Phytochemistry, 1965, Vol. 4, pp. 831 to 843. Pergamon Press Ltd. Printed in England

QUANTITATIVE EXTRACTION AND ESTIMATION OF PLANT NUCLEIC ACIDS

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(*Received* 17 *April* 1965)

Abstract—A study has been made of the methods which have been employed for the quantitative estimation of nucleic acids in plant tissues. Carefully controlled conditions are essential to prevent loss of RNA and DNA during the preliminary extractions. To prevent loss during extraction with hot lipid solvents, the residue remaining after cold acid extraction must be first treated with ethanol saturated with sodium acetate. All three procedures examined (Schneider, Schmidt–Thannhauser, Ogur–Rosen) for the quantitative extraction of nucleic acids failed to provide an RNA extract suitable for estimation. Chromatography of the Schmidt–Thannhauser extract on charcoal and Dowex 1 yields a satisfactory extract. The method is rapid and can deal with very impure extracts.

INTRODUCTION

EVIDENCE has accumulated which shows that the quantitative extraction and estimation of plant nucleic acids present many problems not only of extraction and separation of RNA and DNA, but also of extract contamination. All reported quantitative extractions have been based on one or other of the procedures outlined by Schneider,[1] Schmidt and Thannhauser,[2] or Ogur and Rosen,[3] and originally designed for animal and microbial tissues, but it has been suggested that at least one of these procedures is not quantitative[4] when applied to plants.

A critical study of the quantitative estimation of nucleic acids in plants was therefore undertaken. The three basic procedures have been investigated in an attempt to devise a method which would not only permit a reliable estimate of nucleic acids without the use of elaborate or time-consuming procedures, but also allow several samples to be analysed at the same time. Preliminary extractions are necessary in order to stop enzyme activity when fresh tissue is employed and to remove substances such as coenzymes, mononucleotides, inorganic phosphate, carbohydrates (soluble in cold acid), and phospholipids, all of which would interfere with the final estimation of nucleic acids. Methods for the removal of these interfering substances were investigated before evaluating the methods claiming quantitative extraction of RNA and DNA.

RESULTS AND DISCUSSION

Cold Acid-soluble Substances

The removal of substances soluble in cold acid before the use of lipid solvents is more convenient than the reverse, because in this way a dry powder, suitable for storage over short periods, is obtained. Trichloroacetic and perchloric acids are most commonly employed.

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[1] W. C. SCHNEIDER, *J. Biol. Chem.* **161**, 293 (1945).

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

[3] M. OGUR and G. ROSEN, *Arch. Biochem.* **25**, 262 (1950).

[4] W. C. HUTCHINSON and H. N. MUNRO, *Analyst* **86**, 768 (1961).

Available evidence suggests that the use of 1–2 N $HClO_4$ may result in some loss of nucleic acids,[4,5] whilst 0·2 N $HClO_4$ may fail to extract all non-nucleic acid phosphorus.[4,5] Trichloroacetic acid (10% w/v), on the other hand, appears to extract all non-nucleic acid phosphorus without any detectable loss in RNA and DNA.[6]

Experiments on the removal of non-nucleic phosphorus from rye homogenates with 10 per cent trichloroacetic acid at 0° indicated that three extractions are sufficient (Table 1) and this was the method routinely used. It was generally more satisfactory than the $HClO_4$ method, and this confirms a previous conclusion.[4]

TABLE 1. EXTRACTION OF NON-NUCLEIC ACID PHOSPHORUS FROM RYE SEEDLINGS WITH 10% TRICHLOROACETIC ACID AT 2°

Extract number	μg Phosphorus extracted
1	61·4
2	8·0
3	4·8
4	0·0
5	0·0

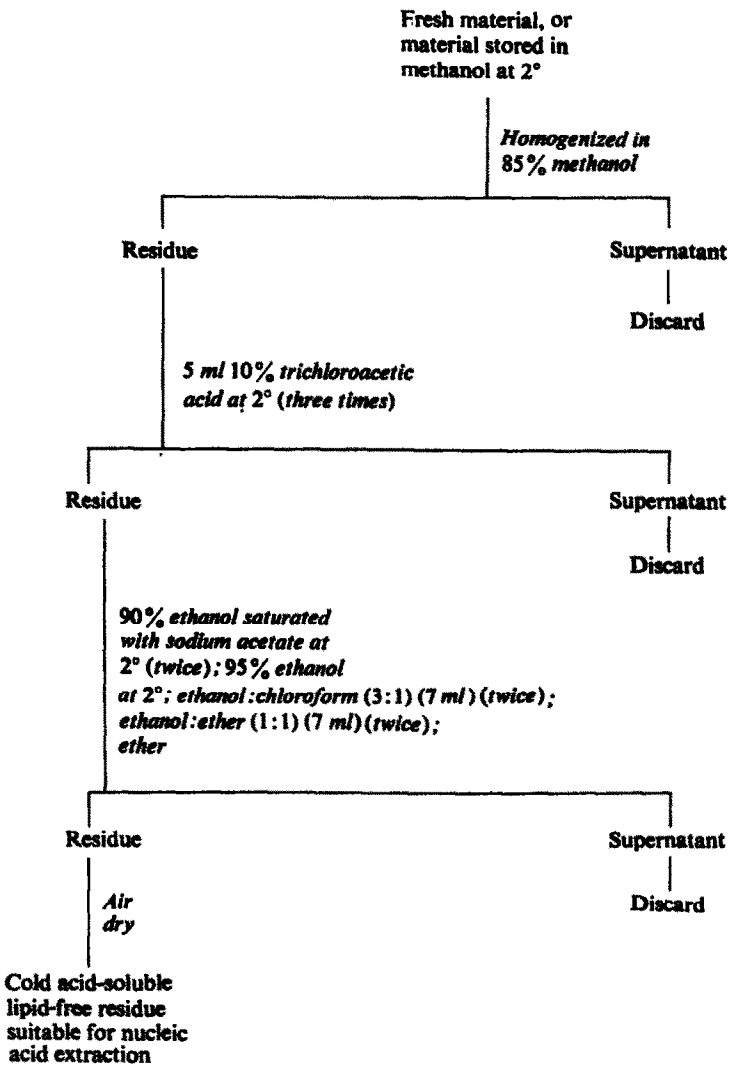
Rye seedlings (approx. 250 mg dry weight of 3 day seedlings) were homogenized in methanol and the residue suspended in 5 ml cold 10 per cent trichloroacetic acid and agitated for 5 min at 0–2° with a glass rod. The glass rod and sides of the tube were washed into the suspension with 2 ml of the cold acid after which the suspension was centrifuged (17,500 g) in a Servall RC-2 refrigerated centrifuge for 5 min. The extraction was repeated on the residue four times. 2 ml aliquots from each supernatant were used for total phosphorus determinations.[7]

It should be emphasized here that in spite of this extraction orcinol-positive material still appears in extracts of RNA and DNA. It appears that some carbohydrate components of plants which are insoluble in cold acid are degraded to smaller units when subjected to the methods used to extract RNA and DNA and thus extracted along with these compounds.

Extraction of Lipid Substances

When extraction of phospholipids with hot solvents follows the preliminary cold acid extraction, care must be taken to avoid creating conditions under which any acid remaining could degrade the nucleic acids. Loss of RNA and DNA in this extraction has been observed by Steele *et al.*[8] Furthermore, Marko and Butler[9] demonstrated that two cold ethanol washes were insufficient to remove all the acid left in the residue; enough trichloroacetic acid remained to degrade some DNA to apurinic acid during the following hot ethanol:ether extraction. These workers removed acid by using ethanol saturated with sodium acetate for the first wash. Hutchinson and Munro[4] also found that this treatment prevented DNA breakdown in acid-washed and lipid-free dry residues left at room temperature. In our initial experiments with rye it was confirmed that two cold ethanol washes were insufficient to remove all the tri-

[5] R. M. SMILLIE and G. KROTKOV; *Can. J. Botany* **38**, 31 (1960).
[6] J. N. DAVIDSON, S. C. FRAZER and W. C. HUTCHINSON, *Biochem. J.* **49**, 311 (1951).
[7] R. J. L. ALLEN, *Biochem. J.* **34**, 858 (1940).
[8] W. J. STEELE, N. OKAMURA and H. BUSCH, *Biochim. Biophys. Acta* **87**, 490 (1964).
[9] A. M. MARKO and G. C. BUTLER, *J. Biol. Chem.* **190**, 165 (1951).



SCHEME 1. SCHEME FOR REMOVAL OF COLD ACID-SOLUBLE AND LIPID SUBSTANCES FROM BIOLOGICAL TISSUE PRIOR TO QUANTITATIVE EXTRACTION OF NUCLEIC ACIDS

The final residue is air-dried; it can be stored in a deep-freeze before analysis. Before addition of nucleic acid solvents the residue must be finely ground with a glass rod.

chloroacetic acid; it was found that if the residue from acid extraction was washed immediately with 90 per cent aqueous ethanol saturated with sodium acetate at 0–2°, no acid remained. However, as a further precaution it was decided to omit heat treatment if possible during lipid extraction. Complete extraction is only achieved if chloroform is used as extractant. Table 2 shows that treatment with chloroform:ethanol following the more conventional solvent mixtures[10–12] extracts some 6–8 per cent more lipid-phosphorus. Although this had previously

[10] J. K. HEYES, *Proc. Roy. Soc. London, B.* **152**, 218 (1960).
[11] D. J. OSBORNE, *Plant Physiol.* **37**, 592 (1962).
[12] L. LEDOUX, P. GALAND and R. HUART, *Biochim. Biophys. Acta* **55**, 97 (1962).

TABLE 2. EXTRACTION OF LIPID-PHOSPHORUS FROM PLANT TISSUES

Extracts*	Phosphorus extracted (μg/100 shoots)
1. Combined methanol, ethanol, and ethanol:ether (1:1) extracts	833·0
2. Chloroform:ethanol (1:3)	54·6
chloroform:ethanol (1:3)	0·0
3. Ether	0·0

* See text for details.
After the second ethanol saturated with sodium acetate extract, all extractions were carried out at room temperature, for 10 min each.

been recommended for animal tissues[13] it has rarely been used. Extraction of lipids at room temperature or lower makes it essential that chloroform is used as one of the solvents. The sequence of solvents eventually found to be most suitable for lipid extraction is outlined in Scheme 1.

Extraction of Nucleic Acids

The Schneider procedure. Schneider[1] proposed the extraction of RNA and DNA from the cold acid-insoluble and lipid-free residue with 5 per cent trichloroacetic acid at 90°, and later he recommended the use of $HClO_4$. Although originally developed for animal tissues this procedure has been used on plant material[5, 10, 14] and the RNA and DNA levels were obtained by measuring the ribose and deoxyribose content of the extract.[5, 10, 14] Subsequent workers have not always found assays by carbohydrate content satisfactory and have used other methods. Heyes[10] estimated total nucleic acid by phosphorus determination and used the diphenylamine test for an estimate of DNA-P, RNA-P being obtained by difference. Cherry,[14] on the other hand, found phosphate values unreliable, and estimated total nucleic acid by direct ultra-violet absorption.

The efficiency of trichloroacetic acid and $HClO_4$ to extract nucleic acids under the conditions suggested has also been questioned. For example, De Deken-Grenson and De Deken[15] used 0·5 N $HClO_4$ at 70° and 90° for two 15-min extractions and found that the yield of DNA at 90° was 10 per cent lower; in contrast, all the RNA may be extracted from liver during a 20 min incubation in 0·5 N $HClO_4$ at 70°, but a higher acid concentration was required for maximal yields of DNA. A full discussion of this problem has been presented by Hutchinson and Munro[4] and it is clear that the best conditions for DNA extraction must be found by experiment on each tissue examined.

The Schneider procedure was tested on rye tissue by extracting the residue remaining after the preliminary extractions with 0·5 N $HClO_4$ at 70°. Three extractions of 20 min each, with frequent agitation, were made. Estimates of nucleic acid levels were determined by estimating the phosphate, ribose and deoxyribose contents; the u.v. spectra of the extracts were also obtained. The shape of spectra and the absorptivity ratios (280 mμ/260 mμ) showed considerable contamination with u.v. absorbing material, the extent of which varied with the source of the extract (Fig. 1). Values for the 280/260 ratio ranged from 0·66 for whole unsoaked seeds to 1·00 for shoots. The u.v. data were therefore of no value for quanti-

[13] R. DAUOST and C. E. S. HOOPER, *Can. J. Biochem. Physiol.* 35, 721 (1957).
[14] J. H. CHERRY, *Plant Physiol.* 37, 670 (1962).
[15] M. DE DEKEN-GRENSON and R. H. DE DEKEN, *Biochim. Biophys. Acta* 31, 195 (1959).

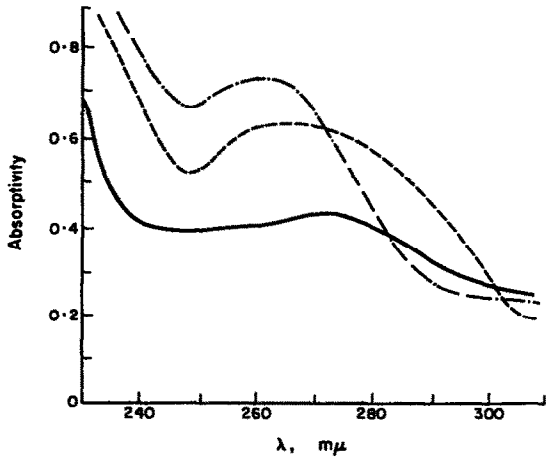


FIG. 1. ULTRAVIOLET SPECTRA OF SOME NUCLEIC ACID EXTRACTS PREPARED FROM RYE BY THE SCHNEIDER PROCEDURE.

RNA and DNA were extracted from the cold acid-soluble lipid-free residue with three 20 min incubations in 5 ml 0·5 N $HClO_4$ at 70°. The extracts were combined and made to 25 ml with 0·5 N $HClO_4$. —— 2-day root; ----- 2-day shoot; -·-·- 0-day seed.

tative estimations in rye, in spite of Cherry's report.[14] Similarly the orcinol reaction gave values over 20 times higher than expected; this is consistent with previous reports. Determinations based on phosphorus content of the extracts were compared with those obtained by the Schmidt–Thannhauser method (see below), the DNA–P content was reasonable but the RNA–P was far too high (Table 3). The excess phosphorus is probably derived from phosphoprotein.

TABLE 3. COMPARISON OF THE VALUES FOR NUCLEIC ACID CONTENT OF RYE SHOOTS OBTAINED BY TWO DIFFERENT EXTRACTION PROCEDURES

Procedure†	No. of analyses	Nucleic acid content* Total	DNA	RNA
Schneider	10	11·5	1·3	10·2
Schmidt and Thannhauser	5		1·1	2·7

* μg/seed determined by the phosphate procedure.
† See text for details.

The Ogur and Rosen procedure. The Ogur and Rosen procedure[3] is based on the claimed differential solubility of RNA and DNA in acid; RNA is extracted from corn root tips with cold 1 N $HClO_4$ overnight after which extraction of DNA was achieved with two 20 min incubations in 0·5 N $HClO_4$ at 70°. RNA and DNA estimation by u.v. spectrophotometry was satisfactory and compared favourably with the values obtained by the orcinol and diphenylamine methods. Subsequently it was found[16] necessary to change the conditions for

[16] M. OGUR, R. O. ERICKSON, G. ROSEN, K. B. SAX and C. HOLDEN, *Exp. Cell Research* 2, 73 (1951).

plant buds; RNA was extracted in 18 hr with cold 1 N $HClO_4$ at 4° and DNA with 1 N $HClO_4$ at 80° for 20 min.

Although Holmes *et al.*[17] obtained essentially the same results for root tips with the Ogur and Rosen procedure and with the Schmidt and Thannhauser method, Woodstock and Skoog[18] found it necessary to lower the extraction temperature to 55° for extracting DNA from roots, in order to minimize accumulation of interfering substances. For a similar reason Ledoux *et al.*[12] changed the DNA-extracting solution to a mixture containing 100 ml concentrated hydrochloric acid, 200 ml water and 700 ml ethanol.

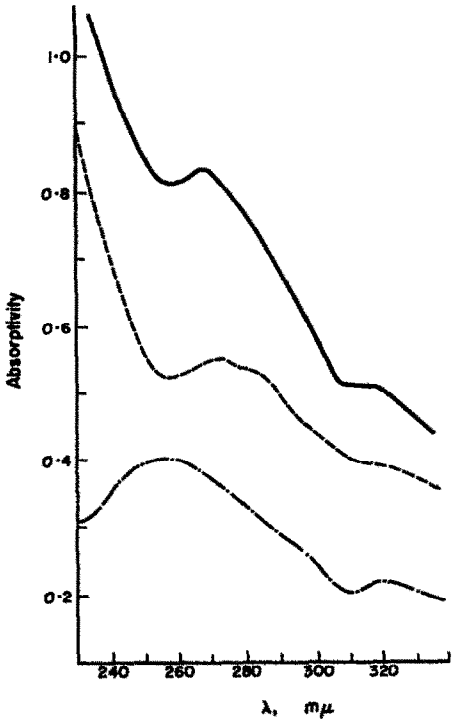


FIG. 2. ULTRAVIOLET SPECTRA OF RNA EXTRACTS FROM RYE OBTAINED BY THE PROCEDURE OF OGUR AND ROSEN.

RNA was extracted from each cold acid-soluble lipid-free residue with 10 ml 0·5 N $HClO_4$ at 2° for 18 hr. Each residue was washed twice with cold 0·5 N $HClO_4$, washings and supernatants were combined and made to 25 ml with $HClO_4$. —— 0-day seeds; – – – 0-day endosperm; –·–·– 0-day embryo.

In our investigations 0·5 N $HClO_4$ at 2° for 18 hr was employed to extract RNA from rye tissue (250 mg dry weight) homogenates. The extract was centrifuged at 0° and the residue washed twice with cold 0·5 N $HClO_4$. The combined supernatant and washings were made to 25 ml. The u.v. spectra (Fig. 2) of extracts of seeds, endosperm and embryo all showed the presence of contaminating material, whilst both the phosphate and orcinol tests on the same extracts indicated at least a five-fold contamination of non-nucleic acid material.

[17] B. E. HOLMES, L. K. MEE, S. HORNSEY, and L. H. GRAY, *Exp. Cell Research* **8**, 101 (1955).
[18] L. W. WOODSTOCK and F. SKOOG, *Am. J. Botany* **47**, 713 (1960).

The failure of cold acid extraction to produce a "clean" RNA extract is not the only criticism which can be brought against this procedure. Many reports disclaim the differential solubility of RNA and DNA in acid. For example, Kessler and Engelberg[19] have shown by base analysis that no combination of time and temperature allowed complete extraction and separation of RNA from DNA in developing apple leaves. Heyes[10] also found RNA in the DNA fraction from root tips, and that some degradation of DNA had taken place during cold acid treatment. There appears to be substantial evidence that there is a cold-acid resistant RNA core which is dissolved only after long periods of digestion[20], or by increasing acid concentration and temperature.[21]

The Schmidt and Thannhauser procedure. This procedure[2] is based on the differential solubility of RNA and DNA in alkali. RNA, with a 2'-vicinal hydroxyl on ribose, is susceptible to alkaline hydrolysis which produces a mixture of 2' and 3' mononucleotides plus some dipurine nucleotides. DNA is unaffected by alkali and is precipitated out of the reaction mixture by lowering the pH to 1. The conditions used for the alkaline hydrolysis have been variable. For conversion of RNA into acid-soluble units a 1 hr digestion with 0·3 N KOH at 37° has been most popular. For complete depolymerization 0·3 N alkali at 37° for 16–18 hr,[5, 11, 22] 1 N alkali at room temperature for 16–20 hr, and 0·5 N alkali at 26–37° for 16–20 hr[15, 23] have all been used successfully.

In the present study the conditions used were 0·5 N KOH at 28° for 16 hr. Contrary to the report of Schmidt and Thannhauser a residue was always found after the alkaline incubation. This residue was removed by centrifuging, a centrifugal force of up to 35,000 g being necessary for endosperm digests which tended to form gels. Any colloidal layers remaining after centrifuging the endosperm digests were removed with the supernatants. (All alkaline extracts, except those from 0 and 1 day embryonic tissue, were straw to brown in colour.) All residues were washed three times with 2–5 ml portions of 0·5 N KOH at room temperature and in all cases the third wash failed to exhibit u.v. absorption.

The precipitation of DNA from the alkaline digest has in general been achieved by acidification to pH 1, at 0–2°. A variety of acids have been used for the precipitation stage, but $HClO_4$ is probably the most convenient because it removes potassium ions as the insoluble perchlorate. In some cases during the present investigation addition of $HClO_4$ to the alkaline digest of rye failed to produce a clear supernatant. The use of magnesium ions[5, 24] did not improve the situation, but addition of 2 vol. of cold 95 per cent ethanol enabled a clear, RNA-containing supernatant to be obtained. In general the RNA extract from rye tissue was very impure, as shown by the u.v. spectra (Fig. 3), and was presumably a result of contamination with protein degradation products. These impure extracts are similar to those obtained from other plant tissues,[15, 19, 25] and it is claimed that 50 per cent of the total tissue protein is found in the RNA fraction.[11] The orcinol reaction and phosphate estimation, also demonstrated the presence of substantial contaminating materials, and a further purification was clearly necessary before any quantitative estimation of RNA could be carried out.

[19] B. KESSLER and N. ENGELBERG, *Biochim. Biophys. Acta* 55, 70 (1962).
[20] P. O. P. Ts'o and C. S. SATO, *Exp. Cell Research* 17, 227 (1959).
[21] M. HOLDEN, *Biochem. J.* 51, 433 (1952).
[22] J. BONNER and J. A. D. ZEEVAART, *Plant Physiol.* 37, 43 (1962).
[23] H. FUKASAWA, *Exp. Cell Research* 25, 276 (1963).
[24] R. MARKHAM, *Modern Meth. Plant Anal.* IV, 246, (1955).
[25] S. P. MONSELISE, A. COHEN and B. KESSLER, *Plant Physiol.* 37, 572 (1962).

Purification of the RNA Extract

Purification of the RNA fraction has been claimed by absorbing the nucleotides onto Dowex-1-Cl^-, and, after washing, eluting them in a relatively strong acid solution;[5,15] RNA was then assayed by the orcinol reaction and by u.v. absorption. In our hands this procedure gave only a partial purification, the orcinol and phosphate values being high. The u.v. spectra of the extracts were also unsatisfactory. These observations confirm those recently made by Brown[26] and Ingle.[27]

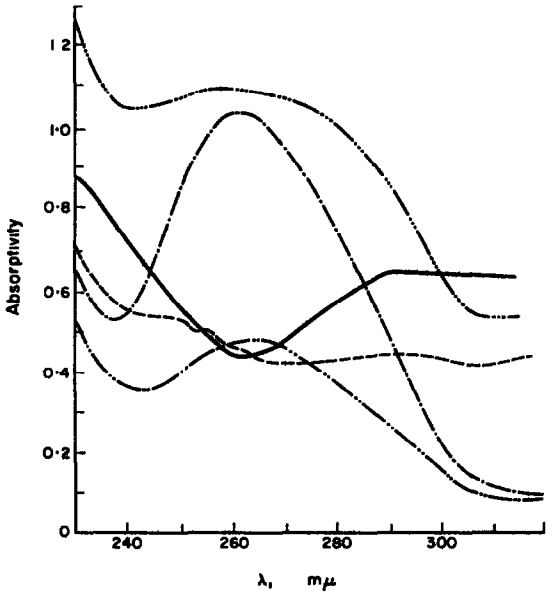


FIG. 3. ULTRAVIOLET SPECTRA OF THE RNA EXTRACTS FROM VARIOUS TISSUES OF RYE BY THE SCHMIDT AND THANNHAUSER PROCEDURE.

Extracts were diluted with suitable volumes of 0·2 N $HClO_4$ to obtain u.v. spectra. —— 3-day root; – – – – 5-day root; –·–·– 0-day shoot; –···–···– 3-day shoot; – ·–··– 5-day shoot.

This problem can be overcome by an initial purification with charcoal.[7,28–29] Adsorption of the RNA hydrolysate from *Xenopus laevis* eggs onto charcoal and elution with ethanol: pyridine followed by chromatography on Dowex-1-Cl– has been made recently, but no indication of the percentage yield was given.[30] Although Bergkvist[28] claimed a quantitative recovery of nucleotides from charcoal by repeated elution with alcoholic ammonia (25 per cent aqueous ethanol containing 0·5 per cent ammonia), Sebesta and Šorm[29] found a 7·5 per cent loss of nucleotides on Karborafin and 38·2 per cent loss on Norit A, and Brown[26] found losses ranging from 20–28 per cent, depending on the nucleotide, on Norit OL.

Our experiments with charcoal showed that recovery of a yeast RNA alkaline hydrolysate from Norit OL columns (1·2 × 2·5 cm), prepared according to Brown,[26] using 10 ml ethanolic ammonia (25 per cent aqueous ethanol containing 0·5 per cent ammonia) as eluant was

[26] E. G. BROWN, *Biochem. J.* **85**, 633 (1962).
[27] J. INGLE, *Phytochem.* **2**, 353 (1963).
[28] R. BERGKVIST, *Acta Chem. Scand.* **10**, 1303 (1956).
[29] K. SEBESTA and F. ŠORM, *Collection Czech. Chem. Communs.* **24**, 2781 (1959).
[30] M. DECROLY, M. CAPE and J. BRACHET, *Biochim. Biophys. Acta* **87**, 34 (1964).

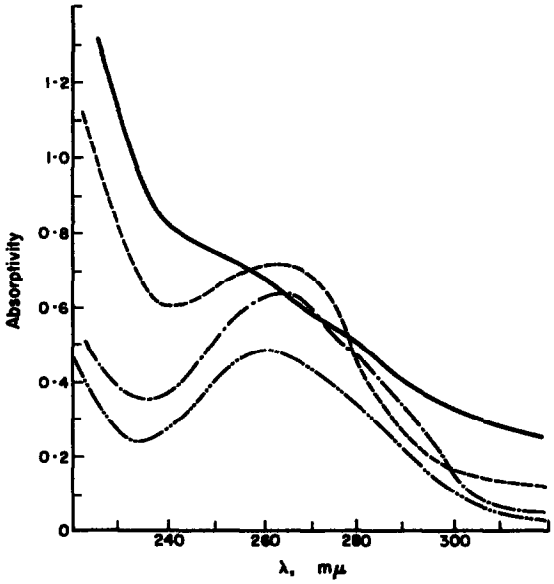


FIG. 4. ULTRAVIOLET SPECTRA OF RNA EXTRACTS OF RYE AFTER PURIFICATION BY CHARCOAL. All extracts obtained by the Schmidt and Thannhauser procedure. Solvent ethanolic ammonia. —— 16-hr endosperm; ----- 16-hr scutellum; -·-·- 16-hr root; -··-·· - 16-hr shoot. (See text for details.)

73·3 per cent, based on both u.v. absorption and the orcinol reaction. The same recovery was obtained with synthetic mixtures of 2′, 3′-nucleotides of varying concentrations. Further washings with ethanolic ammonia failed to elute further u.v. absorbing material. Addition of protein to the yeast RNA hydrolysate had no effect on the recovery of nucleotides.

TABLE 4. RNA CONTENTS OF RYE TISSUES AS ESTIMATED BY PHOSPHATE, CARBOHYDRATE CONTENT, AND BY U.V. SPECTROPHOTOMETRY OF THE SCHMIDT AND THANNHAUSER EXTRACTS

Plant part	μg RNA/plant part as determined by		
	Phosphate	Carbohydrate	u.v.*
0 day endosperms	25·5	40·9	10·8
0 day shoot	11·5	7·0	5·9
0 day root	10·5	7·4	6·6
2 day endosperm	13·7	20·8	8·5
2 day shoot	14·5	12·2	14·6
2 day root	10·1	17·6	13·5
5 day shoot	—	105·0	48·5
6 day shoot	—	150·1	73·9

* Absorptivity at 265 mμ − that at 290 mμ.

The RNA extracts from the Schmidt and Thannhauser procedure were purified by chromatography on charcoal and Dowex 1 × 4 Cl^- ion-exchange resin. Samples were assayed for phosphorus by the procedure of Allen,[7] for purine bound ribose by the orcinol reaction,[10] and by u.v. absorption at 265 mμ and 290 mμ. Highly polymerized yeast RNA similarly treated was used as standard.

The spectra of various tissue extracts after adsorption and elution from charcoal columns are given in Fig. 4 which shows that the older the root and shoots the less pure were the eluates.

Further purification of the extracts was performed by ion-exchange chromatography. The eluates from charcoal columns were taken to dryness in a rotary evaporator at 30°, and after dissolving in very dilute alkali (pH 8) were further purified by adsorption onto and elution from a Dowex 1 × 4 chloride column (2·5 × 1 cm)[5] and subsequent elution with 25 ml of a mixture of sodium chloride and hydrochloric acid (20 ml 10 N hydrochloric acid and 5·6 g sodium chloride in 240 ml water).

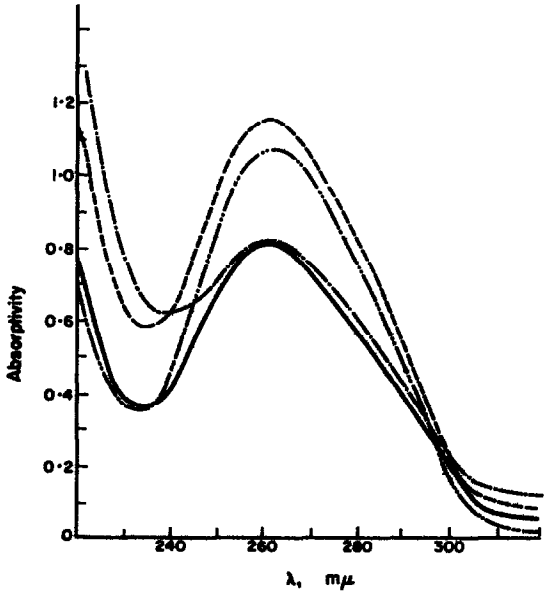
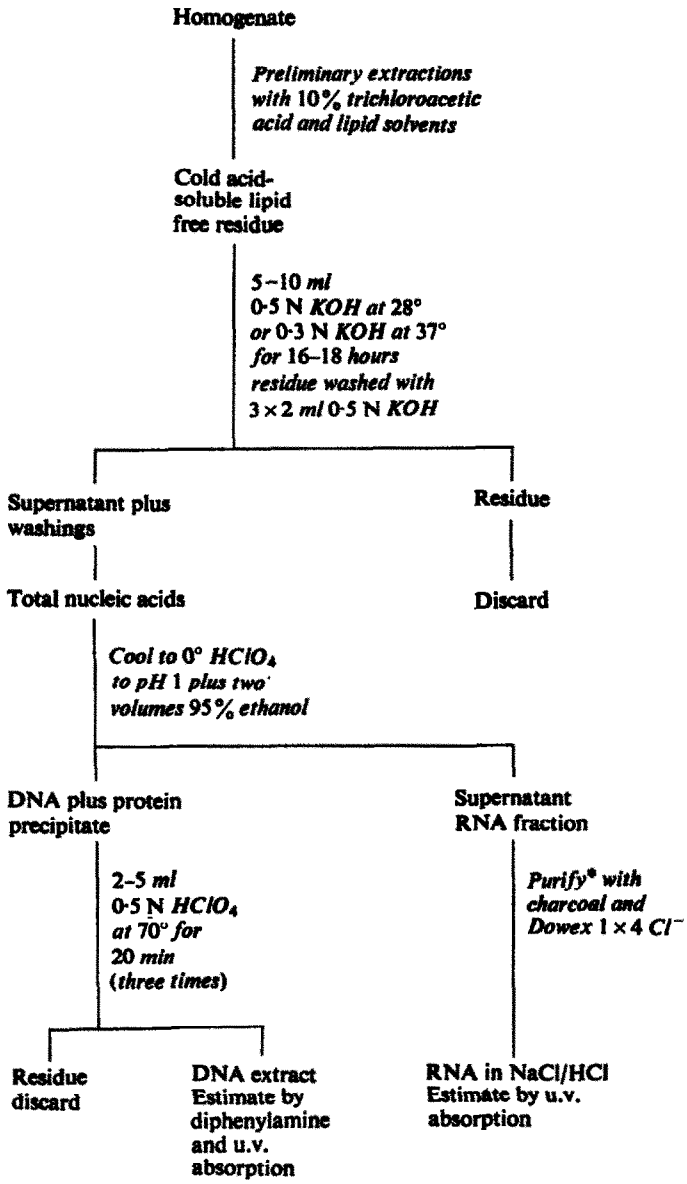


FIG. 5. ULTRAVIOLET SPECTRA OF RNA EXTRACTS AFTER TWO-STEP PURIFICATION ON CHARCOAL AND DOWEX 1 × 4 Cl^-.
—— 2-day root; ----- 2-day scutellum; -·-·- 2-day endosperm; -··-·· 2-day shoot. (See text for details.)

Even after charcoal treatment endosperm RNA still contained non-RNA phosphate and orcinol-reacting material, and in general the older the root and shoots the greater the variation between the three methods of assay used (phosphate, orcinol and u-v). Typical results illustrating this are given in Table 4 and Fig. 5.

Extraction of DNA

Many papers, reviewed in detail by Hutchinson and Munro,[4] have discussed the extraction of DNA from the Schmidt and Thannhauser acid-insoluble precipitate. One common difficulty is destruction of deoxyribose in acid at 90° which results in low values when DNA is estimated by the diphenylamine reaction. The effect of temperature on the extraction of DNA from rye tissue with 0·5 N $HClO_4$ was therefore tested. Three 20 min extractions at 70° were found to give maximum yields; raising the temperature to 90° reduced the yield by 6 per cent. The u.v. spectra of the DNA extracts at 70° and 90° from a range of tissues were not pure, and, in general, u.v. absorption measurement gave slightly higher values than those obtained by the diphenylamine test. Phosphate analysis gave estimates for DNA consistently



SCHEME 2. A MODIFIED FORM OF THE SCHMIDT AND THANNHAUSER PROCEDURE FOR THE EXTRACTION OF NUCLEIC ACIDS FROM PLANT TISSUES

* See text for details.

Volumes of solutions used must be such to ensure a good suspension of the homogenate.

higher than those obtained by u.v. and carbohydrate analyses. The diphenylamine reaction, being most specific, was assumed to give the most accurate estimates. Attempts to extract DNA with dilute alkali were fruitless with the tissues under examination because a sticky unmanageable mass was produced.

The sequence of operations for the extraction and purification finally found to be most suitable for the quantitative analysis of RNA and DNA in rye tissues is summarized in Scheme 2.

When gross contamination of the Schmidt and Thannhauser RNA extract exists, such as extracts from mature tissues, ion-exchange resins cannot achieve a satisfactory degree of purification unless a relatively large column and time-consuming separation is performed.[19,27] An initial purification with charcoal removes a large proportion of the impurities, but this technique has a major drawback in that nucleotide recovery is not quantitative, however, the percentage loss of nucleotides appears to be constant with the same batch of charcoal. It can therefore be allowed for, and thus a reliable estimate of the RNA content can be made after charcoal and ion-exchange chromatography, by computing the value from the differences in absorptivity at two wavelengths e.g. either 260–290 mμ or 260–230 mμ. Assays of the doubly purified extracts by orcinol and phosphate are not reliable, the extent of phosphate and orcinol reacting contaminants tend to increase with the maturity of the tissue analysed.

The procedures outlined for the preliminary extractions (see Scheme 1) have proved reliable, and providing temperatures are kept low and the final cold acid extraction is immediately followed by treatment in the cold with ethanol saturated with sodium acetate no loss of RNA or DNA should be experienced. The proposed modifications to the Schmidt and Thannhauser procedure (see Scheme 2) should provide a reliable estimate of the RNA and DNA contents of higher plants and allows several samples to be analysed with ease at the same time. Our procedure has been satisfactorily used on rye, wheat and bean tissues.

METHODS AND MATERIALS

Materials

All chemicals used were of analytical grade. Nucleotides, highly polymerized RNA and DNA were obtained from Calbiochem. U.S.A. Norit OL charcoal from Messrs. Hopkin & Williams, Chadwell Heath, Essex. Rye var. King II seeds were kindly provided by R. Gunston (Seeds) Ltd., London.

Preliminary Treatments

Seeds were germinated by soaking in water for 24 hr and then sown in vermiculite: water (5:2 v/v). At harvest seedlings were killed by plunging into boiling methanol and stored in methanol at 0–2° until analysed.

All analyses were done on the approximate equivalent to 250 mg dry weight of whole seedlings, endosperm, scutella, shoot and roots ranging in age from 12 hr to 9 days after imbibition started. After careful dissection the seedlings were homogenized under methanol in a mortar and pestle; silver sand was used to aid the grinding of endosperms and older more woody tissues.

The residues remaining after centrifuging the homogenate were extracted consecutively with approximately 7 ml of each of the acid and lipid solvents listed above. All cold acid extractions were performed at 2° or below. Lipid solvents were used at room temperature except the extractions with ethanol saturated with sodium acetate and 95 per cent ethanol which were performed at 0–2°. All centrifugations were performed in a Servall RC-2 refrigerated centrifuge.

Extraction of Nucleic Acids

All extractions of nucleic acids were made on cold acid-soluble lipid-free dry powders. The conditions used for each extraction have been given in the appropriate results section.

Preparation of Charcoal

Norit OL charcoal was prepared as described by Brown;[26] columns (1·2 × 2·5 cm) were prepared from 250 mg charcoal plus 400 mg Hyflo-super-cel. The charcoal and super-cel were mixed and suspended in water and the slurry was supported by a sintered glass disc covered with 2–3 mm Hyflo-super-cel.

Samples were prepared for absorption onto charcoal by neutralizing to pH 8 by the addition of potassium hydroxide or $HClO_4$, cooled and the resulting $KClO_4$ crystals removed; the supernatant was then adjusted to pH 4 with acetic acid. This solution was passed through the charcoal column under slight positive pressure. After the adsorption stage the charcoal was washed with deionized water until the effluent was neutral. The nucleotides were then eluted with 10 ml ethanolic ammonia (25 per cent aq. ethanol containing 0·5 per cent ammonia).

Preparation of Samples for Ion-exchange Chromatography

Eluates from charcoal were rotary evaporated at 30° to remove ethanol and redissolved in basidified water (pH 8). These solutions were then further purified by the bulk elution method using Dowex 1 × 8 chloride 200–400 mesh columns (1 × 2·5 cm).[5]

Estimation of Nucleic Acids

In all cases the nucleic acid extracts were assayed for phosphate and carbohydrate content and for u.v. absorption. Phosphate was determined by the amidol procedure;[7] the volumes of reagents used were scaled down to give a final volume of 5 or 10 ml. The Burton[31] modification of the diphenylamine reaction was used to estimate the 2-deoxyribose in the DNA extracts. The procedure of Dische and Schwartz[32] was used for the estimation of RNA by reaction with orcinol. Ultra-violet absorption of both the RNA and DNA extracts was measured with a Unicam SP 500 spectrophotometer and a Perkin-Elmer Uvicord. In all cases highly polymerized RNA and DNA, used as standards, were treated in the same manner as the extracts.

Acknowledgement—This investigation was supported by the Agricultural Research Council.

[31] K. Burton, *Biochem. J.* **62**, 315 (1956).
[32] Z. Dische and K. Schwartz, *Mikrochim. Acta* **2**, 13 (1937).