A Comparative Investigation of the Acid-Soluble Nucleotides of *Penicillium daleae* and *Penicillium urticae*

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This investigation was undertaken to determine the nucleotide composition of some moulds grown on synthetic medium and to compare the results with one another and with those obtained from investigations on other fungi grown out of doors under natural field conditions. The comparison between the two *Penicillium* species chosen was thought to be especially interesting as the moulds show entirely different production pictures, *P. daleae* excreting primarily kojic acid, *P. urticae* on the other hand only aromatic compounds.

The method of isolation of the nucleotides included extraction of the mycelia with dilute perchloric acid in the cold. The extract was freed from accompanying impurities through an adsorption procedure (charcoal) and then extraction with diethyl ether. The individual nucleotides of the mixture purified in this way were separated by ion exchange chromatography followed by paper chromatography and/or ionophoresis. Further identification of the isolated nucleotides was carried out by means of ultra-violet absorption measurements, paper chromatography, ionophoresis and chemical and enzymic analyses.

About thirtythree different nucleotides were isolated, most of which were fully identified. A 2'- or 3'-UMP-peptide complex not previously described was found in the *P. daleae* extract. The nucleotide composition of *P. daleae* was found to be very similar to that of *P. urticae*, and to those of fungi previously investigated by others. These results are discussed.

During the course of studies in this laboratory on acid-soluble nucleotides of plant materials¹⁻⁴ it was thought to be of interest to investigate some moulds, grown on synthetic medium, with respect to their contents of acid-soluble nucleotides. *Penicillium daleae* (Zalesni) and *Penicillium urticae* (Bainier) R-989 were chosen because a second purpose of this investigation was to see whether the differences in the production spectra of these moulds (see below) were determined by differences in their nucleotide contents. Significant

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differences in the amounts of a nucleotide or simply a new nucleotide derivative in one of the moulds could be the sign of a demand for this nucleotide as co-enzyme in a step in the intermediary metabolism of one of the produced substances.

P. daleae mycelium growing on Czapek-Dox medium at 28°C in the dark will produce large amounts of kojic acid, which is found in the medium (predominantly), but no aromatic compounds. *P. urticae* mycelium, on the other hand, growing under the same conditions, will produce aromatic compounds (*e.g.* 6-methyl-salicylic acid) but no kojic acid or other pyrones. Investigations on the biosynthesis of these materials with the help of ^{14}C -acetate and ^{14}C -glucose have shown that kojic acid is synthesized from glucose, presumably with the carbon chain intact, 6-methyl-salicylic acid on the other hand *via* acetate.⁵⁻⁸

In this connection it must be stressed that certain co-enzymes, such as NAD, NADP, FAD, and CoA, cannot be isolated with the method used in this paper.* Further, in a comparative study like this, the variations of biological materials (even from the same source) must be born in mind.

The generally accepted methods for extraction of nucleotides often fail when plant materials are involved. This depends on the very complex composition of plant tissues in general. Thus, nucleotide extracts from plant tissues

Table 1. The eluting agents used for the ion exchange chromatography.

Symbol	Composition	
	Formic acid	Sodium formate
A	0.02 M	—
B	0.05 M	—
C	0.1 M	—
D	0.1 M	+ 0.05 M
E	0.1 M	+ 0.1 M
F	0.1 M	+ 0.2 M
G	0.1 M	+ 0.3 M
H	0.1 M	+ 0.4 M
I	0.1 M	+ 0.6 M
K	0.2 M	+ 0.8 M
L	0.5 M	+ 1.0 M
M	1.0 M	+ 1.0 M

Table 2. Molar extinction (ϵ) and extinction ratios at pH 1 for the nucleosides, and the corresponding nucleotides.

Nucleoside	$\epsilon \times 10^{-3}$ at 260 m μ	Extinction ratios	
		E_{250}/E_{260}	E_{270}/E_{260}
Adenosine	14.5	0.84	0.70
Inosine	7.2	1.68	0.56
Guanosine	10.8	0.93	0.73
Uridine	10.0	0.76	0.87
Cytidine	6.3	0.47	1.71

* All abbreviations in this paper are in accordance with the recommendations in *Report of the Commission on Enzymes of the International Union of Biochemistry* (Pergamon Press, London 1961).

generally contain a variety of organic acids, pigments and other substances (many of which are U.V. light-absorbing), not or to a lesser extent existing in animal tissues. If nucleotide extracts are not freed from most of these substances before the separation of the individual nucleotides (by ion exchange chromatography, other chromatographic methods and/or ionophoresis), most of the nucleotides will "disappear" in the "background" extinction caused by these concomitant non-nucleotide substances.* However, with the method described by Bergkvist it is possible in most cases to obtain extracts sufficiently pure for further separation of the individual nucleotides.¹

The two moulds investigated in this work behaved as troublesome plant material in general. Thus, the nucleotide fractions obtained by ion exchange chromatography of the purified extracts had to be separated further by large scale paper chromatography and/or paper ionophoresis. Fig. 3 demonstrates the complexity of some of the fractions. A third mould, *Penicillium islandicum* (Sopp), originally intended to be included in this investigation, had to be excluded as all purification methods tested (and numerous modifications) failed to give sufficiently pure extracts.

The ion exchange chromatography system used was a modification of the system reported by Bergkvist and Deutsch.⁹ In addition to the preliminary identification obtained from the elution positions the following criteria and analyses established the identity of the isolated nucleotides. (1) Paper chromatography in the systems I, II, III, and IV (see Table 3) and paper ionophoresis at pH 4.15 in comparison with authentic samples. (2) Indication of the nature of the riboside component by registration of the U.V. light absorption spectra between 220 and 320 m μ at acid and alkaline pH values. (3) Quantitative determination of the contents of total and acid-labile phosphate per mole of nucleotide. (4) Confirmation of the presence of ribose by paper chromatography in the systems V and VI after acid hydrolysis. (5) Quantitative determination of the pentose contents. (6) Determination of the base component of the nucleotides after acid hydrolysis, by means of paper chromatography in the systems VI and VII and U.V. light absorption spectra at acid and alkaline pH values. (7) Enzymic and chemical establishment of the position of the phosphate group on the ribose moiety of the nucleotide. The liberation of

Table 3. Solvent systems for paper chromatography.

Symbol	Composition
I	propanol-conc.ammonia-water (60:30:10)
II	96 % ethanol-1 M ammonium acetate buffer, pH 7.5 (75:30)
III	isobutyric acid-conc.ammonia-water (66:1:33)
IV	isopropanol-saturated ammonium sulphate solution-water (2:79:19)
V	butanol-glacial acetic acid-water (40:10:50), the upper phase
VI	butanol, saturated with water
VII	isopropanol-conc.hydrochloric acid-water (97:25:28)
VIII	phenol, saturated with water and containing 0.03 % 8-hydroxyquinoline

* The localization of the nucleotides in the chromatograms and on ionophoresis is based on U.V.light-absorption measurements. However, the same statement is valid for other analytical methods, all of which are more laborious and less sensitive than the optical method (e.g. phosphate analysis).

phosphate from a nucleotide by the action of lyophilized venom from the rattlesnake *Crotalus adamanteus* was taken as evidence of the presence of a 5'-phosphate.^{1,10} No action indicated the presence of a 2'- or 3'-phosphate. If the two vicinal 2'- and 3'-hydroxy groups in the ribose moiety of a nucleotide both are free, they may be oxidized by periodate. Thus, if upon treatment with periodate this is consumed, the phosphate most probably is attached to the ribose molecule at the 5'-position. If no periodate is consumed, one or both of the vicinal hydroxy groups is blocked, most probably by a phosphate ester bond. (8) Identification of the split products of UDP-glucose, UDP-2-acetyl-amino-2-deoxy-D-glucose, GDP-mannose and related substances after very mild acid hydrolysis. The hexoses were identified by paper chromatography in the systems V, VI, and VIII and by paper ionophoresis in 0.1 M borate buffer at pH 9.2. The nucleotide diphosphates were identified according to point (1) above.

RESULTS

The results of the investigation are tabulated in Tables 4 and 5 and are commented below. See also Figs. 1 and 2.

The nucleotides in most cases appeared as "peaks" in the ion exchange chromatogram; often more than one nucleotide were found in the same "peak" or fraction, cf. Fig. 3. All nucleotides except 2'- and 3'-GMP appeared in the

Fig. 1. Ion exchange chromatogram of acid-soluble nucleotides from 2300 g of *Penicillium daleae* mycelium. Exchanger: Dowex 1, X-10, 200-400 mesh, formate form. Dimensions of the column: Length = 120 cm, diameter = 2.1 cm. Flow rate: 2.7 ml/min. Eluting agents: See Table 1.

Fig. 2. Ion exchange chromatogram of acid-soluble nucleotides from 575 g of *Penicillium urticae* mycelium. Exchanger: Dowex 1, X-10, 200-400 mesh, formate form. Dimensions of the column: Length = 120 cm, diameter = 2.1 cm. Flow rate: 2.7 ml/min. Eluting agents: See Table 1.

expected succession in the ion exchange chromatograms. 2'- and 3'-GMP usually appear before UDP-2-acetyl-amino-2-deoxy-D-glucose. All other criteria, however, guarantee the identity of these substances.

Fraction 2 contained a cytidine derivative which gave rise to 5'-CMP after hydrolysis in 1 M HCl at 100°C for one hour.* Hydrolysis in 0.1 M HCl at 100°C for 30 min did not affect the substance appreciably. R_{FII} was 0.20 if R_{FI} for 5'-CMP was 0.14. (R_{FI} means the R_F value in system I etc., see Table 3.) The velocity in ionophoresis at pH 4.15 was 0.8 of the velocity of 5'-CMP. The small amounts of the substance did not permit a more complete investigation. It was not found in *P. urticae*.

Between 5'-AMP and 2'-AMP (fractions 15 and 9') in the chromatograms a cytidine derivative appeared which probably is identical with the 2'- or 3'-CMP-peptide complex previously found by Bergkvist in *Polyporus squamosus*.¹¹ The following data support this view: (1) The U.V. spectra of the substance were identical with those of cytidine nucleotides. (2) U.V. spectrophotometry and paper chromatography of the base established cytosine. (3) Phosphate analysis revealed the nucleotide as a stable monophosphate. (4) After hydrolysis in 0.05 M HCl at 100°C for 30 min 2'- and 3'-CMP were established by paper chromatography in the systems II and IV and by paper ionophoresis at pH 4.15. (5) Periodate was not consumed by the substance. (6) After hydrolysis in 6 M HCl at 120°C for 24 h and subsequent two-dimensional paper chromatography in the systems VIII and V the presence of the amino acids leucine, valine, alanine, glycine, glutamic acid, and aspartic acid was indicated.

A uridine derivative which resembled the CMP-peptide complex strikingly appeared immediately after ADP (fraction 38; see also Fig. 3). This substance was not found in *P. urticae*. It was investigated in the same way as the CMP-peptide complex and was found to be a derivative of 2'- or 3'-UMP containing a peptide. The two-dimensional paper chromatogram of the hydrolyzed UMP-

* Fraction figures without stress-marks refer to *P. daleae* and fraction figures with stress-marks to *P. urticae*.

Table 4. The nucleotides isolated from *Penicillium daleae* mycelium; see Fig. 1.

Nucleotide	$\mu\text{mole}/1000 \text{ g of}$ mycelium, fresh weight	Found in fraction No.	Eluting agent
Cytidine derivative ($R_{FII} = 0.20$) *	1.6	2	A
5'-CMP	0.9	4	»
2'-CMP	20.5	7	»
3'-CMP	28.4	9	»
5'-AMP	22.8	14	C
CMP-peptide	12.4	15	»
2'-AMP	13.0	16	»
3'-AMP	24.0	20	D
5'-UMP	68.3	22	»
2'- and 3'-UMP	42.7	24 and 25	»
5'-IMP	0.6	25	»
CDP	3.5	26	E
Adenosine derivative ($R_{FIV} = 0.14$; $R_{FII} = 0.15$)	0.3	26	»
5'-GMP	2.5	28	»
UDP-2-acetylamino-2-deoxy-D- glucose	118.0	29 and 30	»
UDP-glucose	4.4	31	F
2'-GMP	3.1	32	»
UDP	32.6	34	H
3'-GMP	18.5	34-36	»
Cytidine derivative ($R_{FII} = 0.02$)	4.0	35 and 36	»
GDP-mannose	2.5	36	»
ADP	45.5	36-38	»
CTP	3.9	38	»
Uridine derivative ($R_{FII} = 0.10$)	5.9	38	»
Adenosine derivative ($R_{FII} = 0.025$)	4.0	38	»
GDP	7.2	41	I
UTP	3.7	42	»
Adenosine derivative ($R_{FIV} = 0.06$)	4.0	40-43	»
Adenosine derivative ($R_{FIV} = 0.17$)	7.0	40-43	»
ATP	66.3	44	K
Adenosine derivative (Mobility in ionophoresis at pH 4.15 = 1.1 $\times$ mobility of ATP)	1.0	45 and 46	»
GTP	13.9	47	L

peptide complex seemed to be identical with that of the hydrolyzed CMP-peptide complex.

Comparison between some data for the CMP-peptide and UMP-peptide complexes:

R_{FII} for the UMP-pept. compl. = 0.10; R_{FII} for UDP = 0.09.

» » » CMP- » » = 0.07; » » CDP = 0.07.

The velocity for the UMP-peptide complex in ionophoresis was 1.2 times higher than the velocity for 2'- or 3'-UMP. The corresponding figure for the corresponding cytidine derivatives was 1.3. The biochemical function of these

* Abbreviations: R_{FI} means the R_F -value in system I, etc; see Table 3.

Fig. 3. Contact prints in U.V. light of large scale paper chromatograms of the ion exchange fractions Nos. 34, 35, 36, and 38 (*P. daleae*; cf. Fig. 1). Amounts chromatographed: 59.0, 62.2, 69.0, and 61.3 optical units at 260 $m\mu$ of the fractions, respectively. Paper: Whatman No. 1, 45 $\times$ 57 cm. Solvent system: No. II, cf. Table 3. Running time: About 90 h. Type of paper chromatography: Descending, outflowing (The lower edge of the paper was cut in a saw-tooth like pattern to allow uniform dripping from the paper). Temperature: 20°C.

The R_{FII} values for the authentic substances: UDPAG (= UDP-2-acetyl amino-2-deoxy-D-glucose): 0.29; 5'-AMP: 0.14; 5'-GMP: 0.10; UDP: 0.09; CTP: 0.04.

Identity and R_{FII} values of the separated substances: 34a = 3'-GMP (0.12), 34b = UDP (0.09), 35a = 3'-GMP (0.12), 35b = Cytidine derivative (0.02). 36a = GDP-mannose (0.14), 36b = 3'-GMP (0.12), 36c = ADP (0.07), 36d = Non-nucleotide substance, 36e = Cytidine derivative (0.02). 38a = Uridine derivative (0.10), 38b = ADP (0.07), 38c = CTP (0.04), 38d = Adenosine derivative (0.025).

substances is obscure. They are hardly involved in the synthesis of pyrones or aromates.

The other unidentified nucleotide derivatives were not investigated.

Fraction 44 which was ATP appeared with a "shoulder"; see Fig. 1. Both parts of the "peak" were ATP. The phenomenon is not uncommon.

The adenosine derivatives in the fractions 26, 40–43, and 45–46 are probably identical with those in the fractions 14', 21'–23', and 25', respectively. Further, paper chromatography of the *P. daleae* fractions 32–33 and 35–36 indicates the presence of small amounts of U.V. light-absorbing substances which might be identical with the adenosine derivatives in the fractions 17' and 19'. The qualitative differences between the two moulds were thus confined to the cytidine derivatives of fractions 2 and 35–36, the uridine derivative of fraction 38, and the adenosine derivative of fraction 38, 5'-CMP and CDP of fractions 4 and 26, respectively, left out of account (*cf.* the introductory remarks of the discussion).

The non-nucleotide substances which contaminated the purified nucleotide extracts appeared in the ion exchange chromatograms partly as a more or less even "background" (*cf.* the introduction) and partly as well-

Table 5. The nucleotides isolated from *Penicillium urticae* mycelium; see Fig. 2.

Nucleotide	$\mu\text{mole}/1000 \text{ g of}$ mycelium, fresh weight	Found in fraction No.	Eluting agent
2'- and 3'-CMP	1.2	6'	C
5'-AMP	5.4	8'	»
CMP-peptide	0.8	9'	»
2'-AMP	0.7	10'	D
3'-AMP	1.3	10'	»
5'-UMP	25.0	12'	E
2'- and 3'-UMP	2.3	14'	F
5'-IMP	0.8	14'	»
Adenosine derivative ($R_{FIV} = 0.14$; $R_{FII} = 0.15$)	1.3	14'	»
UDP-2-acetylamino-2-deoxy-D- glucose	112.0	15'	G
UDP-glucose	14.3	16'	»
2'-GMP	0.2	17'	H
Adenosine derivative ($R_{FII} = 0.21$)	0.4	17'	»
UDP	8.2	18'	I
Adenosine derivative ($R_{FII} = 0.21$)	0.5	19'	K
3'-GMP	1.1	19'	»
GDP-mannose	1.5	19'	»
ADP	12.0	20'	»
CTP	2.2	20'	»
UTP	5.4	22'	»
GDP	1.8	23'	»
Adenosine derivative ($R_{FIV} = 0.06$)	0.7	21'–23'	»
Adenosine derivative ($R_{FIV} = 0.17$)	0.7	21'–23'	»
ATP	39.0	24'	L
Adenosine derivative (Mobility in ionophoresis at pH 4.15 = 1.1 $\times$ (mobility of ATP))	1.0	25'	»
GTP	4.9	26'	»

defined "peaks". Among these substances kojic acid (fraction 1) and benzoic acid (both moulds) were identified. Benzoic acid was found in small amounts in all fractions eluted with the buffer solutions B to I. The acids were identified by paper chromatography in the systems II and IV, ionophoresis at pH 4.15, and by their U.V. light-absorption spectra in acid and alkaline solution. Several fractions (5, 13, 15, and 39, none of which contained phosphate), whose U.V. spectra in acid and alkaline solution were closely related and similar to those of kojic acid, appeared in the *P. daleae* chromatogram. This chromatogram contained also other homogeneous "peaks" consisting of non-nucleotide substances, viz., 17, 19, and 21. The fractions 2, 3, 46, and 48 were very complex. They probably contained only non-nucleotide substances. The *P. urticae* chromatogram contained only one homogeneous non-nucleotide "peak", viz. the first part of fraction 16'. Among the most complex fractions, attention is directed especially to 1', 2', and 3' as they nevertheless appeared as well shaped "peaks".

DISCUSSION

When the results of a comparative study like this are discussed, it must be born in mind that artefacts, in spite of carefulness, may arise during the purification and isolation procedures. (The substances are rather sensitive to both acid and alkaline treatment which they encounter in the experimental procedures, though at low temperature.) For example, at least a part of the mono- and diphosphates may have been formed by hydrolysis of the corresponding di- and triphosphates and diphosphate derivatives as UDP-glucose and GDP-mannose. Further, it cannot be excluded that small amounts of a nucleotide may be overlooked in very complex fractions. This may be the reason why some nucleotides found in small amounts in one of the moulds were lacking in the other.

The results of this investigation are strikingly similar to those of the investigations of Bergkvist on the nucleotide compositions of some mushrooms.^{3,4} Qualitatively the composition is nearly the same. The differences are restricted to some of the nucleotides which were found in very small amounts, e.g., the UMP-peptide complex and IMP, and hence they are not significant. Quantitatively the results are in agreement. Thus, the contents of uridine and adenosine phosphates are much higher than those of guanosine and cytidine phosphates. Furthermore, uridine phosphates are always in excess over adenosine phosphates, and guanosine phosphates over cytidine phosphates.

Nucleotides	μ Moles of nucleotides/1000 g of mycelium, fresh weight	
	<i>P. daleae</i>	<i>P. urticae</i>
Adenosine-5'-phosphates	135	57
Inosine-5'-phosphates	1	1
Guanosine-5'-phosphates	26	8
Uridine-5'-phosphates	227	165
Cytidine-5'-phosphates	10	2

The differences in the amounts of the 2'- and 3'-monophosphates (Tables 4 and 5) are not significant as very large variations are commonly found in different preparations of the same species. These nucleotides most probably have been formed by hydrolysis of RNA during the isolation procedure. The great difference between *P. daleae* and *P. urticae* concerns their total nucleotide contents. See above. This difference is explained by the assumption that the amount of connective tissue was higher in the *P. urticae* mycelium. (The water contents of the two moulds are nearly the same, as seen by the dry weight determinations.)

Thus, the qualitative and quantitative differences of the nucleotide contents of *P. daleae* and *P. urticae* are small. Therefore, it is not adequate to interpret these differences as the cause of the differences in the production spectra of the moulds, though such an interpretation is not completely excluded.

EXPERIMENTAL

Materials

The nucleotides used as standard substances were obtained from Sigma Chemical Company. The charcoal used for adsorption (S XII special) was obtained from R. Grave (Stockholm, Sweden). Before use the charcoal was washed several times with 50 % aqueous ethanol containing 2 % concentrated ammonia, with 1 M hydrochloric acid and with water. Other chemicals used were of *p.a.* grade. The moulds, *Penicillium daleae* (Zalesni) and *Penicillium urticae* (Bainier) R-989, were originally obtained from London School of Hygiene and Tropical Medicine.

Culture conditions

Sterile medium (500 ml portions in 1000 ml Fernbach flasks) was inoculated with spore suspensions of *Penicillium daleae* (Zalesni) and *Penicillium urticae* (Bainier) R-989, and incubated at 28°C in the dark. The incubation time was 17 and 20 days, respectively. The glucose contents of the medium at the end of the incubation were about 1 % of the original value. The composition of the Czapek-Dox' type medium was as follows:¹²

Sodium nitrate	2.0 g
Potassium chloride	0.5 g
Magnesium sulphate, $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$	0.5 g
Ferrous sulphate, $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$	0.01 g
Potassium phosphate, K_2HPO_4	1.0 g
Zinc sulphate, $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$	0.01 g
Copper sulphate, $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$	0.005 g
Glucose	50.0 g
Distilled water	to 1000 ml

The mean yield was about 40 and 20 g wet mycelium/flask of *P. daleae* and *P. urticae*, respectively. The dry weight was 18.7 % and 14.5 % of the wet weight, respectively, when determined by heating the samples (20 g) at 110°C until constant weight.

Isolation of the nucleotides

Extraction of the nucleotides and purification of the extracts was performed according to Bergkvist except for minor modifications.¹ The features of the method employed are outlined below. 2300 g of *P. daleae*-mycelium and 575 g of *P. urticae*-mycelium were

rinsed cautiously with cold water, blotted between filter papers and extracted twice through homogenization with perchloric acid at 0°C (15 % and 10 %, respectively). The nucleotides of the extracts were separated from a part of the accompanying disturbing substances through adsorption on charcoal (0°C, 45 min. 3 g of charcoal/100 g of mycelium) and subsequent elution with 25 % ethanol containing 0.5 % concentrated ammonia. The charcoal was eluted until the eluate did not contain any more organic phosphate (about 15 times). The cold eluates were concentrated and neutralized through blowing with air. The neutralized eluates were stored at -25°C until the elution was completed. The bulk of the remaining impurities of the nucleotide-containing eluates (essentially organic acids) was removed through extraction with ether in the cold after acidification (Kutscher-Steddel apparatus). The nucleotide-containing water phase was freed from ether by aeration. The nucleotides of the solution purified in this way were separated by ion exchange chromatography followed by paper chromatography and/or paper ionophoresis.

Ion exchange chromatography was performed essentially as described by Bergkvist.¹ The diagrams of Figs. 1 and 2 and the extinction ratios shown in Table 2 were used as guides in the selection of appropriate fractions for pooling. The pooled fractions were acidified and concentrated through adsorption on a minimum of charcoal and elution with small amounts of 50 % aqueous ethanol containing 0.5 % ammonia. Neutralization was performed as above by aeration. The concentrates were stored at -25°C. As most of the fractions were impure the concentrates had to be subjected to paper chromatography and/or paper ionophoresis before further analysis.

Analytical methods

Volumetric measurements. The required accuracy in measurements of small volumes (≤ 2 ml) was obtained by the use of calibrated constriction pipettes ("Carlsberg pipettes"). Other pipettes (Voll pipettes) and vessels used for volumetric measurements were also calibrated.

U.V. light absorption measurements were performed in a Beckman Spectrophotometer, Model DU, with photomultiplier and an attachment for measurements on small volumes (down to 300 μ l). Light path: 1.00 cm. All quantitative determinations of the nucleotides were based on U.V. spectrophotometry. The molar extinction and extinction ratios used in this work are tabulated in Table 2. The concept "optical unit" * was used as a convenient measure of the contents of light absorbing substances of a fraction. The U.V. light absorption curves of the nucleotides were determined at acid and alkaline pH values in the range 220–320 $m\mu$.

Phosphate determination. Hydrolysis of the polyphosphates in 1 M HCl at 100°C for 10 min led to the formation of nucleoside monophosphates and inorganic orthophosphate (acid-labile phosphate). On combustion of the nucleotides at 280°C in 70 % perchloric acid all phosphate was obtained as inorganic orthophosphate.

The inorganic orthophosphate was determined colourimetrically according to Allen.¹³ The method was adapted for microscale work. (The final volume of reagents and sample was 5.0 ml.)

The accuracy was better than ± 5 % when the samples contained 0.1–0.5 μ mole of phosphate.

The pentose contents were determined with the orcinol reagent according to Albaum and Umbreit.¹⁴ Authentic substances were run as standards in each series of experiments. The uridine nucleotides gave small and therefore uncertain values. The cytidine nucleotides could not be determined by this method.

The periodate oxidation was performed according to Dixon and Lipkin.¹⁵ The 5'-nucleotides consumed periodate within ± 5 % of the theoretical values (which were calculated from spectrophotometric data). The 2'- and 3'-nucleotides consumed maximally one tenth of the theoretical values. Thus, the 5'-nucleotides on the one hand and the 2'- or 3'-nucleotides on the other behave significantly different when analyzed by this method (at least if they are unsubstituted).

* (Number of opt. units) λ = (Extinction) λ · (ml of solution); thus, X opt. units at λ $m\mu$ equals X/ϵ mmoles. ϵ = molar extinction at λ $m\mu$.

The reaction with snake venom (*Crotalus adamanteus*) was performed as described in the paper by Bergkvist.¹ The venom splits the 5'-nucleotides according to the following scheme: (a) Nucleoside-P-P-P = Nucleoside-P + P-P; (b) Nucleoside-P = Nucleoside + P; Thus, all phosphate of 5'-monophosphates and 5'-diphosphates was obtained as inorganic orthophosphate but only one third of the phosphate of 5'-triphosphates. This is important as the reaction was followed by analysis of inorganic orthophosphate (the Allen method). The results occasionally deviated up to 20 % from the calculated values, too low results were always obtained. 2'- and 3'-phosphates remained completely unaffected by the venom enzymes.

Paper chromatography. Whatman filter paper No. 1 was used both for the usual and preparative paper chromatography. The papers were allowed to equilibrate with the solvent mixtures before the start of the chromatograms. The temperature during the runs was 20°C. The descending technique was always used. Authentic substances were run as standards in every chromatogram.

The U.V. light absorbing substances were located on the papers by means of contact prints which were made in ultraviolet light from a common mercury sterile lamp (maximal light intensity at 253.7 mμ). Very hard photo paper was used; see Fig. 3. The substances were left intact by this localization procedure and, therefore, they could be subjected to further analysis after elution (eluting agent: 0.1 M HCl). For blank solutions, paper areas as large as those of the spots were cut out from the same height of the same chromatogram and eluted exactly as the actual spots. Elution conditions: Continuous shaking for one hour.

The sugar chromatograms were developed by dipping in an 0.1 M solution of aniline hydrogen phthalate in acetone, drying, and subsequent heating at 100–110°C. Pentoses gave red colour with the reagent, hexoses brown, and 2-acetyl amino-2-deoxy-D-glucose green.

The amino acid chromatograms were developed by spraying with a ninhydrine reagent (0.3 % ninhydrine and about 0.2 % glacial acetic acid in 96 % ethanol), drying, and heating, as above.

Paper ionophoresis was performed by the method of Markham and Smith¹⁶ as modified by Bergkvist.^{17,18} Excellent results were obtained if the filter paper used (Whatman No. 1) was first washed two times with 1 M HCl (3 h each time), then with distilled water to neutrality, and thereafter dried. Untreated paper mostly gave bad results. The voltage gradient was about 20 V/cm. Two hours was a suitable time for a run when a paper sheet, 50 cm long, was used. A sheet breadth of about 23 cm usually caused a current of 100 mA. The ionophoresis of nucleotides was always performed in a sodium acetate buffer with pH 4.15 and the ionic strength 0.1. As in paper chromatography, the positions of the unknown substances were compared with those of simultaneously run authentic substances. The paper was moistened with buffer in the following way: After application of the substances on the starting line, the paper was dipped into the buffer solution from one end up to about 2 cm from the starting line and then withdrawn from the buffer. The buffer was allowed to be soaked up to about 5 mm from the starting line. The excess buffer solution was then removed by blotting between two clean filter papers. The procedure was repeated with the opposite end of the paper sheet. After some minutes the buffer solution fronts met at the starting line and the paper was ready for ionophoresis.

Hydrolysis conditions. The hydrolysis conditions for determination of total and acid-labile phosphate of nucleotides are described above.

To obtain the free bases, purine nucleotides were hydrolyzed in 1 M HCl at 100°C for 1 h, pyrimidine nucleotides in 80 % formic acid at 180°C for 1 h in sealed tubes.

The nucleotide-sugar derivatives were split nearly quantitatively into nucleotide and sugar by hydrolysis in 0.01 M HCl at 100°C for 5 min.

Peptides were hydrolyzed to amino acids by heating in 6 M HCl at 120°C for 24 h in sealed tubes.

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