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# Some aspects of pyrimidine nucleotide synthesis in excised pea plants

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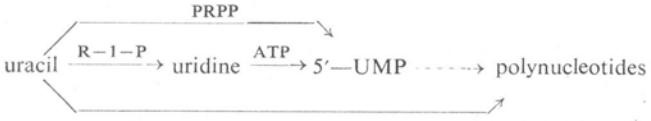
In previous studies we have established that uracil and uridine independent of the orotic acid path may be precursors of pyrimidine nucleotides in higher plants (Wasilewska and Reifer 1967a, b). We have postulated that synthesis of RNA pyrimidines from uracil may proceed on 3 alternative paths. Two of them would lead to the formation of 5′-UMP (by ribosidation of uracil, followed by phosphorylation of the produced uridine, or by direct ribotidation) and the third leading directly to the formation of polynucleotides. Scheme 1 illustrates the 3 alternative paths:




Results previously reported concerning anabolic changes of uracil to 5′-UMP have indicated a direct relation between the endogenous content of uridine in plant material and the path of synthesis observed. Namely, when the content of uridine was relatively high (0.3—0.5 μmoles/g of fresh weight) then the two-step synthesis of 5′-UMP dominated. On the other hand when the content of uridine was low (0.07 or less μmoles/g of fresh weight) then predominance of direct ribotidation has been observed (Wasilewska and Reifer 1967 a). Postulating that the content of uridine may be a regulating factor for the path of uracil anabolism, we have assumed that the diurnal rhythm may be responsible for the changing content of uridine in plant material. We have therefore studied the influence of the time of day on the quantities of endogenous pyrimidines and on the anabolism of $^{14}$C-uracil in young excised pea plants. At the same time we have investigated the influence of glucose on the metabolism of uracil, uridine and orotic acid. In addition, basing on our preliminary reports that the concentration of uracil has a distinct effect on its anabolism (Buchowicz et al 1963) we have studied the influence of uracil concentration fed into the plant on the direction and the rate of anabolic changes of this pyrimidine base.

## MATERIAL and METHODS

Reagents. 2-$^{14}$C-uracil: Philips-Duphar, Amsterdam, Netherland; 2-$^{14}$C-uridine and 6-$^{14}$C-orotic acid: The Radiochemical Centre, Amersham, England; uracil, uridine, orotic acid, 5′-UMP, 5′-CMP, 2′(3′)-UMP and 2′(3′)-CMP: Nutritional

Bioch. Corp. Ohio, USA. All other reagents were of Polish production, distributed by Biuro Obrotu Odczynnikami, Gliwice, Poland.

Plant material, feeding and experimental procedure. Young 14-day old pea plants, variety Perła Szlachetna were used. The plants were grown, excised and starved for 36 hours as previously described (Wasilewska and Reifer 1967 c). In experiments where the influence of glucose was to be determined, the excised plants were immersed with their cut off ends in a 0.1 M glucose solution at 37° in light or darkness for 36 hours. Uninjured, whole plants were etiolated for 36 hours at 37° and cut off just before use. Control samples from the same harvest were also cut off just before use. Feeding, analytical procedure, quantitative determinations of pyrimidine derivatives and their specific activities were carried out as already described (Wasilewska and Reifer 1967 a, b). In some cases 5′-UMP and 5′-CMP were determined as sum of the mono-, di-, triphosphates and their derivatives after hydrolysis in 0.3 N $HClO_4$ for 15 minutes in a boiling water bath.

## RESULTS and DISCUSSION

Figure 1 illustrates the influence of time of day upon the intensity of $^{14}C$-uracil incorporation into pyrimidine derivatives of starved, etiolated and control plants respectively. Plants were fed at 7 a.m. 11 a.m., 3 p.m. and 7 p.m. Highest specific activity of the pyrimidine derivatives was found invariably in plants fed at 11 a.m. Comparing the starved and etiolated plants against the controls, it can be seen that uracil was utilised with highest intensity in the starved plants. Unexpectedly the etiolated plants have incorporated uracil at a much lower rate.

It should be noted that we succeeded for the first time to isolate cytidine from pea plants (0.03—0.05 μmole/g of fresh weight) with specific activity equal to the one of uridine.

No relation between the diurnal rhythm and quantities of endogenous uridine could be detected and consequently no differences in the biosynthetic path could be observed. The content of uridine was very high right through (0.6—0.8 μmole/g) irrespective of time of feeding or pre-treatment prior to feeding. The specific activity of uridine was considerably higher than in 5′-UMP, which was in agreement with our previous results (Wasilewska and Reifer 1967 a).

The observed dependence of intensity of uracil metabolism on the time of feeding may be due to diurnal fluctuations in activities of the enzymes responsible for the utilisation of the base in the synthesis of RNA pyrimidines. The influence of the diurnal rhythm on the metabolism of nucleic acids is still largely obscure, yet Karakashian and Hastings (1962) have shown that the mechanism of circadian variability in Gonyaulax may be dependent on the synthesis of a certain defined fraction of RNA, inhibited by actynomycin D. Our working hypothesis which attempted to link uridine content in the plant with the diurnal rhythm, has found no experimental confirmation. In addition, the identical quantities of uridine in starved, etiolated and control plants would seem to exclude such possibility.

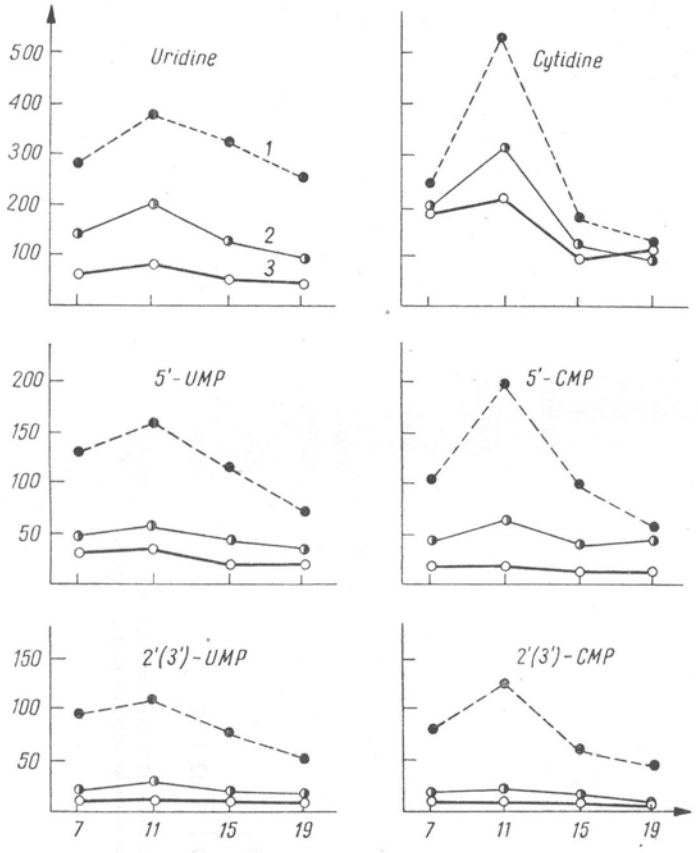



Fig. 1. The effect of time of day on the rate of uracil anabolism in excised pea plants

*1* – starved; 2 – etiolated; *3* – control plants. Spec. act. of 2-$^{14}$C-uracil- 24.000 counts/sec./μmole. Time of feeding – 2 hours. On abscissa is given time of day at which the samples were fed; on ordinate – spec. act. of products of 2-$^{14}$C-uracil anabolism – expressed in counts/sec/μmole

As the diurnal rhythm has no effect upon the uridine level in the plant, we have attempted to lower the content of endogenous uridine by feeding the plant with glucose. We assumed that this may cause increased synthesis of UDPG and by lowering the content of uridine it may stimulate direct ribotidation of uracil to 5′-UMP. Results in table 1 show indeed, that pre-treatment with glucose had a marked effect upon synthesis of uridylic derivatives from uracil. Excised plants fed with glucose in the dark had incorporated $^{14}$C-uracil to UMP (sum of UMP, UDP, UTP and UDPG) with 5-fold intensity over the controls and 3 times over the starved plants. Unfortunately we missed the main objective of this experiment i.e. to decrease the content of endogenous uridine by pre-treatment with glucose, as this time uridine was also very low in the controls (>0.08 μmole/g). We only confirmed again our previous results, namely that at low uridine content in the plant, ribotidation dominates over the two step path of 5′-UMP formation from uracil.

Table 1

Anabolism of 2-$^{14}$C-uracil in starved or glucose pretreated pea plants

| Treatment | $^{14}$C-Precursor*) | Intake counts/ sec./ sample | Uracil | | Uridine | | 5′-UMP**) | | 5′-CMP**) | | 2′(3′)-UMP | | 2′(3′)-CMP | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | μmole/g | counts/ sec./ μmole | μmole/g | counts/ sec./ μmole | μmole/g | counts/ sec./ μmole | μmole/g | counts/ sec./ μmole | μmole/g | counts/ sec./ μmole | μmole/g | counts/ sec./ μmole |
| Control | 2-$^{14}$C-Uracil | 10180 | 0.044 | 9218 | 0.060 | 268 | 0.190 | 302 | 0.098 | 192 | 0.752 | 53 | 0.630 | 19 |
| Starved | ,, | 7680 | 0.068 | 6205 | 0.080 | 289 | 0.157 | 439 | 0.090 | 317 | 0.534 | 530 | 0.455 | 537 |
| Glucose (darkness) | ,, | 12480 | 0.036 | 11080 | 0.058 | 923 | 0.180 | 1393 | 0.092 | 314 | 0.840 | 202 | 0.695 | 84 |
| Glucose (light) | ,, | 9340 | 0.041 | 9780 | 0.042 | 481 | 0.255 | 684 | 0.084 | 322 | 0.678 | 64 | 0.562 | 21 |

*) Amount of 2-$^{14}$C-uracil – 0.12 μmole/1 ml/2 g of fresh weight. Spec. act. – 120 000 counts/sec./μmole. Time of feeding – 2 hours.
**) 5′-UMP and 5′-CMP represent the sum of mono-, di- and triphosphates of nucleosides and their derivatives.

Exceptionally marked differences were observed in the specific activities of the pyrimidines in the polynucleotide fraction. The pyrimidine nucleotides of the 2′(3′)-series in the starved plants have shown specific activities 10—25 times of the activity in the controls, which again confirmed older results (Wasilewska and Reifer 1967 c). Pre-treatment with glucose in the dark has shown less radioactivity in the RNA pyrimidines and glucose in the light has shown no effect when compared with the control plants.

The stimulation of uracil anabolism under conditions of starvation and glucose feeding respectively was followed up by studying the anabolism of uridine and orotic acid under identical conditions. Results in table 2 permit to draw the following conclusions: pre-treatment with glucose led to highest stimulation of incorporation of radioactivity to UMP when uracil was the substrate (6-fold stimulation over the control, to a smaller degree when uridine was the substrate (2-fold stimulation) and least with orotic acid (25 %). Starvation of the plants has led to similar effects: with uracil as precursor, the specific activity of 5′-UMP was 4 times higher and 2′(3′)-UMP even 10 times higher than in the corresponding controls. With uridine as precursor there was a 2-fold stimulation both in 5′-UMP and 2′(3′)-UMP. No stimulation was observed when starved plants were fed with orotic acid.

Table 2

Comparison of metabolism of uracil, uridine and orotic acid in starved or glucose pretreated pea plants

| Treatment | $^{14}$C-Precursor*) | Intake (counts/sec./sample) | Spec. act. of pyrimidine derivatives (in counts/sec./μmole) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Uracil | Uridine | 5′-UMP**) | 5′-CMP**) | OA***) | 2′(3′)-UMP | 2′(3′)-CMP |
| Control | 2-$^{14}$C-Uracil | 11200 | 8880 | 79 | 87 | 33 | — | 6 | 4 |
| Starved | ,, | 9000 | 6904 | 252 | 389 | 179 | — | 56 | 44 |
| Glucose (darkness) | ,, | 9850 | 7401 | 116 | 538 | 101 | — | 33 | 25 |
| Control | 2-$^{14}$C-Uridine | 9700 | 946 | 3038 | 406 | 205 | — | 34 | 26 |
| Starved | ,, | 9800 | 575 | 5647 | 719 | 258 | — | 72 | 60 |
| Glucose (darkness) | ,, | 9850 | 812 | 4661 | 871 | 216 | — | 45 | 32 |
| Control | 6-$^{14}$C-Orotic acid | 9700 | 2019 | 8880 | 1733 | 463 | 622 | 132 | 43 |
| Starved | ,, | 9780 | 891 | 7680 | 1206 | 139 | 1316 | 91 | 47 |
| Glucose (darkness) | ,, | 9000 | 631 | 3549 | 2183 | 83 | 1172 | 110 | 42 |

*) Amount of $^{14}$C-substrates — 0.5 μmole/1 ml/2 g of fresh weight. Spec. act. 24 000 counts/sec./μmole. Time of feeding — 2 hours.

**) 5′-UMP and 5′-CMP represent the sum of mono-, di- and triphosphates of nucleosides and their derivatives.

***) Radioactivity of OA is expressed in counts/sec./sample.

The main aspect arising from this experiment is the undoubted fact that the classical orotic acid path by far dominates over any path in the synthesis of pyrimidine

nucleotides in higher plants. Under normal conditions when plants were not pretreated in any way, the proportion of synthesis of uridylic RNA derivatives produced by orotic acid and uracil amounted to 20 : 1. When however the plants were starved prior to feeding with the radioactive compounds, little change was noticed in the pyrimidine synthesis of the orotic acid fed plants, but feeding with uracil caused an extraordinary high increase in the incorporation of the label and the proportions from 20 : 1 changed to 2 : 1. Cytidylic derivatives of RNA in starved plants have shown identical radioactivities when fed with $^{14}$C-uracil or $^{14}$C-orotic acid. It would appear that starving of the plants activates strongly the alternative uracil path of pyrimidine synthesis, which under normal conditions of growth plays only a subordinate and supplementary role.

This observation confirms earlier results from allied fields, namely that rapidly regenerating tissue and cancerous cells show very intensive formation of RNA pyrimidines from uracil and uridine under conditions of accelerated synthesis of nucleic acids (see review by Schulman 1961).

The production of considerable quantities of uracil and uridine from orotic acid as shown in this paper confirms unequivocally earlier results (Wasilewska and Reifer 1967 b) that orotic acid undergoes decarboxylation to a marked extent at stages prior to orotidine-5′-monophosphate formation. Buchowicz (personal communication) has shown that uracil and even more so uridine, but not 5′-UMP is produced, when acetone powder extracts from the lupine were incubated with $^{14}$C-OA. Finally Balazs et al (1966) investigating the metabolism of $^{14}$C-OA in brain and liver tissue have also observed considerable formation of uracil and uridine, with specific activities much above those of 5′-UMP.

The effect of stimulation of pyrimidine mononucleotides and pyrimidine RNA synthesis with uracil or uridine, followed after starvation or glucose feeding, but no stimulation with orotic acid as precursor, may probably be due to 2 independent reasons: starvation accelerates nucleotide synthesis on the uracil path, but not on the orotic acid path, because under conditions of increased synthesis of nucleic acids, the uracil path takes over, acting as "salvage mechanism", according with the suggestion of Reichard (1959). Enzymes that utilise uracil, acting also under normal conditions, may undergo a process of adaptation at periods of increased requirements of nucleic acids, whereas the enzymes of the orotic acid path retain their normal activity. On the other hand glucose feeding may lead to privileged UDPG formation, which on hydrolysis, as UDP may be the precursor of polynucleotide pyrimidines, either by way of polynucleotide phosphorylase catalysis or after phosphorylation to UTP it may be the substrate for RNA polymerase.

Basing on a chance observation dated several years ago (Buchowicz et al. 1963), namely that low concentrations of uracil (0.05 mM $^{14}$C-uracil) fed into plants led to incorporation of the base into polynucleotides, with the omission of the mononucleotide stage and on the the other hand at high uracil concentrations (2mM) the bulk of the label was found in 5′-mononucleotides, we have decided to study systematically the influence of uracil concentration on the pathway and on

intensity of its anabolic changes. We have tentatively assumed that higher uracil concentrations were inhibiting its direct incorporation into polynucleotides.

Plants were fed with $^{14}$C-uracil at rising concentrations from 0.06 to 1.2 mM. Results presented in fig. 2 seem to confirm indirectly our previous assumption. Rising concentrations of $^{14}$C-uracil led primarily to considerable increases of the 5′-UMP fraction, having a much smaller effect on the incorporation of the label

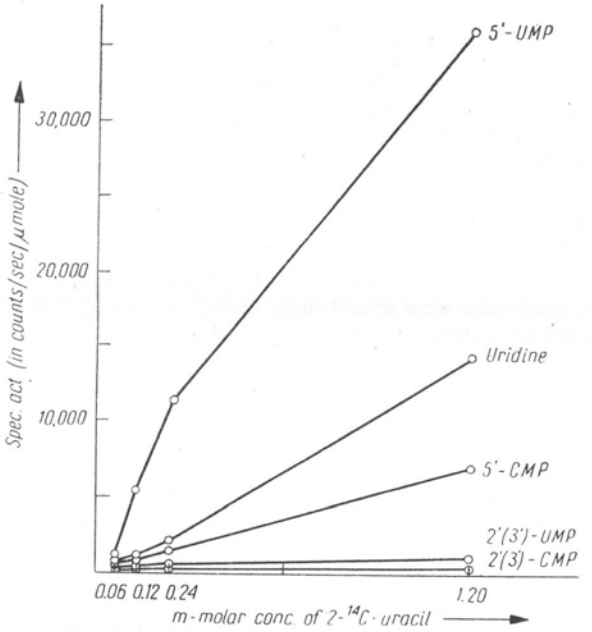



Fig. 2. The effect 2-$^{14}$C-uracil conc. on incorporation of the label into its anabolic products (green pea)
Spec. act. of 2-$^{14}$C-uracil-6.000 counts/sec/μmole. Time of feeding — 2 hours

into pyrimidines of the polynucleotides. A 20-fold increase of $^{14}$C-uracil concentration led to a 20-fold increase of the specific activity of 5′-UMP, whereas the specific activity of 2′(3′)-UMP rose only 4 times.

In this paper the attempt was made to influence in some way the proportions of the 3 paths of uracil anabolism (scheeme proposed in the introduction) and particularly to ascertain the reasons favouring sometime the one step, and some other time the two-step anabolism of uracil into UMP. In this we have largely failed and despite work extending for several years, we still do not know the causes for the observed differences in the level of endogenous uridine in the plant, which seems to be responsible for the domination of one path over the other.

The mentioned difficulties have speeded up our decision to discontinue the work in vivo in view of the multiple complications which largely are beyond the control of the investigator. Studies in vitro on direct incorporation of uracil into polynucleotides have already been reported (Buchowicz and Reifer 1967).

Presently we are endeavouring to isolate the 3 enzymes from plant material, that are responsible for uracil anabolism into 5′-UMP and the first tentative results are presented in the next paper of this journal.

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## SUMMARY

Variations of the rate of uracil anabolism depend on the diurnal rhythm. Synthesis of pyrimidine derivatives was highest at 11 a.m. The diurnal cycle has however no effect on the direction of uracil anabolism and does not influence the content of endogenous pyrimidine derivatives, particularly of uridine. Starving of the plants as well as pre-treatment with glucose intensify the synthesis of pyrimidne nucleotides from uracil and uridine, but not from orotic acid.

It has been established that synthesis of mono- and polynucleotides depends upon concentrations of uracil fed into the plants. Increased concentrations of uracil used, led to proportional increases of specific activity of 5′-UMP, uridine and 5′-CMP, whereas the increases of specific activity of the pyrimidine polynucleotides were several times lower.

The presented results confirm indirectly that the anabolism of uracil in higher plants may proceed on 3 alternative paths.

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## *Niektóre aspekty syntezy nukleotydów pirymidynowych w odciętych kiełkach grochu*

Streszczenie

Badano wpływ niektórych czynników na metabolizm uracylu, urydyny i kwasu orotowego w odciętych kiełkach grochu.

Stwierdzono, że szybkość anabolizmu uracylu ulega wahaniom dobowym. W porze przedpołudniowej (11°°) obserwuje się najbardziej intensywną syntezę pochodnych pirymidynowych z tego prekursora. Pora dnia nie ma wpływu na kierunek przemian uracylu, ani na zawartość endogennych pochodnych pirymidynowych, a zwłaszcza urydyny. Zarówno głodzenie roślin, jak i dokarmianie glukozą prowadzi do wzmożonego wcielania radioaktywności do nukleotydów pirymidynowych z uracylu i urydyny, ale nie ma wyraźnego wpływu na szybkość anabolizmu kwasu orotowego.

Stwierdzono zależność pomiędzy stężeniem uracylu użytego do dokarmiania, a kierunkiem jego anabolizmu. Obserwuje się proporcjonalność pomiędzy wzrostem koncentracji uracylu a wzrostem aktywności właściwej 5′-UMP, 5′-CMP i urydyny, podczas gdy aktywność właściwa pirymidyn polinukleotydów wzrasta kilkakrotnie wolniej w porównaniu ze wzrostem stężenia uracylu użytego do dokarmiania.

Uzyskane wyniki dostarczają dalszych pośrednich dowodów na istnienie 3 alternatywnych torów anabolizmu uracylu w roślinach wyższych.