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IDENTIFICATION OF HELMINTH SPECIES BY MEANS OF DISC ELECTROPHORESIS

L. MIKULÍKOVÁ

Institute of Parasitology, Czechoslovak Academy of Sciences, Prague

Abstract. Soluble proteins of 25 helminth species of the classes Trematoda, Cestoidea and Nematoda, were separated by disc electrophoresis using polyacrylamide gel columns. Differences between the species were investigated on the basis of R_m values of the bands. Protein spectra were complemented by the detection of lipoproteins and glycoproteins and by identification of LDH, SDH, peroxidase, esterase and alkaline phosphatase. On the basis of comparison of protein spectra of parasitic worms belonging to three taxonomic classes it was found by means of numerical taxonomy that individual classes are characterized by a certain number of proteins of the same migration properties.

With the development of new, rapid methods in biochemistry and histochemistry attempts are being made to apply these methods in the taxonomy of helminths. This new approach in the species identification has been dealt with by several authors: Klimenko and Seranov (1972) Vasilev and Osikovski (1974), Vasilev et al. (1975), Bedford and Leader (1977).

Yoshimura (1968) used the method of electrophoretic separation of proteins to differentiate the adult specimens of the species *Schistosoma japonicum* and *Schistosoma mansoni*. In a further paper (Yoshimura 1969 a), the differences in the electrophoretic profile of proteins of the species *Paragonimus westermani*, *P. ohirai* and *P. myizakti* were described. Another paper (Yoshimura et al. 1970) demonstrated the differences in protein content of the species *Paragonimus kellicoti* from *P. myizakti*. The definitive morphological characteristic of adults has not yet been given for these two species. The author used for his experiments metacercariae obtained from experimental invasion. Electrophoresis in polyacrylamide gel was also applied by Klimenko (1969, 1972) for differentiation between *Fasciola hepatica* and *Fasciola gigantica* on the basis of their protein fractions. In another paper, Klimenko (1969) found marked differences in protein content of several species of cestodes belonging to the family Anoplocephalidae. Similar investigations were also carried out by Naumycheva and Sushchenko (1969) using the species *Ascaris suum*.

In our paper, we identified the differences in somatic proteins and some enzymes of different species of parasitic worms. The aim of our study was to compile the data and particulars of the differences in protein spectra of helminth species belonging to different genera and families.

MATERIAL AND METHODS

The species of parasitic worms of the classes Trematoda, Cestoidea and Nematoda used in our experiments were obtained at autopsy of birds and mammals, from slaughtered cattle, or they were expelled from the intestines of hosts after treatment with anthelmintics*).

*) A survey of helminths investigated and the abbreviations used are given in Table 1.

Preparation of extracts: Fresh material, no longer than 3 hours after removal from the hosts, was used in our experiments. The specimens were washed several times with physiological saline (0.9 % NaCl), then with distilled water, dried, weighed and homogenized in cooled Potter-Elvehjen homogenizer with teflon cylinder. Proteins were extracted with O.1M Tris-glycine buffer, pH 8.3. Homogenate was centrifuged for 25 min. at 20 000 g. Supernatant was stored in test-tubes at 20 °C. The samples were prepared from 5—15 worms of the same species from the same collection.

Electrophoresis: For the separation of acid proteins the method of Ornstein and Davis (1962) was used, demonstration of basic proteins was made using the method of Gesteland and Staehelin (1967). 200 μg of proteins were applied on the gel column (8 × 0.6 cm). Electrophoresis was conducted at constant flow 2.5 mA/column and terminated when the marker dye migrated 4 mm from the lower margin of the gel. The gels were stained with 1 % solution of Amido-Black B in 7 % acetic acid for 60 min and destained electrophoretically. A modification of histochemical methods was used for identification of enzyme activities. The concentration of proteins applied on gel for specific detection of enzymes was increased 3-times (about 500—700 μg). The samples were concentrated with Aquacid II (Calbiochem, USA) at 4 °C. Lipoproteins were detected according to Gebot (1969). Glycoproteins were stained using the method of Arai and Wallace (1969) modified in such a manner that the gels were transferred from the buffer solution to an alcohol row into the 95 % alcohol which prevents a sudden dehydration and damage of the gel.

Histochemical detection of enzymatic activities: The following methods were used for the detection of individual enzymes: dehydrogenase of lactic acid — Schrauwen (1966), dehydrogenase of succinic acid — Schrauwen (1966), cytochrome-oxidase — Lawrence et al. (1960), catechol-oxidase — Lysenko and Málek (1969), catalase — Brewer and Sing (1970), peroxidase — modification of Sahulka (1970), arylesterase — Lojda (1958), lipase — Brewer and Sing (1970), alkaline phosphatase — Lawrence et al. (1960), acid phosphatase — Beneš and Opatrná (1964), protease — Kučera and Lysenko (1968).

Location of the individual protein bands is further expressed as RM (relative mobility). The taxonomic relations of the species examined were determined by means of numerical taxonomy. If each of the taxons examined shows a specific protein pattern of different RM, then the individual proteins of this set may be considered characteristic for distinguishing the species. This is true under the assumption that the characteristics (in our case protein bands) are of the same taxonomic value (Adaenson's principle) (see Sneath 1969). In our investigation, the bands showing the same RM may be regarded as identical and those of different RM as different. Then two sets (in our case two taxons) may be compared and their resemblance may be expressed numerically as % of similarity (% S). For calculation we used the equation

$$\% S = \frac{ni}{ni - nd}$$

where ni = number of identical bands, and nd = number of different bands.

RESULTS

The orientation comparison of acid (pH 4.5) and alkaline (pH 8.3) buffer used for protein separation revealed that the alkaline system is more suitable. Further separation was therefore carried out at pH 8.3. Protein spectra of the helminth species examined are shown in Figs. 1, 2, 3. On the basis of a similarity between the protein spectra of the individual species, the proteins of the same RM, which were found in all species within a higher taxon, may be excluded. The results are shown in Tables 1, 2, 3.

Table 1. Helminth species examined (abbreviations used in the text)

Trematoda (T)
Hypoderaeum conoideum (T_1)
Echinoparyphium recurvatum (T_2)
Dicrocoelium dendriticum (T_3)
Fasciola hepatica (T_4)
Fascioloides magna (T_5)

Cestoidea (C)
Moniezia expansa (C_1)
Moniezia benedeni (C_2)
Hymenolepis fraterna (C_3)
Rodentolepis erinacei (C_4)
Fimbriaria fasciolaris (C_5)
Diploposthe laevis (C_6)
Paricterotaenia porosa (C_7)
Dilepis undula (C_8)
Dipylidium caninum (C_9)
Taenia hydatigena (C_{10})
Taenia pisiformis (C_{11})
Taeniarhynchus saginatus (C_{12})

Nematoda (N)
Heligmosomum halli (N_1)
Crenosoma striatum (N_2)
Ascaris suum (N_3)
Ascaris lumbricoides (N_4)
Ascaridia galli (N_5)
Toxascaris leonina (N_6)
Toxocara canis (N_7)
Toxocara mystax (N_8)

Table 2. RM values of proteins present in all species examined belonging to the class Trematoda

a	b	c	d
0.01	0.01	0.01	0.01
0.06		0.06	
0.07			
	0.10		
0.12	0.12	0.12	0.12
		0.18	
0.20		0.20	
0.24	0.24		
0.30	0.30	0.30	
	0.35	0.35	
0.40	0.40	0.40	
*0.53—0.60	0.52	0.52	0.52
	0.56	0.56	
	0.68	0.68	
	0.80	0.80	
	0.88	0.88	

a — Echinostomatidae, b — Dicrocoeliidae, c — Fasciolopsidae, d — all species of the class Trematoda, * — difuse band

The numerical comparison of protein spectra shown in Fig. 1 is shown graphically in Figs. 4, 5, 6. Since the activities of enzymes provide only poor spectra, and, moreover, the bands are not exactly bordered, these results were not included in the numerical evaluation. Moreover, we failed to detect with the applied methods the activity of tyrosinase, catalase, protease, and cytochromoxidase. However, the negative results of our experiments do not exclude the possibility that these enzymes might be detected if another arrangement is used.

TREMATODA

A survey of proteins common for all species characterizing the families of the class Trematoda is given in Table 2. Nine proteins were found to be present both in T_1 and T_2 species belonging to the family Echinostomatidae (Table 2a), 13 proteins were present in each specimen of T_4 and T_5 species belonging to the family Fasciolopsidae (Table 2c), while in the species T_3, belonging to the family Dicrocoeliidae (Table 2b) there were the same proteins as in the groups a and c. The proteins which were found in all families a, b, c are mentioned in Table 2 as d and may be regarded as characteristic for the whole class Trematoda. These relations are consistent to a considerable extent also with the enzymatic activities (Figs. 2, 3).

Numerical evaluation of the relations within the class Trematoda, based on all of the species investigated (Fig. 1), is shown graphically in Fig. 4. These results reveal

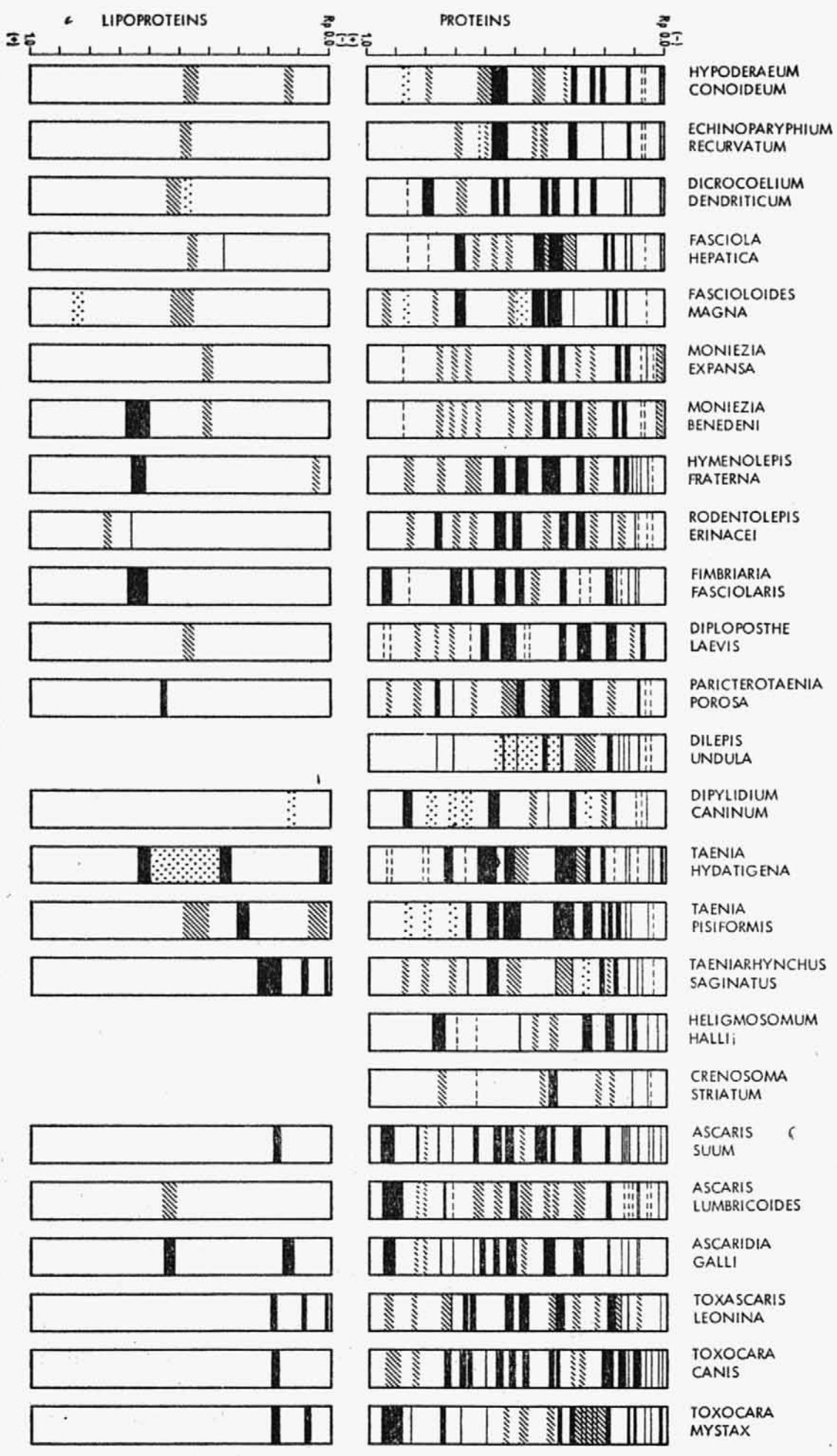

Fig. 1. Graphical illustration of protein and lipoprotein spectra of helmnith species examined. RM — relative mobility. Black fields — intensive staining, cross-hatched field — medium staining, dotted fields — light staining.

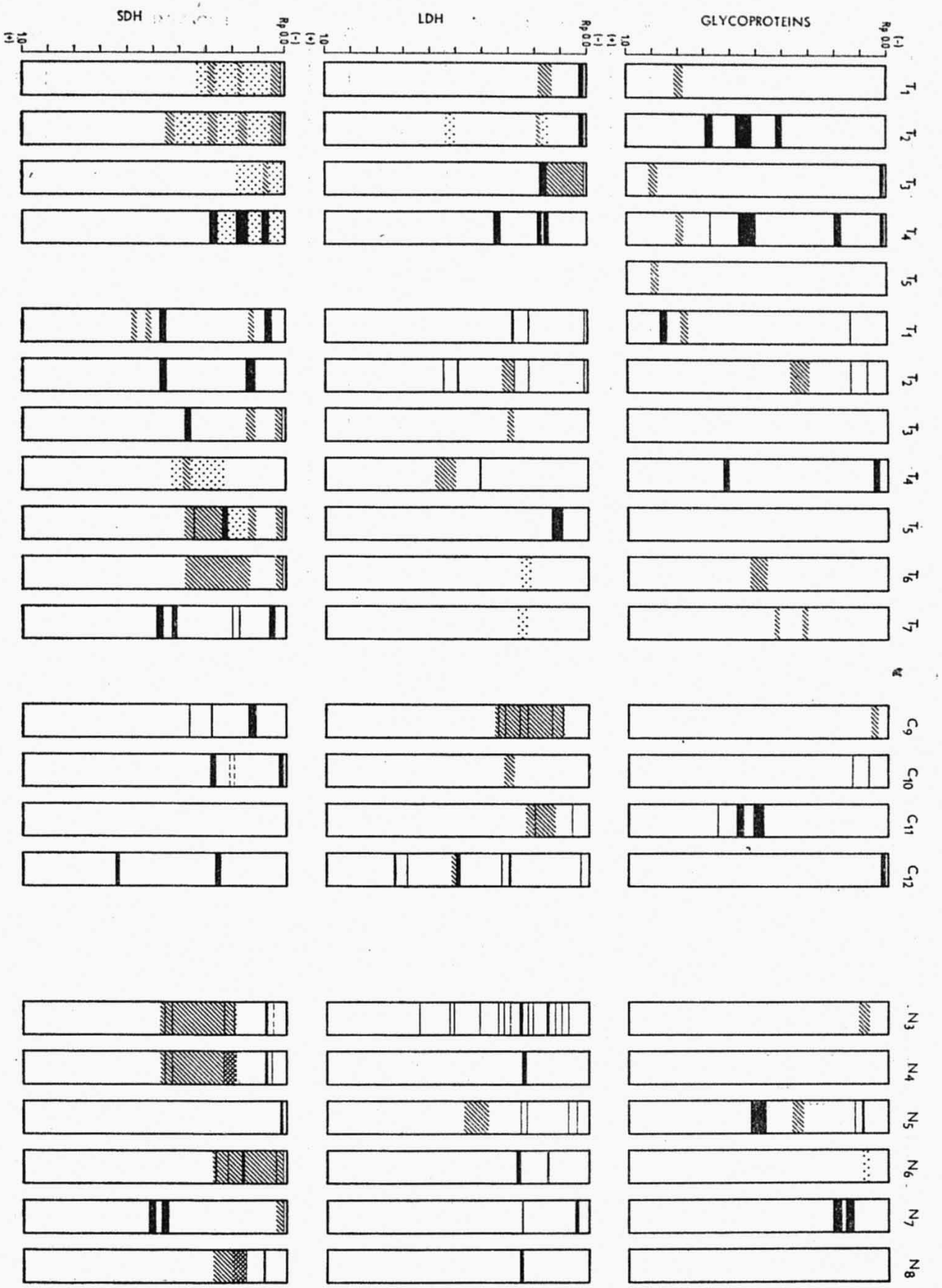

Fig. 2. Graphical illustration of glycoprotein spectra and LDH and SDH activities of helminth species examined. LDH — dehydrogenase of lactic acid, SDH — dehydrogenase of succinic acid. T, C, N — individual families and species (see Table 1).

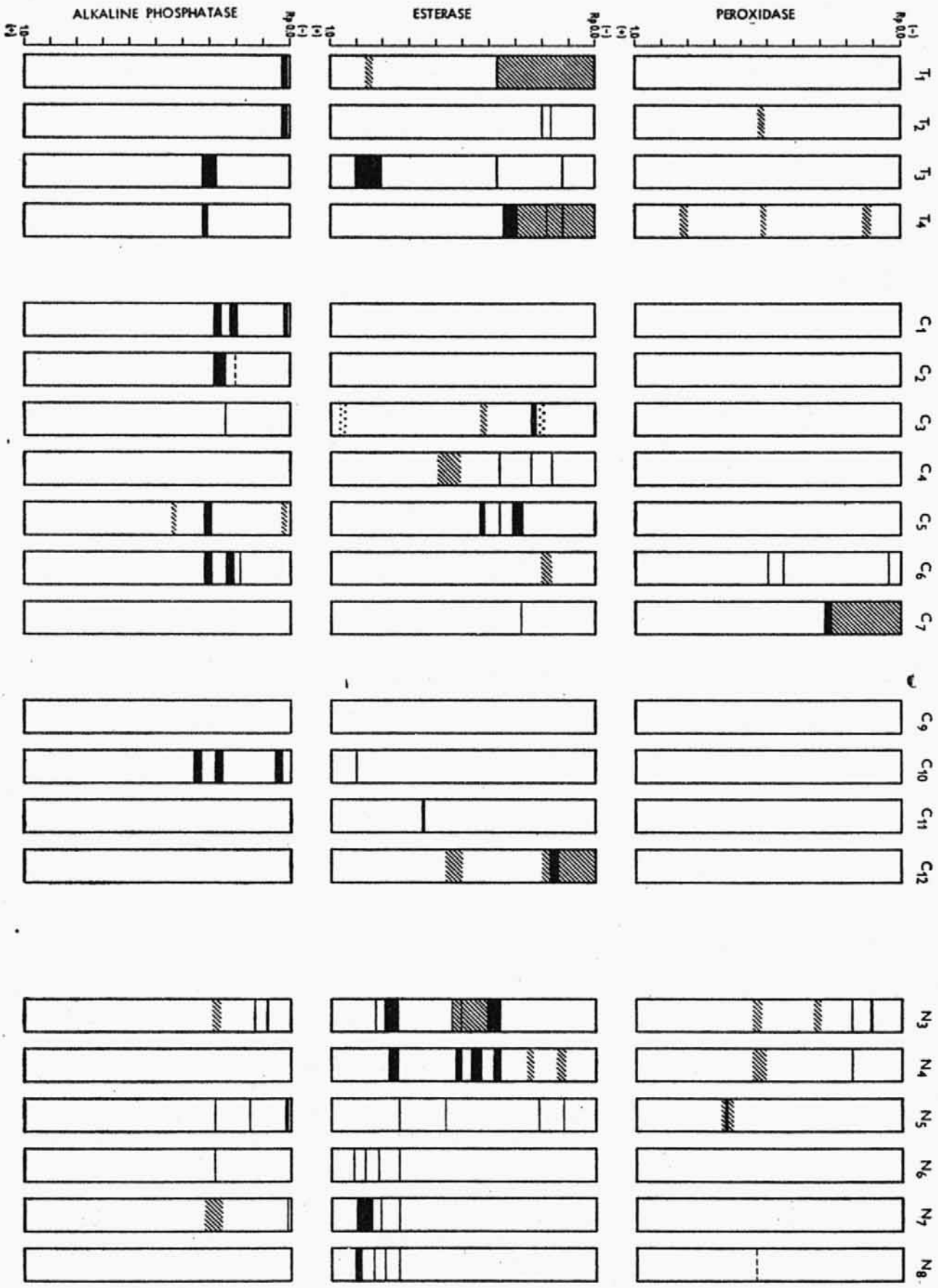

Fig. 3. Graphical illustration of activities of peroxidase, esterase, and alkaline phosphatase of helminth species examined.

Table 3. RM values of proteins present in all species examined belonging to the class Cestoidea

a	b	c	d	e	f	g
0.01						
0.03	0.03				0.03	
0.05	0.05		0.05		0.05	
				0.06		
			0.07	0.07		
	0.08	0.08		0.08		
0.11		0.11	0.11	0.11	0.11	0.11
			0.12		0.12	
	0.13		0.13			
			0.14			
0.15						
	0.18		0.18	0.18		
		0.19				
				0.20		
		0.21				
					0.23	
0.24						
	0.25					
				0.26		
0.29	0.29	0.29	0.29	0.29	0.29	0.29
0.35	0.35	0.35				
			0.36			
	0.38					
				0.39		
0.40						
			0.42			
0.46		0.46				
				0.48		
0.50	0.50	0.50	0.50—0.55	0.50	0.50	0.50
	0.56					
		0.60		0.60	0.60	
0.62						
0.65	0.65	0.65	0.65	0.65	0.65	0.65
			0.66		0.68	
0.70	0.70—0.72	0.70	0.70	0.70	0.70	0.70
0.75	0.75					
			0.76	0.76		
		0.78				
		0.81			0.81	
			0.82			
0.88	0.88	0.88	0.88	0.88	0.88	0.88
		0.92				
			0.93			
		0.95	0.95			

a — Anoplocephalidae, b — Hymenolepididae, c — Diploposthidae, d — Dilepididae, e — Dipylidiidae, f — Taeniidae, g — Cestoidea

a similarity of T_1 and T_2 within the family Echinostomatidae and T_4 and T_5 within the family Fasciolopsidae. The species T_3 (and thus also the family Dicrocoeliidae) forms a link between these two mentioned families.

The proteins which are present in all species and form thus a pattern of higher taxon (family) are shown in Table 3. The species C_1 and C_2 belonging to the family Anoplocephalidae differ in one protein only (Fig. 1). The species C_3, C_4 and C_5 belonging to the family Hymenolepididae have 16 proteins in common (Table 3 b). The species C_5 in the RM range 0.0–0.10, which seems to be characteristic for the determination of relative species, is not consistent with the species C_3 and C_4, but with C_6 (Fig. 1). The species C_6 of the family Diploposthidae has proteins which were also present both in C_5 and C_7. The species C_7 and C_8 assigned to the family Dilepididae correspond with each other in 19 proteins (Table 3 d). As to the family Dipylidiidae, we obtained only a single representative of C_9 showing a characteristic protein spectrum and only in the last third of the gel has the same proteins as C_{10-12} (Fig. 1). The species C_{10}, C_{11}, C_{12} of the family Taeniidae have 14 proteins in common (Table 3 f). The proteins which were present in all families a, b, c, d, e, f (in Table 3 as g) are characteristic for the class Cestoidea. The conformity of protein spectra of individual species within the respective families confirm to considerable extent also the enzymatic activities (Fig. 3). Numerical evaluation of the conformity of individual species belonging to Cestoidea is shown in Fig. 5. Their interrelations are essentially consistent with the results of graphical evaluation and with the systematic determination. Only the species C_5 showed a 60 % similarity both to species C_3 and C_4 belonging to the family Hymenolepididae and to C_6 of the family Diploposthidae, which gives evidence of the independent position of the species *Fimbriaria fasciolaris* (C_5). This species has been placed by Yamaguti (1959) in the family Hymenolepididae together with the species *Hymenolepis fraterna* (C_3) and *Rodentolepis erinacei* (C_4). The species C_5, C_6, C_7, C_8, shown in Fig. 5, parasitize birds. The electrophoretic profile of the worms parasitizing birds and mammals did

4

T_2	100				
T_1	81	100			
T_4	53	65	100		
T_5	44	47	75	100	
T_3	47	60	69	50	100

5

C_1	100											
C_2	94	100										
C_3	53	55	100									
C_4	58	55	80	100								
C_5	53	50	60	60	100							
C_6	48	48	41	53	62	100						
C_7	50	48	58	58	55	57	100					
C_8	53	52	58	59	58	53	65	100				
C_9	58	58	58	50	58	47	56	49	100			
C_{10}	55	56	48	50	57	59	50	59	49	100		
C_{11}	55	52	58	58	53	50	55	58	50	82	100	
C_{12}	53	52	58	55	53	48	42	43	46	65	80	100

6

N_3	100							
N_4	95	100						
N_5	76	76	100					
N_6	65	60	67	100				
N_7	58	58	61	66	100			
N_8	59	59	59	68	75	100		
N_2	49	46	48	47	45	45	100	
N_1	33	33	30	31	35	38	40	100

Figs. 4, 5, 6. Graphical illustration of similarity of different species. **Fig. 4.** — Trematoda, **Fig. 5.** — Cestoidea, **Fig. 6.** — Nematoda. T_1 — T_5, C_1 — C_{12}, N_1 — N_8 — species (see Table 1), numbers — percentage of similarity of individual species (see text).

not reveal any differences in the protein composition of these two groups of parasites. When calculating the RM values, the proteins of RM 0.93 present in the species C_5, C_6 and C_7 (parasitizing birds) and trace amount also in C_{10}, were excluded. The protein of RM 0.58 was identified in the helminth species parasitizing mammals. In order to make conclusions on the differences in protein pattern of parasites from different hosts, further studies of a larger number of species would be necessary.

NEMATODA

In the group of parasitic worms, marked N, the species N_1 of the family Trichostrongylidae and N_2 belonging to Protostrongylidae differ at the first sight from the other species of the family Ascarididae in their small number of proteins detected (12, 9). In the species of Ascarididae about 20 proteins are present (Table 4, Fig. 1). For the

Table 4. RM value of proteins present in all species examined belonging to the class Nematoda

a	b	c	d
	0.03		
		0.04	
0.05			
0.06	0.06	0.06	
0.10	0.10	0.10	0.10
0.12	0.12		
0.20	0.20	0.20	0.20
		0.22	
	0.25		
0.30			
0.38	0.38	0.38	0.38
	0.44	0.44	
0.48	0.48		0.48
0.53			
0.56			
0.65	0.65	0.65	0.65
0.70	0.70		
0.72	0.72	0.72	0.72
0.85			
0.93			

a — Ascarididae, b — Trichostrongylidae,
c — Protostrongylidae, d — Nematoda

lack of material we were unable to study the enzymatic activities of the species N_1 and N_2. For the species $N_3 - N_8$ belonging to the family Ascarididae the common proteins were detected (Table 4 a) and their resemblance was also confirmed by the determination of enzymatic activities (Figs. 1, 2, 3). The proteins which were found to be present in all species belonging to the class Nematoda are shown in Table 4 as d.

The results obtained by numerical evaluation (Fig. 6) confirm the graphical one and they are also consistent with the systematic classification of the species investigated.

DISCUSSION

When considering the application of disc electrophoresis in taxonomy, it may be stated that 1) this method is well-founded, 2) its modifications used in our investigation give comparable results. The absence of some enzymes does not exclude their possible presence if different arrangement is used. Since no quantitative determination of enzymes was carried out, the negative results may not be regarded as definitive. Therefore these tests were not used for numerical evaluation. The application of numerical taxonomy for determination of protein spectra of the species investigated proved to be expedient for it enables to compare a larger number of characters and their abstraction. This method, however, should also be regarded as auxiliary. Although the known taxonomic relationships were confirmed, it would not seem to be appropriate to make definitive taxonomic corrections on the basis of this metod only. The papers dealing with the electrophoretic profile of proteins and identification of enzymatic activities described only the protein composition of the lymph or tissue of the species investigated (Dubovsky 1973, Kurilenko 1971, Krasnoshchekov and Tomilovskaya 1975, Nizami et al. 1975, Roychowdhury 1973, Ruff et al. 1973). Our experiments show another possibility of application of this method showing the differences in the electrophoretic profile of different families and classes of helminths. We have exempted the proteins commonly occurring in the species of a certain family and obtained thus the electrophoretic profile of proteins which was characteristic for this family. Similarly, the protein pattern occurring in several families belonging to the same class was characteristic for all species included in this class. When comparing the species inside the different classes (Trematoda, Cestoidea, Nematoda) we observed a numerical similarity of the individual species. Thus the protein pattern of the families belonging to the class Cestoidea revealed that the species *Fimbriaria fasciolaris* represents a transition between the families Hymenolepididae and Diploposthidae. This fact was also confirmed by numerical methods. Yamaguti (1959) placed *Fimbriaria fasciolaris* in the family Hymenolepididae on the basis of the morphological characters. Skryabin and Matevosyan (1945), who considered the similar life cycles of the species *Fimbriaria fasciolaris* and *Diploposthe laevis*, assigned these two species to two subfamilies of the family Hymenolepididae, namely Fimbriariinae and Diploposthinae, consistently with the results of our analyses.

Yoshimura (1968, 1969 a, b) using the method of disc electrophoresis identified the differences in the electrophoretic profile of proteins of the species which could be hardly distinguished on the basis of their morphology. In our opinion, the differentiating ability of this method is so high that even the quantitative changes and qualitative difference in a single protein may be disclosed in different species. In our experiments we found the difference in a single protein between the species *Moniezia expansa* and *M. benedeni*, as well as *Ascaris suum* and *A. lumbricoides* (Mikulíková 1976). *Moniezia expansa* and *M. benedeni* belong to the same genus and differ from one another both in their morphology (shape of eggs, position of intersegment glands) and seasonal dynamics. The difference in their electrophoretic profile of proteins confirms that their specific determination was correct. The species *Ascaris suum* and *A. lumbricoides* are identical in their morphology. It has not yet been decided whether two species or a single one specific for a certain host is concerned. Due to the fact that there was a difference in a protein both in males and females of the same species (Kobara 1970) and that the enzymatic activities are also different, the specific determination under the name *Ascaris suum* and *A. lumbricoides* does not seem to be correct. If we compare the protein spectra and enzymatic activities of *Moniezia benedeni* recovered from sheep and cattle, the influence of host organism on the parasite may be excluded. Therefore

it may be inferred that the difference between the species *Ascaris suum* and *A. lumbricoides* is not caused by the host.

Disc electrophoresis is a sensitive and rapid method enabling to compare a larger number of samples at the same time. The enzymatic activities may be detected with the common histochemical methods. An advantage of disc electrophoresis is the fact that a small quantity of material may be used. It may be summarized: If standard procedures are used, the results are comparable and in questionable cases they may help to correct the identification.

ИДЕНТИФИКАЦИЯ ВИДОВ ГЕЛЬМИНТОВ ПРИ ПОМОЩИ ДИСКОВОГО ЭЛЕКТРОФОРЕЗА

Л. Микуликова

Резюме. Растворимые протеины 25 видов гельминтов, относящихся к классам Trematoda, Cestoidea и Nematoda, разделяли при помощи дискового электрофореза в полиакриламидном геле. Разницы между видами изучали на основе величин R_m отдельных полос. Белковые спектры дополнили обнаружением липопротеинов и гликопротеинов и идентификацией LDH, SDH, пероксидазы, эстеразы и щелочной фосфатазы. При сравнении белковых спектров гельминтов, относящихся к трем таксономическим классам, с помощью численной таксономии было обнаружено, что отдельные классы характеризованы определенным количеством протеинов, обладающих одинаковыми свойствами миграции.

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L. M., I. gynekologická klinika, U nemocnice 1, 120 00 Praha 2, ČSSR