The Acid-Soluble Nucleotides of Mature Pea Seeds

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(Received 13 July 1962)

Although there are several reports concerned with the nucleotides of seedlings (e.g. Bergkvist, 1956, 1957; Šebesta & Šorm, 1959; Abdel-Wahab & El-Kinawi, 1959, 1960; Cherry & Hageman, 1961), little is known of the free nucleotides of mature seeds. The aims of the work described in this paper are to identify the major nucleotide components in mature pea seeds, to estimate their relative concentrations and to examine the seeds for significant change in nucleotide pattern during storage. The metabolism of mature seeds is especially interesting in that they respire, albeit at a very low rate, over long periods of storage with little other detectable metabolic activity.

EXPERIMENTAL

Materials

Seeds. Seeds of *Pisum sativum* L. var. Meteor were supplied by Suttons Seeds Ltd., Reading, seeds of *Lupinus luteus* L. var. Weiko III by Dunns Farm Seeds Ltd., Salisbury, and seeds of *Phaseolus vulgaris* L. var. Canadian Wonder by Thompson and Morgan Ltd., Ipswich.

Chemicals. ADP and ATP were obtained from C. F. Boehringer und Soehne, Mannheim, Germany. NAD and NADP were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. The remaining nucleotides, nucleosides and purine and pyrimidine bases were supplied by L. Light and Co. Ltd., Colnbrook, Bucks. Hyflo Super-cel and Norit OL were obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex.

Methods

Extraction. Pea seeds were milled in a Glen Creston C580 microhammer mill (Glen Creston Ltd., Stanmore, Middlesex), a screen of 0.1 mm. diameter mesh being used. The milled seed (10 g. samples) was homogenized in a MSE homogenizer for 3 min. at 0° with 0.3N-perchloric acid (25–30 ml.) and the homogenate filtered under vacuum through a sintered-glass Buchner funnel (porosity 3) into a flask surrounded by crushed ice. Extraction from the almost dry residue was assisted by pressing with an inverted wide-topped glass stopper. The homogenizer flask was rinsed out with four successive 10 ml. portions of 0.15N-perchloric acid and these washings were used to effect four further extractions of the seed residue in the funnel. The clear filtrate was adjusted to pH 7.2 with 20% (v/v) potassium hydroxide solution and, after standing at 0° for 45 min., was centrifuged to remove potassium perchlorate.

Adsorption on charcoal. It was advisable to adsorb the nucleotides on charcoal before ion-exchange chromatography to remove much of the ultraviolet light-absorbing

non-nucleotide material present in the extract. Ion-exchange chromatography of untreated extracts gave essentially the same pattern as the treated but the background ultraviolet absorption of the chromatogram was much higher, indicating a steady elution of ultraviolet-absorbing material over long periods.

The extract was adjusted to within the range pH 3.5–4.0 with acetic acid and passed slowly through a column, 1.2 cm. diameter, containing a mixture of acid-washed Norit OL charcoal (500 mg.) and Hyflo Super-cel (800 mg.) supported on a 2 mm.-thick bed of Hyflo Super-cel on a sintered-glass disk (porosity 3). The column was washed with 20 ml. of water and eluted with 15 ml. of 25% (v/v) ethanol containing 0.5% (v/v) of aq. ammonia (sp.gr. 0.88). The charcoal mixture was not allowed to dry out at any stage of the procedure. Percolation of solutions through the column was facilitated by application of a positive air pressure of 1–2 lb./in.²

Ion-exchange chromatography. The elute from the charcoal column was passed through a column (19 cm. × 0.9 cm.) of Dowex 1 (formate; X8; 200–400 mesh) at 0.5 ml./min. This was followed by 5 ml. of water, a volume that would displace any non-exchangeable material; washing with larger volumes of water was avoided as it eluted material that would otherwise resolve in the first few fractions. Chromatography was begun with a system of stepwise gradient elution based on the procedure of Hurlbert, Schmitz, Brumm & Potter (1954), but modified to produce a series of linear gradients with no sudden change in the composition of the eluent. The gradients were produced by an apparatus of Parr (1954). The mixing bottle initially contained 455 ml. of water and, for range 1, the reservoir contained 455 ml. of 4N-formic acid. For range 2 the reservoir solution was changed to 4N-formic acid containing 0.2M-ammonium formate; for range 3, to 4N-formic acid containing 0.4M-ammonium formate; for range 4, to 4N-formic acid containing 0.8M-ammonium formate; for range 5, to 4N-formic acid containing 1.6M-ammonium formate. At the end of each range the mixing bottle was refilled with solution of the same composition as that remaining in it. The eluate from the column (flow rate 0.5 ml./min.) was collected in 10 ml. fractions and $E_{265m\mu}$ of each fraction measured.

Spectrophotometric examination of fractions. All measurements were made with a Unicam SP. 500 instrument. The absorption spectra of fractions direct from the column could not be examined satisfactorily owing to the relatively high extinction of formic acid in the lower range of the ultraviolet spectrum. For preliminary examination of absorption spectra, it was sufficient to remove the formic acid by evaporation *in vacuo* in a Towers rotary film evaporator and to redissolve the residue in water before adjusting the pH. With fractions suspected of being more labile, rotary film evaporation was replaced by freeze-

drying. For more detailed examination of absorption spectra, fractions were desalted by charcoal adsorption and, where necessary, further purified by paper chromatography or paper electrophoresis, or both.

Paper chromatography. Nucleotides were separated in these solvent systems: (1) 0.1M-sodium phosphate buffer, pH 6.8-ammonium sulphate solution-propan-1-ol (Pabst Laboratories, 1961); (2) disodium hydrogen phosphate solution-3-methylbutan-1-ol (Carter, 1950); (3) ammonium acetate solution (containing sodium borate and EDTA)-ethanol (Plesner, 1955); (4) ammonium sulphate solution-propan-2-ol (Markham & Smith, 1952). Nucleotides, nucleosides and free bases were separated in solvent (5), butan-1-ol-acetic acid (Partridge, 1948), and free bases chromatographed in solvents (6), water at pH 10 (Levenbook, 1953), and (7), ammonium bicarbonate solution (Hems, 1959). All chromatograms were ascending on Whatman no. 1 paper with the exception of those run in solvent (2), which were descending on the same grade of paper.

Purines and pyrimidines and their derivatives were detected by viewing the chromatograms directly in ultraviolet light from a Hanovia Chromatolite lamp. With chromatograms exhibiting faint spots or where a record of the chromatogram was required, a contact print was made on photographic paper as described by Markham & Smith (1949).

Paper electrophoresis. This was done on strips (7.5 cm. $\times$ 60 cm.) of either Whatman no. 1 or no. 3MM chromatography paper, no. 1 paper being used for characterizing substances by measurement of electrophoretic migration, and no. 3MM paper for preparative separations of larger amounts of material. The apparatus used was constructed as described by Markham & Smith (1951, 1952) with minor modifications. For most purposes an electrode potential of 1000 v was maintained, giving a gradient of approx. 20 v/cm. during a standard run of 2 hr.

Elution from paper chromatograms and electrophoresis strips. The capillary elution technique of Dent (1947) was employed in eluting spots and bands from paper. Except where otherwise indicated, the eluent was 0.01N-hydrochloric acid. Where a spot or band was to be eluted for spectrophotometric examination, an area of the same dimensions and R_F was cut from the chromatogram and eluted to provide a spectrophotometric blank.

Detection and estimation of phosphate. Phosphorylated compounds were detected on paper by the method of Hanes & Isherwood (1949) as modified by Burrows, Grylls & Harrison (1952). Phosphate estimations were made by the colorimetric procedure of Fiske & Subbarow (1925), modified to use stannous chloride as the reducing agent. Labile-phosphorus estimations were conducted on samples hydrolysed according to Hurlbert *et al.* (1954) and total phosphorus assays were done after digestion of the samples by the procedure of LePage (1949).

Detection and estimation of pentose. Pentose was estimated according to Hurlbert *et al.* (1954). For qualitative purposes a micromodification was used in a total volume of 0.3 ml. Pentose was indicated by a green colour after heating for 15 min. at 100°.

Hydrolysis of nucleotides. For identification of purine and pyrimidine constituents of nucleotides, the free bases were liberated by acid hydrolysis and separated by paper chromatography. Purine nucleotides were hydrolysed, in

sealed tubes, with N-hydrochloric acid for 1 hr. at 100° (Markham & Smith, 1950) and pyrimidine nucleotides similarly hydrolysed with concentrated formic acid at 175° for 2 hr. (Vischer & Chargaff, 1948). After hydrolysis, the materials were evaporated to dryness *in vacuo* and dissolved in a small volume of water for paper chromatography.

Estimation of nicotinamide nucleotides. The cyanide addition reaction of Colowick, Kaplan & Ciotti (1951) was employed.

Assessment of viability of seeds. Batches of 100 seeds were germinated at 27° on vermiculite moistened with tap water. The vermiculite had been washed well with water and autoclaved for 15 min. at 15 lb./in.²

RESULTS

Separation and identification of nucleotides

A typical anion-exchange chromatogram of the acid-soluble nucleotides of mature pea seeds (10 g. samples) is shown in Fig. 1. Elution was carried out through all five solvent ranges described under Methods. No ultraviolet-absorbing material was detected in the effluent from the column during ranges 4 and 5 and these ranges are not shown in Fig. 1. The fractions comprising the various peaks were examined and, where possible, identified as indicated below. For the minor peaks, sufficient material for examination was obtained by pooling the corresponding fractions from several separations, storage of fractions between these separations being at -17°.

Peak 1. Paper electrophoresis of this peak at pH 3.6 in a formate buffer gave a single band, which, in a potential gradient of 20 v/cm., migrated 2.5 cm. towards the anode. This was eluted, freeze-dried and, after being dissolved in a small volume of water, was chromatographed on paper in solvent (5). A single band again resulted and was eluted for spectrophotometric examination (Table 1). The material gave positive tests for pentose and phosphate, but did not appear to be any of the more usual purine or pyrimidine derivatives.

Peak 2. The fractions comprising the peak were pooled, the mixture was evaporated *in vacuo* and the residue dissolved in about 0.5 ml. of water before electrophoresis on Whatman no. 3MM paper in 0.5M-ammonium formate buffer, pH 3.6. After electrophoresis for 2 hr. in a voltage gradient of 20 v/cm., a single band was observed to have migrated 3.5 cm. towards the anode. This band was not homogeneous, as darker zones could be seen within it in ultraviolet light. The band was therefore eluted from the paper, freeze-dried and, after solution in 0.2 ml. of water, streaked as a band on to a paper chromatogram for subsequent development in solvent (5). Four dark bands were observed on viewing the developed chromatogram in ultraviolet light: each was eluted together with a

Fig. 1. Anion-exchange chromatogram of the acid-soluble nucleotides of mature pea seeds. Separation of the extract, equivalent to 10 g. of seeds, was on Dowex 1 (formate) in a formate system. The number of 10 ml. fractions collected and the points at which the solvent was changed are indicated on the abscissa. $E_{265\text{ m}\mu}$ for peaks 2, 3 and 4 were 2.65, 0.95 and 2.40 respectively.

Table 1. Ultraviolet-absorption characteristics of unidentified peak components

Complex peaks were resolved, as indicated, by electrophoresis at pH 3.6 in ammonium formate-formic acid buffer in a potential gradient of 20 v/cm., followed by paper chromatography in solvent (5).

Peak component	Electrophoretic migration towards the anode (cm.)	R_F in solvent (5)	Ultraviolet absorption							
			pH 2				pH 12			
			$\lambda_{\text{max.}}$	$\lambda_{\text{min.}}$	$\frac{E_{250}}{E_{260}}$	$\frac{E_{280}}{E_{260}}$	$\lambda_{\text{max.}}$	$\lambda_{\text{min.}}$	$\frac{E_{250}}{E_{260}}$	$\frac{E_{280}}{E_{260}}$
1	2.5	0.35	265, 269*	240	0.64	0.25	265, 269*	240	0.75	0.36
2b	3.5	0.68	265	222	0.70	0.76	247	228	1.10	0.39
2c	3.5	0.23	262	230	0.85	0.50	—	—	—	—
2d	3.5	0.92	265	240	0.69	0.75	237*	—	1.15	0.49
5a	3.9	—	248	228	1.30	0.45	240	225	1.60	0.66
5b†	9.6	0.35	252, 287	235, 277	1.13	0.41	252, 287	235, 277	1.15	0.44
		0.41	252, 279*	238	1.07	0.78	252, 279*	238	1.05	0.75
		0.63†	250, 285	238, 270	1.12	0.93	—	—	—	—
		—	268	248	0.92	0.93	245, 260*	—	—	—
6a	3.3	—	258	232	0.96	0.31	260	238	0.83	0.40
6b	9.7	0.06	Identical with material of R_F 0.35 from 5b							
	11.0	0.38	Identical with material of R_F 0.41 from 5b							
	11.8	0.46	Identical with material of R_F 0.63 from 5b							
	—	0.55	265	235	0.74	0.71	268	235	0.71	0.85
12a	17.2	0.10	261	230	0.69	0.63	268	240	0.57	0.96
		0.05	257	235	0.79	0.54	261	223	0.62	0.49
		0.04	272	245	0.83	1.13	276	245	0.87	1.42
17a†	—	0.00†	305	277	1.42	0.77	—	—	—	—

* Inflexion.

† Blue fluorescence.

‡ R_F in solvent (4).

corresponding blank for the spectrophotometer (see Methods section). The ultraviolet-absorption spectra of the major band (2a) corresponded closely at both pH 2 and pH 12 to those of the nucleoside uridine. The ratios $E_{250\text{ m}\mu} : E_{260\text{ m}\mu}$, $E_{280\text{ m}\mu} : E_{260\text{ m}\mu}$ and $E_{290\text{ m}\mu} : E_{260\text{ m}\mu}$ were 0.74, 0.36 and 0.03 respectively at pH 2, and 0.83, 0.29 and 0.02 respectively at pH 12. Pentose was present but no phosphate was detected. Confirmation of the identification was obtained by paper chromatography in solvents (5) and (6) in both of which the material, 2a, corresponded to an authentic sample of uridine.

Band 2b, the second most intense band, as judged visually in ultraviolet light, and the two minor bands 2c and 2d were not identified. The ultraviolet-absorption characteristics of these substances are shown in Table 1.

Peak 3. This consists mainly of xanthosine together with a small amount of NAD. The former was identified by its characteristic ratios $E_{250\text{ m}\mu} : E_{260\text{ m}\mu}$, $E_{280\text{ m}\mu} : E_{260\text{ m}\mu}$ and $E_{290\text{ m}\mu} : E_{260\text{ m}\mu}$ (0.75, 0.29 and 0.03 respectively at pH 2 and 1.3, 1.14 and 0.62 respectively at pH 12). It gave a positive orcinol test for pentose but no phosphate was detected. Hydrolysis with *N*-hydrochloric acid, as described in the Methods section, followed by paper chromatography in solvents (5) and (6) showed a spot in each case corresponding to xanthine. NAD was identified by paper-chromatographic comparison with authentic samples in solvents (1) and (4) and by the cyanide addition reaction of Colowick *et al.* (1951).

Peak 4. The values obtained for the ultraviolet-absorption ratios $E_{250\text{ m}\mu} : E_{260\text{ m}\mu}$, $E_{280\text{ m}\mu} : E_{260\text{ m}\mu}$ and $E_{290\text{ m}\mu} : E_{260\text{ m}\mu}$ (0.85, 0.24 and 0.04 respectively at pH 2) indicated an adenine nucleotide. This was confirmed by the spot test of Gerlach & Döring (1955) for adenine compounds. Paper chromatography in solvent (4) followed by elution and more detailed spectrophotometric examination showed that the sole component of the peak was adenylic acid. Analysis gave adenine:ribose:phosphate as 1.0:0.98:1.05. Further paper chromatography in solvents (2) and (3), which separate the isomeric adenine nucleotides, showed that this peak consists entirely of the 5'-isomer, i.e. adenosine 5'-monophosphate.

Peak 5. Paper electrophoresis for 2 hr. at pH 3.6 in ammonium formate-formic acid buffer and under a voltage gradient of 20 v/cm. showed two bands, designated 5a and 5b, which had migrated 3.9 and 9.6 cm. respectively towards the anode: 5a appeared as a dark band under ultraviolet light whereas 5b had an intense blue fluorescence. Neither the main band, 5a, nor the band 5b could be identified with any known purine or pyrimidine derivative; their ultraviolet-absorption properties are listed in Table 1.

Peak 6. Like the previous peak, with which it overlaps to some extent, this peak was found by electrophoresis at pH 3.6 to be heterogeneous. Bands were observed, in ultraviolet light, having anodic migration of 3.3, 9.7, 11.0 and 11.8 cm., under a potential gradient of 20 v/cm., the most intense being that at 3.3 cm. This was eluted and its spectrophotometric properties were examined (see 6a, Table 1). The three remaining bands were too close together on the electrophoresis strip to be eluted separately and were therefore eluted together as one band (designated 6b in Table 1) and subsequently separated again by paper chromatography in solvent (5). The latter procedure separated the mixture into five discrete bands, three of which had ultraviolet-absorption spectra identical with three bands of similar R_f values previously isolated from the contiguous peak 5. These are indicated in Table 1, together with the spectrophotometric properties of the remaining two bands. None of these substances was identified.

Peak 7. This contained a mixture of GMP and a much smaller amount of NADP, both being identified by paper chromatography and spectrophotometry. Chromatograms run in solvents (1) and (4) showed spots, corresponding to GMP and NADP, which were eluted, freeze-dried and taken up in 0.75 ml. of 0.01*N*-hydrochloric acid for spectrophotometric examination in 1 cm. microcells of 0.5 ml. capacity. After examination, the solutions were again freeze-dried and taken up in 0.75 ml. of *mN*-sodium hydroxide for examination of the spectra in alkali. The spectra in both acid and alkali were in good agreement with GMP and a nicotinamide nucleotide. The identification of the latter was confirmed by the increase in $E_{325\text{ m}\mu}$ in *m*-potassium cyanide (Colowick *et al.* 1951).

Peak 8. The main component of this peak was identified by paper chromatography in solvents (3) and (4), and by its ultraviolet-absorption spectra at pH 2 and 12, as inosine 5'-monophosphate. There also appeared to be a trace of material on the chromatograms corresponding to cytidine diphosphate, and, although too little material was present for further examination, the observation of Hurlbert *et al.* (1954) that inosinic acid and cytidylic acid run together in their similar chromatographic procedure would support this tentative identification.

Peak 9. This peak gave a spectrum exhibiting λ_{max} at 258 m μ but paper chromatography in a band, however, showed that the material was not homogeneous, and consisted of two bands of approximately equal intensity in ultraviolet light. The furthest running band (R_f 0.14) corresponded to a marker of authentic UMP and upon elution exhibited, in both acid and alkali, the typical ultraviolet-absorption spectra of a uridine deriva-

tive. The identification of the material as UMP was confirmed by further paper chromatography together with authentic samples of this nucleotide in solvents (2) and (4). Analysis for uracil:ribose:phosphate gave 1.00:0.98:1.02.

The slower-running band in solvent (5) (R_F 0.04) exhibited, after elution, ultraviolet-absorption spectra characteristic of an adenine nucleotide and, further, gave a positive spot test for an adenine derivative (Gerlach & Döring, 1955). Its R_F in solvent (5) had implied that it might have been contaminating ADP from the ensuing peak 10; however, analysis for adenine:ribose:phosphate gave 1.00:1.98:2.02, adenine having been shown, by acid hydrolysis and paper chromatography, to be the sole ultraviolet-light-absorbing component. Comparison with similar ion-exchange chromatograms of animal tissue (cf. Papenberg, 1961), indicated that the material, from its position in the elution sequence, and its base:ribose:phosphate analysis, was ADP-ribose. Paper chromatography in solvent (5) and in ethanol-ammonium acetate solution, pH 7.5 (Pabst Laboratories, 1961), together with authentic samples of ADP-ribose confirmed this identification.

Peak 10. The spot test of Gerlach & Döring (1955) and the ratios at pH 2 ($E_{250\text{ m}\mu}:E_{260\text{ m}\mu}$, 0.85; $E_{280\text{ m}\mu}:E_{290\text{ m}\mu}$, 0.23; $E_{290\text{ m}\mu}:E_{260\text{ m}\mu}$, 0.04) indicated an adenine nucleotide. Paper chromatography in solvents (2) and (4) showed that the material was ADP, no other ultraviolet light-absorbing spots being detected. After elution from the chromatograms, detailed spectral examination at pH 2 and 12 and chemical analysis (adenine:ribose:phosphate, 1.0:1.05:1.95) confirmed the identification. Hydrolysis of the compound, as described for labile phosphate in the Methods section, yielded an adenylic acid which by paper chromatography in solvents (2) and (3) was demonstrated to be the 5'-isomer, i.e. peak 10 is composed of ADP.

Peak 11. This did not appear to be nucleotide material since it showed non-specific absorption between 220 and 300 $\text{m}\mu$.

Peak 12. Like the preceding peak, this was found to have a high content of non-nucleotide material, but paper chromatography with solvent (5) revealed two very weak spots that were not identified owing to the very small amount available. The spectral data obtained are recorded in Table 1.

Peak 13. Like the two contiguous peaks, 11 and 12, a high proportion of material exhibiting non-specific ultraviolet absorption was found in this peak. However, paper chromatography in solvent (5) revealed a trace of material which upon subsequent elution exhibited nucleotide-like absorption spectra (Table 1). Too little was available for further investigation.

Peak 14. This peak consisted mainly of UDP-glucose together with a trace of UDP-galactose. The pooled fractions showed spectra typical of uridine nucleotides ($E_{250\text{ m}\mu}:E_{260\text{ m}\mu}$ and $E_{280\text{ m}\mu}:E_{260\text{ m}\mu}$ were 0.74 and 0.38 respectively at pH 7). Paper chromatography in solvents (1) and (3) showed spots corresponding to UMP and UDP; the presence of sugars was also indicated by an aniline hydrogen phthalate spray reagent. Paper chromatography of the sugar components in solvent (5) and in propan-2-ol-water (Smith, 1960) revealed a well-defined spot corresponding to glucose and a much weaker spot at the position of galactose. These are not likely to represent free sugars from the original seed extract as they would have been removed during the charcoal-adsorption step. It is much more likely that they result from the decomposition of the labile UDP-sugars during the removal of formate (Hurlbert *et al.* 1954), which in this case had been achieved by freeze-drying followed by vacuum sublimation.

Peak 15. An adenine compound was indicated by the spot test of Gerlach & Döring (1955) and the ultraviolet-absorption data ($E_{250\text{ m}\mu}:E_{260\text{ m}\mu}$, $E_{280\text{ m}\mu}:E_{260\text{ m}\mu}$ and $E_{290\text{ m}\mu}:E_{260\text{ m}\mu}$ of 0.83, 0.21 and 0.04 respectively at pH 2, and 0.79, 0.15 and 0.01 at pH 12). Hydrolysis in N-hydrochloric acid followed by paper chromatography in solvents (5) and (6) produced only one ultraviolet-light-absorbing spot, which was subsequently identified as adenine. Estimation of the ratio adenine:ribose:phosphate gave 1.0:1.81:2.85 for the original material, indicating an adenine triphosphate. Paper chromatography in solvent (3) distinguished it from ATP and revealed the presence of two minor spots, both of which also appeared to be adenine nucleotides. On the basis of these observations, the peak would seem to be similar to the fraction AdX of Hurlbert *et al.* (1954), peak 11 of Šebesta & Šorm (1959) and the ATP-X of Schnitger *et al.* (1959). These peaks originate from the acid decomposition of reduced NADP during the initial perchloric acid extraction of the nucleotides (Pressman & Lange, 1957; Papenberg, 1961). In support of this identification, paper chromatography of peak 15 in solvent (3) showed that the main spot coincided with a spot from a reduced NADP preparation that had been kept in 0.3N-perchloric acid at 4° for 2 hr.

Peak 16. This peak was very small but sufficient material was obtained by pooling the corresponding peaks from five separations to identify it as UDP. The identification was made on the basis of paper chromatography in solvents (1) and (3) and ultraviolet-absorption data, the ratios $E_{250\text{ m}\mu}:E_{260\text{ m}\mu}$, $E_{280\text{ m}\mu}:E_{260\text{ m}\mu}$ and $E_{290\text{ m}\mu}:E_{260\text{ m}\mu}$ being 0.79, 0.16 and 0.05 respectively at pH 7.

Peak 17. This peak was found to consist almost entirely of ATP. The ratios $E_{250\text{ m}\mu}:E_{260\text{ m}\mu}$,

$E_{280\text{ m}\mu}:E_{260\text{ m}\mu}$ and $E_{290\text{ m}\mu}:E_{260\text{ m}\mu}$ were 0.85, 0.22 and 0.03 respectively at pH 2, and 0.80, 0.15 and 0.01 respectively at pH 12. The material gave a positive spot test for adenine (Gerlach & Döring, 1955), and on paper chromatography in solvents (2) and (4) corresponded to an authentic sample of ATP. Analysis for the proportion of base:ribose:phosphate gave the ratio 1.0:0.98:2.91 (total phosphate) and 1.0:0.98:1.98 (labile phosphate). A sample of the acid hydrolysate produced during the estimation of labile phosphate was chromatographed, on paper in solvents (2) and (3), to define the adenylic acid isomer. This was shown to be AMP, indicating that the original material was the 5'-isomer, namely ATP.

Paper chromatography of peak 17 in solvent (4) had shown that, apart from the main spot of ATP and concomitant traces of AMP and ADP, the only component was a faint, pale-blue fluorescent spot that remained at the origin and gave a negative spot test for adenine. This was eluted from the paper and examined in the spectrophotometer but the data obtained, and recorded in Table 1 (peak component 17a), did not correspond to any of the common purine or pyrimidine derivatives.

Estimation of the major nucleotides

Nucleotides comprising the main peaks of the anion-exchange chromatogram (Fig. 1) were estimated as indicated in Table 2. Šebesta & Šorm (1959), however, reported appreciable losses of nucleotides during charcoal adsorption and subsequent elution; hence the charcoal-column technique used in the present work and described in the Methods section was examined for such losses. A representative series of nucleotide solutions were prepared and assayed by spectrophotometry both before adsorption and after subsequent elution. The observed losses in recovery (%) were: AMP 20, ADP 19, ATP 20, UMP 28, UDP 26, GMP 22, IMP 28 and NAD and NADP both 22. These figures appear to be about midway between the figures found by Šebesta & Šorm (1959) for the two charcoals, Norit A and Karborafin. As the losses shown above were reproducible, corrections for them were applied to the concentrations found for the nucleotides listed in Table 2.

Estimation of nicotinamide nucleotides

Nicotinamide nucleotides were estimated as described in the Methods section; the reduced nicotinamide nucleotides were, however, estimated by reference to the concentration of their acid-decomposition products, i.e. the adenine nucleotides of peak 15 (Table 2) and ADP-ribose. These substances have been shown by several workers (e.g. Papenberg, 1961) to be derived from NADPH₂

and NADH₂ respectively. Further, as Papenberg (1961) had indicated, these acid-fission products are produced stoichiometrically and hence their concentration may be taken as a measure of the concentration of the respective reduced nicotinamide nucleotides. Papenberg (1961) has shown that such estimation is in excellent agreement with more conventional enzymic assay. The results of the present estimation of the nicotinamide nucleotides of mature pea seeds are recorded in Table 3. They show that the total NAD (oxidized + reduced) exceeds the total NADP, and that with both NAD and NADP it is the reduced form that predominates.

Effect of storage on the nucleotide pattern

The nucleotide pattern of mature pea seeds was found to be virtually unchanged after storage for

Table 2. *Predominant free nucleotides of mature pea seeds*

Nucleotides separated as in Fig. 1 were estimated, for homogeneous peaks, by computation of $E_{265\text{ m}\mu}$ of the fractions comprising the peak, allowance being made for $E_{265\text{ m}\mu}$ of the eluent; substances in heterogeneous peaks were also estimated by spectrophotometry but only after they had been resolved by paper chromatography or electrophoresis. With mixtures composed entirely of two defined nucleotides, differential spectrophotometry was applied directly. Nicotinamide nucleotides were estimated by a cyanide addition reaction (see Methods section). The adenine nucleotides of peak 15 were estimated as AMP. All values represent the mean of five separations. With the very small peaks 7, 8, 14 and 15 the corresponding peaks from the five separations were pooled to provide sufficient material for analysis. Figures given in the third column show nucleotide concentration after correction, as follows, for recovery losses sustained during charcoal adsorption and elution (see text): adenine nucleotides $\times 1.25$, guanine nucleotides $\times 1.28$, uracil nucleotides $\times 1.39$, nicotinamide nucleotides $\times 1.28$ and inosinic acid $\times 1.39$.

Nucleotide	Content in seeds ($\mu\text{moles}/10\text{ g.}$)	
	Found	Corrected for loss on charcoal
NAD	0.01	0.01
AMP	3.58	4.47
GMP	0.10	0.13
NADP	0.02	0.03
IMP	0.11	0.15
UMP	0.41	0.57
ADP-ribose	0.31	0.39
ADP	1.74	2.18
UDP-glucose } *	0.57	0.79
UDP-galactose }		
Adenine nucleotides of peak 15	0.11	0.14
ATP	0.69	0.86

* Mixture not resolved for estimation but consists mainly of UDP-glucose.

Table 3. *Nicotinamide nucleotide content of mature pea seeds*

Nicotinamide nucleotides were estimated by the cyanide addition reaction of Colowick, Kaplan & Ciotti (1951). The reduced forms of the nucleotides were estimated as their acid-decomposition products (see text). All values are corrected for recovery loss during charcoal adsorption and elution as indicated in Table 2.

Nicotinamide nucleotide	Content in seeds (μ mole/10 g.)
NAD	0.01
NADH ₂	0.40
NADP	0.03
NADPH ₂	0.14
	0.41 (total)
	0.17 (total)

12 months in the dark at room temperature. Estimation of the adenine nucleotides from three separations (10 g. samples) gave remarkably good agreement with the results obtained for the seeds before storage. Stored seed gave mean values of 4.30 μ moles, 2.02 μ moles and 0.88 μ mole for AMP, ADP and ATP respectively, as compared with 4.47 μ moles, 2.18 μ moles and 0.86 μ mole respectively for the original seed (Table 2). As the amounts of most of the other major nucleotides, as listed in Table 2, had originally proved to be very small, no attempt was made to compare figures for the stored seed, for such comparisons would not be within the accuracy of the method. However, no qualitative difference was observed in either the distribution of peaks or their relative sizes. During storage a check was kept on the viability of the seeds, as measured by germination on moist vermiculite (see under Methods). Germination fell by about 8 % during the 12 months, the figures being 88 % germination with the freshly supplied seeds and 80 % germination after storage of the same seeds for 12 months.

Examination of some other mature seeds

Mature seeds of yellow lupin (*Lupinus luteus* L. var. Weiko III) and dwarf kidney beans (*Phaseolus vulgaris* L. var. Canadian Wonder) were also examined by anion-exchange chromatography during the present investigation. They were not examined quantitatively for nucleotide content but the chromatograms obtained from 10 g. samples showed essentially the same nucleotide pattern as that found for mature pea seeds.

DISCUSSION

The nucleotide pattern of mature pea seeds reveals several interesting features, especially when examined in relation to the hypothesis expounded by Schmitz (1961) that a correlation exists between the free nucleotide pattern of a tissue and its

metabolic activity. The two extreme categories of ion-exchange chromatograms cited by Schmitz are (a) the *energiety* (e.g. chromatogram of heart-muscle tissue), in which those nucleotides most intimately concerned with energy metabolism, i.e. adenine and nicotinamide nucleotides, constitute about 90 % of the total nucleotide content, and then mostly as the energy-rich di- or tri-phosphates, and (b) the *stoffwechselt* (e.g. chromatogram of regenerating liver tissue), which is characterized by its lower content of adenine and pyridine nucleotides (< 50 % of the total nucleotide content), and its increased content of nucleotides more especially concerned in biosynthesis and biochemical transformation such as those of uridine, cytidine and guanosine. The chromatogram of the acid-soluble nucleotides of mature pea seeds (Fig. 1) trends towards the *energiety*, having a relatively low content of UDP, GDP and CDP, whereas about 83 % of the total nucleotide content consists of adenine and nicotinamide nucleotides. This is consistent with both the virtual absence of synthesis and chemical transformation in the mature non-germinating seeds, and the fact that such seeds are respiring. Chromatograms of young seedlings of various species (cf. Bergkvist, 1956; Šebesta & Šorm, 1959; Abdel-Wahab & El-Kinawi, 1960) appear to resemble more closely, as might be expected, the *stoffwechselt* pattern. Calculations based on figures for nucleotide analysis reported by these authors give values for adenine and nicotinamide nucleotide content of about 30 % of the total nucleotide content. One divergence from the trend towards the *energiety* pattern shown by the pea-seed chromatogram is the relatively small proportion of ATP in comparison with AMP, the present results (see Table 2) giving a ratio of ATP:ADP:AMP of 1.0:2.5:5.2, whereas in the examples of *energiety* nucleotide pattern cited by Schmitz (1961) this ratio is reversed, ATP predominating. Calculation from Table 2 indicates that AMP constitutes about 46 % of the total free nucleotide pool of mature pea seeds; this emphasizes the quantitative importance of this substance in these seeds. The high content of AMP may represent a latent pool of ADP and ATP available for the rapid and extensive increase in respiratory activity that accompanies germination and subsequent growth. Of many different types of animal tissues that have been examined, one of the few that contains more AMP than ATP is embryonic tissue (e.g. Novikoff, Potter & LePage, 1948).

Estimation of the nicotinamide nucleotide content of mature pea seeds (Table 3) showed that NAD + NADH₂ predominates over NADP + NADPH₂ and that both coenzymes are present mainly in their reduced form. Similar findings have been made by others working with animal mito-

chondria and employing similar techniques to those used here, e.g. Pressman & Lange (1957). Nevertheless, it is difficult to interpret such findings in terms of the redox-state of nicotinamide nucleotides *in situ*, especially as, according to Pressman & Lange (1957), an opposite picture may be obtained if, instead of perchloric acid, a non-ionic reagent composed of urea and Triton detergent is used to extract the nucleotides.

On storage of mature pea seeds for 12 months the viability fell by 8% but there was no apparent change in the nucleotide pattern. There was no accumulation of ATP despite the fact that such seed is respiring, although slowly. The figures of Smith & Gane (1935) for the respiration of air-dried peas (3 ml. of carbon dioxide/kg./24 hr.) indicate that a 10 g. sample of seed would be expected to produce between 1.3 and 13.0 μ moles of ATP/day, depending upon the relative extents of aerobic and anaerobic respiration. Yet, even after storage for 12 months the ATP content of 10 g. samples of pea seeds was found to be only 0.86 μ mole. Thus ATP formation is closely paralleled by ATP utilization, although the purpose for which the mature seed required ATP during storage is at present obscure.

SUMMARY

1. Anion-exchange chromatography of the acid-soluble nucleotides of mature pea seeds is described. Stepwise gradient elution involving a series of linear gradients in a formate system has been employed.

2. The adenine and nicotinamide nucleotides together constitute 83% of the total acid-soluble nucleotides. The largest single contribution to this is made by adenosine 5'-monophosphate, which constitutes 46% of the total. The nicotinamide nucleotides were present largely in their reduced form, NAD + NADH₂ predominating over NADP + NADPH₂.

3. Several unidentified substances were resolved and their ultraviolet-absorption spectra recorded.

4. The nucleotide pattern of the seeds did not change significantly over a period of storage for 12 months at room temperature in the dark. Loss of viability over this period amounted to some 8%.

5. The nucleotide patterns of mature seeds of *Lupinus luteus* and *Phaseolus vulgaris* were qualitatively similar to that obtained from pea seeds.

6. Results are discussed in relationship to the metabolic activity of mature pea seeds. It is suggested that the metabolism of these seeds is primarily concerned with the production of energy.

I am indebted to Mrs M. Hughes for skilled technical assistance throughout this work.

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