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Free Nucleotides in the Primary Root of Maize

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Dedicated to Academician S. Prát on the occasion of his 80th birthday

Abstract. Free nucleotides of the primary root of maize were extracted with 5% $HClO_4$ and separated on a column of Dowex 1 × 8 ion exchanger in $HCOO^-$ cycle. A two-step elution gradient (HCOOH, $HCOOHNH_4$) was used for the elution of the nucleotides. The incorporation of ^{32}P into the nucleotides was followed at different time intervals and also in young and more mature root tissues. The nucleotides AMP, GMP, ADP, GDP, and ATP were identified. Labelled phosphorus was found in ATP after 30 s, in ADP after 3 min, and in AMP after 5 min incubation of the roots. More mature roots (18 days) contained higher amounts of AMP than the young ones (3 days).

The velocity of incorporation of inorganic phosphate into organic compounds in the roots represents the measure of metabolism taking place in this organ of uptake and transport of substances. ATP is the first organic compound in which an inorganic phosphate ion appears. The velocity of phosphate incorporation can be expressed in seconds (LOUGHMAN and SCOTT-RUSSEL 1957, KURSANOV and VYSKREBENTSEVA 1960). When following the velocity of incorporation of inorganic phosphate it is also possible to characterize the differences in the metabolism of plant tissues differing in maturity. In this paper we summarize some methodical findings obtained during the investigation of free nucleotides in maize roots as well as observed differences found when comparing tissues at different stages of maturity.

Material and Methods

Preparation of Plant Material

The primary root of maize (*Zea mays* L., cv. 'TA 45 S') served as the experimental object in our experiments. Maize seeds were soaked for 24 h in running tap water and then germinated on wet filtration paper in a thermostat at 25 °C. The three days old seedlings obtained were either analyzed or transferred to a 1/2 Knop nutrient solution and grown in a controlled environment plant growth chamber (at 20 °C and 12 h photoperiod). For the determination of the incorporation velocity of phosphate, labelled phosphorus was applied in Knop nutrient solution ($KH_2{}^{32}PO_4$, 2.4 μCi ml^{-1}) without carrier. The more mature plants were grown before

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transferring them to the nutrient solution containing labelled phosphorus for one day in 1/2 Knop nutrient solution from which PO_4^{3-} was omitted. The roots of the seedlings were then submerged into the radioactive solution. Both the 3 days old and the 18 days old roots were incubated in the radioactive solution for a time ranging from 30 s to 2 h.

Extractions of Free Nucleotides

10 g f.w. samples of 1.5 cm long segments of seminal root were taken from the 3 days old maize seedlings and 10 g f.w. samples of 6.5 cm long segments of seminal root were taken from the older maize seedlings. The roots were frozen in liquid nitrogen and extracted with 5% $HClO_4$ (2 ml g^{-1} f.w.) for 30 min at 5 °C. The homogenate was centrifuged for 20 min at 10 000 g at 0 °C. The supernatant was decanted, lipophile substances were removed by means of ether extraction, and at 0 °C carefully adjusted to pH 6.2 with 10% KOH. $KClO_4$ precipitate was removed by means of centrifugation at 5000 g for 10 min at 0 °C and the supernatant obtained was applied to the column of ion exchanger.

Preparation of Ion Exchange Column and the Separation of Free Nucleotides

The standards of the nucleotides and the extracts of plant material were separated on anionic ion exchanger. Dowex 1 × 8, 200—400 mesh was floated after HAMILTON (1958); the fraction with particle size from 20 to 34 μm was used. The exchanger was then transferred to the $HCOO^-$ cycle. A concave two-step elution gradient described by INGLE (1962) (H_2O — 4M HCOOH, 4M MCOOH + 0.8 M $HCOOHNH_4$) was used for the elution of the nucleotides from the column. Eluate absorbance was recorded automatically at 260 nm and besides that it was further measured in 1 cm quartz cuvettes on the "Spektromom 203" spectrophotometer. When working with the radioactively labelled material, 1 ml samples were taken from each fraction, dried and their radioactivity was determined using an end-window counter (mass of the window 3.1 mg cm^{-2}).

Identification of the Nucleotides

The fractions were purified by means of absorption onto and consequent elution from activated charcoal after FRIČ and HORVATOVIČOVÁ (1967). The elution system consisted of 96% ethanol, pyridin, 25% ammonium solution and water (50 : 5 : 4 : 4.1, v/v). The eluates were chromatographed on Whatman 1 paper using the solvent mixtures proposed by Pabst Laboratories (Circular No 10). Chromatographic spots were detected under an UV lamp (UV Lampe Camage) at 264 nm, eluted with 0.1 N HCl and absorption spectra of the eluates were then recorded in the range from 200 to 300 nm on a UNICAM SP-800 spectrophotometer.

Results and Discussion

The determination of free nucleotides in green plant parts is complicated because of the low content of free nucleotides and because pigments are present in these tissues. During the extraction procedure, when different methods are used, considerable losses occur. This is also the reason why the number of papers which describe methods trying to overcome these difficulties is so high.

For the determination of free nucleotides either the extraction with diluted ethanol or the extraction with diluted perchloric or trichloroacetic acids, or a combination of both, proved satisfactory. The use of diluted acids (0.2—0.6 N) proved to be better for tissues containing pigments, because pigments are extracted simultaneously with ethanol. Acid extracts from plant tissues containing nucleotides also contain other compounds which must be removed from the extracts. The nucleotides can be precipitated

Abbreviations used: CMP... cytidine monophospate, AMP... adenosine monophosphate, GMP... guanosine monophosphate, ADP... adenosine diphosphate, GDP... guanosine diphosphate, ATP... adenosine triphosphate.

in the form of insoluble salts. Because big losses in nucleotide content occur during precipitation, extraction methods using organic solvents by means of which most pigments and lipophile compounds, increasing the absorbance of the extract by 50 to 70%, can be removed, proved to be superior. Ion exchange chromatography was used for the first time for the separation of

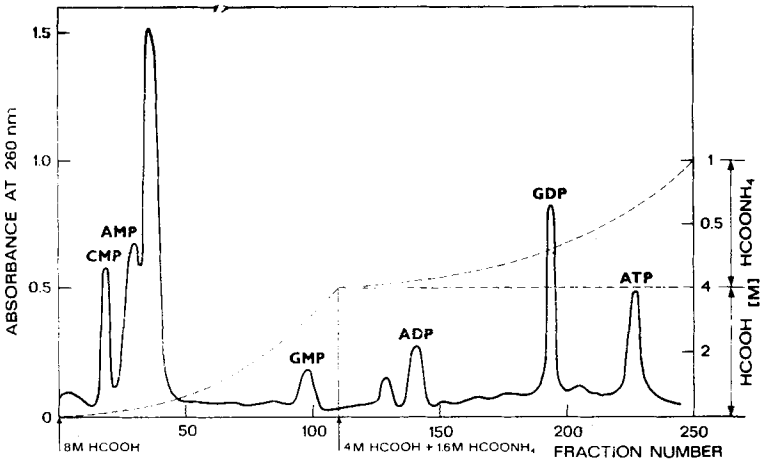

Fig. 1.: Separation of nucleotides extracted from the roots of 3 days old maize seedlings by means of ion exchange chromatography. Column 0.8 × 15 cm, Dowex 1 × 8, 20—34 μm ($HCOO^-$), concave elution gradient, collected fractions 3.4 ml per 5 min.

nucleotides by COHN (1950). Individual nucleotides could be eluted from the column only by means of gradient elution (HURLBERT and SCHMITZ 1954). The method described by INGLE (1962) and modified for plant material proved to be best in our experiments. A relatively good separation is achieved by means of concave elution gradient with a very mild slope at the beginning of elution and a very steep one at the end, which prevents the overlapping of chromatographic peaks at the beginning of chromatography (Fig. 1). Fine exchanger particles with smaller differences in particle size contribute to a better separation. By means of these methods we were able to identify the following nucleotides in acid extracts of maize roots: CMP, AMP, GMP, ADP, GDP, ATP.

A suitable method for the separation of nucleotides enables the study of many physiological processes taking place in plant cells, because many physiological processes and metabolic changes are reflected in quantitative changes of the content of nucleotides. These metabolites play the main role in the mediation and use of energy released during respiration and in the synthesis of sugars, lipids, proteins and nucleic acids. Very little is known about the content and about dynamic changes in the concentration of adenine nucleotides in plant tissues during plant growth and development. Adenine derivatives prevail in maize roots. Adenine nucleotides at all the three energetic levels, AMP, ADP, and ATP, could be demonstrated and separated in the extracts from maize roots (both 3 and 18 days old). We then followed

the velocity of incorporation and the amount of ^{32}P after different times of incubation (30 s—2 h) of experimental plants in radioactive solution. During this short-term incorporation (up to 2 h) of ^{32}P by plant roots most of the ^{32}P can be found in acid-soluble fractions, above all in the nucleotides. During the first 30 s ^{32}P could be found only in ATP, later in ADP (3 min)

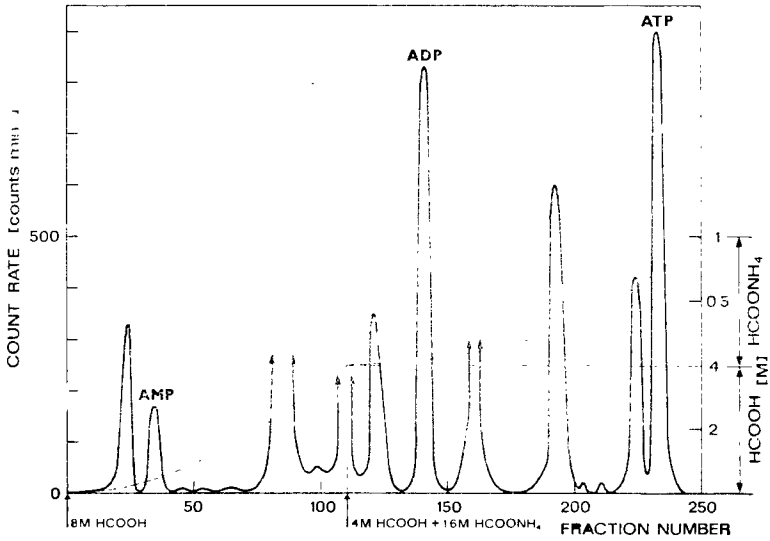

Fig. 2: Measurement of radioactivity in the eluate from the column of the ion exchanger when 3 days old maize seedlings were incubated in the solution of $KH_2{}^{32}PO_4$, 2.4 μCi ml^{-1} h^{-1}. Other conditions are the same as in Fig. 1.

and AMP (5 min). The inclusion of inorganic phosphate into the cycle of biochemical transformations in the root begins with its entry into the nucleotide molecule, predominantly into ATP which is an unavoidable donor of phosphate groups for the other acceptors in phosphorylation reactions catalyzed by the corresponding enzymes.

TABLE 1.

THE AMOUNT OF ^{32}P INCORPORATED BY THE SEMINAL ROOT OF 3 DAYS OLD MAIZE SEEDLINGS INTO AMP, ADP, AND ATP, EXPRESSED IN %, AFTER DIFFERENT TIMES OF INCUBATION OF THE SEEDLINGS IN RADIOACTIVE SOLUTION.

Time [min]	AMP [%]	ADP [%]	ATP [%]
3	—	13.2	86.8
5	5.7	20.7	73.6
60	10.8	42.3	46.9

To compare the amount of the incorporated ^{32}P into the nucleotide molecule, we evaluated diagramatically individual fractions as a function of activity (Fig. 2) for individual times of exposition. The total area corresponding to adenine nucleotides determined planimetrically from the diagram was considered to be 100% and the content of ^{32}P in the individual adenine nucleotides was related to it (Table 1). In the roots of 3 days old maize seedlings, 86% of the ^{32}P incorporated into the nucleotides could be found in ATP after a 3 min exposition in the solution containing $KH_2{}^{32}PO_4$. After a longer exposition, the portion of the incorporated ^{32}P into ATP diminishes and that into AMP increases. This relation is in agreement with the results obtained by other authors (KURSANOV and VYSKRIBENTSEVA 1960). The difference in the incorporation of ^{32}P into free nucleotides in young and more mature root tissues is also of interest. After a 60 min exposition, 10.8% of labelled phosphorus was found in AMP in 3 days old roots whereas as much as 17.7% was found in 18 days old roots. From these data it is possible to deduce that the role of nucleotides in the mediation and utilization of respiratory energy and in the synthesis of sugars and other metabolic products diminishes. This fact was established in senescent bean leaves by WEINSTEIN *et al.* (1969). It is true that there are no qualitative differences in the level of free nucleotides in more mature tissues, however, the difference in quantity is due to the fact that a considerable amount of nucleotides is used in young tissues for growth processes, which holds especially for the root as the organ of absorption, transformation and transport of mineral substances.

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Po vypracovaní vhodnej metódy delenia nukleotidov iónomeničovou chromatografiou podarilo sa z pletív koreňa kukurice (3 a 18dňových) izolovať a identifikovať nasledovné nukleotidy: CMP, AMP, GMP, ADP, GDP a ATP. Sledovala sa rýchlosť inkorporácie ^{32}P do AMP, ADP a ATP. Fosfor sa inkorporoval po 30 s do ATP, po 3 min do ADP a po 5 min do AMP. Porovnaním množstva inkorporovaného ^{32}P do nukleotidov u starších a mladších rastlín kukurice bolo pozorované, že množstvo ^{32}P v AMP bolo u starších rastlín vyššie a znížilo sa množstvo ^{32}P inkorporovaného do ADP a ATP.