PROMOTION OF CELL DIVISION BY EXTRACTS FROM PEA SEEDLINGS*

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[*Manuscript received June* 21, 1962]

Summary

Water extracts of tobacco leaves, dried barley roots, and pea seedlings, when added as supplements to a sucrose mineral medium, promoted cell division in explants of subcultured callus tissue originally prepared from stems of *Nicotiana tabacum* cv. Wisconsin No. 38.

The active material from the pea seedlings was concentrated. Basic lead acetate precipitated this active material in the crude extract. Much of the active material could be extracted from the basic lead acetate precipitate with hot water. Mercuric acetate also precipitated the active material, but barium acetate and silver nitrate under both acid and alkaline conditions were ineffective.

Some of the active material of the basic lead acetate precipitate fraction was adsorbed by "Dowex-50"–H^+ from which it could be eluted with 1·5N NH_4OH and with 4N, but not 2N, HCl. Some activity was also found in the column effluent. Other resins were ineffective. The active material of the water extract of the basic lead acetate precipitate fraction was not adsorbed to "Dowex-50"–H^+.

A portion of the active material of the basic lead acetate precipitate fraction was adsorbed by "Norite". Some of the active material was eluted with ethanol but most with pyridine after washing the "Norite" with ethanol–ammonia–water. This procedure gave an extract active at a concentration of 15 mg/l and was 1600 times more active on a seed weight basis than the starting material.

Attempts to extract the active material into organic solvents were unsuccessful.

I. Introduction

Many workers (van Overbeek 1942; Haagen-Smit, Siu, and Wilson 1945; Mauney *et al.* 1952; Steward and Caplin 1952; Nétien and Beauchesne 1954; Shantz and Steward 1957; Goldacre and Bottomley 1959; Steward and Shantz 1959; Nitsch 1960) have detected in plant extracts of various species the presence of substances which promote cell division in a range of tissues such as tobacco pith and callus and carrot root phloem. Several procedures have been used to concentrate the active material in these extracts. Although outlines of these procedures have been published (Shantz and Steward 1954, 1955; Steward and Shantz 1954; Pollard, Shantz, and Steward 1959) only in a few cases have the details of the methods used been indicated (Mauney *et al.* 1952; Miller 1961; Pollard, Shantz, and Steward 1961).

This paper describes the detection from some sources of substances promoting cell division and also methods of obtaining one of these substances in concentrated form.

* This work formed part of a thesis by J. A. Zwar for the degree of Ph.D in the University of Wisconsin.

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II. Methods and Materials

(a) *Bioassay*

Cell division activity was determined in bioassays with pieces of tobacco callus cultured *in vitro*.

(i) *Tissue Employed in the Assay.*—The callus used in the assay was a strain which had been maintained in stock culture for some years, and which had originally been derived from stems of *Nicotiana tabacum* cv. Wisconsin No. 38 by the method of Miller (1953) and Miller and Skoog (1953).

In a routine transfer, 3 by 3 by 2 mm pieces were cut from callus which had been grown for 3–4 weeks subsequent to the previous transfer. Three pieces were transferred aseptically to the surface of the agar in a 125-ml Erlenmeyer flask.

(ii) *Medium for Stock Callus Growth.*—The composition of the medium used to maintain the stock culture was:

Inorganic Salts

	mg/l		mg/l
NH_4NO_3	400·0	$MnSO_4.7H_2O$	6·5
$Ca(NO_3)_2.4H_2O$	144·0	Fe (as sodium ferric ethylenediaminetetra-acetate)	3·0
KNO_3	80·0	$ZnSO_4.7H_2O$	2·7
$MgSO_4.7H_2O$	72·0	H_3BO_3	1·6
KCl	65·0	KI	0·75
KH_2PO_4	12·5		

Organic Compounds

	mg/l		mg/l
3-Indolylacetic acid	2·0	Nicotinic acid	0·5
Kinetin	0·2	Pyridoxine hydrochloride	0·5
Thiamine hydrochloride	0·1	Glycine	2·0

Sucrose at 20 g/l was used as an energy source and 10 g/l of Difco Bacto agar was added to solidify the medium. The agar was dissolved into the medium by autoclaving for 5 min at 230°F and, after removal from the autoclave, the pH was adjusted to 5·0 with 1N HCl while the agar remained molten. Fifty-ml aliquots of the medium were then dispensed into 125-ml Erlenmeyer flasks and sterilized by autoclaving at 230°F for 10 min.

(iii) *Medium for Testing Extracts.*—Kinetin was omitted from all media in which extracts were being tested. In these cases the concentrations of all the inorganic salts (except sodium ferric ethylenediaminetetra-acetate, which was unchanged) were four times higher than in the callus stock medium. The pH was adjusted to 6·0 before the agar was dissolved.

(iv) *Assay Procedure.*—Because of the dependence of initiation of cell division on concentration of extract and on the presence of toxins, all assays were carried out at 3–5 dilutions. Four replicate cultures were used for each concentration of extract and for controls.

Two types of control cultures were prepared: one contained basal medium without extract addition and was designated as the basal control, and the other

contained kinetin in three concentrations, usually 10, 50, and 100 μg/l. The cultures were grown in diffuse white light from overhead fluorescent lamps at *c.* 27°C and high relative humidity.

After 3–4 weeks growth the cultures were examined. As neither fresh weight nor dry weight of callus tissue provides a satisfactory measure of cell division, the activity of any fraction was assessed by a visual comparison of the size, colour, and texture of the callus tissue which was grown in the presence of extract with that grown on the basal medium alone or together with known concentrations of kinetin.

(*b*) *Purification of Extracts*

The purification achieved by any given treatment was expressed as the ratio of the dry weights of the extracts required for the same activity before and after the treatment. Such comparisons were made between those concentrations which were judged to be equal in activity to a given concentration of kinetin, usually 10 μg/l.

For convenience the concentration of any given supplement was expressed in arbitrary units, representing the number of grams of plant tissue originally extracted for its preparation, per 100 ml of medium.

III. Results

(*a*) *Active Material in Crude Extracts*

Cell division activity was detected when the following crude extracts were tested as described in Section II:

(1) A water extract of tobacco leaves gave a positive reaction in the assay in a concentration of 10 g leaf fresh weight equivalent per 100 ml of medium. The active material was insoluble in ether under alkaline conditions, and was not recovered from the cation-exchange resin "Dowex-50" in the H^+ form.

(2) An active water extract was prepared from a dried barley root preparation obtained as a by-product of the brewing industry. The active material was dialysable and showed good activity at 0·5 and 5 g equivalents of root preparation per 100 ml of medium. Higher concentrations were toxic to the callus.

(3) Extract from pea seedlings, which was used for concentration studies, was prepared in the following manner: "Little Marvel" peas were germinated either in darkness in vermiculite, or in a humid chamber in the light for 60 hr, until the primary roots were about 1 cm long. They were then placed in plastic bags and frozen. On removal from the freezer they were ground in a hammer mill and then thawed by the addition of water at 60°C with constant stirring. When thawing was complete, the juice was expressed with a hand-press, or by passage through a large basket-centrifuge. After clearing in a Sharples supercentrifuge, the juice was concentrated in a Mojonnier evaporator until 1 g of the original seed weight was represented by 1 ml of extract. When tested at this stage the extract was toxic to the

callus tissue. It was found, however, that the toxins could be partially removed from this crude extract by the following procedure:

An equal volume of 95% ethanol was added to the crude extract, and after cooling overnight the precipitate was removed in a Sharples centrifuge. After halving the volume by concentration under reduced pressure, an amount of 30% ammonia, equal to one-tenth the volume of the extract, was added. Following cooling overnight the precipitate was filtered off via "Celite" and the filtrate was concentrated under reduced pressure until the volume was such that 1 ml of extract was equivalent to 4 g of seed weight. This step also removed ammonia. The extract prepared in this way was assayed at a concentration of 5 g seed weight equivalent per 100 ml and at this concentration it was approximately equal in activity to 10 μg/l kinetin (Fig. 1). The dry weight necessary to produce this response was 0·28 g per 100 ml.

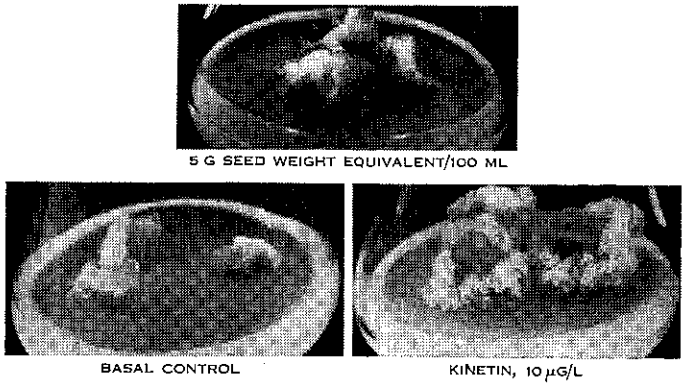

Fig. 1.—Effect of detoxified crude pea seedling extract on growth of tobacco callus tissue after 28 days.

(*b*) *Concentration Methods*

(i) *Basic Lead Acetate Precipitation of Active Material.*—In a typical experiment the procedure was as follows: 625 ml of detoxified crude extract (2500 g seed weight equivalents) were adjusted to pH 7·0 with HCl. Residual protein was precipitated by the addition of 625 ml of 12% trichloroacetic acid and overnight cooling. The precipitate was filtered off via "Celite" and discarded. The filtrate was concentrated to 625 ml under reduced pressure and the pH readjusted to 7·0 with 10N NaOH solution. The basic lead acetate precipitate was then prepared by the addition of 1125 ml of a 30% solution of basic lead acetate, with constant stirring. After overnight cooling it was filtered off. The precipitate was washed with 350 ml of basic lead acetate reagent, and the filtrate and washings were discarded. The washed precipitate was homogenized with 1 l. of water and H_2S was bubbled through the suspension for 90 min with constant stirring. The lead sulphide was filtered off via "Celite", and the precipitate and "Celite" were washed with 1 l. of H_2S-saturated water. The combined filtrate and washings were concentrated to dryness under

reduced pressure and then made up for assay. As shown in Figure 2, activity was observed in the basic lead acetate precipitate fraction in concentrations from 5 to 50 g seed weight equivalents per 100 ml medium. The lowest concentration had about the same activity as 10 μg/l kinetin, and contained 0·13 g dry weight per 100 ml thus representing a 2·2-fold purification over the detoxified crude extract.

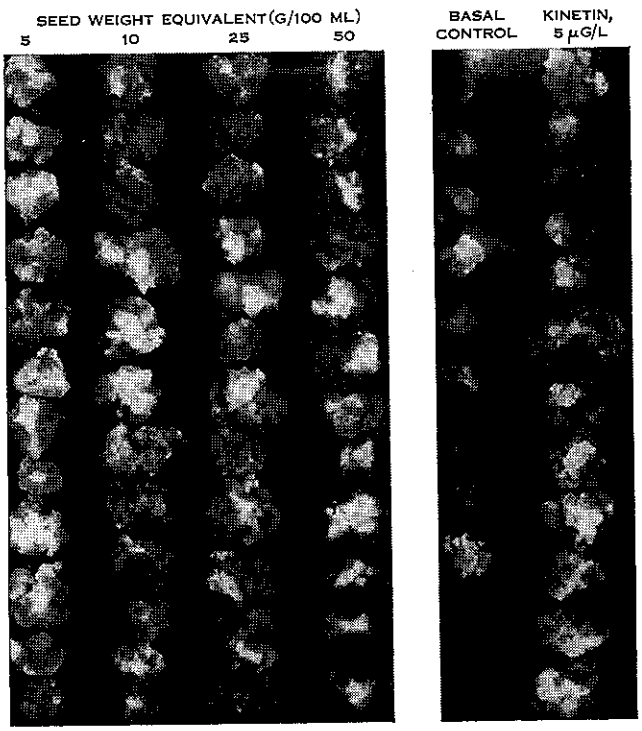

Fig. 2.—Effect of basic lead acetate precipitate fraction prepared from pea seedling extract on growth of tobacco callus tissue after 24 days.

It is well known that some low molecular weight substances, although precipitated by basic lead acetate from complex solutions, can be extracted from the precipitate with water. Accordingly a basic lead acetate precipitate was prepared as described above from a crude solution containing 685 g seed weight equivalents. This precipitate was homogenized with 500 ml of water at 80°C, cooled, and then filtered. This procedure was repeated three times. The combined filtrates were then freed of lead with H_2S as described above, concentrated to dryness under reduced pressure, and prepared for assay.

The results of the assay are shown in Figure 3. The hot-water extract was active and, at a concentration of 5 g seed weight equivalents per 100 ml of medium, had approximately the same activity as 10 μg/l kinetin. This concentration contained 0·03 g dry weight per 100 ml medium and represented a 4·4-fold purification over the basic lead acetate precipitate fraction.

(ii) *Precipitation of Active Material with other Heavy Metals.*—The effect of several other heavy metal precipitants was tested. Silver nitrate at low and at high pH, and barium acetate both failed to precipitate the active material. However, mercuric acetate was found to be an effective precipitant, the activity being recovered in the mercury-freed precipitate fraction. The purification over the detoxified crude extract was, however, negligible.

(iii) *Ion-exchange Fractionation of Active Material.*—The active fraction obtained by basic lead acetate precipitation of crude extract was used as starting material in fractionations on ion-exchange resins.

(1) *"Dowex-50"–H^+ X2, 200–400 Mesh:* A 50 by 5·5 cm column of cleaned and charged resin was washed with 2 l. of HCl at pH 3·0. A basic lead acetate precipitate fraction (1000 g seed weight equivalents) was adjusted to pH 3·0, and added to the column which was then washed with 4 l. of HCl at pH 3·0. The resultant effluent was collected and concentrated to dryness under reduced pressure to remove HCl.

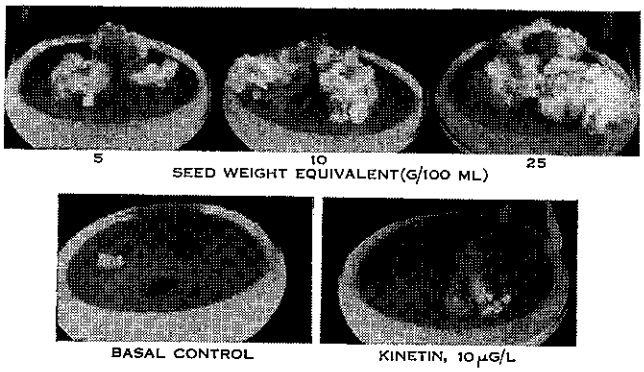

Fig. 3.—Effect of hot-water extract of basic lead acetate precipitate fraction on growth of tobacco callus tissue after 36 days.

The column was eluted with 4 l. of 1·5N ammonium hydroxide and collection of eluate was begun when it became alkaline. The eluate was concentrated to dryness to remove ammonia. As shown in Figure 4, active material was present in both effluent and eluate. The greatest activity was found in the latter at a concentration of 100 g seed weight equivalents per 100 ml. The dry weight necessary for this activity was the same as that for equal activity in the basic lead acetate precipitate fraction. Thus this procedure gave no purification, but suggested the possibility of purification by stepwise elution from this resin.

Stepwise elution was tested with a second 100 ml (1000 g seed weight equivalents) sample on an identical column. After adding the solution at pH 3·0 as described above, the column was eluted with 2 l. portions respectively of 0·5N, 1N, 2N, and 4N HCl. After washing with water until the effluent was neutral, the column was eluted with 2 l. of 1·5N NH_4OH. Eluates were collected separately, the collection of each was begun when the appropriate eluent was added to the column. Each was then prepared for assay as previously described. Of the eluates, only that collected

after the addition of 4N HCl was active, and it was best at a concentration of 100 g seed weight equivalents per 100 ml medium (Fig. 5). This concentration showed about the same activity as 10 μg/l kinetin and 0·013 g dry weight were required to give this activity. This represented a 10·0-fold purification over the basic lead acetate precipitate fraction of equal activity.

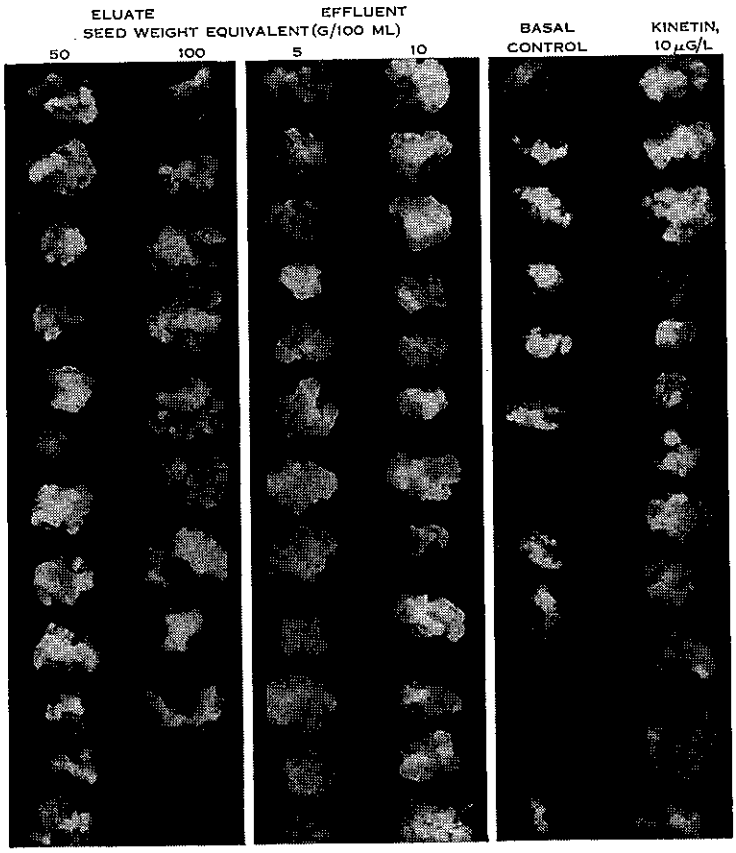

Fig. 4.—Effect of 1·5N NH_4OH eluate fraction and of effluent fraction obtained by displacement of basic lead acetate precipitate fraction from "Dowex-50"–H^+ on growth of tobacco callus tissue after 21 days.

(2) *Other Resins:* Other ion-exchange resins used included the weak cation exchanger "Amberlite CG-50"–H^+, the strong anion exchanger "Dowex 1" in both the formate and chloride forms, and the weak anion exchanger "Amberlite IR-45" in the formate form. The recovery of activity from all of these resins was poor.

(iv) *Adsorption of Active Material to Charcoal.*—A preliminary experiment had shown that the active material of the basic lead acetate precipitate fraction could be adsorbed to "Norite" from which it could be eluted with pyridine. This procedure resulted in a 2·8-fold purification.

Greater purification was obtained by the following procedure: 15 g of "Norite" which had been washed with pyridine and reactivated by heating to 100°C were added to 75 ml (750 g seed weight equivalents) of a lead acetate precipitate fraction. After thorough mixing, the "Norite" was filtered off and washed with 4×100 ml of water. The washings were combined with the filtrate and concentrated for assay.

The "Norite" was then eluted successively with 50% ethanol, 2×190 ml; 95% ethanol–water–30% ammonia (5 : 4 : 1 by vol.), 2×190 ml; and pyridine, 2×100 ml. The two eluates from each treatment were combined, freed of solvents by evaporation under reduced pressure, and prepared for assay together with the

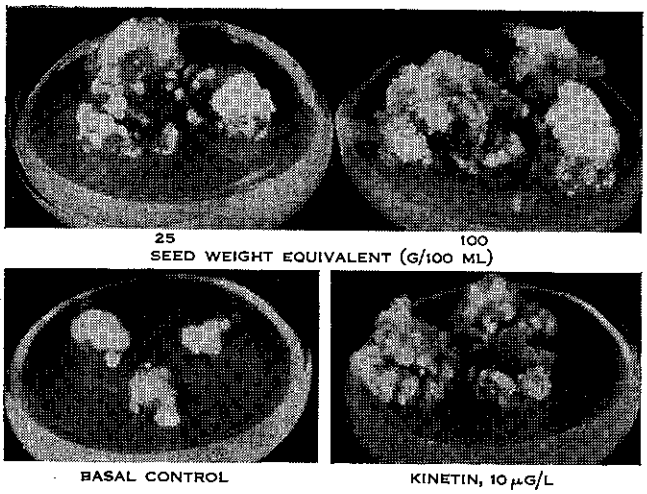

Fig. 5.—Effect of fraction obtained by elution of basic lead acetate precipitate fraction from "Dowex-50"–H^+ with 4N HCl on growth of tobacco callus tissue after 39 days.

starting material and controls. The assay showed some activity in the ethanol eluate, but the best was found in the pyridine eluate in concentrations of 50 and 100 g seed weight equivalents per 100 ml (Fig. 6). The former concentration was approximately equal in activity to kinetin at 10 μg/l and represented a 32-fold purification over the starting material of the experiment. The dry weight needed for perceptible activity was 0·0015 g per 100 ml.

(v) *Organic Solvent Extraction.*—Attempts to extract the active material of the basic lead acetate precipitate fraction into organic solvents including diethyl ether, methanol, 95% ethanol, n-butanol, and acetone gave negative or inconsistent results.

IV. Discussion

Extracts which provoked the proliferation of tobacco callus were obtained from tobacco leaves, dried barley roots, and pea seedlings. It seems probable that extracts with the same activity can be obtained from many sources. However, the presence in crude extracts of substances toxic to the assay tissue frequently masks

the activity and it is necessary to remove these, at least in part, before the crude material can be assayed. The pea extract was partially freed of toxins by addition of ammonia and filtration of the precipitate so obtained. The nature of the toxins is unknown.

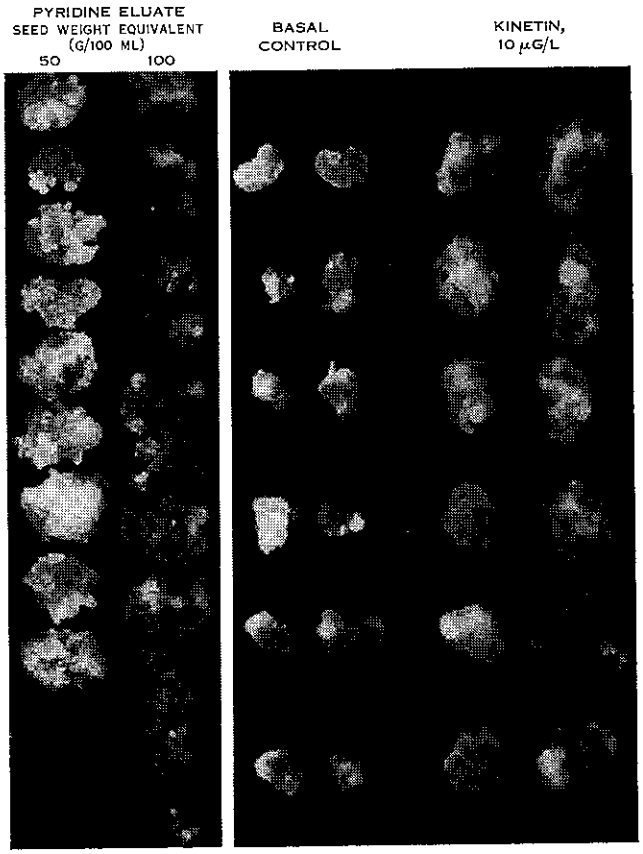

Fig. 6.—Effect of fractions obtained by elution with pyridine of active material from "Norite" on growth of tobacco callus tissue after 23 days.

Growth of the callus tissue for 3–4 weeks was necessary before activity could be detected. This severely limited the rate of progress of the work. However, the assay is satisfactory in another respect as in tests with known substances it has been found that no proliferation of the tissue takes place unless kinetin or one of its analogues is added. In particular, callus tissue does not react to additions of amino acids, mineral salts, or carbohydrates. Therefore it seems probable that an extract which promotes proliferation contains at least one substance which in this test has the same biological function as kinetin. Despite this probability, the experiments reported have not excluded the possibility that several substances may act in conjunction to produce the effect.

On the other hand there is evidence that the extract contains several substances or groups of substances which are separately active. In the ion-exchange experiment with "Dowex-50"–H^+ an active extract could be eluted from the column with ammonia or 4N HCl. However, the water effluent also contained active material. It seems unlikely that the same substance or substances give rise to the activity in both fractions, for a substance which could only be eluted with 4N HCl would not be expected to pass through the column under mildly acid conditions. Moreover, the capacity of the column was many times in excess of the dry weight added.

Further support is given to this view by the observation that much of the active material of the basic lead acetate precipitate fraction is adsorbed to "Dowex-50"–H^+, but only a negligible amount of the activity of the water extract of this precipitate is so adsorbed. Perhaps the active fraction of the "Dowex-50"–H^+ effluent contains the same active substance or substances as the water extract.

Miller (1961) has obtained a very considerable concentration of a cell-division promoter extracted from corn grain in the milk stage. As with the pea extract some activity is adsorbed to "Dowex-50"–H^+, from which it may be eluted with ammonia, and some is found in the water effluent. There is thus in this case also, evidence that more than one active substance is present. In both cases, moreover, one of the active materials is adsorbed to "Dowex-50"–H^+ from which it can be eluted with 4N HCl, but not lower normalities.

The properties reported here are insufficiently specific to throw light on the chemical nature of the active substance or substances. However, that kinetin itself is not the main active component may be inferred from the failure to obtain active ether extracts at alkaline pH or to precipitate active material with silver nitrate under acid conditions.

The most active fraction obtained in the course of this work (by pyridine elution of charcoal-adsorbed material) promoted proliferation at a concentration of 15 mg/l and was more active at 30 mg/l. In the former concentration, this extract is 1600 times more active than the starting material but still about 1000 times less active than kinetin.

V. Acknowledgments

This work was carried out in the Botany Department of the University of Wisconsin while one of us (J. A. Zwar) was the holder of a C.S.I.R.O. Overseas Studentship. Part of the financial support was provided from grants to F. Skoog by the Research Committee of the Graduate School of the University of Wisconsin with funds from the Wisconsin Alumni Research Foundation, and by grant G5890 from the National Science Foundation.

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