Phytochemistry, 1971, Vol. 10, pp 1251 to 1260. Pergamon Press Printed in England

INDUCED CHANGES IN THE NUCLEOTIDE PATTERN AND METABOLIC STATE OF PEA ROOT TISSUE

E. G. BROWN and A. C. CASSELLS

Department of Botany, University College, Swansea, Wales

(*Received* 23 *July* 1970, *in revised form* 30 *September* 1970)

Abstract—Purine and pyrimidine derivatives were used in attempts to induce selective changes in the nucleotide pattern of pea root tissue. The most specific effects were obtained with 6-substituted purines; those produced by 6-furfurylaminopurine (kinetin) were examined in detail. An improved method for the separation of nucleotides, using DEAE-cellulose columns, is described. Kinetin caused a 21% increase in UTP concentration, 43% increase in AMP concentration and a generalized decrease in concentration of the other purine and pyrimidine nucleotides. The most marked changes were in individual concentrations of pyridine nucleotides; whereas NADH decreased, the others increased substantially. The changed nucleotide pattern was associated with an increase in tissue glyceride content and a decrease in that of sucrose and ascorbic acid. Except for glutamic acid and serine, the free amino acid content fell. NADP-dependent isocitrate dehydrogenase increased by 44% whereas the activity of the NAD-dependent enzyme remained unchanged.

INTRODUCTION

BECAUSE of their several roles as coenzymes, precursors of coenzymes, allosteric effectors, and constituent moieties of nucleic acids, nucleotides occupy a central position in metabolism and its integration. Thus, as most nucleotides are present in tissues in limiting amounts, fluctuations in their individual concentrations exert important directional influences on metabolism. The diagnostic value of nucleotide patterns in determining the type and extent of the predominant metabolism of a tissue has been discussed both for animal tissues[1–3] and plant tissues.[4–7] The aim of the work described here was to induce artificial, selective, changes in the nucleotide pattern of a tissue with the long-term objective of relating these changes to associated alterations in the metabolic balance of that tissue.

The most direct way of inducing a selective change in the nucleotide pattern of a tissue would be to raise the endogenous concentration of an individual nucleotide constituent by feeding that substance into the tissue. In our experience with plant tissues, this approach is largely futile owing to the widespread distribution of highly active non-specific phosphatase activity which rapidly dephosphorylates nucleotides during their introduction into the tissue. The practical problems raised by this activity in plant tissues have been discussed by Bieleski.[8] In the present approach, substances were thus sought which, whilst themselves not nucleotides, might effect selective changes in the nucleotide pattern. A number of purine and pyrimidine derivatives were screened for this purpose using pea root tissue as the

[1] P. MANDEL, *Prog. Nucl. Acid. Res* **3**, 299 (1964)
[2] D. E ATKINSON, *Ann Rev. Biochem.* **35**, 85 (1966).
[3] D. E. ATKINSON, *Biochemistry* **7**, 4030 (1968).
[4] E. G. BROWN, *Biochem. J.* **85**, 633 (1962).
[5] E G BROWN, *Biochem J.* **95**, 509 (1965).
[6] E. G. BROWN and K C. SHORT, *Phytochem* **8**, 1365 (1969)
[7] E. G BROWN and B S. MANGAT, *Phytochem.* **9**, 1859, (1970).
[8] R L. BIELESKI, *Anal Biochem.* **9**, 431 (1964)

experimental system. Adenine, uracil, 5-nitrouracil, 6-furfurylaminopurine, and 6-benzylaminopurine were examined, each at several concentrations. The three most promising of these compounds were shown to be adenine, already known to affect root growth,[9] 6-furfurylaminopurine (kinetin) and 6-benzylaminopurine (benzyladenine). In selecting suitable plant material for the investigation, photosynthetic tissue was avoided because of the added complications to nucleotide metabolism of photosynthetic phosphorylation. The tissue was required to be metabolically active, devoid of significant amounts of pigments and phenolics which could interfere with nucleotide analyses and enzyme assays, and easily grown in quantity. Preliminary experiments indicated the suitability of 6-day pea seedling roots. This choice had an additional advantage in that a large amount of information is available concerning the biochemistry of pea seedling tissues, including their free-nucleotide components.[4–7,10]

RESULTS

Preliminary Screening of Some Adenine Derivatives

In the search for an agent which would selectively modify the free-nucleotide pattern of 6-day pea root tissue, adenine, benzyladenine, and kinetin were examined at arbitrary concentrations. These substances were applied to 6-day pea seedlings which were harvested and analysed 16 hr after treatment. Nucleotides were separated by chromatography on DEAE-cellulose using triethylammonium bicarbonate buffer (pH 7·5) (Fig. 1). Adenine

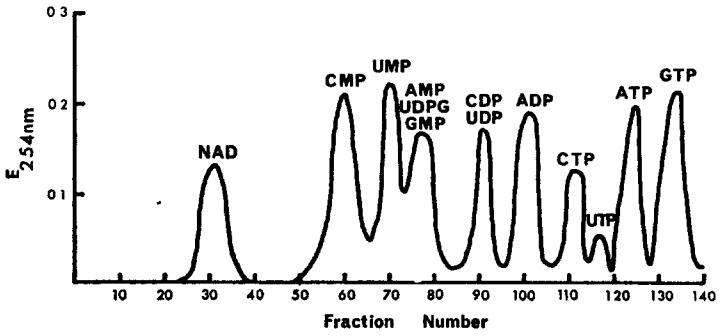

FIG 1. SEPARATION OF A KNOWN MIXTURE OF NUCLEOTIDES ON A COLUMN OF DE 52 CELLULOSE IN A GRADIENT OF TRIETHYLAMMONIUM BICARBONATE AT pH 7·5.

(1·5 mM) was found to markedly stimulate root growth in a manner similar to that previously described;[9] at a lower conc. (0·3 mM) no obvious effects were seen. Examination of the free-nucleotide pattern of roots from seedlings treated with the higher concentration of adenine showed significant differences from that of the controls (Table 1). Treatment of seedlings with benzyladenine (0·1 mM) produced effects on both root growth and nucleotide pattern generally similar to those obtained with 1·5 mM adenine. One noticeable difference however was the fall in UDP-glucose concentration following treatment with benzyladenine in contrast to the increase produced by adenine (Table 1).

[9] J G TORREY, *Physiol Plantanum* **9**, 370 (1956)
[10] E G BROWN and B. S MANGAT, *Biochim Biophys Acta* **148**, 350 (1967).

TABLE 1. FREE NUCLEOTIDE CONTENT OF ROOT TISSUE FROM CONTROL PLANTS AND PLANTS TREATED WITH ADENINE DERIVATIVES 16 hr PREVIOUSLY

Treatment*	Nucleotide† (μmoles/10 g dry wt.) AMP	ADP	ATP	UDPG	UTP	CTP	GTP
Control	14·9 (100)	7 9 (100)	44 0 (100)	64·0 (100)	8·6 (100)	5·7 (100)	2·0 (100)
Adenine	43·0 (307)	9 8 (124)	96 0 (218)	79·0 (122)	19·5 (227)	6·3 (111)	3·9 (195)
6-Benzyladenine	60 0 (428)	8·4 (106)	127·0 (289)	50·0 (78)	36 3 (422)	8 1 (142)	3·9 (195)
Kinetin	20·0 (143)	5·0 (63)	26·0 (59)	49 0 (78)	10 4 (121)	1·3 (23)	1 3 (65)

* Aqueous solutions (0·2 g/l) were used as described in the Experimental section Figures in parentheses are results expressed as % of control value.

† Only the quantitatively important purine and pyrimidine nucleotide constituents of the root tissue are given; traces of others did occur. Pyridine nucleotide values are given in Table 2

Seedlings treated with a 0·1 mM kinetin solution produced shorter but thicker roots than those of the controls: analysis of the root tissue from kinetin-treated seedlings showed that with the exception of UTP, which increased in concentration by 21 % and of AMP which increased by 43 %, there was a general decrease in the free purine and pyrimidine nucleotide content (Table 1). Comparison of the two elution diagrams revealed that the ATP peak from treated tissue had a marked inflexion which was not noticeable in the corresponding peak from control tissue. Paper chromatography and electrophoresis of this peak showed that the second component was the adenosine nucleotide, formerly designed ADP-X, now known to be adenosine-2′-phosphate-5′-diphosphate-ribose (2′-PADPR). This originates from the acid decomposition of NADPH[11] and has been previously found in extracts of pea seeds and seedlings.[4,5] The molar ratio of 2′-PADPR to ATP was found to be 1:5 in untreated seedlings and 1:1 in treated seedlings. This ratio indicates an increased amount of NADPH in roots of seedlings treated with kinetin. Furthermore, although accurate estimation of oxidized pyridine nucleotides following adsorption on charcoal is not possible because of their lability in the alkaline eluant, a substantial increase in NAD^+ content was also observed in root tissue of treated plants. A more detailed and accurate assessment of the pyridine nucleotide pattern in treated and untreated seedlings is described below.

As the main change in nucleotide pattern produced by adenine and benzyladenine was a generalized increase whereas kinetin appeared to affect more selectively AMP, UTP and the pyridine nucleotides, it was considered that the latter treatment afforded a more promising system for study of the relationships between nucleotide pattern and metabolic activity.

Examination of the Effects of Kinetin on the Pyridine Nucleotide Content of Root Tissue

Following the preliminary observations on the effect of kinetin upon the pyridine nucleotide content of pea root tissue, a more detailed examination was made of this effect. For this purpose, pyridine nucleotides were extracted separately from the other nucleotides and assayed enzymically by the procedures described in the Experimental. The results of this analysis (Table 2) confirmed the preliminary findings that the pyridine nucleotide content is significantly altered. From Table 2, it is seen that the total pyridine nucleotide content of root tissue from kinetin-treated plants is double that of control tissue. As the endogenous concentrations of pyridine nucleotides, especially total NADP, are rate

[11] H W HELDT, M KLINGENBERG and K. PAPENBERG, *Biochem. Z.* **342**, 508 (1965).

limiting factors in plant metabolism and play key roles in metabolic control,[12–14] it would be expected that the observed changes in the present plant system would have important metabolic repercussions. This was examined by surveying the concentrations of some of the quantitatively more important constituents of root tissue.

TABLE 2. PYRIDINE NUCLEOTIDE CONTENT OF ROOT TISSUE FROM CONTROL PLANTS AND PLANTS TREATED 16 hr PREVIOUSLY WITH KINETIN (0·1 mM)

Nucleotide	Concentration (nmoles/g dry wt.)		$\frac{K \times 100}{C}$
	Control tissue (C)	Kinetin-treated tissue (K)	
NAD^+	18	52	289
NADH	8	6	75
NAD^++NADH	26	58	223
$NADP^+$	3	9	300
NADPH	16	28	175
$NADP^+$+NADPH	19	37	195
Total pyridine nucleotides	45	95	211

Changes in Metabolic Flow Associated with the Changes in Pyridine Nucleotide Pattern

Examination of the gross lipid fraction of root tissue from treated seedlings showed a reproducible 13% increase in this fraction over that of the control tissue. This was attributable to an increase in the saponifiable fraction but excluding phospholipids (Table 3). Changes in carbohydrate metabolism were indicated by a 60% fall in sucrose concentration. CO_2 output was also found to have fallen by 30%.

During estimation of the pyridine nucleotides, it was noticed that 2,6-dichlorophenol-indophenol, used as an electron-acceptor in the assay, was reduced much more quickly by extracts of untreated root tissues than by those obtained from treated tissues. That the reduction was due to endogenous ascorbic acid was confirmed by paper chromatographic comparison of extracts with an authentic sample. Further examination revealed that root tissue from kinetin-treated seedlings had an ascorbic acid content 12% lower than that of the control root tissue (Table 3).

Analysis of the free amino acid pools of roots from treated and untreated seedlings (Table 3) showed that kinetin had induced a generalized decrease in amino acid concentration; serine and glutamate were exceptions to this, increasing by 36% and 18% over the control, respectively.

Following the observed changes in pyridine nucleotide pattern, the activities of some enzymes dependent on pyridine nucleotide coenzymes were examined in roots of treated and untreated seedlings. The results are shown in Table 4; trends were reproducible. A marked change was seen in the activity of NADP-dependent isocitrate dehydrogenase (E.C.1.1.1.42) which showed a 44% increase over that of the control whereas the activity NAD-dependent isocitrate dehydrogenase remained unaffected. There was little significant change in the activity of malate dehydrogenase (NAD dependent; E.C.1.1.1.37) and glyceraldehyde-3-phosphate dehydrogenase (NADP-dependent; E.C.1.2.1.9).

[12] Y. YAMAMOTO, *Plant Cell Physiol.* **2**, 277 (1961).
[13] Y YAMAMOTO, *Plant Physiol.* **38**, 45 (1963).
[14] Y. YAMAMOTO, *Plant Physiol.* **41**, 519 (1966).

TABLE 3. CONCENTRATION OF SOME QUANTITATIVELY IMPORTANT METABOLITES IN ROOT TISSUE FROM CONTROL PLANTS AND FROM PLANTS TREATED 16 hr PREVIOUSLY WITH KINETIN (0 1 mM)

Metabolites	Concentration (mg/10 g dry wt) Control tissue (*C*)	Kinetin-treated tissue (*K*)	$\frac{K \times 100}{C}$
Carbohydrates·			
Sucrose	180	100	55
Glucose	680	645	95
Ascorbic acid	70	61	87
Lipids			
Glycerides	325	380	117
Phospholipids	25	25	100
Non-saponifiable fraction	70	70	100
Free Amino Acids:			
Lysine	4 96	1 39	28
Histidine	6 64	5 04	76
Arginine	4·08	0 98	24
Aspartic acid	20·0	16·8	84
Serine	11 0	15 0	136
Glutamic acid	3 62	4 28	118
Glycine	1 4	1 12	80
Alanine	3 28	2·48	75
Valine	4·34	4·08	94
Isoleucine	0 38	0 32	84
Leucine	0 47	0 32	68
Tyrosine	0·08	0 07	87
Phenylalanine	1·6	1 17	73

Sequence of Changes

In an attempt to establish the sequence of the kinetin-induced changes described above, pyridine nucleotides were determined at intervals of 2 hr, or less where appropriate, as were also the concentrations of the metabolites previously assayed at 16 hr only. The total period over which these assays were made was extended to 20 hr from the time the seedlings were treated with kinetin. The results, shown diagrammatically in Fig. 2, revealed no significant changes after 30 min. Between 30 min and 4 hr after treatment, an oscillation

TABLE 4 A TYPICAL SET OF RESULTS SHOWING ACTIVITIES OF SOME PYRIDINE NUCLEOTIDE DEPENDENT ENZYMES IN ROOT TISSUE FROM CONTROL PLANTS AND PLANTS TREATED WITH KINETIN

Enzyme	Activity (mU*/ml of extract†) Control (*C*)	Treated (*K*)	$\frac{K \times 100}{C}$‡
NADP-dependent			
Isocitrate dehydrogenase (E C 1 1 1 42)	180	260	144
Glyceraldehyde-3-phosphate dehydrogenase (E C 1 2 1.9)	10	11	110
NAD-dependent			
Isocitrate dehydrogenase (E.C.1 1 1 41)	340	330	97
Malate dehydrogenase (E C 1 1 1 37)	641	760	119

* U = E.C standard unit (i e. μmoles of pyridine nucleotide reduced/min/25°)

† 1 ml of extract is equivalent in each case to 1 g fr. wt. of tissue.

‡ Replication with separately prepared extracts gave values within ±4% of those shown in this column.

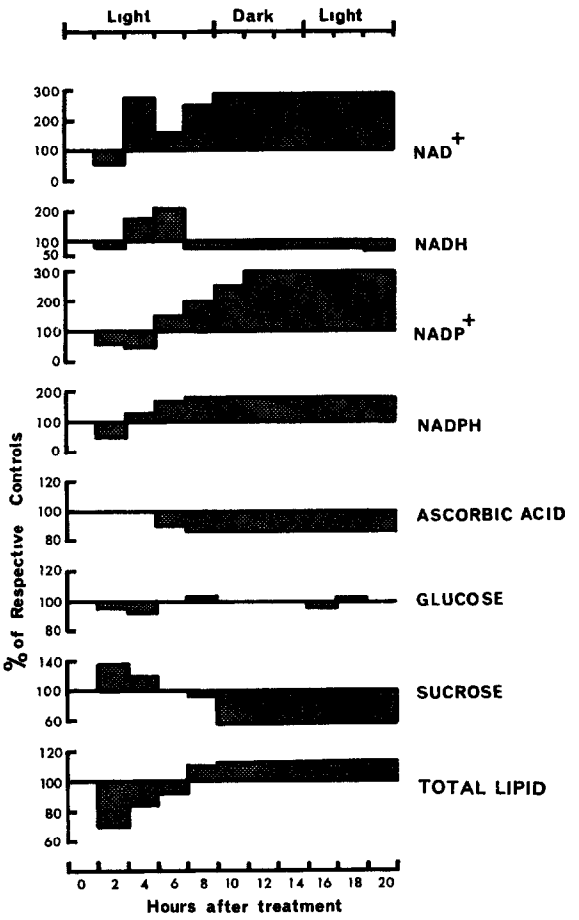

FIG. 2. SEQUENCE OF CHANGES IN KINETIN-TREATED TISSUE RELATIVE TO THOSE OCCURRING IN UNTREATED TISSUE OVER THE SAME PERIOD.
Control values (i e concentrations of specified metabolites in untreated tissue) are shown as 100% at each time interval Treatment of seedlings was at zero time

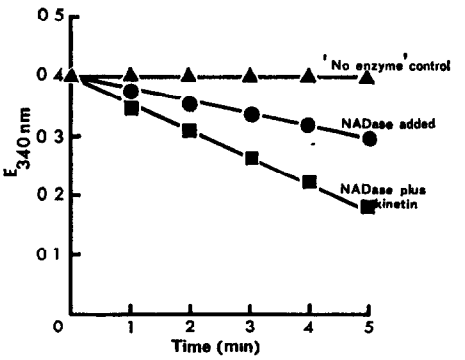

FIG 3. EFFECT OF KINETIN ON NAD NUCLEOSIDASE ACTIVITY.
NAD remaining at each sampling time was estimated by reduction with alcohol dehydrogenase (ΔE_{340nm})

was seen in the concentration of nearly all the compounds. During this, most metabolites underwent a fall in concentration followed by an overcompensated increase. A more persistent pattern emerged between 4–6 hr and it is this which was seen at the previous 16 hr assays. This state was still apparent at the time of the final determinations at 20 hr. During the 4–6 hr period, NAD^+, $NADP^+$ and NADPH began to increase in concentration towards a steady high value relative to the controls. That increases in NAD^+ concentration could be ascribed to inhibition, by kinetin, of NAD nucleosidase (NADase) was considered unlikely following experiments with a commercially available NADase preparation (see Fig. 3).

After 6 hr, the concentration of NADH fell to a value some 25% below that of the control, and after a further 2 hr the sucrose concentration began to decrease, reaching its final value, 44% below the control, by 10 hr. The sucrose concentration during the 20 hr period appeared to be inversely related to the glyceride concentration. For 4 hr after treatment, the ascorbic acid content of the treated roots remained the same as that of untreated tissue but after a further two hour period, the concentration in the treated tissue fell to a persistent low level (Fig. 2).

DISCUSSION

Following treatment with kinetin, the main changes in the nucleotide pattern of pea seedling roots involved the pyridine nucleotides; after 16 hr, NAD^+, $NADP^+$ and NADPH had all increased in concentration whereas that of NADH had decreased. With the exceptions of UTP and AMP, the other nucleotide components underwent a generalized decrease in concentration. The kinetin-induced changes in nucleotide pattern were accompanied by more general metabolic changes which included increased accumulation of glycerides, and of serine and glutamate, with decreased amounts of sucrose, ascorbic acid, and most amino acids. Most of these changes appeared to be initiated within the first 2 hr following kinetin treatment.

Also associated with the observed changes in pyridine nucleotide pattern was a 44% increase in the activity of NADP-dependent isocitrate dehydrogenase whilst that of the NAD-dependent enzyme remained unaffected. Stadtman[15] has suggested that the balance between these two activities could be functionally significant, the NADP-linked enzyme competing with the NAD-linked enzyme for isocitrate and consequently channelling electrons towards biosynthesis of fatty acids on the one hand and towards oxidative phosphorylation on the other. The observed rise in the glyceride content of the tissue is consistent with this suggestion, as is also the increased AMP content. As NAD-dependent isocitrate dehydrogenase is activated allosterically by AMP at low isocitrate concentrations[16,17] the rise in AMP concentration would represent a move towards containing the metabolic shift. Increased activity of NADP-linked isocitrate dehydrogenase also affords explanation for the significant rise in glutamate concentration (Table 3) since this amino acid largely arises from the direct amination of α-oxoglutarate, the product of isocitrate dehydrogenase activity.

Following recent work by Yamamoto[18] concerning the effects of exogenously supplied $NADP^+$ on the metabolism of cotyledon slices and subcellular fractions of *Vigna*

[15] E. R STADTMAN, *Advan Enzymol* **28**, 41 (1966)
[16] J. A. HATHAWAY and D E ATKINSON, *J. Biol. Chem.* **238**, 2875 (1963).
[17] D. E. ATKINSON, J. A HATHAWAY and E. C. SMITH, *J Biol Chem.* **240**, 2682 (1965).
[18] Y. YAMAMOTO, *Plant Physiol.* **44**, 407 (1969).

sesquipedalis, it is concluded that the increased activity of NADP-linked isocitrate dehydrogenase in the present experiments is attributable to the raised concentration of $NADP^+$ induced by kinetin. Yamamoto found that exogenously supplied $NADP^+$ produced similar increases in both NADP-linked isocitrate dehydrogenase activity and in lipid content to those observed here.

As Fig. 2 shows, the changes in pyridine nucleotide pattern were not confined to NADP. NAD^+ showed a rapid initial increase in concentration to a level 2–3 times that of the control. Similar reports of increased NAD^+ content have been made with respect to the effects of kinetin on leaves.[18, 19] The origin of the changes in pyridine nucleotide content are at present obscure but the time sequence of these changes (Fig. 2) shows that the first of the prolonged effects is a rise in total NAD content followed by a more gradual rise in the concentration of total NADP. The latter increase is ascribed to raised NAD^+ kinase activity in the presence of high concentrations of NAD^+ which stimulates it; NADH acts as an inhibitor modulating the effect.[20] That the initial increase in NAD concentration is not due to direct inhibition of NADase by kinetin is shown by Fig. 3. On the contrary, kinetin appears to have some stimulatory effect.

The present work, whilst not primarily concerned with the mode of action of kinetin, highlights some metabolic effects of this agent but, in so doing, poses the question of how kinetin causes the observed alterations in the free nucleotide pattern.

EXPERIMENTAL

Cultivation and Treatment of Pea Seedlings

Seeds of *Pisum sativum*, var. meteor, were surface sterilized by dusting with Harvesan (Boots Ltd). Treated seeds were washed in running water for 1 hr, germinated in the dark for 20 hr, and then sown in trays (48 × 37 cm) of moist vermiculite (8 cm depth) Seedlings were grown in a constant temperature room at 25° in a light regime of 16 hr light (*ca* 700 lx) alternating with 6 hr dark periods. Up to the third day, trays of seedlings were watered daily with 500 ml of water per tray. At a fixed time on the evening of the fifth day, some trays were watered as before (controls), others were treated with a solution of the test substance· roots were harvested 16 hr after this. Care was taken to ensure that the standard conditions described did not produce waterlogging of the seedling roots at any stage since this could affect their nucleotide pattern [21]

Extraction of Nucleotides

Using a Waring blender, roots were homogenized in 5% (v/v) $HClO_4$ at 4° (approximately 1 ml/g fr wt of tissue) The crude extract was filtered through muslin and the residue re-extracted with 2·5% (v/v) $HClO_4$ The pH of the combined filtrates was adjusted to 7 2 using 40% KOH, and the extract chilled at 4° for 1 hr The $KClO_4$ precipitate was removed by centrifuging and the extract slowly percolated through a column (20 × 3 cm) containing a mixture of charcoal (Norit OL, 3 g) prepared as previously described,[4] and Celite Hyflo Super-cel (4 g) A column of these dimensions was used for extracts derived from up to 100 g fr wt of roots It was generally found more convenient to use a number of such columns for larger-scale work rather than scale-up the column dimensions After adsorption of the extract, the column was washed with 50 ml of water followed by 50 ml of EDTA solution (0 05 M), nucleotides were eluted with 100 ml aq ethanol (50%) followed by 125 ml aq. ethanol containing 0 5 ml of ammonia (spec gr 0 88) The ammoniacal eluate was neutralized with acetic acid and combined with the first ethanolic eluate Nucleotide–glycosyl compounds, which are particularly susceptible to alkaline hydrolysis were eluted in the first eluate. The recovery loss for each nucleotide was determined so that corrections could be applied during subsequent analyses, such losses are reproducible providing the same batch of prepared charcoal is used for each analysis [4] The combined eluate was evaporated to dryness and re-dissolved in 10 ml of water for anion-exchange chromatography

[19] D. MISHRA and E R. WAYGOOD, *Can J Biochem* **46**, 167 (1968).
[20] D K APPS, *European J. Biochem* **5**, 444 (1968)
[21] S H. WEST, *Plant Physiol.* **37**, 565 (1962).

Anion-exchange Chromatography

Nucleotides were separated on a column (20 × 1·9 cm dia) of Whatman DE 52 microgranular DEAE-cellulose. DE 52 cellulose was prepared for use as described by the manufacturers and packed in 50% ethanol. Triethylammonium bicarbonate (0 35 M; pH 7·5) was used as the eluting buffer; the separation given by this was similar to that obtained with the triethylammonium acetate buffer used by Nilsson and Sjunnerson[22] with a column of ECTEOLA-cellulose. The bicarbonate buffer had the advantage of being considerably more volatile than the acetate buffer and could be more quickly removed from nucleotide fractions by azeotropic distillation with methanol. Elution was effected in a linear gradient in which the mixing vessel initially contained water (1 l) and the reservoir contained the buffer (1 l.). The flow rate was 0 5 ml/min and 10 ml fractions were collected The resolution (Fig 1) was comparable to that obtained with an anion-exchange resin in a formate system[4] but had the dual advantage of speed, taking approximately half the time required by the resin-formate system, and relative ease of recovery of nucleotides UMP, AMP and some sugar-nucleotides tend to elute at the same point but this peak was easily resolved further by re-chromatography on the more conventional Dowex-1 formate column [4] In practice, the first 700 ml of the eluate from the DE 52 column was passed directly onto a column of Dowex-1 formate (×8; 200–400 mesh) and the latter eluted with the first two ranges of the formate system previously described.[4] This second separation was run simultaneously with the elution of the remainder of the nucleotides from the DE 52 column and, thus, did not affect the time advantage of the cellulose column. All column chromatographic separations were monitored automatically for elution of ultraviolet-absorbing substances at either 265 nm or 254 nm.

Identification and Estimation of Purine and Pyrimidine Nucleotides

Fractions from the ion-exchange columns were chromatographed on paper (Whatman No. 1) in one or more of the solvent systems, (i) isobutyric acid–ammonia (spec. gr 0 88)–water (33:1·66, by vol.),[23] (ii) ethanol–ammonium acetate (M) (70 30, v/v),[24] (iii) *n*-butanol–acetic acid–water (60:15:45, by vol),[25] (iv) isopropanol–HCl (2 M) (65:35, v/v).[26] Solvents (i) and (ii) were also used as a two-dimensional system to separate sugar-nucleotides from nucleoside monophosphates and to separate mixtures of nucleoside triphosphates Separations were also effected by high-voltage electrophoresis on paper (Whatman No. 1). A voltage gradient of 120 V/cm applied for 30 min was routinely used; UMP was run simultaneously as a reference substance The buffers used were (i) formic acid–acetic acid (1 5 M; pH 2),[27] formic acid–ammonium formate (0 05 M, pH 3 5),[28] and borate (0 05 M; pH 9) Nucleotides were tentatively identified from their position in the elution sequence by comparison with a standard nucleotide mixture, from their u v. absorption characteristics, and from their paper chromatographic and electrophoretic behaviour Confirmation was obtained by co-chromatography with authentic samples Purine and pyrimidine nucleotides were estimated spectrophotometrically After correcting for losses during the charcoal adsorption procedure (see above), recoveries of all the nucleotides shown in Fig 1 were quantitative (98–100%) when these were added to the extracts

Extraction and Estimation of Pyridine Nucleotides

Root tissue was dropped into liquid N_2 and immediately freeze dried NADH and NADPH were extracted from the powdered material with 0 1 M KOH[29] whereas NAD^+ and $NADP^+$ were extracted with 0 5 M $HClO_4$ [30] The extracted pyridine nucleotides were estimated by the method of Slater and Sawyer[31] as modified by Heber and Santarius [30] Recovery of NAD^+, $NADP^+$ and NADPH added to the extracts was quantitative (98–100%), but NADH consistently gave a slightly lower value (94–98%)

Estimation of Lipids, Carbohydrates and Amino Acids

Gross lipid was extracted in isopropanol followed by chloroform–isopropanol as described by Kates and Eberhardt[32] and fractionated by the method of Rosowsky *et al* [33] Phospholipids were estimated by digesting a sample in 60% $HClO_4$ and determining the phosphate content using the method of Fiske and

[22] R Nilsson and M Sjunnerson, *Acta Chem Scand* **15**, 1017 (1961)
[23] Pabst Laboratories, *Circular OR*-17, Pabst Laboratories, Milwaukee, Wisconsin, U S A (1961)
[24] A. C Paladini and L F Leloir, *Biochem J* **51**, 426 (1952)
[25] S M Partridge, *Biochem J* **42**, 238 (1948)
[26] G R Wyatt, *Biochem J* **48**, 584 (1951)
[27] M L Efron, *Biochem J* **72**, 691 (1959)
[28] R. Markham and J. D Smith, *Biochem J* **52**, 552 (1954).
[29] G E Glock and P McClean, *Biochem J* **61**, 381 (1955)
[30] U W Heber and K A. Santarius, *Biochim Biophys Acta* **109**, 390 (1965).
[31] T F. Slater and B Sawyer, *Nature* **193**, 454 (1962)
[32] M. Kates and F M Eberhardt, *Can J Botany* **35**, 894 (1957)
[33] A. Rosowsky, A. C Crocker, D H Trites and E J. Modest, *Biochim. Biophys Acta* **98**, 617 (1965).

Subbarow[34] Soluble sugars were extracted in boiling aq. ethanol (80%, v/v) and reducing sugars estimated by the Somogyi method:[35] sucrose was estimated after acid hydrolysis. Ascorbic acid was extracted from freeze dried root tissue in metaphosphoric acid (5%, w/v) and assayed by titration with 2,6-dichlorophenolindophenol solution. Amino acids were extracted in boiling aqueous ethanol (80%, v/v) and assayed using a Beckman Model 120C Amino Acid Analyser.

Assays of Enzymic Activity

Using a pre-cooled mortar and pestle, root tissue samples (25 g fr. wt) were ground in 25 ml of the appropriate buffer at 4° (0·1 M Tris, pH 7·6, for isocitrate dehydrogenase; 0·1 M glycine, pH 7·6, for malate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase). Each extract was pressed through muslin, centrifuged at 165,000 *g* for 60 min at 4°, and the clear supernatant used for immediate assay.

For pyridine nucleotide-dependent enzymes, the rate of reduction of NAD or NADP was determined by coupling the reduced coenzyme, through phenazine methosulphate (PMS), with 2,6-dichlorophenolindophenol (DCIP)[29] and automatically recording ΔE_{660nm} The reaction cuvette contained 0·5 ml of buffered enzyme extract, 0·1 ml of pyridine nucleotide solution (15 mM), 0·1 ml of PMS solution (1 mg/ml), 0·1 ml of DCIP solution (17 5 mg/ml) and 0·1 ml of 0·1 M $MgCl_2$. The total volume was made to 2 9 ml with buffer (see above) and E_{660nm} monitored until constant, i.e until supply of endogenous substrate had been exhausted. The reaction was started by addition of 0 1 ml of substrate solution (15 mM) and the rate recorded over the first 5 min. Glyceraldehyde-3-phosphate dehydrogenase was assayed by the method of Margulies,[36] modified to include PMS and DCIP

The effect of kinetin on NAD nucleosidase (Fig 3) was examined using a preparation of the enzyme from *Neurospora crassa* (Sigma Chemical Co Ltd., London). NAD was assayed by reduction with yeast alcohol dehydrogenase (Boehringer Corporation Ltd., London) NAD nucleosidase activity was measured at pH 7. (0 05 M phosphate buffer) using 2 mM NAD; kinetin was used at a final concentration of 1 mM.

Acknowledgements—The authors thank Miss J Pickford for skilled technical assistance. A.C.C. gratefully acknowledges the Award of a National University of Ireland Travelling Scholarship during tenure of which, this work was carried out

[34] C H FISKE and Y. SUBBAROW, *J. Biol. Chem* **81**, 629 (1929).
[35] M. SOMOGYI, *J Biol. Chem* **195**, 19 (1952)
[36] M. M MARGULIES, *Plant Physiol.* **39**, 579 (1966)