CYTOKININS FROM APPLE EXTRACT AND COCONUT MILK

By J. A. Zwar* and M. I. Bruce*†

[*Manuscript received September* 29, 1969]

Summary

Cytokinin containing extracts of young apples and of coconut milk were purified and subjected to chemical treatments designed to modify the chemical natures of the cytokinins. Chromatographic comparisons were then made between the cytokinins of the treated and untreated extracts and zeatin and zeatin nucleoside.

The results suggest that although zeatin may be one of the cytokinins of coconut milk it does not occur in apple extract. Some evidence was obtained that the apple cytokinin is a derivative of zeatin but it does not seem to be zeatin nucleotide.

I. Introduction

Zeatin, a naturally occurring cytokinin, was isolated from sweet corn extracts (Letham 1963) and later characterized (Letham, Shannon, and McDonald 1964, 1967) as an N^6-substituted adenine. Subsequently two compounds provisionally identified as the nucleoside and the nucleotide of zeatin have been detected in maize milk (Miller 1965) and isolated (Letham 1966*a*, 1966*c*).

Some of the methods used in the isolation of zeatin were necessarily severe and the possibility that a biologically active artefact may have been produced was recognized. However, recent evidence (Miller 1965; Letham and Miller 1965; Letham 1966*b*) supports the belief that zeatin as such occurs in maize milk and in extracts of young plums. The results of chromatographic comparisons between zeatin and the partially purified cytokinins of apple extract and of coconut milk reported here suggest that free zeatin may occur in the latter but not in the extract of apple fruitlets.

II. Materials and Methods

(*a*) *Source*

Cytokinins were obtained from an aqueous ethanol extraction of Granny Smith apple fruitlets harvested between 7 and 21 days after pollination (Zwar, Bottomley, and Kefford 1963). This extract originally of 3000 litres was concentrated 36-fold by evaporation of water and ethanol. The minimum concentration showing cytokinin activity was then 3 mg dry weight per millilitre.

(*b*) *Bioassay*

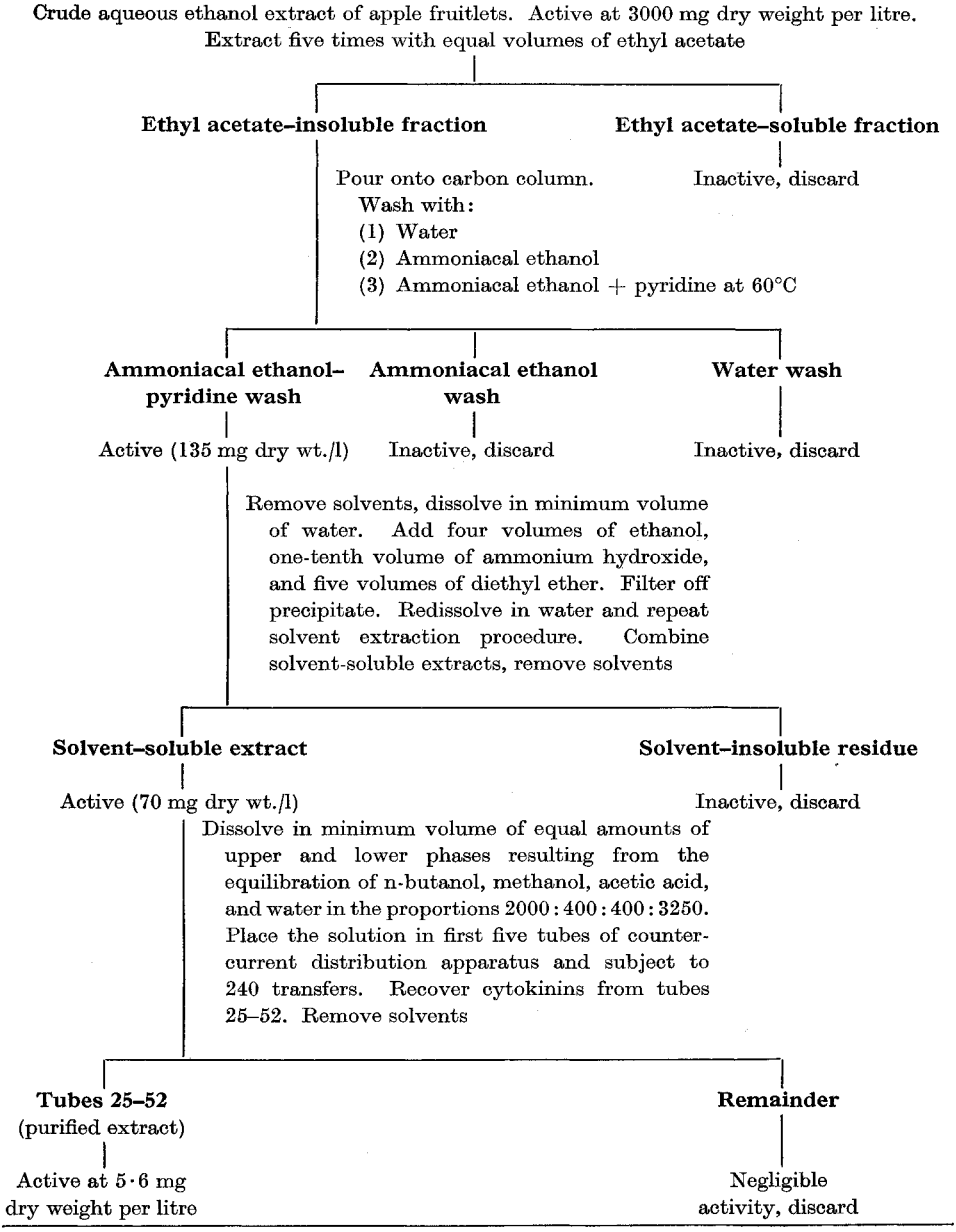
The tobacco pith test for cell division (Bottomley *et al.* 1963) was used to assay cytokinin activity. A measurement of activity at any stage of purification was obtained by bioassay of a dilution series and determination of the dry weight of residue present at the greatest dilution giving perceptible cell division. Activities obtained in this way were used for the calculation of purifications achieved between steps.

* Division of Plant Industry, CSIRO, P.O. Box 109, Canberra City, A.C.T. 2601.

† Present address: School of Chemistry, University of Bristol, England.

TABLE 1

PURIFICATION OF APPLE EXTRACT



(c) *Purification of Apple Extract*

The purification steps are shown in the flow diagram (Table 1). The preparation and running of the carbon column has been described (Zwar, Bottomley, and Kefford 1963). The other two procedures gave high yields of activity and were easily adapted to large-scale use. Moreover none of these steps exposed the extract to strong acids which have been shown to change the chemical identity of cytokinins without destroying the activity (Zwar *et al.* 1964).

(d) *n-Butanol Extraction of Purified Apple Extract*

A 1-ml sample of purified apple extract was extracted six times with equal volumes of water-saturated n-butanol. The extracts were combined, the n-butanol was removed by evaporation under reduced pressure, and water was added to 1 ml. The butanol-insoluble extract was freed from butanol by evaporation under reduced pressure and made to 1 ml.

(e) *Acid Treatment of Apple Extract*

To 1 ml of purified extract (representing 200 g of apple fruitlets) 4 ml of 1N hydrochloric acid were added and the mixture was placed in a boiling water-bath for 1 hr. After cooling, the mixture was diluted 20 times with water and poured onto an 8 by 1 cm column of Dowex-50 in the H^+ form. When this had percolated into the column it was washed with water until the effluent was neutral. The active material was then eluted from the column with 200 ml of 1N ammonium hydroxide and after removal of the ammonia the eluate was made to 10 ml with water.

Acid treatment of purine nucleotides and nucleosides generally results in the production of the corresponding free bases. However, it has been shown (Hall and Srivastava 1968) that when the cytokinin N^6-isopentenyladenosine is treated with acid the free base is not obtained but instead a substance identified as N^6-(3-methyl-3-hydroxybutyl)adenine which has cytokinin activity. Less is known about the effects of acid treatment on zeatin and its derivatives, but it has been found (Hall, personal communication 1969) that nothing comparable to N^6-(3-methyl-3-hydroxybutyl)adenine is formed. Therefore in this work it has been assumed that zeatin would be among the products of acid treatment of zeatin derivatives.

n-Butanol-soluble and -insoluble fractions were also acid-treated with Dowex-50-H^+. A resin slurry (0·5 ml) was mixed with the equivalent of 1 ml of extract and held for 150 sec in a boiling water-bath. The resin was filtered off and the filtrate was then added to the top of a Dowex-50-H^+ column and the active material recovered by ammonia elution as in the case of hydrochloric acid treatment.

(f) *Lanthanum Chloride Treatment of Purified Extracts*

The aqueous solutions of n-butanol-soluble and -insoluble materials (see above) were treated by the method of Bacher and Allen (1951) for the dephosphorylation of nucleotides.

The treated fractions within each of the two classes were extracted six times with n-butanol, the extracts were combined, and the butanol was removed. After dissolving in water the extracts were chromatographed by thin-layer chromatography.

Parallel tests of this procedure with 5′-adenylic acid showed that in this case the product was almost entirely adenosine and that only a trace of adenine could be detected. From this and the data of Bacher and Allen it was assumed that if the active material of apple extract were a nucleotide this procedure would produce the nucleoside.

(g) *Purification and Acid Treatment of Coconut Milk*

To 3380 ml of milk drained from 18 coconuts ethanol was added to give a 70% solution and the precipitated protein was filtered off and discarded. The solute was recovered, redissolved in water, and purified by extraction with ethyl acetate and passage through a carbon column as with the apple extract. It was then passed through a column of nylon deposited on kieselguhr (Carelli, Liquori, and Mele 1955) and the water effluent which contained the active material was concentrated to 68 ml. 1 ml of this solution contained 7·6 μg dry weight of solute and corresponded to 50 ml of the original milk. 20 ml of 1N HCl were added to 5 ml of this solution and this mixture was heated and the active material recovered from Dowex-50-H^+.

(h) Thin-layer Chromatography

Chromatography was carried out on layers of Avicel microcrystalline cellulose (Wolfrom, Paton, and de Lederkremer 1964). To prepare plates (usually 20 by 20 cm) 25 g of Avicel were slurried with 110 ml of water in an Omnimix blender for 40 sec and layered with a Desaga spreader to a thickness of either 0·25 or 1 mm. The plates were allowed to dry overnight at room temperature.

Extracts were streaked onto a line drawn 2·5 cm from one edge of a plate. Four streaks each about 4 cm long could be accommodated on each plate, leaving sufficient space between so that no mixing occurred during development.

TABLE 2

R_F VALUES × 100 OF ZEATIN, KINETIN, AND OF CYTOKININS FROM APPLE EXTRACT AND HYDROCHLORIC ACID-TREATED APPLE EXTRACT

Solvent	Zeatin	Kinetin	Purified Apple Extract	Acid-treated Apple Extract	
				Expt. I	Expt. II
n-Butanol–methanol–ammonia–water (5 : 2 : 1 : 1 v/v)	69–86	—	40–63	69–86	69–86
t-Amyl alcohol–n-propanol–water (4 : 1·5 : 1·5 v/v)	80–96	—	23–45	85–96	85–96
n-Butanol–acetic acid water–(4 : 1 : 1 v/v)	69–91	—	0–51	74–85	69–85
n-Butanol–piperidine–digol–water (9 : 3 : 2 : 4 v/v)	74–85	74–85	45–69	—	80–85
Diisopropyl ether–acetic acid–methanol–water (25 : 10 : 2 : 5 v/v)	45–68	74–91	0–11	—	45–57
Chloroform–methanol–water–acetone (30 : 15 : 5 : 15 v/v)	49–74	68–90	3–23	—	49–68

After development in a suitable solvent, plates were dried in a stream of hot air and the part of the plate over which each streak had run was divided into three longitudinally, and again divided in the transverse direction. The cellulose from the resulting strips, each 0·9 by 1·3 cm was then removed with a razor-blade and transferred to an assay vial. When a comparison of a treated extract with zeatin or zeatin nucleoside was made a larger section of the plate was used for the extract. In such a case the area over which the extract was run was divided into four longitudinally and after the transverse division the resultant cellulose strips were 0·9 by 4 cm. The zeatin and zeatin nucleoside were applied as spots and after development were located by their ultraviolet absorption.

A piece of filter paper to support the pith block and medium without added cytokinin were also placed in the vial and the tubes were assayed after sterilization (Bottomley *et al.* 1964).

When R_F comparisons were made, all the compared values were obtained from a single plate. This avoided errors caused by small changes in R_F observed in chromatograms of the same substance run on different occasions. The width of the strip corresponded to a difference in R_F values of about 0·06.

pH control in the aqueous s-butanol solvent was achieved by equilibrating s-butanol with 0·1M Tris buffer (pH 8·0) or 0·05M potassium acid phthalate (pH 4·0). The organic phase was used for chromatography in each case.

III. Results

(a) *Comparisons of Apple Extract and of Acid-treated Apple Extract with Zeatin and Kinetin*

The R_F values of the cytokinins of apple extract, acid-treated apple extract, and zeatin and kinetin are shown in Table 2. The values describe the limits of the active areas of the chromatograms as revealed by bioassays of the 0·9-cm wide strips. The two samples of acid-treated apple extract were prepared on different occasions.

Although the results shown in Table 2 do not demonstrate the presence of more than one cytokinin in purified apple extract, n-butanol extraction of the purified extract gave activity in both the extract and the residue (Table 3).

Table 3

R_F values × 100 of cytokinins from treated apple extract, zeatin nucleoside, and zeatin chromatographed in aqueous s-butanol

Fraction Chromatographed and Assayed	pH	Apple Cytokinins*	Zeatin Nucleoside	Zeatin
n-Butanol-soluble	8·5	18–41	88–94	94–front
		37–50	81–94	87–94
		23–47	76–82	82–88
	4·0	13–40	80–87	87–93
Lanthanum chloride-treated; n-butanol-soluble	8·5	73–86	73–80	80–86
	4·0	30–37	76–88	88–94
		82–88		
Dowex-50-H^+-treated; n-butanol-soluble	8·5	82–88	76–82	88–94
	4·0	80–87		80–93
n-Butanol-insoluble	8·5	12–23	82–88	94–front
		6–20	80–86	86–93
	4·0	6–26	80–87	87–94
Lanthanum chloride-treated; n-butanol-insoluble	8·5	87–front	75–87	81–94
		81–front	—	—
		(35–41; 82–88)	76–88	82–88
	4·0	40–53	—	
		40–60	82–88	88–front
		(31–38; 87–93)	81–94	86–93
Dowex-50-H^+-treated; n-butanol-insoluble	8·5	41–47	82–88	88–front
		47–53	—	88–94
	4·0	Not seen	—	—

* Unbracketed values show replicate determinations of cytokinin R_F values. Bracketed values show zones of activity on the same chromatogram.

Hence either the apple extract contained more than one cytokinin or a single cytokinin with low solubility in n-butanol. The differences in chromatographic

behaviour between the treated products of the n-butanol-soluble and -insoluble fractions (see below) strongly suggest that more than one cytokinin was present.

Hydrochloric acid treatment changed the R_F of the cytokinin of apple extract and also decreased the amount of activity. However, there was no increase in the number of cytokinins with this treatment. In all six solvents the R_F values of the cytokinins of the acid-treated extract were different from those of the starting material, and in every case the R_F values of the cytokinins of the acid-treated extract were indistinguishable from those of zeatin.

If the cytokinin of the acid-treated material was not zeatin but a substance chemically similar to it, chromatographic separations might be difficult. Solvents with the capacity to separate zeatin from the related substance kinetin might be suitable systems for achieving such a separation. The last two solvents separate zeatin and kinetin but the R_F values of the cytokinins of acid-treated extract remain indistinguishable from those of zeatin. Thus there is good evidence for the production of zeatin in the acid-treated extract.

TABLE 4

R_F VALUES × 100 OF ZEATIN, KINETIN, AND OF CYTOKININS FROM COCONUT MILK AND ACID-TREATED COCONUT MILK

Solvent	Zeatin	Kinetin	Coconut Milk	Acid-treated Coconut Milk
n-Butanol–digol–piperidine–water (9 : 2 : 3 : 4 v/v)	70–88	76–88	22–34 46–52 58–70 82–88	58–64 76–88
Diisopropyl ether–acetic acid–methanol–water (25 : 10 : 2 : 5 v/v)	46–70	64–94	0–22 52–64	0–22 52–76
Chloroform–methanol–water–acetone (30 : 15 : 5 : 15 v/v)	52–76	58–82	0–40 58–76	4–10 22–40 52–76 82–94

(*b*) *Comparisons between the n-Butanol-soluble Fraction of Apple Extract, the n-Butanol-insoluble Fraction, and Lanthanum Chloride- and Dowex-50-H^+-treated Material from Each of these with Zeatin Nucleoside and with Zeatin*

Table 3 shows these comparisons, all of which were made in the solvent s-butanol saturated with buffer. These results show that:

(1) The R_F values of zeatin and its nucleoside were unaffected by pH.

(2) Neither the n-butanol-soluble fraction nor the -insoluble fraction contained zeatin or its nucleoside.

(3) A product was produced by lanthanum chloride treatment of the n-butanol-soluble fraction which chromatographed indistinguishably from zeatin nucleoside in this solvent. The R_F of this product was the same at the two pH values used.

(4) Dowex-50-H^+ treatment of the n-butanol-soluble fraction gave a product which, within limits of experimental error, was indistinguishable from zeatin. The R_F did not change with pH.

(5) Lanthanum chloride treatment of the n-butanol-insoluble fraction gave rise to a cytokinin which had a different R_F from the starting material and the R_F of which changed with pH. At high pH most of the product was seen at high R_F, at low pH most of it was at a lower R_F.

(6) Dowex-50-H^+ treatment of the n-butanol-insoluble fraction gave a product which had an R_F markedly different from zeatin at pH 8·0. At pH 4·0 in two experiments the activity could not be detected, presumably because it was dispersed.

(c) *Comparisons of Coconut Milk and of Acid-treated Coconut Milk with Zeatin and Kinetin*

The R_F values of the cytokinins in coconut milk before and after hydrochloric acid treatment and of zeatin and kinetin are compared in Table 4.

Both acid-treated and non-acid-treated coconut milk contained several cytokinins. Acid treatment resulted in elimination of some, the production of some new ones, and the reduction of activity of others. In the three solvents, untreated coconut milk contained a cytokinin with the same R_F as zeatin and this was also true for acid-treated coconut milk.

IV. Discussion

From these results it would seem unlikely that zeatin itself occurs in apple extract. Letham (1966*a*) has reported that both zeatin and its nucleoside are soluble in n-butanol. Both of these substances would therefore have been expected to run near the front in the counter-current distribution step of the purification procedure. No activity was found in these fractions although amounts equivalent to large weights of apples were processed. Despite this expectation an n-butanol-soluble cytokinin was found in the purified extract. However, Table 3 clearly shows that this was not zeatin or its nucleoside. Therefore, if present at all zeatin and its nucleoside must be minor components of the total cytokinin content.

The R_F values of the cytokinins changed on hydrochloric acid treatment of the unfractionated apple extract. In all six solvents only one zone of cytokinin activity could be detected in acid-treated extract and in every one its R_F was indistinguishable from that of zeatin. This was true even in those solvents which separate zeatin and kinetin and which might distinguish between zeatin and other N^6-substituted purines. It seems likely therefore that the cytokinin present in hydrochloric acid-treated apple extract is zeatin.

Dowex-50-H^+ treatment has been used to hydrolyse polysaccharides (Ivacenko, Zajac, and Kouril 1963). The effect of this treatment on the n-butanol-soluble fraction was to produce a cytokinin with an R_F indistinguishable from zeatin. The effect on the n-butanol-insoluble material, on the other hand, was to produce a cytokinin which was clearly not zeatin. As hydrochloric acid treatment of the whole extract produced only one cytokinin zone with an R_F identical to zeatin (Table 2),

the two treatments cannot have effected the same reactions. Perhaps the cytokinin of the n-butanol-insoluble material contains more than one sugar residue attached to the active portion of the molecule and the gentler Dowex-50-H^+ treatment removed only some of the sugar moieties whereas the more vigorous hydrochloric acid treatment removed all of them.

Although lanthanum chloride treatment of the n-butanol-soluble fraction produced a cytokinin with an R_F indistinguishable from zeatin nucleoside in one solvent, this cannot be accepted as evidence of the occurrence of the nucleotide, for the latter is not soluble in n-butanol (Miller 1965). Lanthanum chloride treatment of the n-butanol-insoluble fraction gave a cytokinin the R_F of which changed with pH and which therefore could not be zeatin nucleoside. Again it seems unlikely that the butanol-insoluble fraction contained the nucleotide.

Miller (1965) has shown that there are three cytokinins in maize milk and has presented evidence that they are zeatin, its nucleoside, and its nucleotide. The nucleotide (insoluble in n-butanol) can be converted to the nucleoside by treatment with calf intestine alkaline phosphatase, but in several experiments not reported here identical enzymatic treatment failed to change the R_F of apple extract cytokinin. An acid phosphatase which was effective in producing adenosine from 5′-adenylic acid was also without effect on the observed R_F.

It would seem then that although the low R_F cytokinins of apple extract may be zeatin derivatives, from which zeatin can be produced by sufficiently severe acid treatment, they do not contain the nucleotide of zeatin.

Burrows *et al.* (1968) have isolated from *Escherichia coli* *t*-RNA a cytokinin which they identified as 2-methylthio-N^6-isopentenyladenosine, and Harada *et al.* (1968) have shown that it is a minor nucleoside of tyrosine *t*-RNA from *E. coli*. Although none of this nucleoside was available for testing, the R_F of the cytokinin from lanthanum chloride-treated n-butanol residue (0·6–0·7) in solvent A of Harada *et al.* was sufficiently different from that of authentic 2-methylthio-N^6-isopentenyladenosine given by these authors (0·9) to make it very unlikely that the two could be identical. It therefore seems unlikely that the corresponding nucleotide occurs in the n-butanol residue.

The identity of the cytokinin of young apples therefore remains unknown although this paper presents some evidence that it is a derivative of zeatin. Letham (1966*b*) found that when gentle extraction methods are used practically all of the cytokinin activity of an extract of young plums is soluble in n-butanol and is chromatographically identical with zeatin. The situation in apples thus differs from that in plums.

From the evidence it would seem that coconut milk, like maize milk, probably contains zeatin as such. However, it also contains several other cytokinins the identity of which are at present unknown.

V. Acknowledgments

We are grateful to Dr. G. Shaw of Bradford University for the gift of samples of zeatin and zeatin nucleoside and to Dr. P. R. Whitfeld who supplied the acid phosphatase. Drs. L. T. Evans and J. V. Jacobsen kindly read the manuscript.

VI. References

Bacher, J. E., and Allen, F. W. (1951).—Dephosphorylation of nucleotides; their analysis by counter-current distribution. *J. biol. Chem.* **188**, 59–63.

Bottomley, W., Kefford, N. P., Zwar, J. A., and Goldacre, P. L. (1963).—Kinin activity from plant extracts. I. Biological assay and sources of activity. *Aust. J. biol. Sci.* **16**, 395–406.

Burrows, W. J., et al. (1968).—Cytokinin from soluble RNA of *Escherichia coli*: 6-(3-Methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine. *Science, N.Y.* **161**, 691–3.

Carelli, V., Liquori, A. M., and Mele, A. (1955).—Sorption chromatography of polar substances on polyamides. *Nature, Lond.* **176**, 70–1.

Hall, R. H., and Srivastava, B. J. S. (1968).—Cytokinin activity of compounds obtained from soluble RNA. *Life Sci.* **7**, 7–13.

Harada, F., et al. (1968).—2-Methylthio-N^6(Δ-isopentenyl)adenosine: a component of *E. coli* tyrosine transfer RNA. *Biochem. Biophys. Res. Commun.* **33**, 299–306.

Ivancenko, D., Zajac, P., and Kouril, P. (1963).—Starch hydrolysis with ion exchangers. *Chem. Abstr.* **59**, 1033.

Letham, D. S. (1963).—Zeatin, a factor inducing cell division isolated from *Zea mays*. *Life Sci.* **2**, 569–73.

Letham, D. S. (1966*a*).—Purification and probable identity of a new cytokinin in sweet corn extracts. *Life Sci.* **5**, 551–4.

Letham, D. S. (1966*b*).—Regulation of cell division in plant tissues. II. A cytokinin in plant extracts: Isolation and interaction with other growth regulators. *Phytochemistry* **6**, 269–86.

Letham, D. S. (1966*c*).—Isolation and probable identity of a third cytokinin in sweet corn extracts. *Life Sci.* **5**, 1999–2004.

Letham, D. S., and Miller, C. O. (1965).—Identity of kinetin-like factors from *Zea mays*. *Plant & Cell Physiol.* **6**, 355–9.

Letham, D. S., Shannon, J. S., and McDonald, I. R. (1964).—The structure of zeatin, a factor inducing cell division. *Proc. chem. Soc.* **1964**, 230.

Letham, D. S., Shannon, J. S., and McDonald, I. R. C. (1967).—Regulators of cell division in plant tissues. III. The identity of zeatin. *Tetrahedron* **23**, 479–86.

Miller, C. O. (1965).—Evidence for the natural occurrence of zeatin and derivatives: compounds from maize which promote cell division. *Proc. natn. Acad. Sci. U.S.A.* **54**, 1052–8.

Wolfrom, M. L., Paton, D. L., and Lederkremer, R. M. de (1964).—Thin-layer chromatography on micro-crystalline cellulose. *J. Chromat.* **17**, 488–94.

Zwar, J. A., Bottomley, W., and Kefford, N. P. (1963).—Kinin activity from plant extracts. II. Partial purification and fractionation of kinins in apple extract. *Aust. J. biol. Sci.* **16**, 407–15.

Zwar, J. A., Bruce, M. I., Bottomley, W., and Kefford, N. P. (1964).—Comparison of the kinins of apple and coconut milk and of the modifications produced by purification procedures. Proc. 5th Int. Conf. Plant Growth Reg. Gif-sur-Yvette, 1963. pp. 123–30.