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Biochemical investigations on endosperm development in *Iris pseudoacorus* L.

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Abstract

In the successive development phases the nitrogen and protein fractions were determined and ion exchanger chromatography of globulins was performed on DEAE cellulose in the endosperm of the chalasal and micropylar poles of *Iris pseudoacorus* seeds. The phosphorus fraction were also analysed and RNA was separated by means of electrophoresis on coloured agar gel. Wide quantitative differences were found in the content of the compounds investigated between the two poles and the successive development phases.

INTRODUCTION

During the development of the embryo and the whole seed of *Iris pseudoacorus* its nutrient tissue — the endosperm — is differentiated into two poles: the micropylar one in the region of the embryo and the one on the opposite side — the chalasal pole. Chalase plays an important role in the process of food supply to the seed. A great number of compounds with high physiological activity are not formed in the seed, but are supplied from the mother organism through the vascular bundles which reach to the chalasal part of the endosperm. A number of investigations has been carried out on the endosperm of *I. pseudoacorus*, its ultrastructure and the cytochemistry of this tissue at various phases of development as well as on the transformation of nuclear into cellular endosperm (Olszewska, Gabara 1966; Mikulska et al. 1967, Olszewska et al. 1968; Gabara, Modrzejewski 1971). These studies demonstrated that the chalasal pole has a higher mitotic activity and initiates a mitotic gradient along the endosperm. It is, moreover the site where cells start to form in the nuclear endosperm (Olszewska, Gabara 1966).

It is noteworthy that so interesting an object as is the endosperm of *I. pseudoacorus* owing to its polar differentiation has so far not been the object of attention of biochemists. In the present work seeds of *I. pseudoacorus* were chosen as material for study because they are known as regards to their histology and histo- and

cytochemistry. Besides, the seeds of this plant are relatively large and distinctly differentiated into two poles at the time of development of the embryo and endosperm. Among the quite extensive literature concerning the physiology or cytology of development of the seeds (Grzesiuk 1967; Grzesiuk et al. 1962; Cinger 1958; Ingle et al. 1965; Kołobkova 1958; Kuran, Marciniak 1969; Öpik 1968; Singh 1968; Pritchard 1964a, b) but few papers may be found in which the problem of the biochemistry of seed development would be related to cytological, cyto- and histochemical investigations.

MATERIAL AND METHODS

The material for investigation was the endosperm of the chalasal and micropylar poles of the seeds of *Iris pseudoacorus* in their successive phases of development:

I — seeds 4–4.5 mm long with nuclear endosperm and central vacuole; the embryo consists of a dozen cells or so;

II — seeds 5–6 mm long; the endosperm transforms from nuclear to cellular; the central vacuole continues to be present; the embryo comprises several score of cells;

III — seeds 7–8 mm long; the entire seed is filled with white endospermic tissue; the embryo comprises several hundred cells;

IV — seeds 8–9 mm long; endosperm yellow; embryo ca. 2 mm long;

V — ripe seeds; endosperm becomes brown; embryo reaches its final size (ca. 4 mm).

Since the experiments corresponded to the vegetation season during which the seeds had to be collected at the successive stages of their development, and the endosperm prepared out, and since some of the analyses are long in performing, the seeds were preserved in a delipiding mixture consisting of ethanol and ether (3:1). Control experiments demonstrated that ca. 3% of protein (as compared with total) of the seeds passes into the ethanol-ether-mixture. Of this 90% consists of prolamins, 9% of albumins 0.5 of globulins. Thus, the losses in the material investigated did not exceed the values of experimental error. Up to phase IV endosperm was analysed together with the embryo which was extremely small. It was removed only from dry seeds (phase V). The endosperm from the micropylar pole constituted 2/3 of the length of this tissue, and that of the chalasal pole 1/3.

In the micropylar pole endosperm and separately in that of the chalasal pole, nitrogen compounds were fractionated by the method of Thimann and Loos (1957) and Thimann and Laloraya (1960). Nitrogen in the particular fractions was determined by Kjeldahl's micromethod. Total protein was extracted after Fletcher and Osborne (1965) and determined by Lowry's method (after Mejbaum-Katzenellenbogen, Mochnacka 1968). Proteins were fractionated according to the method of Pleszkov (1968) and globulins were separated on

a cellulose ion exchanger DEAE (Serva) according to Gorman and Levine (1961). Pleszkov (1968) and Coates and Simmonds (1961).

Moreover the contents of phosphorus fractions were determined after Holden (1952) and Holden and Pirie (1955) and electrophoresis of ribonucleic acids was run in coloured agar gels by the method described by Miczyński (1967a, b).

The experiments were repeated in the course of three vegetation seasons (1966–1968) with three replications from each seed harvest. The results showing relatively small differences were subjected to statistical analysis according to the split-plot system described by Elandt (1964).

RESULTS AND DISCUSSION

Histological, cytochemical and ultrastructure investigations demonstrated that endosperm development in both the poles occurs at first differently (Olszewska, Gabara 1966; Mikulska, Gabara, Olszewska 1967). These data are confirmed by biochemical results.

In phase I wide differences were noted in the content of nitrogen compounds in both poles. With the development of the seeds the amount of acid-soluble nitrogen increases (phases II, III, IV) (Table 1). The rise of the nitrogen level in this fraction is connected with the intensive metabolism in the period of seed development. Compounds of acid-soluble nitrogen supplied by the mother plant cannot be utilised at once by the forming endosperm and embryo. Similar results were obtained by Grzesiuk and Kulka (1960) in the case of cereal seeds. In ripe seed the amount of acid-soluble nitrogen greatly decreases and becomes almost equal at both poles. Probably these compounds are used for protein synthesis both in the endosperm and embryo which in phase V reaches its final size. In the developing endosperm the amount of proteins also increases. When converted to dry weight this increase is not large, and in ripe seed a distinct decrease and equalisation of the differences between the poles are noted. As regards to absolute protein content, it increases during the entire time of endosperm development (Table 2). In ripe seeds it is maximal. These data are confirmed by the investigations of Ingle et al. (1965) who report that synthesis of reserve protein in the endosperm of maize occurs mainly in the later development phases. The cytochemical investigations of Olszewska and Gabara (unpublished) also showed accumulation of reserve proteins in the later phases of development of the seed (phases III, IV, V), and the studies of Gabara and Modrzejewski (1971) confirmed that storage protein is the main component of ripe *I. pseudoacorus* seeds. The twice higher protein content in the chalasal pole in the first phase of endosperm development changes in favour of the micropylar pole in further phases. The higher protein content in the micropylar pole may be explained by the different amounts of endospermal tissue falling to the chalasal and micropylar parts.

The proteins of *I. pseudoacorus* are characterised by a preponderance of albumins and glutelins over globulins and prolamins (Table 3).

Table 1

Nitrogen compounds content in the endosperm of *Iris pseudoacorus* in the successive phases of development expressed in mg/100 mg of dry weight

Fractions	Poles									
	I Chala-sal	I Micro-pylar	II Chala-sal	II Micro-pylar	III Chala-sal	III Micro-pylar	IV Chala-sal	IV Micro-pylar	V Chala-sal	V Micro-pylar
Acid soluble N	0.32	0.11	0.47	0.27	0.47	0.28	0.46	0.48	0.02	0.03
Nucleic acid N	0.39	0.34	0.31	0.32	0.37	0.26	0.36	0.35	0.24	0.24
Protein N	3.12	2.48	3.14	2.55	3.29	2.20	2.37	2.75	1.22	1.12
Total	3.83	2.93	3.92	3.14	4.13	2.74	3.19	3.58	1.48	1.39

Table 1a

Analysis of variance in the split-plot system for the data of table 1

Variability	Degree of freedom	Sum of squares	Mean square	$F_{cal.\ (I)}$	$F_{cal.\ (II)}$
Blocks	2	0.052	0.026		
Fractions (A)	3	188.649	62.883	31441**	16.72**
Error E (a)	11	0.020	0.002		
Poles (B)	1	23.646	23.646	16.23**	4.53*
Interaction A×B	3	15.627	5.209	3.57*	3.57*
Error E (B)	119	173.381	1.456		
Total	139	401.375			

* Significance at p = 0.05.
** Significance at p = 0.01.
$F_{tab.}$ for 3; 11 deg. fr. ($\alpha = 0.05$) = 3.59; ($\alpha = 0.01$) = 6.22.
$F_{tab.}$ for 1; 119 deg. fr. ($\alpha = 0.05$) = 3.94; ($\alpha = 0.01$) = 6.90.
$F_{tab.}$ for 3; 119 deg. fr. ($\alpha = 0.05$) = 2.70; ($\alpha = 0.01$) = 3.98.

The level of all the above mentioned proteins increases as the seed ripens. The increase in globulin content during endosperm development is, however, the highest (Tables 3, 4). Klimienko and Leonov (1967) found that during ripening of maize seeds first glutelins appear, followed by globulins and albumins, and last comes zeine that is maize seed prolamin. It results therefrom that the protein characteristic for the seeds of the given plant is synthesised in this case at the end. These investigations demonstrated that globulins increased most intensively in the last phases of development. The question arises whether this kind of protein (globulin) is characteristic for the seeds of *I. pseudoacorus*. In view of this eventuality and of the

Table 2

Nitrogen compounds content in endosperm of *Iris pseudoacorus* in successive phases of development expressed in mg/100 seeds

Fractions	Poles									
	I Chalasal	I Micro-pylar	II Chalasal	II Micro-pylar	III Chalasal	III Micro-pylar	IV Chalasal	IV Micro-pylar	V Chalasal	V Micro-pylar
Acid soluble N	0.026	0.004	0.030	0.037	0.393	0.552	0.700	1.600	0.430	0.870
Nucleic acid N	0.012	0.002	0.074	0.082	0.690	0.322	0.866	1.976	3.680	5.180
Protein N	0.233	0.104	0.360	0.675	2.060	4.370	2.870	11.200	23.000	32.000
Total	0.271	0.110	0.464	0.794	3.143	5.244	4.436	14.776	27.110	38.050

Table 3

Protein fractions content in endosperm of *Iris pseudoacorus* in successive phases of development expressed in mg/100 mg of dry weight

Fractions	bieguny									
	I Chalasal	I Micro-pylar	II Chalasal	II Micro-pylar	III Chalasal	III Micro-pylar	IV Chalasal	IV Micro-pylar	V Chalasal	V Micro-pylar
Albumins	9.00	7.63	10.25	8.12	9.75	6.62	9.50	8.75	4.92	4.15
Globulins	0.20	0.11	0.62	0.62	1.25	0.68	1.06	1.00	1.00	1.10
Glutamins	12.43	10.90	9.68	7.75	10.75	6.25	6.87	7.18	4.16	4.80
Prolamins	0.08	0.06	0.09	0.17	0.14	0.14	0.12	0.11	0.12	0.18
Total	21.71	18.70	20.64	16.66	21.89	13.69	17.55	17.04	10.20	10.23

Table 4

Protein fractions content in endosperm of *Iris pseudoacorus* in successive phases of growth expressed in mg/100 seeds

Protein fractions	Poles									
	I Chalasal	I Micro-pylar	II Chalasal	II Micro-pylar	III Chalasal	III Micro-pylar	IV Chalasal	IV Micro-pylar	V Chalasal	V Micro-pylar
Albumins	0.37	0.23	1.47	3.63	5.11	8.42	8.90	29.60	60.11	84.70
Globulins	0.08	0.01	0.59	0.43	0.78	0.86	1.14	5.20	12.20	15.10
Glutamins	0.50	0.33	1.39	3.38	5.70	7.94	8.63	30.20	63.50	89.50
Prolamis	0.03	0.01	0.13	0.11	0.87	1.71	1.42	4.70	10.30	11.00
Total	0.98	0.58	3.58	7.55	12.46	18.93	20.09	69.70	146.11	200.30

fact of intensive globulin increase with the growth and development of the endosperm, further studies were devoted to these proteins.

Globulins of the endosperm in mature seeds were separated into four fractions by way of ion exchanger chromatography on DEAE cellulose (Diagrams 1, 2). Fraction one is earliest synthesised and prevails above the others in the entire course of development. The remaining fractions appear as the endosperm maturates, and fraction four reaches a high level in the end phase. Fractions two and three have only low peaks. Similar investigations have been carried out by Jennings and Morton (1963) on the developing endosperm of wheat. They determined, moreover, the amino acid composition of the particular fractions. These authors suggest that the amino acid composition may change during the growth and development of the seed. It would have been interesting in the present study to determine the amino acids of the particular globulin fractions. Only then would the characteristic of the globulins of *I. pseudoacorus* seeds in the successive developmental phases be full. It should be mentioned that in the course of globulin separation on the cellulose column a somewhat different rate of increase of these fractions was noted at both poles in the particular phases of endosperm development. Besides, in the end phase of seed development, a somewhat larger amount of the globulin fractions one and two were found in the micropylar endosperm. This result is rather unexpected. It would seem that in the ripe seed the endosperm does not exhibit any more bipolarity. This observation may be explained by the fact that the micropylar pole endosperm surrounds the embryo, and perhaps this kind of protein is necessary to the embryo during germination.

Investigations on the phosphorus content in the acid-soluble fraction showed that the orthophosphate amount is highest when converted to dry weight in phase I in the chalasal pole (Table 5). Those compounds are supplied from the mother plants and take part in the intensive metabolism. These data confirm the cytochemical and biochemical studies which revealed a considerable amount of acid phosphatase in the chalasal part of the endosperm in the initial period of its development (Gabara et al. 1972). With advancing maturation the orthophosphates content decreases. On the other hand, the absolute amounts of these compounds first increase up to phase III, remain in phase IV at about the same level and then fall (Table 6). Similarly, Jennings and Morton (1963) found that in the period of reduced water supply vigorous phytin synthesis went on in the wheat endosperm with a simultaneous fall of the inorganic phosphorus level. The differences between the poles in orthophosphate content disappear in ripe seeds.

Phosphorus (when converted to dry weight values) bound in the chalasal pole remains more or less at the same level. In the micropylar pole a distinct increase is noted, particularly in phase III, and then a decrease. In ripe seeds the amount of bound P in the micropylar pole reaches the same value as in the chalasal pole (Table 5). The absolute bound P content including also ATP increases with maturation of the seed, reaching maximum at the end phase.

The lack of differences between the poles in lipid P content is supported by the investigations of Olszewska and Gabara (unpublished) who demonstrated on

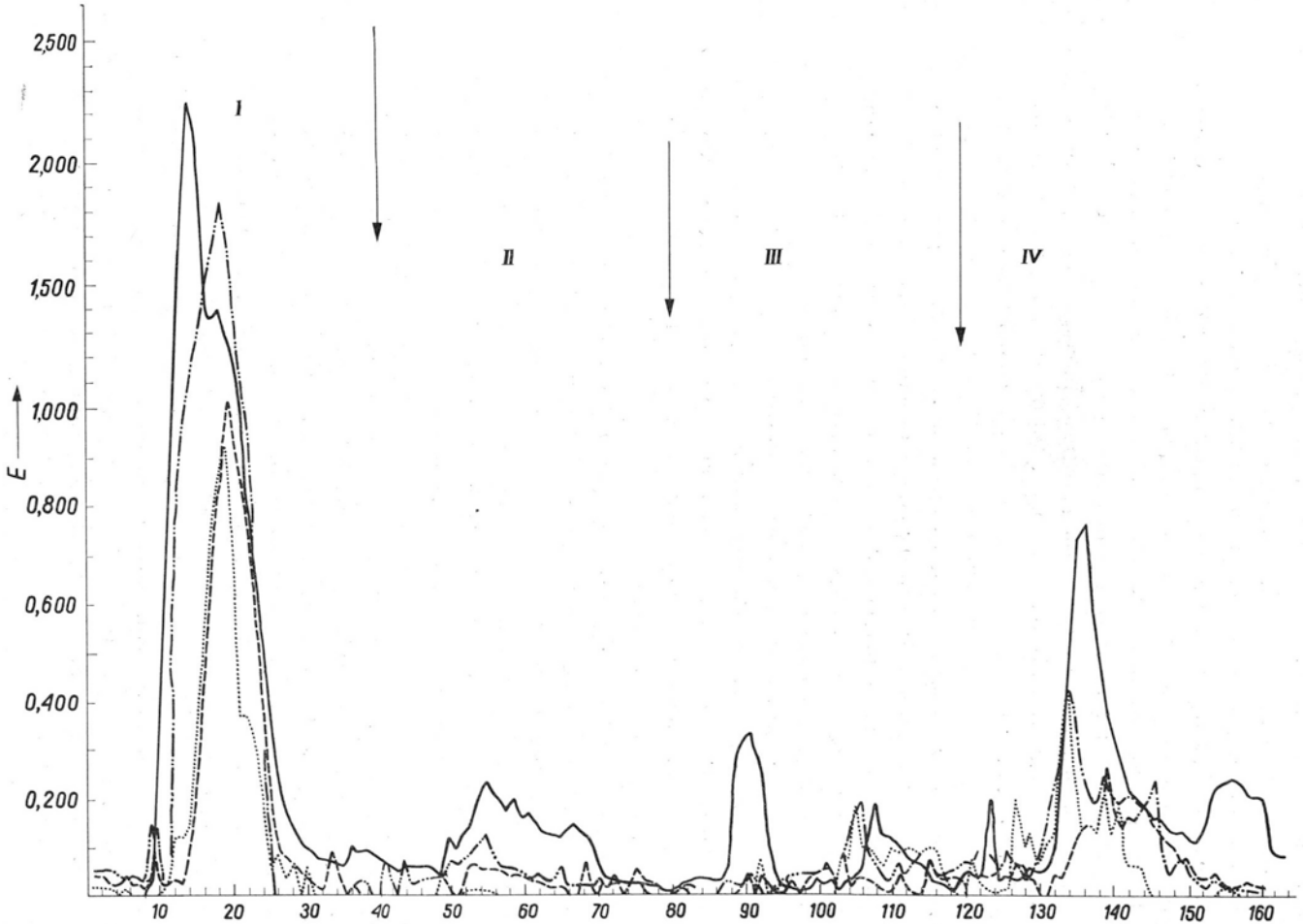

Diagram I. Chromatographic profile of globuline separation in the endosperm of *Iris pseudoacorus* (chalasal pole)

I — 0.2 M NaCl; II — 1 M NaCl; III — 0.1 M NaOH; IV — 1% NaOH

. . . . . II stage; — — — — — III stage; — . . — . . — . . — IV stage; ———— V stage

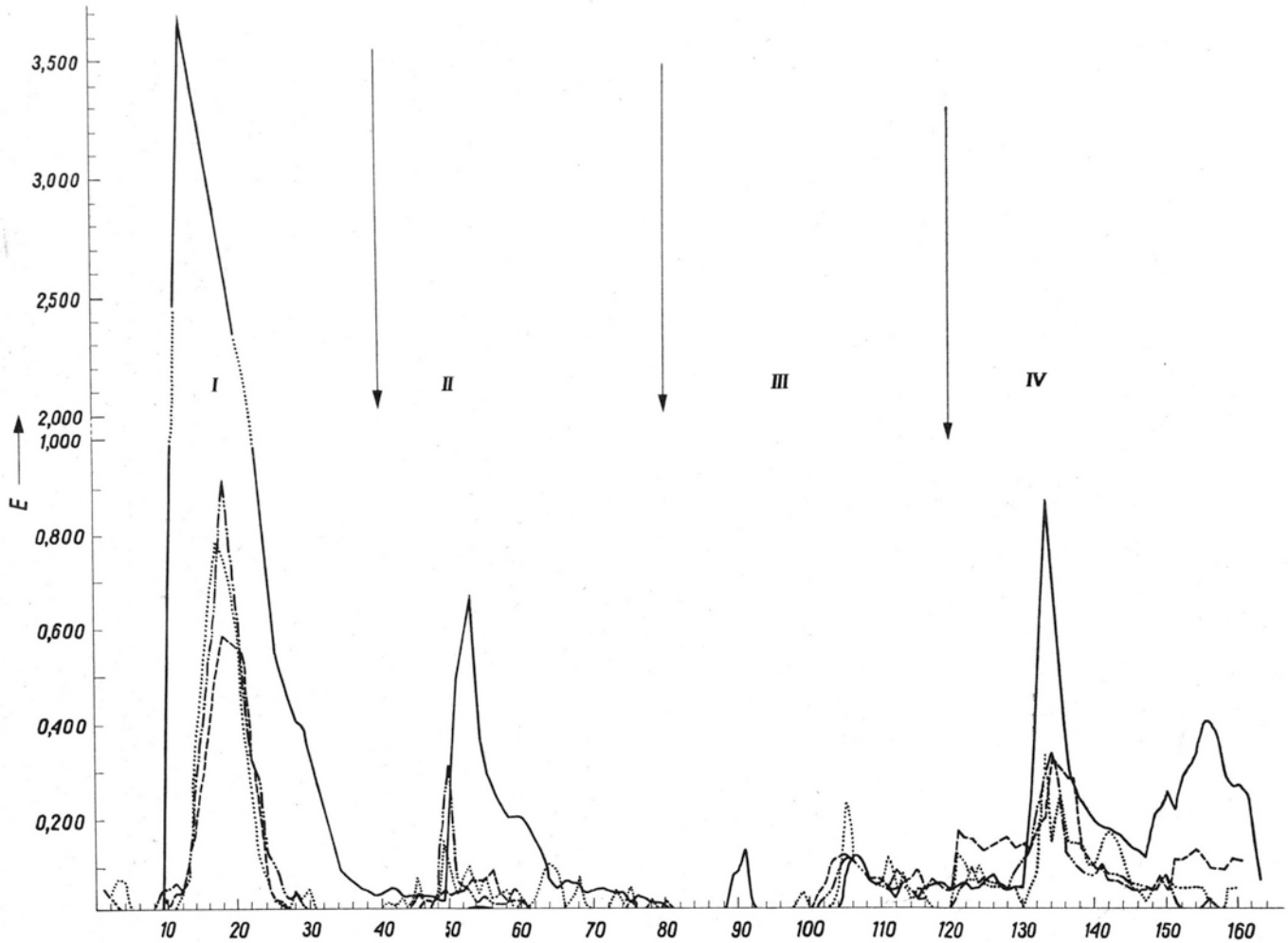

Diagram II. Chromatographic profile of globuline separation in the endosperm of *Iris pseudoacorus* (micropylar pole)

Legend as in Diagram I

the basis of sudanophily of the endosperm cells in *I. pseudoacorus* that lipids accumulate in both poles with equal intensity.

The RNAP content remains almost the whole time at the same level, only in phases II and III a drastic increase is noted in the chalasal pole. Probably in these periods synthesis of nucleic acids occurs associated with intensive cell division (Olszewska, Gabara 1966). The absolute RNAP amount increases as the seeds ripen reaching maximum at full ripeness. During the entire period of development up to phase IV this fraction prevails in the chalasal pole. It is only in the endosperm of mature seeds that there is more RNAP in the micropylar pole. The increase in RNA content in the initial phase of development of maize seed was also established by Ingle et al. (1965), and in the seeds of *Papaver somniferum* by Johri and Maheshvari (1966). The rise of the RNAP and DNAP levels in the chalasal pole in phase II is elucidated by investigations concerning the mitotic gradient in *I. pseudoacorus* by Olszewska and Gabara (1966). These authors found that the chalasal pole is characterised up to phase II by a more intensive mitotic activity, and is the site where the mitotic wave is initiated. Then the gradient of mitotic activity subsides and the endosperm cells divide asynchronically. Jannings and Morton (1963) also found that in wheat seeds intensive cell division is connected with the rise of the RNA and DNA levels. A larger amount of DNAP in the chalasal pole may be connected with the polyploidisation of the nuclei in this zone (Olszewska, Gabara 1966). This phenomenon characteristic for trophic plant tissues leads to an enhanced metabolic activity of such cells (Cinger 1958). The increase of the absolute amount of RNAP and DNAP with growth and development of the seeds is also a confirmation of the data of Grzesiuk (1967) who reports that the RNA and DNA amounts recalculated to 100 wheat and oat seeds increase with their development and reach maximum in the dough phase, the synthesis being, however, slower in the endosperm than in the embryos.

In view of the important role played by nucleic acids in the growth, development and metabolism of ripening seed, it seemed purposeful to perform a precise analysis of ribonucleic acid. By means of electrophoresis on coloured agar gel it was possible to demonstrate in ripe seeds three fractions (scheme). Probably the fraction closest to the starting line with high molecular weight and of brick-orange colour in the UV represents ribosomal ribonucleic acid (rRNA), and it is possible that the light green in UV low-molecular-weight fraction is soluble ribonucleic acid (sRNA), whereas the medium-molecular-weight fraction, lemon-coloured in the UV would be messenger ribonucleic acid (mRNA). During seed development, however, all fractions are not detectable, probably on account of their occurrence in very small quantities. In the chalasal pole in phase II, only low-molecular weight ribonucleic acid is detectable, and in the micropylar pole medium-molecular weight ribonucleic acid. In phase III small amounts of high-molecular weight RNA are detectable in both poles, the differences between the poles remaining, however, considerable. In the chalasal pole there is more medium-molecular-weight RNA, and in the micropylar pole more high-molecular-weight RNA and a detectable amount of the low-molecular weight fraction. The occurrence of larger amounts

Table 5

Phosphorus compounds content in endosperm of *Iris pseudoacrous* in successive phases of growth expressed in mg/g dry weight

Fractions		Poles									
		I Chalasal	I Micro-pylar	II Chalasal	II Micro-pylar	III Chalasal	III Micro-pylar	IV Chalasal	IV Micro-pylar	V Chalasal	V Micro-pylar
Acid-soluble	Orthophosphatate	5.20	1.28	3.00	1.20	3.20	2.30	1.90	1.50	0.10	0.09
	Bound P	2.40	0.60	2.20	0.80	2.30	1.80	1.20	0.80	2.30	2.40
Phospholipid P		1.20	0.72	1.00	0.91	0.51	0.65	0.31	0.34	0.12	0.12
RNAP		0.70	0.50	1.99	0.84	1.50	0.60	0.91	0.58	0.63	0.62
DNAP		0.17	0.12	0.42	0.23	0.40	0.21	0.28	0.16	0.19	0.19
Total		9.67	3.14	8.61	3.98	7.91	5.56	4.60	3.38	3.34	3.42

Table 6

Phosphorus compounds content in endosperm of *Iris pseudoacorus* in successive phases of growth expressed in mg/100 seeds

Fractions		Poles									
		I Chalasal	I Micro-pylar	II Chalasal	II Micro-pylar	III Chalasal	III Micro-pylar	IV Chalasal	IV Micro-pylar	V Chalasal	V Micro-pylar
Acid-soluble	Orthophosphatate	0.041	0.004	0.062	0.052	0.310	0.130	0.370	0.190	0.170	0.180
	Bound P	0.026	0.002	0.031	0.024	0.140	0.120	0.240	0.150	3.620	5.600
Phospholipid P		0.012	0.028	0.047	0.072	0.053	0.110	0.091	0.190	0.190	0.260
RNAP		0.005	0.003	0.028	0.027	0.094	0.061	0.182	0.089	1.014	1.394
DNAP		0.002	0.001	0.006	0.006	0.025	0.016	0.056	0.024	0.289	0.420
Total		0.086	0.038	0.174	0.181	0.622	0.437	0.939	0.643	5.283	7.854

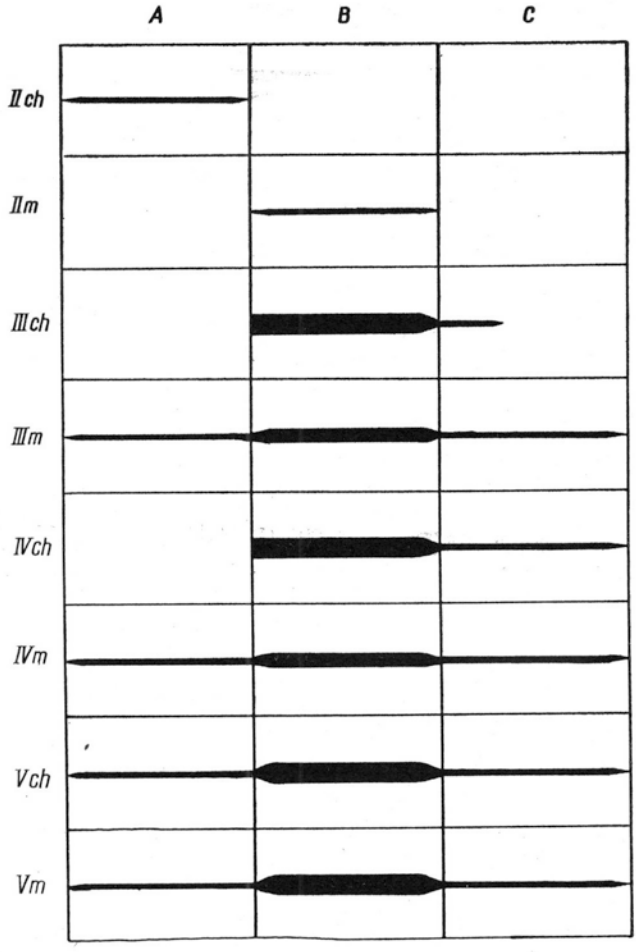

Fig. 1. Scheme of electropherograms

ch – chalasal pole; *m* – micropylar pole; *C*, *B*, *A* – successive fraction from starting line; I–V – successive phase of endosperm development

of high-molecular weight RNA in the micropylar part of the endosperm is probably associated with the formation of the embryo in this part. In phase IV the amount of RNA (probably ribosomal) increases further in the endosperm of the chalasal pole. In the ripe seeds there are no more noticeable differences in the content of the particular fractions between the endosperm of the two poles. These changes in the content of the particular RNA fractions in both poles are no doubt associated with the wide differences in the structure of the endosperm itself and the rate of cell division (Olszewska, Gabara 1966).

The present paper is an attempt at elucidation of the biochemical changes occurring in the course of development of the endosperm of *Iris pseudoacorus*

seeds against the background of histo- and cytochemical data. The investigations confirmed the differences between the endosperm of the chalasal and micropylar poles, demonstrated previously at the cellular and subcellular levels. The results may serve as base for further investigations concerning the relations between the structural and biochemical changes in the cell.

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Biochemiczne badania rozwoju bielma Iris pseudoacorus L.

Streszczenie

W bielmie bieguna chalazalnego i mikropylarnego nasion *Iris pseudoacorus* w kolejnych fazach rozwoju oznaczono zawartość frakcji azotu, białek oraz przeprowadzono chromatografię jonowymienną globulin na DEAE celulozie. Ponadto przeanalizowano frakcje fosforu i rozdzielono RNA na drodze elektroforezy w barwnych żelach agarowych. Względna ilość azotu obniża się, a bezwzględna wzrasta w miarę rozwoju bielma. Większą zawartością N charakteryzuje się biegun chalazalny. W białkach przeważają gluteliny i albuminy. Najintensywniej jednak w miarę rozwoju bielma przyrastają globuliny. Poza tym stwierdzono znaczne różnice ilościowe we frakcjach globulin w obu biegunach.

Bezwzględna ilość ortofosforanów w początkowym okresie rozwoju wzrasta, po czym maleje, a P kwasorozpuszczalnych związków organicznych, RNAP i DNAP w miarę rozwoju nasion rośnie. Względna zawartość RNAP za wyjątkiem II i III fazy utrzymuje się na jednakowym poziomie. W trakcie rozwoju bielma stwierdzono znaczne różnice w zawartości frakcji RNA w obu biegunach.

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