[Jap. J. Parasit., Vol. 30, No. 1, 37-43, Feb., 1981]

Electrophoretic Studies on Enzymes in *Paragonimus* spp.
I. Comparison of Isozyme Patterns between *P. ohirai* and *P. miyazakii*

TAKESHI AGATSUMA AND NORIJI SUZUKI

(Received for publication; June 2, 1980)

Key words: *Paragonimus*, electrophoresis, enzyme

Introduction

Electrophoresis has been used in studies of enzymes in many parasites. This technique is relatively easy to hand and also highly reproducible. Furthermore, it is sufficiently appreciated that enzyme forms provide useful markers in identification of parasitic organisms (Al-Tagi and Evans, 1978; Carter, 1973, 1978; Gardener *et al.*, 1974). Thus, electrophoresis may be of great advantage in differentiating closely allied parasite species which have similar morphological characteristics, and/or in elucidating genetic relationships among natural populations. Many enzymes have been studied electrophoretically in parasitic protozoans (Carter, 1973; Tsukamoto, 1974; Godfrey and Kilgour, 1976; Miles *et al.*, 1977), and helminths (Zee and Zinkham, 1968; Coles, 1970; Ross *et al.*, 1978; Pappas and Schroeder, 1979).

As far as the authors know, however, there is little electrophoretic study on enzymes in the genus, *Paragonimus*. Yoshimura (1969a, b) first applied electrophoretic procedures to differentiate several species of *Paragonimus*, but he performed studies on only non-enzymic proteins in *Paragonimus*. The present paper reports preliminary results of investigations dealing with the enzymes in *Paragonimus* for the purpose of attempting to characterize and classify *Paragonimus* spp. by means of enzyme patterns using starch gel electrophoresis.

This study was supported in part by Scientific Research Grant (No. 577206) from the Ministry of Education, Japan.

Department of Parasitology, Kochi Medical School, Kohasu, Oko-cho, Nankoku City 781-51 Kochi, Japan.

Materials and Methods

Parasite

The metacercariae of *P. ohirai* were obtained from the intermediate host, *Sesarma dehaani*, collected in Sendai, Kagoshima Prefecture during summer, 1979 and the adult worms were harvested from the lungs of rats 40–50 days after oral inoculations of the metacercariae. *P. miyazakii* metacercariae were obtained from the crabs, *Geothelphusa dehaani*, collected at Okuyanai, Kochi Prefecture during summer, 1979 and the adult worms were harvested from rats 90–100 days after oral inoculations of the metacercariae.

Sample Preparation

All of the adult worms were washed with 0.8% physiological saline, and then subsequently stocked in a deep freezer at −80 C until used. The extracts were prepared by homogenizing about 20 worms of each

species in 2.0 ml of 0.1 M phosphate buffer solution (pH 7.5) with a teflon homogenizer in an ice water bath. The homogenized worms were centrifuged at 3,000 rpm for 3 min at room temperature. The supernatant obtained was used in this study. At the control running of each band, the sample of normal rat lung extracts was also prepared in the same way as that of the parasites.

Electrophoresis

Gels for electrophoresis were prepared from hydrolysed potato starch (Connaught Laboratories, Ontario). The amount of starch used was usually 12.0 g/100 ml of gel buffer. The gels were prepared according to Smith's method (1968), by heating the suspension of starch and buffer until a clear solution was obtained. Samples were absorbed on to 7×6 mm strips of filter paper before insertion into gels. Six enzymes were studied as shown in Table 1. The buffer systems and conditions of electrophoresis for each enzyme (Shaw and Prasad, 1970; Nozawa *et al.*, 1977) are given in Table 2.

Table 1 Enzymes examined

Enzyme	Abbreviation
Adenylate kinase	AK
Esterase	EST
Phosphogluconate dehydrogenase	6PGD
Hexokinase	HK
Glucosephosphate isomerase	GPI
Glucose-6-phosphate dehydrogenase	G6PDH

Location of enzyme activity on starch gel

The incubation solution for six enzyme examined (Brewer and Sing, 1970; Shaw and Prasad, 1970) are listed in Table 3. The solution for the enzyme under study was poured on the surface of the cut gel and incubated at 37 C until the bands of enzyme activity developed fully.

Results

As shown in Fig. 1 and 2, all of the parasite enzymes examined migrated anodally and showed their own patterns and mobilities different from those of the host, rat lung. The band patterns obtained here were highly reproducible, that is, all of the four repeated experiments brought about the same results except for EST.

Adenylate kinase (AK)

In *P. ohirai* extract, three enzyme bands were detected. The slowest band near the origin was most intense in color. The intermediate band was less intense than the slowest, and the most anodal band was very faint. On the other hand, in *P. miyazakii*, two or three enzyme bands were found. The slow band was strongly intense, while the fast one faint. But, the most anodal band

Table 2 Buffer systems used in starch gel electrophoresis

Enzyme	Gel Buffer	Electrode Buffer	Condition
6PGD AK	0.01 M Tris	0.155 M Tris	60 mA Current
HK	0.003 M Citrate	0.043 M Citrate	constant, 4h
EST	0.003 M Citrate	0.1 M LiOH	40 mA Current
	0.014 M Tris	0.38 M Borate	constant, 4h
	0.01 M LiOH		
	0.038 M Borate		
GPI	0.017 M Tris	0.25 M Tris	100 V Voltage
	0.003 M Citrate	0.057 M Citrate	constant, 12h
G6PDH	0.05 M Tris	0.5 M Tris	200 V Voltage
	0.097 M Borate	0.646 M Borate	constant, 4h
	0.002 M Na_2EDTA	0.016 M Na_2EDTA	

Table 3 Enzyme assay systems for specific staining on electrophoresis

Enzyme	Substrate	Coenzyme	Other additions			Buffer condition
AK	Glucose 36.6 mg	NADP 2.0 mg	MTT 2.0 mg	PMS 2.0 mg	ADP 10 mg	0.1 M Tris-HCl (pH 8.0) 20 ml
			$MgCl_2$ 74.0 mg	G6PDH 2 U	HK 2 U	
EST	α-naphthyl acetate 60 mg	—	Fast Blue RR 100 mg			0.2 M Tris-HCl (pH 7.1) 100 ml
6PGD	Ba-6-phospho-gluconate 5.0 mg	NADP 2.0 mg	MTT 2.0 mg	PMS 2.0 mg		0.1 M Tris-HCl (pH 8.0) 20 ml
HK	Glucose 36.6 mg	NADP 2.0 mg	MTT 2.0 mg	PMS 2.0 mg	ATP 9.7 mg	〃
			$MgCl_2$ 74 mg	G6PDH 2 U		
GPI	Fructose-6-phosphate 3.0 mg	NADP 2.0 mg	MTT 2.0 mg	PMS 2.0 mg		〃
			$MgCl_2$ 74 mg	G6PDH 2 U		
G6PDH	Glucose-6-phosphate 5 mg	NADP 2.0 mg	MTT 2.0 mg	PMS 2.0 mg		〃

NADP, nicotinamide adenine dinucleotide phosphate. MTT, 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyl tetrazolium bromide. G6PDH, glucose-6-phosphate dehydrogenase. PMS, phenazine methosulfate. Tris, tris (hydroxymethyl) amino methane. HK, hexokinase.

was not always reproducible, while other two bands were reproducible. On the whole, the relative intensity of each band was different between the two parasite species, although the mobilities of bands of *P. miyazakii* were similar to those of the corresponding bands of *P. ohirai*.

Esterase (EST)

A single band was recognized in both parasites. *P. miyazakii* enzyme migrated almost as fast as that of *P. ohirai*. But, the distinct difference in mobility was not always found between the two species.

Phosphogluconate dehydrogenase (6PGD)

Both parasites showed three-banded pattern, but their relative mobilities were different from each other as shown in Figures.

Hexokinase (HK)

Both parasites possessed the same band pattern and the very similar mobility. Thus, this enzyme pattern was not capable of distinguishing between them.

Glucosephosphate isomerase (GPI)

Both parasites possessed a major band and several minor bands. The mobilities of the major band were different between the two species.

Glucose-6-phosphate dehydrogenase (G6PDH)

Single band was observed in both parasites. *P. ohirai* enzyme was relatively slower and broader than that of *P. miyazakii*.

Discussion

Recently, several taxonomical problems have been actively discussed in the Japanese lung flukes, *Paragonimus* spp. The problems arise mainly because of the classification based on the comparative morphology which has been extensively performed in parasitic organisms. In order to try to overcome these difficulties, characteristics other than morphological features have been used in attempts to discriminate each species among morphologically similar parasites in the genus, *Paragonimus*.

Sakaguchi and Tada (1976) studied the karyotype of two species of the lung flukes, *P. ohirai* and *P. miyazakii*, and Terasaki (1977) also performed karyotypic analyses on all of the five species of the lung flukes, *P. westermani, P. miyazakii, P. ohirai, P. iloktsuenensis* and *P. sadoensis* for an interest of their phylogeny and cytology. However, they found that the species mentioned above were karyotypically indistinguishable from each other except for *P. westermani*, of which karyotype was triploid.

Yoshimura (1969b) conducted electrophoretic studies on the whole body proteins of *P. westermani, P. ohirai* and *P. miyazakii*. He concluded, from the results obtained, that each of the three lung flukes has a species-characteristic electrophoretic patterns.

In general, however, when the whole body proteins are compared among species in electrophoretic studies, it is not easy to recognize the respective homologous protein bands among different species. Moreover, in this case, genetic aspect of each band is as a whole vague. Therefore, more detailed quantitative analyses seem to be troublesome. On the other hand, electrophoretic studies of enzymes are of great advantages in identifying and/or classifying the parasite species, that is, a given enzyme reflects the specific genetic loci, which are mostly inherited in a simple mendelian fashion (Markert, 1975).

In the present paper, electrophoretic patterns of six enzymes in *P. miyazakii* and *P. ohirai* were reported. This work is the first attempt to use enzymes to compare *Paragonimus* species.

It might be considered that the host enzymes would be involved in parasite samples, when the worm extracts were used. This probability, however, was completely denied by obtaining the distinct band numbers and mobilities of the parasite enzymes from that of the host enzymes.

In electrophoretic systems used in the present study, both host- and parasite-forms of GPI tended to produce a series of sub-bands running in an anodal direction, while in the case of parasites the sub-band was also found in cathodal from the main band. AK, 6PGD and HK, like GPI, also formed sub-bands on electrophoresis for both parasite enzymes but not significantly for the host lung forms. Similar phenomenon in the enzyme forms has been also found in *Plasmodium* spp. (Carter, 1978) and other animals (Shotake *et al.*, 1977) using the same electrophoretic system as in the present investigation. It can be presumed that these sub-bandings are due to polymerization of molecules in the enzyme proteins (Carter, 1978).

EST generally constitute a family of enzymes with overlapping substrate specificity. Indeed, it has been demonstrated in *Ascaris* (Zam, 1973) and other many animals (Markert, 1975) that EST showed multiple enzyme forms. Therefore, it is very noticeable that *Paragonimus* species examined were detected as only a single band activity of EST. But, the failure to detect the multiple band pattern may be only a quantitative effect.

The results obtained here showed that *P. ohirai* and *P. miyazakii* can be distinguished from each other by the enzyme forms. Totally out of six enzymes, four enzymes, AK, 6PGD, GPI and G6PDH, were different between the two species in number, mobility, or intensity of their enzyme bands. EST did not always show the difference between the two species. This may be caused by intra-species variation in the materials used in each experiment. HK showed no difference in every experiment.

This would suggest that electrophoretic enzyme forms may provide us additional criteria for the classification and/or characterization of *Paragonimus* species.

Summary

Preliminary studies dealing with the enzymes in *Paragonimus* spp. were carried out for the purpose of attempting to classify *Paragonimus* species by the enzyme pattern using starch gel electrophoresis. Six enzymes, adenylate kinase, esterase, phosphogluconate dehydrogenase, hexokinase, glucosephosphate isomerase and glucose-6-phosphate dehydrogenase, of *P. ohirai* and *P. miyazakii* and their host (rat) were compared by means of horizontal starch gel electrophoresis. The electrophoretic patterns of all of the six enzymes were different between parasite and host.

From the results obtained here, at least four enzymes examined were distinctive in the number and mobility of the bands between the two parasite species. This might suggest that the enzyme forms provide us with additional criteria for classification and/or identification of the *Paragonimus* spp.

Acknowledgements

The authors are greatly indebted to Drs. K. Nozawa and T. Shotake, Primate Research Institute, Kyoto University, for their encouragement and advices, and to Mr. Y. Kawamoto, Kyoto University, for his technical assistance in electrophoretic examinations. They are also grateful to Dr. Y. Hashiguchi, Kochi Medical School, for his critical reading of the manuscript and to Mr. H. Kumazawa and Miss K. Nishimura, Kochi Medical School, for their supports throughout this study.

References

1) Al-Taqi, M. and Evans, D. A. (1978): Characterization of *Leishmania* spp. from Kuwait by isozyme electrophoresis. Trans. Roy. Soc. Trop. Med. Hyg., 72, 56–65.
2) Brewer, G. J. and Sin, C. F. (1970): An Introduction to Isozyme Techniques, 1st ed., Academic Press, New York, 186 p.
3) Carter, R. (1973): Enzyme variation of *Plasmodium berghei* and *Plasmodium vinckei*. Parasitol., 297–307.
4) Carter, R. (1978): Studies on enzyme variation in the murine malaria parasites *Plasmodium berghei, P. yoelii, P. vinckei* and *P. chabaudi* by starch gel electrophoresis. Parasitol., 76, 241–267.
5) Coles, G. C. (1970): A comparison of some isozymes of *Schistosoma mansoni* and *Schistosoma haematobium*. Comp. Biochem. Physiol., 33, 549–558.
6) Gardener, P. J., Chance, M. L. and Peters, W. (1974): Biochemical taxonomy of *Leishmania*. II. Electrophoretic variation of malate dehydrogenase. Ann. Trop. Med. Parasit., 68, 317–325.
7) Godfrey, D. G. and Kilgour, V. (1976): Enzyme electrophoresis in characterizing the causative organism of Gambian trypanosomiasis. Trans. Roy. Soc. Trop. Med. Hyg., 70, 219–224.
8) Markert, C. L. (1975): Isozymes IV. Genetics and Evolution, 1st ed., Academic Press, New York, 965 p.
9) Miles, M. A., Toye, P. J., Oswald, S. C. and Godfrey, D. G. (1977): The identification by isozyme patterns of two distinct strain-groups of *Trypanosoma cruzi* circulating independently in a rural area of Brazil. Trans. Toy, Soc. Trop. Med. Hyg., 71, 217–225.
10) Nozawa, K., Shotake, T., Ohkura, Y. and Tanabe, Y. (1977): Genetic variations within and between species of Asian macaques. Japan. J. Genet., 52, 15–30.
11) Pappas, P. W. and Schroeder, L. L. (1979): *Hymenolepis microstoma*; Lactate and malate dehydrogenases of the adult worm. Exp. Parasit., 47, 134–139.
12) Ross, G. C. (1976): Isoenzymes in *Schistosoma* spp.; LDH, MDH and acid phosphatases separated by isoelectric focusing in polyacrylamide gel. Comp. Biochem. Physiol., 55B, 343–346.
13) Ross, G. C., Southgate, V. R. and Knowles, R. J. (1978): Observations on some isozymes of strains of *Schistosoma bovis, S. matthei, S. margrebowiei* and *S. leiperi*. Z. Parasitenkd., 57, 49–56.
14) Sakaguchi, Y. and Tada, I. (1976): A comparative karyotype study of lung flukes, *Paragonimus ohirai* and *P. miyazakii*. Jap. J. Parasit., 25, 5–7.
15) Shaw, C. R. and Prasad, R. (1970): Starch gel electrophoresis of enzymes—A compilation of recipes. Biochem. Genet., 4, 297–320.
16) Shotake, T., Ohkura, Y. and Ishimoto, G. (1977): Genetic polymorphisms of blood proteins in the troops of Japanese macaques, *Macaca fuscata*; V. Erythrocyte phosphohexose isomerase polymorphism. Primates, 18, 285–290.
17) Smith, I. (1968): Starch gel electrophoresis.

In Chromatographic and Electrophoretic Techniques, by Smith, I., Heinmann Medical Books Ltd., New York, 217–324.
18) Terasaki, K. (1977): Studies on chromosomes of the lung flukes in Japan. Jap. J. Parasit., 26, 222–229.
19) Tsukamoto, M. (1974): Differential detection of soluble enzymes specific to a rodent malaria parasite, *Plasmodium berghei*, by electrophoresis on polyacrylamide gels. Trop. Med., 16, 55–69.
20) Yoshimura, K. (1969a): *Paragonimus*; electrophoretic fractionation of whole body proteins as an aid in specific identification of a species from Sado Island, Japan. Exp. Parasit., 25, 107–117.
21) Yoshimura, K. (1969b): *Paragonimus westermani, P. ohirai,* and *P. miyazakii;* electrophoretic comparison of whole body proteins. Exp. Parasit., 25, 118–130.
22) Zam, S. M.(1973): Disc electrophoresis of *Ascaris suum* female reproductive system esterase enzymes. Comp. Biochem. Physiol., 46B, 797–803.
23) Zee, D. S. and Zinkham, W. (1968): Malate dehydrogenase in *Ascaris suum*: Characterization, ontogeny, and genetic control. Arch. Biochem. Biophys., 126, 574–584.

肺吸虫類アイソザイムの電気泳動法による研究
I. 大平肺吸虫と宮崎肺吸虫のアイソザイムパターンの比較

吾妻　健　　鈴木了司
(高知医科大学寄生虫学教室)

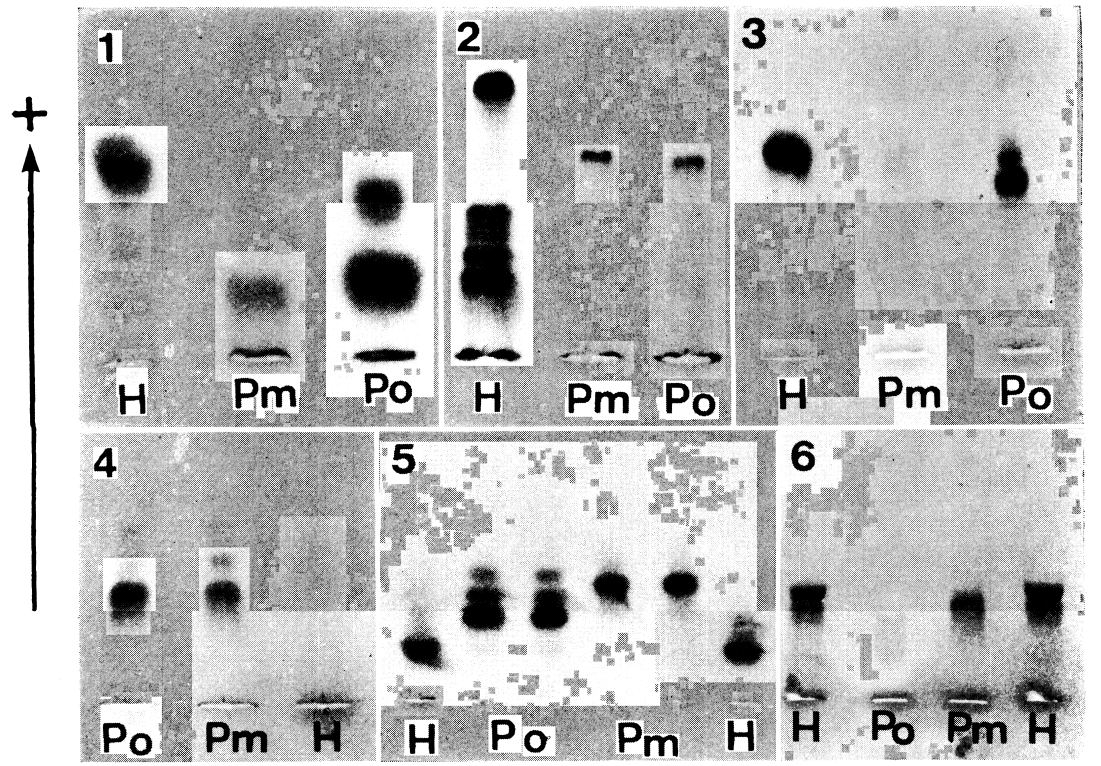
酵素タンパクの電気泳動パターンを利用して，種の同定や分類を行う研究は，最近とくに寄生原虫類において多く見られてきた．一方,寄生蠕虫類においては，若干の報告は，見られるものの，まだ少く，肺吸虫類においてはほとんど未知の状態である．本研究では，肺吸虫類を酵素の泳動パターンを利用して分類することを目的とする試みの予備的研究として，大平肺吸虫及び宮崎肺吸虫の6種類の酵素，adenylate kinase, esterase, phosphogluconate dehydrogenase, hexokinase, glucose-6-phosphate dehydrogenase, glucosephosphate isomerase の泳動パターンをデンプンゲル電気泳動法により比較した．寄生虫サンプル中の宿主酵素の有無を調べるために，同一ゲル上で，寄生虫と宿主(ラット)のサンプルの比較を行ったが，すべての酵素において一致するバンドは見られなかった．

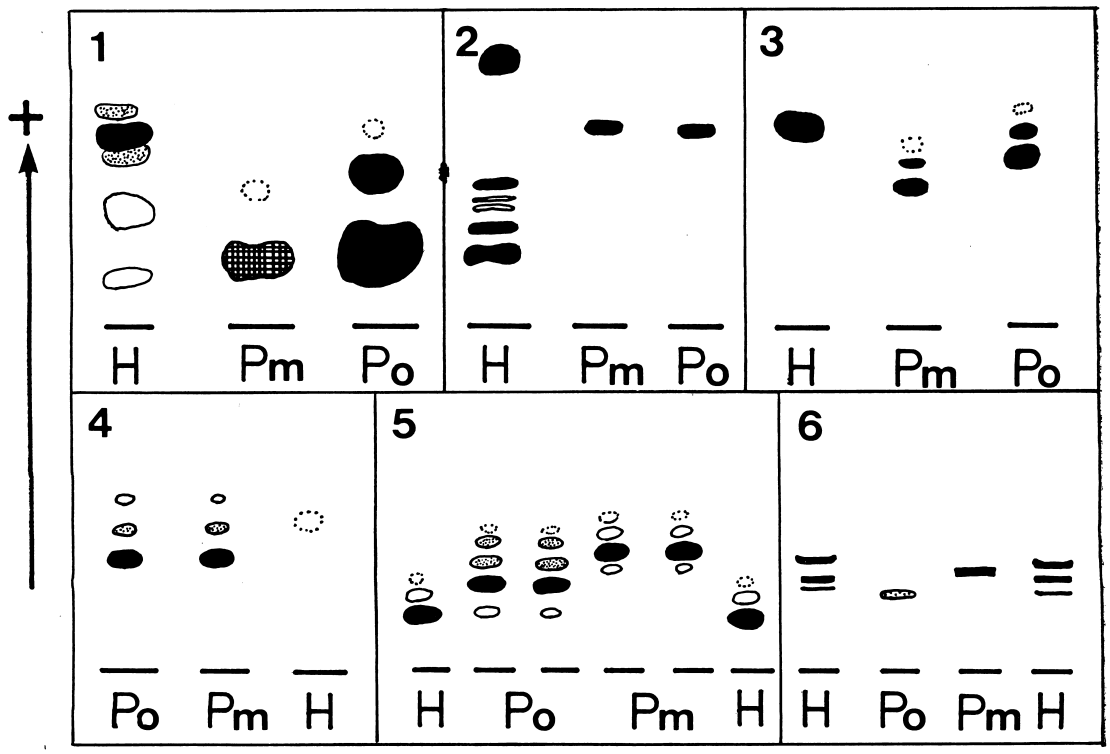
両肺吸虫の6種類の酵素のうち少くとも，4種類の酵素において，その移動度やバンドの数等に違いがみられた。本研究で検出された酵素の泳動パターンは，極めて再現性が高く，種の同定等に利用できると思われる．

Explanation of Figures

Fig. 1 Photograph of starch gel electrophoretic patterns of six enzymes in *P. miyazakii*, *P. ohirai* and the host, rat. 1, AK; 2, EST; 3, 6PGD; 4, HK; 5, GPI; 6, G6PDH. Pm, *P. miyazakii*; Po, *P. ohirai*; H, host (rat lung).

Fig. 2 Diagrammatic representation of starch gel electrophoretic patterns of six enzymes in *P. miyazakii*, *P. ohirai* and the host, rat. Same numberings and abbreviations as that of Fig. 1 are used.


+
1
H
Pm
Po
2
H
Pm
Po
3
H
Pm
Po
4
Po
Pm
H
5
H
Po
Pm
H
6
H
Po
Pm
H


+
1
H
Pm
Po
2
H
Pm
Po
3
H
Pm
Po
4
Po
Pm
H
5
H
Po
Pm
H
6
H
Po
Pm
H