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REGULATORS OF CELL DIVISION IN PLANT TISSUES—II.

A CYTOKININ IN PLANT EXTRACTS: ISOLATION AND INTERACTION WITH OTHER GROWTH REGULATORS*

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Abstract—Two factors which markedly promote cell division have been purified from plum fruitlet extracts. One factor was a cytokinin obtained in chromatographically homogeneous form, while the other was identified as *myo*-inositol. These two factors interacted synergistically to promote cell division in carrot phloem explants. A synergism also existed between the cytokinin and indol-3-ylacetic acid but not between *myo*-inositol and indol-3-ylacetic acid. The cytokinin was distinguished from kinetin by chromatographic behaviour and division-promoting activity. Sweet corn kernels were found to contain a cytokinin (termed zeatin) which was indistinguishable from that purified from plums. The crystalline free base was found to induce cell division at 0·1 μg/l. Kinetin was inactive at this concentration. Zeatin was chromatographically indistinguishable from an active substance detected by bioassay in plum fruitlet and corn extracts prepared after enzyme inactivation. Zeatin does not appear to be an artefact produced by enzymic or chemical degradation of another cytokinin.

INTRODUCTION

SUBSTANCES which resemble the synthetic compound kinetin (6-furfurylaminopurine) in inducing cell division in certain excised plant tissues have been termed kinins.[2] Since animal physiologists apply the same term to substances causing muscle contraction, other terms have been proposed for these stimulants of plant cell division.[1,3,4] Skoog *et al.*,[5] who first referred to these compounds as kinins, now intend to use the term cytokinin which is preferred by the present writer.[1] Cytokinin will, therefore, be used instead of the term kinin in this and in subsequent papers.

Certain synthetic 6-substituted purines,[6] 1-substituted adenines[7] and substituted ureas[8] exhibit cytokinin activity, but little is known about the chemical nature of the cytokinins which occur naturally in plants. Attempts have been made to isolate cytokinins or similar factors from immature maize seeds,[9–11] germinating peas,[12] apple fruitlets,[13] tomato fruits,[14]

* For Part I, see Ref. 1.

[1] D. S. LETHAM, *New Zealand J. Botany* **1**, 336 (1963).
[2] C. O. MILLER, F. SKOOG, F. S. OKUMURA, M. H. VON SALTZA and F. M. STRONG, *J. Am. Chem. Soc.* **78**, 1375 (1956).
[3] K. V. THIMANN, *Ann. Rev. Plant Physiol.* **14**, 1 (1963).
[4] K. MOTHES, *Regulateurs Naturels de la Croissance Vegetale*, p. 131. C.N.R.S., Paris (1964).
[5] F. SKOOG, F. M. STRONG and C. O. MILLER, *Science* **148**, 532 (1965).
[6] F. M. STRONG, *Topics in Microbial Chemistry*, p. 98. Wiley, New York (1956).
[7] H. Q. HAMZI and F. SKOOG, *Proc. Natl Acad. Sci. U.S.* **51**, 76 (1964).
[8] M. I. BRUCE, J. A. ZWAR and N. P. KEFFORD, *Life Sci.* **4**, 461 (1965).
[9] G. BEAUCHESNE, M. LEBOEUF and R. GOUTAREL, *Regulateurs Naturels de la Croissance Vegetale*, p. 119. C.N.R.S., Paris (1964).
[10] E. M. SHANTZ and F. C. STEWARD, *Regulateurs Naturels de la Croissance Vegetale*, p. 59. C.N.R.S., Paris (1964).
[11] C. O. MILLER, *Proc. Natl Acad. Sci. U.S.* **47**, 170 (1961).
[12] J. A. ZWAR and F. SKOOG, *Australian J. Biol. Sci.* **16**, 129 (1963).
[13] J. A. ZWAR, W. BOTTOMLEY and N. P. KEFFORD, *Australian J. Biol. Sci.* **16**, 407 (1963).
[14] E. MAIA, *Regulateurs Naturels de la Croissance Vegetale*, p. 103. C.N.R.S., Paris (1964).

yeast,[2] coconut milk,[10,15–17] the cellular endosperm of the coconut,[2,6] *Aesculus* fruits,[10] crown-gall tumour tissue[18] and corn steep water.[19] This work achieved only a partial purification of certain cytokinins. Factors perhaps related to cytokinins have been purified from coconut milk by Shantz and Steward,[20] three crystalline products termed A, B and C being obtained. These factors promoted cell division in carrot phloem explants in the absence of supplied auxin[20] and thus appear to differ from the typical cytokinins. Compound A has been identified as 1,3-diphenylurea[21] which possesses negligible cytokinin activity[6,22] and could have arisen from solvent impurities.[21] Evidence to establish the purity of products B and C was not presented by Shantz and Steward.[20] The u.v. absorption spectra in acid and base reported[20] for B are identical with those recorded[23] for xanthine which does not promote the growth of carrot phloem explants.[24] Product B may have been impure xanthine.

Previous reports[1,25] from this laboratory have established that fruitlets of several species yield extracts with strong cytokinin activity. Since high activity was associated with the cell division period in both apple and plum fruits,[1] it appeared that cytokinins may play an important role in the regulation of cell division in developing fruits. The activity of plum fruitlet extracts was shown to be due to at least two synergistic factors.[1] Purification of these factors is reported in detail in this paper. Fractions were tested for cytokinin activity with the carrot phloem growth test previously described.[1] A cytokinin in plum fruitlet extract has been obtained in chromatographically homogeneous form and factors which enhance its activity have been identified. A substance indistinguishable from the plum cytokinin has been isolated in crystalline form from sweet corn extracts. Chemical and biological characteristics of this substance are presented. Brief accounts of some aspects of this investigation have previously been published.[26–28]

RESULTS AND DISCUSSION

Separation of Plum Fruitlet Extract into Active Fractions

Fractionation with ion-exchange resins proved a convenient method for the initial purification of the factors. The active water-soluble fraction of the alcoholic extract of plum fruitlets was extracted with ethyl acetate as previously described[1] to remove inhibitors. The resulting purified water-soluble fraction (PWS) was passed through a column of a polystyrene sulphonic acid resin ("Zeo-Karb" 225) and then through a column of a weakly basic resin ("De-Acidite" G or "Amberlite" IR-4B). Evaporation of the resulting effluent yielded

[15] J. E. LOEFFLER and J. VAN OVERBEEK, *Regulateurs Naturels de la Croissance Vegetale*, p. 77. C.N.R.S., Paris (1964).
[16] A. KOVOOR, *Regulateurs Naturels de la Croissance Vegetale*, p. 83. C.N.R.S., Paris (1964).
[17] J. R. MAUNEY, W. S. HILLMAN, C. O. MILLER, F. SKOOG, R. A. CLAYTON and F. M. STRONG, *Physiol. Plantarum* **5**, 485 (1952).
[18] H. N. WOOD, *Regulateurs Naturels de la Croissance Vegetale*, p. 97. C.N.R.S., Paris (1964).
[19] J. E. FOX and C. O. MILLER, *Plant Physiol.* **34**, 577 (1959).
[20] E. M. SHANTZ and F. C. STEWARD, *J. Am. Chem. Soc.* **74**, 6133 (1952).
[21] E. M. SHANTZ and F. C. STEWARD, *J. Am. Chem. Soc.* **77**, 6351 (1955).
[22] W. BOTTOMLEY, N. P. KEFFORD, J. A. ZWAR and P. L. GOLDACRE, *Australian J. Biol. Sci.* **16**, 395 (1963).
[23] G. H. BEAVEN, E. R. HOLIDAY and E. A. JOHNSON, *The Nucleic Acids* **1**, p. 493. Academic Press, New York (1955).
[24] F. C. STEWARD, *Symp. Soc. Study of Develop. Growth*, **19**, 193 (1961).
[25] D. S. LETHAM and E. G. BOLLARD, *Nature* **191**, 1119 (1961).
[26] D. S. Letham, *Life Sci.* **2**, 152 (1963).
[27] D. S. LETHAM, *Life Sci.* **2**, 569 (1963).
[28] D. S. LETHAM, *Regulateurs Naturels de la Croissance Vegetale*, p. 109. C.N.R.S., Paris (1964).

fraction N. In the first experiments, the "Zeo-Karb" column was eluted with 70% ethanol and then with 1·5 N NH_4OH. Evaporation of the effluent ethanol and ammonia yielded *fractions* C_1 and C_2 respectively. The basic column was eluted with 1·5 N NH_4OH and the effluent evaporated yielding *fraction A*. *Fraction* C_1 appeared to contain mainly phenolic compounds adsorbed on the cation exchanger. Activity was confined to *fractions* C_2 and N; the activity of each alone was slight, but in combination they induced growth increments similar to those given by PWS and by a mixture of *fractions* C_1, C_2, A and N (Table 1). In these assays, the media were sterilized by autoclaving. When solutions of C_2 and N were sterilized by filtration and added aseptically to autoclaved basal medium, the activities of these fractions were unchanged. *Fractions* C_1 and A failed to show activity when added singly and in combination to basal medium, to basal containing C_2, and to basal containing N. No activity was eluted from the basic column when 0·3 N HCl was used as eluent instead of 1·5 N NH_4OH.

TABLE 1. WEIGHT AND CELL NUMBER OF CARROT EXPLANTS AFTER GROWTH IN THE PRESENCE OF FRACTIONS DERIVED FROM PWS

Additions to basal medium*	Mean explant weight† (mg)	Mean cell number per explant† (in thousands)
None	12	40
Fraction C_1	12	45
Fraction C_2	18	78
Fraction A	12	37
Fraction N	16	55
Fractions C_2+N	44	310
Fractions C_1+C_2+A+N	44	289
PWS	49	298

* The concentration at which all fractions were tested was the equivalent of 1 g of tissue per 100 ml of medium.

† Each value is the mean for 60 explants derived from three carrots.

The relation between *fractions* C_2 and N has been studied. Addition of C_2 and N separately to the basal medium to give higher concentrations induced increments in weight and cell number similar to those produced at the concentrations used previously. Passing N through a second "Zeo-Karb" 225 column did not partition its activity. For these two reasons, it was concluded that the activities of *fractions* C_2 and N were due to different factors which acted synergistically to induce cell division. This synergism occurred in the presence of auxin (IAA), and, with explants from some carrots, was completely dependent on supplied auxin (Table 2). The synergism between growth-promoting fractions corresponding to N and C_2 prepared by Pollard *et al.*[29] from coconut milk was, in contrast, exhibited only in the absence of auxin. Table 2 also shows that a synergism exists between C_2 and indol-3-ylacetic acid (IAA) but not between N and IAA. In this respect C_2 and N resemble the corresponding fractions obtained from coconut milk.[29]

Other methods for eluting activity from the "Zeo-Karb" column were also studied. After elution with 70% ethanol, activity could be eluted with phenol–acetic acid–water (1:2:18, w/v/v) and with pyridine–water–ethanol (1:1:9, by vol.). Since this activity was

[29] J. K. POLLARD, E. M. SHANTZ and F. C. STEWARD, *Plant Physiol.* **36**, 492 (1961).

TABLE 2. WEIGHT AND CELL NUMBER OF CARROT EXPLANTS AFTER GROWTH IN THE PRESENCE AND ABSENCE OF IAA AND FRACTIONS C_2 AND N

Additions to basal medium*	Weight† (mg) and cell number† (in thousands, in parentheses)	
	IAA	
	Absent	Present (2 mg/l.)
None	6 (25)	19 (100)
Fraction C_2	9 (69)	37 (329)
Fraction N	7 (43)	16 (91)
Fractions $N+C_2$	12 (62)	62 (550)

* C_2 and N equivalent to 1.5 g of tissue was added to 100 ml of medium.
† Each value is the mean for 20 explants.

considerably less than that in *fraction* C_2, these eluates were not further studied. Following successive elution of the column with ethanol and 1·5 N NH_4OH, further activity could be eluted with 1·5 N piperidine, 1 N NaOH or 6 N HCl. The activity recovered by either of the first two eluents was considerably less than that already eluted with 1·5 N NH_4OH, but the 6 N HCl eluate equalled the ammonia eluate in activity. The 6 N HCl eluate contained much ammonium chloride. After the ammonia elution, washing the column with 0·4 N and then with 1·5 N HCl removed all the ammonium ions, but little activity, from the resin. Elution with 6 N HCl and evaporation of the effluent then gave a very active eluate termed *fraction* C_3. The specific activity (growth response/weight of dry matter added to medium) of *fraction* C_3 was about five times that of C_2. *Fraction* C_3 and the 1·5 N HCl eluate both acted synergistically with *fraction N* and thus resembled *fraction* C_2.

Kinetin, but not gibberellic acid, was found to substitute for *fractions* C_2 and C_3 and a synergism could be demonstrated between kinetin and *fraction N*. Hence *fractions* C_2 and C_3 appeared to contain cytokinins, while *fraction N* contained some factor which enhanced their activity.

The possibility of purifying the factors in PWS by adsorption onto and elution from charcoal was also studied. Charcoal deactivated with palmitic acid was used and elution was effected with phenol–acetic acid–water (1:2:18, w/v/v), the phenol being removed by ether extraction after elution. An eluate was obtained which acted synergistically with the fraction not adsorbed onto the charcoal and with *fraction N*. Since some loss of activity accompanied this fractionation, ion-exchange fractionation was adopted for the initial purification of the extract.

The Factor in Fraction N

This factor did not adsorb onto charcoal. *Fraction N* was chromatographed on paper using solvent 7 (see Experimental) and the chromatogram assayed for activity. One region exhibited activity which coincided with a component (R_f 0·17) detected by the silver nitrate sprays of both Trevelyan *et al.*[30] and Hough.[31] This component, which appeared to be chromatographically homogeneous, did not absorb u.v. light appreciably and was not

[30] W. E. TREVELYAN, D. P. PROCTER and J. S. HARRISON, *Nature* **166**, 444 (1950).
[31] L. HOUGH, *Nature* **165**, 400 (1950).

detected with the aniline–diphenylamine–phosphoric acid[32] or benzidine reagents[33] commonly used to locate sugars. Treatment with 6 N HCl at 100° did not alter the chromatographic behaviour or biological activity of this component. The above observations indicated that the active substance could be either a cyclitol or a sugar alcohol. Subsequently it was found that the substance reacted positively in a modified Scherer test considered specific for inositols.[34] The substance was isolated in crystalline form (see Experimental section) and identified as *myo*-inositol. The optimum concentration of *fraction N* in the culture medium was about 150 mg/l. which was estimated to give a *myo*-inositol concentration of approximately 12 mg/l. At 10–50 mg/l., authentic *myo*-inositol induced growth increments in weight and cell number only slightly less than those caused by an optimum concentration of *fraction N*. Therefore the activity of this fraction was almost entirely accounted for by the *myo*-inositol content. The basal medium of the present investigation contained *myo*-inositol at a concentration similar to that used by Henderson *et al.*[35] This was, however, insufficient to stimulate the growth of carrot explants.

When this investigation was initiated, the importance of inositols in the nutrition of excised tissues had not been demonstrated. Although *myo*-inositol was at that time frequently added to media in greatly varying concentrations, it had been found to promote the growth of only three tissues, all of which grew actively in the absence of the vitamin.[36, 37] While the present investigation was in progress, it was reported that *myo*-inositol and certain other cyclitols considerably enhance the growth of two additional excised tissues.[29, 38] Of particular relevance is the demonstration by Pollard *et al.*[29] that the ability of coconut milk to promote the growth of carrot phloem explants is partly due to its content of *scyllo*-inositol, *myo*-inositol and sorbitol. Sorbitol, however, was found inactive when tested in this laboratory.

The work reported here shows that *myo*-inositol can greatly stimulate cell division in carrot phloem explants when a cytokinin-containing fraction is also present in the media. *myo*-Inositol and related compounds could, therefore, function as naturally-occurring regulators of cell division.

The Factors in Fraction C_2

For preparation of *fraction* C_2 on a large scale, the ethanol extract was passed directly through a "Zeo-Karb" 225 column. The advantage gained by evaporation of the ethanol and extraction with ethyl acetate, as done for small-scale preparations, was insufficient to justify the additional effort. The two methods of preparation yielded fractions with very similar activity. The optimum concentration of both fractions in the medium was about 40 mg/l. In assays for the factors of *fractions* C_2 and C_3, *fraction N* or *myo*-inositol was added to all media.

An attempt to purify the factors in *fraction* C_2 by adsorption onto charcoal deactivated with palmitic acid and elution with phenol–acetic acid–water (1:2:18, w/v/v) resulted in a partial loss of activity. The factors in C_2 could, however, be precipitated by silver ions and recovered from the precipitate by extraction with dilute HCl without detectable loss of activity. The fraction not precipitated by silver ions was freed from silver by addition of

[32] I. Smith, *Chromatographic Techniques*, p. 168. Heinemann, London (1958).
[33] G. Harris and I. C. MacWilliam, *Chem. & Ind. (London)* 249 (1954).
[34] Y. Nagai and Y. Kimura, *Nature* **181**, 1730 (1958).
[35] J. H. M. Henderson, M. E. Durrell and J. Bonner, *Am. J. Botany* **39**, 467 (1952).
[36] D. Paris, *Année Biol.* **31**, 15 (1955).
[37] A. C. Braun, *Proc. Natl Acad. Sci. U.S.* **44**, 344 (1958).
[38] C. Steinhart, L. Anderson and F. Skoog, *Am. J. Botany* **37**, 60 (1962).

chloride and found to possess negligible activity. When the fraction (C_2A, very active at 0·3 mg/l.) recovered from the silver precipitate was chromatographed on paper (solvent 1), strong activity was located at about R_f 0·70 (see Fig. 1), but the factor could not be detected by any of a variety of chemical methods. With explants from some carrots, slight activity was detected at about R_f 0·40 apparently associated with a component (C_2A_2) located by u.v. printing (method of Markham and Smith[39]). By cellulose column chromatography, these factors were purified on a much larger scale. C_2A_2 was then isolated in crystalline form and identified as adenine. This accounted for the slight activity detected at R_f 0·40. The factor of R_f 0·70 was concentrated in a very active fraction (C_2A_1, 9 mg from 30 kg of plums) which at 0·01 mg/l. of medium induced marked growth increments. The specific activity of C_2A_1 was over 100,000 times that of the dry matter in the original ethanol extract of plums.

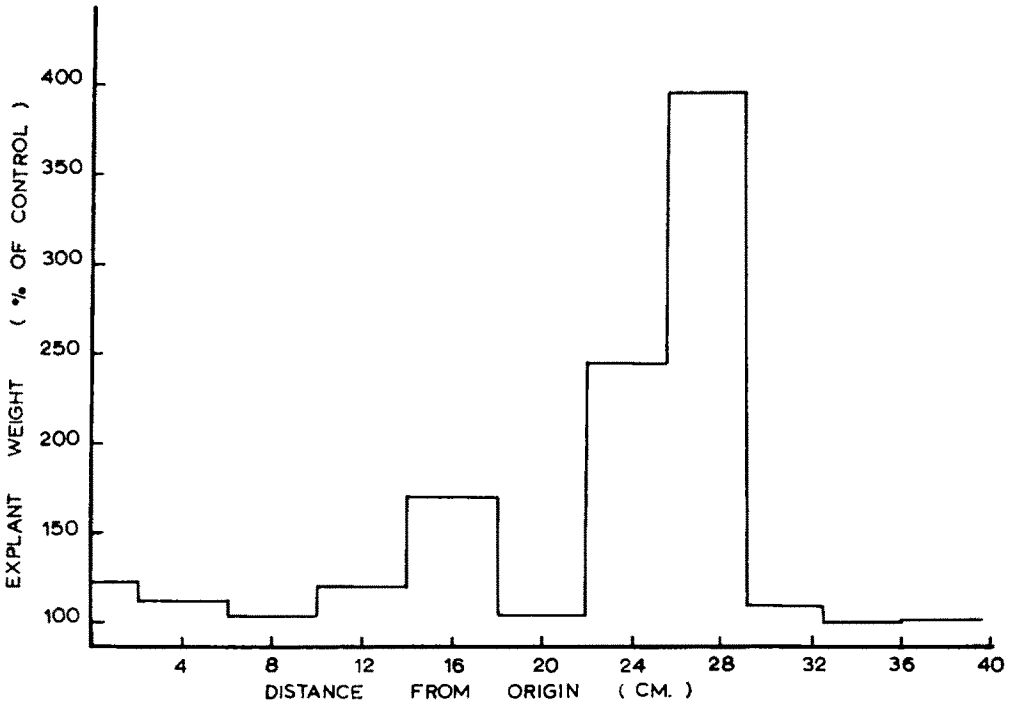


FIG. 1. A HISTOGRAM SHOWING ACTIVITY OF ZONES OF A PAPER CHROMATOGRAM (SOLVENT 1) OF FRACTION C_2A.

When *fraction* C_2A_1 was subjected to two-dimensional paper chromatography (solvent 1 followed by 2), two components (C_2A_1A and C_2A_1B) were detectable by u.v. printing.[39] These components were clearly separated from other substances which fluoresced under u.v. light but did not show on the u.v. print of the chromatogram and from ninhydrin-reacting substances. The R_f values for C_2A_1A and C_2A_1B are given in Table 3. Only one portion of the two-dimensional chromatogram possessed activity and this coincided exactly with the location of C_2A_1A (see Fig. 2). C_2A_1A eluted from other chromatograms was rechromatographed on paper using solvents 3, 4, 5 and 7, and on cellulose phosphate paper (H form) with 0·1 N acetic acid, N formic acid and N HCl as solvents. Each chromatogram was cut

[39] R. MARKHAM and J. D. SMITH, *Biochem. J.* **45**, 294 (1949).

TABLE 3. R_f AND R_A* VALUES FOR C_2A_1A, C_2A_1B AND KINETIN

Solvent†	C_2A_1A		C_2A_1B		Kinetin	
	R_f	R_A	R_f	R_A	R_f	R_A
1	0·74	1·48	0·71	1·42	0·83	1·66
2	0·80	1·54	0·54	1·04	0·83	1·60
3	0·77	1·88	0·21	0·50	0·85	2·07
4	0·59	2·36	0·41	1·64	0·70	2·80
5	0·75	1·63	0·17	0·37	0·82	1·78
6	0·59	2·03			0·87	3·00
0·1 N acetic acid	0·02	1·25			0·02	1·25
N formic acid	0·09	1·30			0·09	1·30
N HCl	0·65	1·12			0·65	1·12

* R_A = movement relative to adenine.

† For identity of solvents 1–6, see experimental section. Solvents 1–6 were used with Whatman No. 1 paper. The remaining solvents were used with Whatman cellulose phosphate paper (P 20) in the hydrogen form.

into zones which were assayed for division-promoting activity. On all seven chromatograms, activity coincided exactly with C_2A_1A which was not resolved by any of the solvents used. Assays for three of the chromatograms are represented in Figs. 3–5 as examples of the results obtained. In nine chromatographic systems, therefore, activity was associated with C_2A_1A which was concluded to be the active factor in *fraction* C_2A_1.

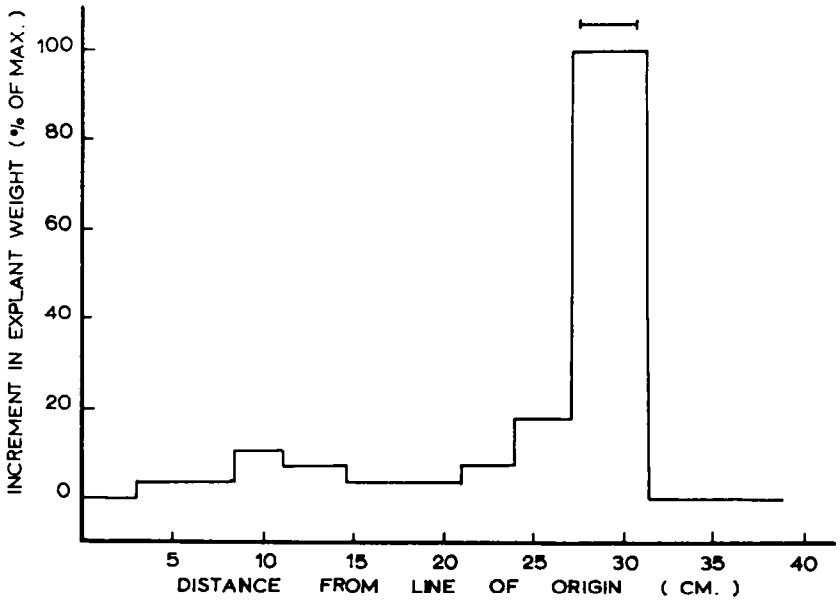
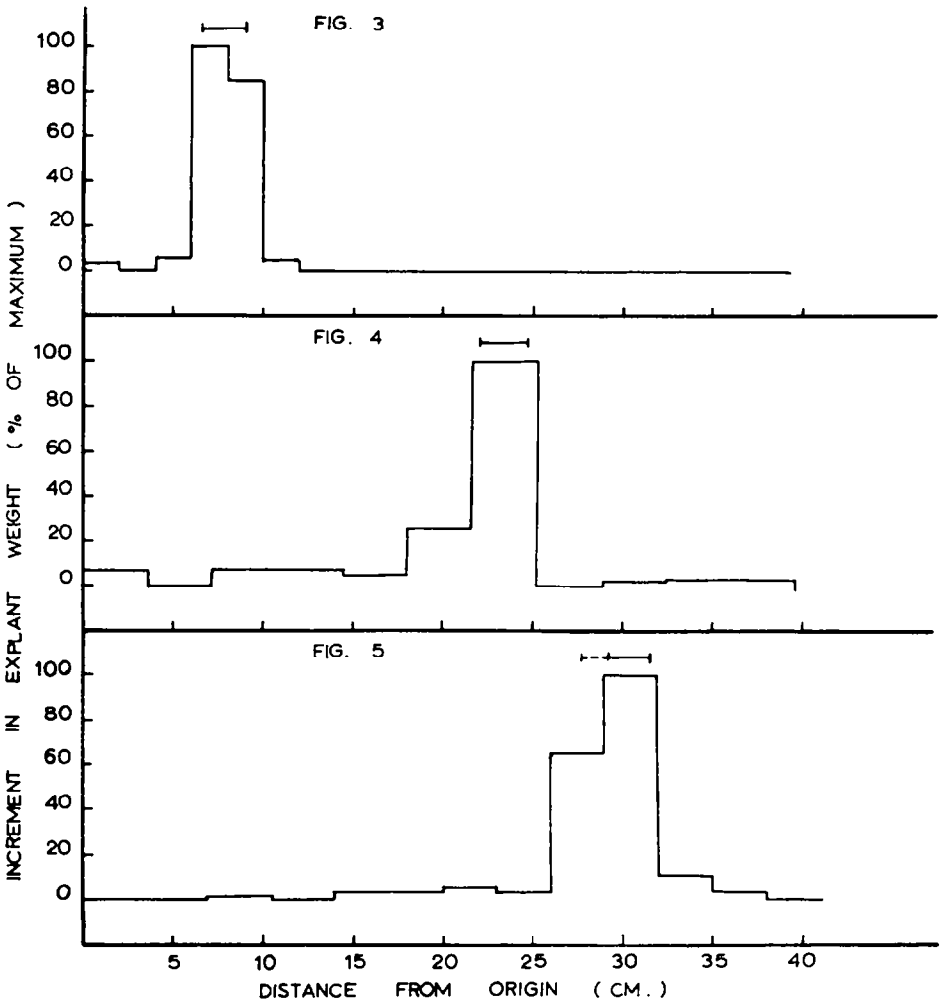


FIG. 2. A HISTOGRAM SHOWING ACTIVITY OF ZONES OF A STRIP CUT ACROSS A TWO-DIMENSIONAL PAPER CHROMATOGRAM OF FRACTION C_2A_1.

The solvents used were 1 followed by 2. The strip ran in the direction of flow of solvent 2 and passed through component C_2A_1A, the location of which is indicated by the line at the top of the histogram. Growth increments are expressed as a percentage of the increment induced by the most active zone.



FIGS. 3–5. HISTOGRAMS SHOWING THE ACTIVITY OF ZONES CUT FROM PAPER CHROMATOGRAMS OBTAINED BY RECHROMATOGRAPHING COMPONENT C_2A_1A USING THREE SOLVENTS.

Fig. 3: N formic acid (cellulose phosphate paper). Fig. 4: solvent 4. Fig. 5: solvent 3. Solvents 3 and 4 were used with Whatman No. 1 paper. The chromatogram of Fig. 3 was developed by allowing the solvent to flow to the bottom of the sheet and then to drip from a serrated edge. The location of C_2A_1A is indicated by the line at the top of each histogram. A broken line (Fig. 5) indicates streaking of C_2A_1A.

Component C_2A_1A, but not C_2A_1B, gave a red spot on paper chromatograms with the reagents of Reguera and Asimov,[40] a reaction considered specific for purine bases.[40] Absorption spectra for C_2A_1A have already been published[27,28] and chromatographic data are listed in Table 3. The factor is seen to differ from kinetin in chromatographic behaviour. Since the amount of C_2A_1A in the chromatogram spots was not exactly known, it was not possible to relate growth increments induced to definite concentrations of the factor. At a concentration definitely less than 0·01 mg/l. (probably less than 0·001 mg/l.) in the presence

[40] R. M. REGUERA and I. ASIMOV, *J. Am. Chem. Soc.* **72**, 5781 (1950).

of *fraction N* and IAA, C_2A_1A usually increased the weight of explants about three times and the cell number about six times. These increases in weight and cell number were appreciably greater than those induced by kinetin at 0·01 mg/l. In the above bio-assays, the media containing C_2A_1A was sterilized by autoclaving. When a solution of C_2A_1A was sterilized by filtration and added aseptically to autoclaved basal medium, the factor exhibited the same activity.

The effects which IAA, *myo*-inositol and adenine had on the activity of C_2A_1A were also studied (Table 4). IAA and *fractions* C_2 and N were previously found to interact synergistically in a characteristic way. If C_2A_1A and *myo*-inositol accounted for the activity of *fractions* C_2 and N, these isolated factors and IAA should also interact in a similar way. The results in Table 4 show that this did occur. The synergism between C_2A_1A and *myo*-inositol occurred in the presence of auxin, was accentuated by auxin and with explants from some carrots (e.g. carrot 2) was almost completely dependent on the presence of auxin. A synergism existed between C_2A_1A and IAA but not between IAA and *myo*-inositol. With explants

TABLE 4. WEIGHT AND CELL NUMBER OF EXPLANTS (DERIVED FROM TWO CARROTS) CULTURED IN THE PRESENCE OF IAA, C_2A_1A, *myo*-INOSITOL AND ADENINE SEPARATELY AND IN COMBINATIONS

	Average explant weight† (mg) and cell number† (in thousands, in parentheses)			
	Absence of IAA		Presence of IAA	
Additions to basal medium*	Carrot 1	Carrot 2	Carrot 1	Carrot 2
None	19 (100)	10 (66)	35 (148)	10 (66)
Fraction C_2A_1A	22 (161)	10 (67)	35 (450)	13 (112)
myo-Inositol	27 (127)	10 (68)	35 (150)	11 (69)
myo-Inositol + *fracton* C_2A_1A	47 (373)	11 (99)	70 (740)	33 (360)
myo-Inositol + adenine			36	11
myo-Inositol + *fraction* C_2A_1A + adenine		10	87	32

* Concentrations (mg/l.) of additives: C_2A_1A probably less than 0·001, *myo*-inositol 15, and adenine 3.

† Each value is the mean for at least 10 explants.

from some carrots only, a synergism could be demonstrated between adenine and C_2A_1A (see Table 4) and a slight enhancement of C_2A_1A activity resulted from the addition of enzymic hydrolysate of casein (250 mg/l.) to the culture media. Other workers have previously reported the ability of casein hydrolysate to increase the activity of synthetic cytokinins.[41]

Plum fruitlets, therefore, appeared to contain a cytokinin (C_2A_1A) which interacted with *myo*-inositol and auxin to induce cell division and which was considerably more active than kinetin. The amount of this cytokinin extracted from plum fruitlets was estimated to be about 0·6 mg/100 kg. Isolation of this factor in crystalline form in amounts needed for structural study was not possible because of the difficulty in obtaining the necessary quantities of plums of diameter 7–11 mm. Other plant materials such as coconut milk, immature wheat heads and immature sweet corn kernels were then extracted to see if they contained components similar or equivalent to C_2A_1A in amounts sufficient for isolation in a state of purity.

[41] E. M. SHANTZ, K. MEARS and F. C. STEWARD, *Plant Physiol.* 33, Suppl., xvi (1958).

Coconut milk and wheat heads did not appear to contain any appreciable amounts of components comparable to C_2A_1A, but extracts of corn kernels yielded a factor which appeared identical with C_2A_1A. This work is outlined later in this section.

The Factors in Fraction C_3

Fraction C_3, which was very active at about 8 mg/l. of medium, was further fractionated by extracting an aqueous solution (pH 4·2) with butan-1-ol. The extracts contained most of the activity and yielded on evaporation *fraction* C_3A (9 mg from 100 g of plums) which induced marked increments in explant weight at 1 mg/l. The *fraction* C_3B (71 mg from 100 g of plums) in the extracted aqueous solution induced only small increments even at 15 mg/l.

Fraction C_3A was chromatographed on paper using solvent 1. Two zones of activity were detected, one centred about R_f 0·30 and one about R_f 0·70. The components of these zones were separated by chromatography in other solvents.

The active zone of lower R_f was found to contain three u.v.-absorbing substances detectable by the Markham and Smith procedure[39] and also three ninhydrin-reacting compounds. Slight activity was associated with one of the former compounds, but the remainder of the above components were inactive. The substance or substances responsible for most of the activity of the zone of R_f 0·30 occurred at positions on the chromatograms where no component could be detected by chemical or physical methods.

The active zone of R_f 0·70 was found to contain three substances which fluoresced under u.v. light and a compound detected by an iodine spray or by Dragendorff reagent.[42] The activity of the zone was not associated with any of these components. The active substance was precipitated by silver ions and possessed chromatographic properties similar to those of *component* C_2A_1A.

The activity of *fraction* C_3, therefore, appeared to be due to highly active factors present at levels too low for detection by methods other than bio-assay.

Isolation of a Cytokinin from Sweet Corn Kernels

The fractionation procedure used with plum extract was applied to immature sweet corn kernels. When the cellulose-column fraction corresponding to C_2A_1 was subjected to two-dimensional chromatography (solvent 1 followed by 2), the method of Markham and Smith[39] detected a *component* M which was chromatographically indistinguishable from C_2A_1A in solvents 1, 2, 4 and 7. Chromatogram eluates containing M showed strong activity. Ultraviolet absorption spectra of factor M (Fig. 6) are almost identical with those recorded[27, 28] for plum *factor* C_2A_1A. The very small differences can be attributed to error in the spectra of C_2A_1A which were determined with chromatogram eluates of low optical density. C_2A_1A and M showed identical pK_a values (4·4 and 9·8). Both factors were revealed as red spots on chromatograms by the spray procedure of Reguera and Asimov,[40] but neither were located by ninhydrin or by a Dische spray[43] used to detect kinetin. Thus the two factors appeared to be identical. The amount of factor in corn extract was about five times the amount in plum extract.

By the methods described in the experimental section, *factor* M was isolated as a crystalline picrate which was converted into free base. The yield of crystalline picrate from 60 kg of kernels varied between 0·4 and 4 mg depending on the type of corn used and the period of storage at −20°. This growth stimulant has been named zeatin[27] and is the first cytokinin to be isolated in a state of purity from a plant.

[42] H. M. BREGOFF, E. ROBERTS and C. C. DELWICHE, *J. Biol. Chem.* **205**, 565 (1953).
[43] J. G. BUCHANAN, *Nature* **168**, 1091 (1951).

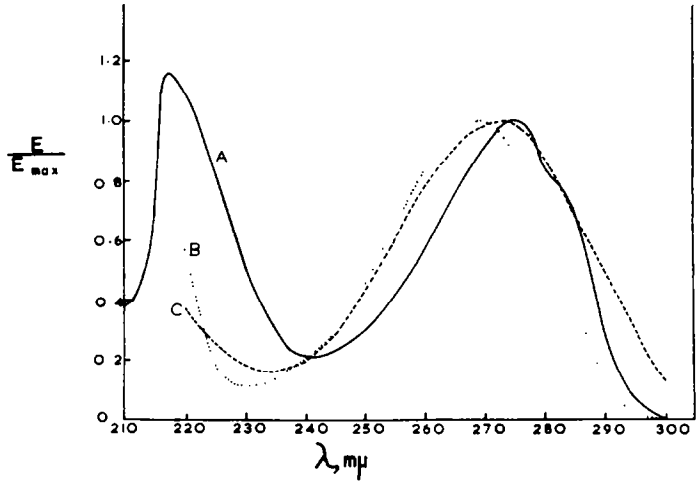


FIG. 6. ULTRAVIOLET ABSORPTION SPECTRA OF CORN FACTOR *M* IN THREE SOLVENTS.
A, 0·1 N NaOH; B, 95% ethanol; C, 0·1 N HCl.

The growth-promoting activities of zeatin, kinetin and 1,3-diphenylurea are compared in Table 5. In other experiments similar results were obtained. 1,3-Diphenylurea at 2 mg/l., the concentration found effective by Shantz and Steward,[21] did not appreciably promote growth. At 1 μg/l., kinetin induced only very small increments in weight and cell number and at 0·1 μg/l. was inactive. Zeatin, however, was very effective at 1 μg/l. and even at 0·1 μg/l. induced marked growth increments. Table 5 shows that explants derived from different carrots vary in their response to zeatin. The ratio, mean weight of explants cultured in the

TABLE 5. WEIGHT AND CELL NUMBER OF CARROT EXPLANTS CULTURED IN THE PRESENCE OF 1,3-DIPHENYLUREA, KINETIN AND ZEATIN

Additions to basal medium†	Average weight* (mg) and cell number* (in thousands, bracketed figures) of explants from four carrots (C1–C4)			
	C1	C2	C3	C4
None	14	17 (104)	15 (64)	44 (175)
1,3-Diphenylurea‡ (2 mg/l.)	14	16 (110)	13 (65)	53 (170)
Kinetin (10 μg/l.)	39	48	33	70
Kinetin (1 μg/l.)	18	23 (143)	18 (64)	54 (242)
Kinetin (0·1 μg/l.)	14	17	15	45
Zeatin (10 μg/l.)	56	68 (670)	30 (488)	82 (744)
Zeatin (1 μg/l.)	53	69 (692)	33 (435)	69 (730)
Zeatin (0·1 μg/l.)	42	37	24	58
CH§ (300 mg/l.)	14	19	15	40
CH + zeatin (10 μg/l.)	69	98	40	80
CH + zeatin (0·1 μg/l.)	44	48	33	61

* Each value is the mean for twenty explants.
† The basal medium contained *myo*-inositol (50 mg/l.) in addition to the usual components.
‡ The 1,3-diphenylurea used was purified by recrystallization.
§ CH = Casein hydrolysate (enzymic) supplied by Nutritional Biochemicals Corporation, Cleveland, U.S.A.

presence of zeatin (10 μg/l.)/mean weight of explants cultured on basal medium, for seven additional carrots was 4·3, 2·3, 1·8, 6·5, 3·0, 2·2 and 4·4. This indicates the type of variation to be expected. Table 5 shows that zeatin induced percentage increments in cell number which were considerably greater than the percentage increments in explant weight. The activity of zeatin was usually enhanced by the presence of enzymic hydrolysate of casein in the media. Photographs of explants cultured in the presence of zeatin, kinetin and coconut milk have previously been presented.[28] With explants from some carrots, zeatin at 1 μg/l. induced increments in explant weight which approached those given by coconut milk at 10% by volume. Usually, however, the increments in weight were about 50% of those induced by coconut milk. Filter-sterilized and autoclaved zeatin exhibited identical activity.

Zeatin as a Naturally-occurring Cytokinin

To determine whether zeatin was a naturally-occurring cytokinin or an active degradation product, the experiments outlined below were carried out.

Plum fruitlets (dia. < 11 mm) were frozen rapidly to −20° immediately after picking. After 24–48 hr, the fruit was extracted in the following three ways: (1) with 70% ethanol at 2°; (2) with 70% ethanol at room temperature; (3) with 70% ethanol at room temperature after being sliced while still frozen and then held in ethanol at 68–70° for 6 min.

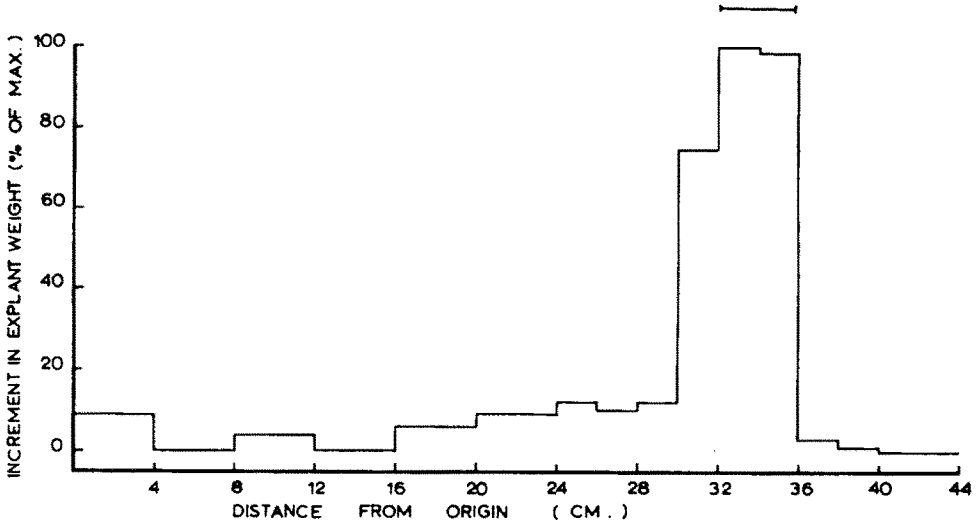


FIG. 7. A HISTOGRAM SHOWING THE ACTIVITY OF ZONES OF A PAPER CHROMATOGRAM (SOLVENT 2) OF THE BUTANOL FRACTION DERIVED FROM PLUM EXTRACT (3).

The position to which zeatin moved when chromatographed with the butanol fraction is shown by the line at the top of the figure.

The extracts were evaporated under vacuum ((1) and (2) at 30°, (3) at 40–45°) and the residues extracted with water. The resulting aqueous solutions were centrifuged and extracted with ethyl acetate (pH 3·4) and then butan-1-ol (pH 7). The butanol fraction from each extract was chromatographed in two solvents. On each chromatogram, one zone of activity was located and this coincided with the position to which added zeatin ran. The assay of one chromatogram is represented in Fig. 7 as indicative of the results obtained. A chromatogram (solvent 1) of the ethyl acetate fraction derived from extract (3) was also

assayed. Inhibitors known to occur in this fraction were located at R_f 0·2–0·5 while at about R_f 0·75 (the position to which added zeatin ran) weak growth-promoting activity was detected. In assays of the complete ethyl acetate fraction reported previously,[1] this cytokinin activity was apparently masked by the inhibitors.

Sweet corn kernels were excised at their base with a scalpel and immediately extracted in the following three ways: (1) with 70% ethanol at room temperature after being held in ethanol maintained at 70° for 6 min; (2) with 70% ethanol at 2°; (3) with 70% ethanol at room temperature after inactivation of phosphatase according to Bieleski[44] with methanol–chloroform–formic acid at −20°.

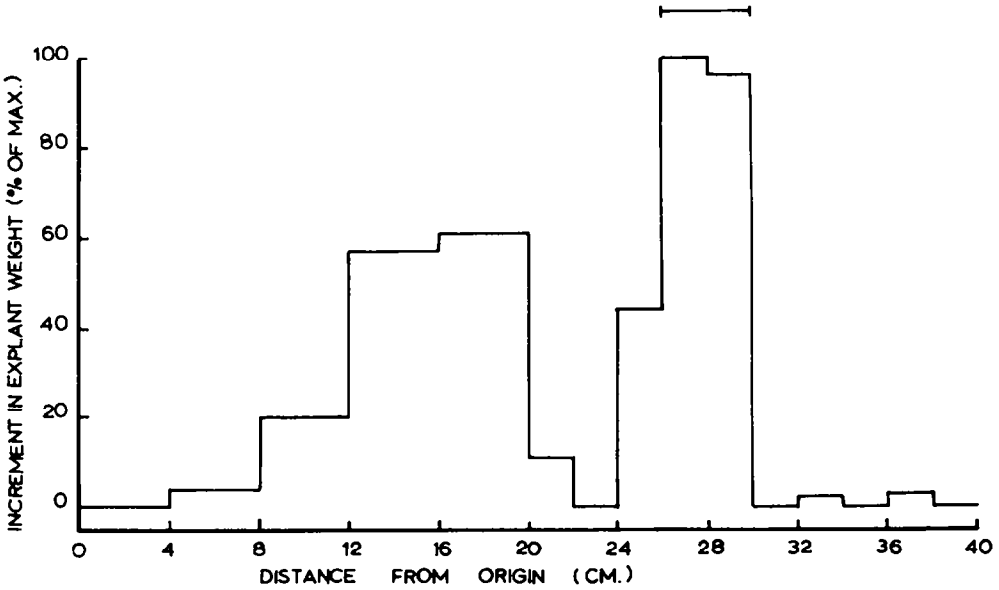


FIG. 8. A HISTOGRAM SHOWING THE ACTIVITY OF ZONES OF A PAPER CHROMATOGRAM (SOLVENT 1) OF THE BUTANOL FRACTION DERIVED FROM SWEET CORN EXTRACT (1).

The position to which zeatin moved when chromatographed with the butanol fraction is shown by the line at the top of the figure.

The extracts were evaporated under vacuum ((1) at 40°; (2) and (3) at 25°) and the residues were extracted with water. The resulting solutions were centrifuged and extracted with butan-1-ol at pH 7. The butanol fraction from extract (1) was chromatographed in solvents 1, 5 and 6, while the fraction from extract (2) was chromatographed in solvents 1 and 5. The fraction derived from extract (3) was chromatographed in solvent 1. A zone of activity, which coincided with the position to which added zeatin moved, was located on each chromatogram. Active substances of lower R_f were also detected on each chromatogram. The distribution of activity on one chromatogram is presented in Fig. 8 as indicative of the results obtained.

Plums and sweet corn kernels, therefore, appear to contain a cytokinin which is chromatographically indistinguishable from zeatin. This indicates that zeatin is not an artefact formed by enzymic or chemical degradation of another cytokinin. It has already been shown[28] that

[44] R. L. BIELESKI, *Biochim. Biophys. Acta* **74**, 135 (1963).

zeatin was not formed from a 1-substituted adenine by use of weakly alkaline solutions during purification.

The butanol fraction derived from corn extract (1) was found to be considerably less active than the fraction which did not partition into butanol. Since zeatin accounted for only part of the activity of the butanol fraction, most of the cytokinin activity in corn extract is due to substances other than zeatin. Zeatin does, however, appear to account for most of the cytokinin activity in plum fruitlet extract.

EXPERIMENTAL

Materials and Methods

Plum fruitlets and corn kernels. Plum fruitlets (variety Sultan) and immature sweet corn kernels (variety unknown) were frozen at −20° until required for extraction. The corn cobs were harvested immediately after the kernels turned yellow. Kernels used for the isolation of *factor M* were removed from the cobs with a corn stripping machine.

Solvents and paper chromatography. All solvents were purified before use. Percolation through a "Zeo-Karb" 225 (H) column was used to purify ethanol for the large-scale extractions. Impurities, which would have been adsorbed with the active substances when the extract was passed through such a column, were removed. All other solvents were redistilled.

The following solvent mixtures were used frequently for chromatography: (1) butan-1-ol–acetic acid–water (12:3:5); (2) propan-2-ol–water (4:1), 0·1 ml of conc. ammonia for each litre of tank volume was added to a beaker on the tank bottom; (3) butan-1-ol–N NH_4OH (10:7), upper phase; (4) propan-2-ol–conc. HCl–water (16:1:3); (5) butan-1-ol saturated with water; (6) butan-2-one saturated with water; (7) propan-2-ol–water (4:1).

Chromatograms which were to be bioassayed were run on paper sheets which had been washed by allowing N formic acid to flow down them for several days. This paper was also used when separated components were to be eluted for spectral study. An equal area of adjacent paper was eluted to provide a blank. Whatman No. 120 and 3MM papers used in the isolation of *factor M* were also washed in the above way. Whatman cellulose phosphate paper (P 20) was converted to the acid form by washing with N HCl and then with water.

Evaporation of solvents. All solvents were evaporated under reduced pressure (15–20 mm) using a rotary film evaporator for small volumes and a cyclone evaporator for large volumes.

Preparation of deactivated charcoal. B.D.H. activated charcoal was deactivated by the method of Synge and Tiselius[45] using palmitic acid in place of stearic acid. The deactivated product contained 3 per cent of palmitic acid.

Bioassay of cytokinins. Extracts and fractions were assayed for cytokinin activity by determining their ability to promote the growth of carrot secondary phloem explants cultured as previously described.[1] Growth increments were determined after 21 days. The culture tubes were exposed to continuous fluorescent lighting in a room maintained at 24°. Media were normally sterilized by autoclaving (15 lb for 0·5 hr) after addition of the material being tested. On occasions, however, a solution of the material for assay was filter sterilized using a Jena sintered glass filter (grain size 5f) and added aseptically to autoclaved basal medium. Unless otherwise stated, all media contained IAA at 2 mg/l.

Zones of chromatograms were usually assayed for activity by placing portions of them directly in the culture tubes prior to autoclaving. At times, however, the zones were eluted (usually with ethanol) and the eluate tested for activity.

[45] R. L. M. SYNGE and A. TISELIUS, *Acta Chem. Scand.* 3, 231 (1949).

Explant cell number was determined after chromic acid maceration.[46]

Melting-point determinations. All m.p. are corrected. When only small amounts of product were available, m.p. were determined with a Kofler apparatus. The stage was heated to about 15° below the m.p. before the sample was inserted.

Extraction of Plum Fruitlets and Ion-exchange Fractionation

Frozen fruitlets (240 g, dia. 7–11 mm) were blended with 85% ethanol (1·1 l.) and the resulting macerate was stirred for 0·5 hr. After the extract had been filtered through muslin and then centrifuged, it was concentrated to 120 ml at 40°. Material which separated from solution was centrifuged down and stirred with water (120 ml). The resulting suspension was centrifuged and the supernatant liquid added to the concentrated extract. The resulting solution (pH 3·3) was shaken with three 480-ml volumes of ethyl acetate. The extracted aqueous solution was freed from ethyl acetate at 40° under reduced pressure, adjusted to the original volume and passed through a column (dia. 3 × length 20 cm) of "Zeo-Karb" 225 (H form, 14–52 mesh, 8% DVB) which was then washed with water (350 ml). The effluent was percolated through a column (dia. 3 × length 16 cm) of either "De-Acidite" G or "Amberlite" IR-4B (free base form, 20–50 mesh) which was washed with water (480 ml). The effluent from the latter columns contained 3·1 g (*fraction N*). The "Zeo-Karb" 225 column was eluted with 70% ethanol (750 ml), water (350 ml), 1·5 N NH_4OH (1·1 l.), water (350 ml), 0·4 N HCl (750 ml), 1·5 N HCl (550 ml) and 6 N HCl (500 ml). The effluent alcohol, ammonia and 6 N HCl when evaporated yielded *fractions* C_1 (62 mg), C_2 (0·82 g) and C_3 (0·20 g) respectively. The weakly basic columns were eluted either with 0·3 N HCl (600 ml) or with 1·5 N NH_4OH (1 l.). Evaporation of the effluent ammonia yielded *fraction A*.

Isolation and Identification of the Factor in Fraction N

Fraction N (660 mg, the equivalent of 55 g of plums) was dissolved in water and applied as a narrow streak (length 30 cm) to washed Whatman seed-test paper (thickness $\frac{1}{16}$ in.). The chromatogram was developed with solvent 7 and, immediately after removal from the tank, was placed on a glass plate and overlaid with a sheet of Whatman No. 1 paper. The edge of a Petri dish was rolled firmly over the sheets in the direction of the solvent flow. Parallel rollings were made at intervals across the sheets. The locations of the zones on the chromatogram were determined by drying and treating the No. 1 paper with the reagents of Trevelyan *et al.*[30] The active zone (R_f 0·17) was located between a much fainter zone (R_f 0·23) and a zone containing phenolic compounds which streaked from the origin. The latter zone was also detectable with diazotized sulphanilic acid reagent and with ferric ammonium sulphate spray. The material eluted from the active zone with water was crystallized from propan-2-ol–water to give 26 mg of crude product containing impurities difficult to remove by recrystallization. Removal of these was effected by the method used by Hawthorne[47] for purifying inositols involving acid treatment and resin purification. The purified product was crystallized from propan-2-ol–water to yield thick needles (18 mg).

The following observations established that this compound was *myo*-inositol. The substance (Found*: C, 39·8; H, 6·6; O, 53·4. Calc. for $C_6H_{12}O_6$: C, 40·0; H, 6·7; O, 53·3%) was chromatographically indistinguishable from *myo*-inositol in three solvents and had m.p. 225–226·5° unchanged by admixture with authentic *myo*-inositol (m.p. 225–226·5°).

* Analysis by A. Bernhardt, Max-Planck-Institut fur Kohlenforschung, Mulheim, Germany.

[46] R. Brown and P. Rickless, *Proc. Roy. Soc. London* B, 136, 110 (1949).
[47] J. N. Hawthorne, *Biochem. J.* **81**, 425 (1961).

Acetylation (acetic anhydride and zinc chloride) and recrystallization from ethanol yielded an acetate, m.p. 217·5–218·5° unchanged by admixture with *myo*-inositol hexaacetate (m.p. 217·5–218·5°). In a growth test, the isolated factor and *myo*-inositol induced identical increments in explant weight.

Preparation of Fraction C_2 *on a Large Scale and Purification of Active Compounds*

Frozen plum fruitlets (30 kg) of dia. 8–15 mm were blended with purified 95% ethanol (90 l.). The macerate was stirred for 0·5–1 hr before being filtered first through muslin and then through a perforated basket-type centrifuge rotor lined with filter paper. The resulting solution (110 l., dry matter content 1·92 kg) was halved and each half percolated through a column (dia. 7·5 × length 75 cm) of "Zeo-Karb" 225 (H form, 14–52 mesh, 8% DVB) which was washed with 70% ethanol (16 l.) and with water (5 l.) and then eluted with 1·5 N NH_4OH (16 l.). The effluent ammonia from the two columns was combined and evaporated at 30° yielding *fraction* C_2 (102 g). A solution of C_2 in water (2 l.) was adjusted to pH 3·3, mixed with $AgNO_3$ solution (100 g/l., 600 ml) and cooled to 1°. After 3 hr, the precipitate was centrifuged down and washed at 1° with $AgNO_3$ solution (10 g/l., 300 ml). Activity could be recovered from the precipitate by dilute HCl at room temperature but more completely at about 45°, the factors recovered at the two temperatures being chromatographically indistinguishable. The HCl could be removed without loss of activity or change in the R_f of the factor by rapid evaporation *in vacuo* at 40° or by extraction with a chloroform solution of tri-*n*-octylamine. Removal by use of the weakly basic resin "De-Acidite" G (free base form) resulted in a partial loss of activity. A suspension of the precipitate obtained above in 0·2 N HCl (1·5 l.) was, therefore, stirred for 2 hr at room temperature and for 0·5 hr at 40–45° before being centrifuged. Volumes of 100 ml were then evaporated to dryness *in vacuo* at 40° using a rotary film evaporator fitted with a 5-l. evaporation flask. The residues were dissolved in 20-ml volumes of water and re-evaporated to render the removal of HCl more complete. Bulked solutions of the final residues were stirred with sufficient "De-Acidite" G (free base) to adjust the pH to 3·1 and then evaporated yielding *fraction* C_2A (0·86 g). This pH adjustment with resin was not accompanied by a detectable loss of activity.

Fraction C_2A was stirred with solvent 1 (30 ml) and the insoluble tar filtered off. Equal volumes (15 ml) of the solution were each applied to a carefully packed column (dia. 5 × length 42 cm) of Whatman ashless cellulose powder (material passing an 80-mesh sieve, 340 g) which had been exhaustively washed with solvent 1. The chromatograms were developed with solvent 1 and the effluent collected in 15-ml volumes. Fractions 63–70 (from one column only) containing C_2A_2 were combined and evaporated. To a solution (pH 3) of the residue in water, an excess of a saturated aqueous solution of picric acid was added. The precipitate was centrifuged down, washed with water and crystallized from water to give long slender needles of C_2A_2 picrate (88 mg) with m.p. 296–297° (decomp.). Part of this product was converted to C_2A_2 hydrochloride which was crystallized from 95% ethanol to yield needles of m.p. 286–287° (decomp.). These m.p. are identical with those found for adenine picrate and hydrochloride; mixed m.p. showed no lowering. C_2A_2 and adenine possessed identical u.v. spectra and were indistinguishable when chromatographed on filter paper (solvents 1, 2 and 4) or on cellulose phosphate paper (H form; solvents: 0·1 N and 1 N HCl, N formic acid).

Fractions 42–45 from both cellulose columns were very active and were all combined to yield on evaporation *fraction* C_2A_1 (9 mg). For two-dimensional chromatography (solvents 1 and 2), 0·3–0·6 mg of C_2A_1 was applied to each sheet of washed Whatman No. 1 paper. C_2A_1A and C_2A_1B were revealed by u.v. printing[39] as very faint spots.

Isolation of Factor M *from Sweet Corn Kernels*

Two procedures used for isolating *factor M* are described below.

Procedure 1. Frozen immature corn kernels (60 kg) were blended with purified 95% ethanol (180 l.) to give a macerate which was stirred for 2–3 hr and then filtered through muslin. The extract was clarified using a basket-type centrifuge rotor lined with filter paper and then percolated through a column (dia. 10·2 cm × length 85) of "Zeo-Karb" 225 (H form, 14–52 mesh, 8% DVB) which was washed first with 70% ethanol (18 l.) and then with water (9 l.). The column was eluted with 1·5 N NH_4OH (30 l.) which was evaporated at 35°. To a solution of the residue in water (1·8 l.; solution pH adjusted to 3·95 with dilute HNO_3), $AgNO_3$ solution (100 g/l.; 800 ml) was added and the resulting mixture was held at 2° for 2–3 hr. The precipitate was centrifuged down, washed with $AgNO_3$ solution (10 g/l.; 350 ml) at 2° and stirred with 0·2 N HCl (1 l.) at room temperature for 2 hr and then at 45° for 0·5 hr. Volumes of 100 ml of the centrifuged acid extract were evaporated rapidly at 40° using a rotary evaporator fitted with a 5-l. evaporation flask. Solutions of the residues were bulked (total volume 500 ml), adjusted to pH 7 and shaken with three 1-l. volumes of butan-1-ol. The residue obtained by evaporation of the butanol extracts was stirred with 95% ethanol (40 ml) at room temperature. Undissolved material was centrifuged down and stirred with a further 20 ml of 95% ethanol. The ethanol extracts were combined and streaked across the bottom of nine sheets (width 40 cm) of washed Whatman No. 120 paper. The chromatograms were developed with solvent 1 and examined under u.v. light. The absorbing zones at about R_f 0·7 were eluted with N formic acid which was then evaporated. A solution of the residue in ethanol was streaked across the bottom of 8 sheets (width 40 cm) of washed Whatman No. 3MM paper which were developed with solvent 2. Two u.v.-absorbing zones were present on the chromatograms. The one of higher R_f (about 0·8) was eluted with N formic acid which was then evaporated. To a solution (pH 3) of the residue in water (0·5 ml), a saturated aqueous solution of picric acid (0·6 ml) was added yielding a precipitate which was centrifuged down and washed twice with water (0·5 and 0·3 ml). Crystallization from water yielded clusters of fine needles which were centrifuged down, washed with water (0·8 ml) and dried *in vacuo* at room temperature over P_2O_5. When the resulting product (2·4 mg) was chromatographed only two components were detectable—picric acid and *factor M*. The crystals melted at 189–190° (Kofler apparatus) after softening at about 186°. An u.v. absorption spectrum is shown in Fig. 9.

Procedure 2. Extract from 60 kg of corn kernels (for preparation see procedure 1) was percolated through a column of "Zeo-Karb" 225 (identical with that used in procedure 1) which was washed first with 70% ethanol (16 l.) and then with water (9 l.) before being eluted with 4 N HCl (20 l.). Evaporation of the acid at 30° yielded a syrup which was dissolved in water (0·9 l.). The solution was adjusted to pH 7 and shaken with four 1-l. volumes of butan-1-ol which were combined and evaporated at 50°. To a solution (pH to 3·9 with HNO_3) of the residue in water (600 ml), $AgNO_3$ solution (200 g/l., 520 ml) was added and the mixture held at 0° for 3 hr. After the precipitate which formed had been washed at 2° with $AgNO_3$ solution (10 g/l., 400 ml), it was stirred with 0·2 N HCl (1 l.) at room temperature for 2–3 hr and then at 40–45° for 0·5 hr. The acid extract was evaporated rapidly in 100-ml volumes as in procedure 1. Water was added to the residues and evaporated to render the removal of acid more complete. An aqueous solution (300 ml) of the combined residues was adjusted to pH 2·9 by the addition of "De-Acidite" G (free base) which was then filtered off and washed with water. The washing and solution at pH 2·9 were combined and evaporated to give a

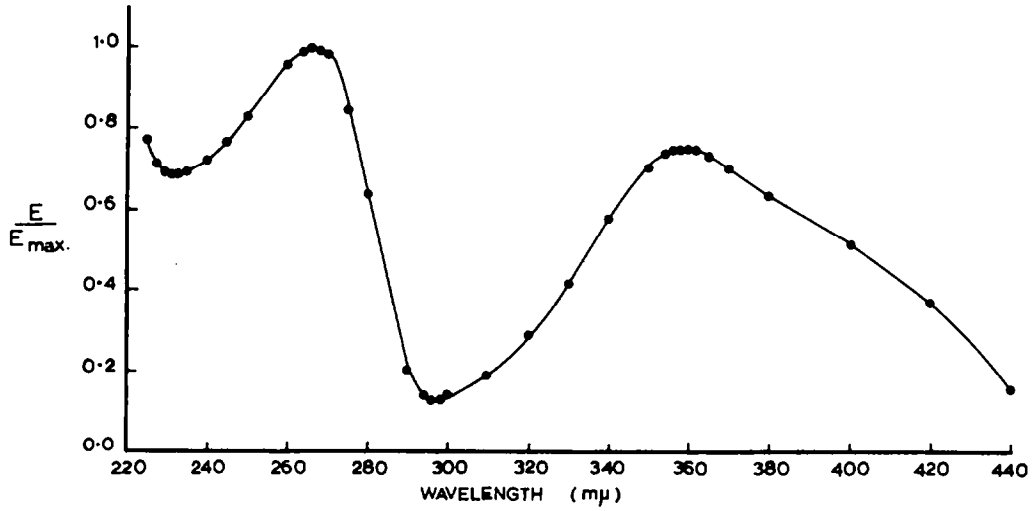


FIG. 9. ABSORPTION SPECTRA OF PICRATES OF FACTOR *M* IN ABSOLUTE ETHANOL.
The spectrum of picrate prepared by procedure 1 (see text) is represented by the series of points. The curve defines the spectrum of picrate prepared by procedure 2.

residue which was stirred at room temperature with 95% ethanol (25 ml). Undissolved material was centrifuged down and washed with 95% ethanol (15 ml). The ethanol solutions were combined and evaporated yielding a residue which was chromatographed on paper with solvents 1 and 2 as described in procedure 1. *Factor M* was then eluted and precipitated as a picrate as in procedure 1. Crystallization from water yielded clusters of fine needles which were washed with water and dried *in vacuo* at room temperature over P_2O_5. The yield was 3·6 mg with m.p. 188–190° (Kofler) preceded by some softening at 186°. Recrystallization did not alter the m.p., growth-promoting activity or the u.v. spectrum (see Fig. 9) of the picrate. This spectrum is identical with that of the picrate obtained in procedure 1.

In the two isolations described above, the same batch of corn kernels was used. This had been stored at −20° for about 4 weeks. Some of the same batch of corn was stored at −20° for a further 8 months and then processed according to procedure 2. From 60 kg, only 1 mg of crystalline picrate was obtained. A sample (60 kg) of immature cattle maize yielded about 0·4 mg of crystalline picrate by procedure 2.

Factor M picrate was converted into the free base by the procedure next described. Picrate (2 mg) was dissolved in the minimum volume of water at 50° and the solution acidified to pH 3 with acetic acid. "De-Acidite" FF (acetate form, 100–200 mesh) was added with stirring until the solution was free from picric acid. The resin was filtered off and the solution was evaporated at 50° to small volume under vacuum before being filtered into a micro centrifuge tube. Evaporation was completed at 50° in a current of nitrogen. The residue was left in a vacuum desiccator with NaOH pellets for several days and then crystallized from absolute ethanol to give clusters of short needles. These were centrifuged down, washed with hexane–absolute ethanol (1:3) and dried *in vacuo* over P_2O_5. The yield was 0·42 mg, m.p. 208–209° (Kofler).

Acknowledgements—Thanks are due to Miss J. K. Grigor and Miss M. E. Page for assistance with the bio-assays and to Mr. O. Winn for help in collecting plum fruitlets.