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# Correlated Changes of some Enzyme Activities and Cofactor and Substrate Contents of Pea Cotyledon Tissue during Germination

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1. The activities of six enzymes (hexokinase, phosphoglucose isomerase, phosphofructokinase, aldolase, glucose 6-phosphate dehydrogenase and amylase) in extracts of pea cotyledons were determined. The activities during the first 10 days after germination showed individual and characteristic changes that indicate a specific control of both synthesis and destruction of enzymes. 2. Tissue contents of glucose, inorganic phosphate, glucose 6-phosphate, fructose 6-phosphate, ATP, ADP, AMP, NAD and NADP were also determined, and a correlation is reported between the substrate concentrations at day 1 and the subsequent enzymic activity. 3. The initial $NAD^+$/NADH ratio value of 1 changed to about 3 by day 4; the NADP content was lower and changes in the oxidation state were less striking. The ratio of ATP to ADP and AMP remained virtually constant.

The tissue content of substrates, cofactors and enzymes is maintained as a consequence of dynamic equilibria that are known to respond to external stimuli by a rapid redistribution of carbon atoms among endogenous metabolite pools and more slowly by changes in enzyme content. During development the dynamic equilibria are perturbed, and the fundamental problem is to understand how a given metabolic stage can evoke the development of a later stage. The release of genetic information is in itself insufficient to explain the timing of events and the quantitative extent of the changes.

In feeding experiments the results are limited by the selection of certain substrates for investigation and by the assumption that the compound selected is taken up unchanged. In the early stages of seed germination only water and respiratory gases are exchanged with the environment. Seed storage tissues are well supplied with endogenous food reserves, and as cell division and enlargement are both completed before dormancy (Varner, 1961) the biochemistry of development can be studied in germination without the complications of mitosis or specialized carbohydrate metabolism associated with cell-wall formation.

There is abundant evidence from the effects of inhibitors of protein synthesis on plant tissues to show that increase in enzyme activity during development is associated with new synthesis, and in the present work preincubation with actinomycin D prevented the increase in glucose 6-phosphate dehydrogenase activity. It thus seems reasonable to suppose that an abundance of endogenous substrate at a given stage of development could result in the synthesis of enzyme and thus modify the pattern of development. In direct support of this Marre, Cornaggia, Alberghina & Bianchetti (1965) showed that slices of germinating castor-bean cotyledon incubated in certain sugars (which included glucose and fructose) showed enhanced kinase activity towards these substrates.

Decrease in activity of an enzyme during development is commonly observed as a decline after a peak value has been attained, but in the present work phosphofructokinase and aldolase activity decreased from the onset of germination. Though other mechanisms might be concerned the most direct interpretation of the loss of enzyme activity would be specific proteolysis. Protein turnover in *Escherichia coli* has been studied by Mandelstam (1960) and Willets (1967), who consider that it has a significant role in development. Specific proteolysis is considered by Schimke, Sweeney & Berlin (1965) to be due to greater resistance of enzyme–substrate complexes to proteolytic attack. If this idea is applicable then a deficiency of substrate might result in net destruction of enzyme, provided that the substrate concentration in the tissue approximated to the dissociation constant of the relevant enzyme–substrate complex, and an abundance of substrate would result in net synthesis.

In the present work determinations of enzyme activity extracted from cotyledon tissue were conducted *in vitro* under conditions of optimum substrate concentration. Cofactor and substrate

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contents were also determined. A preliminary report of this work has been published (Wray & Brown, 1966).

## MATERIALS AND METHODS

*Chemicals.* Glucose 6-phosphate (sodium salt), fructose 1,6-diphosphate (sodium salt), ATP, ADP, AMP, fructose 6-phosphate (barium salt) and $NADP^+$ were obtained from Boehringer Corp. (London) Ltd., London, W. 5. NADH, $NAD^+$ and NADPH were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. [1-$^{14}$C]- and [6-$^{14}$C]-Glucose were obtained from The Radiochemical Centre, Amersham, Bucks. Hyamine hydroxide (1 M in methanol), 2,5-diphenyloxazole, 1,4 - bis - (5 - phenyloxazol - 2 - yl)benzene and naphthalene were obtained from Nuclear Enterprises Ltd., Edinburgh, as scintillation grade. The gift of actinomycin D from Merck, Sharp and Dohme Inc., Rahway, N.J., U.S.A., is gratefully acknowledged. All other reagents were standard commercial preparations.

*Enzymes.* Glucose oxidase test kits and enzymes used in the determinations of hexokinase and phosphofructokinase were obtained from Boehringer Corp. (London) Ltd.

*Germination conditions.* Seeds of *Pisum sativum* var. Meteor were purchased from Sutton and Sons Ltd., Reading. The seeds were grown at 25–30° in moist vermiculite (Vermiculite Products Ltd., Bristol) in glass dishes placed at a window facing north. Daylight was supplemented by a mercury fluorescent light source (Phillips HPLR. 700. 572261. G/93) providing 25000 lux and a 250 w tungsten-filament bulb.

*Tissue extraction for enzyme determinations.* The testa and root–shoot axis were removed from the cotyledons and discarded. The cotyledons (about 2g.) were then weighed and, after preliminary grinding in a chilled mortar, were homogenized in a Potter–Elvehjem homogenizer with 10ml. of cold 20mM-sodium glycylglycine buffer, pH 7·4. The supernatant obtained after centrifuging in an MSE Super-Speed 40 Centrifuge at 100000$g$ for 60min. was used for enzyme assays. Experience with a large number of samples over a period of many months showed that reproducible results were obtained.

*Enzyme assay procedures.* Enzymes were assayed at 25° and at pH 7·4 under optimum conditions.

Amylase was assayed by the method of Bernfeld (1955), which follows the formation of reducing sugar. Starch was omitted from control incubations, and maltose was used for calibration.

Hexokinase was assayed by a method based on that of DiPietro & Weinhouse (1960). The assay medium contained the following final concentrations, in a total volume of 1·5ml.: sodium glycylglycine buffer, pH 7·4 (20mM); $MgCl_2$ (5mM); ATP (5mM); $NADP^+$ (5mM); glucose (100mM); glucose 6-phosphate dehydrogenase (20$\mu$g.). ATP was omitted from the control cuvette. The increase in $E_{340}$ after adding 0·05ml. of extract was followed in a modification (Gilford Instruments) of the Unicam SP. 500 spectrophotometer. The cotyledon supernatant fraction contained 6-phosphogluconate dehydrogenase, so that for every molecule of glucose 6-phosphate formed two molecules of $NADP^+$ were reduced. The validity of this was confirmed by showing that the addition of commercial 6-phosphogluconate dehydrogenase to the assay cuvette did not cause a significant increase in activity.

Phosphoglucose isomerase activity was assayed by a procedure based on that of Ramasarma & Giri (1956) for measuring the production of fructose 6-phosphate. The assay medium contained the following final concentrations, in a total volume of 1·0ml.: sodium glycylglycine buffer, pH 7·4 (20mM); glucose 6-phosphate (15mM) (this concentration of glucose 6-phosphate is suboptimum; see the Results section). After 5min. incubation with 0·1ml. of extract the reaction was stopped by the addition of 1·0ml. of 10% (w/v) trichloroacetic acid. After centrifuging, a 1·0ml. sample was removed for the determination of fructose by the method of Roe (1934). The tissue extract was added to the control incubations after the trichloroacetic acid.

Phosphofructokinase was assayed by a modification of the method of Axelrod, Saltman, Bandurski & Baker (1952). The assay medium contained the following final concentrations, in a total volume of 1·5ml.: sodium glycylglycine buffer, pH 7·4 (20mM); $MgCl_2$ (5mM); NADH (0·1mM); fructose 6-phosphate (20mM); ATP (5mM); aldolase (50$\mu$g.); glycerol 3-phosphate dehydrogenase (5$\mu$g.); triose phosphate isomerase (5$\mu$g.). The decrease in $E_{340}$ after addition of 0·02ml. of extract was followed in a Beckman model DB recording spectrophotometer. All assays were compared with controls from which fructose 6-phosphate was omitted.

Aldolase was assayed by the procedure of Sibley & Lehninger (1949) in which triose phosphate is measured as alkali-labile phosphate. The incubation contained, in a total volume of 2·5ml.: sodium glycylglycine buffer, pH 7·4 (20mM); hydrazine (56mM); fructose 1,6-diphosphate (20mM); 0·1ml. of extract. The incubation was terminated after 30min. by the addition of 1ml. of 20% (w/v) trichloroacetic acid. After centrifugation 1ml. of supernatant was taken for inorganic phosphate determination (Fiske & Subbarow, 1925). Triose phosphate was assayed as phosphate released from 1ml. of deproteinized supernatant by treatment for 20min. at room temperature with 1·2ml. of 2M-NaOH.

Combined glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities were assayed as follows. The assay medium contained the following final concentrations, in a final volume of 1·5ml.: sodium glycylglycine buffer, pH 7·4 (20mM): glucose 6-phosphate (5mM); $MgCl_2$ (5mM); $NADP^+$ (5mM). The increase in $E_{340}$ after addition of 0·02ml. of extract was followed as described above for the hexokinase assay. Glucose 6-phosphate was omitted from the control cuvette.

*Treatment with actinomycin D.* Dry seeds were vacuum-infiltrated with water for 15min. to facilitate removal of the testa. The surface of each cotyledon was then abraded and the cotyledons (with shoot–root axis still attached) were incubated in actinomycin D (50$\mu$g./ml.) for 18hr. at 23° in the dark. The seeds were then germinated in vermiculite under the conditions given above.

*Preparation of tissue slices.* The seeds were divested of the testa and root–shoot axis. The cotyledons were placed on filter paper moistened with 20mM-sodium glycylglycine buffer, pH 7·4, which was placed on a glass plate over ice. The tissue was held in position with filter paper and 1mm. slices were cut freehand with a razor blade.

*Determination of $^{14}CO_2$ production and the C-6/C-1 ratio.* After being sliced, one cotyledon from each pea was incubated with 25$\mu$moles of [1-$^{14}$C]glucose (150000 dis-

integrations/min.) and the other cotyledon with 25 μmoles of [6-$^{14}$C]glucose (130000 disintegrations/min.) in Warburg flasks for 3hr. at 25°. Each flask contained a total of four cotyledons. The centre well contained 0·2ml. of 1 M-Hyamine hydroxide in methanol. At the end of the incubation period the Hyamine solution was transferred by pipette into a vial of scintillation fluid and counted (MacLennan, Beevers & Harley, 1963) in a Nuclear–Chicago model 725 liquid-scintillation counter. Counts were corrected for quenching to 100% efficiency.

Total $CO_2$ production was similarly measured with cotyledon slices incubated in distilled water.

*Determination of starch.* This was carried out by the method of Nielson & Gleason (1945); starch prepared from 1-day-old cotyledons by the method of Nielson (1943) was used as a standard.

*Determination of dry weight.* This was done by drying 1 mm. cotyledon slices at 97–105° for 24 hr.

*Tissue extraction for metabolite determinations.* After rapid removal of the root–shoot axis and testa, the cotyledons were weighed in ice-cold 0·3 M-$HClO_4$, and were then ground in a mortar with more ice-cold acid and acid-washed silver sand. The resultant brei was centrifuged at 30000 g for 10 min. The residue was washed twice with 0·15 M-$HClO_4$, and the combined extracts were neutralized to pH 5·5 with 4 M-KOH. Determinations of metabolites (inorganic phosphate, glucose 6-phosphate, fructose 6-phosphate, ATP, ADP, AMP and glucose) were performed immediately.

Extraction of $NAD^+$ and $NADP^+$ was conducted as above but with 1 M-$HClO_4$. Determinations were carried out immediately on the neutralized extracts.

Extraction of NADH and NADPH was performed with 0·1 M-NaOH. The combined extracts were heated for 1 min. in boiling water to destroy the oxidized coenzymes and, after cooling, were neutralized to pH 8·5 with 1 M-potassium phosphate. Determinations were carried out immediately.

*Metabolite determinations.* Glucose was assayed with glucose oxidase and o-dianisidine (Huggett & Nixon, 1957).

Glucose 6-phosphate was assayed spectrophotometrically by the method of Barker, Jakes, Solomos, Younis & Isherwood (1964).

Fructose 6-phosphate was determined by difference, the glucose 6-phosphate content of the extract being assayed (as above) in the presence and absence of 5 μg. of phosphoglucose isomerase.

ATP was assayed by the method of Lamprecht & Trautschold (1963). The assay system contained the following, in a final volume of 3 ml.: sodium glycylglycine buffer, pH 7·4 (80 mM); $MgCl_2$ (10 mM); $NADP^+$ (1 mM); glucose (100 mM); 1·5 ml. of neutralized extract. Addition of glucose 6-phosphate dehydrogenase (20 μg.) caused a change in $E_{340}$ corresponding to the glucose 6-phosphate content of the extract. When $E_{340}$ was constant hexokinase (20 μg.) was added, and the resultant change in $E_{340}$ due to the ATP measured.

The assay system for ADP and AMP contained the following, in a final volume of 3 ml.: 0·5 M-triethanolamine–HCl buffer, pH 7·4 (0·5 ml.); 10 mM-NADH (0·1 ml.); 0·1 ml. of a solution containing 10 mM-phosphoenolpyruvate, 1·3 M-KCl and 0·4 M-$MgSO_4$; neutralized extract (2 ml.); 20 μg. of lactate dehydrogenase. When $E_{340}$ was constant, 20 μg. of pyruvate kinase was added. The subsequent decrease in $E_{340}$ was due to ADP. When the decrease had ceased, 20 μg. of myokinase (adenylate kinase) was added to give a further decrease in $E_{340}$ due to AMP.

NAD, NADP, NADH and NADPH were assayed by the methods of Slater, Sawyer & Sträuli (1964).

## RESULTS

In Fig. 1 the values obtained for hexokinase (EC 2.7.1.1), aldolase (EC 4.1.2.7) and the combined glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) activities are plotted against the time in days from sowing. Similarly in Fig. 2 the values are given for phosphofructokinase (EC 2.7.1.11), phosphoglucose isomerase (EC 5.3.1.9) and also

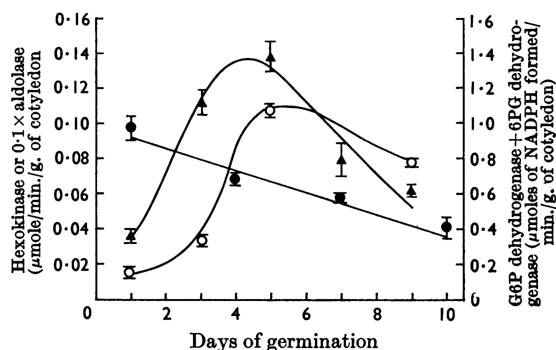


Fig. 1. Hexokinase (○), aldolase (●) and combined glucose 6-phosphate (G6P) dehydrogenase and 6-phosphogluconate (6PG) dehydrogenase (▲) activities of the cotyledon supernatant during germination. Each point represents the mean of three or more determinations. The vertical bar denotes ± S.E.M. when large enough to record.

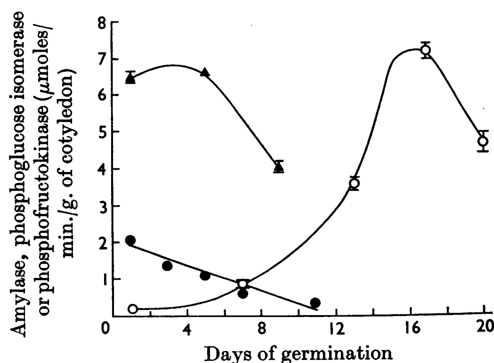


Fig. 2. Amylase (○), phosphoglucose isomerase (▲) and phosphofructokinase (●) activities of the cotyledon supernatant during germination. Each point represents the mean of three or more determinations. The vertical bar denotes ± S.E.M. when large enough to record.

Table 1. *Effect of substrate concentrations on pea cotyledon phosphofructokinase activity*

The enzyme was assayed by the spectrophotometric method, measuring the consumption of NADH. Details are given in the Materials and Methods section.

| Concn. of fructose 6-phosphate (mM) | Concn. of ATP (mM) | Rate ($\mu$mole/min.) |
|---|---|---|
| 1·5 | 1* | 0·155 |
| | 5 | 0·120 |
| 20 | 4* | 0·325 |
| | 10 | 0·28 |

* These ATP concentrations gave the maximum rates attainable for these given concentrations of fructose 6-phosphate.

amylase. [Amylase activity in the pea cotyledon is predominately $\alpha$-amylase (EC 3.2.1.1), according to Swain & Dekker (1966).]

Saturating substrate and cofactor concentrations were employed except with phosphoglucose isomerase, the substrate affinity of which is very low (the $K_m$ for glucose 6-phosphate was found to be 17mM). For standard assays of this enzyme 15mM-glucose 6-phosphate was adopted.

Dehydrogenase activity was determined with glucose 6-phosphate and $NADP^+$ as substrates. The results are presented as $\mu$moles of NADPH formed; it is assumed on the basis of tests in which commercial 6-phosphogluconate dehydrogenase was added that this enzyme was never present in limiting amounts in the cotyledon extracts, i.e. in a given assay half the NADPH formed reflected the glucose 6-phosphate dehydrogenase activity.

For assays of phosphofructokinase the conditions for pea seeds of Axelrod *et al.* (1952) of 5mM-ATP and 20mM-fructose 6-phosphate were tested and found to be suitable. Inhibition by ATP could be demonstrated (Table 1) and was relieved by an increased concentration of fructose 6-phosphate. Dennis & Coultate (1966) have also observed ATP inhibition of phosphofructokinase from a plant tissue (carrot root), which was relieved by inorganic phosphate but not by ADP or AMP. They also found inhibition by citrate, which was not investigated here.

The results of these assays have been presented on the basis of fresh weight of cotyledon. To permit comparisons with other published information the fresh weight, dry weight, protein content and starch content per pair of cotyledons are given in Fig. 3 as a function of time in days. Until day 5 the plumule of the young seedling is surmounted by unexpanded leaves held tightly in a bud with only a small area exposed to light, and by day 8 some expansion occurs and the leaves unfold. This

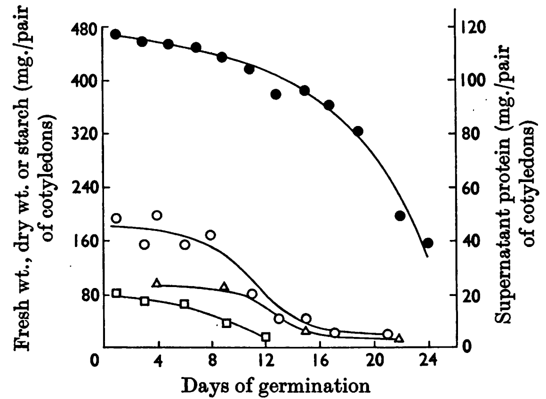


Fig. 3. Changes in the fresh weight (●), dry weight (○), starch (△) and supernatant protein (□) during germination of the cotyledon pair.

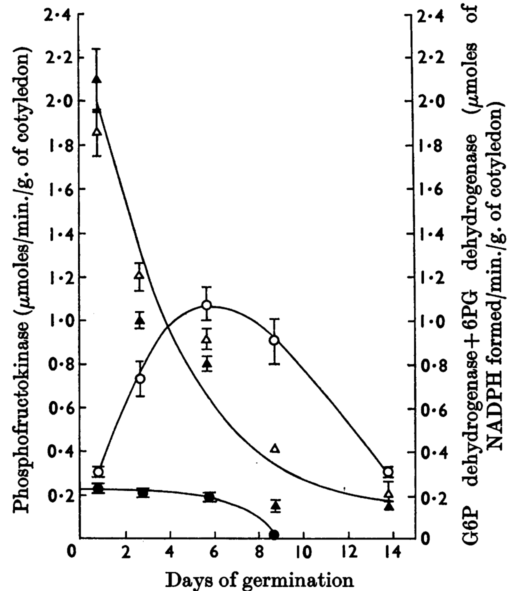


Fig. 4. Effect of presoaking in actinomycin D on the phosphofructokinase and combined glucose 6-phosphate (G6P) dehydrogenase and 6-phosphogluconate (6PG) dehydrogenase activities of the cotyledon supernatant during germination. The cotyledons with the root–shoot axis still attached were soaked for 18hr. in darkness in actinomycin D (50$\mu$g./ml.) before being planted out in vermiculite. Control tissue was presoaked in distilled water for 18hr. before planting. Phosphofructokinase activity: control (△); actinomycin D-treated (▲); combined glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities: control (○); actinomycin D-treated (●). Each point represents the mean of from three to six determinations. The vertical bar denotes ±S.E.M. when this is large enough to record.

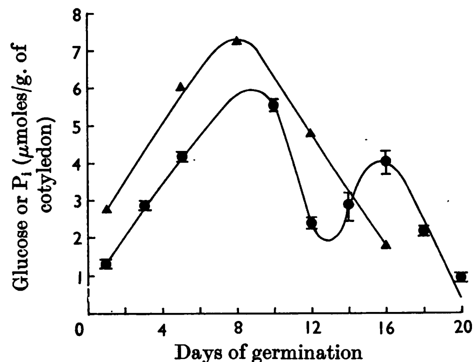


Fig. 5. Glucose (●) and inorganic phosphate (▲) contents of the cotyledon during germination. Each point represents the mean of two determinations. The vertical bar denotes ± S.E.M. where this is large enough to record.

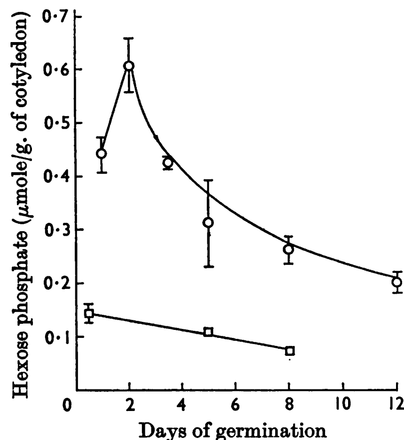


Fig. 6. Glucose 6-phosphate (○) and fructose 6-phosphate (□) contents of the cotyledon during germination. Each point represents the mean of from two to four determinations. The vertical bar denotes ± S.E.M. when this is large enough to record.

process is complete in a few hours and may be taken as the start of significant photosynthetic contribution to the carbohydrate balance of the seedling.

In Fig. 4 the effect of preincubating seeds in actinomycin D on the development of the dehydrogenase activity towards glucose 6-phosphate is shown, with the effect on the phosphofructokinase content serving as a comparison. The results are consistent with the hypothesis that DNA-directed new synthesis of dehydrogenase enzyme is responsible for the enhanced activity observed in the untreated tissue. The effect on the phosphofructokinase activity was very slight, and probably not significant. The downturn in dehydrogenase activity occurred at the same time in actinomycin D-treated tissue as in the untreated material. The onset of this change is thus probably not due to a concurrent DNA-directed synthesis of proteolytic enzyme. The response to actinomycin D suggests that protein molecules in the cotyledons may not turn over rapidly.

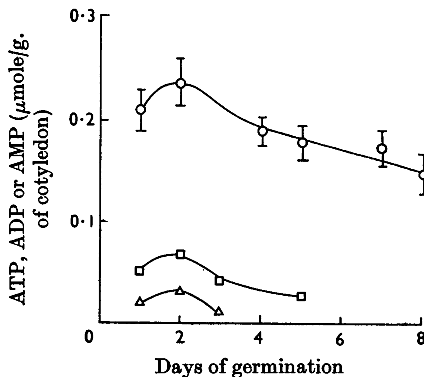


Fig. 7. ATP (○), ADP (△) and AMP (□) contents of the cotyledon during germination. Each ATP point represents the mean of from two to four determinations. Each ADP point represents the mean of from either two (3 day), four (2 day) or seven (1 day) determinations. Each AMP point represents the mean of either two (3 and 5 day), four (2 day) or seven (1 day) determinations. The vertical bar denotes ± S.E.M. when this is large enough to record.

The glucose and inorganic phosphate contents of the tissue are plotted in Fig. 5. Both these compounds were present in substantial amounts throughout the period of investigation, and showed a maximal level at about day 8. The glucose content also showed a subsidiary peak at approximately day 16, coincident with the high amylase activity shown in Fig. 2.

The glucose 6-phosphate and fructose 6-phosphate contents are shown in Fig. 6. The initial rise in glucose 6-phosphate content indicates that during this period glucose and ATP must be accessible to hexokinase, and possibly promote synthesis of this enzyme. Subsequently the glucose content rises and the glucose 6-phosphate content falls, which led us to consider the tissue ATP content.

The enzymes and substrates of the glycolytic sequence are generally regarded as occurring in the soluble phase of the cell, but the cofactors are known to occur in the mitochondria as well. The extraction technique adopted here is believed to be sufficiently vigorous to disrupt mitochondria and other organelles, so that Fig. 7 shows the total

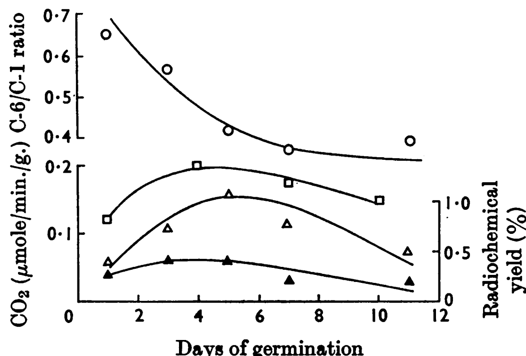


Fig. 8. Radiochemical yield of $^{14}CO_2$ released by cotyledon slices during germination. Cotyledon slices were incubated for 3hr. with either [1-$^{14}$C]glucose (△) or [6-$^{14}$C]glucose (▲) and the radiochemical yields were plotted. The ratio of radiochemical yields, C-6/C-1, was also plotted. Each point represents the mean of three determinations. The respiratory rate of cotyledon slices, as represented by the total $CO_2$ output in µmole/min./g. of tissue, was also measured and plotted (□).

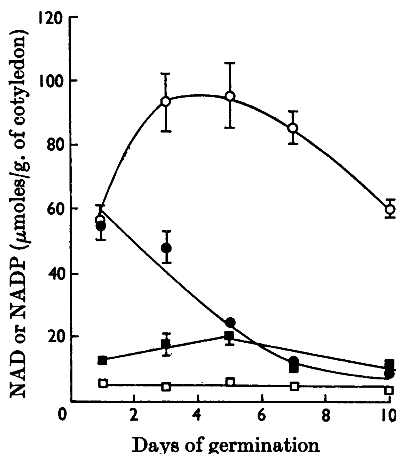


Fig. 9. $NAD^+$ (○), NADH (●), NADP (□) and NADPH (■) contents of the cotyledon during germination. Each point represents the mean of from three to nine determinations. The vertical bar denotes ±S.E.M. when this is large enough to record.

ATP, ADP and AMP of the tissue. There is no evidence to suggest a marked shift in the ratio of ATP to ADP or AMP, so that the kinetic parameters of enzymes susceptible to activation or inhibition by particular adenine nucleotides are probably not dramatically altered during this 8-day period.

The initial rise in the adenosine nucleotide contents during the first 2 days coincides with the increase in glucose 6-phosphate, and is attributed to synthesis. Subsequently the amount falls, so that by day 8 the ATP content is approx. 70% of the initial value. Such a fall could have a significant effect on the rate of amino acid incorporation into proteins (Villa-Trevino, Shull & Farber, 1963).

Gibbs & Beevers (1955) have pointed out that in young plant tissues the pentose phosphate-pathway contribution to glucose catabolism is always small, but may increase substantially with age; Wang, Doyle & Ramsey (1960) conjectured that in tomato fruit at ripeness possibly as much as 27% of the glucose catabolism occurs by this pathway. The simple method of Bloom & Stetten (1953) was used to investigate the effects of [1-$^{14}$C]glucose and [6-$^{14}$C]glucose on $^{14}CO_2$ production in pea cotyledons, though this has been shown to underestimate the pentose pathway contribution when this is greater than 5–10% (Wood, Katz & Landau, 1963). The results obtained with 1mm.-thick slices of cotyledon tissue are presented in Fig. 8. The C-6/C-1 ratio decreased rapidly from day 1 to day 5, but then more slowly until determinations ceased at day 12. The change indicates an increasing dependence on the pentose phosphate pathway in accord with the generalization above, and suggests that by day 5 an appreciable part of glucose catabolism is by this route. The $K_m$ of phosphoglucose isomerase (17mM) is much higher than that for glucose 6-phosphate dehydrogenase (0·6mM), so that in competing for substrate the dehydrogenase is favoured. The pentose phosphate-pathway activity is often limited by the availability of $NADP^+$, and can generally be increased by the addition of a reducible dyestuff to reoxidize the NADPH formed. This can be demonstrated in pea cotyledon slices (A. P. Brown & R. Gingell, unpublished work).

Fig. 9 shows the tissue contents of NAD and NADP, in both oxidized and reduced states. The results how an initial increase in total NADP; the $NADP^+$/NADPH ratio fell until day 5 and then slowly rose. The evidence suggests that development of the pentose phosphate pathway might perhaps be correlated with the NADPH content.

The initial $NAD^+$/NADH ratio was about unity, and increased to about 3·2 by day 4. Yamamoto (1963) has shown previously that the NAD and NADP contents of plant tissues are subject to considerable change during development, and the redox state of these cofactors may change widely from the ratios found in animal tissues generally. A low $NAD^+$/NADH ratio in the early stages of pea germination is compatible with the accumulation of ethanol (Cossins & Turner, 1959; Cossins, 1962) and lactate (Cossins, 1964) at this time.

Table 2. *Relation of observed tissue substrate and cofactor concentrations to $K_m$ values of the enzymes*

The ratio of tissue concentration to $K_m$ in column 4 is compared with the observed change in enzyme activity (day 1–day 5) in column 5. The values are derived from the data of Figs. 1–9. References: [a] Saltman (1953), enzyme from wheat germ; [b] present work; [c] Glaser & Brown (1955), enzyme from yeast; [d] Axelrod *et al.* (1952), enzyme from pea seeds.

| Enzyme and reactant | $K_m$ (mM) | Tissue concn. at day 1 ($\mu$moles/g. of water) | Ratio: $\frac{\text{tissue concn.}}{K_m}$ | Change in enzyme activity (day 1–day 5) |
|---|---|---|---|---|
| Hexokinase | | | | 6·9× |
| Glucose | 0·44[a] | 2·05 | 4·65 | |
| ATP | 0·87[b] | 0·33 | 0·38 | |
| Glucose 6-phosphate dehydrogenase | | | | 3·75× |
| Glucose 6-phosphate | 0·6[b] | 0·69 | 1·15 | |
| NADP | 0·02[c] | 0·009 | 0·48 | |
| Phosphoglucose isomerase | | | | 1·08× |
| Glucose 6-phosphate | 17[b] | 0·69 | 0·04 | |
| Phosphofructokinase | | | | 0·5× |
| Fructose 6-phosphate | 7·1[d] | 0·22 | 0·03 | |
| ATP | 2·4[e] | 0·33 | 0·014 | |

## DISCUSSION

In the past attempts have been made to interpret tissue metabolism and development in terms of tissue enzyme activity, but this may change (e.g. as a result of new synthesis) without changing the metabolic rate. Thus the control of metabolism during development in some cases at least does not lie in enzyme activity, even for enzymes commonly regarded as potentially rate-limiting. Hess (1963) related the endogenous substrate pools of tissue at rest to equilibrium constants for the reactions considered, and he demonstrated that metabolic reactions *in vivo* may be driven by substrate concentration. This prepares the ground for a change in viewpoint from enzyme activity to substrate pool size as possibly a controlling influence in tissue development.

In determining substrate concentrations errors may arise as a result of compartmentation in the tissue. However, estimates made from substrate and cofactor determinations and the tissue water content (taken as the difference between fresh weight and dry weight) are presented in Table 2, and may be compared with $K_m$ values of the appropriate enzymes from pea cotyledon, supplemented where necessary with reported values for other plant tissues.

As often happens, some of the substrate concentrations *in vivo* calculated in the present work are lower than the $K_m$ values, which implies that the enzymes may operate at lower efficiency in the tissue than in experimental assays. This is also seen by comparing the enzyme content of the tissue with the metabolic rate; for example at day 1 the tissue content of glucose 6-phosphate dehydrogenase is sufficient to account for all the carbon dioxide respired being derived from pentose phosphate pathway activity, which is clearly not the case.

Table 2 suggests a correlation between the degree of substrate saturation *in vivo* and the change in enzyme activity during germination. This may perhaps be due to enzyme–substrate complexes being protected from destruction, e.g. by proteolytic attack, acting in conjunction with the promotion of enzyme synthesis by free substrate. Whether this or some other interpretation is appropriate the evidence suggests that substrate and cofactor concentrations may perhaps influence the enzyme activities subsequently developed.

The falling content of adenine nucleotides is the only metabolic indication of incipient senescence observed during this period of germination.

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