ACTA CHEMICA SCANDINAVICA 11 (1957) 1457—1464

The Acid-soluble Nucleotides of Barley and Oat Plants

ROLF BERGKVIST

Institute of Biochemistry, University of Lund, Sweden

The acid-soluble nucleotides of barley and oat plants are isolated and fractionated by ion exchange chromatography; they are shown to include the 5′-triphosphates of adenosine, guanosine, cytidine and uridine besides mono- and diphosphates. The identifications are based on ultraviolet absorption measurements and chromatographic and ionophoretic evidence before and after hydrolysis together with chemical and enzymic analysis.

Besides as a constituent of nucleic acids, adenosine-5′-phosphate has been known for a long time to occur in combined forms in smaller molecules which act as coenzymes in many metabolic processes. None of the other three nucleotides from ribonucleic acid had been found to occur in biological material either free or forming part of small molecules. Seven years ago Leloir and coworkers [1] studying the galactose metabolism in yeast, and Park and Johnson [2] studying the effects of penicillin on bacteria, observed independently the occurrence of acid-soluble uridine-containing compounds in living tissues. Following these important contributions to the progress of nucleotide biochemistry, derivatives of uridine-5′-phosphate, guanosine-5′-phosphate and cytidine-5′-phosphate have been isolated from different sources with the result that the number of derivatives of these nucleotides known to occur in living tissues is about the same as that in the adenosine-5′-phosphate series, so that the latter compounds have now lost the unique position, as building blocks of coenzymes, which they were believed to have.

The main difficulties encountered in the demonstration of these compounds in plants have centered around the problem of their isolation free from interfering substances and their separation. Methods developed primarily for the isolation of the nucleotides from animal tissues have proven unsatisfactory for the isolation of these compounds from plants. From mung beans, which have a low content of disturbing substances, an ATP *-like compound [3] and

* Abbreviations used: A, adenosine; G, guanosine; U, uridine; C, cytidine; MP, monophosphate; DP, diphosphate; TP, triphosphate; UDPG, uridine diphosphate glucose; UDPAG, uridine diphosphate acetylglucosamine; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide.

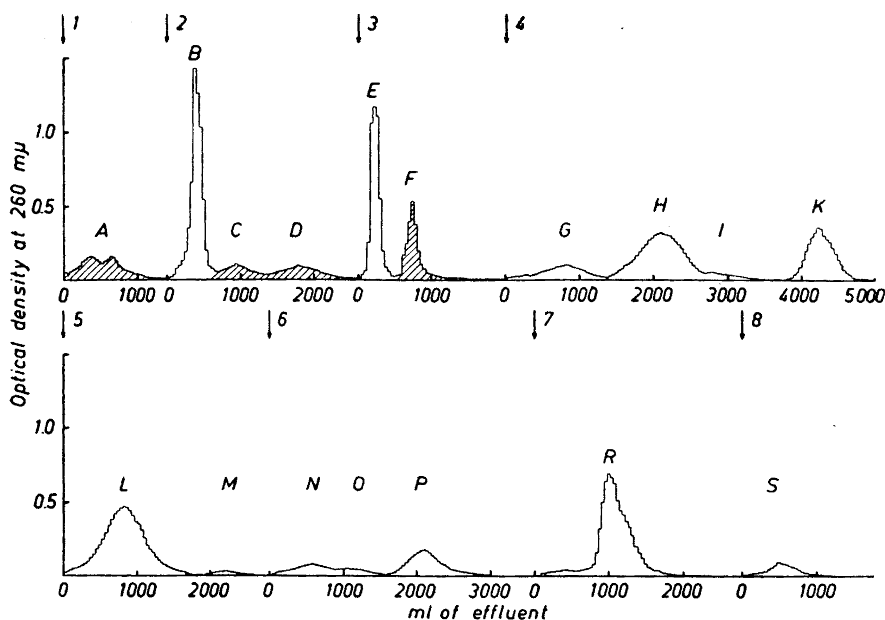

Fig. 1. Ion exchange chromatogram of acid-soluble nucleotides from 500 g of barley plants. The crossed fractions varied in amounts in different preparations.

Exchanger: Dowex 1, X-10, 200 to 400 mesh, 1.6×40 cm., formate form; flow rate 1.5 ml/min.

Eluting agents:

1) 0.02 M formic acid
2) 0.1 M » »
3) 0.1 M » » + 0.05 M sodium formate
4) 0.1 M » » + 0.3 M » »
5) 0.1 M » » + 0.4 M » »
6) 0.1 M » » + 0.6 M » »
7) 0.2 M » » + 0.8 M » »
8) 0.5 M » » + 1.0 M » »

Identity of peaks:

A) 2'- and 3'-CMP
B) 5'-AMP
C) 2'-AMP
D) 3'-AMP
E) 5'-UMP
F) 2'- and 3'-UMP; 2'- and 3'-GMP; CDP and TPN.
G) UDPAG
H) UDPG
I) ADP derivative
K) UDP
L) ADP
M) CTP
N) GDP
O) ATP derivative
P) UTP
R) ATP
S) GTP

quite recently a mixture of uridine diphosphate sugar compounds [4] have been isolated. Paper chromatographic evidence for the presence of adenosine and uridine containing compounds in plants has been presented by several authors [5-7].

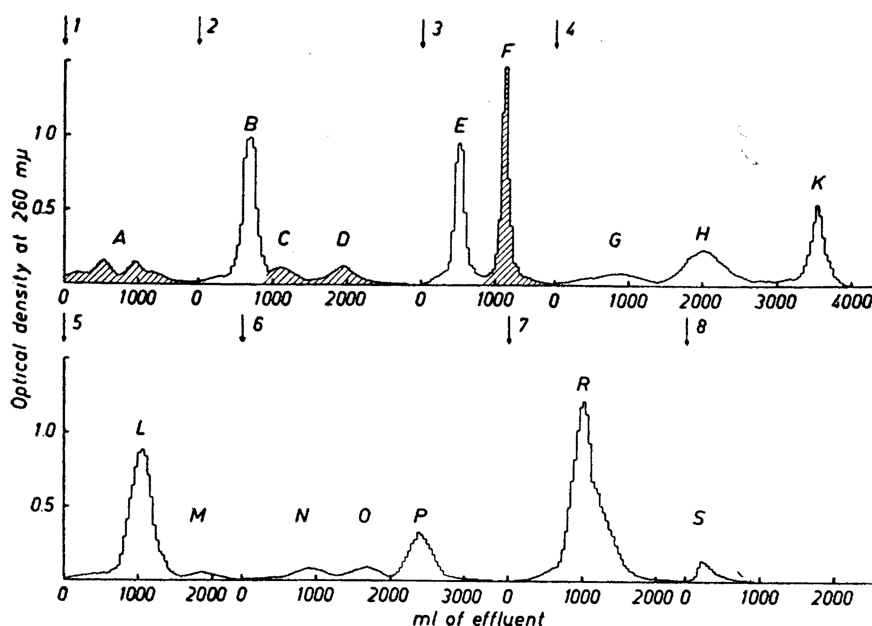

Fig. 2. Ion exchange chromatogram of acid-soluble nucleotides from 500 g of oat plants. The crossed fractions varied in amounts in different preparations.

Exchanger: Dowex 1,X-10, 200 to 400 mesh, 1.6 × 40 cm., formate form; flow rate 1.5 ml/min.
The eluting agents and identity of peaks are the same as in Fig. 1.

An improved method for the isolation and quantitative estimation of the acid-soluble nucleotides from plant tissues has been described in a previous paper [8]. The application of this method to wheat plants established the occurrence of all the four ribonucleosides in the form of acid-soluble 5'-mononucleotides at different levels of phosphorylation [8]. Of interest is the predominance of uridine, besides adenosine derivatives in wheat plants, in contrast to the dominating role of the latter compounds in animal tissue.

The present paper deals with the isolation and identification of the acid-soluble nucleotides of two other kinds of grain, namely oats and barley. Because of very marked similarities in nucleotide content the two plants are described together. From oats, the isolation in low yield of a nucleotide of low purity which appeared to be related to, but not identical with 5'-ADP, had been reported by Albaum and Umbreit [9].

The acid-soluble nucleotides were isolated by homogenizing the plant material in cold perchloric acid and freed from other phosphorus-containing or ultraviolet-absorbing substances according to the method previously elaborated. After adsorption on a column of 200 to 400 mesh strong base anion exchanger in the formate form, the individual nucleotides were eluted with

formic acid solutions containing increasing concentrations of sodium formate. Elution of the nucleotides was followed by the changes in optical densities of the eluate at 250, 260 and 270 mμ. The optical densities at 260 mμ of the effluent fractions were plotted against effluent volume to obtain the chromatographs shown in Figs. 1 and 2.

The elution position and the absorbance ratios gave a preliminary identification of the individual fractions. Since the nucleotides from the column were distributed in large volumes of formate buffer, it was desirable to concentrate them and remove the excess salt by readsorption on norite and elution 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 and ionophoretic behaviour with that of the corresponding authentic nucleotides. Further characterization of the nucleotide fractions was obtained by the following criteria: (a) ultraviolet absorption spectra at different pH values; (b) determination of total phosphorus content and of phosphorus content and phosphorus liberated after hydrolysis for 10 min with 1 N hydrochloric acid; (c) determination of the sugar content; (d) paper chromatographic identification of the sugars; (e) paper chromatographic behaviour of the bases after hydrolysis with acids; and (f) ultraviolet absorption curves of the products of hydrolysis.

The position of the phosphate groups was confirmed by oxidation with periodate and by the action of rattle snake venom. All these analyses have already been described in detail [8]. In addition, special analyses of certain nucleotides were undertaken, which are described later.

The quantities of the nucleotides were calculated by using molecular extinction coefficients at 260 mμ of 14 500 for the adenine nucleotides, 10 800 for the guanine nucleotides, 6 300 for the cytosine nucleotides, and 10 000 for the uracil nucleotides. The amounts of the different components are summarized in Table 1.

An amount of ultraviolet-absorbing material, representing about 2—4 % of the total absorbancy of the sample was not adsorbed on the column but was recovered in the wash water. This fraction contained nucleosides and free bases and as main components non-nucleotide material.

Peaks A, C and D appeared to contain the 2'- and 3'-phosphates of cytidine and adenosine, respectively, together with larger amounts of ultraviolet absorbing non-nucleotide material. These nucleotides could, however, easily be separated from interfering substances and estimated by paper chromatography or ionophoresis.

The main component of the sixth peak (F) comprising 60—80 % of the total nucleotide material of that peak was found to be TPN. The minor components behaved ionophoretically and chromatographically like CDP, 2'- and 3'-UMP and 2'- and 3'- GMP.

Analysis of the nucleotides of peaks G and H gave spectra similar to that of uridine; they contained 1 μmole of acid-stable and 1 μmole of acid-labile phosphorus per μmole of base. Treatment with acid at pH 2 during 5 min at 100°C led to the formation of UDP and acetylglucosamine and glucose, respectively. With stronger acid one phosphate group was split off leaving

Table 1. Amounts and identity of effluent fractions obtained on ion exchange chromatography of the acid-soluble nucleotides from barley and oat plants. The 2′- and 3′-phosphates were obtained in varying amounts and the maximum values are given.

Compound	Identity	μmoles/1 000 g of barley plants	μmoles/1 000 g of oat plants
A	2′- and 3′-CMP	11	13
B	5′-AMP	33	35
C	2′-AMP	4	7
D	3′-AMP	5	8
E	5′-UMP	37	38
F	2′- and 3′-UMP	4	9
F	2′- and 3′-GMP	6	4
F	TPN	9	12
F	CDP	3	4
G	UDPAG	14	12
H	UDPG	44	38
I	ADP derivative	3	
K	UDP	29	30
L	ADP	43	47
M	CTP	5	8
N	GDP	8	7
O	ATP derivative	4	6
P	UTP	18	26
R	ATP	40	76
S	GTP	7	8
Adenosine 5′-phosphates		123	164
Uridine 5′-phosphates		142	144
Guanosine 5′-phosphates		15	15
Cytidine 5′-phosphates		8	12

5′-UMP. The sugars were identified by chromatography in three solvent systems and by ionophoresis in borate buffer at pH 9.2. The sugar moiety of the substance in peak G gave the same spectrum as that from authentic UDPAG after treatment with dilute alkali and *p*-dimethylaminobenzaldehyde.

The unhydrolyzed samples from the two peaks behaved also ionophoretically and chromatographically like UDPAG and UDPG, respectively.

The acid-soluble nucleotides of barley included a small amout of an adenosine diphosphate derivative obtained as peak I, which followed closely UDPG. Both plant extracts contained another adenosine derivative, which could either be eluted between GDP and UTP or overlapped partly these fractions. The three substances could, however, be separated by ionophoresis at pH 4.15. The adenosine derivative contained 3 μmoles of phosphorus per μmole of base. A typical adenosine spectrum was obtained from both adenosine derivatives and no spectral change was observed after addition of cyanide. Paper chromatography of the base gave in both cases R_F values identical with that of adenine. Amounts sufficient for detailed structural studies have not been available yet of either substance.

The results of the present investigation establish further the occurrence in plant tissues of 5′-ribonucleotide derivatives of all the four bases present in ribonucleic acids. A preponderance of uracil derivatives besides those of adenine confirms previous findings on wheat plants. These nucleotides — without which metabolic processes would come to a standstill — are apparently involved in metabolism as coenzymes, as precursors of coenzymes and as building blocks for nucleic acids, and as such they may participate in the energy-requiring process of protein synthesis. Ever since Caputto *et al.* have shown that UDPG participates in the interconversion of D-glucose and D-galactose in yeast, it has become more and more apparent that the sugar nucleotides and specially 5′-UDP derivatives play a fundamental role in carbohydrate metabolism, and it is therefore according to expectations that these nucleotides are present or even dominate in plant tissues.

Only very small and varying amounts of the 2′- and 3′-monophosphates were detected in some experiments; they originate presumably from the degradation of ribonucleic acids. No quantitative estimation of DPN and TPN was attained with the present method; for the determination of these nucleotides other methods are available.

EXPERIMENTAL

Isolation and separation of acid-soluble nucleotides

Only the aerial portion of mature plants grown out of doors under normal field conditions, which had begun to fruit when harvested, were investigated. 1 000 g of fresh plants were homogenized in 3 000 ml of ice cold 10 % perchloric acid in a Waring Blendor and after re-extraction with 1 000 ml of 5 % perchloric acid the nucleotides were isolated and freed from disturbing substances according to the method[8] previously elaborated.

Fractionation of the isolated nucleotide mixture was carried out by ion exchange chromatography using Dowex 1, X-10, 200 to 400 mesh in the formate form. The individual nucleotides were eluted with increasing concentrations of formic acid or a mixture of formic acid and sodium formate. The eluting agents and the column size are seen from Figs. 1 and 2. The nucleotides were recovered from the pooled fractions by norite adsorption and elution with small volumes of 50 % aqueous ethanol containing 0.5 % ammonia.

Analytical methods

Ultraviolet absorption. The chromatographic fractions were analyzed by measurement of their light absorption at 250, 260 and 270 mμ in a Beckman spectrophotometer, Model DU, with photomultiplier. Each peak was also identified by determining its optical density over the range 220 to 320 mμ in acid and alkaline solution. The molar absorbancy and the absorbance ratios E_{250}/E_{260} and E_{270}/E_{260} were given in an earlier report [8].

Phosphate. Phosphorus was determined by the method of Allen [10] adapted to a total volume of 5.0 ml, and acid-labile phosphorus was determined after 10 min hydrolysis in 1 N hydrochloric acid at 100°C.

Pentose. Pentose estimations were carried out with the orcinol reagent according to Albaum and Umbreit [11]. The results were calculated by use of authentic samples as standards.

Acetylglucosamine. Mild acid hydrolysis of UDPAG liberated the hexosamine, which gave a positive Morgan-Elson reaction according to the modified test by Reissig *et al.*[12]

Periodate oxidation. Oxidation with periodate was performed according to Dixon and Lipkin [13].

Action of snake venom. The action of rattle snake venom was studied as previously described [8].

Paper chromatography

Whatman No. 1 filter paper was used, and the papers were in equilibrium with the solvent mixture before the runs were started. Standard substances were run in every chromatogram. The temperature was 20 to 22°C.

The nucleotides were located in ultraviolet light of 254 mμ photographically, eluted, and estimated spectrophotometrically. For paper chromatography of the nucleotides the following solvent systems were used: (I) *n*-propanol-ammonia-water (60:30:10); (II) ethanol-1 M ammonium acetate, pH 7.5 (75:30); (III) ethanol- 1 M ammonium acetate buffer, pH 3.8 (75:30); (IV) *iso*propanol-saturated ammonium sulphate solution-water (2:79:19); and (V) *iso*butyric acid-ammonia-water (66:1:33).

The bases obtained after hydrolysis by heating at 100°C for one hour in 1 N hydrochloric acid for the purines, and in 70 % perchloric acid for the pyrimidines, were identified with the following systems: (VI) *iso*propanol-hydrochloric acid (*d* 1.180) — water (65:17:18); (VII) *n*-butanol-acetic acid-water (4:1:5); and No. IV.

The sugar released on mild acid hydrolysis was identified by chromatography in the following solvents: (VIII) ethyl acetate-pyridine-water (2:2:1); (IX) phenol-water saturated at room temperature; and system No. VII. The positions of the sugar spots were demonstrated by spraying with aniline hydrogen phthalate or benzidine.

Paper ionophoresis

Ionophoresis on paper was performed with the technique described in the following paper [14]. Separation of the nucleotides was obtained using the acetate buffer of ionic strength 0.1 and pH 4.15. The sugars were studied in 0.1 M sodium tetraborate buffer at pH 9.2.

Materials

The nucleotides used as standard substances were obtained from Sigma Chemical Company.

This work has been supported by a grant from the *Swedish Natural Research Council*, and financial support for purchase of the reference substances has been obtained from *Sigma Chemical Company*.

REFERENCES

1. Caputto, R., Leloir, L. F., Cardini, C. E. and Paladini, A. C. *J. Biol. Chem.* **184** (1950) 333.
2. Park, J. T. and Johnson, M. *J. Biol. Chem.* **179** (1949) 585.
3. Albaum, H. G., Ogur, M. and Hirshfeld, A. *Arch. Biochem.* **27** (1950) 130.
4. Ginsburg, V., Stumpf, P. K. and Hassid, W. Z. *J. Biol. Chem.* **223** (1956) 977.
5. Buchanan, J. G., Lynch, V. H., Benson, A. A., Bradley, D. F. and Calvin, M. *J. Biol. Chem.* **203** (1953) 935.
6. Simonis, W. and Schwinck, L. *Naturwiss.* **40** (1953) 245.
7. Sebesta, K. and Sorm, F. *Chem. Listy*, **48** (1954) 1585.
8. Bergkvist, R. *Acta Chem. Scand.* **10** (1956) 1303.
9. Albaum, H. G. and Ogur, M. *Arch. Biochem.* **15** (1947) 158.
10. Allen, R. J. L. *Biochem. J. London* **34** (1940) 858.
11. Albaum, H. G. and Umbreit, W. W. *J. Biol. Chem.* **167** (1947) 369.
12. Reissig, J. L., Strominger, J. L. and Leloir, L. F. *J. Biol. Chem.* **217** (1955) 959.
13. Dixon, J. S. and Lipkin, D. *Anal. Chem.* **26** (1954) 1092.
14. Bergkvist, R. *Acta Chem. Scand.* **11** (1957) 1465.

Received June 5, 1957.