irradiation. After approximately 4 hr., budding of some cells was noted. The percentage of budded cells was uniformly greater in irradiated than in control suspensions (10.7 and 6.6% respectively at the end of 5 hr. in preparation 13. 12. 41).

Table 1. *Variation in cell number per ml. during course of injury by ultra-violet radiation as determined by haemocytometer counts*

Time, hr. after start of irradiation	% deviation from mean for each series			
	Prep. 8. 11. 41		Prep. 13. 12. 41	
	Non-irradiated	Irradiated	Non-irradiated	Irradiated
	%	%	%	%
0	+5.5	+5.0	-6.0	-5.7
1.5	-2.5	-4.9	—	—
3.0	—	—	+6.1	+1.2
5.0	—	—	+7.6	+10.4
6.5	-3.1	+4.8	—	—
8.0	+0.1	-4.9	-7.7	-5.9

Average cell counts in all instances were approximately 10^6 cells per ml. Small buds occasionally observed attached to mother cells late in the irradiation periods were not counted as separate cells.

A larger percentage of budded than of unbudded cells in the irradiated suspensions, but not in the controls, stained with methylene blue. At the 5 hr. observation of the 13. 12. 41 irradiated preparation, approximately two-thirds of the budded cells, but only half of the unbudded cells, were stained. This might be explained by greater susceptibility of the budded cells to the lethal radiation, or it may indicate a correlation between injury and tendency to bud formation.

3. *Results of tests for presence of protein.* The intercellular fluids gave negative biuret and phosphotungstic acid precipitation tests, in agreement with previously reported results for other preparations [Loofbourow, Cook & Stimson, 1938]. Spectra of the fluids showed no maxima in the region 2700–2900 Å. characteristic of proteins, tyrosine, or tryptophan. Davidson [1940] found his extracts from irradiated cells to give a biuret reaction. Their protein nitrogen content was much less than that from similar suspensions of autoclaved cells (8.7 mg./100 ml. as compared with 75.6 mg./100 ml.) but was higher than that of control extracts (2.1 mg./100 ml.). It is possible that the discrepancies between his results and ours were due to more drastic irradiation of his preparations.

4. *Evidence regarding changes in membrane permeability.* The effects of injury and death in increasing the permeability of cell membranes are well known, and it has been suggested that this may account for the release of proliferation promoters by injured cells [Loofbourow, Dwyer & Cronin, 1941]. The following observations substantiate this view. Preliminary studies with radioactive phosphorus indicated that permeability is increased during the course of irradiation. Cells removed from the irradiated suspensions late in the irradiation period appeared shrunken, as observed by Davidson [1940]. The stained cells were generally smaller than the unstained ones. When yeast cells were irradiated continuously on the stage of a quartz ultra-violet microscope so that the same individual cells could be observed from time to time, there was a progressive decrease

Table 2. *Changes in cell volume during the progress of injury by ultra-violet radiation. Values listed are % of total suspension-volume occupied by cells*

Time, hr. after start of irradiation	Prep. 8. 11. 41		Prep. 13. 12. 41	
	Non-irradiated	Irradiated	Non-irradiated	Irradiated
0	11.5	11.5	11.0	11.0
1.0	—	—	11.5	10.5
1.5	12.0	11.0	—	—
2.0	—	—	11.5	10.5
3.0	—	—	11.5	10.5
5.0	—	—	11.5	9.0
5.5	12.0	9.0	—	—
8.0	12.0	8.5	12.0	7.5

in cell size [Loofbourow & Joyce, 1941; and unpublished data]. Finally, haematocrit determinations on preparations in distilled water showed progressive decrease in the total volume of irradiated cells as compared with an increase in the volume of control cells (Table 2).

There is evidently an increased permeability of the membranes of the injured cells, accompanied by loss therefrom of water-soluble crystalloidal substances and reduction in turgor.

5. *The possible role of direct photochemical action in the production of proliferation-promoting factors.* It has been suggested from time to time that the proliferation-promoting factors found in the intercellular fluids from ultra-violet injured cells might arise as a result of direct photochemical action. In view of the fact that the intercellular fluids from cells injured mechanically, chemically, or by subjection to CO₂ [Loofbourow & Dwyer, 1938; 1939; Loofbourow, Cook *et al.* 1939] show similar proliferation-promoting activity, direct photochemical conversion of an inactive into an active substance can hardly be considered as uniquely responsible for this type of phenomenon. Nevertheless, the possibility had to be considered that direct photochemical activation might have been involved in those instances in which ultra-violet radiation was employed as the injuring agent.

In order to determine whether such a mechanism could be considered reasonable from the point of view of energetics, the following measurements and computations were made. Radiation of longer wave-length than about 2950 Å., which is the long-wave limit of the lethal ultra-violet region, was found to be ineffective in causing the release of proliferation-promoting factors. From energy measurements with a calibrated Hanovia ultra-violet meter and transmission measurements with a monochromator and thermopile, it was determined that the absorbed energy in the region $\lambda < 2950$ Å., neglecting surface reflectance, was 4.23×10^8 erg/ml. of suspension for the 8 hr. irradiation periods employed. The surface reflectance was calculated to be about 5% from data of Supplee & Dorcas [1934], a fraction too small to be of significance in the computations. To determine the approximate number of quanta absorbed, the energy of the quantum at 2500 Å. was used, since the source of ultra-violet was a low-pressure mercury arc having a large proportion of its energy output in the range $\lambda < 2950$ Å. concentrated in the 2537 Å. mercury resonance line. On this basis, the number of quanta absorbed per ml. was $\frac{4.23 \times 10^8}{7.96 \times 10^{-12}} = 5.31 \times 10^{19}$. The growth-stimulating potency of a typical preparation was 106 growth units*/ml. greater than that of the corresponding control. Hence the number of quanta per additional growth unit was $\frac{5.31 \times 10^{19}}{106} = 5.0 \times 10^{17}$.

If the photochemical activation process had a quantum efficiency of 1, with no reduction in efficiency due to absorption of interfering substances, 5.0×10^{17} molecules of activated material would, therefore, correspond to one growth unit. If, as is more likely, the absorption of energy by interfering substances greatly reduced the overall efficiency, the number of activated molecules per growth unit would be much less. For purposes of approximation, it may be assumed that the conditions are analogous to the photochemical activation of sterols to vitamin D, which for ergosterol in solution has a quantum efficiency of about 0.2–0.5 [Marshall & Knudson, 1930] but for milk has a quantum yield of 7×10^{-6} [Ellis, Wells & Heyroth, 1941, p. 844], the overall efficiency

* Our experimentally determined potencies are based on the quantity of material which must be added to each ml. of a yeast suspension of 0.064 mg./ml. (wet weight) in Reader's medium to give a 24 hr. crop of 1.6 mg./ml. (approximately 6,250,000 cells/ml.) greater than the control crop, the cultures being grown at 30° under aerated conditions. This standard biological effect is defined as a 'growth unit'. The quantity of material in mg. or ml., necessary to give this effect having been determined, the reciprocal of this quantity then gives the number of growth units per mg. or per ml. [Loofbourow, Dwyer & Lane, 1940].

being reduced by 2×10^{-5} because of the dissipation of energy by non-activable absorbing substances in the milk. Assuming a similar factor for the effect of interfering substances in the yeast suspension, the number of activated molecules per growth unit would be $(5.0 \times 10^{17}) (2 \times 10^{-5}) = 1.0 \times 10^{13}$.

Such considerations, then, lead to the deduction that if direct photochemical activation accounts for the proliferation-promoting factors, the number of activated molecules per growth unit is of the order of 1.0×10^{13} to 5.0×10^{17} , the lower value being more probable.

It is now possible to determine whether the growth-stimulating activity of such an hypothetical substance would be of reasonable order of magnitude. Data of Williams, Eakin & Snell [1940], recalculated to our potency basis, and unpublished experiments of our own, indicate that pantothenic acid has a growth potency of about 4.8×10^{-5} mg. per growth unit under our assay conditions. This is equivalent to

$$\frac{4.8 \times 10^{-5} \times 10^{-3}}{229} \times 6.06 \times 10^{23} = 1.27 \times 10^{14} \text{ molecules per growth unit.}$$

Comparing this with the computed values for the hypothetical photoactivation product, one finds for the latter a potency of the order of 2.5×10^{-3} to 12 times that of pantothenic acid, depending upon whether the higher or the lower of the two assumed quantum yields is employed. These are not unreasonable values, and the possibility of direct photochemical activation giving rise to the proliferation-promoting factors cannot, therefore, be ruled out by a *reductio ad absurdum* argument on the grounds of energetics alone.

The significance of these computations lies in their relation to the question of whether or not the effects observed by the ultra-violet cell-injury technique are characteristic of cell damage generally or are specifically related to ultra-violet injury. Although, as stated above, the observation of similar increases in the growth potencies and ultra-violet absorption of intercellular fluids from cells damaged in other ways indicates that the phenomenon is not specifically characteristic of ultra-violet injury, the above computations suggest the need of caution in making too broad generalizations from ultra-violet injury experiments. Lethal radiant energy is convenient and easily applied in investigating cell-damage phenomena, but the results obtained with it should be checked by other methods.

6. *Effects of monochromatic ultra-violet radiation.* A preliminary investigation of the effects of monochromatic ultra-violet radiation on wound hormone production was carried out as follows: a General Electric H-6 1-kW. high-pressure water-cooled mercury arc in a quartz envelope was used as a source with a Bausch and Lomb constant-deviation double-prism quartz monochromator. A calibrated thermopile and galvanometer served for energy measurements. The yeast employed was suspended in distilled water at a concentration of 0.873 mg. wet weight/ml. It was necessary to use this low concentration, as compared with 100 mg./ml. used in full ultra-violet preparations, in order to obtain appreciable killing in the 10 hr. irradiation period with the low ultra-violet intensities available from the monochromator system. The yeast suspensions were irradiated in a quartz cell of 2 cm. thickness by 3 cm. height and 3 cm. width, inside dimensions. The volume of irradiated and control suspensions was 12 ml. in each experiment. Both suspensions were stirred continuously with motor stirrers throughout the irradiation periods.

The effects of three wave-length regions were investigated, namely 2900–2800 Å, 2740–2560 and 2483–2417 Å. These are designated in what follows by the wave-lengths at the centres of the bands, respectively 2850, 2650 and 2450 Å. The band widths were chosen so that the same total energy (1.39×10^7 ergs) was absorbed in the irradiated suspension in all preparations. Immediately following irradiation, the control and irradiated suspensions were filtered through Seitz filters; thus the extraction period was uniformly 10 hr. in all instances. The results are given in Table 3.

Table 3. *Effects of monochromatic ultra-violet radiation on the production of wound hormones*Time of irradiation 10 hr. in each instance. Total energy absorbed in 12 ml. of suspension, 1.39×10^7 erg.

Wave-length Å.	Absorbed quanta per ml.	% dead cells*			% of total volume occupied by cells		
		A	B	B - A	A	B	B - A
		Before irradiation	After irradiation		Before irradiation	After irradiation	
2850	$\{1.61 \times 10^{17}$	1	5	+ 4	0.06	0.04	- 0.02
(2900-2800)	{Control	1	1	—	0.06	0.07	+ 0.01
2650	$\{1.50 \times 10^{17}$	1	41	+ 40	0.07	0.02	- 0.05
(2740-2560)	{Control	1	1	0	0.07	0.07	0
2450	$\{1.34 \times 10^{17}$	1	2	+ 1	0.07	0.04	- 0.03
(2483-2417)	{Control	1	1	0	0.07	0.07	0

Wave-length Å.	Absorbed quanta per ml.	Potency of extracts. Growth units per ml.		Quanta per growth unit of increased potency due to irradiation	Extinction at 2650 Å.	
		Irradiation	- non- irradiation		Irradiation	- non- irradiation
2850	$\{1.61 \times 10^{17}$	1.1	- 0.1	—	1.6	+ 0.4
(2900-2800)	{Control	1.2	—	—	1.2	—
2650	$\{1.50 \times 10^{17}$	2.2	+ 1.1	1.36×10^{17}	3.0	+ 1.8
(2740-2560)	{Control	1.1	—	—	1.2	—
2450	$\{1.34 \times 10^{17}$	1.2	0	—	1.7	+ 0.2
(2483-2417)	{Control	1.2	—	—	1.5	—

* Methylene blue test.

Of the three bands employed, only the 2650 Å. band yielded sufficiently potent preparations for quantitative growth assay. At this wave-length, the number of quanta per growth unit of increased potency following irradiation was 1.36×10^{17} , which compares with 5.0×10^{17} reported above for full ultra-violet radiation in the range $\lambda < 2950$ Å. On an equal energy basis, the 2650 Å. band was markedly more effective than the 2850 or 2450 bands in bringing about (a) death of cells, (b) shrinkage of cell volume, (c) increase in growth potency of intercellular fluids, and (d) increased absorption of intercellular fluids at 2650 Å. The maximum lethal effectiveness of ultra-violet for yeast [Oster, 1934] and bacteria [Gates, 1930] lies near 2600 Å. For *B. coli*, Gates found maximum effectiveness at 2650 Å., a minimum at 2400 Å., and rapid decrease in effectiveness from 2650 Å. toward longer wave-lengths, the relative effect at 2850 being about the same as that at 2400 Å. Thus the preliminary results on effects of monochromatic radiation indicate a correlation between the lethal effectiveness of various wave bands and their ability to bring about the release of proliferation-promoting factors and nucleotide-like substances.

7. *Total yields from wound hormone extracts and autolysed residues.* In order to obtain more conclusive data as to whether or not synthesis of growth factors and nucleotide-like substances in the living, injured cells accompanies the loss of such substances to the intercellular fluids, growth-stimulating potencies and absorption spectra were determined on autolysates of the residues following irradiation and extraction as well as on the original extracts. 400 g., wet weight, of yeast were washed twice and suspended in distilled water to make a total volume of 4 l. The suspension was divided into two portions of 2 l. each, one of which was irradiated for 8 hr. with full ultra-violet, the other serving as a control. After irradiation, both suspensions were centrifuged, and the supernatants were decanted and filtered through Seitz E.K. filter pads. The manipulations up to this point followed precisely those previously described in detail [Loofbourow, Webb, Loofbourow & Abramowitz, 1942]. The residues from the centrifuged suspensions were set aside under

toluene and allowed to autolyse for 11 days. The autolysates were then autoclaved and centrifuged. The supernatants were decanted, filtered through Seitz E.K. filter pads, and made up to 400 ml. each with distilled water.

Absorption spectra and growth potencies were determined on the original extracts and on the filtrates from the autolysed residues. In the case of growth tests of the autolysates, difficulty was encountered in evaluating the potencies accurately because the relationship between 24 hr. growth and concentration was decidedly non-linear for both preparations. A further complication was introduced by the fact that at concentration levels higher than about 0.003 ml. of autlysate per ml. of culture, the autlysate from the irradiated residue was more potent than that from the non-irradiated, while at levels lower than the above value the autlysate from the non-irradiated residue was the more potent

Fig. 1. Growth test of residue autolysates from injured and uninjured cells.

(Fig. 1). This difference in behaviour of the two autolysates was established by repeated assays at concentrations ranging from 0.0045 to 0.1 ml./ml. of culture. It indicates that the two autolysates differed qualitatively (i.e. in composition) as well as quantitatively.

The autolysate of the injured-cell residue was more potent than that of the non-injured cell residue throughout approximately 97% of the concentration range tested. The autolysate potencies in Table 4 are averages of those for concentrations corresponding to increased 24 hr. yeast crops of 1.6 and 2.4 mg./ml. above the control crop. This is in the range in which the autolysate from uninjured cells showed the greatest relative potency, and yields more conservative values than would be obtained by computing the potencies from higher concentration levels.

The total growth potency of extract plus residue autolysate from injured cells, as shown in Table 4, was greater than that from uninjured cells by approximately 2 to 1. This strongly suggests that synthesis of growth-stimulating materials took place in the injured cells. The potencies of the two autolysates were very nearly the same, the difference in potencies of injured- and uninjured-cell preparations lying primarily in the original intercellular fluids. Evidently resynthesis of proliferation-promoting factors just about kept pace with loss of material to the intercellular fluids throughout the course of injury.

Table 4. *Growth potency and ultra-violet absorption of wound hormone extracts and residue autolysates*

Preparation	Growth units per ml.*	Volume V, in ml.	Total growth units*	$K = \frac{1}{d} \log_{10} \frac{I_0}{I_d}$ $\lambda = 2650 \text{ \AA.}$	KV
A. Extract from injured cells	84.7	1815	153,731	12.25	22,234
B. Autolysate of injured-cell residue	364	400	145,600	375	150,000
Totals A + B	—	—	299,331	—	172,234
C. Extract from uninjured cells	3.0	1815	5,445	2.1	3,812
D. Autolysate from uninjured-cell residue	378	400	151,200	115	46,000
Totals C + D	—	—	156,645	—	49,812

* Average values computed for two concentration levels, as explained in the text.

The spectra of all samples showed absorption curves characteristic of nucleotides, or their derivatives. The 2650 Å. extinction coefficient on a volume basis ($K = \frac{1}{d} \log_{10} \frac{I_0}{I_d}$; d in cm.) is shown for each sample. Since this is proportional to the concentration of material absorbing at 2650 Å., multiplication of K by the total volumes, V , of the preparations results in values proportional to the total yields of nucleotide-like substances present. The KV value for the autolysate from injured cells was about thrice that for the autolysate from uninjured cells, while the values for the original intercellular fluids had a ratio of approximately 6 to 1 in the same direction. Hence only about 15% of the nucleotide-like material appeared in the intercellular fluids from injured cells whereas slightly more than half the growth potency was found in the intercellular fluids. It appears that, while there was marked synthesis of nucleotide-like materials in the injured cells, the greater part of the synthesized material could not permeate the cell membranes, whereas synthesized growth-stimulating materials were released into the intercellular fluids with comparative ease.

It seems clear from the data that not all of the nucleotide material was involved in growth-stimulating activity. It is conceivable that a large portion of the inactive nucleotide-like material, which failed to pass through the cell membranes, was yeast nucleic acid itself. Previous experiments have shown it to be comparatively inactive [Loofbourow, Dwyer & Lane, 1940]. Furthermore, we have obtained evidence that the yeast nucleic acid content of yeast is increased during injury by ultra-violet radiation [Loofbourow, Englert & Dwyer, 1941; Loofbourow, Webb, Loofbourow & Lisco, 1942].

The greater nucleotide-like content of irradiated cell autolysates than of non-irradiated cell autolysates is evidence against the possibility that the effect of ultra-violet irradiation of the cells is simply to break down nucleic acid-like materials already present in the cell and thus to permit their diffusion outward through the cell membrane.

SUMMARY AND CONCLUSIONS

Washed suspensions of *S. cerevisiae* in distilled water were subjected to slow injury by ultra-violet radiation over periods of 8 or 10 hr. for the purpose of studying the mechanism of the release of proliferation-promoting factors by damaged cells into the intercellular fluids. The following observations resulted.

1. Inappreciable change in cell number throughout the irradiation periods indicated that the proliferation-promoting factors in the intercellular fluids were released through the membranes of grossly intact cells.

2. There was no evidence of cytolysis throughout the irradiation periods, substantiating the above conclusion.

3. Negative biuret and phosphotungstic acid tests on intercellular fluids from damaged cells indicated substantial freedom from protein, and supported the conclusion that cytolysis was negligible.

4. Preliminary studies with radioactive P have indicated an increase in membrane permeability during irradiation. This was confirmed by haematocrit determinations of cell volume, which showed an average decrease of 26% in injured-cell volume as against an increase of 8% in control suspensions. It is concluded that permeability changes play an important role in the release of proliferation-promoting factors by damaged cells.

5. It was computed from energy determinations that if the active proliferation promoter were a photochemically activated substance, it would be expected to have a potency of the order of 10^{-3} to 10 times that of pantothenic acid.

6. Preliminary studies with monochromatic wave bands centring at 2850, 2650 and 2450 Å. shows the 2650 Å. band to be the most effective in bringing about the release of proliferation-promoting factors, cell death, loss of cell volume, and increase in 2600 Å. absorption of the intercellular fluids.

7. After the removal of intercellular fluids from injured and uninjured cells, the residues were autolysed under toluene for 11 days, autoclaved, centrifuged, and the supernatants tested for growth activity and ultra-violet absorption. The total activity of original intercellular fluids plus residue autolysates was approximately twice as great for injured as for uninjured cells, whereas the activities of the autolysed residues alone were approximately the same. It is concluded that resynthesis of growth-stimulating materials occurred in the living, damaged cells at a rate comparable with the loss of such materials to the intercellular fluids. Similar evidence indicated the synthesis of substances having ultra-violet absorption characteristic of nucleotides, but at a rate greater than that of their diffusion into the suspension medium.

The above observations indicate that the mechanism of the release of proliferation-promoting factors by slowly-injured cells involves an increased membrane permeability arising as a result of injury, accompanied by a diffusion of various non-protein substances into the intercellular fluids and a resynthesis of certain of these materials within the living, injured cell as their concentration is disturbed by diffusion.

I wish to acknowledge the kind assistance of Mrs Rosaline K. Abramowitz in carrying out the growth tests, of Mr Roy Slaunwhite in preparing the monochromatically-irradiated materials, of Mr Robert Sinsheimer for assistance in the experiments with autolysates, and of Dr Dorothea G. Loofbourow for assistance in obtaining the absorption spectra of various preparations.

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