Changes in the Free Nucleotide and Nucleoside Pattern of Pea Seeds in Relation to Germination

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1. Major changes in the free nucleotide and nucleoside pattern of germinating pea seeds are described. 2. During the imbibition phase of germination (0–16 hr.) there was a 250% increase in ATP content and a parallel fall in AMP content without detectable change in ADP content. Metabolic implications are discussed. 3. The main nucleoside changes during imbibition were a 93% increase in xanthosine content and a 39% fall in adenosine content. 4. During the last phase of germination, leading to the emergence of the radicle, there is a general fall in free nucleotide content. AMP, ADP and ATP contents decreased 73, 48 and 52% respectively. Acetyl-3'-dephosphocoenzyme A concentration fell by 53%. However, the $(\text{NADP}^+ + \text{NADPH})/(\text{NAD}^+ + \text{NADH})$ ratio increased, and except for uridine content (52% decrease) the nucleoside pattern changed little. 5. A sixfold increase in the concentration of an unidentified UDP-glycosyl compound occurs at this stage, although the content of UDP-glucose and UDP-galactose remained unchanged. 6. No free purine or pyrimidine bases could be detected at any stage of germination.

As previously discussed (Brown, 1962), the central role played by nucleotides in metabolism and especially in its regulation suggests that the free nucleotide pattern of a tissue provides an index to the nature and extent of the predominant metabolic processes in that tissue. Accordingly, the aim of the present investigation has been to define the nucleotide and related nucleoside pattern of seeds at different stages of germination with a view to obtaining information relating to the major biochemical events of germination. In the present paper the term 'germination' is used to describe that period commencing with water uptake and ending with the penetration of the seed coat by the developing radicle; subsequent development is treated as 'growth'. The work is an extension of previous studies of purine and pyrimidine derivatives in seeds (Brown, 1962, 1963).

EXPERIMENTAL

Seeds. Seeds of *Pisum sativum* L. var. Meteor were obtained from Suttons Seeds Ltd., Reading, Berks. Viability, assessed as described by Brown (1962), was 90%.

Chemicals. ATP, ADP and CoA were purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Other nucleotides and nucleosides were supplied by L. Light and Co. Ltd., Colnbrook, Bucks.

Germination of seeds (imbibition phase). Seeds (10g. samples) were covered to a depth of 2–3 cm. with deionized

water and kept in the dark overnight for 16 hr. at 25°. The selection of a 16 hr. soaking period followed preliminary experiments in which determination of the water uptake by tared seed samples showed that imbibition was just about complete at this point.

Germination of seeds (second phase). For examination of the second phase of germination, the entire seed sample was transferred at the end of the imbibition phase to moist filter paper and kept for a further 24 hr. in a dark constant-temperature room at 25°. At the end of this phase 83% of the seeds of a typical sample had radicles 1–5 mm. in length, 7% had radicles apparently close to emergence and 10% were showing no signs of germination.

Extraction of seeds. Dry seeds were extracted at 0° with 0.3N-perchloric acid as described by Brown (1962, 1963). Imbibed seeds were extracted in the same way except that they were not milled but homogenized directly in 0.3N-perchloric acid for a total of 4 min. at 0°.

Separation and estimation of nucleotides and nucleosides. The methods used to separate nucleotides were based on anion-exchange chromatography on Dowex 1 (X8; formate form; 200–400 mesh) and employed stepwise gradient elution involving a series of linear gradients (Brown, 1962). Nucleosides were separated by the method of Brown (1963) and involved anion-exchange chromatography on Dowex 1 (X10; Cl⁻ form; 200–400 mesh), elution being by charge adjustment. All ion-exchange chromatograms were repeated on at least four separate samples of seeds to ensure reproducibility. Ion-exchange chromatography of the many different extracts was facilitated by the use of a Vanguard Automatic UV-Analyzer, model 1056A (Vanguard Instrument Co., La Grange, Ill., U.S.A.) to monitor the column effluent. Nucleosides and nucleotides in chromatographic

peaks were estimated after further resolution where necessary by spectrophotometric methods (Brown, 1962, 1963). Results are expressed in relation to the original weight (10g.) of the dry seed samples before the start of germination.

RESULTS

Changes in nucleotide and nucleoside pattern induced by imbibition. A typical anion-exchange chromatogram of the acid-soluble nucleotides of pea seeds (samples weighing 10g. before soaking) after a 16hr. imbibition period was compared with that obtained from an equivalent sample of untreated pea seeds of the same batch. Peak constituents of this latter chromatogram have been identified and described by Brown (1962). The corresponding peaks in the chromatogram of imbibed seeds were examined by the same spectrophotometric and paper-chromatographic techniques as before and found to be qualitatively similar to those from the chromatogram of untreated seeds. Quantitatively, however, there were significant changes. Constituents of peaks undergoing such changes were assayed spectrophotometrically (Brown, 1962) both before

and after imbibition, and the results are recorded in Table 1. The peaks mainly involved in the changes were those of the adenosine nucleotides, AMP and ATP. The concentration of the former fell by about 43% whereas that of ATP increased by about 250%. In contrast, the amount of ADP remained unaltered, as did also the concentrations of the nicotinamide nucleotides (Table 2).

The nucleosides of imbibed pea seeds (samples weighing 10g. before soaking) were separated by anion-exchange chromatography and a representative chromatogram was compared with that obtained from an equivalent sample of untreated seeds from the same batch. As with the nucleotides, after imbibition, the nucleoside pattern of pea seeds showed changes that were essentially quantitative, no difference in the qualitative picture being observed. By methods described by Brown (1963) the components of peaks showing changes were assayed spectrophotometrically both before and after imbibition (Table 1). The main changes were an increase of 93% in the concentration of xanthosine and a 39% fall in that of adenosine.

Changes in the nucleotide and nucleoside pattern

Table 1. *Changes in the concentration of the major free nucleotides and nucleosides of pea seeds during germination*

Nucleotides and nucleosides, separated by anion-exchange chromatography as described by Brown (1962, 1963), were estimated, for homogeneous peaks, by computation of $E_{265m\mu}$ of the fractions comprising the peak, allowance being made for the $E_{265m\mu}$ of the eluent; substances in heterogeneous peaks were also estimated by spectrophotometry but only after they had been resolved by paper chromatography or electrophoresis. Mixtures composed entirely of two defined nucleotides or nucleosides were analysed directly by differential spectrophotometry. All values represent the mean of four separations, reproducibility being good. Corrections have been applied (Brown, 1962) for recovery losses of nucleotides sustained during charcoal adsorption and elution. The compound UDP-X, obtained from peak 5 of the chromatographic separation on Dowex 1 (Cl⁻ form), was estimated as UMP. Conditions under which the seeds were germinated are described in the text.

Substance	Content in seeds (μ moles/10g.*)		
	Dry mature seeds	At end of imbibition phase (16hr.)	At initial protrusion of radicles (40hr.)
Acetyl-3'-dephospho-CoA	1.95	2.00	0.94
Adenosine	1.52	0.93	0.90
ADP	2.18	2.16	1.12
AMP	4.47	2.56	0.69
ATP	0.69	2.41	1.16
Guanosine	0.66	0.68	0.65
GMP	0.13	0.13	0.11
IMP	0.15	0.15	0.08
Uridine	1.98	1.89	0.90
UDP-glucose } †	0.79	0.73	0.61
UDP-galactose }			
UDP-X of 'peak 5' Dowex 1 (Cl ⁻ form) separation	0.34	0.34	2.15
UMP	0.57	0.53	0.30
Xanthosine	0.91	1.76	1.78

* Each sample of seeds weighed 10g. before soaking.

† Mixture not resolved for estimation but consists mainly of UDP-glucose at all three stages.

Table 2. *Nicotinamide nucleotide content of pea seeds during germination*

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 (Brown, 1962). All values are corrected for recovery loss during charcoal adsorption and elution (Brown, 1962). Seeds were set to germinate at zero time. At the end of the second phase of germination the seed coats had just been penetrated by the radicles.

Nicotinamide nucleotide	Content in seeds (μ moles/10g.*)		
	Dry mature seeds	End of imbibition phase (16 hr.)	End of second phase (40 hr.)
NAD ⁺	0.01	0.02	0.02
NADH	0.40	0.35	0.25
NADP ⁺	0.03	0.03	0.08
NADPH	0.14	0.14	0.16
	0.41 (total)	0.37 (total)	0.27 (total)
	0.17 (total)	0.17 (total)	0.24 (total)

* 10g. was the weight of the dry seed samples before setting to germinate.

during the second phase of germination. During the second phase of germination, i.e. the period commencing when the seed has become fully imbibed and ending with the initial penetration of the radicle through the seed coat, further marked changes occurred in the nucleotide and nucleoside pattern of pea seeds (Table 1). The concentrations of all three adenosine nucleotides, AMP, ADP and ATP, showed relatively large decreases (73, 48 and 52% respectively), as did that (53% decrease) of the nucleotide coenzyme derivative acetyl-3'-dephospho-CoA, identified in mature dry pea seeds by Brown (1963). Changes were also recorded in the concentrations of the nicotinamide nucleotides (Table 2). That of total NAD (NAD⁺ + NADH) decreased whereas that of the total NADP (NADP⁺ + NADPH) increased. The (NADP⁺ + NADPH)/(NAD⁺ + NADH) ratio, initially 0.41 in mature dry seeds, increased approximately twofold during germination to reach a value of 0.88 at the end of the second phase. With the exception of uridine, which decreased in amount by 52%, the nucleosides showed little change during this phase.

In addition to the changes described, an increase was observed during the second phase of germination in 'peak 5' of the chromatographic separation on Dowex 1 (Cl⁻ form) (Brown, 1963). This peak had been previously shown to contain, as its main component, a uridine nucleotide of the type UDP-X (Brown, 1963). Paper chromatography and electrophoresis of the pooled fractions constituting peak 5 showed a similar qualitative pattern at the end of the second phase to that found in mature dry seeds. Visual comparison of the chromatograms in ultraviolet light indicated that the increase in this peak was mainly attributable to a rise in the amount of the uridine derivative. The chemical structure of this substance is being investigated but it does not appear to be any of the more commonly occurring UDP-glycosyl compounds. Assuming that it has

Table 3. *Balance sheet of main changes in the free nucleotide and nucleoside pattern of pea seeds during germination*

Increases are shown as positive values and decreases as negative values. Experimental details are as given for Table 1. The second phase of germination (second column) is that beginning with the completion of imbibition and ending with the protrusion of the radicle. UDP-X is the unidentified nucleotide of peak 5 in the separation on Dowex 1 (Cl⁻ form). This substance was assayed spectrophotometrically as UMP.

	Net changes (μ moles/10g.* of seed sample)	
	0-16 hr. (imbibition phase)	16-40 hr. (second phase)
Acetyl-3'-dephospho-CoA	+ 0.05	- 1.06
Adenosine	- 0.59	- 0.03
ADP	- 0.02	- 1.04
AMP	- 1.91	- 1.87
ATP	+ 1.72	- 1.25
UDP-X	0.00	+ 1.81
UMP	- 0.04	- 0.23
Uridine	- 0.09	- 0.99
Xanthosine	+ 0.85	+ 0.02
Sum:	+ 2.62	- 2.65
		Sum: + 1.83 - 6.47

* Seed samples weighed 10g. before being put to germinate.

the same molecular extinction coefficient as UMP, the amount of this compound in the standard 10g. sample of seed analysed (Table 1) rises from 0.34 μ mole before and during the imbibition phase to 2.15 μ moles at the end of the second phase of germination.

Balance sheet of changes in the nucleotide and nucleoside composition of pea seeds during germination. To assess the degree of supplementation or depletion of the free nucleotide and nucleoside pool

of pea seeds during germination, a balance sheet was drawn up (Table 3) of the major changes in the concentrations of free nucleotides and nucleosides. During the imbibition phase, this showed a good balance between μ moles of total nucleotide plus nucleoside material disappearing and μ moles of that material newly appearing. During the second phase of germination, however, there was a substantial deficit between these two amounts, i.e. a net loss in total free nucleotide plus nucleoside material. Despite the efficacy of the employed ion-exchange techniques in separating free purine and pyrimidine bases (Brown, 1963), no such compounds were detected at any stage of germination.

More detailed examination of the changes in concentration of AMP, ADP and ATP during germination. As the major changes in the nucleotide pattern of germinating pea seeds (Tables 1 and 3), especially during the imbibition phase, appeared mainly to involve the adenosine nucleotides, a more detailed examination of the variations in the concentrations of these compounds was undertaken by the anion-

exchange methods described above. The results (Fig. 1) show that after the first 4 hr. of imbibition, during which no significant changes occur, there is a period of rapid ATP formation with a concomitant disappearance of AMP. This period terminates at about the time the seed has become fully imbibed (16 hr.). Examination of the analytical data shows that, although no significant change in the concentration of ADP occurred during imbibition (Table 1), there was a quantitative correlation between the production of ATP and the disappearance of AMP (Table 4).

DISCUSSION

The foregoing results show that, during imbibition, the first clearly discernible phase of germination, rehydration of the seed, is accompanied by well-defined changes in the concentrations of certain nucleotide and nucleoside constituents. The main changes involve the adenosine derivatives, with the notable exception of ADP. The biggest single increase is the 250% increase in the content of ATP (Table 1). This is probably related to the increase in the rate of respiration that is known to parallel imbibition during seed germination (see, e.g., Stiles, 1960), and confirms the suggestion (Brown, 1962) that the nucleotide pattern of mature dry pea seeds is indicative of a system mainly committed to 'energy production'. Šebesta & Šorm (1954) have reported an increase in the ATP content of germinating seeds of *Phaseolus vulgaris*, a material possessing a similar nucleotide and nucleoside complement to that of pea seeds (Brown, 1962, 1963). Since the formation of ATP inevitably involves phosphorylation of ADP, it seems paradoxical that, despite the large increase in the ATP content of the seeds, the ADP content remains remarkably constant (Fig. 1). This observation implies the existence of a mechanism for replacing ADP lost by phosphorylation to ATP. Present theories implicating ADP concentration as the controlling factor of tissue respiration (see, e.g., Chance & Williams, 1956) suggest that this mechanism, by preventing depletion of ADP, is a

Fig. 1. Metabolism of endogenous AMP, ADP and ATP during germination of pea seeds. The nucleotides were separated by ion-exchange chromatography and estimated spectrophotometrically. Germination of mature dry seeds (10g. samples) was initiated at zero time by putting seeds to soak. At 40 hr. the radicles were just penetrating the seed coats. ○, ATP; ●, ADP; □, AMP.

Table 4. *Correlation of the disappearance of AMP with the production of ATP during the imbibition phase of germination*

At zero time, 10g. samples of dry mature seeds were put to soak in water (see the Experimental section) for 4, 6, 8, 12 and 16 hr. respectively. After extraction and separation by anion-exchange chromatography (Brown, 1962), AMP and ATP were estimated spectrophotometrically.

Time-interval (hr.)	Decrease in AMP content (μ mole/initial 10g. sample)	Production of ATP (μ mole/initial 10g. sample)
0-4	0.00	0.00
4-6	0.21	0.20
6-8	0.27	0.25
8-12	0.64	0.63
12-16	0.70	0.66

key factor in maintaining the rising respiratory rate during imbibition. The close correlation between the decreasing content of AMP and the formation of ATP (Table 4) points to this mechanism being the phosphorylation of AMP. Most probably this would be through the action of ATP-AMP phosphotransferase (adenylate kinase), an enzyme found in the mitochondria of various plants (Mazelis & Stumpf, 1955) and known to occur in pea seeds (Kirkland & Turner, 1959). The enzymic reactions interrelating the adenosine nucleotides during the imbibition phase of germination would then be as shown below. Reaction (1) is that catalysed by ATP-AMP phosphotransferase and reaction (2) is the formation, during respiration, of ATP by either oxidative or substrate phosphorylation.

The overall effect of coupling reactions (1) and (2) is consistent with the results of the present investigation, i.e. a net fall in AMP content equivalent to the net gain in ATP, with no overall change in ADP. During the rapid rise in respiratory rate observed in germinating seeds (see, e.g., Stiles, 1960), the tendency of the increasing level of phosphorylation (Fig. 1) to deplete the ADP pool would be countered by the equilibrium of the ATP-AMP phosphotransferase system. As indicated in the sequence:

this effect would be reinforced by a feedback from the increasing pool of ATP. AMP, present in relatively high concentration (Table 1), would probably be non-limiting.

The main changes in the nucleoside pattern of pea seeds during the imbibition phase of germination were a 93% increase in xanthosine concentration and a 39% decrease in that of adenosine. Table 3 shows that the increase in xanthosine is approximately equivalent on a molar basis to the net loss in purine derivatives at this time. Xanthosine may thus represent a common point on a degradatory pathway for the purine nucleotides and nucleosides.

By taking into account the main changes in free nucleotide and nucleoside composition during the imbibition phase, there appears to be a good balance on a molar basis between total amount of free nucleotide and nucleoside material that has disappeared and the total amount that has been newly produced (Table 3). This suggests a closed system of free nucleotides and nucleosides during imbibition, with no new material being introduced either by degradation of nucleic acid or by new synthesis

of the purine and pyrimidine ring systems. This is in marked contrast with the balance sheet (Table 3) at the end of the second phase of germination, which appears to be characterized by a general decrease in the amount of nucleotide and nucleoside compounds. Such depletion probably results from the onset of a period of rapid synthesis of nucleic acids and other bound forms of purines and pyrimidines not extracted in the present procedures. Šebesta & Šorm (1956) found a similar fall in the ATP content of germinating beans (*Phaseolus vulgaris*) after the initial increase of the imbibition phase. In the present work, the nucleotide constituents most affected during the second phase were again the adenosine compounds, this time including ADP. A substantial fall (53%) in the concentration of acetyl-3'-dephospho-CoA was also recorded during this phase, although, like that of ADP, its concentration had undergone little change during imbibition (Table 1). It was suggested by Brown (1963) that acetyl-3'-dephospho-CoA may be the immediate source of energy for cell division, being a 'high-energy' acyl thiol of the type discussed by Swann (1957). The observed relatively large decrease in the content of this substance during the last stage of germination is compatible with this suggestion, for it is at this time, when the radicle is just penetrating the testa, that cell division and growth proper are most probably starting.

From the observed changes in the concentrations of the nicotinamide nucleotides during germination (Table 2), the $(\text{NADP}^+ + \text{NADPH})/(\text{NAD}^+ + \text{NADH})$ ratio is seen to undergo a significant increase during the second phase. Such an increase might be expected with the development of the embryo for, according to Yamamoto (1963), this ratio is low in the storage tissues of a variety of young seedlings but high in the growing parts.

Most of the known UDP-glycosyl compounds are precursors of polysaccharides, the formation of which must be an essential part of growth, especially of cell walls, in higher plants. Thus the increase in concentration of the UDP-X compound of 'peak 5' during the second phase is consistent with the suggested transition of the metabolism of the seed during the late stages of germination and early stages of growth from a system predominantly concerned with energy production to one of biosynthesis and chemical transformation (Brown, 1962, 1963). However, UDP-glucose and UDP-galactose, which have been detected and assayed in pea seeds (Brown, 1962), do not show significant increase in concentration (Table 1) during germination as the term is defined in the present paper, but increases in these compounds have been shown to occur very early in the growth of young seedlings, i.e. immediately after germination (see, e.g., Abdel-Wahab & El-Kinawi, 1960). The increase in UDP-X concen-

tration during the second phase of germination in pea seeds appears to be balanced in part by an accompanying decrease in those of UMP and uridine, particularly the latter (Table 3). This tends to confirm a suggestion (Brown, 1963) that the free nucleosides of mature dry pea seeds represent a reserve supply of corresponding nucleotides required in the late stages of germination and early stages of growth.

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