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ELECTROPHORETIC PATTERNS OF PROTEINS AND ENZYMES IN *PARAMPHISTOMUM (P.) MICROBOTHRIUM* AND *CARMYERIUS GREGARIUS*

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ABSTRACT

The electrophoretic patterns of total soluble proteins, esterases, Glutamic-oxaloacetate transaminase (GOT) and malate dehydrogenase (MDH) are determined in two paramphistomes, infecting cows and buffaloes, namely *Paramphistomum (P.) microbothrium* (Fischoeder, 1901) and *Carmyerius gregarius* (Liiss, 1986). The results obtained indicate that the biochemical methods may provide an important promising tool for identification of paramphistomes at the generic level.

INTRODUCTION

The taxonomy of digenea, and indeed all the other helminth parasites, impose a number of problems due to the great variability encountered in the morphological and biological characteristics particularly at the lower taxonomic levels. The consensus of opinion amongst helminth taxonomists is that valid classification of these animals must depend on the collective characteristics of the parasite population, including morphology, life cycle, geographical distribution together with their cytological and biochemical features. Experimental methods, involving biochemical characterization of parasite populations, as an important tool in taxonomy, have gained wide interest among workers in this field with utmost use of techniques developed for the taxonomy of other animals e.g. birds (Sibley, 1960) and insects (Throckmorton, 1962).

Extensive use of electrophoresis in the characterization of the chemical constituents of organisms began around 1960, when it became common to separate protein and/or enzyme fractions electrophoretically followed by staining with specific methods to identify and study the electrophoretically separated protein bands and/or the enzyme and/or the isoenzyme activities to be used as a biochemical taxonomical criterion.

In this respect, Yoshimura (1968) reported that *Schistosoma japonicum* and *S. mansoni* have a species-specific disc electrophoretic patterns of proteins and enzymes, which could be easily used for their differentiation. Moreover, the same author reported that the disc electrophoretic patterns of proteins and enzymes could possibly be employed as an aid for the objective differential diagnosis of various species belonging to the genus *Paragonimus* (Yoshimura, 1969 a and b).

Similarly, Fripp (1970) suggested that the isoenzymes which hydrolyzed alpha- naphythyl acetate could be used to separate cercariae of *S. haematobium* those of *S. mattheei.* Mahon and Shiff (1978) have found that the phosphoglucomutase system (P.G.M.) could be more reliable in differentiating cercariae of the above two species. Ruff *et al.* (1973) demonstrated differences between the Japanese, Philippine and Formosan strains of *S. japonicum* based on polyacrylamide disc gel electrophoresis of total soluble proteins. Similar results were obtained for strains of other species of schistosomes (Wright *et al.,* 1972; Southgate and Knowles, 1975 and Ross *et al.,* 1978). Fletcher *et al.* (1980) used starch gel electrophoresis of 10 enzyme systems to differentiate *Schistosoma mekongi* from four strains of *S. japonicum.*

Fletcher and Loverde (1981) and Boissezon and Jelnes (1982) used the differences in the mobility of certain enzymes to distinguish between the cercariae of *S. rodhaini* and *S. mansoni.* These authors believed that the mobility of cercarial phosphoglycomutases provided a fast and dependable way to differentiate between the adults and cercariae of the two species.

Haque and Siddiqi (1982) reported a histochemical and electrophoretic study on the isoenzymes of acid and alkaline phosphatases in four trematodes, including *Gigantocotyle explanatum, Gastrothylax crumenifer, Echinostoma malayanum and Fasciolopis buski.*

Experimental studies in the field of the biochemical taxonomy of paramphistomes are scarce. Wright *et al.* (1979) studied some enzymes of the intramolluscan larval stages of *P. (P.) microbothrium in Bulinus senegalensis.* They also determined the isoelectric points for some enzymes of the adult worm of that species. Oskovskii *et al. (1977)* made a brief comparative electrophoretic of prptein fractions in *P. microbothrium* form different hosts. Wright *et al.* (1979) stated that in view of the

difficulties encountered in making specific identification of adult paramphistomes, studies on the enzyme analysis of adult worms would contribute to the solution of some of the taxonomic problems in this group.

The aetiological agents of paramphistomiasis in Egypt include two species, namely *Paramphistomum (P.) microbothrium* (Fischoeder, 1901) and *Carmyerius gregarius* (Looss, 1896). The present work was done to devlop valid biochemical methods to distinguish between these two species of paramphistomes. It is hoped that studies of this nature would extend beyond regional limits to contribute towards solving taxonomic problems frequently encountered in most helminth parasites.

EXPERIMENTAL

Adult specimens of two paramphistomes: *Paramphistomum (P.) microbothrium* (Fischoeder, 1901) from cows and buffaloes and *Carmyerius gregarius* (Looss, 1986) from cows, are obtained alive from the rumen and reticulum of their respective hosts that are slaughtered at the Cairo Abattoir.

1. Preparation of Samples for Electrophoresis of Proteins

Adult worms are washed in 0.7 % saline solution as soon as they are collected and blotted with filter paper to absorb adhering water. A pool of equal number of worms from each group is placed in a micro-test tube and homogenized, using a rotating glass rod. Centrifugation at 18,000 r.p.m. for 2 minutes gives a clear supernatent which is ready for electrophoretic application.

2. Preparation of Samples for Enzyme Electrophoresis

The blotted worms are homogenized in an equal volume of 0.1 M Trist-HCI (pH = 7.0) with an enzyme stabilizer such as dithiothreitol, e-aminocaproic acid and ethylenediaminetetra acetic acid (EDTA), each to a concentration of 1.0 mM (Wright *et al*, 1979). The resulting suspension is centrifuged at 50,000 g at 4° C for 1 minute. The clear supernatent fluid is ready for immediate application to the cellogel strips.

By using cellogel, the complications frequently met on different electrophoretic supporting media are eliminated. Accordingly, a high degree of standardization and reproducibility of electrophoresis are achieved.

Cellogel is available in the wet state in air-tight plastic bags. After the bag is opened, it is important not to let the strips in the open air. Once the bag is opened, the strips are directly immersed in 30% methanol in a plastic container until they are used.

3. a. **Buffer**

Sodium veronal is a very safe and efficient buffer and can be used in most electrophoretic separations. The buffer formula is a follows:10.3 gm Na barbiturate 1.84 gm diethyl barbituric acid. The pH of the buffer is adjusted at 8.6, using a pH meter. One compartment of the Gillman electrophoresis tank is filled with the buffer and tilted at one end to produce an even level in both compartments. Strips of cellogel are immersed in the buffer; a good impregnation of the strips is achieved after about 10 minutes.

b. **Electrophoresis Procedure**

i. While the strips are impregnated with the buffer, the test samples and control applicators are prepared.

ii. After 10 minutes impregnation, the strips are blotted between two filter paper sheets and then immediately placed on the bridge. The strips must be evenly stretched and arranged parallel to each other across the bridge. They are usually held in place by a plastic clip.

iii. The bridge is transferred into the tank and the samples are applied 2 cm fromthe cathodic edge; or from the anodic edge to separate the anodic migrating and the cathodic migrating protein fractions. The aplied volume is aproximately 10 microlitres. The tank is covered and connected to the power supply. The voltage is adjusted at 200V and the run starts and continues for 60-70 minutes after which the power supply is disconnected.

c. **Staining**

The strips are taken away from te bridge and immersed in Ponceau-S staining solution (0.5 gm in 100 ml 5 % trichloroacetic acid for 5 minutes). Destaining is affected by washing the strips into 3 baths of 5 % acetic acid after which the protein fractions become clearly visible. The strips may be preserved in 5 acetic acid.

d. **Densitometry**

The strips are rendered transparent before densitometry using the following simple procedure:

i. Dehydrate the strips for 1 minute in a pure methanol bath.

ii. The methanol is discarded and substituted with the transparentizing solution formed of 6 ml methanol, 14 ml acetic acid and 0.1 ml glycerol. The strips are left in that solution for about one minute.

iii. The strips are placed on a glass plate and the excess solution is then removed. They are transferred to a drying oven at 50-70° C for about one hour after which the

strips are allowed to cool at room temperature. They can be packed in an envelope for densitometry.

iv. The strips are scanned quantitatively using a Digital DG16 Gillman Scanning Densitometer.

4. **Electrophoresis of Isoenzymes**

Whenever, cathodic-migrating bands of enzymes are studied, the sample from adult worms is applied at the middle of the strips. In other cases, the sample is applied from the cathodic edge.

The same procedure described before for the separation of proteins is applied, except for staining that takes place using a reactive substratum specific for each enzyme (Jelnes, 1979). The strips can be preserved for some time in 4 formaldehyde or can be made transparent for densitometry after immersion in 30 % acetic acid in water and warming at 70°C.

The enzymes separated using cellogel include esterase, glutamate-oxalocetate transaminase and malate dehydrogenase.

Enzyme Staining

All enzyme staining took place in the dark at 37°C. The recipes for enzyme staining were as follows:

1. **Esterase (EST)**

250 ml	Tris buffer pH: 8.0
2 ml	1 % Alphanaphthyl acetate in acetone
100 mg	Fast Red

2. **Glutamate-oxaloacetate transaminase (GOT)**

3.8 g.	K_2HPO_4
1 g.	Polyvinyl pyrilidone
100 mg.	EDTA (Ethylene diaminetetraacetic acid)
74 mg.	2 oxoglutaric acid
266 mg.	L-aspartic acid
100 ml	Water

Shake well in order to dissolve chemicals and just before use add 250 mg Fast Violet B-salt and shake again.

3. **Malate dehydrogenase (MDH) (Wright *et al*, 1979).**

30 ml	0.2 M tris hydrochloric acid buffer. (pH 8.0)
5 ml	2.0 M maleate (Stock solution)
268.2 g	DL-malic acid

100 ml	Water
900 ml	4.0 M Sodium hydroxide

pH is ajdusted to 7.0 with sodium hydroxide

20 mg	NDP (nicotinamide adenine dinucleatide phosphate oxidized)
10 mg	NAD (niocotinamide adenine diculeatide oxidized)
20 mg	NBT (Nitro blue tetrazolium)
5 mg	PMS (Phenazine methosulphate)

Incubate in the dark from 30 minutes to 2 hours.

RESULTS

A key for trematodes under biochemical investigation is given in Table (1).

I. Total Soluble Body Proteins

1. Anodic-Migrating Bands

The electrophoretic mobility of anodic-migrating total soluble body proteins of *Carmyerius gregarius* from cows (P_1), *Paramphistomum (Paramphistomum) microbothrium* from buffaloes (P_2) and *P. (P.) microbothrium* from cows (P_3) are studied.

Table 1

Key of Trematodes Under Investigation

Trematodes	Host	Abbreviations
Carmyerius gregarius	Cows	P_1
Paramphistomum (P.) microbothrium	Buffaloes	P_2
Paramphistomum (P.) microbothrium	Cows	P_3

The number and relative distribution of different bands of anodic-migrating proteins in paramphistomes are shown in Table (2) and Fig (1).

Table 2

Densitometry of Anodic-Migrating Total Soluble Body Proteins In Paramphistomes

	Protein Bands					
TREMATODES	I	II	III	IV	V	VI
P_1	5.90	7.65	12.59	34.23	36.98	1.86
P_2	5.98	50.03	27.80	4.35	10.69	1.03
P_3	7.65	5.36	46.97	29.38	9.45	1.08

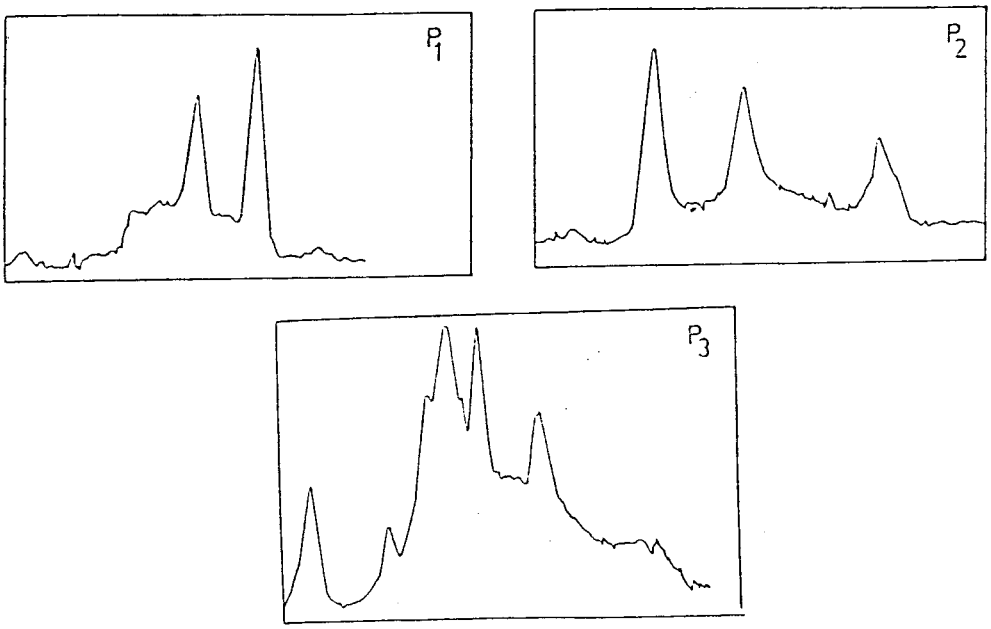

Fig. 1: Scan of Electrophoretic Patterns of Anodic-Migrating Total Soluble Proteins in Paramphistomes.

P_1 - *Carmyerius gregarius* from cows
P_2 - *Paramphistomum (P.) microbothrium* from buffaloes
P - *Paramphistimum (P.) microbothrium* from cows.

All trematodes showed five anodic migrating protein bands but the relative distribution and density of the different bands are different. In *Carmyerius gregarius* (P_1) there are two main bands (IV and V) representing 34.23 % and 36.98 % of the total proteins respectively. In *Paramphistomum (P.) microbothrium* from buffaloes (P_2) and *P. (Paramphistomum) microbothrium* from cows (P_3) one band (band II in P_2 and band III in P_3) constitutes 50.03 % and 46.97 % of the proteins respectively.

2. Cathodic-Migrating Bands

The number and the relative distribution of different bands of cathodic-migrating proteins in paramphistomes are shown in Table (3) and Figures (2).

In all trematodes there are 4 cathodic-migrating protein bands. In *Carmyerius gregarius*(P_1) bands I and II represent 36.78% and 56.78 % respectively. In *P. (P.)*

Table 3

Densitometry of Cathodic-Migrating Soluble Body Proteins In Paramphistomes

Trematodes	Protein Bands (%)			
	I	II	III	IV
P_1	36.78	56.41	2.19	4.61
P_2	10.04	44.00	40.10	5.60
P_3	17.0	27.43	29.22	26.15

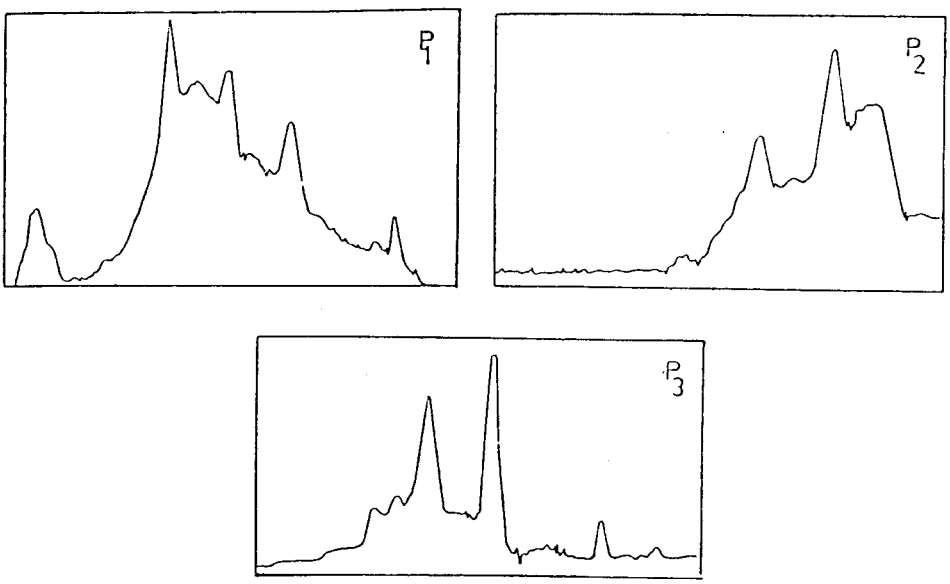

Fig. 2: Scan of Electrophoretic Patterns of Cathodic-Migrating Soluble Proteins in Paramphistomes.

P_1 - *Carmyerius gregarius* from cows.

P_2 - *Paramphistomum (P.) microbothrium* from buffaloes

P_3 - *Paramphistomum (P.) microbothrium* from cows.

microbothrium from buffaloes (P_2) the highest density resides in bands II and III which constitute 44.00% and 40.10% of total proteins respectively. In *P. (P.) microbothrium* from cows (P_3) the relative density of bands II, III and IV are nearly equal, being 27.43%, 29.22% and 26.15% respectively while band I constitutes 17.0% of the total.

II. Non Specific Esterases

The number, density and relative distribution of different fractions of anodic-migrating non specific esterases in paramphistomes are shown in Table 4 and Fig. (3).

Table 4

Densitometry of Anodic - Migrating Non Specific Esterases in Paramphistomes

Trematodes	Enzyme Fractions (%)				
	I	II	III	IV	V
P_1	--	30.63	47.00	10.98	11.35
P_2	--	22.89	47.26	16.03	13.79
P_3	12.29	28.26	46.98	14.68	7.85

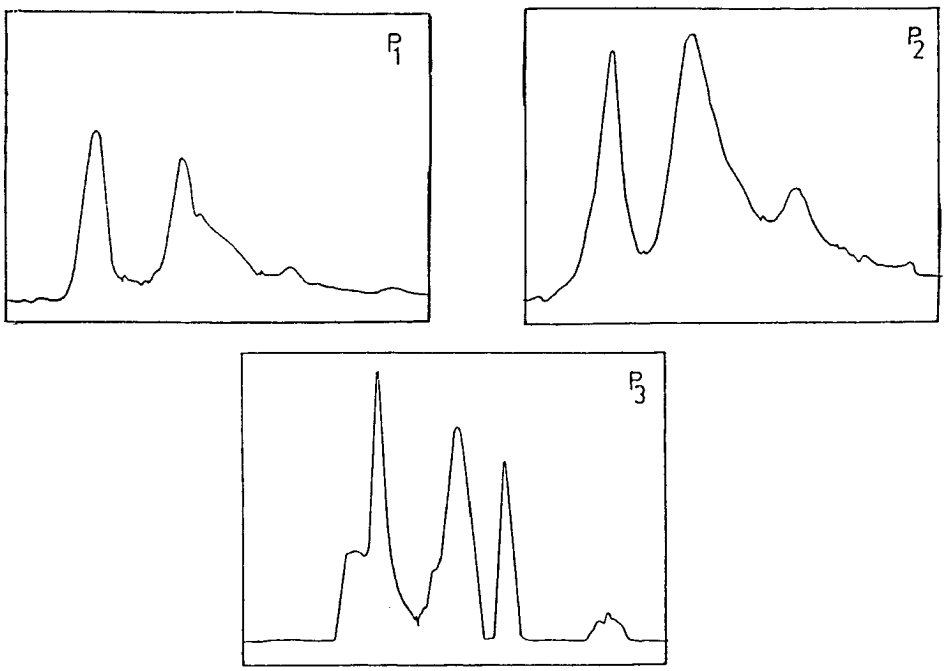

Fig. 3: Scan of Electrophoretic Patterns of Anodic-Migrating Non Specific esterases in Paramphistomes.

P_1 - *Carmyerius gregarius* from cows.

P_2 - *Paramphistomum (P.) microbothrium* from buffaloes.

P_3 - *Paramphistomum (P.) microbothrium* from cows.

In (P_1) and (P_2), there are three enzyme bands while a fourth band stays at the origin. The same picture is seen in (P_3) but an additional faint fast-moving band (band I) is present which is absent in (P_1) and (P_2). Moreover, the resolution of the enzyme bands is best in *P. (P.) microbothrium* from cows (P_3) followed by *P. (P.) microbothrium* from buffaloes (P_2) while it is less resolved in *Carmyerius gregarius* from the cows (P_1).

III. Glutamate - Oxaloacetate Transaminase (GOT)

The number, density and relative distribution of different fractions of anodic-migrating glutamic oxaloacetic transaminases in paramphistomes are shown in Table 5 and Fig, (4).

Table 5

Densiometry of Anodic-Migrating Glutamic Oxaloacetic Transaminase (GOT) In Paramphistomes

Trematodes	Enzyme Fractions ( )		
	I	II	III
P_1	56.92	34.37	7.74
P_2	64.12	27.65	8.21
P_3	60.58	34.51	4.87

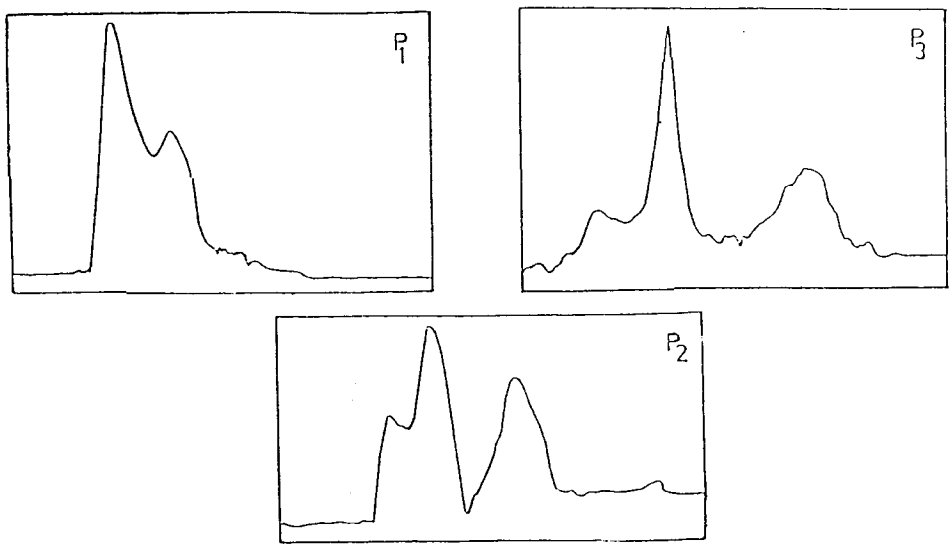

Fig. 4: Scan of Electrophoretic Patterns of Anodic-Migrating GOT in Paramphistomes.

P_1 - *Carmyerius gregarius* from cows

P_2 - *Paramphistomum (P.) microbothrium* from buffaloes.

P_3 - *Paramphistomum (P.) microbothrium* from cows.

In all trematodes two anodic-migrating bands of enzyme activity are detected with an additional small band remaining nearly at the origin. In (P_2) and (P_3) the two bands of enzyme activity are well resolved from each other but in (P_1) the resolution is not complete and the two fractions are very close to each other. In (P_2) band I is far away from fraction II.

IV. **Malate Dehydrogenase (MDH)**

The number and the relative distribution of different fractions of anodic and cathodic-migrating malate dehydrogenase in paramphistomes are shown in Table 6 and Fig. (5).

Table 6

Densitometry of Anodic and Cathodic-Migrating Malate Dehydrogenase (MDH) In Paramphistomes

Trematodes	Ezyme Fractions (%)				
	Anodic Migrating				Cathodic Migrating
	I	II	III	IV	
P_1	4.15	19.0	53.45	4.96	18.39
P_2	40.75	15.45	30.56	9.13	4.08
P_3	21.61	5.30	64.83	--	8.25

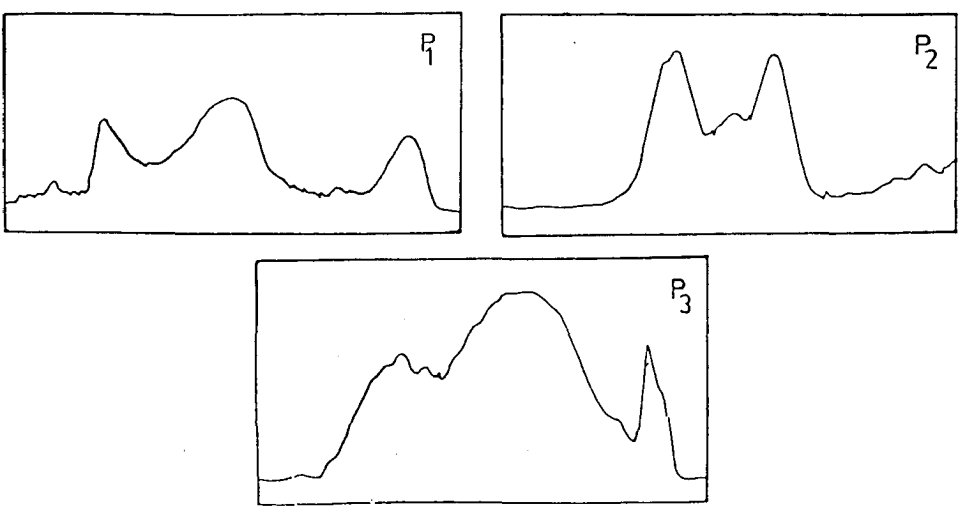

Fig. 5: Scan of Electrophoretic Patterns of Malate Dehydrogenase in Paramphistomes.

P_1 - *Carmyerius gregarius* from cows.

P_2 - *Paramphistomum (P.) microbothrium* from buffaloes.

P_3 - *Paramphistomum (P.) microbothrium* from cows.

In all trematodes there is one cathodic-migrating fraction of nearly similar electrophoretic mobility. The anodic migrating bands are of better resolution in Carmyerius gregarius from cows (P_1) than in P. (P.) microbothrium from buffaloes (P_2) and P. (P.) microbothrium from cows (P_3). Four anodic migrating-fractions are present in (P_1) and (P_2) but only three fractions are present in (P_3).

DISCUSSION

Members of the family Paramphistomatidae have been always difficult to identify on the basis of conventional morphological and anatomical features. Nasmark (1937) proposed a system of classification in paramphistomes which depended on the microstructure of certain bodily structures as observed in serial histological sections. Although Nasmark's system offered some consistent and reliable basis for adult paramphistome taxonomy, it was less useful in the identification of immature trematodes which were quite often responsible for paramphistomiasis in domestic ungulates (Horak, 1971).

In the last few years, some interest was shown in the biochemical taxonomy of paramphistomes using electrophoresis of proteins and enzymes (Osikovskii *et al.*, 1977, and Wright Rollinson and Goll, 1979). Wright *et al. (1979) suggested that enzyme analysis of adult paramphistomes derived from naturally infected hosts of known identity would contribute to the resolution of taxonomic problems of the group. It is found presently that there are some differences in the number and relative distribution of anodic-migrating protein bands between the two species of paramphistomes as well as betweenP. microbothrium* collected from buffaloes and cows (Table 2). However, in P_2 and P_3 which are paramphistomes of the same species *(P. microbothrium)* but grown in different hosts, buffaloes and cows, the electrophoretic patterns of the anodic-migrating protein fractions are more similar than the picture in P_1 which is a different species of paramphistomes *(Carmeyrius gregarius)* grown in the same host as P_3, cows. Only four bands of cathodic-migrating soluble protein fractions are found in both species of paramphistomes. There are some differences in the relative densities of the various protein bands between species as well as between *P. microbothrium* collected from the two hosts under consideration (Table 3). However, in P_1 and P_2 which are different species developing in different hosts, the highest protein percentage resides in two bands with only a small amount of protein in the other two bands, whereas in P_3 which is the same species as P_2 but developing in another host the percentages of the proteins in three fractions II, III and IV are nearly similar with a smaller percentage in band I. This result indicates that although P_1 and P_2 are from two different hosts, yet the pictures of the cathodic migrating protein bands are more

similar than the picture present in P_3 which is the same species as P_2 but grown in a different host.

The resolution of the different bands of esterases are found to be best in *P. microbothrium* from the cow (P_3) followed by that from buffaloes while it is least in *Carmyerius gregarius* grown in cows (Fig. 3). In P_3 five bands of enzyme activity are resolved whereas in P_1 and P_2 only four bands are present. This again shows similarities between two species grown in different hosts than between the same species grown in different hosts.

In case of GOT, there is similar picture in the resolution of the different isoenzymes fractions in the two species of paramphistomes where three isoenzymes are obtained in P_1, P_2, P_3. However, the resolution of GOT isoenzyme bands is best in *P.microbothrium* grown in cows and in buffaloes (P_3 and P_2) and less so in *Carmeyrius gregarius* (P_1) (Fig. 4 and Table 5). Here the same species grown in different hosts (P_3 and P_2) showed a more similar picture of the isoenzyme bands than the picture present in the different species grown in the same host. (P_1 and P_3).

The mobility of malate dehydrogenase exhibits a differential picture. The anodic migrating bands of M D are better resolved inCarmyerius gregarius than in *P.microbothrium* (Figs 5 and Table 6). Four anodic-migrating bands are present in *C.gregarius* (P_1) and *P.microbothrium* from buffaloes (P_2), while there are only 3 fractions in the latter species from cows (Table 6). This again shows similarities between two different species grown in different hosts. Thus, it is evident from the above discussion that biochemical taxonomy of paramphistomes is very complicated but it might provide a promising tool for the identification of paramphistomes at the lower taxonomic levels. The present results thus indicate that each species of paramphistomes exbibits specific characteristic electrophoretic pattern of proteins and isoenzymes. The interaction between the parasite and the host resulted in differences in the electrophoretic patterns in the proteins and isoenzymes between paramphistomes from the same species but grown in different hosts. Sometimes the differences are even more pronounced than the differences between the picture in paramphistomes from different species. Accordingly, more work is certainly needed to define more differences in protein and enzyme systems. However, it must be always emphasized that experimental taxonomy is not an alternative to classical taxonomy but merely an extension of classical taxonomy which makes use of certain new developments in the biological sciences (Jelnes, 1979).

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طرز الحمل الكهربي (الكترو فوريسس) للبروتينات والإنزيمات في الأمفيوستومات
من نوعي بارمفستومم (ب) ميكروبوثريم وكار ميريس جريجاريس

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يختص هذا البحث بدراسة طرز البروتينات وبعض الإنزيمات لنوعين من الأمفستومات التي تصيب الماشية : برامفستومم (ب) ميكروبوثريم وكارميريس جريجاريس ، وباستعمال طرق الحمل الكهربي (الكتروفوريسيس) تم فصل البروتينات الكلية الذائبة إلى مكوناتها المختلفة وكذلك فصلت مكونات إنزيمات الاستريز والجلوتاميك أوكسالو أستيك ترانسأمينيز والمالات ديهيدروجيينز . ولقد أوضحت نتائج تلك الدراسة أن الطرق الكيميائية الحيوية تعتبر من الوسائل الواعدة التي يمكن الاعتماد عليها في تعريف وتصنيف أجناس الأمفستومات .