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Nucleic Acid Determination in Storage Tissues of Higher Plants¹

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Little information is available on the function of nucleic acids in storage tissues of plants. It has generally been assumed that the nucleic acids of storage tissues are a nucleotide reserve for the growing seedling (16). This has been based on the available evidence (4, 6, 12, 13) that there is a loss of RNA³ from storage tissues on germination. But there is no information on the synthesis, if any, of RNA during germination or the changes which take place in nucleic acid content in the very first stages. It is possible that a specific biochemical function such as protein synthesis may require a type of RNA in the early stages of germination for the formation of

enzymes. After this function has been satisfied, the nucleic acids may be degraded and transported to the growing portion of the seedling. We have been interested in exploring this matter further and have selected the peanut as the experimental material.

A method (2, 4, 5, 6, 8) employing the extraction of nucleic acids with hot HClO₄ had proven to be satisfactory for the estimation of RNA in corn seedlings. Preliminary investigations showed that this method was not adequate for measuring nucleic acids in peanut cotyledonary tissue. A comparative study, therefore, was made of the suitability of the above method and a new method (15), slightly modified, for estimating nucleic acids in storage tissues of three species of germinating seedlings.

In this paper, the details of the two methods are presented. Ultraviolet absorption spectra, data on the quantitative extraction of nucleic acid from peanut cotyledon, corn scutellum, and castor bean endosperm, and results of column chromatography of the soluble nucleotides from peanut cotyledons are compared for the two methods. This study points up the fact that methods for analysis of nucleic acids must be tailored to the tissue. For storage tissues which have a low nucleic acid content and a high content of storage materials (lipids, carbohydrates, or proteins), a more refined technique is required.

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³ The following abbreviations will be used: AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; UMP, uridine monophosphate; UDP, uridine diphosphate; UTP, uridine triphosphate; GTP, guanosine triphosphate; CMP, cytosine monophosphate; CTP, cytosine triphosphate; DPN, diphosphopyridine nucleotide; RNA, ribonucleic acid, and DNA, deoxyribonucleic acid.

Materials & Methods

Virginia 56-R peanut seed (1961 crop), WF9XM14 hybrid corn seed and Baker 296 castor bean seed were lightly dusted with Spergon⁴ and germinated in a dark, humid atmosphere at 30 C in vermiculite. Total phosphate was measured by the method of Fiske and Subbarow (7) following digestion in H₂SO₄ with H₂O₂. Burton's (1) modification of the diphenylamine reaction was used for measuring deoxyribose. Ribose was estimated by a modification of the orcinol reaction (11). Soluble nucleotides were extracted and chromatographed on Dowex-1×8 (formate) by the method of Cherry and Hageman (3).

All data presented in this paper represent the averages of two or more experiments.

Results

► Preliminary Work With Peanut Cotyledons: Ultraviolet absorption spectra of the nucleic acids extracted by a method satisfactory for corn seedlings (2, 4, 5, 6, 8) from the hypocotyl, radicle, and cotyledon of the germinating peanut seedlings are shown in figure 1A, 1B, and 1C, respectively. Note that the spectra from the embryonic tissues (hypocotyl & radicle) are typical for nucleic acids. However, the spectrum for the nucleic acids extracted from the peanut cotyledon had a maximum absorbency at about 275 m μ and appeared to be contaminated with interfering substances. Since the measurement of nucleic acid (primarily RNA) depends on the difference in absorption at 260 to 290 m μ , it appeared quite obvious that this method, found satisfactory for corn seedlings and embryonic tissue of the peanut, was unsatisfactory for peanut cotyledons.

► Procedures for Estimating Nucleic Acids: Smillie and Krotkov (15) compared several different methods for estimating nucleic acids in a number of algae and higher plants. Most of them appeared inadequate, hence they devised a method more suitable for estimating RNA and DNA from plant tissues. In this paper, the method used by Hanson (8) for mitochondria will be referred to as method I and compared with the method of Smillie and Krotkov (15), slightly modified, referred to as method II. Details of the two methods are described below and shown schematically in figures 2 and 3, respectively.

In method I the tissue (about 100–200 mg) is homogenized in 2 ml of H₂O or 0.5 M sucrose with an ice-jacketed glass homogenizer and a power-driven Teflon pestle. The homogenate is then cleared of cellular debris by centrifugation. Aliquots of the homogenate are adjusted to 0.2 M HClO₄ and the precipitate isolated and washed with cold 0.2 M

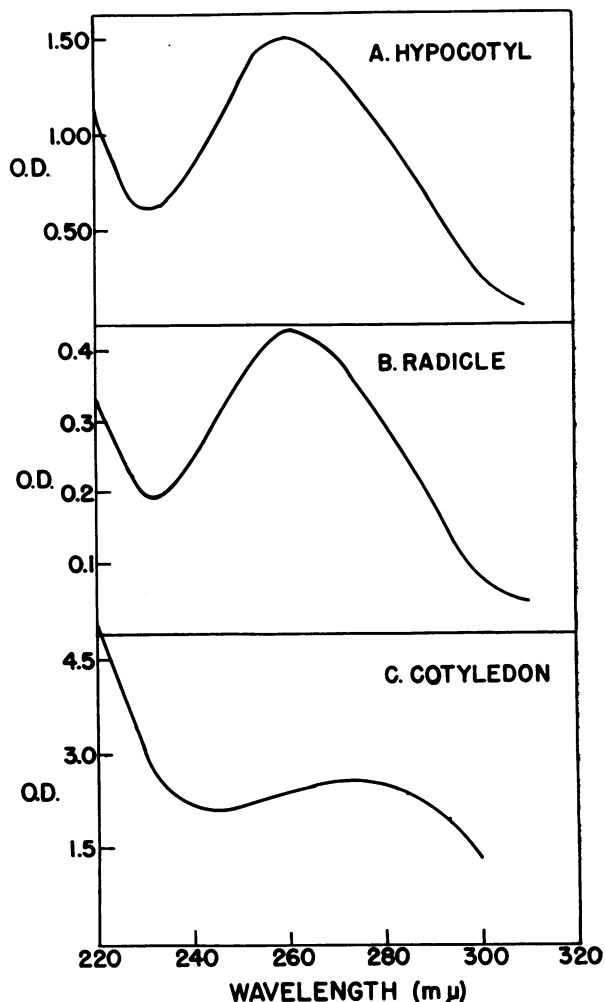


Fig. 1. Spectra of the nucleic acids extracted from various parts of 4-day-old peanut seedlings. The tissues were homogenized in 0.5 M sucrose and the spectra made on the nucleic acid fraction in 0.5 M HClO₄ as prepared by method I (see fig 2 for details).

HClO₄. The precipitate is next defatted with ethanol: ether: chloroform (2:2:1). Nucleic acid (primarily RNA, since the cellular debris is discarded) is then extracted with 0.5 M HClO₄ at 70 C for 40 minutes, and is estimated by referring absorbancy differences at 260 and 290 m μ to a standard curve obtained with similarly-treated yeast RNA.

The major difference between methods I and II is that in method II the tissue is homogenized in methanol instead of in water or a sucrose solution. After homogenization in methanol, the insoluble material is sedimented by centrifugation and washed twice with ice-cold methanol. The methanol-insoluble material is extracted twice with 0.2 M HClO₄ to remove acid-soluble phosphates. Perchloric acid was used instead of trichloroacetic acid as described

⁴ Trade names are given as part of the exact experimental conditions and not as endorsement of the products over those of other manufacturers.

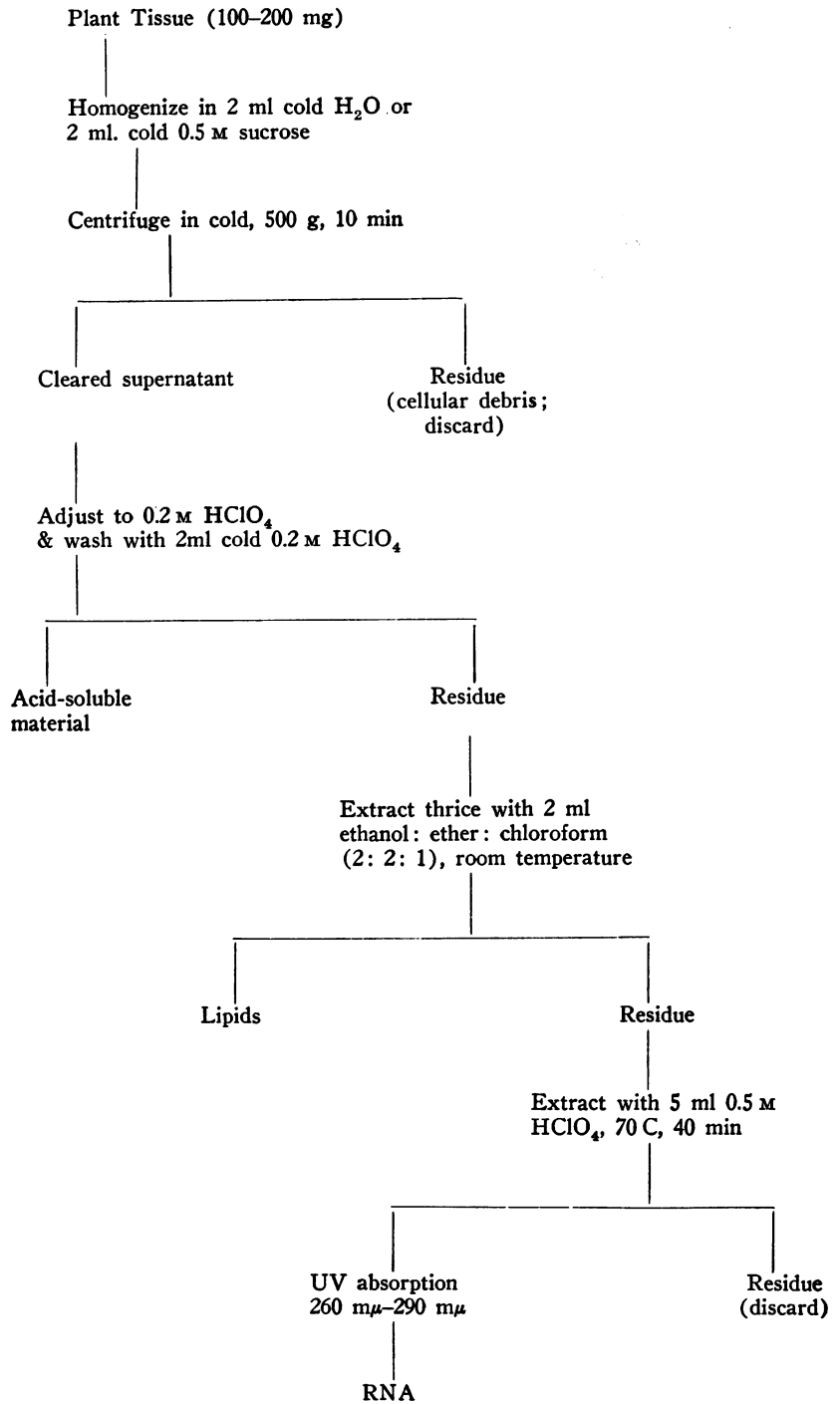


Fig. 2. Extraction procedure for the estimation of RNA in corn seedlings (Method I).

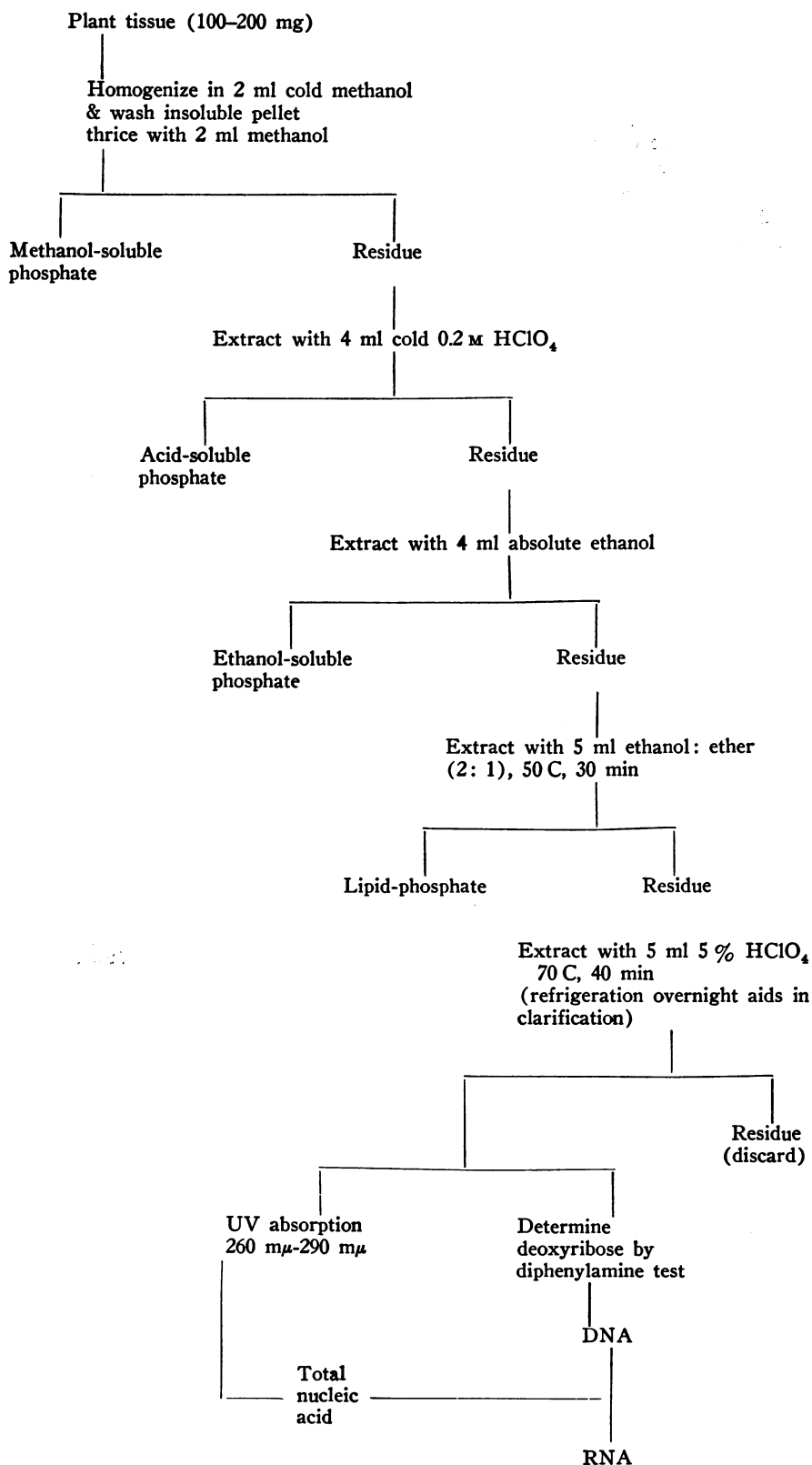


Fig. 3. General extraction procedure for the estimation of nucleic acids in storage tissues of plants (Method II).

by Smillie and Krotkov (15) to permit determination of the ultraviolet absorption spectra. The precipitate was next extracted with ethanol to remove the acid and other soluble materials. This was followed by extraction with ethanol: ether (2:1) at 50 C for 30 minutes to remove lipids as described by Kupila et al. (10). The lipid-free residue was incubated with 5 ml of 5% HClO_4 at 70 C for 40 minutes to remove the nucleic acids; the HClO_4 hydrolysates were placed in the refrigerator overnight to aid in removing a substance (probably phospho-protein) which interfered with the ultraviolet determination. Absorbency difference at 260 and 290 $\text{m}\mu$ of the cleared supernatant was referred to a standard curve obtained with similarly-treated yeast RNA to estimate the content of nucleic acid. A factor of 57 was used to multiply the O.D. value to calculate μg nucleic acid. DNA was determined by measuring the deoxyribose (1); the RNA content was obtained by difference.

► Spectra of Nucleic Acid Extracts: The ultraviolet absorption spectra of the nucleic acids extracted from the storage tissues of three different plant species are shown in figure 4. The nucleic acid spectra obtained from the peanut cotyledon (fig 4-C) and the castor bean endosperm (including the cotyledons) (fig 4-E) using method II appear quite satisfactory.

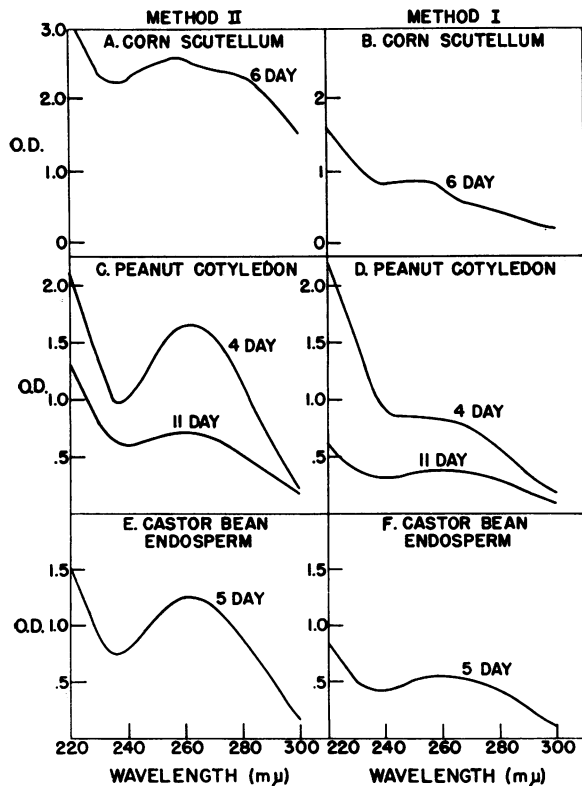


Fig. 4. Spectra of the nucleic acids extracted from the storage tissues of three different kinds of germinating seedlings using method I and method II. All spectra were determined in 0.5 M HClO_4 .

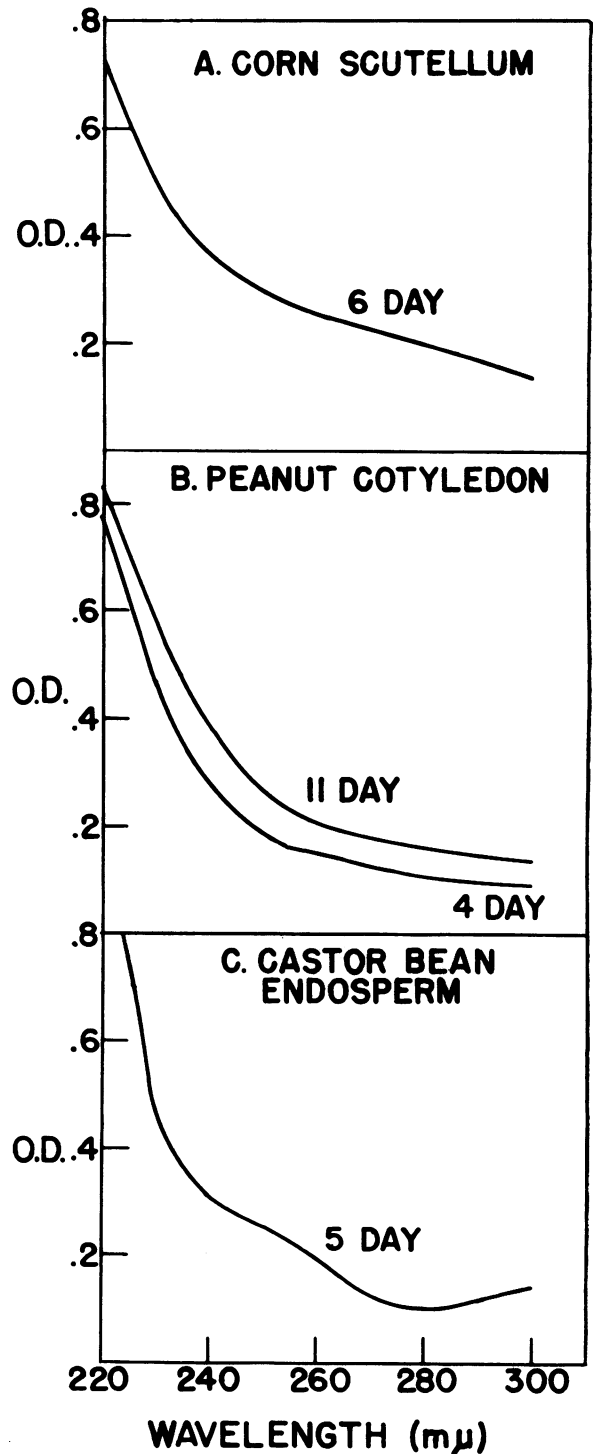


Fig. 5. Spectra of the cold 0.2 M HClO_4 soluble materials extracted from plant tissues using method I. The spectra were made in 0.2 M HClO_4 .

However, the spectra obtained with method I are not typical and there was a large loss in nucleic acids (fig 4-D & 4-F). For the corn scutellum (fig 4-A & 4-B) neither method appeared adequate. Method II extracted, in addition to the nucleic acids, an interfering substance with a maximum absorbency at 280 $m\mu$. Evidently, method I does not allow for complete extraction of the nucleic acids. The apparent reduction in nucleic acid content in the peanut cotyledon (fig 4-C & 4-D) with germination is primarily due to an increase in moisture content of the tissue.

► **Spectra of Extracts of Soluble Materials:** The above evidence indicates that method II is more satisfactory for peanut cotyledon and castor bean endosperm than method I. Therefore, a comparison was made of the soluble ultraviolet absorbing material extracted by methods I and II. The ultraviolet spectra of the cold acid-soluble materials extracted in 0.2 M $HClO_4$ by method I are unspecific (fig 5), not resembling typical nucleotide spectra. However, when the tissue was homogenized and washed in cold methanol (method II), materials were extracted from corn scutellum (fig 6-A) and peanut cotyledon (fig 6-C) which gave maximum absorption at 280

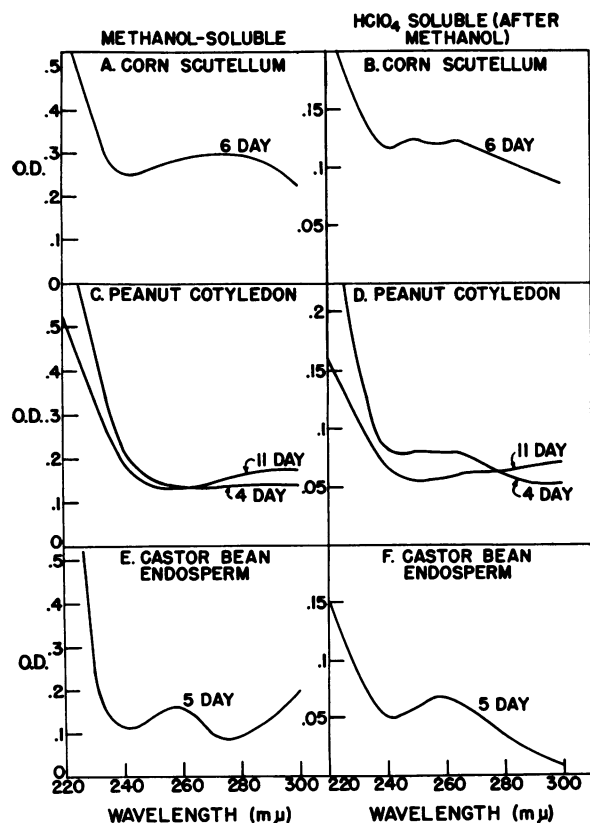


Fig. 6. Spectra of the materials extracted from plant tissues with cold methanol and subsequently with cold 0.2 M $HClO_4$ using method II. Note change in scale. All spectra were determined in 0.2 M $HClO_4$.

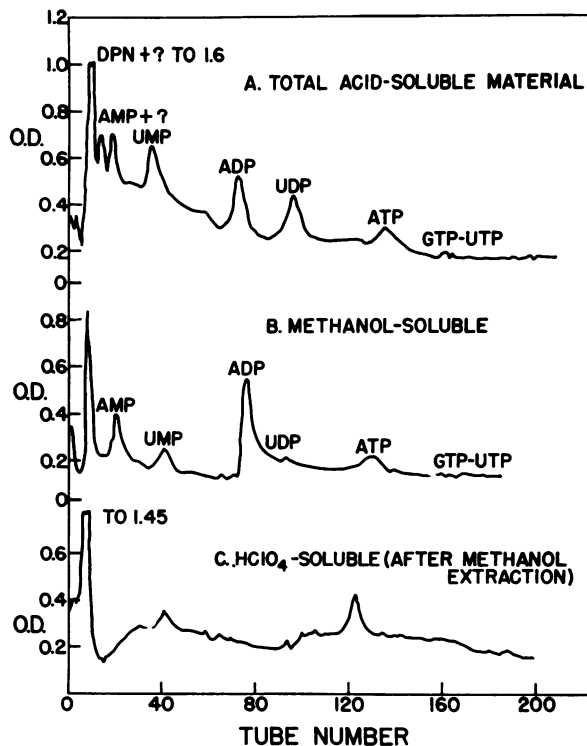


Fig. 7. Elution chromatograms of the acid-soluble nucleotides extracted with various solvents from 10-day-old peanut cotyledons. Each chromatogram represents 10 g fr wt of material. The nucleotides were collected in 10 ml fractions and the absorbency of each determined at 260 $m\mu$.

$m\mu$ and 290 $m\mu$, respectively. Note for castor bean endosperm (fig 6-E) that cold methanol probably extracted nucleotides (maximum absorption at 260 $m\mu$) and some other materials which absorbed between 280 to 300 $m\mu$.

The spectra of the materials extracted from the methanol-insoluble pellets in cold 0.2 N $HClO_4$ by method II for corn, peanut, and castor bean tissues are presented in figure 6-B, 6-D, and 6-F, respectively. All the spectra obtained from the different tissues except for 11-day-old peanut cotyledon had maximum absorption peaks at about 260 $m\mu$ similar to that of nucleotides.

► **Chromatography of Nucleotides:** The evidence presented in figure 6 indicates that most of the ultraviolet absorbing materials in the tissues are extracted in cold methanol. However, the materials extracted in cold $HClO_4$ more closely resemble nucleotides. Therefore, the various fractions were chromatographed on Dowex-1 \times 8 to determine the effect of methanol extraction and to determine which fraction contained the so-called acid-soluble nucleotides (fig 7). A typical elution chromatogram of the acid-soluble nucleotides from 10-day-old peanut cotyledon extracted in cold 0.6 M $HClO_4$ and subsequently chromatographed as previously described (3) is given in

Table I

Phosphates Extracted From Homogenates of Peanut Cotyledons Using Method I & Method II*

Fraction	4-day-old Peanut cotyledon		11-day-old Peanut cotyledon	
	Method I	Method II	Method I	Method II
	μ moles Phosphate/g fr wt			
Methanol	...	13.6	...	10.1
HClO ₄	18.4	25.1	23.3	15.8
Ethanol	...	2.6	...	1.6
Ethanol-ether (lipid phosphate)	1.8	0.4	1.3	0.5
Hot HClO ₄ extract (nucleic acids)	9.0	20.8	8.6	5.0
Total	29.2	62.5	33.2	33.0

* Cotyledon tissues were homogenized and the various fractions were analyzed for phosphate. See figures 2 and 3 for details of the two methods.

figure 7-A. The identification of the nucleotides was tentatively made by the order and position on the chromatogram, as reported for corn seedlings (3). Further identification was made by adsorbing the nucleotides from peak tubes on charcoal and eluting with ethanol:NH₄OH:H₂O (40:1:59) (pH 11) after washing the charcoal free of salt with H₂O. Spectra of the ethanol:NH₄OH:H₂O elutes were determined from 220 to 300 $m\mu$. Positive identification was made by comparing the spectra and ratios at 250 $m\mu$:260 $m\mu$ and 280 $m\mu$:260 $m\mu$ at pH 11 to those given by Pabst (14).

Homogenization and washing with cold methanol extracted most of the so-called acid-soluble nucleotides (fig 7-B). The base line and the height of the first peak (tubes 7-11), the UMP peak and the UDP peak were reduced to considerably less than that given in figure 7-A. Extraction of the methanol-insoluble residue with cold 0.2 M HClO₄ and subsequent chromatography gave an atypical elution chromatogram (fig 7-C). There was a large peak between tubes 6 to 10 corresponding to the smaller peak in figure 7-B. A large amount of material was eluted from the column, but there were only two other major peaks. The peaks at tube 41 and tube 123 may correspond to the smaller UMP and UDP peaks

shown in figure 7-B. The high base line given in figure 7-C may be due to gradual elution of polynucleotides.

► Phosphate Analysis: Analysis of the total phosphates extracted by the various solvents by methods I and II are compared in table I. About twice as much total phosphates were extracted from 4-day-old peanut cotyledons by method II than by method I. However, there was no difference in total phosphates extracted by the two methods from 11-day-old tissue. Most of the phosphates were extracted by cold HClO₄ (acid-soluble) and hot HClO₄ (nucleic acid). These data show a decrease in total phosphates with germination, especially in the hot HClO₄ extract.

► Nucleic Acid Analysis: Estimation of RNA and DNA from the storage tissues of three different kinds of germinating seedlings by methods I and II is given in table II. These data show that much less nucleic acid was extracted by method I than by method II. The largest discrepancy was found with 4-day-old peanut cotyledons. Only a small amount of the DNA was extracted by method I, which proceeded on the assumption that little or no DNA was present in the debris-free homogenate.

Table II shows that water homogenates, free from cellular debris and nuclei, contain about 1/6 to 1/4 of the total DNA of peanut cotyledons and corn scutella. These data also indicate that a large amount of the total RNA is lost by discarding the cellular debris (method I). In other experiments, the extent of this loss was determined by comparing the amount of nucleic acids extracted by method I on fresh tissue (cytoplasmic), by method II on debris from method I (cellular debris), and by method II on an acetone powder of the entire cotyledons (total). It was found that 20% to 50% of the total RNA and about 70% of the total DNA are removed with the cellular debris and thus not measured by method I. Similar results were obtained on peanut hypocotyl tissue.

Since the estimation of RNA by method II depends on analysis by difference, a comparison of extraction and estimation of RNA and DNA by other standard procedures was made and is summarized in table III. The direct extraction of RNA with 0.5 M KOH and subsequent estimation of content by ultraviolet light closely agree with the values obtained by

Table II
RNA & DNA Estimation From Homogenates of Plant Tissues Using Method I & Method II*

Tissue	μ g RNA/g fr wt		μ g DNA/g fr wt		RNA: DNA		
	Method		Method		Method		
	I	II	I	II	I	II	II
4-day-old Peanut cotyledon	550	1360	90	240	6.1		5.7
11-day-old Peanut cotyledon	340	830	50	160	6.8		3.3
6-day-old Corn scutellum	620	870	100	570	6.2		1.5
5-day-old Castor bean Endosperm	390	660	0	60	...		11.0

* The storage tissues were homogenized and the nucleic acids were estimated. See figures 2 and 3 for details of the two methods.

Table III
Extraction & Estimation of Nucleic Acids
by Several Procedures*

Procedure	Age (days)	
	4	11
	$\mu\text{g RNA/g fr wt}$	
(a) UV—Direct (extracted with 0.5 M KOH)	1,400	880
(b) UV—Difference (Method II)	1,350	990
(c) Phosphate determination	4,070	2,830
(d) Ribose determination	4,720	4,920
	$\mu\text{g DNA/g fr wt}$	
(a) UV—Direct	200	150
(b) Deoxyribose determination	170	150
(c) Phosphate determination	410	210

* Peanut cotyledons were homogenized in and the pellets washed in cold methanol. These pellets were subsequently washed with 0.2 M HClO_4 and defatted as outlined by figure 3. In case (a) the RNA was extracted with 0.5 M KOH at room temperature for 24 hours, the extract was acidified with HClO_4 , the KClO_4 was removed in the cold and the RNA content determined by ultraviolet light measurements. DNA was then extracted with 5% HClO_4 and the content determined by ultraviolet light measurements. The RNA estimated by difference (case b) was by the same procedure as outlined in figure 3 (method II). Phosphate, ribose, and deoxyribose were determined (see methods).

difference (total nucleic acid extracted in 5% HClO_4 -DNA determined by diphenylamine). The direct estimation of DNA by ultraviolet light also closely agrees with values obtained by the diphenylamine reaction. However, the estimation of RNA by phosphate and ribose analysis gave values three- to fivefold higher than ultraviolet light measurement which agrees with the finding of Smillie and Krotkov (15) and reflects measurement of nonnucleotide phosphorus and ribose.

Discussion

Evidence presented shows that a slight modification of the method of Smillie and Krotkov (15) is satisfactory for determining nucleic acids in peanut cotyledons. This method is judged satisfactory by the characteristic spectra and by increased values for nucleic acids. One discrepancy is that the nucleic acid content calculated from phosphorus and ribose analysis is manifold higher than obtained from ultraviolet light measurements (table III). This discrepancy was previously encountered by Smillie and Krotkov (15), who found that the contaminating nonnucleotides phosphorus and ribose could be removed with Dowex-1 resin.

Some evidence indicates that nucleic acid analysis on acetone powders of peanut cotyledons might be a satisfactory procedure. Analysis of acetone powders

shows slightly lower values for RNA and slightly higher values for DNA than those obtained on fresh tissue using method II. Extraction of both methanol-washed acetone powders and methanol-insoluble cotyledon homogenates with dimethylformamide or dimethylsulfoxide, known nucleic acid precipitants, decreased the amount of nucleic acids extracted by method II with no improvement in spectra.

One reason that method II is more satisfactory than method I may be the high lipid content of storage tissues which would interfere with nucleic acid analysis. Homogenizing and extracting in methanol would remove some of the lipids prior to acid precipitation of nucleic acids; thus, there would be less interfering material to complicate the ultraviolet light measurements. Nucleic acid analysis on acetone powders using method I and method II gave comparable results which further indicate that lipids interfere.

The data show that there is a large loss of nucleic acids when the cellular debris is discarded. In some previous reports (4, 9) of analysis by method I, the values for RNA were correctly termed cytoplasmic rather than total. The author believes, from previous experience (2, 4), that this method (method I) is satisfactory for RNA analysis of various cytoplasmic fractions (mitochondria, microsomes, & high-speed supernatant), but this paper shows that it is not adequate for total homogenates of storage tissues. In the case of homogenates of meristematic tissue (reference to 5 mm corn root tips) (2), there are little cellular debris and interfering substances; thus, method I was satisfactory for RNA analysis of cytoplasmic fractions and for nucleic acid analysis of isolated nuclei.

In addition to the comparison of methods for the determination of nucleic acids in storage tissues, a relationship between nucleic acid metabolism in storage tissues and seed germination is indicated. There is an apparent reduction in nucleic acid content in the peanut cotyledon during germination (fig 4-C & table II). However, the decrease in RNA content is primarily due to an increase in moisture content. The decrease in ratio of RNA : DNA indicates a loss of RNA in comparison to DNA. Other reports show a decrease in RNA content in corn scutella (4, 6) and bean cotyledons (13) during germination. But our present results indicate that the RNA content of the peanut cotyledon does not entirely serve as reserve of nucleotides. The RNA content doubles with the 1st week of germination, followed by a decrease. The activity of mitochondria and certain enzymes seems to follow the pattern of RNA; this work will be reported later.

It is to be noted that the ratio of RNA : DNA in the storage tissues of the three species of plants varied from 1.5 to 11 (table II).

Summary

Two methods for the extraction and determination of nucleic acids were applied to the storage tissues

of three species of germinating seedlings. Ultraviolet absorption spectra of the materials extracted by various solvents were studied. The efficiency of extraction of phosphates by the two methods were compared.

Column chromatography of the soluble nucleotides extracted by cold methanol or cold 0.2 M HClO₄ revealed that the elution patterns were nearly the same. However, the elution pattern for the methanol extractable nucleotides had a lower base line and the heights of three peaks were reduced. Subsequent extraction and chromatography of the methanol-insoluble residue with cold 0.2 M HClO₄ gave an atypical elution chromatogram, suggesting that the acid extracts contained materials which were not mononucleotides.

A slight modification of the method of Smillie and Krotkov proved to be satisfactory for the extraction and determination of nucleic acids in the storage tissues of seedling plants.

There were large differences in the ratios of RNA to DNA in the storage tissues of the three species of plants.

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