63. PROLIFERATION-PROMOTING INTER-CELLULAR HORMONES

2. EVIDENCE FOR THEIR PRODUCTION BY LIVING CELLS AS A RESPONSE TO INJURY

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Previously published investigations from these laboratories indicate that when yeast, various other micro-organisms, living tissues of rat, mouse and chicken embryos, and living adult newt tissues are subjected to such varied forms of injury as lethal ultraviolet radiation, X-rays, mechanical injury, chemical irritation and oxygen lack, there appear in the intercellular fluids factors stimulating cellular proliferation [Fardon *et al.* 1937; Norris & Ruddy, 1937; Sperti *et al.* 1937, 1, 2; Loofbourow *et al.* 1938; 1939; 1940]. We have called these factors 'proliferation-promoting intercellular hormones'.[2] Davidson [1940] has recently confirmed our results in the case of proliferation-promoters induced by the irradiation of yeast with lethal ultraviolet.

The possibilities that intercellular hormones from injured cells may act as the initial stimulus in wound healing, and that they may play a part in over-growth and neoplasia, are obvious reasons for interest in this problem. The conception of a relationship between the products of injured tissues and the repair processes following injury is not new. There is, in fact, an extensive literature regarding this question [for example, see Wiesmer, 1892; Carrel, 1930; Arey, 1936; Menkin, 1940; Loofbourow, 1941]. The techniques employed by our colleagues and ourselves [Sperti *et al.* 1937, 1, 2, 3; Loofbourow *et al.* 1938; 1939; 1940] make possible the controlled comparison of the proliferation-promoting effects of products from injured and uninjured tissues, and hence afford a new quantitative approach to the problem.

In investigating this problem, it seems of importance to distinguish between (*a*) factors released as a result of cell disintegration; (*b*) factors, already in the cell, released as a result of increased membrane permeability; and (*c*) factors synthesized by living, injured cells and released by them into the intercellular fluids. *A priori*, it is conceivable that the proliferation-stimulating activity found in the intercellular fluids of suspensions of injured cells might be attributable entirely to (*a*) or to (*b*) and (*a*). As will appear from the following, however, the experimental evidence seems to indicate that factors of the (*c*) type play a major part in the phenomenon.

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[2] The term 'intercellular hormone' was suggested by Loofbourow & Morgan [1938] for factors from cells which act through the intermediary of the intercellular fluids in affecting the metabolism of the cell community.

Experimental methods and results

In the following experiments, yeast (*S. cerevisiae*, Fleischmann Bakers' strain) was both the organism injured and the test organism for determining the proliferation-promoting activity of the intercellular fluids. Staining with methylene blue was used to estimate the number of cells killed [Richards, 1932] in the suspensions subjected to injury.

The technique of the growth tests has been described in detail elsewhere [Loofbourow, Dwyer & Morgan, 1938]. The potencies are evaluated in terms of growth units per mg. of dry material or per ml. of fluid, one growth unit being the amount of material (mg. or ml.) which must be added to 1 ml. of culture to cause an increased 24-hr. yeast crop of 1·6 mg./ml. (wet weight) as compared with the controls, under the standard conditions adopted for the growth tests. Since the control crop is normally about 0·16 mg./ml., one growth unit is also (approximately) the quantity of material which must be added to each ml. of culture medium to increase the 24-hr. crop by 1000 %.

1. *Effect of rapid killing compared with that of slow injury.* If proliferation-promoting factors arise in the intercellular fluids primarily as a result of mechanism *a* (disintegration of dead cells), one would expect yields to be nearly the same from the same number of dead cells, whether they were killed rapidly or slowly. If they arise as a result of *b* (increased permeability) or *c* (production of factors by living injured cells), greater yields might be expected from cells killed slowly.

To test this point, yeast was suspended in isotonic salt solution at a concentration of 20 g./l. Aliquots were irradiated with full ultraviolet from a quartz mercury arc for times of 20, 40, 80, 160 and 320 min. The intensities were adjusted (by varying the distances of the samples from the arc) so that approximately 90 % of the cells were dead in each aliquot at the end of its irradiation period. Corresponding control aliquots stood without irradiation for the same lengths of time. Immediately following each irradiation period, the irradiated suspension and the control suspension corresponding to it were filtered through Berkefeld filters, and the cell-free filtrates were taken to dryness. The residues of cells were discarded. For growth tests, the dried products were made up in distilled water to one-fifth their original volume, and assayed by the usual techniques (loc. cit.).

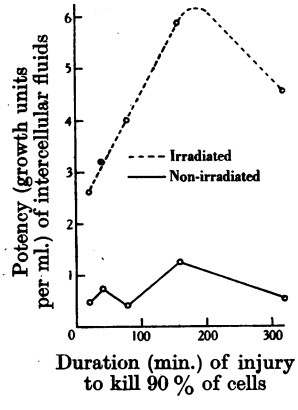

Fig. 1. Effect of decreased rate of killing on increased yield of proliferation-promoting factors.

The results of such an experiment are shown in Fig. 1. In the case of irradiated suspensions, the potency of the extract was approximately a linear function of the duration of injury up to 160 min., after which there was a marked decrease, the cause of which has not been established. Since the same numbers of cells were killed in all instances, these results indicate that the production of active factors was probably not primarily by mechanism *a* (disintegration of dead cells). In these particular experiments, the longer irradiation periods corresponded, also, to longer extraction periods. Hence either mechanism *b* or mechanism *c* could account for the results.

2. *Relation of yield to number of cells killed.* In a series of seven experiments, yeast suspended in isotonic salt solution was irradiated with full ultraviolet at

intensity levels which varied from experiment to experiment. Aliquots were removed at intervals for the determination of percentages of dead cells by methylene blue staining, and of the potencies of the intercellular fluids. Two examples, which are representative, are summarized in Fig. 2. The maximum staining referred to in each instance was between 97 and 100%.

It will be noted from these examples that from 15 to 20% of the maximum potency was attained by the time 2% of the cells were killed, and roughly one-third of the maximum potency by the time 10% of the cells were killed. This

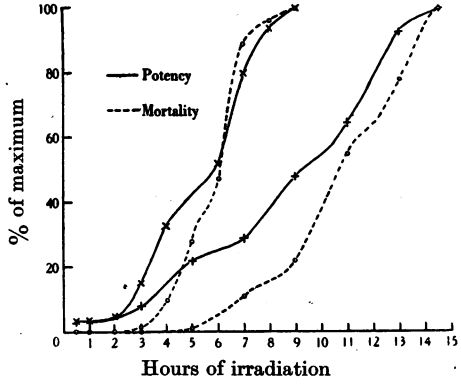

Fig. 2. Relation of yield of proliferation-promoting factors to mortality.

rather rapid rise in potency during the early periods of irradiation, before appreciable killing took place, seems unattributable to mechanism *a* and seems more easily explained by mechanism *b* or *c*.

3. *Effect of injuring cells in various suspension media.* Cells suspended in distilled water or isotonic salt solution have no source of building materials for the synthesis of proliferation-promoting factors other than their own protoplasm. Consequently, if mechanism *c* (production of active materials by living, injured cells) accounts in part for the phenomena observed, one might expect to obtain greater yields of active materials from cells suspended in media containing available sources of nitrogen, phosphorus, carbohydrate etc.

Yeast was suspended at a concentration of 100 g. (wet weight) per l. in the following media: distilled water, isotonic NaCl, Reader's medium[1] [Reader, 1927], 95% alcohol, and Ringer's glucose phosphate solution.[2] These suspensions were irradiated simultaneously in quartz test tubes with full ultraviolet at a distance of 15 cm. from a quartz mercury arc until 100% of the cells in the salt solution stained when tested with methylene blue. Under these conditions, practically complete killing was obtained in all suspensions. Similar suspensions were kept standing without irradiation during the same interval as controls. All suspensions were then filtered through Berkefeld filters, and the cell-free fluids were taken to dryness. The dried products were dissolved in distilled water to one-fifth their original volume, taking care to suspend all the material. These solutions were assayed in the usual way. Table 1 shows the results.

[1] The composition of Reader's medium per 100 ml. is as follows: $(NH_4)_2SO_4$, 0·3 g.; $MgSO_4$, 7HO, 0·07 g.; KH_2PO_4, 0·10 g.; K_2HPO_4, 0·016 g.; NaCl, 0·05 g.; $Ca(NO_3)_2$, 0·04 g.; glucose, 0·5 g.

[2] The composition per 100 ml. was as follows: NaCl, 0·09 g.; $CaCl_2$, 0·024 g.; KCl, 0·001 g.; $Na_2H_2P_2O_7$, $6H_2O$, 0·006 g.; $NaHPO_3$, $3H_2O$, 0·205 g.; glucose, 0·02 g.

It will be noted that the most highly potent irradiation product and the least potent non-irradiated extract were those from cells suspended in Reader's medium. In the case of alcohol, the combined lethal effects of alcohol and irradiation resulted in lower potency per ml. of filtrate than extraction with alcohol alone. Since alcohol is an effective solvent for extracting growth-promoters of the bios type [e.g. Narayanan, 1930], it is not surprising that the alcohol preparations showed high activity.

All cells were killed in the various irradiated suspensions. Hence it is difficult to attribute the observed results to mechanism *a* alone. If increased permeability accounted entirely for the difference in potency of the irradiated and non-irradiated extracts, why was the difference greatest in the case of Reader's medium? If, on the other hand, mechanism *c* played a part in the phenomenon, the increased effect in Reader's medium is understandable because of the carbohydrate, nitrogen, phosphorus etc. supplied by it to the cells.

Table 1. *Effect of the suspension medium on the yield of proliferation-promoting factors from injured cells*

Suspension medium	Potency G.U. per ml. Not irrad.	Irrad.
Distilled water	2·95	11·8
Isotonic NaCl	2·17	9·52
95% alcohol	13·3	10·9
Reader's medium	0·77	15·4
Ringer's G.P. solution	2·67	14·3

4. *Effects of CO_2 and O_2.* Because of the fermentation-stimulating effect of filtrates from irradiated as compared with non-irradiated cells [Cook & Loofbourow, unpublished], it was believed of interest to determine the effects of CO_2 and O_2 on the production of proliferation-promoting factors by yeast [Loofbourow & Dwyer, 1939]. In six experiments, yeast, at a concentration (wet weight) of 100 or 200 g./l., was suspended in isotonic NaCl. The suspensions were divided into three portions of 75 ml. each. Through one portion, CO_2 was bubbled continuously; through another portion O_2. The third portion had nothing bubbled through it. After 24 or 48 hr. of such treatment, the suspensions were filtered through Berkefeld filters, and the cell-free fluids were dried and assayed.

In these experiments, the cells were not killed. The maximum staining observed at the ends of the periods was of the order of 2%. The greatest potencies were in the filtrates from yeast exposed to CO_2, the next in the control filtrates, and the least in the filtrates from the cells exposed to O_2. Table 2 is from a

Table 2. *Effect of CO_2 on the production of proliferation-promoting factors by yeast*

Sample	Cell-free filtrates Potency G.U. per ml.
CO_2	6·6
Untreated	3·1
O_2	1·5

representative experiment. Since no appreciable killing took place in these experiments, mechanism *a* appears improbable, and mechanisms *b* and *c* more likely. The effect of CO_2 on membrane permeability is well known. Hence one cannot say from these experiments whether *b* or *c* or both were involved.

The possible effect of accumulated CO_2 in leading to production of proliferation-promoting factors must evidently be considered when investigating the action of various injuring agents in inducing the production of such factors.

5. *Comparison of yields from cells fragmented by grinding before and after irradiation with lethal ultraviolet.* The following experiments were suggested by Prof. A. J. Carlson of the University of Chicago. They are based on the consideration that if active factors are produced by living cells as a response to injury (mechanism *c*), the yield should be less from cells ground until they are fragmented and then irradiated dead than from cells irradiated while alive and then ground. In both instances the end-products would, theoretically, be the total water-soluble cell contents freed from the cell—hence no questions of membrane permeability or efficiency of extraction should be involved.

Two equal weights of moist yeast (10–30 g.) were taken. One of these was ground with sand in a mortar until the cells appeared to be well fragmented. Both portions were suspended in isotonic NaCl at a concentration of 100 g./l. and irradiated in quartz test tubes with full ultraviolet until 50–100 % (the percentage varied from experiment to experiment) of the cells in the unground suspension were killed. The suspensions were centrifuged, and the supernatant fluids decanted. The unground cells were then ground in a mortar, after which both residues were resuspended in their respective fluids. The suspensions were later filtered through Berkefeld filters, and the cell-free filtrates dried and assayed. The purposes of filtration were to remove insoluble residues which might interfere with the optical estimation of yeast growth and to render the solutions sterile.

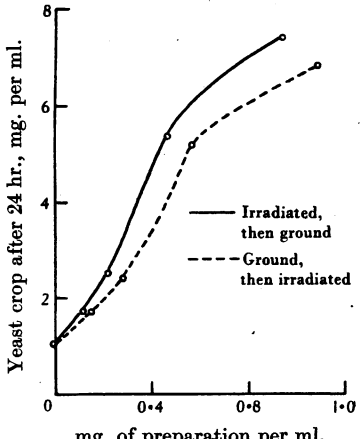

Fig. 3. Effect of killing cells by grinding before irradiation.

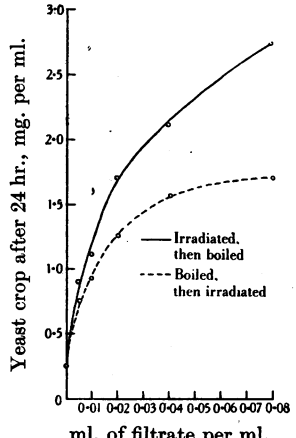

Fig. 4. Effect of killing cells by boiling before irradiation.

The products from cells irradiated while alive were slightly more active, both on a weight basis and equivalent filtrate volume basis, but the differences were small. Fig. 3 is a representative assay. The weight yields averaged from 20 to 30 mg./ml. in various experiments (compared with 2–5 mg./ml. in techniques in which unfragmented cells were used). Considerable difficulty was encountered in fragmenting all the cells by the method used.

The higher yields in the case of cells irradiated while alive seem to indicate that mechanism *c* was involved in the production of active factors.

6. *Comparison of yields from cells killed by boiling before irradiation and boiled after irradiation.* As an alternative to the above procedure, yields from cells killed by boiling were compared with yields from cells irradiated while alive. Yeast was suspended in isotonic NaCl, at 100 g./l. and divided into two aliquots. One of these was boiled for 3 min. to kill the cells. Both were then irradiated in quartz test tubes with full ultraviolet until all cells in the unboiled suspension were killed (methylene blue test). The irradiation time in the experiment illustrated in Fig. 4 was 4·25 hr. The distance from the quartz mercury arc was 15 cm. Following irradiation, the unboiled suspension was boiled for 3 min. Both suspensions were filtered through Berkefeld filters, and their filtrates were dried and assayed.

The suspensions irradiated before boiling gave more potent filtrates. This may be interpreted as indicating that proliferation-promoting factors are produced in living cells during injury (mechanism *c*) or that previous boiling interferes with extraction and results in lower yields from suspensions boiled before irradiation. The later interpretation does not seem to explain the phenomenon completely, however, because the filtrates from the suspensions irradiated while alive were more potent per unit weight as well as per unit volume.

Discussion

The question as to whether the increased proliferation-promoting effect of intercellular fluids from injured cells may be attributed in part to factors produced by living cells as a specific response to injury is an extremely difficult one to answer with finality. Certainly, there exist in yeast cells many substances stimulating yeast growth, such as amino-acids, pantothenic acid, vitamin B_1, vitamin B_6, biotin etc., as has already been pointed out repeatedly in connexion with this problem [Cook *et al.* 1940; Davidson, 1940; Loofbourow & Morgan, 1940]. When cells are injured or killed, the release into the intercellular fluids of such substances present in the uninjured cell might be expected to complicate any attempt to determine the presence of factors specifically produced as a result of injury. Nevertheless, the greater potencies of preparations from cells injured slowly rather than killed rapidly, the rapid increase in such factors in the intercellular fluids before appreciable killing, the appearance of such factors in suspensions of living cells subjected to CO_2, and finally, the greater yields from cells fragmented or killed by boiling after rather than before prolonged injury all suggest that living, injured cells do produce proliferation-promoting factors (intercellular wound hormones) while undergoing injury.

Various evidence that proliferation-promoting intercellular hormones from injured cells are related to nucleic acids has been presented [Loofbourow, Cook & Stimson, 1938; Cook *et al.* 1939]. The results of Davidson [1940] in which assays of extracts from irradiated cells showed markedly higher nucleoside- and free purine-nitrogen than extracts from non-irradiated or autoclaved cells suggest this same conclusion. It is difficult to explain Davidson's results as entirely due to increased membrane permeability following irradiation or to the release of dead cell disintegration products as a result of irradiation, for the nucleoside- and purine-nitrogen was three times as great in his filtrates from irradiated as in his filtrates from autoclaved cells, whereas the dry weights of the materials obtained were roughly 3 to 2 in favour of the autoclaved cell extracts. These results seem to us an argument for the production of nucleic-acid-like materials as a specific response to injury.

The suggestion is frequently made that our intercellular wound hormones may be synthesized by a specific photochemical process in the cell. The fact that

the active materials are obtained from cells injured in other ways and the indications that they are produced in living cells rather than by an effect of ultraviolet on the dead constituents of protoplasm are in our opinion arguments against this possibility.

SUMMARY

In order to determine whether the increase in the proliferation-promoting effect of intercellular fluids following cell injury is due to (*a*) release of dead cell disintegration products, (*b*) increased permeability of the cell membrane or (*c*) formation of proliferation-promoting intercellular hormones by cells as a specific response to injury, the following types of experiments were tried.

(1) A comparison was made of the effects of rapid killing and of slow injury. The yield of proliferation-promoting intercellular hormones was, within limits, closely proportional to the time required to kill the cells.

(2) The yield of active factors was compared with the number of cells killed and found to increase rapidly before appreciable killing took place.

(3) The yields from cells injured in various media were compared and found to be highest when the medium contained carbohydrate, nitrogenous substances, phosphoric acid etc.

(4) CO_2 was found to increase the yield of active factors in the intercellular fluids without killing the cells.

(5) Cells exposed to lethal ultraviolet before grinding yielded more potent preparations than cells irradiated after grinding.

(6) Cells irradiated with lethal ultraviolet after they were killed by boiling yielded less potent preparations than those irradiated before boiling.

The results suggest that in addition to possible factors arising in the intercellular fluids as a result of mechanisms *a* and *b*, at least part of the potency must be attributable to factors arising from mechanism *c*.

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