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PYRIMIDINE METABOLISM IN GERMINATING SEEDLINGS[1, 2, 3]

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In the course of an inquiry into the biochemical events associated with germination and dormancy in seeds an attempt was made to assess the turnover or new production of ribonucleic acid and deoxyribonucleic acid in germinating rape seeds. The use of uracil and thymine, both labelled in the 2-position with C^{14} has disclosed that at least in the early stages of germination, these two pyrimidines, the first of which is found in ribonucleic acid and the second in deoxyribonucleic acid, are only sparingly incorporated into polynucleotides although the bases are rapidly decomposed by a sequence of events suggestive of that found in higher animals.

MATERIALS AND METHODS

CHEMICALS USED: Uracil and thymine were obtained from the Nutritional Biochemical Corp., dihydrouracil (DHU)[4] and dihydrothymine (DHT) from the California Corp. for Biochemical Research and were used without further purification. β-Ureidopropionic acid (BUP) and β-ureidoisobutyric acid (BUIB) were made from DHU and DHT, respectively, by alkaline hydrolysis (10). (mp of BUP found, 170–171° C; reported 169–170° C; mp of BUIB, found, 114–116° C, reported, 116–119° C (10). Uracil-2-C^{14} (0.0263 mc/mg) and thymine-2-C^{14} (0.011 mc/mg) were purchased from the New England Nuclear Corp.

GERMINATION OF SEED: The rape seeds (*Brassica napus* L., var. Dwarf Essex) were obtained from the Crawfordsville Seed Co., Crawfordsville, Ind. Germination was carried out in small cylindrical dishes (1 cm high × 1 cm dia.). A disc of Whatman No. 1 filter paper was inserted into each dish. Fifty μliters of a 0.01 % solution of either uracil-2-C^{14} or thymine-2-C^{14} were pipetted into each dish. Five

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[4] The following abbreviations are used throughout: BUIP, β-ureidoisobutyric acid; BUP, β-ureidopropionic acid; DHT, dihydrothymine; DHU, dihydrouracil.

seeds were placed in each dish which was then covered with an inverted dish of a slightly larger diameter. The dishes were supported on a wire gauze in a covered 10 cm petri dish. Sufficient water was present in the petri dish to insure a high relative humidity throughout the germination period. Germinations were carried out in the dark at an average temperature of 25° C. The filter paper disc was omitted from the germination dishes in experiments in which it was desired to determine the unabsorbed radioactivity.

When seeds were germinated under N_2 the small germination dishes were placed into Thunberg tubes, in which they were supported on small vials containing 0.4 ml of water. The Thunberg tubes were alternately evacuated and filled with N_2 to insure exclusion of air.

Determination of Activity Incorporated: The seedlings were removed from the germination dishes and thoroughly rinsed with distilled water to wash off any radioactive material adhering to their surfaces. They were then homogenized in ice-cold 80 % ethanol with a Potter-Elvehjem glass homogenizer and made up to known volumes. Aliquots of the suspension were transferred to aluminum planchets containing 0.1 ml of a 0.1 % aqueous agar suspension. The homogenate and agar suspension were thoroughly mixed with a fine wire. To obtain reproducible geometry for radio-isotope measurement the sample was carefully spread over a previously inscribed circle on the planchet and dried under an infra-red lamp. In all the samples counted the weight of the plated material was less than 1 mg/cm^2. Counting was done in a windowless gas-flow counter.

When the amount of unabsorbed activity was to be determined, the rinse water from the seedlings was collected and combined with the rinse water from the germination dishes and aliquots were counted.

Chromatography: The homogenates of the seedlings were centrifuged at 680 × *g* for 15 minutes. The supernate was removed by decantation, concentrated in a stream of filtered air, and chromatographed. Chromatography was carried out on Whatman No. 1 paper in cylindrical glass jars (30 cm high × 15 cm dia) in an ascending direction. Development was allowed to proceed for 18 hours using the solvents described by Fink et al (7). The developed chromatograms were dried and scanned for radioactivity using the Forro scanning device in conjunction with a recording rate-meter. Pyrimidines, dihydropyrimidines and β-ureido acids were detected by the procedures of Fink et al (9). Nucleic acid hydrolyzates were neutralized with solid K_2CO_3 and after removing the $KClO_4$ by centrifugation, were concentrated and chromatographed using solvent system I of Kirby (15).

Isolation of Nucleic Acids From Seedling Extracts: The homogenates of the seedlings were fractionated according to the method of Ogur and Rosen (16). The combined nucleic acids were extracted and hydrolyzed to their constituent bases as described by Basler and Commoner (3). Preliminary experiments indicated virtually complete recovery of nucleic acids from the tissue by this procedure.

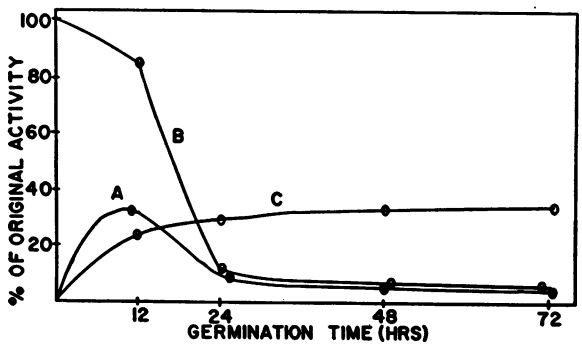
Results

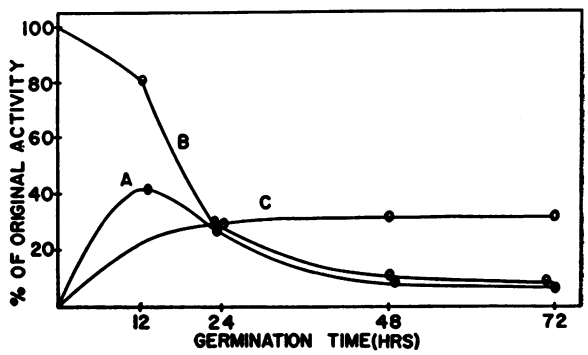
When rape seeds were germinated in solutions of uracil-2-C^{14} or thymine-2-C^{14}, radioactivity appeared in the seedlings reaching a maximum, and then decreasing. Some typical results are shown in figures 1 and 2. That the later decrease was not caused by loss of pyrimidine or radioactive metabolites by leaching is clear from the fact that radioactivity remaining in the germination vessel also declined. It is noteworthy that an appreciable amount of radioactivity from both thymine and uracil was incorporated into the seeds after only 6 hours of contact; the primary shoot was not observed to emerge until after 12 hours of germination. From these experiments and others it is apparent that absorption of the radioactive pyrimidines can occur prior to breaking the testa. With increasing time the activity present in the unabsorbed fraction decreased until it had virtually disappeared. Ultimately about 90 % or more of the original activity was lost; the remainder was found in the seedlings.

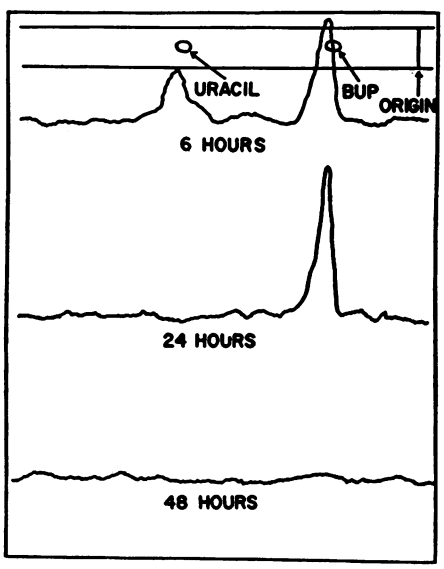
Parallel experiments carried out in the presence of NaOH to trap the CO_2 showed that the dissipated radioactivity was being evolved as $C^{14}O_2$. Thus when ten seeds were germinated for 48 hours in 0.2 ml of a 0.01 % solution of uracil-2-C^{14}, 45 % of the original activity was recovered as carbonate. In a similar experiment with 0.2 ml of a 0.015 % solution of thymine-2-C^{14}, 44 % of the activity was recovered.

Employing solvent 2 (7), chromatography of the extracts obtained from seedlings grown, for various periods in uracil-2-C^{14}, resulted in the appearance of two radioactive regions (fig 3). One of the regions coincided with BUP, while the other corresponded with both uracil and DHU which are not well resolved by this solvent. However, by using solvents 1 and 6 (7), which gives an excellent resolution of these substances, it was found that DHU-C^{14} as well as unreacted uracil-C^{14} were present in seedlings germinated for 12 hours (fig 4). Similarly with seeds which were germinated in thymine-2-C^{14}, solvent 2 (8) resolved the radioactive substance into two areas, thymine-DHT and BUIB-C^{14} (fig 5). Application of solvents 1 and 6 (7) showed the thymine-DHT region contained both thymine-C^{14} and DHT-C^{14} when the extract was obtained from seedlings germinated in thymine-2-C^{14} for 12 hours in air (fig 6).

Although large fractions of the bases were catabolized, some incorporation into nucleic acid occurred. Thus when 15 seedlings obtained by germination on H_2O for 72 hours were transferred to 0.75 ml of a 0.01 % aqueous solution of uracil-2-C^{14} for 24 hours, approximately one percent of the radioactivity was found in nucleic acid. This represented 32 % of the total activity in the homogenate. Radio-







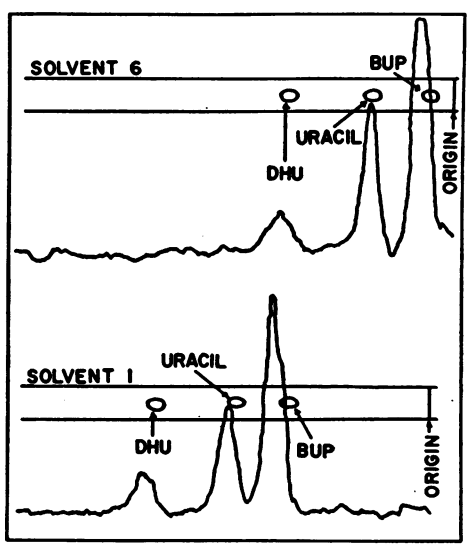
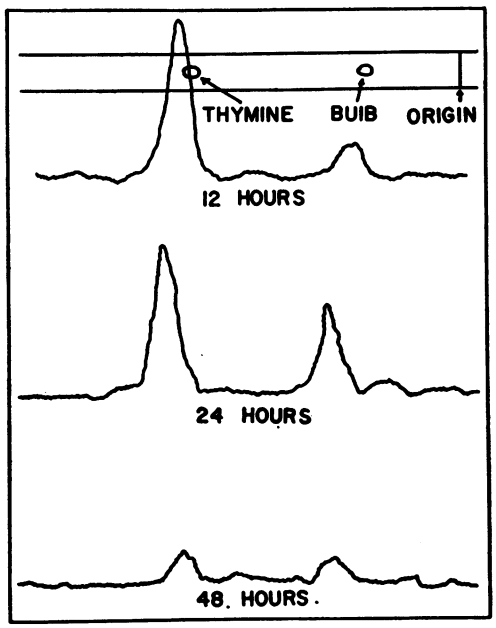


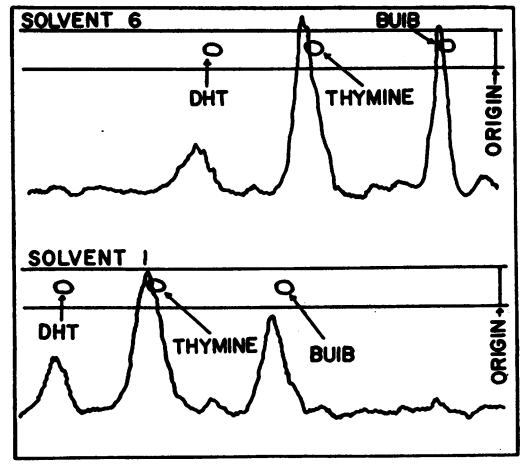
FIG. 1 (*upper left*). Time course of incorporation of radioactivity from uracil-2-C^{14} into germinating rape seeds. Radioactivities are expressed as percent of the total activity originally added to the germination vessel. A: Radioactivity found in seedlings (germination in air). B: Radioactivity remaining in germination vessel (germination in air). C: Radioactivity found in seedlings (germination in N_2).

FIG. 2 (*upper right*). Time course of incorporation of radioactivity from thymine-2-C^{14} into germinating rape seeds. Radioactivities are expressed as percent of the total activity added to the germination vessel. A: Radioactivity found in seedlings (germination in air). B: Radioactivity remaining in germination vessel (germination in air). C: Radioactivity found in seedlings (germination in N_2).

FIG. 3 (*lower left*). Radio-isotope scannings of chromatograms of supernates from homogenates obtained from seeds germinated for 6, 24, and 48 hours in uracil-2-C^{14}; solvent 2 (7).

FIG. 4 (*lower right*). Radio-isotope scanning of chromatograms of supernates of homogenates from seedlings germinated for 12 hours in uracil-2-C^{14}. *Upper figure,* solvent 6; *lower figure,* solvent 1, (7).





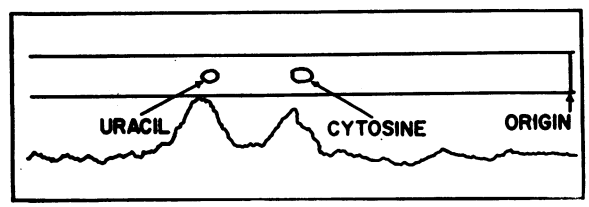


FIG. 5 (*upper left*). Radio-isotope scanning of chromatograms of supernates of homogenates from seedlings germinated for 12, 14, and 48 hours in thymine-2-C^{14}; solvent 2 (7).

FIG. 6 (*upper right*). Radio-isotope scanning of chromatograms of supernates of homogenates from seedlings germinated for 24 hours in thymine-2-C^{14}; solvent 1 and 6 (7).

FIG. 7 (*lower right*). Radio-isotope scanning of chromatograms of the hydrolyzed nucleic acids from seedlings germinated for 72 hours in water followed by 24 hours in uracil-2-C^{14}; solvent 1 (15).

active uracil and radioactive cytosine were identified among the hydrolytic products (fig 7). The formation of cytosine from uracil at the nucleotide level is well known to occur in other organisms. The ratio of counts in uracil to cytosine was approximately 1:1 indicating a substantial conversion. In these experiments no effort was made to separate the DNA from the RNA. When 72 hour-old seedlings were transferred to a 0.01 % solution of thymine-2-C^{14} for 24 hours radioactive nucleic acid was obtained but the low level of activity did not permit the identification of radioactive bases present in the hydrolytic products after chromatography.

DISCUSSION

The production of $C^{14}O_2$ β-ureidopropionic acid and dihydrouracil from uracil-2-C^{14} by germinating rape seeds strongly suggests the following pathway is involved:

Uracil → dihydrouracil → β-ureidopropionate → β-alanine + CO_2 + NH_3

Similarly the formation of $C^{14}O_2$, dihydrothymine and β-ureidoisobutyrate from thymine-2-C^{14} suggests operation of the following catabolic scheme:

Thymine → dihydrothymine → β-ureidoisobutyrate → β-aminoisobutyrate + CO_2 + NH_3

Apparently the catabolic pathways in germinating rape seeds are not unlike those reported in higher animals and in some bacteria (4, 5, 8, 9, 11, 12, 13, 19). In the experiments described here, one would not expect to find labeled β-alanine or β-aminoisobutyrate owing to the loss of the C^{14}-labeled C-2 atom as CO_2. Attempts to demonstrate the formation of these compounds from non-radioactive uracil and thymine have not been successful apparently because of the relatively low enzyme activities in the seedlings.

The presence of these catabolic sequences is consistent with the established decrease of RNA phosphorus in seeds during germination (17).

These reactions are the only ones known to occur in higher plants which can account for the formation of β-aminoisobutyrate which has been found in iris (2). β-Alanine in higher plants (14) could also arise from these reactions, although both squash (18) and various leguminous seeds (1) have been reported to catalyze the evolution of CO_2 from L-aspartate. However, it remains to be shown that in these cases the reaction is caused by an aspartic acid decarboxylase.

Note that degradation of uracil and thymine involve only a hydrogenation step followed by hydrolytic steps. It is therefore interesting that the exclusion of oxygen from germinating seeds represses the catabolism of these substrates. This behavior

may be a secondary effect resulting from the overall inhibition of germination brought about by anaerobiosis and the consequent inhibition of developmental changes such as formation of new enzymes or coenzymes, alterations in subcellular structure, or permeability changes needed to permit the substrate to come in contact with the enzyme.

As far as we know the individual enzymes concerned with each of the steps shown here have not been demonstrated in higher plants. The isolation of the enzymic reactions is under way in our laboratory. Wallach and Grisolia have purified a hydrase from rat liver which attacks dihydrothymine, dihydrouracil, and hydantoin (19). They have suggested that the hydantoin peptidase discovered by Eadie et al (6) some years ago in seeds may also act on the dihydropyrimidines. In a study to be reported later we have partially purified a preparation of dihydrouracil hydrase from pea seeds which also opens the rings of dihydrothymine and hydantoin, but the results thus far do not permit a decision as to whether or not these activities reside in a single enzyme.

The ability of the seedlings to incorporate uracil into nucleic acid suggests, by analogy with other organisms, the action of a nucleoside phosphorylase to produce a nucleoside which is then converted by the action of a nucleoside kinase to a 5′-nucleotide. This in turn would be converted to the di- or triphosphate. It is interesting, but not unexpected, that uracil can serve as a precursor of cytosine.

Summary

I. Germinating rape seeds rapidly absorb and decompose added uracil and thymine.

II. Among the catabolic products, arising from these bases in addition to CO_2, are dihydrouracil and β-ureidopropionic acid from uracil, and dihydrothymine and β-ureidoisobutyric acid from thymine.

III. In addition to these catabolic reactions, incorporation of the pyrimidines into nucleic acids can be shown.

IV. When uracil is incorporated into nucleic acid it gives rise to both uracil and cytosine.

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