IDENTIFICATION OF MAIN NUCLEOLYTIC ACTIVITIES OF THE BEE-GATHERED RAPE POLLEN (*Brassica napus* L.)*

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The bee-gathered pollen from *Brassica napus* L. contains several nucleolytic enzymes which originate from the native pollen and are not artefacts due to contamination of pollen during the collection by bees. The bee-gathered pollen was washed with acetone to remove lipoids, extracted with a neutral aqueous buffer solution, the extract dialysed, the dialysate fractionated with ammonium sulfate (50–70%) and acetone (20–50%), filtered through Sephadex G-100, and chromatographed on Sepharose 4B with bound O-(*p*-aminophenyl) ester of uridine 5′-thiophosphate. Two protein fractions were obtained. One fraction contains nonspecific ribonuclease with predominating transfer activity, decyclizing phosphodiesterase which degrades ribonucleoside 2′,3′-cyclic phosphates to 2′-ribonucleotides, and finally, phosphodiesterase which degrades the (3′→5′)-internucleotidic bonds of the *ribo* series nonspecifically with respect to the base and affords 5′-ribonucleotides as degradation products. The other enzymatic fraction contains 3′-nucleotidase free of further nucleolytic activities and degrading exclusively the 3′-ribonucleotides to the corresponding ribonucleosides.

Nucleolytic enzymes were isolated from phages, bacteria, fungi, plants, and tissues of vertebrata; most investigations relate to enzymes isolated from bacteria and fungi (*cf.*[1–5] and references therein quoted). A lesser number of papers concerns isolation and identification of nucleolytic enzymes from the plant material but notwithstanding, all the typical nucleolytic enzymes, ribonucleases, deoxyribonucleases, endonucleases, exonucleases, decyclizing 2′,3′- and 3′,5′-phosphodiesterases, and nonspecific phosphomonoesterases, 3′-, 2′-, and 5′-nucleotidases have been reported. Papers on extraction and isolation of nucleolytic enzymes from the plant pollen are limited to localisation and qualitative estimations of enzymes in pollen grains[5–10]. Only a paper of Japanese authors[11] reports on a partial purification of nonspecific acidic ribonuclease from the *Cycas revoluta* pollen. The lack of papers from this field is rather curious since pollen represents a readily accessible starting material, especially from plant cultures cultivated on large areas.

* Taken in part from the Thesis of M.P. (Charles University, Prague).

In apiculture, a great amount of the bee-gathered pollen is obtained and used as a source of sugars and proteins for bees as well as in food industry and in cosmetics. In the present paper, we wish to report on extraction of the bee-gathered pollen from rape (*Brassica napus*) and on characterisation of the main enzymatic fractions with nucleolytic activity.

As the starting material for the present investigations, the bee-gathered rape pollen was used as a readily accessible material of a great homogeneity with respect to the plant species. Pollen grains are very compact: only a limited penetration of water into the grain and diffusion of substances from the grain is possible. The rape pollen also contains a considerable amount of lipoids. The bee-gathered pollen differs from the native pollen in the contact of pollen grains with organism of bees (*Apis mellifera*) during the collection. Some contamination of the native material with the nucleolytic enzymes of the insect organism cannot thus *a priori* be excluded. A direct comparison of the native and bee-gathered pollen is hardly to realize in the case of rape. The comparison was therefore performed with the use of the native hazel-tree pollen, a portion of which was brought into contact with bees in an isolated space. The native and bee-contacted pollen was then separately extracted and assayed for enzymatic activity according to Kunitz (degradation of RNA to oligonucleotides and mononucleotides). No significant difference could be observed between the two specimens with respect to the activity level. In further experiments, inert materials such as cellulose powder, diatomaceous earth (Hyflo Super Cel), and talc were brought into contact with bees. Only in the case of talc a sufficient amount of the contacted material was obtained but its extract did not exhibit any significant activity (Table I). To exclude induction of enzymes in digestion and collecting organs of bees by specific features of the native pollen such as colour or grain size, the native pollen was extracted, dried, and ground. This material was then compared with a specimen which was additionally brought into contact with bees: the activity of these two samples

TABLE I

Nucleolytic Activity of Pollen Samples for RNA (measured by Kunitz's method)

Sample	Activity e.u./10 g
Rape pollen (gathered by bees)	61·0
Rape pollen (extracted with water and gathered by bees)	3·8
Hazel-tree pollen natural	16·5
Hazel-tree pollen (gathered by bees)	15·5
Mixed pollen (gathered by bees)	28·0
Talc (gathered by bees)	0

did not differ. Finally, pharynx glands of one hundred bees were ultrasonically disintegrated in a physiological solution and the resulting homogenate assayed for activity towards RNA and uridine 2′,3′-cyclic phosphate; no activity has been observed. The nucleolytic activity of the bee-gathered pollen is thus most probably identical with that of the native pollen; consequently, the nucleolytic enzymes of the insect organism, if any, do not interfere.

The bee-gathered rape pollen was processed as follows. The material was extracted with acetone at −20°C, washed with cold ether, and dried to remove residual solvents. The dry material was extracted with a neutral aqueous buffer solution and the extract dialysed to remove low-molecular components. The dialysate was fractionated by precipitation with ammonium sulfate; total nucleolytic activity was contained in the sediment of the 50−70% saturated fraction.

In preliminary experiments on purification of this sediment by chromatography on a column of DEAE cellulose or CM cellulose with a sodium chloride gradient at pH 5·5 or 7·8, a complete loss of activity was observed. The purification was therefore performed by means of a fractionated acetone precipitation. Thus, the dialysed solution of the above mentioned sediment was precipitated with precooled acetone to the concentration of 20%, the mixture centrifuged, the supernatant adjusted to the concentration 50% of acetone. Both sediments (up to 20% and 50% of acetone) exhibited nucleolytic activity; most contaminating proteins remained in the supernatant.

Both sediments were separately subjected to gel chromatography on a column of Sephadex G-100 in a weakly acidic acetate buffer solution. Fig. 1 shows the elution profiles of proteins and nucleolytic activities as determined by the Kunitz test.

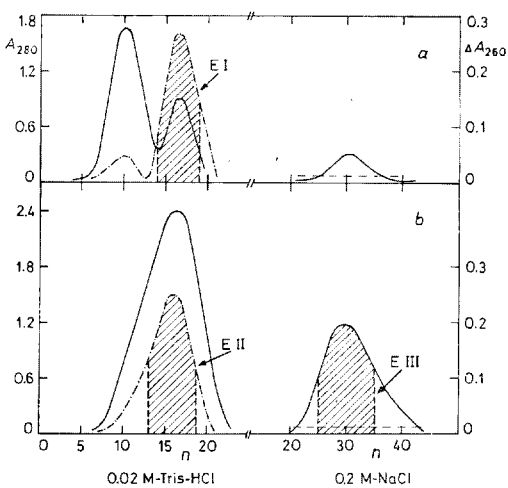

FIG. 1

Chromatography of Acetone Precipitates on Sephadex G-100

a fraction up to 20% of acetone (fraction 3a, Table II); *b* fraction up to 50% of acetone (fraction 3b, Table II). ——— ultraviolet absorption, – · – · – activity according to Kunitz. The hatched area designates fractions used in the next step.

By the gel chromatography, there are removed further portions of inactive proteins which are eluted before or after the fractions exhibiting the enzymatic activity. In addition to the main activity, a small amount of a low-molecular active fraction (which has not been investigated in detail) is removed along with a considerable amount of pigments. The main enzymatically active fractions were pooled and brought with ammonium sulfate up to the 70% saturation, centrifuged, the sediments dissolved in water, and dialysed against a neutral aqueous buffer solution.

In the last purification step, the two dialysates were separately subjected to chromatography on Sepharose 4B containing the O-(*p*-aminophenyl) ester of uridine 5'-thiophosphate bound by mediation of cyanogen bromide (*cf.*[12]). The column was equilibrated with 0·02M-Tris-HCl buffer solution, eluted first with the same buffer solution (retention of the nucleolytic activity) and then with 0·2M-NaCl in the same buffer solution (Fig. 2). With the use of the greater ionic strength of the neutral electrolyte, the bound protein was released from the column. The thus-obtained fractions were dialysed against a neutral volatile buffer solution and the dialysates freeze-dried. The isolation of the three main fractions on modified Sepharose 4B is shown on Table II. The enzymatic activity of eluates was systematically checked by means of the Kunitz test. This detection is limited to enzymes degrading internucleotidic bonds; the enzymes active on monomeric substrates only are not

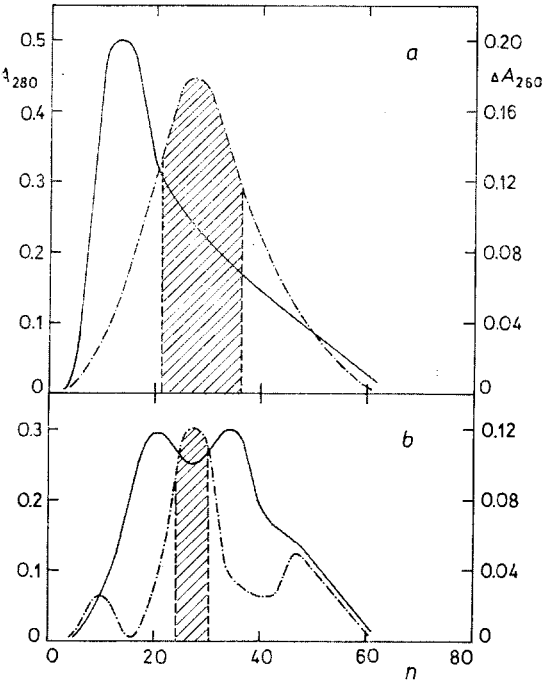

FIG. 2

Chromatography of Purified Fractions of Acetone Precipitates on Modified Sepharose 4B

a Fraction up to 20% of acetone (fraction 5a, Table II); *b* fraction up to 50% of acetone (fraction 5b, Table II). ——— ultraviolet absorption, –·–·– activity according to Kunitz. The hatched area designates fractions used in the next step.

detected. The specific activity of the two nucleolytically active fractions EI and EII (see Table II) was (0·9 and 0·6 e.u. per mg, resp.) similar to that of commercial pancreatic ribonuclease (1·0–1·5 e.u. per mg). The third fraction EIII (Fig. 2*b*) was inactive in the Kunitz test.

Enzymatic activities of these fractions were identified with the use of substrates specific for the particular types of nucleolytic enzymes, namely, RNA (polyribonucleotide with linear (3′ → 5′)-phosphodiester bonds), ribonucleoside 2′,3′-cyclic phosphates (monomolecular cyclic phosphodiesters), 5′-ribonucleotides, and 2′(3′)-ribonucleotides. The degradation by protein fractions was examined after brief and prolonged incubations. As it may be seen from Table III, fractions EI and EII are able to degrade both monomeric and polymeric phosphodiesters. The protein EII degrades the two cyclic phosphodiesters to an almost equal extent whereas the protein EI prefers the adenosine derivative. In both fractions, there are present lower levels of phosphomonoesterase activities which require a longer incubation to dephosphorylate the resulting nucleotides to nucleosides.

TABLE II
Isolation of Nucleolytic Proteins from Rape Pollen

Purification step	E.u. total	Volume, ml
1. Crude extract	6 100	2 240
2. Ammonium sulphate fractionation	4 200	150
3. Acetone precipitation		
a) up to 20%	2 180	12
b) up to 50%	490	15
4. Sephadex G-100 chromatography		
a) up to 20%	1 110	75
b) up to 50%	182	90
5. Ammonium sulphate precipitation		
a) up to 20%	280	10
b) up to 50%	150	10
6. Affinity chromatography		
a) up to 20%	71 (EI	35
b) up to 50%	54 (EII)	25
	0 (EIII)	20
7. Dialysis, freeze drying		
EI	7·2	8 mg
EII	54·0	90 mg
EIII	0	90 mg

The protein EIII does not degrade linear phosphodiesters as also indicated by the negative Kunitz test. The cyclic phosphodiesters (ribonucleoside 2′,3′-cyclic phosphates) are also resistant (Table III). On the other hand, a high activity towards uridine 2′(3′)-phosphate has been observed, since the isomeric uridine 5′-phosphate is resistant to protein EIII even for a long incubation time, the main component of this protein fraction is obviously represented by specific 2′- or 3′-nucleotidase free of nonspecific phosphomonoesterase or 5′-nucleotidase. The occurence of similar enzymes in plants has been reported[13]. A long incubation of the corresponding substrates with the three protein fractions EI – III results in the formation of ribonucleosides (uridine and adenosine; Table III). As shown by electrophoresis of degradation products in a borate buffer solution, the heterocyclic base is not present. All the three fractions are consequently free of nucleoside phosphorylases, nucleoside hydrolases, and/or other enzymes degrading nucleotides with the formation of a base. Furthermore, the occurrence of adenosine as the final product in degradations of adenosine nucleotides demonstrates the absence of adenosine or adenylate deaminase in protein fractions EI – III (neither inosine nor hypoxanthine are formed).

In view of the relative content in pollen extracts, only the fractions EII and EIII were examined in detail. Degradation of monomeric 2′,3′-cyclic nucleotides (type *A*) and the linear (3′ → 5′)-polyribonucleotide (type *B*) may be caused by three types of enzymes. Thus types *A* and *B* may be degraded by ribonucleases, type *B* by phosphodiesterases (exonucleases and endonucleases), and type *A* by decyclizing phosphodiesterases. In order to differentiate these three activities, it is necessary to analyze the degradation course of synthetic substrates and to determine the structure of reaction products.

Degradation of RNA with the protein fraction EII at pH 5·5 affords a mixture of four mononucleotides identified as Up, Cp, Ap, and Gp on comparison with

TABLE III

Nucleolytic Activity (degradation, % after 3 h at 37°C) of Protein Fraction from Rape Pollen

Substrate	EI	EII	EIII
Uridine 2′,3′-cyclic phosphate	11	77	6
Adenosine 2′,3′-cyclic phosphate	81	72	11
Uridine 2′(3′)-phosphate	55	57	58
Uridine 5′-phosphate	30	37	0
Adenosine[a]	0	0	0
RNA[b] (e.u./mg protein)	0·9	0·6	0

[a] Degradation to adenine or inosine: 24 h at 37°C; [b] after 25 min at 25°C.

authentic specimens by paper chromatography, electrophoresis, and UV spectra (we did not observe any resistant residue or the formation of oligonucleotides). The phosphodiesterases of fraction EII are thus nonspecific with respect to the heterocyclic base of the nucleotide unit. As established by analysis of Ap and Gp by paper chromatography and of Cp on Dowex 1 (formate) ion exchange resin (methods for differentiating the nucleotide isomers), isomerically pure 2′-ribonucleotides are formed in all three cases without any admixture of 3′-phosphates or 5′-nucleotides. These 2′-ribonucleotides may arise from internucleotidic (3′ → 5′)-phosphodiester bonds of RNA exclusively *via* cyclic phosphorus intermediates. With the use of a shorter incubation time and a lower concentration of the enzyme, uridine 2′,3′-cyclic phosphate or cytidine 2′,3′-cyclic phosphate were identified (Table IV) as intermediates in degradation of UpU, UpA, and CpU dinucleoside phosphates. These intermediates disappear with the proceeding incubation, a mononucleotide being formed. The formation of stable cyclic intermediates of this type can be exclusively catalysed by a ribonuclease (cyclizing 2′-nucleotidyltransferase), necessarily present in the EII protein fraction.

The presence of a ribonuclease in the EII protein may also be confirmed by resistance of 2′-O-tetrahydropyranyluridylyl-(3′ → 5′)-uridine ($U^{THP}pU$) and uridylyl-(2′ → 5′)-adenosine towards this protein fraction. The first step of ribonuclease-catalysed reactions (the transfer reaction) requires the presence of a *ribo*-oriented 2′-hydroxylic function vicinal to the 3′-phosphate group; on the other hand, ribonucleases are not able to degrade the isomeric unnatural (2′ → 5′)-bond. By this evidence, namely, formation of a 2′,3′-cyclic nucleotide as intermediate, resistance of the (2′ → 5′)-isomer, and the inhibitory effect of a blocking group on the hydroxylic function at position 2′, the ribonuclease-like character of the enzyme in the EII protein fraction is unequivocally established.

TABLE IV

Degradation of Dinucleoside Monophosphates by Rape Pollen Protein EII

Compound	Degradation, %[a]	Products[b,c]
UpU	78	Up, Ucp, Urd, pU
UpA	100	Up, Ucp, Ado, pA, Urd
CpC	100	Cp, pC, Cyd, Ccp
$U^{THP}pU$	0	—
(2′ → 5′)-UpA	0	—

[a] After 3 h at 37°C, pH 7·3; [b] characterized by unequivocal methods; [c] abbreviations: nucleotides and dinucleoside phosphates by one-letter symbolics, nucleosides by three-letter symbolics; $U^{THP}pU$, 2′-O-tetrahydropyranyluridylyl-(3′ → 5′)uridine.

Degradation of ribonucleoside phosphates with the EII protein fraction is more complex than it would correspond to the presence of ribonuclease alone. As shown by examination of degradation products of heterogeneous dinucleoside phosphates, *e.g.*, of ApU, there are formed two pairs of products such as Ap + Urd and Ado + + UMP. The other enzyme present in the EII protein fraction must be a phosphodiesterase similar to the snake venom phosphodiesterase which degrades the (3′ → 5′)--internucleotidic bond with the formation of 5′-ribonucleotides. A similar ambiguous course of the degradation has been also observed in the case of UpA, CpC and the like (see Table IV). In the neutral pH region, both degradation types occur to an almost equal extent. However, degradation of RNA with the EII protein fraction is at variance with the above findings since only 2′-nucleotides are obtained but no 5′-nucleotide. Table V shows the pH-dependence of the ApU dinucleoside phosphate degradation. The phosphodiesterase activity manifests itself exclusively in the alkaline and neutral pH region; it disappears in the acidic region and only the ribonuclease activity remains. Consequently, the degradation of RNA in acidic pH region afforded exclusively the ribonuclease-type degradation products.

For the formation of 2′-nucleotides from RNA or ribonucleoside phosphates (Table IV), only the decyclizing phosphodiesterase (analogous to the enzyme from spleen[14]) may be responsible. In model degradations of 2′,3′-cyclic phosphates of guanosine, adenosine, and cytidine with the EII protein fraction at neutral pH value, the corresponding 2′-ribonucleotides were obtained. On the other hand, the isomeric adenosine 3′,5′-cyclic phosphate is towards EII completely resistant. Thus, the EII protein fraction contains decyclizing 2′,3′-phosphodiesterase nonspecific to heterocyclic bases and yielding 2′-nucleotides as degradation products.

Both from RNA and from ribonucleoside 2′,3′-cyclic phosphates there were exclusively obtained the 2′-nucleotides. It is therefore obvious that the above established ribonuclease catalyses the transfer reaction (making thus possible degradation of internucleotidic bonds) but does not participate on hydrolysis which would ex-

TABLE V

Effect of pH upon the Degradation (%) of ApU by Rape Pollen Protein EII

Degradation product	pH 5·5	pH 7·3	pH 8·5
Uridine 5′-phosphate	4·0	21	21
Adenosine	13	35	34
Adenosine 2′-phosphate	30	11	16
Uridine	43	25	14
ApU	10	8	15

clusively yield the 3′-ribonucleotides. Ribonucleases with predominating transfer effects have been reported in plant materials[15,16]; also the action of the ribonuclease isolated from pollen of *Cycas revoluta* has been claimed to cease in the ribonucleoside 2′,2′-cyclic phosphate stage[11].

The hydrolysis of internucleotidic bonds in RNA and oligonucleotides obviously proceeds according to the following mechanism:

$$\ldots\ \mathrm{XpY}\ \ldots \xrightarrow{\text{PDase}} \ldots + \mathrm{pX} + \mathrm{pY} + \ldots$$

$$\Big\downarrow \text{RNase}$$

$$\ldots + \mathrm{Xcp} + \mathrm{Ycp} + \ldots \xrightarrow{\text{cPDase}} \text{X-2′-p} + \text{Y-2′-p} + \ldots$$

The above mentioned main nucleolytic enzymes of the EII protein fraction are accompanied by 2′- or 3′-nucleotidase which was isolated (free of other activities) from the EIII protein fraction. These four enzymes do not necessarily represent the sole nucleolytic enzymes of rape pollen; additional enzymes that would exclusively degrade monomeric substrates, are not excluded. The occurrence of the above enzymatic activities has also been observed in other pollen samples; as indicated by preliminary experiments, the course of the separation process is analogous.

EXPERIMENTAL

Methods

Paper chromatography was performed by the descending technique on paper Whatman No 1 (preparative runs on paper Whatman No 3 MM) in the solvent systems S_1, 2-propanol–conc. aqueous ammonia–water (7 : 1 : 2); S_2, 2-propanol–conc. aqueous ammonia–0·1M triethylammonium borate pH 7·5 (7 : 1 : 2); S_3, saturated aqueous ammonium sulfate–0·1M sodium acetate–2-propanol (79 : 19 : 2), and S_4, 1-propanol–conc. aqueous ammonia–water (6 : 3 : 1). Paper electrophoresis was performed according to the technique of Markham and Smith[17] on paper Whatman No 3 MM (20 V/cm, 1 h) in the buffer solution E_1, 0·1M triethylammonium hydrogen carbonate (pH 7·5). Spots were detected in ultraviolet light (Chromatolite). The R_F values and electrophoretical mobilities of some substances are summarized in Table VI. Spectrophotometrical measurements were performed in 0·01M-HCl on a Unicam SP 8000 apparatus and quantitative determinations in aqueous solutions on a Spectromom 203 apparatus, always at 260 nm.

Materials

The Analytical Grade reagents were products of Lachema, Brno, Czechoslovakia. Uranyl acetate was product of Sojuzchimreaktiv, Soviet Union. The high-molecular yeast RNA was a gift from Dr G. S. Ivanova, Pushchino on Oka, Soviet Union. The nucleosides and mononucleotides were products of Calbiochem, Los Angeles, U.S.A. The substrates for enzymatical assays were prepared by synthesis[14].

Collection of Pollen and Bee-Contacting of Pollen Imitations

Pollen gathered by bees in the full blossom of the corresponding plants was daily collected, air-dried to the water content of 10—12% (original water content, 18%), and stored at —30°C. Rape pollen was collected in 1974 in Nové Dvorce near Tachov, Czechoslovakia. The pollen from *Taraxacum officinale* WEB and maple (*Acer sp.*) pollen was selected from other pollen species gathered by bees in Nedašov near Valašské Klobouky, Czechoslovakia. Pollen was gathered by *Apis mellifera carnica* POLM. bees which were also used to contact pollen imitations.

Biologically inert materials such as talc, cellulose powder (Whatman), diatomaceous earth (Hyflo Super Cel) or native materials such as hand-collected hazel-tree pollen or rape pollen (previously extracted to remove fats, see below, and powdered) were brought into an artificial contact with drilled bees in a play-flight room (Research Institute for Apiculture, Důl near Libčice on Vltava, Czechoslovakia). From the biologically inert materials, best results were obtained with talc. The size and shape of bee-gathered pollen loads and the artificially bee-contacted pollen loads were similar.

TABLE VI
Chromatography and Electrophoresis

Compound[a]	R_F				EUp[b]
	S1	S2	S3	S4	
Urd	0·50	0·50	—	—	0
Ado	0·57	0·60	—	—	—0·10
Ino	0·45	0·40	—	—	0·08
2′(3′)-UMP	0·15	0·20	—	0·45	1·00
5′-UMP	0·10	0·05	—	0·42	1·00
2′(3′)-CMP	0·15	0·22	—	0·52	0·85
5′-CMP	0·12	0·05	—	0·48	0·85
2′-AMP	0·20	0·25	0·60	0·60	0·90
3′-AMP	0·20	0·25	0·45	0·60	0·90
5′-AMP	0·15	0·07	0·65	0·55	0·90
2′-GMP	0·07	0·12	0·70	0·30	0·94
3′-GMP	0·07	0·12	0·55	0·30	0·94
5′-GMP	0·04	0·02	0·70	0·25	0·94
Ucp	0·42	—	—	—	0·58
Ccp	0·42	—	—	—	0·50
Acp	0·48	—	—	—	0·45
Gcp	0·35	—	—	—	0·50
UpU	0·25	—	—	—	0·30
UpA	0·28	—	—	—	0·28
CpC	0·25	—	—	—	0·25
$Up^{THP}U$	0·50	—	—	—	0·30

[a] For abbreviations see Table IV; [b] mobility in buffer solution E_1 and referred to 3′-UMP.

Nucleolytic Activity Assay according to Kunitz (for modifications see ref.[18])

A solution of 2 mg RNA in 450 μl 0·2M acetate buffer (pH 6·0) was incubated at room temperature for 25 min with 200 μl of the tested sample, the mixture treated with 250 μl of 0·75% uranyl acetate in 25% perchloric acid, diluted with 600 μl of water, and the suspension kept at 0°C for 2 h. The precipitate was filtered off (Schleicher-Schüll filter No 3), and the filtrate diluted with 300 parts by volume of water. The absorbancy of the sample at 260 nm was measured against a blank in which the tested sample was added to RNA solution pretreated with uranyl acetate. With values of $\Delta A_{260} \geqq 0{\cdot}30$, the original sample was diluted with water prior to the determination. The content of the Kunitz enzymatic activity units was calculated from the equation

$$e.u. = 2{\cdot}5 \,.\, \Delta A_{260} \,.\, x \,.\, n ,$$

wherein x is the dilution of the sample prior to the determination and n is the volume of the fraction in milliliters.

Estimation of Nucleolytic Activity Assays of Pollen and Related Materials

A sample of the material (10 g) was extracted under stirring at 0°C with 70 ml of 0·2M-TRIS-HCl buffer solution (pH 7·3) overnight, the suspension subjected to centrifugation (2200) r.p.m. under cooling, the supernatant filtered (Schleicher-Schüll filter No 2), and the activity determined in an aliquot of the filtrate according to the method of Kunitz. For results see Table I. The following samples were assayed: *1*) bee-gathered rape pollen; *2*) bee-gathered rape pollen pre-extracted with a buffer solution (see Isolation of Enzymes), washed with ethanol, dried, powdered, and brought into an artificial contact with bees; *3*) mixed bee-gathered pollen from maple and pear-tree (harvested in 1974); *4*) native hazel-tree pollen (spring 1975); *5*) sample 4 which was brought into an artificial contact with bees; and *6*) talc which was brought into an artificial contact with bees.

Isolation of Enzymes

Bee-gathered pollen (1 kg) from rape (*Brassica napus* L.) was used as the starting material. Prior to the isolation process, the material was stored in polyethylene bags at −10°C. The material was stirred in precooled (−20°C) acetone (2000 ml) for 2·5 h, the suspension filtered through sintered glass funnel, the pollen washed with acetone and ether (2000 ml each), and dried at room temperature overnight. The thus-obtained dry material was stirred at 4°C for 24 h in 1200 ml of a 0·2M Tris-HCl buffer solution (pH 7·3) containing 0·5% of thiourea, and subjected to centrifugation (4°C, 20 min, 1000 *g*). The sediment was extracted once more under the same conditions. The supernatants were combined and filtered through a Büchner funnel lined with cotton cloth. The filtrate was centrifuged again (4°C, 20 min, 9000 *g*) and the supernatant dialysed against two 5000 ml portions of 0·02M triethylammonium hydrogen carbonate (pH 7·5). The dialysate was centrifuged under the above conditions. The supernatant (fraction 1, Table II) was saturated under stirring at 25°C with ammonium sulfate up to the concentration of 50%. The mixture was kept at room temperature overnight, centrifuged (4°C, 20 min, 9000*g*), and the supernatant saturated with ammonium sulfate up to the concentration of 70%. The mixture was processed as above. The second sediment was dissolved in a minimum volume of 0·02M Tris-HCl buffer solution (pH 7·3) and subjected to two dialyses against 5000 ml of 0·02M triethylammonium hydrogen carbonate pH 7·5. The dialysate was centrifuged under the above conditions.

To the previous supernatant (fraction 2, Table II) there was added under stirring in small portions precooled (−20°C) acetone up to the final concentration of 20%, and the sediment

(fraction 3a, Table II) collected by centrifugation (4°C, 20 min, 9000*g*). Acetone was then added under similar conditions to the supernatant up to the concentration of 50% acetone. The suspension was again subjected to centrifugation under the above stated conditions (3b, Table II). Both sediments were dissolved in minimum volumes of 0·1M sodium acetate (pH 6·0), the solutions separately applied to columns (90 × 3 cm) of Sephadex G-100 (medium) equilibrated with 0·1M sodium acetate (pH 6·0). The elution was performed with the same buffer solution at the rate of 20 ml per hour, 5 ml fractions being taken. Content of proteins was determined by measurements of A_{280}. The enzymatic activity was checked in aliquots of fractions by means of the Kunitz method. The separation of the two fractions 3a and 3b is shown in Fig. 1. The fractions exhibiting the enzymatic activity (4a and 4b) were saturated with ammonium sulfate under the above conditions up to the final concentration of 70%. The sediments (5a and 5b, Table II) were dissolved in minimum volumes of water and the solutions dialysed against 2000 ml of 0·02M triethylammonium hydrogen carbonate (pH 7·5). The dialysates were centrifuged (4°C, 20 min, 9000*g*) and applied to a column (21 × 2·8 cm) of Sepharose 4B with bound O--(*p*-aminophenyl) ester of uridine 5′-thiophosphate (ref.[12]) and equilibrated with 0·02M Tris-HCl buffer solution (pH 7·3). The column was eluted with the same buffer solution (100 ml) at the rate of 1 ml per min, 5 ml fractions being taken. The subsequent elution was performed with 0·2M-NaCl in the preceding buffer solution under the same conditions (total 200 ml). The protein content was determined at A_{280} and the enzymatic activity was tested by the method of Kunitz. The elution of the two fractions is shown on Fig. 2. An eluate of fraction 2 with sodium chloride was also isolated (Table II). All the three fractions were separately dialysed against 0·02M triethylammonium hydrogen carbonate (pH 7·5) and the dialysates freeze-dried to afford 8 mg of EI protein from fraction 6a with the use of the basic buffer solution; 90 mg of EII protein from fraction 6b (basic buffer); and 80 mg of EIII protein from fraction 6b (0·2M-NaCl). For the isolation of nucleolytic enzymes from rape pollen *cf.* Table II. Aliquots of EI—EIII proteins were dissolved in 0·1M-NaCl (2 mg/ml) and the activity towards RNA determined by means of the Kunitz test (Table III).

Activity Assays of EI—EIII Fractions on Monomeric Substrates

The incubation mixture contained 3 μmol of the substrate (lithium salt of uridine 2′,3′-cyclic phosphate, ammonium salt of adenosine 2′,3′-cyclic phosphate, sodium salt of uridine 2′(3′)-phosphate or sodium salt of uridine 5′-phosphate), 50 μg of the appropriate protein fraction, and 5 μmol of NaCl in 100 μl of 0·05M Tris-HCl buffer solution (pH 7·3). After the incubation at 37°C for 3 h, a 50 μl of the mixture was chromatographed in the solvent system S_1 overnight, the spots of products and starting materials eluted with 10 ml of water each, and the content determined spectrophotometrically. Blanks were performed in the absence of the protein under otherwise the same conditions. For results see Table III.

Preparative Degradation of RNA with the EII Protein Fraction

The incubation mixture contained 20 mg of RNA and 1·5 mg of the EII protein fraction in 1·5 ml of 0·05M sodium acetate (pH 5·5). After 2 h at 37°C, the mixture was applied to 2 sheets of paper Whatman No 3 MM and chromatographed in the solvent system S_1 for 48 h. The resulting four main UV-absorbing bands were eluted with dilute (1 : 100) aqueous ammonia, the eluates evaporated under diminished pressure, and dried over phosphorus pentoxide. On the basis of UV spectrum and electrophoresis in buffer solutions E_1 and E_2, band 2 was identified as uridine monophosphate; as shown on comparison with authentic specimens in solvent systems S_1 and S_2, only uridine 2′-phosphate or uridine 3′-phosphate are present in this fraction.

Band 3 was subjected to preparative paper electrophoresis in the buffer solution E_1 to afford four separate bands which were identified as UpU, Ucp + Acp, Cp, and Up by means of paper chromatography in solvent systems S_1 and S_2 and electrophoresis in buffer solutions E_1 and E_2 as well as with the use of UV spectra at pH 2 and comparison with tabelated data.

Band 4 was unequivocally identified as uridine by means of paper chromatography in solvent system S_1, electrophoresis in buffer solutions E_1 and E_3, and UV spectrum at pH 2.

Band 1 (a mixture of mononucleotides) was rechromatographed on 2 sheets of paper Whatman No 3 MM in the solvent system S_1 for four days to afford two UV-absorbing bands (1a and 1b) which were eluted with dilute (1 : 100) aqueous ammonia and the eluates concentrated under diminished pressure over phosphorus pentoxide. Preparative paper electrophoresis in buffer solution E_2 of band 1a afforded two bands which were identified as cytidine 2′-phosphate and adenosine 2′-phosphate by means of UV spectra and comparison with authentic specimens in solvent systems S_1 and S_4 as well as buffer solutions E_1 and E_2. Band 1b is chromatographically (S_1, S_2) and electrophoretically (E_1, E_2) homogeneous guanosine monophosphate identified as guanosine 2′-phosphate on comparison with an authentic specimen in the solvent system S_3.

Analytical degradation of RNA *with the* EII *protein fraction*: The above incubation mixture was analysed by chromatography in the solvent system S_4 (72 h). The spots corresponding to authentic mononucleotides were eluted with 0·01M-HCl (10 ml each). The following content in mol% was determined: Up, 29·0; C-2′-p, 29·8; A-2′-p, 12·9; G-2′-p, 28·3.

Degradation of Diribonucleoside Phosphates and Analogues with the EII Protein Fraction

The incubation mixture contained 2 μmol of the appropriate substrate (ammonium salt) and 250 μg of the EII protein fraction in 100 μl of 0·05M Tris-HCl buffer solution (pH 7·3). After the incubation at 37°C for 3 h and 24 h, 50 μl aliquots were withdrawn and analysed by chromatography in the solvent system S_1. Blanks were performed in the absence of the enzyme under otherwise identical conditions. Spots were eluted with water (10 ml each) and the A_{260} value of eluates was measured. For a survey of substrates and degradation data (corrected with respect to the non-enzymatic hydrolysis) see Table IV.

pH-*Dependence of* ApU *degradation.* The incubation mixture contained 1 mg of ApU (ammonium salt) and 250 μg of the EII protein fraction in 100 μl of 0·05M sodium acetate (pH 5·5) or 0·05M TRIS-HCl buffer solution (pH 7·3 or 8·5). After the incubation at 37°C for 3 h, the mixture was analysed by chromatography in the solvent system S_1. Spots of Urd, Ado, Ap, UMP, and ApU were eluted with water (10 ml each) and the A_{260} value of eluates was measured. For the degradation extent and products see Table V.

Evidence for the presence of ribonucleoside 2′,3′*-cyclic phosphates.* The incubation mixture contained 2 μmol of the appropriate substrate (ammonium salt of UpU, CpC or UpA) and 100 μg of the EII protein fraction in 100 μl of 0·05M Tris-HCl buffer solution (pH 7·3). After the incubation at 37°C for 1 h, the mixture was analysed by paper chromatography in the solvent system S_1. The spot corresponding to the 2′,3′-cyclic phosphate of uridine or of cytidine was eluted with a little water and identified by comparison with an authentic specimen by means of electrophoresis in the buffer solution E_1. Parallel quantitative measurements with aliquots of the incubation mixture yielded the following data (substrate, % of total degradation, and % of 2′,3′-cyclic phosphate given): UpU, 78, 22; UpA, 100, 15; and CpC, 100, 10.

Identification of isomeric mononucleotides. The incubation mixture contained 10 μmol of the appropriate substrate (ApU, CpC, Acp, Ccp) and 750 μg of the EII protein fraction in 300 μl of 0·05M-TRIS-HCl buffer solution (pH 7·3). After the incubation at 37°C for 3 h, the mixture

was analysed by chromatography in the solvent system S_1. Bands of adenosine monophosphate or cytidine monophosphate were eluted with a little water and the eluates concentrated under diminished pressure over phosphorus pentoxide. Identification of adenosine monophosphate was effected by rechromatography of the eluate in the solvent system S_3 and comparison with authentic samples of the 2′- and 3′-monophosphates. The present degradation of ApU and Acp was unequivocally demonstrated to afford exclusively adenosine 2′-phosphate. Identification of cytidine monophosphate was effected by chromatography on a column (11 × 1 cm) of Dowex 1 X 8 ion exchange resin equilibrated with 0·02M formic acid[19]. The column was eluted with 0·02M formic acid (1 ml/min) and the elution checked by the Uvicord apparatus (Uppsala, Sweden). Cytidine 2′-phosphate was shown to be the exclusive product of the present CpC and Ccp degradations by means of a comparison with authentic 2′- and 3′-phosphate of cytidine.

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