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STUDIES ON THE FREE NUCLEOTIDE POOL AND RNA COMPONENTS OF DETACHED LEAVES OF *PHASEOLUS VULGARIS* DURING ROOT DEVELOPMENT

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Abstract—Significant changes occur in the free nucleotide pool and RNA complement of bean petioles during the formation of roots by detached leaves. Initially, a general increase occurs in free nucleotide content with specific increases in the nucleoside 5'-triphosphates and UDP-glucose but by the time of root emergence the concentrations of ATP, CTP and GTP have decreased again and UDP-glucose has replaced ATP as the predominant petiolar nucleotide. Increases in the petiolar free-nucleotide pool could not be accounted for by corresponding losses from the leaf laminae. Associated changes occurred in the RNA complement of rooting petioles, the most important of which, quantitively, was a 350% increase in the heavy ribosomal fraction. No changes could be detected in the nucleic acid fractions of the laminae. During the first 24 hr after excision, a large increase occurred in petiolar phosphodiesterase activity against RNA but base analysis of the various RNA fractions gave no evidence for a direct link between depolymerization of RNA and specific changes in the petiolar nucleotide pool.

INTRODUCTION

SINCE free-nucleotides function as coenzymes and allosteric effectors and mostly occur in limiting concentrations, the nucleotide composition of a tissue is clearly related to the characteristic metabolic activity of that tissue. Changes in the nucleotide pool indicate, and to a large extent control, the major trends in metabolism. In consequence, a study of the changing nucleotide pool during the normal developmental processes may be expected to yield valuable information concerning the biochemistry of development. This approach has been usefully applied to a study of germination[1-3] but is difficult to apply to the relatively slow, and frequently slight, metabolic changes associated with the subsequent growth and development of seedlings. In the present work, the aims were: (*a*) to overcome this problem by inducing a sudden, non-lethal but drastic, change in the normal growth of tissue; (*b*) to follow the associated trends in nucleotide pattern; and (*c*) to examine the extent to which the depolymerization of RNA contributes to the free nucleotide pool and hence the feasibility of the hypothesis that rapid switches in metabolism may be programmed by a sequential release of nucleotides from RNA into the free nucleotide pool.[4,5]

In selecting suitable experimental material, two criteria were used: firstly, the plant tissue had to be easily obtained at the same stage of morphological and physiological development for each experiment; and secondly, it had to be capable of marked morphological or

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[2] E. G. BROWN, *Biochem. J.* **88**, 498 (1963).
[3] E. G. BROWN, *Biochem. J.* **95**, 509 (1965).
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metabolic change in response to some simple stimulus, preferably non-chemical, so as to avoid complicating the picture with foreign metabolites. The material eventually chosen was petiolar tissue from primary leaf cuttings of *Phaseolus vulgaris*: these cuttings root readily in water without additional treatment.[6,7]

RESULTS

Trends in the Free-nucleotide Patterns

The changes in nucleotide content of bean petioles following excision and subsequent rooting are shown in Table 1. No detectable leaching of nucleotides from the cut ends of the

TABLE 1. CHANGES IN THE FREE-NUCLEOTIDE CONTENT OF BEAN PETIOLES FROM EXCISION (day 0) TO ROOT FORMATION (day 6)

Nucleotide	Concentration (mμmole/10 g fr. wt.)*			
	Day 0	Day 1	Day 3	Day 6
CMP	10	20	30	50
NAD^+	—	20	30	30
AMP	10	30	30	50
GMP	—	—	—	30
$NADP^+$	—	—	—	10
GR†	10	10	10	10
ADP-ribose	5	10	30	30
UMP	5	60	150	50
ADP	30	30	30	90
GR'†	—	—	—	5
AdR§	10	20	20	40
UDP-glucose	240	270	430	660
NADPH	20	30	40	50
UDP	—	—	—	40
CTP	10	30	50	—
ATP	330	500	770	580
GTP	60	80	110	40
UTP	90	130	160	190
Total	830	1240	1890	1955

* Results are the mean of three separations. Similar trends are seen when concentrations are expressed on a dry wt. basis.

† Unidentified guanine nucleotides.

§ Unidentified adenine nucleotide arising during the alkaline degradation of NAD^+.

petioles occurred during rooting. Analysis of petiolar tissue immediately following detachment from the parent plant (day 0) indicates that the nucleotide complement at this stage consists predominantly of nucleoside 5'-triphosphates and UDP-glucose with relatively low concentrations of nucleoside monophosphates (Table 1). Quantitatively, the most important nucleotide at day 0 is ATP. Over the 6-day period from excision to rooting, there is a general rise in nucleotide concentration, the most significant increases being amongst the nucleoside

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[7] E. C. HUMPHRIES, *Physiol. Plantorum* **13**, 659 (1960).

5′-triphosphates and UDP-glucose. However, with the exception of UTP, the triphosphates show a significant fall before rooting occurs (Table 1) and, by day 6, UDP-glucose has become the major nucleotide component of the petiolar tissue. At all stages there is a preponderance of adenine and uracil nucleotides over those of cytosine and guanine (Table 2).

TABLE 2. CHANGES IN THE BASE COMPOSITION OF THE FREE-NUCLEOTIDE POOL OF BEAN PETIOLES FROM EXCISION (day 0) TO ROOT DEVELOPMENT (day 6)

	Concentration (mμmole/10 g fr. wt.)			
Base	Day 0	Day 1	Day 3	Day 6
Cytosine (C)	20	50	80	50
	} 425	} 690	} 1030	} 930
Adenine (A)	405	640	950	880
Uracil (U)	335	460	740	940
	} 405	} 550	} 860	} 1025
Guanine (G)	70	90	120	85

To examine the extent to which the changes in the nucleotide pattern of petioles can be ascribed to translocation of free nucleotides from the associated leaf laminae, the nucleotide composition of the latter was also determined by the same procedures. The results (Table 3) show that the leaf lamina has a well-defined nucleotide pattern which undergoes no significant change during the period studied. In contrast to the petiolar nucleotide complement (Table 1), that of the laminae is dominated by the adenine nucleotides (Table 3).

TABLE 3. PREDOMINANT FREE NUCLEOTIDES OF BEAN LEAF LAMINAE AT days 0 AND 3

	Concentration (mμmole/10 g fr. wt. of tissue*)	
Nucleotide	Day 0	Day 3
NAD^+	80	66
AMP	120	100
GMP	70	56
$NADP^+$	18	15
ADP-ribose	30	22
UMP	150	136
ADP	180	160
AdR†	20	15
UDP-glucose	240	210
NADPH	70	60
ATP	690	640
GTP	80	80
UTP	140	136
Total	1898	1696

* Parallel trends are seen when results are expressed on a dry wt. basis.

† See Table 1.

The adenine nucleotide, ADP-ribose, listed in Tables 1 and 3 is an acid-decomposition product of NADH. AdR is an unidentified adenine nucleotide found to originate from the alkaline degradation of NAD^+ during elution of the latter from the charcoal column. Because of the previously discussed difficulties[1] associated with quantitative extraction and chromatography of pyridine nucleotides, especially in relation to their original redox state *in situ*, no attempt has been made to interpret the figures for pyridine nucleotides and their products except to say that, in common with the other nucleotides of rooting petioles, they show a general increase in concentration during the period days 0–6.

GR and GR′ (Table 1) are unidentified nucleotides exhibiting u.v. spectra typical, at various pHs, of guanosine derivatives. Upon acid hydrolysis they yielded guanine, identified by co-chromatography with an authentic sample; insufficient material was available for more detailed investigation.

Phosphodiesterase Activity

Consideration of the possibility that depolymerization of RNA could be making a significant contribution to the increasing concentrations of certain nucleotides seen between days 0–6 (Tables 1 and 2) led to examination for petiolar phosphodiesterase activity towards RNA. The results (Fig. 1), which demonstrate the presence of such activity in the petiole at all stages from excision to rooting, reveal a rapid initial increase following excision. Maximum activity of phosphodiesterase, representing a 4-fold increase over the day 0 value, was seen at 24 hr.

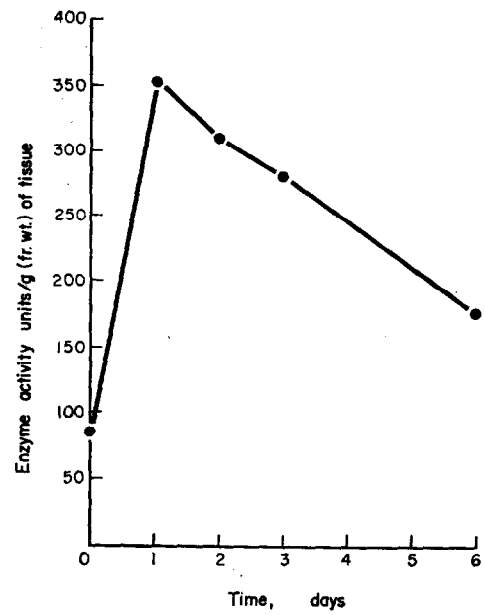

FIG. 1. PHOSPHODIESTERASE ACTIVITY TOWARDS RNA IN EXTRACTS OF BEAN PETIOLES DURING ROOT FORMATION.

Leaves were detached at day 0 and showed root formation by day 6. Assay incubations were at 37° for 1 hr, at pH 5·1: one unit of enzyme activity corresponds to an increase in $E_{260\,nm}$ of 0·01 under these conditions.

Examination of Nucleic Acid Fractions

Following the observations on phosphodiesterase activity, an assessment was made of the relative concentrations of the major nucleic acid fractions, both of leaf laminae and their associated petioles during the rooting process. Using a minor modification of Kirby's phenol method,[8] the nucleic acids were extracted at several stages from excision to rooting and separated on a column of methylated serum albumin/kieselguhr into five fractions, *viz.* sRNA (I), low mol. wt. ribosomal RNA (II), DNA (III), light ribosomal RNA (IV) and heavy ribosomal RNA (V). Although the phenolic extraction procedure entails small losses in recovery, these were found to be reproducible and affected all the nucleic acid fractions

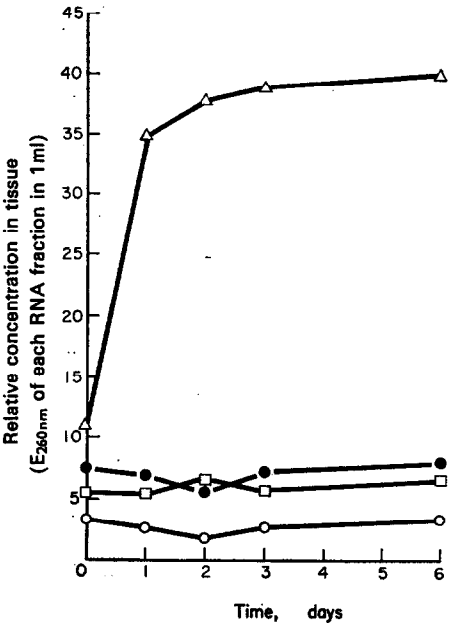

FIG. 2. RELATIVE CONCENTRATION OF THE FOUR FRACTIONS OF RNA EXTRACTED FROM BEAN PETIOLES AT VARIOUS STAGES DURING ROOT FORMATION.

Leaves were detached from the parent plant on day 0 and roots emerged by day 6. ●, sRNA (fraction I); ○, low mol. wt. ribosomal RNA (fraction II); ■, light ribosomal RNA (fraction IV); △, heavy ribosomal RNA (fraction V).

equally: the individual peaks within any one elution profile are thus directly comparable to one another and the pattern as a whole may be compared directly with that obtained from different tissues by the same procedure. Examination of the nucleic acid pattern of petioles during rooting revealed specific changes (Fig. 2), the most important of which was a 3·5-fold increase in the heavy ribosomal fraction, but similar analyses of the associated leaf laminae showed no quantitatively significant changes in their nucleic acid composition during the same 6-day period from excision to rooting. This is exemplified by Fig. 3 which shows the elution profiles obtained by chromatography on MAK of nucleic acid extracts from the laminae of rooting petioles at day 0 and day 1, i.e. over a period during which there is a maximal rise in phosphodiesterase activity (Fig. 1) and a 41 % increase in total petiolar free-nucleotide concentration (Table 1).

[8] K. S. KIRBY, *Biochem. J.* **64**, 405 (1956).

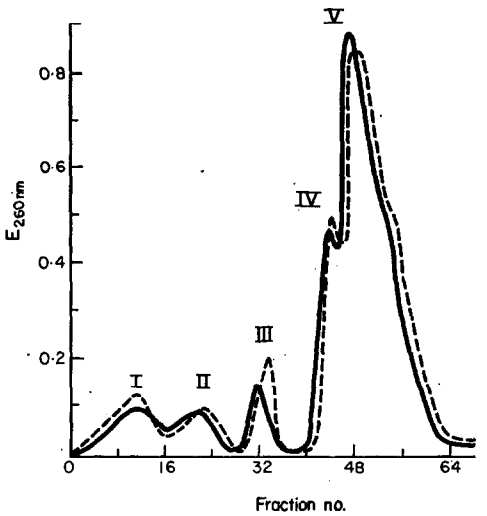

FIG. 3. ELUTION PROFILES OBTAINED AT day 1 (——) AND day 2 (– – –) BY CHROMATOGRAPHY ON MAK OF THE NUCLEIC ACID COMPONENTS OF BEAN LEAF LAMINAE DURING ROOT FORMATION BY THE ASSOCIATED PETIOLES.

Leaves were detached on day 0 and roots emerged by day 6. A linear gradient of buffered NaCl (0·03–1·15 M) was used at pH 6·7 and 3-ml fractions were collected.

In order to see if the changes in petiolar nucleic acid composition could account for a release of free nucleotides in a specific manner and so be responsible for the observed selective re-orientation of the nucleotide pool during rooting, the nucleotide composition of the different molecular species of RNA in the petiole was determined over the 6-day period. DNA was not examined in this context since, (*a*) it is of minor importance quantitatively and (*b*) it would be expected to release deoxyribonucleotides and not ribonucleotides (the major free nucleotides of plant tissues are of the latter type). Alkaline hydrolysis of the individual RNA fractions of the petiole, followed by cation-exchange chromatography of the constituent nucleotides, gave the results shown in Table 4. The overall balance sheet of changes in concentration of individual families of nucleotides, both free and bound as RNA, during the period from excision to rooting (days 0–6) is presented in Table 5.

DISCUSSION

Changes in the nucleotide pool of bean petioles during the period of metabolic re-orientation induced by excision and culminating in rooting, mainly concern the nucleoside 5′-triphosphates and UDP-glucose (Table 1). From days 0–3, the individual concentrations (Table 1) of CTP, ATP, GTP and UTP increase by factors of 5, 2·3, 1·8, and 1·8 respectively but, with the exception of UTP, these values have decreased again by the time the roots begin to emerge (day 6). The initial increase in nucleoside triphosphate concentration is comparable to that seen in several other biological systems during short periods of profound physiological change, e.g. germination of seeds,[3] cells approaching log phase in tissue culture[9,10] and

[9] J. M. VAIL, J. R. C. BROWN and A. D. GLINOS, *J. Cell Biol.* **35**, 135A (1967).
[10] E. G. BROWN and K. C. SHORT, *Phytochem.* **8**, 1365 (1969).

TABLE 4. MONONUCLEOTIDE COMPOSITION OF THE INDIVIDUAL RNA FRACTIONS OF BEAN PETIOLES OVER THE PERIOD FROM EXCISION (day 0) TO THE APPEARANCE OF ROOTS (day 6)

	Concentration (μmole × 10^2/10 g fr. wt. of tissue)*															
	RNA-I †				RNA-II †				RNA-IV †				RNA-V †			
Day	UMP	GMP	CMP	AMP	UMP	GMP	CMP	AMP	UMP	GMP	CMP	AMP	UMP	GMP	CMP	AMP
0	42	43	35	25	24	20	16	15	32	28	22	22	65	66	49	45
1	54	34	28	20	19	14	8	7	33	30	23	24	165	225	170	145
2	34	30	26	18	13	10	9	7	33	33	23	25	195	246	175	165
3	44	42	33	24	19	12	12	10	40	32	22	24	194	246	176	167
6	47	48	40	29	23	18	14	14	40	41	26	29	204	263	183	168

* Each value is the mean of six determinations.

† RNA was separated on MAK into four fractions, *viz.* (I) sRNA, (II) low mol. wt. ribosomal RNA, (IV) light ribosomal RNA, (V) heavy ribosomal RNA. Fraction (III) was DNA.

TABLE 5. FLUCTUATIONS IN THE CONCENTRATIONS OF FREE AND RNA-BOUND NUCLEOTIDES DURING ROOTING OF BEAN PETIOLES

	Concentration (mμmole/10 g fr. wt. of tissue)																			
	Uracil nucleotides					Guanine nucleotides					Cytosine nucleotides					Adenine nucleotides				
	Bound in RNA fraction:*					Bound in RNA fraction:					Bound in RNA fraction:					Bound in RNA fraction:				
Period (day)	Free	I	II	IV	V	Free	I	II	IV	V	Free	I	II	IV	V	Free	I	II	IV	V
0–1	+125	+120	−50	+10	+1000	+20	−90	−60	+20	+1590	+30	−70	−80	+10	+1210	+235	−50	−80	+20	+1000
1–3	+280	−100	0	+70	+290	+30	+80	−20	+20	+210	+30	+50	+40	−10	+60	+310	+40	+30	0	+220
3–6	+200	+30	+40	0	+100	−35	+60	+60	+90	+170	−30	+70	+20	+40	+70	−70	+50	+40	+50	+10

* RNA fractions I, II, IV and V are sRNA, low mol. wt. ribosomal RNA, light ribosomal RNA and heavy ribosomal RNA, respectively.

synchronous cultures of *Tetrahymena pyriformis* before cell division[11,12] and is a reflection of the high level of energy metabolism in these changing systems.

Although the individual concentrations of CTP, ATP and GTP fall after day 3, those of UDP-glucose and UTP increase throughout the rooting of the petiole (Table 1) until UDP-glucose has become the predominant nucleotide constituent. This is compatible with the known role of UDP-glucose in carbohydrate metabolism and the increased synthesis of polysaccharides necessary for the formation of the new root structure. Šebesta and Šorm[13] reported that bean (*Phaseolus vulgaris*) roots have a higher concentration of UDP-glucose than of ATP whereas the ratio is reversed in the green parts and we have noted the same variations (unpublished results) in pea and wheat. Considered in conjunction with these observations, the nucleotide pattern of the rooting petiole can be seen to be changing gradually over the 6-day period to one more characteristic of roots.

During the period day 0–3, there is an increase in the total free-nucleotide concentration (Table 1). This could occur through (i) translocation from the nucleotide pool of the leaf lamina, (ii) enzymic depolymerization of RNA, or (iii) synthesis from non-nucleotide precursors within the petiole. It can be seen (Table 1) that in the first 3 days, the total increase in the nucleotide content of rooting petioles was 1060 mμmoles/10 g fr. wt. whereas the nucleotide content of the associated leaf laminae (Table 3) decreased by only 202 mμmoles/10 g fr. wt. of tissue. Furthermore, no individual compound was specifically involved in this nucleotide loss from the laminae. It is thus concluded that translocation of preformed free nucleotides makes no significant contribution to the petiolar nucleotide pattern during this period.

That the changes in the petiolar nucleotide pool during rooting may be attributable to the controlled release of mononucleotides arising from depolymerization of RNA is an attractive hypothesis in view of the directing role of free nucleotides in metabolism (through allosterism and coenzyme activity) and the "informational" nature of nucleic acids. Similar suggestions involving RNA in metabolic control via the nucleotide pool have been discussed by Strominger[4] and by Soodak.[5] The relatively large increase in phosphodiesterase activity towards RNA during the first 24 hr (Fig. 1) is compatible with these suggestions and compares to similar increased degradation of RNA during the metabolic changes associated with the wounding of leaf tissue,[14,15] with the germination of seeds,[16,17] and with the growth of root cells,[18] yeast cells[19] and various animal cells.[20]

During the increase in phosphodiesterase activity in the present system, the total concentration of RNA also increased, due primarily to a 3·5-fold increase in the concentration of both the light and heavy ribosomal fractions (Fig. 2). Slight decreases in the sRNA and low mol. wt. fractions were observed during the first 2 days (Fig. 2). Table 5 shows, however, that whereas the fall in the concentration of these two RNA fractions (I and II) could account for the increase of free cytosine and guanine nucleotides in the nucleotide pool from day 0–1, it could not explain the relatively large increase in free adenine and uracil nucleotides at this

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[20] J. ROTH, *Cancer Res.* **23**, 657 (1963).

time. That the RNA undergoing depolymerization might not be petiolar but a fraction translocated from the laminae following excision, was eliminated by consideration of the virtually identical MAK elution profiles of the gross nucleic acid from leaf laminae at days 0 and 1 (Fig. 3), for, during this period, the petiolar RNA pattern (Fig. 2) and petiolar nucleotide pattern (Table 1) both change significantly and phosphodiesterase activity is at a maximum (Fig. 1). Present findings thus indicate that free nucleotide patterns in rooting bean petioles are not affected in a specific or quantitatively significant manner by the release of nucleotides from RNA and point to the regulation of these pools through the third possibility outlined above, i.e. synthesis of new nucleotide material from non-nucleotide precursors. However, although none of the RNA fractions that were examined contributed significantly to the free nucleotide pool, a number of recent reports have described RNA fractions that are not extracted by the hitherto standard procedures employed here. For example, Hadjivassiliou and Brawerman[21] have described the isolation from rat liver of an RNA fraction, especially rich in AMP, which is not extracted with the aqueous phase in the phenol-extraction procedure used in the present work. Similarly, Asano[22] described a rapidly labelled RNA fraction which is retained on an MAK column and not eluted by standard procedures; Ewing and Cherry[23] have also found RNA fractions in pea seedlings which behaved in a similar way on MAK columns and which, again, were particularly rich in AMP. Clearly, depolymerization of such fractions, rich in bound AMP, would have important implications if this nucleotide were released in significant amounts into the nucleotide pool, especially if Atkinson's adenylate energy-charge concept[24] is a major regulatory parameter in metabolism. This possibility warrants further investigation.

MATERIALS AND METHODS

Seedlings

Seeds of *Phaseolus vulgaris* L. var. Canadian Wonder, supplied by the Scottish Seed House, Perth, were surface sterilized in 6% (w/v) calcium hypochlorite solution containing 0·2% (v/v) Stergene as a wetting agent, and germinated at 25° in moist Levington potting compost. A light cycle of alternately 18 hr light and 6 hr dark was employed.

Rooting of Petioles

Fully expanded primary leaves of 10-day-old seedlings were excised together with their petioles. Since it hinders rooting,[25] the pulvinus was removed and the cut end of the petiole immersed in tap-water. The water was changed every 24 hr; "used" water was immediately sterilized by filtration, pooled and evaporated to a small volume for examination for the presence of leached nucleotides. Histological examination of the petioles showed organization of meristematic cells to form root primordia by the third day and emergence of roots on the sixth day.

Extraction and Separation of Acid-soluble Nucleotides

Tissue samples (20–25 g fr. wt.) were extracted in ice-cold 0·3 N perchloric acid as described by Brown.[1] The crude extract, from which the perchlorate anion had been removed by precipitation in the cold with KOH, was treated by charcoal adsorption[1] in order to remove the bulk of the u.v.-absorbing, non-nucleotide, impurities which otherwise interfere with the subsequent chromatographic procedure. All nucleotide analyses were routinely corrected for recovery losses during the charcoal treatment.[1] The prepared nucleotide extract was separated on a column (9 mm dia. × 190 mm) of Dowex 1 (formate; ×8; 200–400 mesh) employing the system of linear gradients described previously.[1] In order to minimize alkaline hydrolysis of sugar nucleotides, it was found necessary to carry out both the elution from charcoal and the adsorption on to Dowex

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1 at 4°. Fractions comprising heterogenous peaks were further resolved by paper chromatography and high-voltage electrophoresis. Nucleotides were estimated spectrophotometrically.

Identification and Estimation of Nucleotides

Nucleotides were tentatively identified by their u.v.-absorption characteristics and relative position in the sequence of elution from the anion-exchange column. Further examination was made by paper chromatography in the solvent systems, (i) 2-methylpropionic acid–ammonia (sp. gr. 0·88)–water (57:4:39, v/v/v),[26] (ii) propan-2-ol–2 N-HCl (65:35, v/v),[27] (iii) 2-methylpropan-2-ol–formic acid–water (3:1:2, v/v/v), (iv) butan-1-ol–acetic acid–water (12:3:5, v/v/v),[28] (v) methanol–M-ammonium acetate (7·5:3, v/v; pH 7·5). Solvent (v) is the system described by Palandini and Leloir[29] but modified by replacing ethanol by methanol. High-voltage paper electrophoresis at pH 2 in formic acid–acetic acid buffer[30] was also employed to examine and separate nucleotides. Confirmation of identification was obtained by co-chromatography with authentic samples in the above solvents. Purine and pyrimidine nucleotides were estimated by direct spectrophotometry, pyridine nucleotides were estimated spectrophotometrically by the cyanide addition reaction of Colowick, Kaplan and Ciotti.[31]

Assay of Phosphodiesterase Activity

Using a pre-cooled mortar and pestle, tissue samples (10 g fr. wt.) were finely ground with 3 ml of ice-cold sodium acetate buffer (0·1 M; pH 5); acid-washed sand was used to facilitate grinding. Insoluble debris was removed by centrifuging at 2000 *g* for 15 min. All operations from grinding to centrifuging were carried out at 4°. The volume of the final supernatant was adjusted to 50 ml with the same acetate buffer and the phosphodiesterase activity of aliquots assayed spectrophotometrically by a modification of McDonald's method[32] for pancreatic ribonuclease. Yeast RNA was used as substrate for the assay.

Extraction and Separation of RNA

Weighed tissue samples (1–5 g fr. wt.) were homogenized at 0–4° in an extractant consisting of 10 ml of 6% (w/v) 4-aminosalicylate, 20 ml of aq. phenol (80%, w/v) and 50 mg of bentonite. The homogenate was filtered under vacuum through Whatman No. 54 filter paper and the filtrate separated into two phases by centrifuging at 3000 *g* for 15 min at 0°. The upper aqueous phase was decanted and RNA precipitated from it with 2·5 vol. of ice-cold ethanol–water–10% (w/v) NaCl (25:10:1, v/v/v). After washing twice with cold ethanolic NaCl by re-suspension and recentrifuging, the precipitate was redissolved in NaCl–phosphate buffer[8] for chromatography on columns (20 mm dia. × 100 mm) of methylated serum albumin/kieselguhr (MAK) by the method of Mandell and Hershey.[33]

Hydrolysis and Base Analysis of RNA

Each RNA fraction was desalted by dialysis for 24 hr against distilled water and evaporated to dryness *in vacuo*. The residue was redissolved in 1·5 ml of 0·3 M KOH and hydrolysed for 16 hr at 37°. The hydrolysate was neutralized with 0·6 M $HClO_4$, allowed to stand for 30 min at 4°, and the precipitated $KClO_4$ removed by centrifuging. The neutralized supernatant was then made 0·05 M with respect to HCl and the sample (<3 ml) chromatographed on a column (10 mm dia. × 50 mm) of Dowex $50H^+$ (8×; 200–400 mesh). Elution was effected with 0·05 M HCl, followed by water, as described by Katz and Comb.[34] UMP and GMP emerged as two separate peaks which were estimated by direct spectrophotometry, whereas AMP and CMP eluted together as a single peak and were estimated by differential spectrophotometry.

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