ACTA CHEMICA SCANDINAVICA 13 (1959) 1653–1658

The Acid Soluble Nucleotides of *Vicia Faba*

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The use of small doses of ^{32}P has been found to stimulate the synthesis of the acid soluble nucleotides of *Vicia Faba*. A suitable incorporated activity has been found to be 0.1 μC per seed in a 48 h period, while higher and lower doses seemed to be less effective.

The acid soluble nucleotides of *Vicia Faba* were extracted with 10 % perchloric acid, and fractionated by ion exchange chromatography. They were shown to include the 5′-triphosphates of adenosine, guanosine, cytidine and uridine as well as mono- and diphosphate.

The identification of ten nucleotides was mainly based on the ultraviolet absorption measurements on the extract and pure nucleotides, radioactivity measurements together with chemical and enzymatic analyses. The extraction was carried out on plants exposed to ^{32}P before sowing.

While heavy radiation is damaging to plant life, some authors have claimed that moderate exposure would stimulate plant growth and accelerate the metabolic processes [1]. Bean plants were grown in solutions in which the ^{32}P concentration was varied. The plants were later radiographed, (that is, laid over sheets of X-ray film) showing that the ^{32}P uptake increased within but not beyond a very low concentration range of phosphorus in the soil [1].

Small doses of ^{32}P have been shown to increase the production of nucleoproteins in plants [2].

In the last few years many reports have appeared concerning the metabolism of the acid soluble nucleotides, in yeast [3–5], wheat, oats and barley [6]. As a further continuation of the study of the metabolism of acid soluble nucleotides in different plants, we present here the first results of experiments carried out on *Vicia Faba*.

The main difficulties encountered in the demonstration of these compounds in plants have centred on the problem of their separation from interfering substances and their isolation in pure form. Methods developed primarily for the isolation of the nucleotides from animal tissues have proved to be unsatisfactory for the isolation of these compounds from plants [7].

Cohn was one of the pioneers in using the chromatographic methods for the estimation of the acid soluble nucleotides in yeast [7]. Potter and coworkers [8]

have improved the method of separation on the column and shortened the time for the estimation of the acid soluble nucleotides by the so called gradient elution method.

An improved method for the isolation of the acid soluble nucleotides from plant tissues has been described by Bergkvist [6]. The application of this method to *Vicia Faba* established the occurrence of all the four ribonucleotides, *viz.* the adenosine, guanosine, cytidine and uridine derivatives. Such nucleotides exist in the form of acid soluble 5-ribonucleotides at different levels of phosphorylation. Of great interest is the occurrence of uridine, besides adenosine derivatives in *Vicia Faba*, in contrast to the dominating role of the latter in animal tissues.

In the present paper we have tried to give a complete picture of the acid soluble nucleotides obtained by cold 10 % perchloric acid (PCA) extraction of 250 g of *Vicia Faba* plant, just before fruiting. The yield was found to be low; however, the nucleotides isolated were similar to those which have previously been obtained from yeast under anaerobic conditions [5]. The results given are the average of ten experiments. The seeds of the plants used for the extraction were first exposed to the beta-radiation emitted from small doses of ^{32}P. In a previous paper we have estimated the suitable dose for irradiation to produce no harmful effect on the plant. The morphological appearance of the plant and the analyses of total nitrogen, soluble nitrogen, and protein nitrogen were taken as criteria for the estimation of the suitable dose (Abdel-Wahab and El-Kinawi, unpublished work). A suitable incorporated activity has been found to be 0.1 μC per seed and a 48 h period of exposure.

Both radioactivity and ultraviolet adsorption were measured on the extract. The results obtained for the acid soluble extract of the *Vicia Faba* nucleotides from ultraviolet absorption spectrum, and from radioactivity measurements, were found similar (see Figs. 1 and 2).

Autoradiogaphic experiments have shown that the ^{32}P-uptake is more pronounced in leaves than in shoot or root systems. They have shown also that plants grown in pots afford better material than those grown in the field. In our experiments we have used pot plants four weeks after sowing. At the end of such a period the plant has begun to fruit.

The acid soluble nucleotides were isolated by homogenising the plant material in ice-cold 10 % PCA and freed from other phosphorus-containing or ultraviolet-absorbing substances according to the method described by Bergkvist [6]. After adsorption on a strong base anion exchanger in the formate form, the individual nucleotides were eluted with formic acid solutions containing increasing concentration of sodium formate. Elution of the nucleotides was followed by the change in optical density of the effluent fractions at 260 mμ and 270 mμ. The optical densities of the effluent fractions at 260 mμ were plotted against the effluent volume to obtain the chromatograph shown in Fig. 1. The elution positions as well as the absorbance ratios gave a preliminary identification of the individual fractions. Resolution of the elution position was achieved by plotting the radioactivity against the tube number. The activity was measured in counts per min and gave rise to the results shown in Fig. 2. Figs. 1 and 2 are nearly similar. From these figures one can conclude also that no reduced nucleotides are detected under our

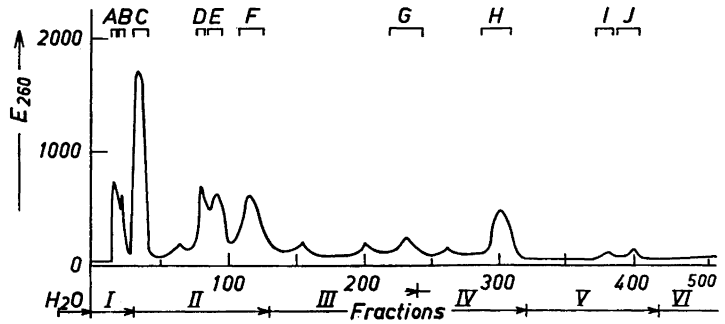

Fig. 1.

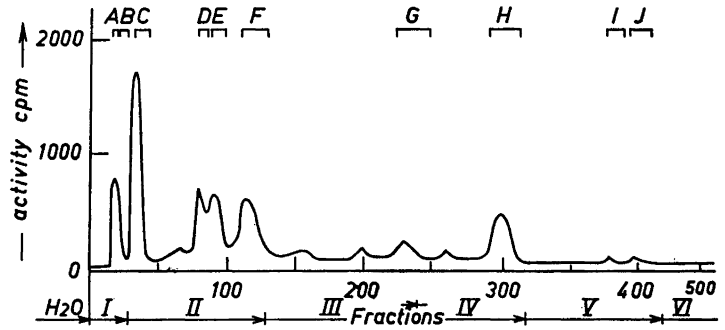

Fig. 2.

experimental conditions in *Vicia Faba*. An attempt to demonstrate such reduced nucleotides in yeast under anaerobic conditions has been carried out by us [5], and proved also their absence in yeast. In case such nucleotides are present in *Vicia Faba* they should only appear in Fig. 2 (radioactivity), but not in Fig. 1 (ultraviolet-absorption), because of the fact that such reduced nucleotides have no absorption in the ultraviolet spectrum.

The R_F values obtained from the paper chromatograms in different solvent systems and the density curves of the free nucleotides eluted from the paper chromatograms were also studied and compared to standard pure nucleotides. Since the nucleotides obtained from the column were dissolved in large volumes of formate buffer, it was desirable to concentrate them and to get rid of the excess salts. In order to remove the excess of salts, the individual peaks were separated, readsorbed on norite and then eluted with ammoniacal ethanol. The solutions thus obtained were used to identify the nucleotides and establish the purity of the peaks by comparison of their chromatographic behaviour with those of the corresponding authentic samples.

Further characterization of the nucleotides was obtained by the following procedures:

a. Ultraviolet absorption spectra in 0.1 N HCl and 0.1 N NaOH.
b. Determination of the total phosphorus content and of the phosphorus liberated after hydrolysis for 10 min in 1 N HCl.
c. Determination of the sugar content in each sample.
d. Paper chromatographic identification of the nucleotides and comparing their R_F values in different solvents with authentic samples.
e. Comparing the optical density curves of the nucleotides with standard curves over a range, 220—320 mμ, in intervals of 5 mμ.
f. The position of the phosphate group on the different nucleotides was confirmed by oxidation with periodate and the action of rattle snake venom [6].

Fig. 1 shows the ultraviolet extinction of 260 mμ of the fractions collected by an automatic fraction collector against the tube number. The results are obtained from 250 g *Vicia Faba* (whole plant), extracted by means of ice cold 10 % PCA and chromatographed on Dowex 1, formate form. The extinction values were obtained by the use of a Beckman spectrophotometer. The formate system used gave rise to peaks up to buffer solution V (*cf.* Experimental). Buffer solutions VI, VII, and VIII did not give rise to new peaks. Each tube represents 5 ml effluent. The diagram indicated the presence of ten distinct peaks as described at the bottom of Table 1 and are arranged in order of appearance.

In Fig. 2 the radioactivity is plotted against the tube number. The activity was measured as counts per min. Solutions used for measurements are those used in Fig. 1. The absence of extra peaks in Fig. 2 denotes that no reduced nucleotides are detected.

Table 1. R_F values of the ten peaks obtained from Fig. 1, compared with the R_F values of the standard pure nucleotides.

	R_F for the peaks appearing in Figs. 1 and 2. / R_F for the pure standard nucleotides									
System 1	0.22 0.20	0.38 0.38	0.45 0.43	0.22 0.21	0.26 0.24	0.29 0.28	0.08 0.07	0.20 0.20	0.20 —	0.05 —
System 2	0.46 0.46	0.40 0.39	0.66 0.67	0.32 0.31	0.18 0.19	0.20 0.20	0.40 —	0.19 0.18	0.62 —	0.50 —
System 3	0.76 0.76	0.28 0.28	0.70 0.70	— —	— —	— —	0.70 —	— —	0.78 —	0.50 —
System 4	0.30 0.29	— —	— —	0.40 0.40	0.26 0.27	— —	— —	— —	— —	— —
System 5	0.88 0.87	0.70 0.69	0.85 0.86	— —	— —	— —	— —	— —	— —	— —
Identified nucleotides	A CMP	B DPN	C AMP	D GMP	E UMP	F ADP	G UDPX*	H ATP	I UTP	J GTP

* UDPX is a sugar derivative of uridine diphospate.

Comparison of the R_F values obtained showed the existence of ten nucleotides identified in Table 1. The density curves obtained for the peaks in acid and alkaline medium and those of the ten pure standard nucleotides, together with the extinction ratios E_{250}/E_{260} and E_{270}/E_{250}, confirmed the presence of ten nucleotides.

EXPERIMENTAL

Irradiation of seeds. 250 g = 400 seeds of *Vicia Faba*, variety Rebaia 34, kindly supplied by the Egyptian Botanical Adminstration, were soaked in 2 l of tap water containing 40 μC ^{32}P. After 48 h, the seeds were removed from the soaking medium washed twice with tap water and used for sowing.

Sowing. Seeds were sowed in pots (3 per pot) and left to grow out of doors. The soil used for sowing was that used for sowing beans and other plants in Egypt. The plants were irrigated daily and after the first week the seedlings began to appear. At the end of four weeks the plants began to fruit. A control experiment was carried out using the same procedure but without using ^{32}P in the soaking medium.

Separation of the acid soluble nucleotides. The whole mature plant, which had begun to fruit, was ready for the extraction at the end of four weeks. 250 g of fresh whole plant were homogenised in 1 000 ml ice-cold 10 % PCA in a Waring Blender and reextracted with 250 ml 5 % PCA. The nucleotides were isolated from the PCA extract by ether extraction. The etherial layer containing the nucleotides was separated and left to evaporate by passing a stream of air through it. The oily residue left after aeration was dissolved in 500 ml CO_2-free redistilled water and brought to pH 8 by dilute ammonium hydroxide.

Fractionation of the nucleotides. Dowex 1 in the formate form was used as fractionating system. The individual nucleotides were eluted with increasing concentrations of formic acid or a mixture of formic acid and sodium formate. The following eluting system and the type of column described, were used.

Eluting Buffers	Formic acid, M		Sodium formate, M
I	0.02		—
II	0.10		—
III	0.10	+	0.05
IV	0.10	+	0.30
V	0.10	+	0.40
VI	0.10	+	0.60
VII	0.20	+	0.80
VIII	0.50	+	1.00

Exchanger. Dowex 1—X 10, 200—400 mesh, strong base anion exchanger, 1.2 × 20 cm formate column, effluent rate 1 ml per min.

The nucleotides were recovered from the pooled fractions by norite adsorption. For each peak the paper chromatographic procedure was carried out as is explained elsewhere.

Ultraviolet absorption. The chromatographic fractions were analysed by measuring their extinction at 250, 260 and 270 mμ in a spectrophotometer, Beckman Model DU with photomultiplier. The extinctions at 260 mμ were plotted against tube number as shown in Fig. 1. Each peak was also further studied by determining its optical density over a range of 220 to 320 mμ in intervals of 5 mμ, in 0.1 N HCl and 0.1 N NaOH solutions. The absorption ratios E_{250}/E_{260} and E_{270}/E_{260} were determined as well and used as criteria for the identification of the different nucleotides.

Phosphorus determination. Each sample was evaporated to a very small volume to remove the solvent used for elution. The samples were then heated with perchloric acid in 25 ml micro-Kjeldahl flasks till they turned colourless. The samples were left to cool and phosphorus was determined by the method of Allen [9], adapted to a total volume of 5 ml, and the acid labile phosphorus was determined after 10 min hydrolysis in 1 N HCl at 100°C.

Pentose estimation. Both samples from *Vicia Faba* extract and standard pure nucleotides were subjected to pentose estimation using the orcinol method of Albrum and Umbereit [10]. The results were calculated by comparison with the standards.

Periodic oxidation. Oxidation with periodate was performed according to Dixon and Lipkin [11], to confirm the position of the phosphate group in the nucleotides present.

Action of snake venom. The action of rattle snake venom was studied according to the method of Bergkvist [6].

Paper chromatography. Schleicher and Schüll, No. 204, 3b, filter paper was used. The Markham and Smith method [12], for localisation of the spots on the paper chromatogam was used. Identification of the R_F of nucleotides present, was achieved by using pure nucleotides and comparing the R_F values obtained (see Table 1).

Both the descending and ascending methods for paper chromatography were used to resolve the nucleotides.

Descending systems.

1. *Iso*butyric acid/NH_3/H_2O (60/1/33).
2. Ethanol/M ammonium acetate (aqueous) (70/30).
3. 0.1 M phosphate buffer (pH 6.8)/saturated ammonium sulphate/*n*-propanol (1/60/40).
4. Butanol/glacial acetic acid/water (40/11/50).

Ascending system.

5. *Iso*amylalcohol/water/citric acid (10/50/0.3 g).

The sample tubes belonging to one peak in the extinction curve (Fig. 1) were collected, shaken with 2 g norite for 20 min, then centrifuged and the supernatent fluid was discarded. The adsorbed nucleotides were then eluted successively with 10 and 5 ml of the ammoniacal ethanol (50 % aqueous ethanol containing 0.5 % ammonium hydroxide, *d* 0.91). The eluent was separated by centrifugation and lyophilised. The dry residues, dissolved in a minimum amount of distilled water (40—80 μl), were used to spot the chromatograms.

Acknowledgment. One of the authors (Abdel-Wahab) is most grateful to prof. Dr. Phil. Hans H. Ussing for his continuous interest and helpful criticism; he wishes also to thank the IAEA in Vienna for the maintenance award which enabled him to visit the Institute of Biological Chemistry in Copenhagen where he continued his work in this field.

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Received May 13, 1959.