# 59. Proliferation-promoting Intercellular Hormones

## 3. Relation of Aeration to the Activity of Proliferation-promoting Factors from Injured Cells*

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(*Received 22 May 1942*)

Comparative assays, by the yeast growth method, of the activities of extracts from injured and uninjured cells showed little difference between the two when tested in stationary culture tubes, but a markedly greater activity of injured cell preparations when tested in cultures aerated by continuous agitation [Loofbourow, Webb & Abramowitz, 1942]. Further studies of this phenomenon are reported in the present paper in view of the important relationship which it must bear to the composition and properties of the proliferation-promoting factors (intercellular wound hormones) as well as to the mechanism of their physiological action.

### Experimental methods and results

The methods of preparation and assay of the materials were similar to those previously employed [Fardon, Carroll & Ruddy, 1937; Sperti, Loufbourow & Dwyer, 1937; Loofbourow, Cook & Stimson, 1938; Loofbourow, Cueto & Lane, 1939; Loofbourow, Dwyer & Lane, 1940] but since they involved certain modifications and improvements in procedure, they will be described in detail. In a typical preparation, 400 g. of moist commercial cake yeast (*S. cerevisiae*, Fleischmann baker's strain) were washed thrice in 400 ml. portions of distilled water, and then dispersed uniformly in distilled water. After removal of an aliquot containing 40 g. wet weight of yeast, the remainder (360 g. wet weight) was made up to 3900 ml. with distilled water. This was divided into two 1950 ml. portions, which were placed in cylindrical pyrex glass vessels. The depth of the suspension was 5·4 cm. in each instance, and the diameter of the vessel at the surface of the suspension was 22·7 cm. Both suspensions were stirred with motor-driven triangular glass paddles, arranged to give equal agitation to the two suspensions. One suspension was irradiated with unfiltered ultraviolet light from a Hanovia D.C. quartz mercury arc operated at 5 amp. with a potential difference of 65–75 V. across the arc. The other suspension served as a control. The perpendicular distance from the arc to the suspension surface was 25 cm., under which conditions approximately $2{\cdot}94 \times 10^7$ ergs per sec. of ultraviolet energy of wave-length shorter than 2900 Å. were incident on the suspension. The total irradiation period was 8 hr. At the end of this time, 98 % of the cells in the irradiated suspension were dead (methylene blue test [Richards, 1932]) as compared with 7 % in the control suspension. There was no appreciable change in cell number in either suspension as judged from haemacytometer counts made at intervals throughout the irradiation period. A stream of cooling water flowing through a glass coil maintained the irradiated suspension at a temperature 2–4° below that of the control suspension (18–20°). No metal was in contact with the suspensions at any time.

After irradiation, the suspensions were centrifuged and the supernatant fluids were decanted and filtered through Seitz EK filters. The filtrates were made up to equal

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volumes (1800 ml.) with distilled water, and divided into 150 ml. aliquots, some of which were autoclaved at 15 lb. for 20 min. in tightly capped bottles and the remainder set aside for use in the immediate assays.

For the assays, *S. cerevisiae*, Fleischmann baker's strain, obtained from 48-hr. slants on Sabouraud's medium was used. The yeast was washed off the slants with Reader's medium, filtered through Whatman No. 1 filter paper, and made up to a concentration of 0·64 mg. wet weight per ml. in Reader's medium. Addition of 1 ml. of this suspension to each culture tube resulted in a final concentration of 0·064 mg. wet weight of yeast per ml. of culture (approximately 250,000 cells per ml.), since the total volume of the cultures, including test materials, was in each instance 10 ml. The cultures were grown in Reader's medium [Reader, 1927] in $\frac{3}{4}$ in. pyrex tubes specially selected for optical uniformity so that they could be employed directly in a photoelectric densitometer. The densitometer readings were calibrated in terms of wet weight of yeast per ml. of suspension [Loofbourow & Dwyer, 1938]. It was thus possible to determine the course of growth periodically without removing material from the tubes or exposing the cultures to contamination. Aseptic technique was used throughout.

For tests of growth under non-aerated conditions, the tubes were suspended in a water bath at $30 \pm 0{\cdot}1°$. For growth conditions approximating the degree of aeration obtained in our earlier experiments in which rocker tubes were employed [Loofbourow, Dwyer & Morgan, 1938], use was made of a roller tube device in which the tubes were inserted in holes near the periphery of a wheel rotating on an axis inclined 12–15° to the horizontal. This angle resulted in a comparatively large air interface at the culture medium surface, but was insufficient to cause wetting of the cotton plugs of the tubes. Speeds of rotation of 40–82 r.p.m. resulted in closely similar growth kinetics. A speed of 62 r.p.m. was finally adopted as standard. The wheel was encased in an insulated cabinet, the interior of which was maintained at a temperature of $30 \pm 0{\cdot}5°$.

Our previous assays of wound hormones were based on the 24-hr. yeast crop, whereas in these experiments the progress of growth was followed at frequent intervals for periods as long as 82 hr. The studies of growth kinetics will be reported later; attention is confined in this paper to the effect of aeration.

### (1) *Assays under non-aerated conditions (stationary tubes)*

In the example shown in Fig. 1, the stationary tube assays showed equal activities for the products from injured and non-injured cells, within the limits of experimental error. This was not, however, uniformly the case in stationary tube experiments, as illustrated in Fig. 2. The growth activities of the same materials exhibited considerable variation, as illustrated in Fig. 2, '*c*' and '*d*', which are assays of the same samples (preparation 12. 31. 41, autoclaved). In view of the results obtained with aerated cultures, described below, it seems possible that such variation may be attributable, at least in part, to difference in degree of aeration. For each growth determination, the tubes were removed from the rack and shaken vigorously to suspend the organisms uniformly before being placed in the densitometer. There was some variation in both the frequency and extent of shaking in different experiments, and this may have affected the aeration appreciably.

Before the roller tube experiments were undertaken, it was repeatedly noted that cultures containing products from injured cells exhibited rings of yeast growth adhering to the tubes at the air-medium interface, while such growth rings were seldom observed in cultures containing non-injured cell products and were never observed in the controls. These observations, together with the consistent lack of appreciable 'wound hormone' activity in stationary tube assays as compared with the marked activities of similar preparations in the earlier rocker tube experiments indicated that aeration might be necessary in order for the 'wound hormone' activity to become evident. Furthermore, Fardon, Carroll & Ruddy [1937], Fardon & Ruddy [1937], and Norris & Ruddy [1937] had

found that extracts from ultraviolet-irradiated cells had marked respiratory-stimulating activity when tested on yeast. This suggested a possible relationship between materials in such extracts and the respiratory enzyme systems of the test organisms. Hence growth assays were undertaken by the roller tube technique of preparations which had exhibited little or no activity in stationary tube assays.

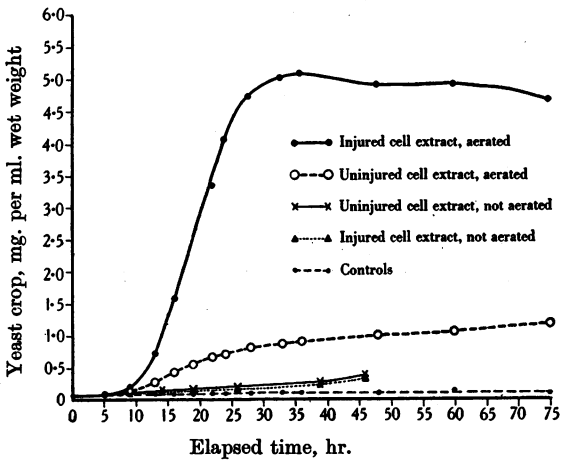


Fig. 1.

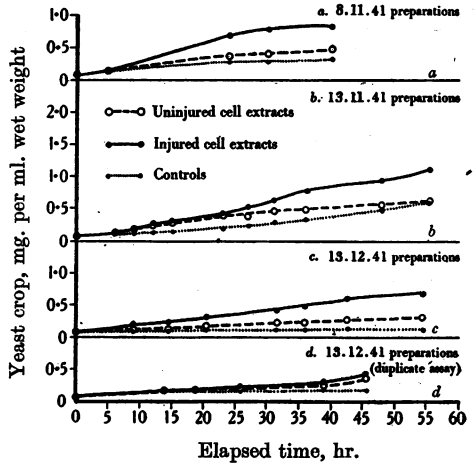


Fig. 2.

Fig. 1. Assays of the proliferation-promoting activities of extracts from injured and uninjured cells under aerated and non-aerated growth conditions. Concentrations employed: 0·1 ml. of extract per ml. of culture, preparations of 13.12.41.

Fig. 2. Representative assays of the proliferation-promoting activities of extracts from injured and uninjured cells under non-aerated growth conditions. Concentrations employed: 0·1 ml. of extract per ml. of culture.

### (2) *Assays under aerated conditions (roller tubes)*

It was to be expected that greater yeast growth would be obtained under conditions of better aeration, because of the well-known relation of aeration to the growth of yeast. The striking effect noted in the roller tube experiments was not simply increased growth, however, but markedly greater increase in growth of cultures containing injured cell products than of cultures containing uninjured cell products. Thus, in a typical experiment (Fig. 1), aeration by the roller tube technique resulted in increasing the 24-hr. yeast crop of cultures containing injured cell products by approximately 27 times, whereas in corresponding cultures containing uninjured cell products, the increase was but 3·5 times. Aeration, therefore, appears essential for the full 'wound hormone' activity to be observed with the test organism, medium and inoculum employed in these experiments.

Successive assays of the same materials by the roller tube method gave closely similar results with reference both to growth kinetics and the relationship of growth to concentration of stimulating cell extracts. This is illustrated in Fig. 3, in which is plotted the relation of the 24-hr. yeast crop to the concentration of added materials for two assays, and in Fig. 4, illustrating growth kinetics at various concentration levels of added materials, in which curves *A* and *B* represent duplicate assays at the 0·1 ml. level.

The relation of concentration to 24-hr. yeast crop was not linear throughout the entire concentration range for the injured cell products (Fig. 3). In previously reported experiments using rocker tube techniques [Loofbourow, Dwyer & Morgan, 1938; Loofbourow, Dwyer &. Lane, 1940], linear crop-concentration relationships were usually found for concentrations up to those corresponding to 24-hr. yeast crops of 4–5 mg. per ml.,

whereas in these experiments departure from linearity occurred at levels corresponding to 24-hr. crops of 2·5 mg. per ml. or less. It is possible that the factor limiting linearity of response at the higher concentration levels in these experiments was insufficient aeration in the roller tubes as compared with rocker tubes.

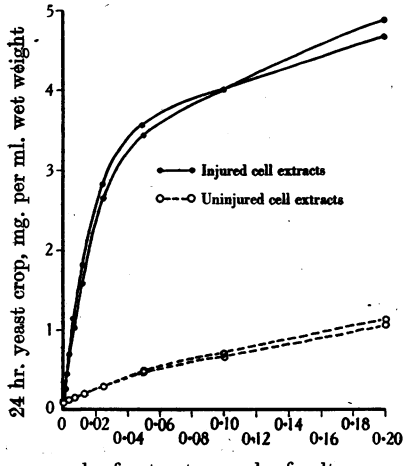


Fig. 3.

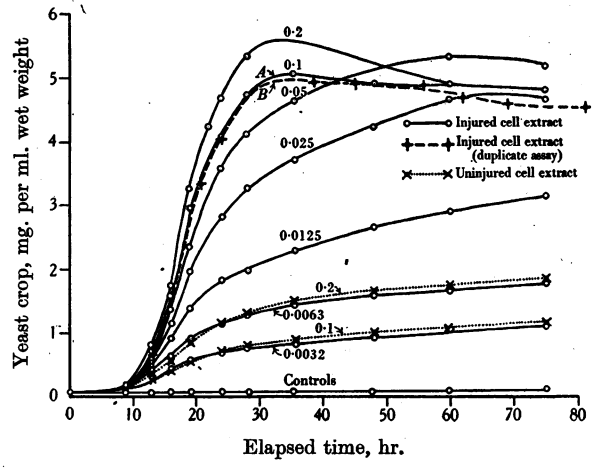


Fig. 4.

Fig. 3. Relation of 24 hr. growth to concentration of injured and uninjured cell extracts. Duplicate assays of 13.12.41 preparations under aerated growth conditions.

Fig. 4. Assays of injured and uninjured cell extracts under aerated conditions at various concentration levels. The numbers on the curves refer to ml. of added material per ml. of culture. Preparations of 13.12.41.

## Discussion

Davidson [1940] obtained increased growth in flask cultures containing irradiated yeast products as compared with cultures containing non-irradiated yeast products, without agitation of the cultures. Our results are not inconsistent with his, however, since (1) the air-medium interface in flask cultures is considerably greater in proportion to the amount of medium employed than in tubes such as those used in our non-aerated experiments, and (2) even in our stationary tube experiments, some wound hormone activity was usually observed (Fig. 2), though the difference in activities of injured cell and uninjured cell preparations was always of markedly lower order than that obtained under aerated conditions.

The evidence of 'wound hormone' activity in aerated cultures of injured cell products which failed to exhibit such activity under non-aerated growth conditions, together with the previously mentioned results of Fardon, Carroll & Ruddy [1937] and Fardon, Norris *et al.* [1937] indicating respiratory-stimulating activity of such materials suggest a relationship to respiratory enzymes of the active factors in preparations from ultraviolet-injured yeast. The ultraviolet absorption spectra of the injured cell products showed a maximum at 2600 Å., characteristic of purines and pyrimidines, with an extinction coefficient markedly higher than that in the spectra of uninjured cell products, in agreement with reports of similar preparations from both yeast and animal tissues [Loofbourow, Cook & Stimson, 1938; Cook, Loofbourow & Stimson, 1939]. The effect of *p*H on the spectra of the injured cell products resembled that on the spectrum of adenine [Loofbourow & Stimson, 1940] rather than that on the spectrum of uracil [Heyroth & Loofbourow, 1931; 1934], cytosine [Loofbourow & Stimson, unpublished), or guanine

[Holiday, 1936; Heyroth & Loofbourow, 1934]. These spectrographic data, together with previously reported chemical tests [Loofbourow, Cook & Stimson, 1938; Cook, Loofbourow & Stimson, 1939] indicating the presence of adenine, pentose and phosphorus in 'wound hormone' preparations, led us to investigate their possible content of diphospho- or triphospho-pyridine nucleotides. On reduction of the preparations with sodium hydrosulphite, the characteristic 3450 Å. band of the reduced coenzymes [Warburg, Christian & Griese, 1935; Warburg & Christian, 1936] failed to appear. Tests of injured cell preparation 13.12.41 for diphosphopyridine nucleotide by the method of Jandorf, Klemperer & Hastings [1941] indicated its content of this coenzyme to be less than 0·5$\mu$g. per ml., as compared with a hypothetical concentration of approximately 300$\mu$g. per ml. calculated from the absorption at 2600 Å. These data, together with previously reported negative chemical tests for pyridine, are presumptive evidence that the substance or substances accounting primarily for the 2600 Å. ultraviolet absorption of the wound hormone preparations are not coenzyme I or II.

The possible identity of the ultraviolet-absorbing moiety with adenosine triphosphate, adenosine diphosphate, muscle adenylic acid, yeast adenylic acid or adenosine is being investigated. A variety of evidence, which will be reported in detail subsequently, indicates that the ultraviolet-absorbing moiety does not account entirely for the proliferation-promoting activity of the preparations, but it appears, none the less, to contribute materially to such activity.

It should be emphasized that the requirement of aeration for full 'wound-hormone activity' in the experiments reported does not necessarily mean that this phenomenon would be observed under other conditions of culture medium, test organism, inoculum or source of wound-hormone material. Further investigations are being carried out to determine the effects of such variables.

## Summary

Growth tests of injured cell products (wound hormones) from yeast as compared with non-injured cell products showed activities of approximately 32 to 1 under aerated culture conditions, while assays of the same preparations in non-aerated cultures showed inappreciable differences in activity. The necessity of aeration for exhibition of 'wound hormone' activity, and the ultraviolet-absorbing characteristics of the preparations suggested the possibility that coenzyme I or II might account for the activity, at least in part. Spectrographic tests (hydrosulphite reduction) for coenzymes I and II, and biological tests for coenzyme I were negative. The possibility that other adenine complexes may account for the ultraviolet absorption and for an appreciable proportion of the activity is suggested.

We wish to acknowledge the kind assistance of our colleague Prof. Irwin Sizer in carrying out the hydrosulphite reductions and of Mr Bernhard Jandorf of the Department of Biological Chemistry, Harvard Medical School, in assaying our preparations for coenzyme I.

## REFERENCES

Cook, E. S., Loofbourow, J. R. & Stimson, M. M. [1939]. *Atti X° Congr. intern. Chim.* **5**, 26.
Davidson, J. N. [1940]. *Biochem. J.* **34**, 1537.
Fardon, J. C., Carroll, M. J. & Ruddy, M. V. [1937]. *Studies Inst. Divi Thomae*, **1**, 17.
Fardon, J. C., Norris, R. J., Loofbourow, J. R. & Ruddy, M. V. [1937]. *Nature, Lond.*, **139**, 589.
Fardon, J. C. & Ruddy, M. V. [1937]. *Studies Inst. Divi Thomae*, **1**, 41.
Heyroth, F. F. & Loofbourow, J. R. [1931]. *J. Amer. chem. Soc.* **53**, 3441.
—— —— [1934]. *J. Amer. chem. Soc.* **56**, 1728.
Holiday, E. R. [1936]. *Biochem. J.* **30**, 1795.
Jandorf, B. J., Klemperer, F. W. & Hastings, A. B. [1941]. *J. biol. Chem.* **138**, 311.

Loofbourow, J. R., Cook, E. S. & Stimson, M. M. [1938]. *Nature, Lond.*, **142**, 573.
Loofbourow, J. R., Cueto, A. A. & Lane, M. M. [1939]. *Arch. exp. Zellforsch.* **22**, 607.
Loofbourow, J. R. & Dwyer, C. M. [1938]. *Studies Inst. Divi Thomae*, **2**, 129.
Loofbourow, J. R., Dwyer, C. M. & Lane, M. M. [1940]. *Biochem. J.* **34**, 432.
Loofbourow, J. R., Dwyer, C. M. & Morgan, M. N. [1938]. *Studies Inst. Divi Thomae*, **2**, 137.
Loofbourow, J. R. & Stimson, M. M. [1940]. *J. chem. Soc.* p. 844.
Loofbourow, J. R., Webb, A. M. & Abramowitz, R. K. [1942]. *Nature, Lond.*, **149**, 272.
Norris, R. J. & Ruddy, M. V. [1937]. *Studies Inst. Divi Thomae*, **1**, 53.
Reader, V. [1927]. *Biochem. J.* **21**, 901.
Richards [1932]. *Arch. Protistenk.* **78**, 263.
Sperti, G. S., Loofbourow, J. R. & Dwyer, C. M. [1937]. *Studies Inst. Divi Thomae*, **1**, 163.
Warburg, O. & Christian, W. [1936]. *Biochem. Z.* **287**, 291.
Warburg, O., Christian, W. & Griese, A. [1935]. *Biochem. Z.* **282**, 157.