THE EXTRACTION AND ESTIMATION OF NUCLEOTIDES AND NUCLEIC ACIDS FROM PLANT MATERIAL*

JOHN INGLE†

Department of Botany,
University of Bristol
(*Received* 14 *December* 1962)

Abstract—The methods available for the extraction and estimation of nucleotides and nucleic acids have been applied to a variety of plant tissues. It has been shown that, with the tissues investigated, either aqueous ethanol or dilute perchloric acid could be used to extract the soluble nucleotides. Components other than nucleotides contributed to the 260 mμ optical density of the extract, and with most tissues only 20 to 30 per cent of the optical density was due to nucleotide material. Although purification of the nucleotides in the extract was attempted by various procedures, such as precipitation of the nucleotides as insoluble salts, extraction of pigments with organic solvents and ion-exchange treatments, no suitable general method was found. The separation into individual nucleotides by ion-exchange chromatography was the only reliable way of estimating the nucleotide content of a tissue.

The Schmidt and Thannhauser procedure was the only general method suitable for the extraction of nucleic acids from plant tissue. Purification of the RNA mononucleotides before estimation was essential, but the anion-exchange treatment described by Smillie and Krotkov did not remove all the material which interfered with the estimation of the RNA by phosphate or ribose content. The separation of the mononucleotides by ion-exchange chromatography provided the most reliable method of estimation. The DNA content of the tissue as estimated by the Schmidt and Thannhauser method was considerably lower than that obtained by the direct, hot acid extraction of Schneider, due in part to an incomplete precipitation of the DNA after alkaline hydrolysis. The Schneider method was suitable for the estimation of RNA under special conditions, such as in tissue homogenates containing no cell debris or nuclei, while the Ogur and Rosen procedure appeared to be more useful as a method of RNA fractionation than for the quantitative measurement of RNA.

INTRODUCTION

DURING an investigation of nucleotide and nucleic acid metabolism in germinating barley and maize, difficulty was experienced in applying the standard methods of extraction and estimation. Despite the large body of literature on this subject, problems still remain in choosing methods for a quantitative analysis of nucleotides and nucleic acids in plant material. Two problems are involved; one, the extraction of the compounds, and the other, the estimation of these compounds in the extract. Although the nucleotides or nucleic acids in an extract may be estimated by the optical density at 260 mμ (260. OD), the phosphate content or the sugar content, none of these methods are entirely specific for the nucleotide or nucleic acid structure, consequently the success of the estimation is dependent on the quantity and the nature of other components present in the extract.

The data, obtained from a comparative study of the extraction and estimation of nucleotides and nucleic acids from plant material by the standard methods, are presented, and the significance of the results discussed.

* Part of this work was done in the Agronomy Department of the University of Illinois, supported by Grant 791 from the Atomic Energy Commission.

† Present address, Department of Botany and Plant Pathology, Purdue University, Lafayette, Indiana.

EXPERIMENTAL RESULTS AND DISCUSSION

The Soluble Nucleotides

Soluble nucleotides are normally extracted from a tissue with either aqueous ethanol or dilute acid. Ethanol, widely used for the extraction of nucleotides from micro-organisms, has not been extensively used for other material, presumably because of difficulties caused by the co-extraction of pigments. Tissues containing pigments are normally extracted with cold dilute acid, either perchloric or trichloracetic acid. The optical density at 260 mμ is then used to calculate the nucleotide content of the extract.

In this section ethanol and cold acid are compared for their efficiency and selectivity in extracting soluble nucleotides, and the use of the 260.OD of an extract as a measure of its nucleotide content is evaluated.

1. *The Extraction of Soluble Nucleotides*

The removal of additional nucleotide material by cold acid from yeast previously extracted with ethanol,[1] suggests that acid may be more efficient than ethanol for the extraction

TABLE 1. THE EXTRACTION OF NUCLEOTIDES AND RIBONUCLEIC ACID FROM *Torulopsis utilis*
The results, expressed as percentages of the total 260.OD extracted, are the mean of two experiments.

Procedure 1			Procedure 2		
Extraction procedure	Fresh *T. utilis*	Lyophilized *T. utilis*	Extraction procedure	Fresh *T. utilis*	Lyophilized *T. utilis*
(Nucleotides)			(Nucleotides)		
5% $HClO_4$, 4°	13·3	20·6	60% Ethanol, 4°	13·7	17·4
Six successive 15-min extraction periods	3·1, 4·7, 2·0, 1·3, 1·2, 1·0	11·0, 3·6, 1·5, 1·4, 1·4, 1·7	Six successive 30-min extraction periods	8·1, 2·4, 1·3, 0·7, 0·6, 0·6	6·8, 3·7, 2·1, 1·6, 1·6, 1·6
			5% $HClO_4$, 4°	19·5	18·5
			Six or eight successive 15-min extraction periods	1·5, 0·8, 1·6, 6·2*, 3·4, 2·5, 1·9, 1·8	2·9, 1·5, 1·4, 2·2, 4·8, 5·7
(RNA)			(RNA)		
5% $HClO_4$, 4°	81·7	37·8	5% $HClO_4$, 4°	61·8	40·7
Three successive 12-hr extraction periods	27·9, 39·9, 13·9	8·1, 10·9, 18·8	Three successive 12-hr extraction periods	22·4, 32·5, 6·9	13·3, 14·1, 13·3
Alkaline hydrolysis†	5·9	41·8	Alkaline hydrolysis	5·2	23·5

* Maximum in extraction series.
† See Table 7 for details.

of soluble nucleotides. Since, however, cold acid of a similar concentration will extract RNA,[2] it is necessary to establish whether such additional material represents part of the soluble nucleotide content, or if it is simply the beginning of RNA extraction.

[1] G. HARRIS, J. W. DAVIES and R. PARSONS, *Nature* **182**, 1565 (1958).
[2] M. OGUR and G. ROSEN, *Arch. Biochem.* **25**, 262 (1950).

extraction procedures, cold perchloric acid (Procedure 1), and cold ethanol followed by cold perchloric acid (Procedure 2) were compared for fresh and lyophilized yeast (Table 1).

Fresh yeast. The same amount of nucleotide material was removed by acid and ethanol from fresh yeast. Additional nucleotide material was, however, removed by following the ethanol treatment with acid extraction (Procedure 2), and the presence of a maximum value in the extraction series (Table 1) suggested a definite nucleotide fraction, rather than the beginning of the extraction of the bulk of the RNA. The ethanol treatment was necessary to

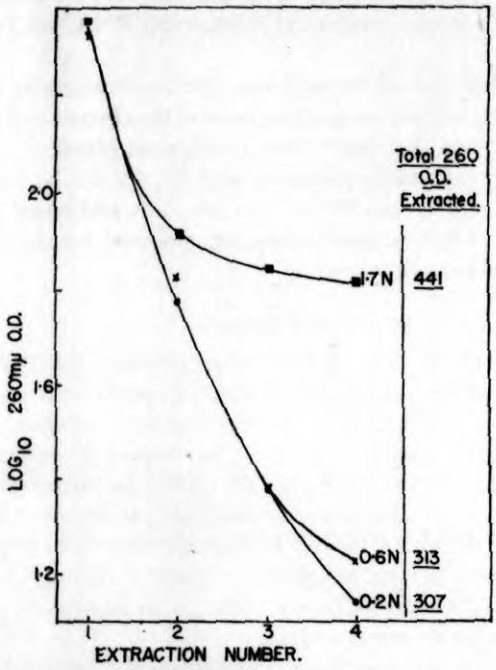

FIG. 1. THE EXTRACTION OF DAY 3 BARLEY EMBRYOS WITH VARYING CONCENTRATIONS OF PERCHLORIC ACID.
8 g fresh weight of tissue was extracted for four successive 15 min periods with 50 ml cold perchloric acid.

render this material acid-soluble, since it was not removed by direct acid extraction. An analysis of the material extracted by acid following an ethanol treatment, showed that it consisted of polynucleotide material,[3] (Gilbert and Ingle, in preparation) and was thus more closely related to the RNA of the cell than to the soluble nucleotides. (This fraction of polynucleotide material is further discussed in the nucleic acid section of this paper.)

Lyophilized yeast. Both acid and ethanol removed more nucleotide material from lyophilized than from fresh yeast. Extraction with acid following the ethanol treatment again removed more nucleotide material, but as there was no maximum value in the extraction series, this represented the beginning of RNA extraction, rather than the extraction of a discrete fraction of material.

[3] J. INGLE, Ph.D. Thesis, Bristol (1961).

The extraction of 3 day-old etiolated corn embryos by these two procedures showed that both acid and ethanol removed similar amounts of ultraviolet absorbing material, but no additional nucleotide fraction was removed with acid following the ethanol extraction.

The rate at which ultraviolet absorbing material was released from 6 day-old barley embryos was compared for three concentrations of perchloric acid (Fig. 1). The two lower concentrations of acid, 0·2 N and 0·6 N, extracted similar amounts of 260.OD, while the highest concentration, 1·7 N, extracted considerably more. An examination of the rate of extraction suggested that whereas with the lowest concentration the extraction approached the theoretical case of a simple partition of material between the extractant and the tissue, at higher concentrations a second component, presumably RNA, was being extracted from the tissue.

With the tissues studied, both ethanol and acid remove similar amounts of ultraviolet absorbing material, and ion-exchange separations of the ethanol and perchloric acid extracts of fresh yeast have shown that both contain an almost identical nucleotide composition. Direct extraction with cold dilute perchloric acid (4°, 0·2–0·6 N) for short periods of time (30–60 min) does not remove any RNA. However, any additional nucleotide material extracted with acid after an initial ethanol treatment, appears to belong to the RNA complement rather than to the soluble nucleotides.

2. *The Estimation of Soluble Nucleotide Content*

(a) *Estimation by 260.OD.* The 260.OD of the extract is usually the basis of methods for estimating nucleotide content, although sometimes a correction is made by taking the difference between the 260 mμ and the 280 or 290 mμ optical densities. Even with an ethanol extract of yeast, the spectrum of which (Fig. 2A) approaches quite closely that of a pure nucleotide mixture, only 60 per cent of the 260.OD of the extract was recovered as nucleotides in the eluate from ion-exchange chromatography, as described by Ingle.[4] This nucleotide recovery was 80–90 per cent if the 260.OD of the extract was corrected for contamination by subtraction of the 280.OD ,but any such correction is arbitrary since the spectral characteristics of the contaminants are unknown. Thus, even with yeast, a nucleotide estimation based on the 260.OD can be very inaccurate.

The nucleotide content of a perchloric acid extract of 6 day-old barley embryos, as estimated from the 260.OD, was several times greater than that determined by ion-exchange separation into the individual nucleotides. The spectrum of the acid extract (Fig. 2B) had a maximum at 270 mμ, instead of 260 mμ, and a second smaller peak at 320 mμ. The shift of this peak towards the yellow, from 320 to 380 mμ when the extract was made alkaline, suggested that flavonoid material was responsible for this absorption. The isolation of this component from the extract[5] showed that it also absorbed heavily at 260 mμ, and in fact accounted for 30 per cent of the 260.OD of the acid extract. The results of anion-exchange separations of acid extracts of day 3 and day 6 seedling tissue are summarized in Table 2. The total recovery of 260.OD from the column was only 68 and 76 per cent respectively, and this was distributed between four major fractions: material washed straight through the column which included non-phosphorylated derivatives of purines and pyrimidines, the flavone peak, peak R (a non-nucleotide peak) and nucleotide material. The total content of purine and pyrimidine derivatives, both phosphorylated and non-phosphorylated, represented only 35 per cent and 21·5 per cent of the 260.OD of extracts of day 3 and day 6 seedlings.

[4] J. INGLE, *Biochim. Biophys. Acta* **61**, 147 (1962).
[5] T. B. GAGE, Q. L. MORRIS, W. E. DETTY and S. H. WENDER, *Science* **113**, 522 (1951).

Estimation of the nucleotide content in a 5 per cent perchloric acid extract of etiolated maize embryos presented an interesting problem, since not only did the extract contain pigments, but the spectral characteristics of the extract changed with time (Fig. 2C). The 260 mμ peak, the value upon which the nucleotide estimation is based, decreased with time, and by 20 hr had disappeared. Changes in the 260–290 mμ absorption, a value which has been used for the estimation of nucleotides in acid extracts of corn seedlings,[6–8] were even more marked than those of the 260.OD. The rate of change of the 260.OD and the 260–290.OD was dependent on the temperature, concentration and pH of the acid extract;

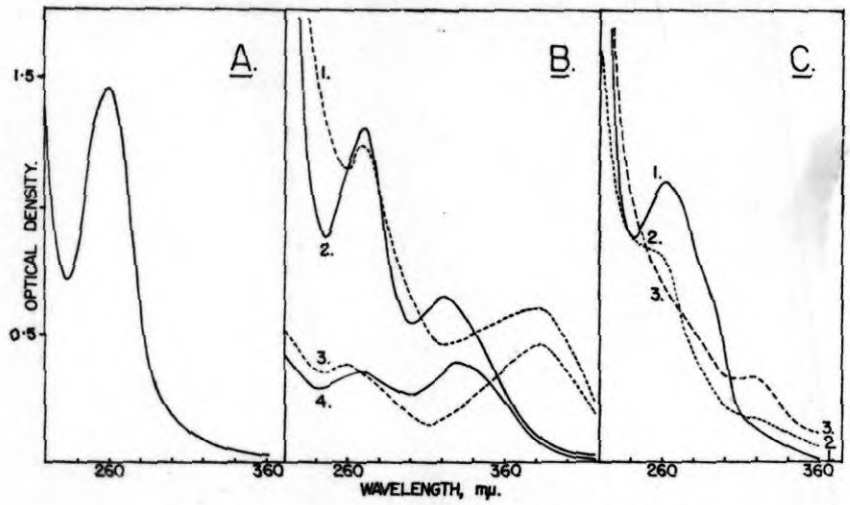

FIG. 2. THE SPECTRA OF SOLUBLE NUCLEOTIDE EXTRACTS.

A. An ethanol extract of *Torulopsis utilis*. (60% ethanol, 4°).
B. A perchloric acid extract of day 6 barley embryos (5% $HClO_4$, 4°).
   1. Extract at pH 12.
   2. Extract at pH 2.
   3. Flavone component of extract, pH 12.
   4. Flavone component of extract, pH 2.
C. A perchloric acid extract of day 3 etiolated corn embryos (5% $HClO_4$, 4°). All spectra were determined at pH 2, and extract was maintained at room temperature (30°).
   1. Determined directly after extraction.
   2. Determined 8 hr after extraction.
   3. Determined 21 hr after extraction.

dilution with water and reduction of the temperature greatly decreased the rate of change. The total recovery of 260.OD from anion-exchange resin, which adsorbed most of the pigment, was only 30–40 per cent, and of this, 45–50 per cent was non-nucleotide material, leaving nucleotides to account for only 20 per cent of the 260.OD of the original extract.

(b) *Estimation by sugar or phosphate content.* Of these two other possible methods of estimating nucleotide content, determination by ribose content was impossible due to the presence of other carbohydrate material which interfered with the orcinol determination. Total phosphate was little better as a method of estimation, since a high percentage of the

[6] J. H. CHERRY and R. H. HAGEMAN, *Plant Physiol.* **36**, 163 (1961).
[7] J. H. CHERRY, R. H. HAGEMAN, J. N. RUTGER and J. B. JONES, *Crop Science* **1**, 133 (1961).
[8] J. B. HANSON, A. E. VATTER, M. E. FISHER and R. F. BILS, *Agron. J.* **51**, 295 (1959).

total phosphate was inorganic, and of the organic phosphate only about 20 per cent was due to nucleotides.

Although neither the 260.OD nor the total phosphate content of an acid extract agree quantitatively with the nucleotide content as estimated by ion-exchange separation (Fig. 3A), these values are frequently used to indicate the qualitative changes in nucleotide content of a

TABLE 2. A SUMMARY OF THE RESULTS OF ION-EXCHANGE SEPARATIONS OF PERCHLORIC ACID EXTRACTS OF BARLEY EMBRYOS

Lyophilized day 3 and day 6 embryo tissue was extracted for three 15-min periods with cold 5% perchloric acid, and the components of these extracts were then separated by ion-exchange chromatography.

	260.OD/100 embryos	
	Day 3	Day 6
Original acid extract	262	830
Ion-exchange separation:		
Water wash;		
Non-nucleotide	40	114
Purine and pyrimidine derivatives	46	70
Eluate from column;		
Flavone	13	223
Peak R	32	120
Nucleotide	46	108
Total recovered	177	635
Total nucleotides + purine and pyrimidine derivatives	92	178

tissue. Figure 3B shows, however, that neither of these values give a very accurate picture of the changes in nucleotide content which occur during the germination of barley.

3. *Purification of the Soluble Nucleotides*

The spectral data and the ion-exchange separations show that several groups of compounds contribute to the 260.OD of the extract, and for the determination of nucleotide content it is therefore necessary to isolate the nucleotides from the other components in the extract.

The insolubility of nucleotides, as the mercury salts in 50% ethanol[9] or as the calcium salts in ethanol–ether,[10] has been used as a method of purification. This method was not, however, readily applicable to ethanol extracts of plant tissue.

Although it is difficult to isolate the nucleotides from the extract, it is possible to remove certain contaminating components. Extraction with ether or chloroform removed most of the pigment from an acid extract, reducing the 260.OD of an etiolated corn extract by 50 to 70 per cent. Attempts to remove flavonoids and brown pigments from the extract with cation-exchange resin[5] proved impracticable due to a concomitant exchange of certain of the nucleoside monophosphates by the resin.

The nucleotides may be purified by absorption and bulk elution from an anion-exchange resin, but the usefulness of this method is dependent on the absence of any other components which would be co-eluted with the nucleotides. The separation of the nucleotides in an ethanol extract of yeast[4] showed that in this case a bulk elution of the nucleotides would give a true

9 R. CAPUTTO, L. F. LELOIR, C. E. CARDINI and A. C. PALADINI, *J. Biol. Chem.* **184**, 333 (1950).
10 H. G. PONTIS and N. L. BLUMSOM, *Biochim. Biophys. Acta* **27**, 618 (1958).

evaluation of the nucleotide content, since only nucleotide material was eluted from the column under those conditions. With an acid extract of barley embyros, however, much of the material eluted from the resin was non-nucleotide in nature.

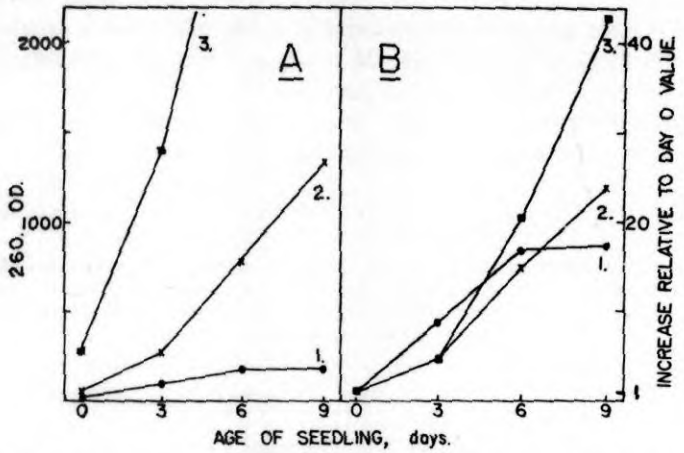

FIG. 3. THE CHANGES IN SOLUBLE CONSTITUENTS DURING THE GERMINATION OF BARLEY.
The soluble constituents were extracted with cold 5% $HClO_4$.

A. The absolute values obtained.
B. The values expressed relative to the day 0 value.
1. Nucleotide content, determined from an ion exchange separation of the extract.
2. 260.OD of the extract.
3. Total phosphate content of the extract. In Fig. 3A the μg P have been converted to a 260.OD value by using an arbitrary millimolar extinction coefficient of 14.

The chromatographic separation of the individual nucleotides has proved to be the most satisfactory method for the estimation of nucleotide content. The variety of interfering compounds in an extract normally prevents the direct estimation of nucleotides by 260.OD, phosphate or ribose content, and no suitable simple purification procedure has been found.

THE NUCLEIC ACIDS

This paper is restricted to problems encountered in the extraction and estimation of nucleic acids from plant material, since an extensive literature exists for the extraction of these compounds from other tissues, as illustrated by the excellent review of Hutchinson and Munro.[11] Nucleic acid extracts from plant material invariably contain high concentrations of substances which interfere with the 260.OD, sugar and phosphate estimations, and the usefulness of an extraction method thus depends largely upon the ease with which the nucleic acid component can be purified before estimation.

The Extraction and Estimation of RNA

Methods which have been developed specifically for the extraction of undegraded nucleic acid are not quantitative,[12,13] and will not be considered here. Quantitative extraction of

[11] W. C. HUTCHISON and H. N. MUNRO, *Analyst* **86**, 768 (1961).
[12] K. YAMANA and A. SIBATANI, *Biochim. Biophys. Acta* **41**, 295 (1960).
[13] F. F. DAVIS and F. W. ALLEN, *J. Biol. Chem.* **227**, 907 (1957).

RNA is usually obtained only by degradation of the macromolecule, using either hot or cold acid, or alkali.

1. *Extraction of RNA with Cold Acid*[2]

Many doubts have been expressed concerning the quantitativeness of RNA extraction with cold acid.[11] Work done in this laboratory suggests that part of the variability of this method is that the rate of RNA extraction is dependent not only on the kind of tissue, but also on the treatment which the tissue has undergone prior to this extraction. This is illustrated by the data in Table 1 (Procedure 1). With fresh yeast, three 12-hr extractions with cold 5%

TABLE 3. THE EFFECT OF DIFFERENT INITIAL TREATMENTS ON THE EXTRACTION OF RNA FROM DAY 3 ETIOLATED CORN EMBRYOS BY COLD PERCHLORIC ACID

Samples were taken from an homogenate of embryo tissue and extracted by one of four procedures. The lipids were removed in Procedure 3 with cold ethanol, followed by hot ethanol–ether (3:1) and hot ether, and in Procedure 4 with ethanol–ether–chloroform (2:2:1) at room temperature. Total nucleic acid was extracted from duplicate samples with 5% $HClO_4$, 70°, 40 min.

		260-OD of Extract			
		Procedure 1	Procedure 2	Procedure 3	Procedure 4
A. Initial Treatment of Sample.					
Soluble nucleotide extraction		3 × 60% EtOH, 0°, 30 min	—	—	—
		3 × 5% $HClO_4$, 0°, 15 min	3 × 5% $HClO_4$, 0°, 15 min	3 × 5% $HClO_4$, 0°, 15 min	3 × 5% $HClO_4$, 0° 15 min
Lipid extraction		—	—	Ethanol–ether	E–E–C
		—	—	Acid wash, 0°	Acid wash, 0°
B. Extraction of Nucleic Acids					
Total nucleic acid		23·2	24·1	22·8	23·4
Extraction of RNA					
5 successive 12 hr extractions with N $HClO_4$, 4°	1	7·4*	8·5*	5·9*	4·2*
	2	3·7*	5·9*	5·4*	6·5*
	3	1·0	1·6*	1·5*	3·3*
	4	0·5	1·1	0·8*	1·7*
	5	0·5	1·1	0·7*	1·3*
† Summation		11·1	16·0	14·3	17·0
% Total nucleic acid		48	66	63	73

* Extract had a spectrum with a 260 mμ peak.
† Only those extracts having a 260 mμ peak were included in the summation.

perchloric acid removed 95 per cent of the RNA, whereas with lyophilized yeast only half of the RNA was removed by comparable extractions.

A further study was made of the effects of different initial treatments on the extraction of RNA from etiolated corn embryos by cold perchloric acid (Procedures 1–4, Table 3). The extraction of a duplicate sample of material with hot acid showed that the different initial treatments did not affect the extraction of total nucleic acid. However, with cold perchloric acid (used for five successive extraction periods of 12 hr each), the largest amount of RNA, representing 73 per cent of the total nucleic acid, was removed from tissue which had been defatted with the ethanol–ether–chloroform mixture (Procedure 4), whereas only 48 per cent of the total nucleic acid was removed from the tissue previously extracted with ethanol and

perchloric acid (Procedure 1). The rates of extraction were such, however, that had 12 rather than 60 hr been used as the extraction period, then Procedure 4 would have removed much less than Procedure 1.

Other experiments have shown that the RNA initially extracted by perchloric acid from lyophilized yeast differed from the bulk of the acid-extracted RNA in base composition, and probably in physical structure (Table 4). These data, together with the high metabolic activity

TABLE 4. THE EXTRACTION OF RNA FROM YEAST WITH COLD 5% PERCHLORIC ACID

Two samples of lyophilized yeast were extracted with 60% ethanol (3 × 30 min, 0°) to remove soluble nucleotides. One sample was then extracted with alkali (N KOH, 25°, 24 hr) to give the total RNA, and the RNA of the other sample was removed by a series of acid extractions (5% $HClO_4$, 4°).

	Percentage of total RNA	Composition of RNA*				Percentage of RNA degraded to mononucleotides during acid extraction
		CMP	AMP	GMP	UMP	
Alkaline Extraction						
Total RNA	—	23·6	24·0	27·2	25·2	—
Acid Extraction						
Fraction 1 (Extraction time of 1 hr)	3·1	15·9	20·6	36·1	27·8	43·5
Fraction 2 (Extraction time of 22·5 hr)	66·9	22·2	20·9	25·4	31·6	14·5

* The composition was determined after alkaline hydrolysis of the RNA, and is expressed as moles per 100 moles of the four nucleotides.

observed for the polynucleotide fraction isolated by perchloric acid from fresh yeast,[3] (Gilbert and Ingle, in preparation), suggest that the extraction of RNA with cold perchloric acid is probably more useful as a method of RNA fractionation[14, 15] than for the quantitative estimation of RNA.

2. *Extraction of RNA with Hot Acid*

The Schneider extraction procedure,[16] in which total nucleic acid is extracted with hot acid, and the RNA and DNA are measured by means of the differential colorimetric sugar reactions, is difficult to apply to most plant materials, since the excess of carbohydrate in such material interfers with the orcinol determination of ribose. In the present investigation with barley embryos, estimates of RNA content based on the orcinol estimation of ribose in a hot perchloric acid extract were up to a 100 times too high. The RNA could not be estimated indirectly as the difference between total nucleic acid and DNA, as estimated by the diphenylamine reaction, since neither phosphate content nor 260 mμ OD gave reliable estimations of the total nucleic acid content of the extract.

Hot acid extraction has, however, been used for the measurement of RNA in plant material. Hanson, *et al.*[8] estimated the nucleic acid content (RNA) of corn mitochondria from the 260 mμ OD of a hot acid extract, and this method has also been applied to the estimation of RNA

[14] E. BASLER and K. NAKAZAWA, *Botan. Gaz.* **122**, 238 (1961).
[15] J. K. HEYES, *Proc. Roy. Soc.* (B) **152**, 218 (1960).
[16] W. C. SCHNEIDER, *J. Biol. Chem.* **161**, 293 (1945).

in corn and soybean seedling homogenates from which cellular debris and nuclei (i.e. DNA) had been removed by low speed centrifugation.[6,7,17] A comparison has been made between RNA content as extracted and estimated by this method, and RNA content as measured by alkaline hydrolysis and anion-exchange purification. The most striking result from this experiment was that clearing the homogenate of cellular debris and nuclei not only removed most (86 per cent) of the DNA, but also removed 55 per cent of the RNA (Table 5, Procedure 1). The percentage of nucleic acid lost in this method could be reduced by a more thorough homogenation, and a situation could be reached where the whole homogenate was put through the extraction procedures, as in the case of the homogenization in perchloric

TABLE 5. A COMPARISON OF THE EXTRACTION OF NUCLEIC ACIDS BY HOT ACID OR BY ALKALI

Samples of 3 day-old etiolated corn embryos were homogenized in 0·5 M sucrose or 5% $HClO_4$. The sucrose homogenate was cleared by centrifugation,[7] and aliquots of the supernate and debris, and of the whole acid homogenate, were extracted by two procedures. Soluble nucleotides were removed with 5% $HClO_4$ (3 × 15 min, 0°) and lipids with ethanol–ether–chloroform (3 × 15 min, 25°). In Procedure 1 the RNA was extracted by alkaline hydrolysis (N KOH, 25°, 24 hr) and the acid precipitate was extracted with hot acid (5% $HClO_4$, 70° 40 min) for DNA. In Procedure 2 both RNA and DNA were extracted with hot acid and then the residue was extracted with alkali to remove any remaining RNA.

The RNA content of the acid extracts was calculated from a standard curve of similarly treated yeast RNA (260–290 . OD × 48), and the alkaline extracted RNA was estimated on the basis of a theoretical tetranucleotide structure (260·OD × 31·7—see Table 7). DNA was estimated by the diphenylamine procedure.

	Procedure 1			Procedure 2		
	Extraction	μg RNA	μg DNA	Extraction	μg RNA	μg DNA
Whole homogenate	KOH	1015	—	$HClO_4$	865	72
	$HClO_4$	0	37	KOH	180	—
	Total	1015	37	Total	1045	72
Supernate	KOH	505	—	$HClO_4$	538	8
	$HClO_4$	0	5	KOH	0	—
	Total	505	5	Total	538	8
Debris	KOH	622	—	$HClO_4$	258	60
	$HClO_4$	0	31	KOH	282	—
	Total	622	31	Total	540	60

acid. Although hot acid extraction (Procedure 2) was sufficient to remove all the RNA from the cleared homogenate (no further RNA was removed by subsequent alkaline hydrolysis), 17 per cent was left in the whole homogenate, and 52 per cent in the homogenate debris.

Although the spectra of the alkaline extracted RNAs were reasonably constant, the spectrum of the hot acid extract varied for the different samples, and the 250/260 and 280/260 optical density ratios, which give a good indication of the shape of the 260 mμ peak and hence the extent of contamination in the extract, are given in Table 6. Only from the cleared homogenate were the spectral ratios of alkaline and acid extracts similar. The spectrum of the acid extract of the homogenate debris had no 260 mμ peak.

Hot acid can be used for the extraction of RNA from a cleared homogenate, but the action of clearing removes a considerable portion of the total RNA. A more thorough homogenation gives a higher recovery of RNA in the supernate, but the inclusion of nuclear fragments necessitates a correction for DNA content. Furthermore, with the inclusion of more cellular

[17] J. L. KEY and J. B. HANSON, *Plant Physiol.* **36**, 145 (1961).

tissue, the hot acid does not extract all the RNA, and in addition, the nucleotide character of the spectrum is lost.

TABLE 6. THE SPECTRAL CHARACTERISTICS OF HOT ACID AND OF ALKALINE EXTRACTS OF ETIOLATED CORN EMBRYOS

The extracts are those of the experiment described in Table 5.

Optical density ratios	Whole homogenate		Supernate		Debris	
	KOH (Procedure 1)	$HClO_4$ (Procedure 2)	KOH (Procedure 1)	$HClO_4$ (Procedure 2)	KOH (Procedure 1)	$HClO_4$ (Procedure 2)
250/260	0·85	0·93	0·80	0·84	0·82	1·02
280/260	0·69	0·87	0·66	0·65	0·67	0·98

The 250/260 and 280/260 ratios of a standard solution containing equimolar proportions of the four RNA nucleotides are 0·78 and 0·65 respectively.

3. *Extraction of RNA with Alkali*

The Schmidt and Thannhauser procedure,[18] based on the difference in chemical structure between RNA and DNA, is theoretically the most satisfactory method for the extraction and separation of the nucleic acids, and the details of this procedure are adequately discussed by

TABLE 7. THE ESTIMATION OF RNA FROM BARLEY EMBRYOS, WITH AND WITHOUT ANION-EXCHANGE BULK PURIFICATION OF THE ALKALINE HYDROLYSATE

Nucleotide-free, lipid-free powder of day 6 embryos was hydrolysed with N KOH, 25°, 24 hr. Part of the hydrolysate was then purified using the procedure of Smillie and Krotkov;[20] the acidified hydrolysate was concentrated to a few ml and run onto a Dowex 1, × 10, Cl^- column, 5 cm × 0·5 cm^2, at pH 7–8. After washing with 0·01 M NaCl, the nucleotides were eluted with N HCl containing 2·3% NaCl.

Estimation based on	μg RNA (tetranucleotide)*	
	Original hydrolysate	Anion exchange eluate
260.OD	Brown solution	965
Total phosphate	1,370	1,305
Ribose	81,000	1,660
Total nitrogen	5,560	1,640

* If RNA is assumed to be a linear chain consisting of tetranucleotide units, each unit containing one molecule each of AMP, GMP, CMP and UMP, then it can be calculated that 1358 μg RNA contain 600 μg ribose (of which 300 μg will react under the normal conditions of the orcinol reaction), 124 μg phosphorus, 210 μg nitrogen and will have a total 260.OD of 42·8.

Hutchinson and Munro.[11] Determination of the RNA content by the direct estimation of 260.OD, ribose or phosphate in the acidified alkaline hydrolysate, while satisfactory for most animal tissues and micro-organisms, is not feasible for plant tissue. Purification of the

[18] G. SCHMIDT and S. J. THANNHAUSER, *J. Biol. Chem.* **161**, 83 (1945).

RNA nucleotides in the hydrolysate is required. Deken-Grenson and Deken[19] and Smillie and Krotkov[20] have used anion-exchange resin for the purification of the nucleotides, and found good agreement for RNA content as estimated by 260.OD, ribose or phosphate after this treatment. The use of a short hydrolysis time, 1 hr instead of 15, to render the RNA acid-soluble without degrading protein,[11] has not been beneficial with the plant tissues studied.

Analyses were made to determine the efficiency of anion-exchange purification[20] of the RNA mononucleotides from the acidified alkaline hydrolysate. Table 7 presents data for the RNA content of an alkaline extract of barley embryos, calculated from 260.OD, phosphate,

TABLE 8. THE ESTIMATION OF RNA CONTENT OF BARLEY EMBRYOS AND ENDOSPERM AFTER ANION-EXCHANGE BULK PURIFICATION

Results expressed as mg RNA (tetranucleotide) per 100 embryos or endosperms.

	mg RNA					
Age of seedlings (days)	0	2	4	6	8	10
Embryo						
Estimated by;						
260.OD	2·6	6·1	12·4	14·2	12·4	9·0
Phosphate	3·1	6·1	11·6	15·4	15·2	10·4
Ribose	7·8	8·0	20·2	23·4	25·6	23·4
RNA (phosphate) / RNA (260.OD)	1·20	1·00	0·94	1·08	1·22	1·16
RNA (ribose) / RNA (260.OD)	3·00	1·31	1·63	1·65	2·07	2·60
Endosperm						
Estimated by;						
260.OD	5·8	5·6	3·4	2·3	2·4	0·8
Phosphate	21·7	14·3	9·2	4·8	3·2	1·2
Ribose	10·7	10·1	9·3	7·1	10·2	7·4
RNA (phosphate) / RNA (260.OD)	3·75	2·56	2·70	2·08	1·34	1·50
RNA (ribose) / RNA (260.OD)	1·85	1·81	2·73	3·09	4·25	9·20

ribose and total nitrogen determinations, assuming the RNA to have a theoretical tetranucleotide structure. For pure RNA these estimates will of course agree closely, but for the alkaline hydrolysate the estimates differed widely, and even after anion-exchange bulk purification the estimates were still different.

Alkaline hydrolysis, followed by anion-exchange bulk purification, was used to follow the changes in the RNA content of the embryo and endosperm during the germination of barley. The data (Table 8) showed that not only did the purified eluates contain interfering phosphate and sugar compounds, but that the degree of interference varied with the stage of development of the tissue. Interference with the ribose estimation was found from both embryo and endosperm tissue, the amount of interference increasing with the development of the tissue. With

[19] M. DEKEN-GRENSON and R. H. DEKEN, *Biochim. Biophys. Acta* **31**, 195 (1959).
[20] R. M. SMILLIE and G. KROTKOV, *Can. J. Botany* **38**, 31 (1960).

the embryos, the phosphate estimate was in reasonable agreement with that of the 260.OD, in contrast the endosperm contained large amounts of interfering phosphate, particularly at the earliest stages of germination.

The changes in the total RNA content of the barley seedlings over the ten-day germination period, shown in Fig. 4, are an excellent example of how the choice of a method can affect one's impression of the metabolism of a tissue. On the basis of ribose estimation the total RNA increased twofold over the germination period; on the basis of phosphate estimation the total RNA decreased by half; and on the basis of 260.OD estimation the RNA increased to a maximum value at day 6 and then decreased.

The RNA from several other sources was extracted, purified and estimated in an identical manner, Table 9. The ratios obtained for the sample of commercial yeast RNA approached unity, as expected for a purified product. The RNA eluates from the samples of *Torulopsis*

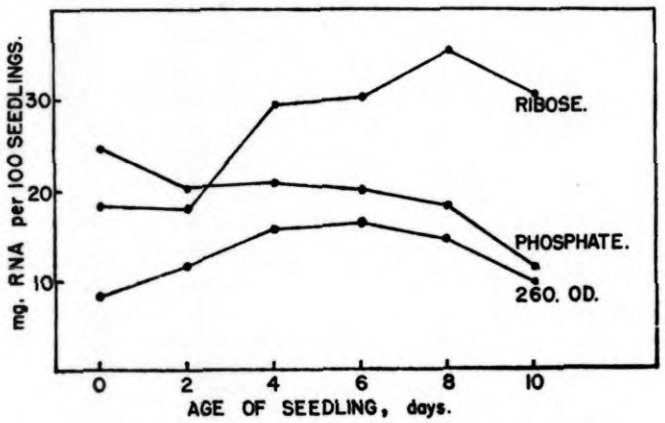

FIG. 4. THE CHANGES IN THE RNA CONTENT OF WHOLE SEEDLINGS DURING THE GERMINATION OF BARLEY.
The RNA was extracted by alkaline hydrolysis, and estimated from 260.OD, ribose and phosphate determinations made on the eluate from anion-exchange bulk purification.

utilis and *Rhizopus sexualis* both contained considerable impurities, particularly phosphate, but much of this interference could be removed by a partial dialysis of the hydrolysate before acidification. The results from pea roots showed that there was still considerable interference with the ribose estimation, but the agreement between phosphate and 260.OD estimates was good.

For the range of material investigated, bulk elution of the RNA mononucleotides from anion-exchange resin gave only a partial purification, and RNA content based on ribose or phosphate estimates was usually considerably higher than that based on 260.OD. The best method of estimating the RNA is to separate and estimate the individual mononucleotides, and the RNA content and composition of a variety of tissues has been determined by the separation of the mononucleotides by ion-exchange chromatography.[3] The 260.OD, phosphate and ribose content of the RNA, as obtained from the summation of the four nucleotide peaks, can be used to calculate the RNA content on the basis of a tetranucleotide structure. The values so obtained, relative to the 260.OD value, are the ratios which would be obtained from the anion-exchange resin treatment if the purification of the nucleotides

by this method was complete. These values are given for a few samples in Table 10, and can be compared with those actually obtained after the anion-exchange bulk purification. In all cases the ratios as calculated from the separated nucleotides approximated to unity, the value expected for a tetranucleotide structure. The 260.OD recovered from the separation as the

TABLE 9. THE ESTIMATION OF RNA CONTENT OF DIFFERENT MATERIALS BY 260.OD, RIBOSE AND PHOSPHATE DETERMINATIONS ON THE ELUATE FROM ANION-EXCHANGE BULK PURIFICATION

	Yeast RNA*	*T. utilis*	*T. utilis*†	*R. sexualis*	Pea roots
$\frac{\text{RNA (phosphate)}}{\text{RNA (260.OD)}}$	1·05	1·58	1·06	2·29	1·08
$\frac{\text{RNA (ribose)}}{\text{RNA (260.OD)}}$	1·11	1·22	1·08	1·69	1·85

* Commercial preparation of yeast RNA from British Drug Houses, Ltd.
† The alkaline hydrolysate was partially dialysed before acidification and purification.
(Dialysis against H_2O for 8 hr at 25°. Twenty per cent of the nucleotides were recovered.)

four nucleotides, relative to the 260.OD recovered from the anion-exchange bulk purification, varied a little, but it was obvious that an RNA estimation based on the 260.OD of the eluate from bulk purification would be within 10 per cent of the true value.

TABLE 10. A COMPARISON OF RNA CONTENT AS BASED ON ANION-EXCHANGE BULK PURIFICATION WITH THAT BASED ON THE SEPARATION OF THE NUCLEOTIDES

Material	Anion-exchange bulk purification		Summation of separated nucleotides		260.OD* Recovery %
	$\frac{\text{RNA (phosphate)}}{\text{RNA (260.OD)}}$	$\frac{\text{RNA (ribose)}}{\text{RNA (260.OD)}}$	$\frac{\text{RNA (phosphate)}}{\text{RNA (260.OD)}}$	$\frac{\text{RNA (ribose)}}{\text{RNA (260.OD)}}$	
Yeast RNA (B.D.H.)	1·05	1·11	0·98	1·06	102
T. utilis	1·48	1·14	1·00	1·03	91·5
Day 3 barley embryo (alkaline extract)	1·02	1·48	1·02	1·08	105
Day 3 barley embryo (perchloric acid extract)	0·93	1·41	1·03	1·02	89

* 260.OD recovered as the four nucleotides relative to the 260.OD recovered from anion-exchange bulk purification.

The Extraction and Estimation of DNA

1. *Extraction*

The DNA, solubilized by alkaline hydrolysis in the Schmidt–Thannhauser procedure, is separated from the RNA mononucleotides by acid precipitation. The precipitation method described by Smillie and Krotkov,[20] where the alkaline hydrolysate was cooled, Mg^{2+} added

and mixed with an equal volume of 95% ethanol, has given reproducible results for the tissues studied. The DNA is then extracted from the precipitate by hot acid extraction. Both 15-min extractions at 90°, and 20-min extractions at 70° have been used, and no major differences have been observed, providing the standard DNA was subjected to the same procedure. The difficulty observed by Kupila *et al.*[21] in the extraction of DNA by hot acid from mature plant tissue was not encountered with barley, and two 15-min extractions at 90° were found to remove more than 93 per cent of the total extractable DNA from both embryo and endosperm tissue.

From Table 5 it can be seen that the estimation of DNA in a hot acid extract made prior to alkaline extraction (Procedure 2) was considerably larger than that estimated in a hot acid extract of material which had been precipitated by acidification of the alkaline hydrolysate (Procedure 1). This is in agreement with the report of Drasher[22] that with some tissues the DNA may be degraded by the alkaline digestion to a stage at which part is no longer acid-precipitable. As pointed out by Hutchinson and Munro,[11] however, this assumes the higher value obtained from the direct hot acid extraction to be the correct value, although this value could possibly be high due to contamination in the extract. To determine if this discrepancy was due to incomplete precipitation of the DNA, a standard sample of DNA was extracted with hot acid (5% $HClO_4$, 70°, 40 min) before and after alkaline hydrolysis (N KOH, 25°, 24 hr). The DNA content after alkaline hydrolysis was consistently lower, by 20 to 25 per cent. It would appear then, that under the conditions used, part of the DNA was lost during the alkaline hydrolysis and acid precipitation.

This loss of 20 to 25 per cent, however, was not large enough to account for the 50 per cent difference found for the corn tissue. A comparison of the spectra of the blue complexes formed by the two corn extracts in the diphenylamine reaction did not suggest that contamination was responsible for the higher value observed with the direct acid extraction. Consequently, it is thought that a direct hot acid extraction gives the better value for the DNA content of the tissue.

2. *Estimation*

The DNA in the hot acid extract may be estimated by either 260.OD, phosphate or deoxyribose content. Extracts of barley embryos and of barley and corn endosperm had no distinct 260 mμ spectra, and although the spectra of corn embryo and yeast extracts did have a slight 260 mμ peak, the estimation of DNA content based on this value was up to fifteen times higher than that based on the diphenylamine estimation.

Since the development of the method by Schmidt and Thannhauser, phosphate determinations have been used to measure the amounts of nucleic acid present in each fraction of the acidified hydrolysate. With growing barley embryo tissue there was reasonable agreement between phosphate and deoxyribose estimations of DNA. This agreement did not exist for the endosperm, which contained considerable non-nucleic acid phosphate as shown in Table 11, where the total phosphate content of the hot acid extract is given as a percentage of the DNA, as estimated by the diphenylamine procedure.

The estimation of deoxyribose content is the only method which will specifically determine DNA rather than RNA. Although not entirely specific for deoxyribose, the diphenylamine reaction is not affected by the abundance of carbohydrate present in the extract, material which interferes so markedly with the ribose determination. Kupila *et al.*[21] have

[21] S. KUPILA, A. M. BRYAN and H. STERN, *Plant Physiol.* **36**, 212 (1961).
[22] M. L. DRASHER, *Science* **118**, 181 (1953).

checked the specificity of the diphenylamine reaction by the microbiological assay method of Hoff-Jorgensen,[23] and with the tissues tested the two methods gave reasonable agreement, showing that there was little interference with the diphenylamine determination of DNA.

Since most of the methods for the extraction and estimation of nucleotides and nucleic acids were originally developed for animal tissues, it is not surprising that inconsistencies should arise with their adaptation to other tissues. Because of the abundance of materials in plant extracts which interfere with sugar, phosphate and 260.OD determinations, no reliable general method for the direct estimation of total nucleotides or nucleic acid is available. Complex purification procedures based on ion-exchange chromatography are necessary for the reliable estimation of these compounds.

TABLE 11. THE PHOSPHATE AND DNA CONTENT OF HOT ACID EXTRACTS OF BARLEY EMBRYO AND ENDOSPERM

Age of seedling (days)	Total phosphate as a percentage of the DNA	
	Embryo	Endosperm
0	12·5	85·8
3	8·8	19·0
6	7·4	11·1
9	7·1	15·7

DNA was estimated by the diphenylamine procedure. Pure DNA contains 7–8% phosphorus.

METHODS AND MATERIALS

Ultraviolet absorption. Optical densities were determined in quartz cells of 1 cm light path, using either a Hilger "Uvispek" or a Beckman "DU" ultraviolet spectrophotometer. The total 260.OD of a sample is the 260 mμ optical density as measured, multiplied by the volume of the sample.

Phosphate estimation. Inorganic phosphate was measured on aliquots containing 0 to 50 μg of phosphorus. The sample, in a 50 ml graduated flask, was made up to approximately 40 ml with water. To this were added 2 ml 60% perchloric acid, 2 ml Deniges solution (5% $(NH_4)_6Mo_7O_{24}.4H_2O$ in 9 N H_2SO_4) and 1 ml stannous chloride (2·5% $SnCl_2$ in 1·2 N HCl). The mixture was thoroughly shaken, made up to 50 ml with water, and after 20 min the intensity of the blue color produced was measured on a Hilger "Spekker" colorimeter, using 1 cm cells and a red (Ilford 608) filter. Organic phosphate was hydrolysed by heating the sample with 2 ml 60% perchloric acid for 1 hr on an electric heating rack, and the total phosphate was then determined as inorganic phosphate, but without any further addition of perchloric acid. The production of the blue complex was very sensitive to the level of perchloric acid present in the reaction mixture, and the reproducibility of the method largely depended on maintaining a constant concentration of perchloric acid.

Ribose estimation. The orcinol method of Mejbaum[24] as modified by Hurlbert *et al.*[25] was used.

[23] E. HOFF-JORGENSEN, *Biochem. J.* **50**, 400 (1952).
[24] W. MEIBAUM, *Hoppe-Seyler's Z. physiol. Chem.* **258**, 117 (1939).
[25] R. B. HURLBERT, H. SCHMITZ, A. F. BRUMM and V. R. POTTER, *J. Biol. Chem.* **209**, 23 (1954).

Deoxyribose estimation. The Dische[26] reaction as modified by Burton[27] was followed. Redistillation of the diphenylamine and of the acetic acid, as recommended by Burton, were found to be unnecessary if Analytical Grade Reagents were used.

Chromatographic Analysis of Soluble Nucleotides and RNA

The soluble nucleotides were separated as described by Ingle.[4] 0·8 M formate in 4 N formic acid was used to elute the nucleotides in bulk from the column.

The RNA mononucleotides produced by alkaline hydrolysis, were separated by ion-exchange chromatography. Although it was possible to use the gradient elution system employed for the soluble nucleotides,[4] it was found more convenient to use a modification of a stepwise-elution separation described by Cohn.[28] With modification of the column dimensions and of the eluting buffers, it was possible to reduce the volume of eluent that had to be collected from 1 l. to 250 ml, (70 tubes of 3·25 ml). Dowex 1, ×8 resin 200–400 mesh, with formate as the exchangeable ion, was used in a 15 cm by 0·6 cm diameter column, 0·23 N and 3·26 N formic acid being used for elution. The first buffer removed CMP and AMP, the two isomers of AMP (2′ and 3′) being completely separated. Any nucleosides or bases appeared immediately prior to CMP. The second buffer immediately brought off a small peak, the identification of which was uncertain, before the elution of GMP. Directly following GMP was another small peak, which, from its spectral characteristics and position[29] could be 5′ribosyluracil phosphate, and finally UMP was eluted. This separation, while being relatively quick and easy to handle, provided the required information on the RNA content and composition.

The method described by Smillie and Krotkov[20] was used for the bulk purification of the RNA mononucleotides.

Biological Material

Barley (Spratt Archer) was germinated as described by Folkes *et al.*[30] After the required period of germination the embryo was dissected from the endosperm, and both portions were ground in solid CO_2 prior to lyophilization. Aliquots of the lyophilized tissue were ground for 1 min in an M.S.E. homogenizer (100 mg/25 ml extractant) at 4° and then extracted for the required time with occasional shaking.

Maize (WF9 × M14) was germinated as described by Cherry and Hageman.[6] At the required time the embryo axis, scutellum, and endosperm were separated by dissection. The fresh embryo or scutellar tissue was immediately homogenized in a conical glass homogenizer maintained at 0°, (1 g/5 ml). Endosperm tissue was homogenized at 0° in a Servall Omni-Mixer (top speed for 2 min). Homogenates were shaken occasionally (Vortex Mixer) during the extraction period (0°).

Torulopsis utilis was cultured as described by Sims (personal communication).[3] The yeast was harvested by filtration through a Millipore filter or a celite pad, and either extracted directly or lyophilized. Extractions (100 mg fresh yeast/25 ml) were made at 4° with occasional shaking.

[26] Z. Dische, *Mikrochemie* **8**, 4 (1930).
[27] K. Burton, *Biochem. J.* **62**, 315 (1956).
[28] W. E. Cohn, *J. Amer. Chem. Soc.* **72**, 1471 (1950).
[29] K. Ichimura, M. Izawa and Y. Oota, *Plant Cell Physiol (Tokyo)* **1**, 317 (1960).
[30] B. F. Folkes, A. J. Willis and E. W. Yemm, *New Phytologist* **51**, 317 (1952).

A sample of nucleotide-free, lipid-free powder of pea roots, taken from seedlings grown for 4 days at 25°, was kindly supplied by Mr. A. J. Abbott. A lyophilized sample of *Rhizopus sexualis*, grown at 20° for 100 hr in static liquid media, was kindly supplied by Mr. M. D. Coley-Smith.

Acknowledgements—The author is indebted to Drs. B. F. Folkes, R. H. Hageman, J. B. Hanson and C. A. Ingle for helpful discussion, and to D.S.I.R. for a Research Grant to support part of this investigation.