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The Acid-soluble Nucleotides of Wheat Plants

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A method is presented for the analysis and isolation of the acid-soluble nucleotides from plant tissue.

The application of this method to wheat plants resulted in the identification of the 5'-triphosphates of adenosine, guanosine, uridine and cytidine beside mono- and diphosphates. The identifications are based on ultraviolet absorption spectra and chromatographic evidence before and after hydrolysis, together with chemical and enzymic analysis.

The demonstration in recent years of the fundamental role of nucleoside polyphosphates in mechanisms of energy transfer and biosynthesis made it desirable to develop methods for their analysis and isolation from plant materials.

The first direct demonstration of the presence of nucleoside polyphosphates in plants originates from Albaum and Umbreit [1], who isolated from germinated oat seedlings an adenine-pentose-pyrophosphate in low yield and of low purity. This compound appeared to be related to, but not identical with 5'-ADP *.

Later, an ATP-like compound was isolated from mung bean sprouts [2], a material chosen because of its low content of polysaccharides, phytic acid and non-specific ultraviolet absorbing substances, which interfered with the isolation from other plant sources. The product obtained originally appeared to be similar to ATP from animal tissue but not identical with it, and was considered [3] to represent a polymerized form of ATP, which with appropriate treatment could be depolymerized to give a compound identical with the ATP found in other tissues.

Among the radioactive compounds observed when algae or green leaves are allowed to photosynthesize in $^{14}CO_2$ Buchanan [4] *et al.* detected a nucleotide fraction, and presented paper chromatographic evidence for the presence of 5'-AMP, 5'-UMP, UDPG, UDPgalactose, an adenosine-containing nucleotide other than ATP, ADP or AMP, and a mannose-containing compound chromatographically similar to a nucleotide.

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

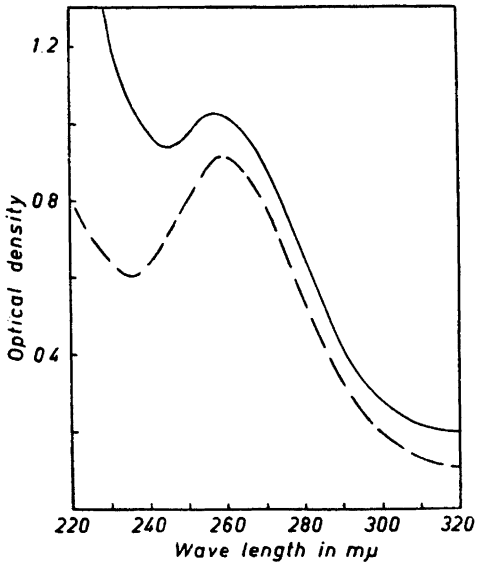

Fig. 1. Norite eluate before ———— *and after* — — — — *ether extraction.*

An ATP-like compound in chlorophyll-containing plants has been detected chromatographically by Simonis and Schwinck [5], and Sebesta and Sorm [6] have determined the ATP content of *Phaseolus vulgaris*.

The determination of the total acid-soluble nucleotide content in plants and isolation of the nucleoside polyphosphates in pure form has generally met with difficulties mainly because the procedures, which were found satisfactory for animal tissue, yielded unsatisfactory results when applied to plant materials [1-3].

The experience of previous authors in this direction was confirmed in the present investigation. Extracts prepared by homogenizing plant material from various sources in cold trichloroacetic or perchloric acids exhibited very complex ultraviolet absorption spectra, and no separation of the nucleotide fraction from other phosphorus-containing or ultraviolet-absorbing substances could be achieved by precipitation of the barium or mercury salts or by any other procedures of purification. Chromatography at this stage proved also impracticable. A crude separation of the nucleotide fraction from non-nucleotide phosphorus compounds could, however, be attained by adsorption on norite, a current method in the nucleotide field. The nucleoside derivatives from the acid extracts were quantitatively retained by norite, and could be recovered by repeated elution with 25 % aqueous ethanol containing 0.5 % ammonia.

Spectrophotometric analysis of the combined eluates showed that the nucleotides were mixed with a large quantity of non-nucleotide material. Precipitation with zinc or barium salts at this point yielded products containing less than 10 % nucleotides. Ion exchange chromatography was also unsuccessful, because of the multitude of ultraviolet absorbing, non-nucleotide fractions. Hence, further purification prior to chromatography was necessary. By the

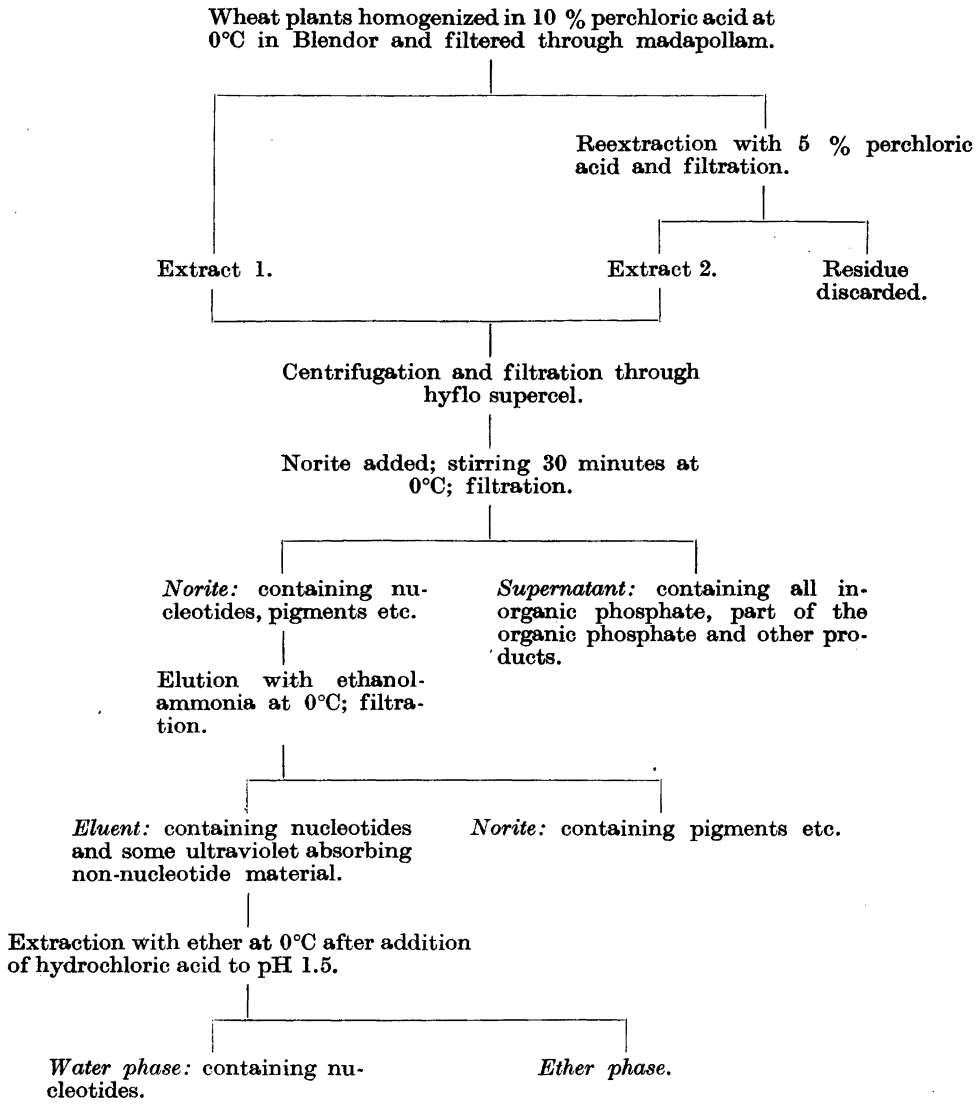

Fig. 2. Isolation of the acid-soluble nucleotide fraction from wheat plants.

addition of hydrochloric acid to pH 1.5 and extraction with ether for several hours the disturbing substances were removed from the norite-eluates. The absorption curves before and after extraction, shown in Fig. 1, indicate the effectiveness of this step in removing ultraviolet absorbing, non-nucleotide material.

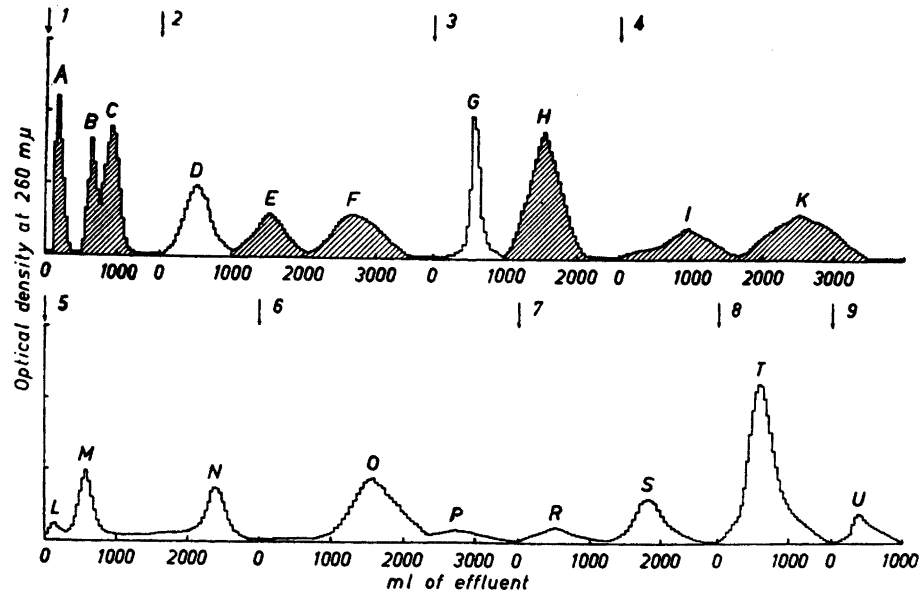

Fig. 3. Ion exchange chromatogram of acid-soluble nucleotides from 500 g of wheat plants. The crossed fractions varied in amounts in different preparations. Exchanger: Dowex 1, X-10, 200 to 400 mesh, 1.6 × 40 cm., formate form; flow rate 1.5 ml/min.

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* » » + *0.1 M* » »
5) *0.1 M* » » + *0.3 M* » »
6) *0.1 M* » » + *0.4 M* » »
7) *0.1 M* » » + *0.6 M* » »
8) *0.2 M* » » + *0.8 M* » »
9) *0.5 M* » » + *1.0 M* » »

By this method, the acid-soluble nucleotide fraction from various plant tissues could be prepared in sufficient purity for further fractionation by chromatography. In the present paper the application of this procedure to wheat plants is reported.

From wheat plants, which had grown out of doors under normal conditions and begun to form grains, only the aerial part was investigated. The acid-soluble nucleotide fraction was isolated as outlined in Fig. 2.

The individual nucleotide compounds were separated by ion exchange chromatography, and the method reported by Bergkvist and Deutsch [7] was used with slight modifications. Because of employing a longer column some changes in the eluting system were necessary.

Fig. 3 shows an ion exchange chromatogram of the acid-soluble nucleotides of wheat plants, as obtained from a formate column.

Table 1. R_F values of effluent fractions.

Solvent systems: (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);
(V) *iso*butyric acid-ammonia-water (66:1:33).
Descending chromatography was used in all systems.
Temperature 20 to 22°C.

Compound	R_F/R_F-5'-AMP	R_F			
	I	II	III	IV	V
B and *C*	1.14	0.17	0.38	0.70	0.39
2'- and 3'-CMP	1.14	0.17	0.38	0.70	0.39
D	1.00	0.14	0.32	0.32	0.44
5'-AMP	1.00	0.14	0.32	0.32	0.44
E	1.18	0.16	0.33	0.28	0.51
2'-AMP	1.18	0.16	0.33	0.28	0.51
F	1.18	0.16	0.33	0.17	0.51
3'-AMP	1.18	0.16	0.33	0.17	0.51
G	0.71	0.18	0.41	0.72	0.23
5'-UMP	0.71	0.18	0.41	0.72	0.23
H	1.00	0.23	0.47	0.70	0.28
2'- and 3'-UMP	1.00	0.23	0.47	0.70	0.28
I	0.69	0.12	0.28	0.54	0.26
2'-GMP	0.69	0.12	0.28	0.54	0.26
K	0.69	0.12	0.28	0.42	0.26
3'-GMP	0.69	0.12	0.28	0.42	0.26
M	0.71	0.23	0.26	0.75	0.24
UDPG	0.71	0.23	0.26	0.75	0.24
N	0.53	0.09	0.20	0.79	0.13
UDP	0.53	0.09	0.20	0.79	0.13
O	0.74	0.07	0.14	0.38	0.32
ADP	0.74	0.07	0.14	0.38	0.32
P	0.52	0.04	0.04	0.82	0.07
CTP	0.52	0.04	0.04	0.82	0.07
R	0.39	0.05	0.10	0.61	0.10
GDP	0.39	0.05	0.10	0.61	0.10
S	0.40	0.04	0.09	0.82	0.08
UTP	0.40	0.04	0.09	0.82	0.08
T	0.56	0.04	0.06	0.47	0.21
ATP	0.56	0.04	0.06	0.47	0.21
U	0.30	0.03	0.04	0.64	0.06
GTP	0.30	0.03	0.04	0.64	0.06

The elution positions and the absorbance ratios E_{250}/E_{260} and E_{270}/E_{260} gave a preliminary identification of the individual fractions.

Peak *A* contained a mixture of purine and pyrimidine bases, which were separated by cation exchange in an acid system according to Cohn [8]. The

Table 2. Analysis of effluent fractions.

Data on absorption spectra, phosphate and ribose content, and base. The chemical data are expressed in terms of moles per mole of base, as determined spectrophotometrically. The absorption spectra were determined in acid and alkaline solutions. The ribose content of the cytosine nucleotides could not be determined by the method of Albaum and Umbreit [15]. The bases were identified by paper chromatography.

Compound	Spectrum type	Phosphate per mole of base		Ribose per mole of base	Base
		Total	Labile		
B	Cytidine	1.00	0	—	Cytosine
C	Cytidine	1.01	0.	—	Cytosine
D	Adenosine	0.96	0	1.00	Adenine
E	Adenosine	1.00	0	1.00	Adenine
F	Adenosine	0.98	0	1.03	Adenine
G	Uridine	1.00	0	0.80	Uracil
H	Uridine	1.01	0	0.86	Uracil
I	Guanosine	0.97	0	1.00	Guanine
K	Guanosine	0.95	0	1.00	Guanine
L	Uridine	—	—	—	Uracil
M	Uridine	2.10	1.00	—	Uracil
N	Uridine	2.01	0.98	0.87	Uracil
O	Adenosine	2.00	1.00	0.97	Adenine
P	Cytidine	3.00	1.98	—	Cytosine
R	Guanosine	2.00	0.97	0.96	Guanine
S	Uridine	2.87	1.90	0.90	Uracil
T	Adenosine	3.00	2.00	0.92	Adenine
U	Guanosine	3.09	2.06	0.94	Guanine

bases adenine, guanine, uracil and cytosine were unequivocally identified by paper chromatography in different solvent systems.

The fractions corresponding to the following peaks were adsorbed on norite with subsequent elution by ethanol-ammonia. The concentrated solutions thus obtained were subjected to paper chromatography in comparison with the authentic products. The results are summarized in Table 1.

Beside elution position, absorbance ratios and paper chromatography, the ultraviolet absorption spectra at different pH values established further the identity of the different fractions.

Total and acid-labile phosphate as well as the pentose content were determined. After hydrolysis and paper chromatography in the *n*-butanol-acetic acid-water (4:1:5) system, the presence of ribose was revealed on development with aniline hydrogen phthalate.

In order to confirm the identity of the bases of the different purine and pyrimidine nucleotide fractions, the free bases were prepared by hydrolysis for one hour at 100°C, in 1 N hydrochloric acid and 70 % perchloric acid, respectively. R_F values identical with those of the corresponding authentic

products were obtained by paper chromatography in three different solvent systems. The ultraviolet absorption curves of the products of hydrolysis were likewise found to be identical with those of the corresponding bases. The results are summarized in Table 2.

Beside uridine and two phosphate groups peak *M* was found to contain glucose. Boiling in 0.01 N acid for 5 minutes liberated practically all the glucose and only a very small portion of the phosphate. After such a treatment uridine diphosphate and glucose could be identified as the breakdown products by paper chromatography and ionophoresis.

Uridine diphosphate glucose was closely associated with another UDP derivative (peak *L*), which on mild acid hydrolysis liberated UDP. This fraction was too small for more complete analysis.

The di- and triphosphates of adenosine were degraded to the monophosphate on heating the free acids for two hours to 100 °C. The uridine polyphosphates were hydrolysed for one hour at 100°C in 1 N hydrochloric acid, which resulted in the formation of the corresponding monophosphate together with orthophosphate, in agreement with the expected behavior of a pyrimidine nucleoside-5'-polyphosphate. Paper chromatography of the products of hydrolysis gave ultraviolet absorbing spots with the same R_F values as 5'-AMP and 5'-UMP, respectively, as seen from Table 3.

The location of the phosphate groups in the different nucleotides was established both by oxidation with periodate [9] and by the action of rattlesnake venom (*Crotalus adamanteus*). Treatment with the venom splits the pyrophosphate linkage between the phosphate group attached to ribose and the next phosphate [10]. The specific 5'-nucleotidase of the venom then splits off the phosphate from any 5'-monophosphate formed, yielding the nucleoside

Table 3. R_F values of the monophosphates obtained by hydrolysis of the different polyphosphates, in comparison with authentic samples.

Solvent systems: (I) ethanol-1 M ammonium acetate, pH 7.5 (75:30); (II) etanol-1 M ammonium acetate buffer, pH 3.8 (75:30); (III) *iso*propanol-saturated ammonium sulphate solution-water (2:79:19). Descending chromatography was used in all systems. Temperature 20 to 22°C.

Compound	R_F		
	I	II	III
AMP from peak *O*	0.14	0.32	0.32
AMP from peak *T*	0.14	0.32	0.32
5'-AMP	0.14	0.32	0.32
2'- and 3'-AMP	0.16	0.33	0.17; 0.28
UMP from peak *M*	0.18	0.41	0.72
UMP from peak *N*	0.18	0.41	0.72
UMP from peak *S*	0.18	0.41	0.72
5'-UMP	0.18	0.41	0.72
2'- and 3'-UMP	0.23	0.47	0.70

Table 4. Periodate oxidation and action of snake venom on effluent fractions.

Periodate oxidation. 0.50 μmole of potassium metaperiodate was added to approx. 0.30 μmole of the sodium salt of the nucleotide in a total volume of 3.0 ml. The amount of periodate consumed was measured at 227 mμ against a blank containing periodate of the same concentration. Reaction time was 60 minutes at 20°C.

Incubation with snake venom. 1 μmole of nucleotide in 1.0 ml of glycine buffer (pH 8.5) and 0.05 ml of 1 M magnesium chloride were treated with 2 mg of lyophilized *Crotalus adamanteus* venom in 0.2 ml of 0.2 % sodium chloride. The mixture was incubated for 60 min. at 38°C. Phosphate was estimated after addition of 0.1 ml of 70 % perchloric acid and centrifugation. The reaction product was isolated from the remaining solution by adsorption on norite and elution with ethanol-ammonia. The products were identified by paper chromatography.

Compound	μmoles of periodate consumed per μmole of nucleotide	μmoles of orthophosphate formed per μmole of nucleotide	Reaction product
B	0.14	0	2'-CMP
C	0.11	0	3'-CMP
D	0.99	0.91	Adenosine
E	0.12	0	2'-AMP
F	0.12	0	3'-AMP
G	1.00	0.90	Uridine
H	0.04	0	2'- and 3'-UMP
I	0.03	0	2'-GMP
K	0.08	0	3'-GMP
M	1.14	0.29	Uridine
N	0.92	0.77	Uridine
O	0.98	0.84	Adenosine
P	0.96	0.30	Cytidine
R	1.04	0.71	Guanosine
S	1.02	0.32	Uridine
T	1.00	0.34	Adenosine
U	0.97	0.20	Guanosine

and orthophosphate. The 2'- and 3'-monophosphates are resistent towards the enzyme and should be unaffected. The reaction products were examined by phosphate analysis and paper chromatography. The results appear in Table 4.

5'-ADP, 5'-ATP, 5-'UTP and 5'-GTP have been isolated as the barium salts; the analyses are shown in Table 5. The results correspond to preparations of about 90 % purity with the exception of GTP, the amount of which was too small for further purification.

As already briefly mentioned, some doubts have arisen in the literature concerning the structural identity of adenosine triphosphate encountered in plants with that from animal tissue. The evidence outlined above leaves little or no doubt about their identity, but as a further test, the reaction with glucose in the presence of hexokinase was studied. ATP from fraction *T* transferred

Table 5. Analysis of the isolated barium salts of nucleotides.

The barium salts were washed with ethanol and ether and dried *in vacuo* over phosphorus pentoxide to constant weight. The base concentrations were calculated from optical densities and ribose concentrations from the values of periodate oxidation. Theoretical values were calculated for barium salts of triphosphates and diphosphate containing 2 and 1.5 barium atoms per mole of nucleotide, respectively, and 4 moles of water per mole of nucleotide.

Compound	Component	Found μmoles/mg	Calculated μmoles/mg	Ratio component/base
ADP from peak *O*	Adenosine	1.18	1.30	
	Total P	2.36	2.60	2.00
	Labile P	1.18	1.30	1.00
	Ribose	1.16	1.30	0.98
ATP from peak *T*	Adenosine	1.05	1.17	
	Total P	3.15	3.51	3.00
	Labile P	2.10	2.34	2.00
	Ribose	1.05	1.17	1.00
UTP from peak *S*	Uridine	1.05	1.21	
	Total P	2.95	3.63	2.81
	Labile P	1.95	2.42	1.86
	Ribose	1.07	1.21	1.02
GTP from peak *U*	Guanosine	0.51	1.15	
	Total P	1.58	3.45	3.10
	Labile P	1.05	2.30	2.06
	Ribose	0.50	1.15	0.98

half of its labile-bound phosphate to glucose, exactly as animal ATP. Furthermore, the effect on the viscosity of actomyosin solutions [11] was identical for ATP from fraction *T* and ATP isolated from muscle.

The qualitative and quantitative analysis of the fractions corresponding to each peak of the chromatogram are summarized in Table 6.

The present work establishes the occurrence in wheat plants of all the four ribonucleosides in the form of acid-soluble 5'-mononucleotides at different levels of phosphorylation, in addition to their occurrence in nucleic acids and in analogy to previous findings on animal tissue.

Nearly 90 % of the acid-soluble nucleotide material in wheat plants consists of adenosine and uridine derivatives, present in practically equimolar amounts. The predominance of uridine, beside adenosine derivatives is of considerable interest and in contrast to the dominating role of adenosine derivatives in animal tissue. But whereas about half of the acid-soluble adenosine and guanosine nucleotides were isolated as the triphosphates, only little over 20 % of the uridine derivatives were found in the form of triphosphate. In the case of cytidine, the triphosphate was the only derivative obtained; neither 5'-CMP and CDP nor 5'-GMP could be detected in any of the experiments. It is probable, that during further isolation work additional nucleotides will be found to occur in small amounts.

Table 6. Amounts and identity of effluent fractions obtained on ion exchange chromatography of the acid-soluble nucleotides from wheat plants. The second column give sthe maximum amounts of 2'- and 3'-phosphates.

Compound	Identity	μmoles/1000 g of wheat plants	μmoles/1000 g of wheat plants
B	2'-CMP		80
C	3'-CMP		80
D	5'-AMP	35	
E	2'-AMP		35
F	3'-AMP		55
G	5'-UMP	44	
H	2'- and 3'-UMP		135
I	2'-GMP		44
K	3'-GMP		89
L	UDPX	4	
M	UDPG	27	
N	UDP	52	
O	ADP	49	
P	CTP	14	
R	GDP	14	
S	UTP	35	
T	ATP	72	
U	GTP	15	
Uridine derivatives		162	135
Adenosine derivatives		156	90
Guanosine derivatives		29	133
Cytidine derivatives		14	160

In addition to nucleoside-5'-polyphosphates, varying amounts of the 2'- and 3'-monophosphates were obtained in some experiments. The fact, that nucleoside 2'- and 3'-derivatives were only encountered in some and not detectable in other experiments, together with the absence of 2'- and 3'-di- and triphosphates in all experiments, makes it possible that the 2'- and 3'-nucleotides originated from the degradation of ribonucleic acid during preparation.

The absence of diphosphopyridine and triphosphopyridine nucleotides in the nucleotide fraction, prepared from wheat plants by the present method, is due to the fact that these compounds, although present in the original acid extract and adsorbed on norite, are not eluted appreciably with ethanol-ammonia. For the isolation of DPN and TPN elution of the norite with pyridine-water is more effective, although this eluent proved unsatisfactory for the isolation of the main bulk of the acid-soluble nucleotides. DPN and TPN have been previously isolated from wheat germ [12] and from other plant materials [13].

EXPERIMENTAL

Isolation of the nucleotide fraction

1 000 g of fresh wheat plants were homogenized in 3 000 ml of ice cold 10 % perchloric acid in a Waring Blendor. The precipitated proteins and fiber masses were separated from the extract by squeezing through madapollam. The residue was re-extracted in the Waring Blendor with 1 000 ml of 5 % perchloric acid and again pressed through madapollam. The combined extracts were further freed from suspended particles by centrifugation and then filtered through hyflo supercel on a Büchner funnel.

The clear, slightly yellow solution was treated with 30 g of norite. The suspension was shaken or stirred vigorously for 30 minutes and then filtered with suction. Experiments with various amounts of norite demonstrated, that under these circumstances all nucleotides were adsorbed on the norite. The norite was washed with water and then dried by suction.

The elution of the nucleotides from the norite was investigated with a number of solvent systems. An aqueous solution containing 25 % ethanol and 0.5 % concentrated ammonia proved to be the most satisfactory for the quantitative elution of the nucleotides with a minimum of disturbing substances. Therefore the norite was suspended in 150 ml of this mixture. The suspension was stirred vigorously in the cold for 30 min. Then the norite was filtered off and the elution repeated. Each fraction was collected in the cold and immediately adjusted to pH 7 with hydrochloric acid. After 5 to 6 extractions no more ultraviolet-absorbing material could be eluted.

The combined fractions were acidified with hydrochloric acid to pH 1.5, and the remaining disturbing substances were removed by continuous liquid-liquid extraction with ether for five hours. The extraction apparatus of Kutscher and Steudel was used, and the solution was cooled with ice during extraction. Thereafter the aqueous phase was freed from the last traces of ether by aeration. Phosphorus analysis before and after extraction showed that no decomposition had occurred. The aqueous phase could now be subjected to ion exchange chromatography.

Ion exchange chromatography

The strong base anion exchange resin Dowex 1, X-10, 200 to 400 mesh, was used. After freeing from fine and coarse particles by sedimentation the resin was converted to the formate form with a mixture of 2 M formic acid and 2 M sodium formate.

A column of 1.6 × 40 cm was supported in a glass tube by a constriction covered with glass wool. The resin was poured as an aqueous slurry and allowed to settle. The column was washed with a large quantity of water until the pH of the effluent was about 7. The column was fed with eluting agent through glass tubing from a container about 1.5 meters above the column.

The nucleotide fraction was put on the column from dilute ammoniacal solution at about pH 10 at the rate of 1.5 ml per min. After washing with water the eluting agents shown in Fig. 3 were used. Fractions of 35 ml were taken with an automatic fraction collector adjustable to different time intervals. Appropriate fractions were pooled and adsorbed on norite (10 mg of norite / μmole of nucleotide), and after washing with water the norite was eluted with small amounts of 50 % aqueous ethanol containing 0.5 % ammonia.

The column could be used several times except for a small layer at the top, which darkened during the run and was replaced each time by fresh resin.

Analytical methods

Ultraviolet absorption. Optical densities were measured in a 1.00 cm quartz cuvette in a Beckman Universal Spectrophotometer, Model DU, with photomultiplier. The course of the elution was followed by measurement of the optical densities of the fractions of chromatogram at 250, 260 and 270 mμ with the suitable eluting agents as the blank. The densities at 250 and 270 mμ assisted in interpretation of the chromatogram since the ratios of the readings E_{250}/E_{260} and E_{270}/E_{260} are characteristic of the various nucleotides.

Table 7. Molar absorbancy and absorbance ratios at pH 1 of the different nucleotides. The values for the polyphosphates followed those obtained for the corresponding monophosphates very closely.

Compound	$a_M \times 10^{-3}$	Absorbance ratios	
		250/260	270/260
Adenosine phosphates	14.5	0.84	0.71
Guanosine phosphates	10.8	0.93	0.73
Cytidine phosphates	6.3	0.47	1.71
Uridine phosphates	10.0	0.76	0.87

The optical densities at 260 mμ of the effluent fractions were plotted against effluent volume to obtain the chromatograph shown in Fig. 3.

The molar absorbancy a_M at 260 mμ and the absorbance ratios in acid solution are shown in Table 7. Each peak was also identified by determining its optical density over the range 220 to 320 mμ in both acid and alkaline solution, compared with authentic samples.

Phosphate. Inorganic phosphate was determined by the method of Allen[14] adapted to a total volume of 5.0 ml (4.0 ml of aqueous sample, 0.40 ml of 70 % perchloric acid, 0.40 ml of amidol-reducer and 0.20 ml of 8.5 % ammonium molybdate). The color was read 15 minutes after mixing, in a Klett-Summerson photoelectric colorimeter with a red filter.

The acid-labile phosphate was determined from the increase in inorganic phosphate after 10 minutes in 1 N hydrochloric acid at 100°C.

For total phosphate, the sample was digested with 0.40 ml of 70 % perchloric acid at 250°C and after cooling the inorganic phosphate was determined without further addition of acid.

Pentose. All fractions were analyzed for pentose with the orcinol reagent according to Albaum and Umbreit[15]. To the sample in 3.0 ml of water, 3.0 ml of 0.1 % ferric-chloride in concentrated hydrochloric acid and 0.3 ml of a reagent, containing 0.4 % orcinol in 95 % ethanol, were added. The reaction tubes were heated for 45 min. in a boiling water bath, cooled, diluted to a total volume of 10.0 ml and read in a colorimeter with a red filter. The results obtained were calculated by use of authentic samples as standards. The values for the uridine nucleotides were very small and therefore uncertain, and the cytidine nucleotides did not give any color.

Paper chromatography

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

The nucleoside derivatives were located by making a contact print in monochromatic ultraviolet light of 254 mμ. For quantitative estimations, spots were cut out and eluted with 0.1 N hydrochloric acid. Standing for a few hours with occasional shaking effected quantitative elution. To allow for ultraviolet absorbing substances in the paper, blanks were cut, equal in area and at equal distances from the starting line, and were eluted and read at the same wave-lengths as the corresponding spots. Aniline hydrogen phthalate was used to demonstrate the position of the sugar spots on the paper chromatograms.

The nucleotides were hydrolysed to the free bases by heating at 100°C for one hour in 1 N hydrochloric acid for the purines, and in 70 % perchloric acid for the pyrimidines. Paper chromatography of the bases was carried out with the following systems: a) *iso*-propanol-hydrochloric acid (*d* 1.180)-water (65:17:18); b) *n*-butanol-acetic acid-water (4:1:5); c) *iso*propanol-saturated ammonium sulphate solution-water (2:79:19).

For paper chromatography of the sugars, the butanol-acetic acid-water system, already described, was used, in addition to ethyl acetate-pyridine-water (2:2:1).

Paper ionophoresis

Ionophoresis on paper was performed with a technique described by Markham and Smith [16] where the paper is kept cold by immersion in carbon tetrachloride. The substances were applied near one end of a long strip of Whatman No. 1 paper (6×50 cm). It was found convenient to wash the paper with 1 N hydrochloric acid and water before use. The carbon electrodes were connected to a d.c. power supply of 900 V thus giving a voltage gradient of about 20 V/cm. Under these conditions a run of 3 h gave good separations with the different buffers used. In ionophoretic as well as in chromatographic experiments, the position on the paper was compared with the position of the authentic substances.

The 5'-phosphate esters of any ribose nucleoside possess one characteristic by means of which they may be separated from the 2'- and 3'-esters of the same nucleoside: the presence of two adjacent hydroxyl groups in the ribose moiety, which can give complexes with boric acid. In 0.1 M sodium tetraborate buffer at pH 9.2 the mobility of the 5'-nucleotide is faster than that of the corresponding 2'- and 3'-nucleotides. The sugars could also be separated with the same buffer.

Separation of mono-, di- and triphosphates was obtained using an acetate buffer of ionic strength 0.1 at pH 5.

Action of snake venom

The estimation was performed as shown in Table 4. Phosphate was determined as described above, but the blue color was read in the colorimeter first after extraction with amyl alcohol, because of interference by magnesium otherwise.

The reaction products were adsorbed on norite and then eluted with small volumes of ethanol-ammonia. The concentrated solutions thus obtained were analyzed by paper chromatography. The solvent systems used were: *iso*propanol-saturated ammonium sulphate solution-water (2:79:19) and 95 % ethanol-1 M ammonium acetate buffer; pH 3.8 (75:30).

Periodate oxidation

Oxidation with periodate was performed according to Dixon and Lipkin [9].

Hexokinase reaction

This reaction was performed according to Colowick and Kalckar [17], and the reaction was followed by the disappearance of the acid-labile phosphate.

0.1 ml of the ATP-solution (10 μmole/ml), 0.1 ml of 0.5 M magnesium sulphate solution, 0.5 ml of 0.04 M sodium bicarbonate solution (pH 7.5), and 0.1 ml of 0.3 M glucose were combined and warmed to 30°C. 0.1 ml of dilute enzyme (10 mg/ml) was added and the mixture was incubated at 30°C for 30 minutes.

The reaction was stopped by the addition of 0.4 ml of 70 % perchloric acid. After centrifugation the acid-labile and total phosphates were determined. The values were compared with similar analyses of a mixture at zero time. Identical results were obtained with ATP from wheat plants and animal tissue.

Effect on actomyosin

The reaction was followed by viscosity measurement in Ostwald viscosimeters at 21°C (time of flow for water, about 40 sec.). The viscosimeters contained 3.5 ml of actomyosin solution in 0.5 M potassium chloride with a protein concentration of approximately 3 mg/ml. 0.4, 0.8 and 1.6 μmoles of triphosphate were added in volumes of 25, 50 and 100 μl.

Materials

The mono-, di- and triphosphates used as standard substances were isolated from muscle as previously described [18,19]. GTP and hexokinase were obtained by courtesy of Sigma Chemical Company.

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