54. PROLIFERATION-PROMOTING INTERCELLULAR HORMONES

I. QUANTITATIVE STUDIES OF FACTORS PRODUCED BY INJURED ANIMAL TISSUE CELLS

By JOHN R. LOOFBOUROW, SISTER CECELIA MARIE DWYER, S.C., and SISTER MARY MICHAELLA LANE, S.C.

From the Research Laboratories of Institutum Divi Thomae, Cincinnati, Ohio, and the Research Laboratory of Good Samaritan Hospital, Dayton, Ohio

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In investigating factors from cells which influence cellular proliferation [Fardon *et al.* 1937; Sperti, Loofbourow & Dwyer, 1937, 1, 2], respiration [Norris & Kreke, 1937; Cook *et al.* 1938], and glycolysis [Schroeder & Cook, 1939] our studies of proliferation-promoting factors from injured animal tissue cells have been carried out with tissue cultures as a means of assaying the products obtained [Sperti, Loofbourow & Lane, 1937; Loofbourow, Cueto & Lane, 1939]. In the case of similar proliferation-promoting factors from injured yeast cells, the assays on yeast have afforded much more accurate quantitative data [Loofbourow, Dwyer & Morgan, 1938]. Since it is desirable to obtain a comparable degree of accuracy in the assay of animal-cell products in order to answer various questions regarding the physiological production and chemical nature of the factors, we endeavoured to see if the animal-cell products could be assayed on yeast. The yeast assay technique having proved to be practicable, we wish to present here the results of quantitative studies by this method of the products from injured animal tissue cells.

The name "intercellular hormones" [Loofbourow & Morgan, 1938] has been suggested for factors controlling cellular metabolism which are released by cells and act from cell to cell through the intermediary of the intercellular fluids. Thus the respiratory, glycolytic and proliferation-controlling factors investigated in our laboratories, as well as Törö's [1937, etc.] corhormone, Wildiers' [1901] bios, Williams' [1933, etc.] pantothenic acid, Bonner's [1937, etc.] traumatin, the auxins and many other such substances are probably all "intercellular hormones" in this sense. The particular proliferation-promoting intercellular hormone discussed in what follows appears to be produced especially by injured cells, and is thus a "wound hormone" in the sense used by Wiesner [1892], Haberlandt [1921], Reiche [1924] and others for growth factors obtained from injured plant tissues. For this reason, we have at times called it an "intercellular wound hormone", but it should be pointed out that the same proliferation-promoting factor appears to occur in the intercellular fluids from uninjured cells, only to a less extent, and the relation to injury is therefore evidently a quantitative rather than a qualitative one.

Experimental

As a means of injuring the tissue cells, lethal ultraviolet from a 500 w. Burdick quartz mercury arc, operated at 65 v. across the arc, was used in all of the following experiments. The irradiation distance was uniformly 25 cm.

Irradiation was carried out in quartz test tubes, with constant stirring. The control and irradiated tissues were always kept at the same temperature. Lethal ultraviolet is a convenient means of producing cellular injury because its effects can easily be controlled.

Three sources of tissue were used: adult newts, 7-day chick embryos and 14-day rat embryos.

In the newt experiments, whole newts were washed and minced in Ringer's solution. The suspensions of minced tissues were divided into two portions, one of which was irradiated for 1–1·25 hr. Following irradiation, both suspensions were centrifuged, and the supernatant fluids were decanted and taken to dryness in an oven.

The chick embryos were removed under sterile conditions, and minced in Drew's solution. Irradiation, centrifuging and drying followed the technique described above.

The rat embryos were removed under sterile conditions from 1-year-old pregnant females. They were minced in Drew's solution or 0·89 % NaCl and washed three or more times in changes of the solution to remove growth factors arising from mechanical injury [Fischer, 1930; Fardon *et al.* 1938; 1939; Loofbourow, Cueto & Lane, 1939]. The tissues were divided into two approximately equal portions, each of which was suspended in 10 ml. of the solution. Irradiation, centrifuging and drying were carried out as above. Special precautions were taken in these experiments to keep the tissues at or near 37° throughout all manipulations.

The assays of the animal tissue products on yeast were made by adding serial dilutions of the preparations to cultures of *S. cerevisiae* grown in Reader's medium [Reader, 1927] in rocker tubes [Norris & Kreke, 1937] for 24 hr. at 30°. The concentration of yeast used for seeding and the crop after 24 hr. were determined photoelectrically in terms of the concentration of yeast protoplasm in mg. per ml. [Loofbourow & Dwyer, 1938, 2]. Haemacytometer counts showed that the increase in protoplasm measured in these experiments is accounted for by increase in cell number rather than by increase in cell size. Consequently, such products as were found to be growth-promoting in the assays here reported were proliferation-promoting factors.

Each tube contained 24 ml. of Reader's medium and 1 ml. of added material (0·89 % NaCl in the case of controls). The yeast used for seeding was washed with Reader's medium from 48 hr. slants on Saboraud's medium and added to the rocker tubes in such dilution as to seed them to a concentration of 0·064 mg. per ml.

When the yield of yeast after 24 hr. was plotted against the concentration of test material added, a linear relationship usually resulted within the range of values used (Fig. 1) as in the case of yeast products [Loofbourow, Dwyer & Morgan, 1938], though for some preparations the relationship was non-linear (Fig. 2), as in the case of certain preparations we have obtained from bacteria [Loofbourow & Morgan, 1939].

As a standard unit of growth, a crop of 1·6 mg. of yeast per ml. greater than the control crop was used, as in previous work [Loofbourow, Dwyer & Morgan, 1938]. The quantity (mg.) of tested material in each ml. of rocker tube contents required to give such a crop was determined from the curves. Thus there was determined the number of mg. of tested material per "growth unit" (the reciprocal of which gave the number of growth units per mg.) in terms of a standard, reproducible biological effect.

In comparing the potencies of materials which give different shapes of growth-concentration curves, it is necessary to beware of false interpretations because

of the fact that the apparent potency ratios of such materials are not the same at different concentration levels. We have not found, as yet, a simple method of avoiding this difficulty, and hence have limited ourselves to pointing out the salient facts about those particular instances in which there was a marked departure from a linear relationship in the concentration ranges employed.

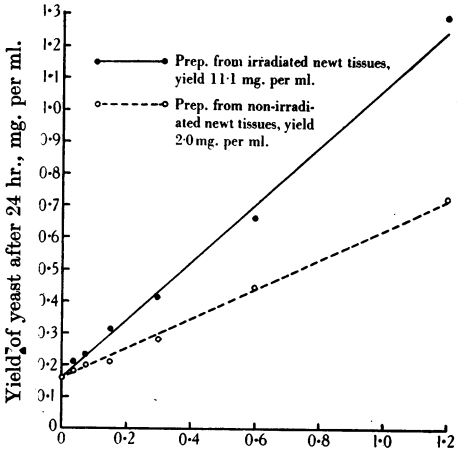

Fig. 1.

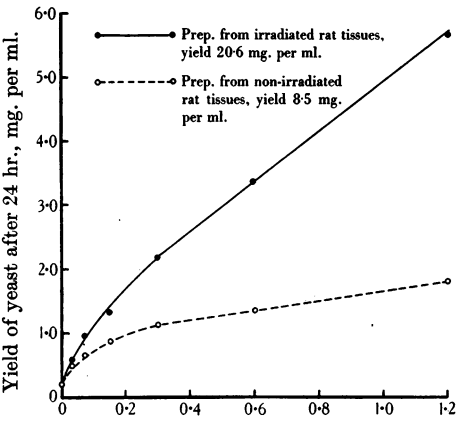

Fig. 2.

Fig. 1. Typical assay of intercellular wound hormone from irradiated newt tissues. It should be noted that not only was the potency of equal weight of material from the irradiated tissues approximately twice that from non-irradiated tissues, but the yield of material from the irradiated tissues was also about 5·5 times as great. Thus, equal volumes of the two filtrates showed growth potency ratios of about 11 to 1.

Fig. 2. Assay of intercellular wound hormone from irradiated rat tissues selected to illustrate the non-linear growth concentration relationship obtained with some preparations. Note increased yield of material from irradiated tissues as well as increased potency per unit weight.

Table 1 summarizes several typical assays of preparations from newts, chick embryos, rat embryos and yeast, selected to illustrate various points.

In all the animal preparations tested, the intercellular fluids from irradiated tissues showed greater growth potencies than those from corresponding non-irradiated tissues. If the ratio of potencies of test and control filtrates (Table 1, column H) of animal tissues is compared with that of the representative yeast preparation listed, it is seen, however, that the ratio for the yeast preparation is roughly nine times as great as the highest ratio for the animal preparations. Reference to columns D, E and F indicates that this is due to the greater potencies and yields of control filtrates in the case of the animal tissue products. We believe this to be explained by the greater production of proliferation-promoting factors in the control animal tissues by mechanical injury, and the greater release in these tissues of dead-cell disintegration products.

The results demonstrate the necessity of the utmost care in washing animal tissues to free them from such growth factors when they are to be used in tissue cultures for growth tests or in the study of the effects of various agents in causing them to produce growth factors [compare Fardon & Sullivan, 1938; Fischer, 1930].

Autoclaving for 20 min. at 15 lb. did not destroy the active factor, as shown by preparation No. 3·7 from newts. Filtration through Berkefeld "N" filters

Table 1. *Results of typical assays of intercellular wound hormone preparations from animal tissues*

A	B	C	D	E	F	G	H	I
Preparation	Irradiation time	Concentration of tissue	Yield mg. per ml.	Growth units per ml.	Growth units per ml.	Growth units per g. tissue	Ratio irrad- to non- irrad. g.u. per ml.	Extinction at 2600 Å.
3·25 from newts	1 hr. 15 min.	—	11·0 n.	0·57 n.	6·3	—	10·5	—
	Non-irradiated	—	2·0 n.	0·29 n.	0·6	—		—
3·7 from newts, autoclaved 20 min. at 15 lb.	1 hr.	—	—	—	6·0	—	—	—
3·29 from chick embryos, unwashed	25 min.	—	—	—	27·2	—	1·3	—
	Non-irradiated	—	—	—	21·8	—		—
11·11 from rat embryos	2 hr. 55 min.	2·24 g. to 1 ml. Drew	41·3	8·9	367·5	164	2·1	2·92
	Non-irradiated	2·66 g. to 1 ml. Drew	29·8	5·8	172·7	65		1·78
11·161 from rat embryos	3 hr.	0·93 g. to 1 ml. saline	25·8	9·6	250·3	272	4·9	—
	Non-irradiated	1·33 g. to 1 ml. saline	13·5	3·8	51·2	39		—
11·162 from rat embryos (11·161 after Berkefeld filtration)	See preparation 11·161		23·5 10·3	9·5 3·8	223·4 39·2	240 29·5	5·7	3·1 1·29
11·252 from rat embryos, Berkefeld filtrate	4 hr.	1·72 g. to 1 ml. saline	18·0	6·9	124·2	72	8·7	2·50
	Non-irradiated	1·89 g. to 1 ml. saline	6·2	2·3	14·3	7·6		0·89
11·2 from rat embryos, Berkefeld filtrate	61 min.	1·77 g. to 1 ml. Drew	20·4	5·8	118·4	67	2·7	1·84
	Non-irradiated	1·84 g. to 1 ml. Drew	23·0	1·9	43·6	23·7		1·16
4·26 from yeast, Berkefeld filtrate	7 hr.	0·1 g. per ml. saline	14·3 n.	5·0 n.	71·5	715	87·2	—
	Non-irradiated		0·59 n.	1·39 n.	0·82	8·2		—

Note. The weights used in calculating yields and potencies were the total weights of the dried filtrates, including the salts, etc., from the solutions in which the cells were suspended, except in those instances marked "n." (for "net"), in which allowance was made for the weight of such materials.

did not remove the factor, as illustrated by comparison of rat preparations No. 11·161 and No. 11·162. These are typical of results obtained repeatedly. If we may presume that the factor active on animal tissues is identical with that active on yeast, we must conclude that our preliminary indications [Loofbourow, Cueto & Lane, 1939] that Berkefeld filtration removes the active factor were in error.

The ultraviolet absorption spectra of the preparations had a maximum near 2600 Å. (Fig. 3) characteristic of purines and pyrimidines of the nucleic acid complex [Heyroth & Loofbourow, 1931; 1934], as in the case of preparations from yeast. On the basis of spectrographic and chemical studies of the yeast preparations [Cook, Loofbourow & Stimson, 1938; Loofbourow, Cook & Stimson, 1938], it has been suggested that the active yeast product contains adenine, guanine, pentose and phosphorus, and is free from pyridine, protein and sulphur. While the spectrographic curves of the tissue products are broader than those of the yeast products, their common maximum near 2600 Å. and the rather close correlation between the extinctions of the animal products at this wave-

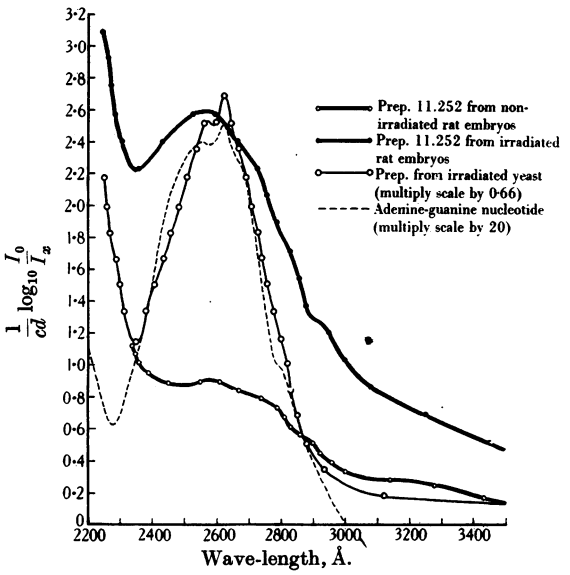

Fig. 3.

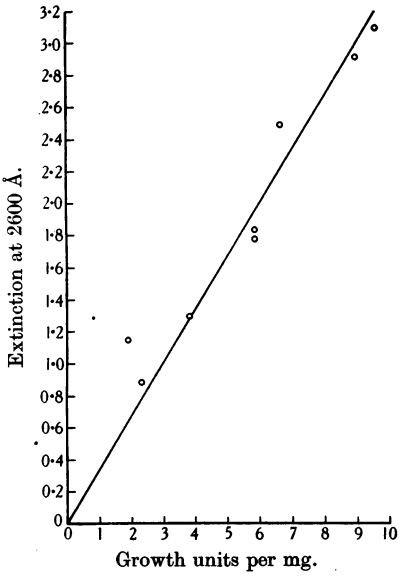

Fig. 4.

Fig. 3. Ultraviolet absorption spectra of typical preparations from rat tissues and yeast. The spectra of the yeast preparation and of the hypothetical guanine-nucleotide-adenine-nucleotide complex are plotted to different extinction scales to facilitate comparison.

Fig. 4. Correlation of potency and ultraviolet absorption at 2600 Å. in various preparations.

Table 2. *Tests of the growth-promoting activities of amino-acids, nucleic acids etc. on yeast*

Material	*p*H adjusted to	Concentration range tested mg. per ml.	Growth units per mg.
Intercellular wound hormone prep. 11·16 from rat embryos	—	0·038–1·2	9·6
Arginine	7·3	0·038–1·2	0·06
Cystine	7·25	0·0038–0·12	Inactive
d-Alanine	7·4	0·096–0·31	Slight activity in low concentrations (see Fig. 5)
l-Alanine	7·2	0·038–1·2	Inactive
dl-Alanine	7·3	0·037–1·18	? Slight activity
β-Alanine	7·4	0·041–1·30	Inactive
Tryptophan	7·3	0·037–1·19	Inactive
Tyrosine	7·4	0·045–1·45	Inactive
d-Glutamic acid	7·3	0·027–0·86	Inactive
Histidine	7·22	0·034–1·1	Inactive
dl-Phenylalanine	7·2	0·019–0·06	Inactive
Glycine	7·4	0·045–1·45	Inactive
l-Leucine	7·36	0·0092–0·29	Inactive
l-Aspartic acid	7·39	0·028–0·88	Slight activity in low concentrations (see Fig. 5)
l-Proline	7·39	0·025–0·8	Inactive
Inositol	7·2	0·033–1·08	Inactive
Yeast nucleic acid	7·25	0·036–1·16	0·02
Thymus nucleic acid	7·39	0·038–1·2	Inactive
Adenosine	—	0·038–1·2	0·03
Aneurin	7·3	0·038–1·2	Inactive
Riboflavin	—	0·038–1·2	Inactive
Nicotinic acid	7·25	0·038–1·2	Inactive
Insulin (8 units per mg.)	—	0·063–0·2	Inactive

length and their potencies (Fig. 4) suggests that the active factors are similar in the yeast and animal preparations.

Since it was possible that the difference in the ultraviolet absorptions of the animal and yeast products might be due to the presence in the former of greater quantities of amino-acid residues from proteins, we undertook a study of the activity of various amino-acids in yeast growth experiments, and at the same time tested certain other materials that might logically be suspected as contributing to the activity. The results are shown in Table 2.

The sensitivity of the method was sufficient to show a growth activity of about 0·01 growth unit per mg. All of the amino-acids tested had activities less than this, except arginine, *l*-aspartic acid and *d*-alanine. The two latter showed some growth activity in small concentrations, which rapidly fell off as the concentration increased. In neither of these could the activity be determined by extrapolation because of the non-linear characteristics of the growth-concentration relationships (Fig. 5). Comparison of their growth curves with those

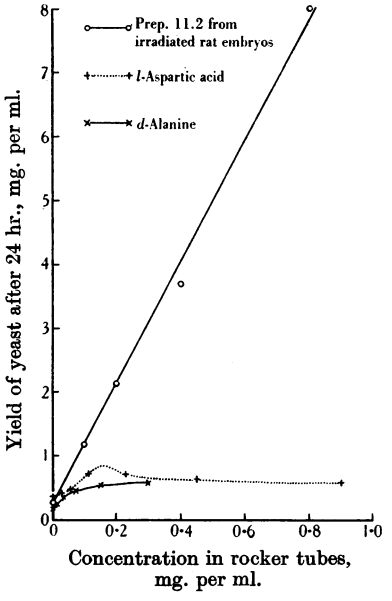

Fig. 5.

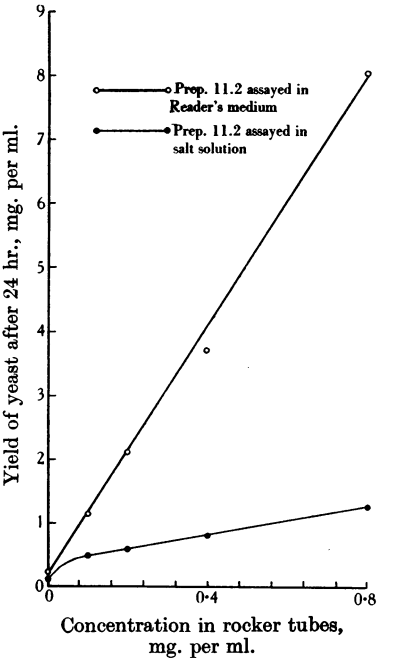

Fig. 6.

Fig. 5. Assays of *l*-aspartic acid and *d*-alanine compared with an assay of preparation 11·2 from rat tissues.

Fig. 6. Assays of rat tissue preparation 11·2 in Reader's medium and in salt solution.

of the animal preparation included on the figure show that they had insufficient effect to account for the activities of the animal preparations. The shapes of their growth-concentration curves suggest that such materials may account for the non-linear growth-concentration relationships at low concentrations in some of the wound hormone assays illustrated by the example shown in Fig. 2.

Arginine had an activity of 0·06 g.u. per mg.; *l*-alanine was inactive; *dl*-alanine showed a slight indication of activity, apparently attributable to

the *d*-alanine component. The only other materials with measurable activity were yeast nucleic acid (0·02 g.u. per mg.) and adenosine (0·03 g.u. per mg.). These values compare with 9·6 g.u. per mg. for the most potent rat preparation and 5·0 g.u. per mg. for the yeast preparation of Table 1.

It is evident that none of the materials tested can account for the activity of the proliferation-promoting intercellular hormone as determined in our growth experiments. This, of course, does not preclude the possibility of such substances acting as active growth factors for other strains of yeast, or for the same strain under other growth conditions, but that is a separate problem with which we were not concerned, our one purpose being to determine if the substances in question could account for the measured activity of the intercellular wound hormone preparations.

The question of whether the active material in the rat preparations acts directly as a nutrient or is effective in increasing the utilization of carbohydrate by yeast was investigated by comparing the growth of yeast in salt solution to which a rat preparation was added with its growth in Reader's medium (of which the nutrient component is dextrose) containing the same factor (Fig. 6). The 24 hr. yield of yeast in the controls was twice as great in Reader's medium as in salt, whereas in the rocker tubes containing 0·8 mg. per ml. of rat preparation No. 11·2, the yield was approximately seven times as great in Reader's medium as in salt. Thus, while there are apparently present in the rat preparations materials which support growth directly, the major part of the growth-promoting effect is evidently due to stimulation of carbohydrate metabolism.

Discussion

It should be emphasized that we do not regard ultraviolet irradiation as having any special significance in the production of the proliferation-promoting intercellular hormone other than that of a cell-injury agent. We have had comparable success in obtaining the factor by injuring cells mechanically [Loofbourow, Cook, Dwyer & Hart, 1939], chemically [Loofbourow & Dwyer, 1938, 1; 1939], by heat (unpublished investigations), by X-rays [Fardon *et al.* 1937], and by subjecting cells to anaerobiosis [Loofbourow & Dwyer, 1939]. Harker [1938] has shown that radium radiation leads to the release by yeast cells into the intercellular fluids of substances accelerating sucrose inversion. There is thus, so far as we know, no special effectiveness of ultraviolet radiation in causing cells to produce intercellular hormones. Ultraviolet irradiation of the cell-free filtrates from non-irradiated cells leads to no increase in proliferation-promoting activity.

The increased yield of the proliferation-promoting factor in the intercellular fluids when cells are injured is apparently due to the production of the factors by living cells as a physiological response to such conditions, and not due to dead-cell disintegration products [Loofbourow, Dwyer & Morgan, 1938; unpublished investigations of Loofbourow & Dwyer]. If the production of proliferation-promoting intercellular hormones by living cells is, as we believe, increased quite generally when cells are injured, these proliferation-promoting factors must be taken into account in considering such physiological processes as wound healing, overgrowth and neoplasia. The possible relationship of such substances to neoplasia has already been considered in a preliminary way [Sperti, Loofbourow & Dwyer, 1937, 2]. We have recently reported investigations in which the injection of the preparations from rat-embryo tissues described in this paper led to the production of local overgrowths, or tumours [Loofbourow, Cueto, Whalen & Lane, 1939].

In earlier publications [Loofbourow, Cook & Stimson, 1938; Cook, Loofbourow & Stimson, 1938] we pointed out that the shapes of the absorption curves of the active preparations are such as to suggest the presence of guanine as well as adenine. Recently Claude [1939], in an interesting study of the active factor of chicken tumour I, has found it to contain a lipin portion and a nucleoprotein portion, the latter being characterized by an absorption spectrum similar to the spectra of our preparations. From the position of the maximum of his spectrum near 2575 Å., and other evidence, he deduced that his nucleoprotein fraction contains guanine as a predominant part of the molecule. This, together with the fact that he has recently obtained similar fractions from chick and rat embryo tissues, and the fact that repeated injection of our proliferation-promoting intercellular hormone apparently leads to the production of tumours in rats, strongly suggests a similarity of the nucleic acid-like component of his preparations to our intercellular wound hormone.

In collaboration with Prof. E. S. Cook and his associates we have obtained preliminary evidence that our intercellular wound hormone stimulates yeast fermentation. That it is apparently produced in larger quantities under conditions of oxygen lack has already been mentioned. Schroeder & Cook [1939] have found that respiration-stimulating intercellular hormones obtained by them from normal cells and tissues lead to stimulation of the respiration and depression of the anaerobic and aerobic glycolysis of certain tumours *in vitro*, as well as of normal tissues. Davis [1937] has produced tumours in rats by ligating the arteries supplying one horn of the uterus, thus bringing about local oxygen want. Orr [1935] has shown that oxygen lack produced in the epithelium by the ligation of arteries or the injection of adrenaline leads to the more rapid production of tumours by dibenzanthracene applied to the affected areas. Cook *et al.* [1939] have demonstrated that dibenzanthracene depresses the respiration of yeast. In viewing these diverse findings in relation to each other, it seems possible that the metabolism of tissues generally may be controlled in large part by a suitable balance of intercellular hormones affecting proliferation, respiration and glycolysis, and that prolonged injury to appreciable volumes of cells, or prolonged oxygen lack, may lead, through the mechanism of an unbalanced production of such factors, to the abnormal proliferation and glycolysis characteristic of many types of tumour tissue.

The evidence thus far accumulated with regard to the chemical nature of our proliferation-promoting intercellular hormone points toward its being a guanine-adenine-nucleotide complex. On this assumption, a combined curve of the hypothetical absorption of guanine-nucleotide and adenine-nucleotide has been plotted for comparison of its shape (the magnitudes of absorption at different wave-lengths cannot of course be compared because the degree of purity of the intercellular hormone preparations is not known) with the shapes of the curves of the yeast and rat preparations (Fig. 6), using unpublished data of Stimson & Loofbourow for the absorption of adenine and guanine at the *p*H characteristic of the intercellular hormone solutions. The resemblance to the yeast preparation is close. Absorbing impurities may possibly account for the high minimum (2375 Å.) and high absorption in the long-wave region by the rat preparation and for the position of the minimum in the yeast preparation at about 2375 Å. The absorption of amino-acids might account for some of these effects. Both tyrosine and tryptophan have minima near 2400 Å. in acid solution [Holiday, 1936]. Other amino-acids with "end absorption" would produce the same effect of shifting the minimum toward longer wave-lengths. It is interesting, however, that, although chemical tests have thus far not indicated its presence

in our intercellular wound hormone preparations, nicotinic acid amide has a minimum at about 2400 Å., and the possibility that it may occur in our preparations cannot be excluded entirely until more highly purified samples have been shown to be free from it.

The extinction at 2600 Å. of the guanine-nucleotide adenine-nucleotide complex is roughly twenty times that of rat preparation No. 11·252. Consequently if the active material is of this nature, one would expect pure preparations to have an activity roughly twenty times that of rat preparation No. 11·252, or 138 g.u. per mg.

Spectrographic analysis would be greatly facilitated by the use of more highly purified preparations. Investigations soon to be published in collaboration with Sister Miriam Michael Stimson and others show that the active material can be obtained in more highly purified form by adsorption on magnesium oxide in Tswett columns. At least two adsorbed fractions are obtained, one fluorescing brilliantly yellow in ultraviolet light, the other fluorescing blue. Of these, the yellow-fluorescing fraction appears to be the more active.

The apparent relationship of the proliferation-promoting intercellular hormone to nucleic acid derivatives is of particular interest in view of Fischer's [1939] recent finding of a nucleoprotein growth factor for tissue cultures in beef embryo extracts, and of Caspersson's [1936; 1939] ultraviolet microscope studies showing increase in the nucleic acid content of cells during mitosis. It should be noted that the factor which Fischer isolated is apparently active only when linked with protein, whereas our yeast product is evidently equally active when protein-free, since it resists boiling and autoclaving and is active after precipitation of its protein content until it gives a negative biuret test.

Summary

1. Tests of the proliferation-promoting effects on yeast of intercellular wound hormones produced by the injury of newt, chick and rat tissues with lethal ultraviolet irradiation showed that the factors are active on yeast and can be assayed by quantitative yeast growth methods.

2. The data indicated that growth factors produced as a result of mechanical injury must be taken into account in considering results with animal tissue products. When the tissues were carefully washed after mincing, lethal ultraviolet irradiation led to fluids with potencies 2 to 9 times as great as those of control fluids from non-irradiated tissues. The active factor was stable to drying and autoclaving, and was not removed by filtration through Berkefeld filters.

3. Absorption spectra of the active preparations showed a maximum near 2600 Å. characteristic of purines and pyrimidines of the nucleic acid complex. The extinction at this wave-length closely paralleled the potency in various preparations. Tests of 15 amino-acids, yeast and thymus nucleic acids, inositol, aneurin, riboflavin, adenosine and insulin showed that all had either undetectable activity, or insufficient activity to account for that of the intercellular wound hormone preparations. It is concluded that the active factor in animal tissue preparations is either identical with or similar to that in yeast preparations (which contains adenine, guanine, pentose and phosphorus and is free from protein, pyridine or sulphur).

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