# DIFFERENTIATION of TAENIID WORMS by BIOCHEMICAL and SEROLOGICAL METHODS

by

PHILIP DAVID LE RICHE, M.V.Sc. (Melbourne),
B.V.M.S. (Glasgow), M.R.C.V.S., D.T.V.M. (Edinburgh)

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## PREFACE

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## SUMMARY

Fresh specimens of Echinococcus granulosus, Hydatigera taeniaeformis, Multiceps multiceps, Taenia hydatigena, Taenia pisiformis, Taenia ovis, Taenia solium and Taeniarhynchus saginatus were collected from natural infections and from experimentally infected animals. Collections were made in the United Kingdom and in Nigeria and a few specimens were also obtained from other countries abroad. Material from the specimens was compared by staining, serological techniques and enzyme electrophoresis.

Staining ova by the Ziehl-Neelsen method was not successful in differentiating ova of any of the species including T. saginatus and T. solium.

Serological techniques that were tried included oval precipitation, agar gel diffusion precipitation, fluorescent antibody, immunoelectrophoresis and the enzyme-linked immunosorbent assay (ELISA). Strobilate, oval and, in some instances, larval cyst antigens, were used in tests against antisera raised in rabbits and other species. With antisera that had been absorbed by one or two heterologous antigens, some success was achieved in agar gel diffusion precipitation tests and with ELISA in differentiating strobilate tissue, but serological techniques were not generally adequate for this purpose.

Success in differentiating taeniid material was achieved by enzyme electrophoresis on thin layer starch gels.

Of the many enzymes tested, five were found to display inter-species zymogram differences in the pattern and mobility of their multiple forms: adenylate kinase, glutamate dehydrogenase, malate dehydrogenase, hexokinase and glucose phosphate isomerase, of which the latter two were the most useful for differentiation.

No differences were seen between larval and adult material except for the presence of host enzymes in the former which were easily identified by comparison with host tissue extracts. No differences were seen between immature, mature and gravid proglottides and no individual variation was seen between worm specimens. Anthelmintic treatment did not affect worm enzyme patterns.

Taeniid species were compared with one another and also with other tapeworm species. The mobility and pattern of the enzyme forms in zymograms differed more between unrelated than between related species. With glucose phosphate isomerase it was possible to identify most of the taeniid species. When this enzyme was preserved in a solution containing enzyme stabilisers, it stored well over a long period especially in liquid nitrogen or freeze dried and kept at -20$^{o}$C. Dilution of samples did not distort zymogram patterns and crude extracts could be identified using the mobility of the slowest moving band as the criterion. This method was used successfully for a survey in Nigeria. Identification was by comparison with a known species. The other enzymes were useful for confirming the diagnosis.

Strain variation between E. granulosus of horse and sheep origin was seen in specimens collected from Belgium and different parts of the United Kingdom. Cysts from oxen were identical to those from sheep, but indirect comparison with cysts from Nigerian camels showed these to be different from both the equine and ovine strains.

# INTRODUCTION AND LITERATURE REVIEW

# CHAPTER ONE

## INTRODUCTION

Tapeworms have been known as parasites of man since ancient times. In 1500 BC the Ebers papyrus described worms that were probably of the Taenia species, but it is likely that prehistoric man knew of their existence before that (Hoeppli, 1956). Cysticerci were also known to man in ancient times, but it was not until 1760 that Pallas recognised the association of the larval cyst and the adult worm (Foster, 1965), and the life cycles of the various taeniid worm species were not unravelled until the middle of the nineteenth century (Rausch, 1975; Muller, 1975). The significance of tapeworms, therefore, was not at first realized.

As the life cycles became known, the public health importance of larval cysts in meat became apparent and this led to the condemnation of organs or whole carcases during meat inspection in order to prevent completion of the parasitic life cycle. The serious consequences to man of parasitization by some larval cestodes, together with the severe economic loss caused by the disease in man and animals (Schwabbe, 1969; Abduladze, 1970; Olivier, 1974), has since led to many national efforts to control or eradicate tapeworm diseases. As early as 1863, Iceland, for instance, invited Krabbe from Copenhagen to advise on hydatid disease and he instituted various measures which eventually led to its eradication (Schwabbe,

1969; Beard, 1973). Many other countries have attempted eradication of echinococcosis and effective national campaigns are currently operating in New Zealand (Begg, 1961; Gemmell, 1973) and the Australian island state of Tasmania (Meldrum & McConnell, 1968). Cyprus has since followed this example (Polydorou, 1977).

All countries suffer economic loss resulting from cysticercosis of livestock and, in those countries whose economies are based to a large extent on livestock production, this loss may be severe. Export of meat from East Africa to Europe, for instance, would be uneconomic due to the number of carcases condemned at slaughter (Kagan, 1974). In 1968, 12.5 per cent of Australian shipments of mutton to the U.S.A., worth $U.S.1,538,000, were rejected. Between 50 and 80 per cent of these rejects were due to Taenia ovis cysts. It was partly due to this that the U.S. and Canadian authorities placed a total ban on mutton imports from Australia in 1970, cutting Australia's export of mutton by 45.2 per cent (Arundel, 1972). In New Zealand where the economic loss from organs condemned for hydatid disease alone amounted to $N.Z.4 m (Schwabbe, 1969), the problem of cysticercosis of sheep is also important and their national hydatid eradication scheme has been extended to include the compulsory treatment of dogs against Taenia hydatigena and Taenia ovis, as well as Echinococcus granulosus (Gemmell, 1973; Anon., 1976). In the U.S.S.R., national schemes for the eradication of cysticercosis are also in force (Abduladze, 1970).

Correct identification of the parasites concerned is essential if epidemiological studies are to be meaningful. At present, the identification of taeniid worms is based on morphological characteristics of the adult and larval stages. This is time consuming and subject to error. Fresh approaches have therefore been made to identify the taeniid worms by different and more reliable methods. This thesis presents the results of this work.

# CHAPTER TWO

## CLASSIFICATION OF TAENIIDS AND THEIR PUBLIC HEALTH AND VETERINARY IMPORTANCE

As new species of adult and larval tapeworms were discovered, they were given generic or specific names, but when their life cycles were revealed, it was found that adults and their larvae had sometimes been classified differently. The confusion caused by this has been perpetuated by the retention of both sets of names even after the life cycles became known, and Pawlowski & Schultz (1972) quite rightly objected to the continuance of this practice and suggested the disuse of the larval name when it differed from that of the adult.

Diversity of larval structures is common in these species and larval morphology cannot in any case be relied upon as a means of distinguishing the species (Esch & Self, 1965; Verster, 1969); nor can their host specificity be relied upon as a distinguishing criterion, as larvae may occasionally establish themselves in unusual hosts (Verster, 1969). Verster found that most features used to distinguish species were subject to some variation and that species could therefore only rarely be identified on the basis of a single feature. Using reliable criteria based on several features of the adult worms, she revised the taeniid worms and was able to reduce the number of species from 70 to 30.

Historically, the taeniid worms have been grouped and classified under a variety of names such as Taenioidea Zwicke, 1841; Taeniadae Baird, 1853; Taeniodea Goldberg, 1855; Taeniadae Carus, 1863; Taeniidae Ludwig, 1886 (Abduladze, 1970).

Within the family Taeniidae the worms have been grouped into various sub-families, genera and sub-genera by different authorities. There has been a lot of conflicting opinion about their generic grouping often depending upon whether morphological differences of larvae were considered important characteristics or not. Modern authorities are still not agreed on the grouping of the taeniid worms. Abduladze (1970) divided the family into two sub-families; Taeniinae Perrier, 1897, embracing all the taeniid worms except Echinococcus species, and Echinococcinae. Yamaguti (1959) divided all the taeniid worms into various genera including the genus Echinococcus. Verster (1965a; 1969) separated the genus Echinococcus Rudolphi, 1801, from the other taeniid worms which she included under one genus, Taenia Linnaeus, 1758. Wardle & McLeod (1968) included ten genera under Taeniidae, including Echinococcus, Taeniarhynchus, Hydatigera, Multiceps and Taenia, but in 1974 (Wardle et al, 1974), whilst largely accepting Verster's revision of the Taenia species, they reclassified the whole group by elevating Taeniidae to ordinal status within the class of true tapeworms, Eucestoda.

Most of the cestodes of public health and veterinary importance are members of the new order Taeniidea in which they are included in only four genera: Echinococcus Rudolphi, 1801; Multiceps Goeze, 1782; Taenia Linnaeus, 1758 and Taeniarhynchus Weinland, 1858. In addition, and of minor importance, the genus Hydatigera Lamarck, 1815 contains Hydatigera taeniaeformis Batsch, 1786, whose host is the cat and whose intermediate hosts are small rodents. This worm has very rarely been found in man (Abduladze, 1970).

The genus Echinococcus comprises at least four species of which three are of public health importance because the larval stage can infect man; Echinococcus granulosus Batsch, 1786; Echinococcus multilocularis Leuckart, 1863 and Echinococcus oligarthrus Diessing, 1863 (Thatcher & Sousa, 1966; Rausch, 1975). Of these three species, E. granulosus is world wide in distribution (Smyth & Smyth, 1964; Anon., 1967; Schwabbe, 1968), E. multilocularis is confined to the northern hemisphere within certain defined areas (Anon., 1967; Rausch, 1968, 1975), being mainly a problem in arctic and sub-arctic regions, and E. oligarthrus is restricted to central and northern South America (Thatcher, 1970; Rausch, 1968, 1975). E. multilocularis has a more limited host range than E. granulosus and the two species do not share any natural intermediate host (Rausch, 1968). Dogs and cats may acquire infection by eating rodents and pass on infection to man and, therefore, E. multilocularis

attains importance as a human pathogen only when man encroaches upon natural foci (Rausch, 1975).
E. oligarthrus is confined geographically to a small area and is associated with wild animals (Leiby & Kritsky, 1972; Rausch, 1975), although domestic cats can be readily infected (Sousa & Thatcher, 1969).

Although infection of man by the hydatid organism of either E. multilocularis or E. oligarthrus is more serious than infection by E. granulosus, because of their restricted geographical and host distributions, these two species are not as important as E. granulosus.

Many sub-species of E. granulosus have been described such as E. granulosus granulosus, E. granulosus canadensis, E. granulosus borealis and E. granulosus equinus, but Rausch (1967) considered them all, with the possible exception of E. granulosus canadensis Cameron, 1960, to be synonymous with E. granulosus granulosus. Likewise Rausch found no adequate evidence to divide E. multilocularis into sub-species and regarded populations that had been described from different geographical areas with peculiar host ranges as exhibiting only strain differences.
It has even been suggested that the division of Echinococcus into species is invalid (Pérez Fontana, 1968).

A critical study of Taenia pisiformis, Multiceps multiceps and Hydatigera taeniaeformis by Esch & Self (1965) confirmed earlier observations that these species could not be separated generically, and suggested they be

included in the genus Taenia. When Verster revised the Taenia genus she included these three species within it (Verster, 1969). However, Wardle et al (1974), on raising Taeniidae to ordinal status, continued to divide it into genera placing Multiceps multiceps Leske, 1780 in a separate genus but ambiguously included serialis Gervais, 1847 and serialis brauni n. comb. Verster, 1969 in both Multiceps and Taenia genera.

M. multiceps is of veterinary importance as it has a dog-sheep life cycle and a wide geographical distribution, although surprisingly it is not found in Australia (Pullar, 1946; Seddon, 1967). It may also affect other ruminants. M. multiceps is also important from the medical aspect as coenuriasis has been recorded in man (Witenburg, 1964; Abduladze, 1970; Wardle et al, 1974). Coenuriasis may also be an important economic problem of livestock in some parts of the world. Shumakovich (1958) reported that in the lower Volga area of U.S.S.R.in 1957, 39.2 per cent of losses due to helminthiasis in sheep were caused by coenuriasis, and other such reports of high incidence and loss due to this disease have been recorded from other parts of the Soviet Union (Abduladze, 1970). Coenuriasis in man has also been caused by M. serialis brauni (Abduladze, 1970; Templeton, 1968, 1971; Wardle et al, 1974), which was previously thought to be a separate species from M. serialis (Verster, 1969).

The genus Taenia Linnaeus, 1758, comprises ten main species according to Wardle et al (1974), of which several are of public health or veterinary importance. The dog is the host of Taenia hydatigena Pallas, 1766, Taenia ovis Cobbold, 1869 and Taenia pisiformis Bloch, 1780. The larval form of T. hydatigena, known as Cysticercus tenuicollis, is found in many ruminants and has occasionally been found in man; that of T. ovis, known as Cysticercus ovis, is found mainly in sheep and that of T. pisiformis, known as Cysticercus pisiformis, is found mainly in rabbits and hares (Abduladze, 1970).

One species, Taenia solium Linnaeus, 1758, parasitises man, and the larval cyst is found in the pig. Man can also act as the intermediate as well as the definitive host, and there are many reports in medical literature of cysticercosis caused by this parasite. A characteristic site of the cysts in man is believed to be the eye and brain (Šlais, 1970), although Muller (1975) states that cysts occur in all organs in proportion to their size. A racemose cyst is sometimes found in cerebral cysticercosis of man, which was at first thought, because of its shape and size, to be a different species, Cysticercus racemosus (Šlais, 1967). Taeniarhynchus Weinland, 1858 contains only one species, Taeniarhynchus saginatus which, like T. solium, parasitises man. The larval form, Cysticercus bovis, found in cattle, is cosmopolitan. This worm was first described by Goetze (1782) and named Taenia saginata.

It was later assigned to a new genus by Weinland who called it Taeniarhynchus saginatus (Stiles & Hassal, 1912) but these names are often confused.

The classification of Wardle et al (1974) has been adopted in this thesis.

# CHAPTER THREE

## MORPHOLOGICAL DIFFERENTIATION

Some of the taeniid worms of domestic animals and man are easily distinguished by obvious characteristics. Thus, *Echinococcus* spp. are all small and have from two to seven segments including mature and gravid segments (Verster, 1965) and *T. saginatus* is the only worm lacking an armed rostellum.

The larval forms of the worms also differ: *Echinococcus* spp. forming a hydatid cyst which is typically unilocular in *E. granulosus* and alveolar in *E. multilocularis* (Abdussalam *et al*, 1968; Rausch, 1968); *Multiceps*, a coenurus cyst; *Hydatigera*, a strobilocercus and the rest large or small cysticerci containing only one scolex. Whereas these larval characteristics may be an aid to identification, classification based on larval morphology is unreliable because larvae may have uncharacteristic forms in unusual hosts (Esch & Self, 1965; Abdussalam *et al*, 1968; Verster, 1969).

In the introduction to the paper revising the taeniid species, Verster (1969) pointed out the deficiencies of many of the criteria that had formerly been used to distinguish them and concluded that differences of a single character could not justify the creation of a separate genus. Therefore she did not approve of the creation of *Taeniarhynchus* as a separate genus, based solely on the

absence of an armed rostellum.

Descriptions of characteristic shapes and sizes of soft organs are often dependent upon the method of fixation and so are unreliable. Furthermore, the shape of some organs, such as the cirrus pouch, may vary in different parts of the strobila. The number of branches of the uterus is subject to variation and the number of testes is difficult to determine due to layering because of the thickness of the segment (Verster, 1967).

Host specificity is shown more strongly by the adult than by the larval stages, but experimental infection of unusual hosts has often been successful; for instance, T. solium may become established in the golden hamster (Gnezdilov, 1957) and in the chacma baboon (Verster, 1965b). Adult worms may establish themselves in unusual hosts, but sometimes the worm may then not reach patency: for instance, Echinococcus granulosus has been recorded in the cat, but in this unusual host it did not reach patency (Rausch & Nelson, 1963). Verster concluded that host specificity can therefore not be relied upon as a sole means of identification.

In man, T. saginatus can be differentiated from T. solium by only a few features that are valid, according to Verster (1967) and Proctor (1972). Of these, the most reliable and easily assessed are:

1. The presence of an armed rostellum in T. solium and its absence in T. saginatus.

2. The presence of three ovarian lobes in T. solium and only two in T. saginatus.

3. The presence of a vaginal sphincter in T. saginatus and its absence in T. solium.

Although ova of T. solium tend to be spherical and of T. saginatus round, Kamalova (1953) has shown that it is impossible to differentiate them on measurements of the eggs, onchospheres or embryos. When only gravid proglottides are available, specific identification can only be based on the presence or absence of a vaginal sphincter. This is not easily identified and proglottides must be carefully stained in order to recognize it.

It is partly because of inaccurate identification that Verster (1967) considered T. solium to be probably more common than formerly supposed, as identification of gravid proglottides had usually been based on the number of branches of the uterus.

Diagnostic characters for distinguishing cestode larvae found in man were summarized by Šlais (1970), based mostly on his own observations. He differentiated cysts by the number of scolices, the presence or absence of hooks and on characters of the bladder wall. These characters depended mostly on histological examination to determine the size of various protruberances and the presence or absence of certain tissues. Šlais pointed out that the cyst can be extremely difficult to distinguish especially if it is incomplete.

In the dog, Echinococcus species can be distinguished easily from other mature taeniid worms by their size (Abdussalam et al, 1968). E. multilocularis can be distinguished from E. granulosus on the basis of several morphological characteristics (Rausch, 1968). Morphological and physiological differences also occur within species and this has led to the assumption that many species and sub-species exist (Sweatman & Williams, 1963; Williams & Sweatman, 1963; Verster, 1965a).

Although Rausch (1967) disagreed with the division of E. granulosus and E. multilocularis into sub-species, he acknowledged that morphological and physiological differences occurred but preferred to assign these differences to strain variation. These strain differences are nevertheless important because of host preferences which characterise each strain. E. granulosus, for instance, is usually infective to man but a strain occurring in Europe and the near East which has a horse-dog life cycle may not be (Thompson & Smyth, 1976). There is also evidence to suggest that this strain may not be infective to sheep and that the strain having a sheep-dog life cycle is probably not infective to horses (Williams & Sweatman, 1963; Hatch & Smyth, 1975).

The other canine taeniid worms are more difficult to distinguish and the shape, size and number of the hooks are the most reliable characters. In addition, a vaginal sphincter muscle is present in T. ovis and M. serialis but absent in T. hydatigena and T. pisiformis. M. multiceps

has a muscular "pad" on the anterior wall of the vagina between the latter and the cirrus pouch. H. taeniaeformis of the cat also has a vaginal sphincter (Verster, 1969). Incorrect identification can easily result from inaccurate measurements of the hooks or the reliance on soft organ features. This has led to conflicting conclusions being drawn from the results of different surveys.

In Australia it was important to establish the role of the fox, the dingo and the dog as the definitive hosts of worms causing cysticercosis of meat in order to plan control measures. In 1946, Pullar reported from a survey of dogs and foxes in Victoria that 13.5 per cent of foxes were infected with T. ovis. He found T. ovis and E. granulosus in dogs, but failed to provide details of the method used in identification (Pullar, 1946). Jackson & Arundel (1971) did a survey of dogs in Victoria in which they found T. pisiformis, T. hydatigena, T. ovis, M. serialis and E. granulosus to be common. Diagnosis was based on the size of the mature worm, measurements of the length of the rostellar hooks in the anterior row, the number of branches of the uterus, and the size of the genital pore which, according to Hall (1919) is characteristically broad in T. ovis. The size of the scolex and neck and the shape of the segments were also used to differentiate borderline species of T. pisiformis with hooks smaller than 210 µm (Arundel, 1972). Dent & Kelly (1974), using Arundel's criteria in a survey of foxes, did not find E. granulosus or, contrary to Pullar's findings,

T. ovis. They found T. pisiformis, M. serialis and also T. hydatigena in 5.2 per cent of 58 foxes. Coman (1973), in another survey of Victorian foxes, found T. pisiformis and M. serialis present but not T. ovis, E. granulosus or T. hydatigena. He cast doubts on the presence of T. hydatigena and T. ovis in foxes as recorded in previous reports. He quoted a further survey by Ryan (unpublished) in which no T. ovis or T. hydatigena had been encountered. Diagnosis had been by hook measurement and he pointed out that accurate hook measurement of T. hydatigena would place it well outside the range of measurements for T. pisiformis and M. serialis.

Beveridge & Gregory (1976) did yet another survey including dogs, foxes and dingoes. They found T. pisiformis and M. serialis present in foxes, T. pisiformis, M. serialis and T. hydatigena in dingoes and T. ovis, T. hydatigena, M. serialis and T. pisiformis in rural dogs. They compared different methods of identification, including characters of the mature proglottis, and suggested a key based on confluency of the testes posteria to the vitellaria, the presence or absence of a vaginal sphincter, and the position of the cirrus sac in relation to the osmo-regulatory vessels. They concluded that this latter relationship was not affected by the method of fixation although histological sections may be necessary to see the morphology of the vagina and the cirrus sac. Gross strobilar morphological characteristics were described, based on the shapes of the scolex, neck and

proglittides after relaxation. They found that the length of the large rostellar hooks was reliable, although they overlapped to some extent, but the small hook length was unreliable except for identifying M. serialis, which is the only worm that may have hooks less than 100 µm long. They confirmed that the number of uterine branches was not a very reliable criterion on which to base a diagnosis.

# CHAPTER FOUR

## IDENTIFICATION BY OTHER METHODS

Apart from morphological examination, other ways of distinguishing taeniid worms are limited to differential staining techniques and serological tests.

### Staining methods

Capron & Rose (1962) described a staining method to differentiate T. saginatus and T. solium. Lichtenberg & Lindenberg (1954) had reported the presence of an alcohol and acid fast substance in Schistosoma mansoni eggs and recommended the staining of stool specimens for their easy detection. Brygoo & Randriamalala (1959) confirmed their findings but noted staining differences between S. mansoni and S. haematobium. Using the technique described (Brygoo et al, 1959), Capron & Rose (1962) examined ova of several helminth species and found that T. saginatus ova retained the stain whereas T. solium ova did not. The embryophoral blocks and cement substance, but not the inner surface of the embryophore, were found to be acid fact (Šlais, 1970).

### Serological methods

The immune response to tapeworm infestation has been recently reviewed by Gemmell & Soulsby (1968), Terry (1968), Dobson (1972), Gemmell & Macnamara (1972) and Gemmell & Johnstone (1977). Most interest has centred on the reaction of the host to somatic infection by the larval

forms of the tapeworms, probably because this form is the most important from the medico-clinical point of view and also because a stronger humoral antibody response is illicited to tissue, rather than to lumen dwelling helminths. This has led to the development of a vast range of tests and techniques directed towards the detection of antibodies. Many reviews of these tests are in the literature (Hiles, 1926; Giunchi, 1960; Kent, 1963; Sorice, Castagnari & Tolu, 1966; Schiller, 1967; Terry, 1968; Kagan, 1968; Miller & Brown, 1969; Denham et al, 1971; Fife, 1971; Capron et al, 1972; Kagan, 1974; Kagan & Norimman, 1974). These tests are useful in the diagnosis of helminth diseases in man or in experimental work with animals, but they are of little value in diagnosing luminal infestations of the alimentary tract or disease in animals. Even the more reliable tests that are used in human medicine vary in their efficacy. The condition of the patient, the strain of the organism, its viability or its location may affect tests (Kagan, 1974) and false positive or false negative results may be frequent enough to render some tests of little value.

Antigen prepared from one species of cestode may have many components in common not only with other cestode species, but also with nematodes and trematodes (Biguet et al, 1962; Capron et al, 1968; Kagan, 1968; Sviridenko-Stepankovskaya et al, 1972). Furthermore, mimicry by the parasite of host antigens has been observed (Gemmell & Macnamara, 1972). In animals,

therefore, the tests pick up only heavily infected animals and are erratic and non-specific. Kagan (1974) found that virtually any cattle in Kenya or Nigeria, whether infected with the cystic stage of T. saginatus or not, were positive in serological tests at the time of slaughter.

Various forms of precipitation test, complement fixation, bentonite flocculation, latex agglutination, indirect immunofluorescent antibody, SAFA (soluble antigen fluorescent antibody), immunoelectrophoresis, counter current electrophoresis, radioimmunoassay and enzyme-linked immunoassay (e.g. ELISA) have been developed for use in the diagnosis of cestode disease (Biagi & Tay, 1958; Castagnari & Sorice, 1971; Beltran & Gomez-Priego, 1973; Torres et al, 1973; Kagan, 1974; Bout et al, 1975; Farag et al, 1975). Of these tests only immunoelectrophoresis as a means of diagnosing hydatid disease is truly specific as only in this disease is one particular arc, designated arc five, seen in the gel (Capron et al, 1970).

All these tests use antigens that have been prepared by different extraction methods from parasite tissues or cyst fluid. Some tests have been described using whole parasites as antigen. Thus, with schistosomiasis, techniques have been described in which the egg, the miracidium or the cercaria are used in such a way. Parasitic tissue sections of Echinococcus protoscolices, or ova of taeniid worms have also been used in fluorescent antibody tests. Although primarily designed to test sera for antibodies, these tests using whole organisms as antigen lend themselves to the identification of parasitic

material.

The circum-oval precipitation test

Visible precipitation that develops around ova, miracidia and cercaria when bathed in homologous antiserum has been the basis of tests that have been widely used and reported in the diagnosis of schistosomiasis in man (Kagan & Pellegrino, 1961).

Oliver-González (1954) first reported the phenomenon of precipitation around the ova of S. mansoni after incubation at 37°C in sera from infected humans and monkeys. There were no cross reactions with Ascaris, hookworm, Trichuris, Entamoeba, Paragonimus intestinalis or Fasciola hepatica infection. He considered the egg antigen to be specific and could not demonstrate its presence in living or lyophilised adults or lyophilised cercariae and no precipitation occurred if sera from mice with single sex schistosome infections were used (Oliver-González, 1954; Oliver-González et al, 1955a, b). The test is not entirely species specific as reactions between schistosome species occur (Oliver-González et al, 1955b; Newsome, 1958). Furthermore, Newsome found that the host origin of both the egg and the sera used in the test determined its success (1958). False positive results can also occasionally occur (Oliver-González, 1954; Newsome, 1958; Kloetzel, 1959). It is sometimes difficult to distinguish precipitates of an immune reaction from other blebs seen on eggs (Anderson, 1963), and this may be the reason for

false positive reactions. The precipitates have been examined under the electron microscope by Sakumoto *et al* (1972). They observed that these were often associated with some defective portion of the egg shell.

Before Oliver-González described the test, Chen (1950) had examined ova and strobilocerci of *H. taeniaeformis* in the presence of immune sera. Although the strobilocerci shrank and collapsed, he did not notice any effect on the ova. Heyneman & Welsh (1959), working with *Hymenolepis nana*, observed the effect of placing each phase of the life cycle in immune serum. The ova agglutinated, precipitates were formed inside the outer shell membrane, there was withdrawal of the central membrane from the embryophore and infectivity of the ova for mice was reduced. The visible reactions, however, were only present in one third of the ova and ten per cent of ova placed in normal serum showed the same effects, except that clumping was not seen. Weinmann (1966), using mouse antiserum, did not confirm these results. Silverman (1955) used activated taeniid onchospheres in a similar test and saw precipitates around the penetrating gland and enclosing membranes. Inactivated immune sera had a lethal lytic effect after one hour but some active and inactivated normal sera produced the same response after three hours. Precipitates were also seen inside the membrane of those onchospheres which had failed to break loose and inactive onchospheres showed a tendency to clump.

Smyth found the embryophoral blocks of *T. pisiformis* ova aggregated *in vitro* in the presence of sera from experimentally infected animals but only if the sera were not inactivated. Aggregation of onchospheral blocks in immune serum was observed by Blundell-Hasell (1971). Heath (1970) also investigated the effect of complement on the immune reactions of *T. pisiformis* onchospheres when they were being liberated from the ova. In its presence they became paralysed and incapable of reorganisation.

The fluorescent antibody test

This has been widely used for the diagnosis of hydatid disease in human medicine. It has also been used for diagnosing cysticercosis due to *T. solium* larvae in man (Daoc *et al*, 1972; Kagan, 1968; 1974), and cysticercosis due to *T. saginatus* in cattle (Euzéby & Dubra, 1970; Calamel & Soulé, 1972). Both the direct and the indirect methods have been studied as well as a soluble antigen fluorescent antibody test (SAFA). Various tissues of taeniids have been used as antigen such as sections of larvae (Vanni & Radice, 1952; Biagi & Piña, 1964; de Pita *et al*, 1965; Panaitesco, 1965; Pozznoli *et al*, 1965; Coudert *et al*, 1966, 1967; Euzéby & Dubra, 1970; Pauluzzi, 1970; Konovalova, 1973; Coltorti & Varela-Díaz, 1974), sections of adult worm (Capron *et al*, 1970; Movesijan & Mladenović, 1971; Soulé *et al*, 1971; Daoc *et al*, 1972; Rydzewski *et al*, 1975)

and ova (Euzéby & Dubra, 1970; Calamel & Soulé, 1972; Machnicka, 1973). Ova of Davainea proglottina and Raillietina cesticillus were also used by Enigk & Sticinsky (1957). Autofluorescence of some tissues, such as the larval hooks (Vanni & Radice, 1952; Biagi & Piña, 1964; Panaitesco, 1965; Machnicka, 1973; Benex, 1974; Coltorti & Varela-Díaz, 1974) and the ova (Calamel & Soulé, 1972; Benex, 1974), is marked. In the diagnosis of echinococcosis, it is now normal practice to use live protoscolices as antigen and the use of germinative epithelium has more or less been abandoned due to the physical difficulties in dealing with sections of this tissue (Benex, 1974). Panaitesco (1965) quenched this autofluorescence with dilutions of Lugol's iodine and counterstaining with Evans blue or rhodamine has enhanced visibility of the reaction (Biagi & Piña, 1964; Machnicka, 1973; Kagan, 1974). Working with T. saginatus larval sections, Euzéby & Dubra (1970) and Calamel & Soulé (1972) found false positive reactions with normal bovine serum, which they attributed to the presence of bovine antigenic material within the cysts. On the other hand, Biagi & Piña (1964) used T. solium larval sections as antigen and obtained very specific reactions which were located in the calcareous corpuscles from which the fluorescence emanated.

This test is one of the more sensitive ones used in the diagnosis of human cysticercosis and echinococcosis but suffers from the high degree of subjectivity required to read it (Fife, 1971; Capron et al, 1972; Kagan, 1974).

As with the other serological tests cross reactions with other cestodes occur (Capron et al, 1970; Euzéby & Dubra, 1970; Calamel & Soulé, 1972; Daoc et al, 1972; Rydzewski et al, 1975).

Immunoelectrophoresis

Of the serological tests, immunoelectrophoresis is quite specific for the diagnosis of hydatidosis. This technique was adapted for use in the study of cestodes by Biguet et al (1962). Capron et al (1967) examined hydatid material and noted the presence of a specific arc designated arc five. This test has now been evaluated (Capron et al, 1970; Quilici et al, 1971) and standardised (Guisantes et al, 1975).

Although sensitive and very specific in diagnosing hydatid disease in man, it has disadvantages in relation to the diagnosis of cestode material. It has not been successful due to non-specificity in cysticercosis in pigs (Morris et al, 1968) and recent work has shown that the arc five antigen is present in T. hydatigena cyst fluid (Vareta-Díaz et al, 1977). Even in hydatid disease in man the arc is not seen in all sera. The preparation of sera is extravagant, requiring lyophilisation and concentration to a third the volume and large amounts of antigen are required to test each sample (Kagan, 1974). The specificity of this technique has not been improved by electrosineresis (Torres et al, 1973).

The basic fault with all the serological tests used in the diagnosis of helminth disease is that the antigens used are complex and may not be those antigens which the host experiences. This has led to the repeated plea for a search for the specific antigens concerned in the stimulation of humoral immunity. This limitation does not necessarily preclude their use for the identification of helminth material by serological methods.

Of the recently developed techniques, the enzyme-linked immunosorbent assay (ELISA) is one of the most sophisticated and sensitive, and enables quantitative estimation of antibodies (Voller et al, 1976). The assay is also easy to perform, economical in the use of antigen and antiserum and a safe alternative to radioimmune assay. Bout et al (1975) and Farag et al (1975) have used this technique in the diagnosis of hydatid disease in human medicine and obtained good results, but cross reactions have been observed between other helminths. When purified hydatid fraction five was used there was no over - lap in extinction values.

Enzyme electrophoresis

The technique of enzyme electrophoresis has been widely used by clinicians and geneticists. Latterly, parasitologists have shown increasing interest in its use as a means of identifying species, sub-species and strains of organisms.

In 1957, Vessel & Bearn reported the heterogeneity of lactate dehydrogenase (1957). Similar findings were published the same year by Wieland & Pfleiderer (1957). They showed that different forms of this enzyme from the same species could be separated by electrophoresis. This led to an escalation in the study of enzyme electrophoresis resulting in the discovery of many enzymes with multimolecular forms (Wilkinson, 1970).

It has been postulated that these multiple forms arise in several different ways. Genetic differences account for the presence of different forms which may be chemically very distinct or merely have slight physical differences. Hybrids may be formed between major types. Multiple forms of non-genetic origin may also occur due to chemical or conformational alteration of molecules (Cann & Goad, 1968; Kaplan, 1968; Masters & Holmes, 1975). This multiplicity of enzyme forms may have a metabolic advantage as certain forms may be better suited to work in particular tissue, in cellular or sub-cellular environments (Masters & Holmes, 1974).

The name "isozyme" was originally proposed by Markert & Møller (1959) to describe the enzyme forms, but "isoenzyme" is now more commonly used. Terminology, however, is being revised and it is proposed that the term "isoenzyme" should be limited to those multiple forms arising from genetically determined differences in primary structure, and not to those derived by modification of the same primary sequence. The term "multiple

forms of an enzyme" should be used as a general term to cover all enzymes with the same activity and occurring naturally in a single species (Masters & Holmes, 1975).

The enzymes present in tapeworms, including taeniids, have been investigated by many workers. Numerous enzymes concerned with carbohydrates and metabolism have been detected. Acid and alkaline phosphatase is present in high concentrations in the tegument of tapeworms and may be involved in glucose transport (Rogers, 1947; Fefevere, 1952; Yamao, 1952a,b; Chowdhury, 1955; Erasmus, 1956a,b; Kilejian et al, 1961; Lee & Tatchell, 1964; Waitz & Schardein, 1964; Arme, 1966; Rothman, 1966; Smyth, 1969; Ždárská, 1973).

Read (1949) was the first to show the presence of glycolytic enzymes and it has since been established that enzymes involved in glycolysis (Embden-Meyerhof pathway), oxidative decarboxylation (Krebs cycle) and the pentose-phosphate pathway are present in tapeworms, although this latter pathway does not appear to be very significant (de Ley & Vercruysse, 1955; Phifer, 1958; Agosin & Aravena, 1959, 1960; Heyneman & Voge, 1960; Rothman & Lee, 1963; Waitz, 1963; Waitz & Schardein, 1964; von Brand, 1966, 1974; Smyth, 1969; Behm & Bryant, 1975a,b,c; Cornish & Bryant, 1975).

Carbohydrate metabolism has been studied more than other metabolic processes, but enzymes involved in lipid metabolism have also been reported, although parasitic worms do not appear to be able to synthesize cholesterol.

They are able to absorb fatty acids and synthesize glycerides (Pylkkö, 1956,a,b; Schwabbe, 1959; Lee, Rothman & Senturia, 1963; Lee & Tatchell, 1964; Schardein & Waitz, 1965; Rothman, 1966; von Brand, 1966, 1974; Smyth, 1969; Bogdanov, 1974). Enzymes involved in protein metabolism have also been reported (Smorodinzew & Bèbèchine, 1936; Daugherty, 1955; Lee & Tatchell, 1964; von Brand, 1966, 1974; Smyth, 1969; Barret, 1975; Parker & MacInnis, 1977). Transaminases have been found (Wertheim et al, 1960; Cornish & Bryant, 1975) but the capacity for transamination is probably limited (Smyth, 1969). In addition, many other enzymes have been detected but their roles remain uncertain. Some in the tegument are associated with inactivation of biochemical processes and may have a use in protecting worms from, for instance, host proteolytic activity. It is also of interest that adsorption of the host enzyme alpha-amylase onto the surface of the tapeworm has been observed in vitro (Read, 1973; Pappas & Read, 1975).

When enzymes undergo electrophoresis, iso-enzymes and other enzyme forms may exhibit differences in directional mobility, the rate of mobility or in band patterns, and thus separate from one another. Under standard conditions, these patterns are characteristic. This has proved a very useful tool in distinguishing genera, species, sub-species or even strains of organisms. In parasitology, it is often important to distinguish pathogens from non-pathogens which may resemble one another morphologically and interest

in biochemical classification is growing. Enzyme electrophoresis has been used to distinguish trypanosomes (Bagster & Parr, 1973; Kilgour & Godfrey, 1973; Steiger et al, 1974), Leishmania (Kilgour et al, 1974), Plasmodium (Carter, 1970, 1973; Carter & McGregor, 1973; Carter & Voller, 1973), Entamoeba (Reeves & Bischoff, 1968), and ascarid worms (Zee & Zinkham, 1975).

Enzyme electrophoresis has not been used for identifying helminths. Although Zee & Zinkham found differences between some ascarid worms, they were primarily concerned with the diagnosis of visceral larva migrans and the differentiation of host and parasite enzymes. The technique of enzyme electrophoresis suggested possibilities for the biological differentiation of cestodes. The work recorded in this thesis concerns the use of this and other techniques for this purpose.

# MATERIALS AND METHODS

# CHAPTER FIVE

## TAPEWORM MATERIAL

Tapeworm material was obtained from three sources: Kenya, Nigeria and the United Kingdom.

a) Kenya: Material was collected and despatched by biologists attached to the Animal Health and Industry Training Institute, Kabete.

Taeniarhynchus gravid proglottides were collected from dispensaries in Kenya, washed and kept in normal saline. These specimens were despatched to Edinburgh at fortnightly intervals. On arrival, the segments were washed and stored in saline and antibiotics (100 U Sodium benzylpenicillin B.P., 100 µg streptomycin sulphate B.P. and two to five µg amphotericin B, U.S.P. per ml) at -20°C. Live ova were collected by dissection of segments before freezing. Ova were washed and stored in saline and antibiotics at 4°C.

b) Nigeria:

Collection methods

Material was collected by the author. Table 1 shows the anthelmintics that were used to expel worms. Some specimens were obtained after spontaneous expulsion or post-mortem. Most of the material was collected live.

Table 1

Method of Collection of Tapeworm Material from Vom, Nigeria

| Species | Arecoline hydrobromide B.Vet.C. | Male Fern B.P. | Mepacrine hydrochloride B.P. | Niclosamide B.P. | Spontaneous | Post-mortem | Unknown |
|---|---|---|---|---|---|---|---|
| T. saginatus | - | 40 | 1 | 1 | 4 | - | 1 |
| T. solium | - | 4 | 2 | - | 1 | - | - |
| T. hydatigena | 4 | - | - | - | 1 | - | - |
| E. granulosus | - | - | - | - | - | 4 | - |
| Cysticercus cellulosae | - | - | - | - | - | All | - |
| Dipylidium caninum | 1 | - | - | - | - | - | - |
| Avitellina centripunctata | - | - | - | - | - | 1 | - |
| Moniezia benedeni | - | - | - | - | - | 1 | - |
| Helictometra giardi | - | - | - | - | - | 1 | - |
| Pyrogynia sp. | - | - | - | - | - | 5 | - |
| Raillietina sp. | - | - | - | - | - | 4 | - |

Human material

Nine hospitals and seven dispensaries in the Plateau State or nearby areas were supplied with normal saline and antibiotics (100 U Penicillin G, B.P., 100 µg dihydrostreptomycin B.P. and 100 U Nystatin B.P. per ml). Instructions were given to store freshly collected human tapeworm material at 4°C or at -20°C.

Frequent visits were made to collect material as fresh as possible. A veterinary technician was also posted to his home village (Miango) for three days to study faecal examination records and to examine faeces brought in by arrangement with the local dispensary. Positive cases were encouraged to receive free treatment to expel tapeworms.

The number of worms collected between May and September 1976 are given in Table 2. Specimens were transported to the United Kingdom on ice.

Canine material

By arrangement with the Vom Veterinary Clinic, arecoline hydrobromide solution (15 mg arecoline hydrobromide B.Vet.C. and 1.5 mg saccharine sodium B.P. per ml) was supplied for a survey on dogs presented there.

Instructions were given for dosage (3.5 mg per kg) and purges were collected for examination. The formulation and dosage were in accordance with the New Zealand National Hydatids Council procedures (Thompson, 1970). Faeces were washed through a succession of sieves down to 100 meshes

Table 2

Tapeworms collected in Nigeria (May to September, 1976)

| Species | Number | Scolices present | |
|---|---|---|---|
| T. saginatus | 44 worms | 9 | Including 3 multiple infections of 4, 4 and 5 respectively |
| T. solium | 7 worms | 2 | |
| T. hydatigena | 5 worms | 3 | |
| E. granulosus | 4 cysts | | From camels |
| Cysticercus cellulosae | many cysts | | All from one pig |
| Dipylidium caninum | 1 worm | 1 | |
| Avitellina centripunctata | 1 worm | 1 | |
| Moniezia benedeni | 1 worm | 1 | |
| Helictometra giardi | 1 worm | | |
| Pyrogynia sp. | 5 worms | 5 | All from one guinea fowl |
| Raillietina sp. | 4 worms | | " " " " " |

per inch and the sediment of each was placed in a black tray and examined with a magnifying glass under a strong light (see Tables 1 and 2).

Slaughter house material

Collections of cysts and tapeworms were made at Vom, Jos and Bukuru slaughter houses (see Tables 1 and 2). Cysts were also collected at Kano slaughter house and despatched to Vom by members of the Parasitology Department of the Ahmadu Bello University, Zaria.

c) Belgium

Echinococcus cysts were collected from a slaughter house by Dr. S. Geerts of the Institute for Tropical Medicine, Antwerp. The hydatid sand in cyst fluid was stored separately from the germinative epithelium or the cysts were stored whole. The material was stored at -20°C and transported to Edinburgh on dry ice.

d) United Kingdom

Collection of material

Worms or cysts were collected at every opportunity from the Veterinary Field Station post-mortem room, from the Gorgie and Glasgow abattoirs and from wild rabbits that had been shot. Some samples were obtained from other research workers within the United Kingdom. Adult tapeworms were also obtained from dogs with natural infections by purging with arecoline acetarsol ("Tenoban", Burroughs Wellcome & Co.*).

The various species of worms collected in the United Kingdom can be seen in Table 3. These figures include experimental infections.

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* Full names of commercial companies and their addresses are given in Appendix III.

Table 3

Species of tapeworms collected in the United Kingdom

| Parasite species | Host species | Number of occasions |
|---|---|---|
| *Anaplocephala perfoliata* | Horse | 1 |
| *Cittotaenia* sp. | Rabbit | 1 |
| *Dipylidium caninum* | Dog | 2 |
| *Dipylidium caninum* | Cat | 1 |
| *E. granulosus* cysts (5 from Belgium) | Horse | 8 |
| *E. granulosus* cysts | Ox | 2 |
| *E. granulosus* cysts | Sheep | 3 |
| *E. granulosus* cysts (sent from Kenya) | Man | 1 |
| *Hymenolepis diminuta* | Rat | 1 |
| *Moniezia expansa* | Sheep | 2 |
| *T. hydatigena* | Dog | 6 |
| *T. hydatigena* cysts | Sheep | 4 |
| *M. multiceps* | Dog | 2 |
| *M. multiceps* cysts | Sheep | 3 |
| *T. ovis* | Dog | 2 |
| *T. ovis* cysts | Sheep | 8 |
| *T. pisiformis* | Dog | 5 |
| *T. pisiformis* cysts | Rabbit | 3 |
| *T. saginatus* (sent from Kenya) | Man | 3 |
| *T. saginatus* cysts | Ox | 2 |
| *T. solium* (sent from Nigeria) | Man | 1 |
| *H. taeniaeformis* | Cat | 11 |
| *H. taeniaeformis* cysts | Mouse | 26 |

Experimental infections

All animals used for experimental infections were kept indoors on University premises and fed on dried or tinned food. Dogs and cats were dosed with bunamidine hydrochloride ("Scoloban", Burroughs Wellcome & Co.) at

the recommended dose rate at least one or two days before infection. If necessary they were also dosed against roundworms and hookworms prior to infection. Sheep were dosed with thiabendazole ("Thibenzole", Merck, Sharp and Dohme Ltd.) at the recommended dose rate prior to infection.

Tapeworms were collected from definitive hosts after dosing with arecoline acetarsol B.Vet.C. Cysts were collected post-mortem. Details of experimental infections are given below.

Hydatigera taeniaeformis

Ova were obtained from Janssen Pharmaceutica, Belgium. Laboratory bred mice and rats were dosed with a suspension of ova in saline directly into the stomach by means of a fine plastic tube attached to a syringe. Cysts that developed to viability were fed to cats, and when infection became patent, the cycle was repeated. Infections are shown in chronological order in Table 4.

Taenia hydatigena and Multiceps multiceps

Viable cysts were obtained from sheep and fed to dogs. Infections are shown in chronological order in Tables 5 and 6.

Table 4

Experimental infections with *H. taeniaeformis*

| Host Species | Age of host (months) | No. of eggs or cysts per host | Period from infection to patency (days) | Period from infection to parasite collection (days) | No. of parasites recovered |
|---|---|---|---|---|---|
| 2 white rats | 6 | 1000 eggs | – | 225 | No cysts |
| 2 white rats | 6 | 750 eggs | – | 225 | No cysts |
| 12 white rats | 1 | 100 eggs | – | 225 | Average 4-5 cysts each |
| Cat | 6 | 10 cysts (degenerating) | – | 81 | None |
| Cat | 6 | 1 cyst (degenerating) | – | 81 | None |
| 6 Brown Swiss mice | 1 | 100 eggs | – | 63 | Average 1.3 live cysts |
| 6 Brown Swiss mice | 1 | 100 eggs | – | 80 | Total 3 live cysts |
| Cat | 5 | 4 cysts | 74 | 74 | 3 gravid worms |
| Cat | 5 | 4 cysts + 3 cysts 39 days later | – | 140 | No worms |
| 9 Brown Swiss mice | 1 | 500 eggs (mostly immature) | – | 120 | Many cysts |
| 3 Brown Swiss mice | 1 | 1000 eggs (old) | – | 120 | Many cysts |
| Cat | 5 | 10-20 live cysts | 90 | | Still infected after 14 months |
| Cat | 5 | 10-20 live cysts | 90 | | Still infected after 14 months |

Table 5

Experimental Infections with T. hydatigena

| Host Species | Age of host (months) | No. of eggs or cysts per host | Period from infection to patency (days) | Period from infection to parasite collection (days) | No. of parasites recovered |
|---|---|---|---|---|---|
| Dog | 5 | *4 cysts | - | 51 | 3 gravid worms |
| Dog | 5 | *4 cysts | - | 51 | 1 gravid worm |
| Dog | 7 | 4 cysts | 52 | 191 | 4 gravid worms |
| Dog | 7 | 7 cysts | 74 | 124 | 5 gravid worms |
| Dog | 4 | 11 cysts | 175 | - | Infection lost after 1 year |

* Cysts kept for 48 hr at 4°C prior to dosing.

Table 6

Experimental infections with M. multiceps

| Host Species | Age of host (months) | No. of eggs or cysts per host | Period from infection to patency (days) | Period from infection to parasite collection (days) | No. of parasites recovered |
|---|---|---|---|---|---|
| Dog | 5 | *3 cysts (many scolices) | 119 | 119 | 370 worms many gravid |
| Sheep | 12 | 2000 eggs | - | - | No evidence of infection after 18 months |
| | | + | | | |
| | | 27000 eggs 7 days later | - | - | |
| Dog | 3 | *1 cyst (many scolices) | 63 | 56 | 2 gravid worms passed spontaneously. Still infected after 7 months |

* Cysts kept for 48 hr at $4^{o}C$ prior to dosing

Taenia ovis

Ova were obtained from Dunedin, New Zealand, and dosed orally to lambs. Two lambs were treated with dexamethasone trimethylacetate ("Opticortenol 0.5%", Ciba-Geigy A G) at the rate of five mg per ten kg live weight twice weekly, starting two weeks before infection with ova and continuing up to the time of slaughter. Viable cysts obtained from the sheep were fed to dogs. Infections are shown in chronological order in Table 7.

Taenia pisiformis

Fresh cysts were obtained from wild rabbits that had been shot. Infections are shown in chronological order in Table 8.

Table 7

Experimental Infections with *T. ovis*

| Host Species | Age of Host (months) | No. of eggs or cysts per host | Period from infection to patency (days) | Period from infection to parasite collection (days) | No. of parasites recovered |
|---|---|---|---|---|---|
| Sheep (Black Face) | 12 | 200 eggs | – | 84 | 90 dead; 1 live cyst |
| Sheep (Black Face) | 12 | 800 eggs | – | 84 | 11 dead; 1 live cyst |
| Sheep (Black Face) | 12 | 1600 eggs | – | 84 | 144 dead; 1 live cyst |
| Sheep (Black Face) | 12 | 3200 eggs | – | 84 | 133 dead; No live cysts |
| Dog | 5 | 1 cyst (ruptured) | – | 77 | None |
| Sheep | 6 | 3000 eggs (old) | – | 54 | None |
| Sheep | 6 | 1500 eggs (old) | – | 91 | 1 dead 1 live cyst |
| Dog | 6 | 1 cyst | – | 70 | None |
| Sheep[+] (Black Face) | 10 | 15000 eggs | – | 42 | Several hundred live cysts |
| Sheep [+] (Black Face) | 10 | 17000 eggs | – | 80 | Several hundred live cysts |
| Sheep (Black Face) | 3 | 10000 eggs | – | 42 | 120-150 cysts (approx. 75% live) |
| Sheep (Black Face) | 3 | 10000 eggs | – | 59 | 5-7 cysts. Poor condition |
| Dog | 6 | 10-20 cysts* | – | 70 | None |
| Dog | 6 | 50 cysts | 56 | – | Still infected after 14 months |
| Dog | 6 | 7 cysts | 56 | – | Still infected after 14 months |

* Held for 4 days at 4°C.
[+] Sheep received "Opticortenol 0.5%" (Ciba-Geigy AG).

Table 8

Experimental infections with T. Pisiformis

| Host Species | Age of host (months) | No. of eggs or cysts per host | Period from infection to patency (days) | Period from infection to parasite collection (days) | No. of parasites recovered |
|---|---|---|---|---|---|
| Dog | 6 | About 60 cysts | 61 | 82 | 53 worms |
| Rabbit | 7 | 500 eggs | - | 147 | 1 cyst |
| Dog | 12 | 1 cyst + 19 cysts 17 days later | 154 | 154 | 4 gravid worms |

# CHAPTER SIX

## PREPARATION AND STORAGE OF TAPEWORM MATERIAL

### Tapeworm ova

Ova were obtained by teasing gravid proglottides apart in normal saline. Ova were washed through a 50 µm sieve and retained by a 30 µm sieve except for the small H. taeniaeformis ova which were retained by a 25 µm sieve. The ova were collected in saline to which antibiotics had been added and were stored at 4°C in medical flat bottles, which were half filled and stored on their sides in order to allow the maximum area for oxygen diffusion. Counting was done with an eel-worm larva counting chamber (a glass chamber holding one ml of fluid in an area three cm by two cm ruled into 24 squares). One ml samples of a suitably diluted ova suspension were counted. Mature, live ova were differentiated from immature and dead ova by appearance. Ova that had onchospheres which had shrunk away from the embryophores were considered dead and those that had thin and light coloured embryophores were considered immature. Only "mature" ova were used for infecting animals.

### Hatching and activation of ova

A slightly modified technique of Gallie & Sewell (1970) was used. Ova were incubated at 37°C for one hour in Solution A and then for at least three hours in Solution B.

Solution A: Pepsin 1 g (BDH Chemicals Ltd.)
Distilled water 100 ml
Concentrated hydrochloric acid 1.75 ml
(May & Baker Ltd.).

Solution B: Pancreatin 1 g (BDH Chemicals Ltd.)
Sodium hydrogen carbonate 1 g (BDH
Chemicals Ltd.)
Trypsin 40 mg (BDH Chemicals Ltd.)
Distilled water 80 ml
Ox bile 20 ml
L - Cysteine hydrochloride (BDH Chemicals
Ltd.) 0.0316 g/100 ml solution.

When ova were transferred to Solution B, the container was filled almost to the top. 0.2 ml of ten per cent hydrochloric acid solution was added and an air-tight lid applied to prevent escape of the carbon dioxide that evolved. Ox bile was obtained under aseptic conditions from freshly slaughtered cattle and stored at -20°C.

Tapeworm cysts

Cysts of T. saginatus from cattle and T. ovis from sheep were collected by cutting meat and offal from the carcase into slices of approximately one cm thickness. All tissue of host origin was removed from the cysts, which were stored in saline with antibiotics at -20°C. Large cysts (T. hydatigena, M. multiceps and E. granulosus) were separated into different components prior to

freezing. Scolices and the cyst wall were washed and stored separately from cyst fluid. Hydatid sand was separated from cyst fluid by centrifugation, washed and stored in saline with antibiotics. Host tissue samples were also collected separately.

Adult tapeworms

Whole tapeworms were washed in several changes of normal saline and stored in a small quantity of saline and antibiotics at -20°C.

## CHAPTER SEVEN

## STAINING TAPEWORM MATERIAL

Gravid proglottides were embedded in paraffin wax and cut into approximately five μm sections by technicians of the Pathology Department and the Centre for Tropical Veterinary Medicine teaching unit. The Ziehl-Neelsen technique was used to stain the sections by the following method, which was adapted from Drury & Wallington (1967):-

a) Stains:

Carbol fuchsin:

| | |
|---|---|
| Basic fuchsin | 1 g (G.T. Gurr) |
| Absolute alcohol | 10 ml |
| 5% phenol in distilled water | 100 ml |

The basic fuchsin was dissolved in alcohol then mixed with the phenol solution and filtered.

Acid alcohol:

1% HCl in 70% alcohol

Methylene blue:

| | |
|---|---|
| Methylene blue | 1 g (G.T. Gurr) |
| Glacial acetic acid | 1 ml |
| Absolute alcohol | 20 ml |
| Distilled water | 80 ml |

b) Procedure:

Sections were dewaxed and rehydrated in two changes of xylene, two changes of absolute alcohol and one change each of 90 per cent alcohol, 70 per cent alcohol and distilled water, each for two minutes.

A piece of filter paper was placed over the section on each slide and carbol fuchsin poured over it. The slides were heated to steaming point for five minutes after which the paper was removed. They were washed with water and then decolourised with acid alcohol for one to three minutes, followed by 20 per cent sulphuric acid for about 20 seconds. They were washed in water again, counterstained with methylene blue for one to three minutes and then washed well in absolute alcohol. They were cleared in xylene for two minutes before mounting in synthetic resin (DePeX, Searle Scientific Services).

Various methods were used to stain ova; they were dried on to a slide or they were suspended in staining fluids in test tubes and washed by means of dilution and centrifugation. A Millipore filter holder (Swinnex, Millipore (U.K.) Ltd.) was also used. The ova were retained in it by a 5 $\mu$m filter (SMWP 02500, Millipore (U.K.) Ltd.). A variation on the above staining technique was also used on the ova. As an alternative to heat, ova were stained with cold carbol fuchsin for up to 30 minutes after the method described by Brygoo, Capron & Randriamalala (1959).

# CHAPTER EIGHT

## SEROLOGICAL TESTS: ANTIGENS AND ANTISERA

### Preparation of antigen

Antigens were prepared from fresh material if possible, otherwise material stored at -20°C was used. Strobilate or metacestode material was dried, weighed and cut up into small pieces with scissors. Phosphate buffered normal saline pH 7.3 (PBS Tablets, Oxoid Ltd.) was added to give ten per cent w/v, and this was then cooled in an ice bath and homogenized at maximum speed (7700 - A homogenizer, MSE Scientific Instruments). Homogenization was done in four two minute bursts so as to avoid heating.

The homogenate was sometimes disintegrated further in an ultrasonicator (100 watt ultrasonic disintegrator No. 7100, MSE Scientific Instruments). Two bursts each of two minutes were given at the eight micron reading of the peak to peak amplitude meter. The sample was kept in an ice bath during this process.

The homogenate was then centrifuged at 2500 g for 30 minutes at 4°C and the supernatant was frozen at -20°C. More PBS was added to the precipitate and the whole process repeated three or four times. The supernatants were then thawed and pooled. A preliminary test for protein content indicated how much concentration was required to bring it to five to ten mg per ml.

Protein estimation was done by the spectrophotometric method of Warburg and Christian. The optical extinction of the extract was measured at 260 nm and 280 nm and from this was calculated the nucleic acid and protein contents (Warburg and Christian, 1941).

Concentration was usually done with polyethylene glycol 6000 (B.D.H. Chemicals Ltd.). The extract was poured into dialysis tubing of a suitable diameter which had been previously soaked in water for about an hour (Visking tubing, Scientific Instrument Centre Ltd.). This was suspended in a cylinder and a reading of the fluid column length noted. The tube was then surrounded by polyethylene glycol and left until the column was reduced to the desired length.

Dehydrated polyacrylamide gel (Lyphogel, Gelman Instrument Co.) was sometimes used for concentrating. A calculated amount of dehydrated gel pieces was placed in the extract. After absorbing water they were then removed. Ultracentrifugation was often necessary, especially after freezing at -20°C in order to clear the solution of colloidal matter. This was done at 4°C for 40 minutes at 100,000 g.

Antigens from tapeworm ova

Different methods were tried. Ova were ultrasonicated in PBS solution for a total of 40 minutes in short bursts. On another occasion, ova were hatched and onchospheres were submitted to ultrasonication (four bursts

of two minutes each). Sometimes ova, in a little normal saline and with some crushed glass, were simply ground with a pestle and mortar.

Solutions were centrifuged, concentrated and the protein content estimated as described for other antigens.

Serum preparation

Blood taken from animals for serum production was held at 37°C for about one hour and then at 4°C overnight. The serum was sucked off the clots and centrifuged at 2500 g for 30 minutes at 4°C. Clear serum was stored at -20°C.

Antiserum production

Laboratory bred young adult rabbits were used for antiserum production. Antigen, ideally containing ten mg protein per ml, was made up with Freund's incomplete adjuvant (Difco Laboratories) in equal proportions.

The antigen was injected into the adjuvant in small quantities and thoroughly mixed by vigorous shaking between each addition until a stable emulsion was produced that did not separate out on standing and remained as a discrete droplet on water. The rabbit was injected with two ml of emulsion containing one ml antigen solution having an estimated protein content of up to ten mg per ml. Half the dose was administered into the thigh of one hind leg, and the other half into the other thigh. Blood was obtained from the rabbit at the same time by ear vein



puncture for checking titre.

This procedure was repeated three weeks and five weeks later. Subsequently the same quantity of antigen, but without adjuvant, was injected subcutaneously as a booster five days before removal of blood.

Bovine antisera were mostly derived from experiments which have formed the basis of a publication (Gallie & Sewell, 1976). Bovine antisera to T. saginatus were produced by the repeated oral dosing of cattle with T. saginatus ova (E10, E674 and E668) or by repeated intramuscular injection of calves with T. saginatus strobilate material followed by oral challenge (E675, E67 and Z155). These antisera were known to have a high titre to T. saginatus antigen in the haemagglutination inhibition and agar gel diffusion tests.

Serum was also collected from sheep that had been orally infected with T. ovis ova (see Table 7, first four sheep), with M. multiceps ova (see Table 6) and a rabbit that had been orally infected with T. pisiformis ova (see Table 8). Collections were made weekly for the first three months of infection.

Goat anti-rabbit and rabbit anti-bovine globulin were obtained from Mrs. L.J.S. Harrison. The former was used for conjugation with fluorescein isothiocyanate and the latter was used in the indirect fluorescent antibody test.

For the former, normal rabbit serum had been precipitated with ammonium sulphate solution at a final concentration of one third saturation. Fifty mg of this normal rabbit serum precipitate in Freund's incomplete adjuvant had been injected intramuscularly into a goat on five occasions, three weeks apart. The goat serum gave a titre of between 1:16 and 1:32 in the gel diffusion test against one mg per ml normal rabbit serum protein.

Rabbit anti-bovine globulin had been produced in a similar way: a rabbit had been hyperimmunized with bovine serum precipitate in Freund's incomplete adjuvant. Ten mg of the precipitate had been given intramuscularly on two occasions, one month apart. Thereafter, a series of intravenous injections had been given at two day intervals. The antiglobulin gave a titre of between 1:16 and 1:32 by the gel diffusion test against one mg per ml bovine serum protein.

# CHAPTER NINE

## PRECIPITATION TESTS

### Circum-oval precipitation test

In this test ova were incubated in the presence of antiserum and examined microscopically for surface precipitates. T. saginatus ova were first washed in normal saline. A suspension of ova was centrifuged at a minimum speed (less than 1,000 rpm on an MSE Super Minor centrifuge) and the supernatant discarded. The ova were resuspended in saline by shaking and were then recentrifuged. Glass slides were prepared by squeezing petroleum jelly onto them through a nozzle so as to form a ring the size of a cover slip. Fifty to 100 ova in 0.1 to 0.5 ml saline were dropped into the centre of the ring, and an equal amount of serum or diluted serum was added to the ova. Anti-T. saginatus rabbit and bovine sera were used. The bovine sera was derived from two animals: one infected by oral dosing with ova (E674) and the other hyperimmunized by injection with strobilate material (Z155). Normal rabbit and normal bovine sera were used as controls. Sera were used neat or in dilutions of 1:4, 1:16 and 1:20. A cover slip was placed over this and pressed onto the petroleum jelly so as to form a seal. The slides were incubated at 37°C for 24 hours but sometimes they were also examined earlier than this. About 0.02 per cent sodium azide was usually incorporated to control

bacterial growth.

Fifty to 100 T. saginatus ova were placed on each slide and ten to 20 ova on each slide were examined microscopically for precipitates. A search was made for chain-like and globular precipitates emerging from the embryophore after incubation with serum. Matter which resembled these two types of precipitate was judged and scored according to the quantity.

Ova were also treated in a variety of ways before use in these tests: they were held in hatching fluid solution A, they were hatched with solution A and then solution B, or they were soaked in 0.2 per cent solution of sodium hypochlorite (20 per cent "Milton", Richardson Merrell Ltd.) for five hours at 37°C so as to remove the outer onchospheral membranes. After incubation the slides were examined under a light microscope.

Micro agar diffusion precipitation test

a) Method

The template technique of Crowle (1961) was used. Microscope slides were cleaned in chromic acid, rinsed and dried. They were then coated with an 0.2 per cent ion agar solution in distilled water to which 0.01 per cent sodium azide had been added. This was done by heating the slides in a flame and then dipping them into the hot agar solution. After drying, each end of the slide was covered with two layers of plastic insulation

tape leaving a two cm wide space in the middle. This provided shoulders about 0.5 mm high for the matrix to lie on. The slides were warmed and a melted one per cent solution of agar in normal saline containing 0.01 per cent sodium azide was poured onto the slide to cover the area in between the tapes. The matrix, lightly greased with silicone grease (Ambersil MS 4, Ambersil Ltd.), was then placed on the agar and rested on the tapes. Care was taken to exclude air bubbles and the slide was allowed to cool. Excess agar that had risen up the matrix holes was removed by suction from a water pump with a sawn-off hypodermic needle as a nozzle. The needle was chosen to fit as closely as possible into the drilled holes of the matrix. The matrix dimensions were 2.5 cm by 2.5 cm and three mm thick. Four wells were positioned equidistant from each other and five mm from a centre well. The wells were three mm diameter at the top, tapering at a depth of two mm to 1.5 mm diameter.

These slides were prepared immediately prior to use. A dilution of antigen or antiserum was delivered into each matrix well with a Pasteur pipette that had been drawn out to an extra fine point. Care was taken to exclude air bubbles by filling with the Pasteur pipette touching the bottom of the well. The filled slides were kept at room temperature (about 20$^{o}$C) in moist conditions. A plastic sandwich box was used as a moist chamber: blotting paper lightly soaked with water was

inserted into the lid and the box was sealed with tape to prevent evaporation. Incubation lasted until lines developed, usually after four days. After incubation, the matrix was removed. This was done by carefully lifting it so as not to disrupt the agar, or by drying it at 37°C until the agar had dehydrated and shrunk away from the matrix.

The grease was removed before staining by gently rubbing with a moist cotton wool swab. Recording of precipitation lines was done before and after staining.

The slides were sometimes soaked for a short period in 0.0125 per cent cadmium acetate to increase visibility of the lines and then washed in two changes of PBS (ten minutes in the first, 30 minutes in the second).

b) Staining

Stain - 0.1% thiazine red R (R.A. Lamb), a protein stain dissolved in 100 ml one per cent acetic acid.

The slides were soaked in each of the following fluids for ten minutes:

1. Distilled water
2. Distilled water
3. Thiazine red stain
4. One per cent acetic acid
5. One per cent acetic acid
6. One per cent acetic acid + one per cent glycerol

They were then allowed to dry thoroughly at 37°C.

c) Antigens and antisera

The technique was used to measure the titre of rabbit and sheep antisera during hyperimmunization and during development of infection in animals dosed with ova. It was also used to test cross reactions between different worm species and for absorption studies.

In experiments IP3, IP4, IP5, IP6, IP7 and IP8, antigens and antisera were used neat as well as in doubling dilutions. Dilutions from 1:2 to 1:16 were usually made. Normal saline was used instead of antigen and normal bovine, sheep or rabbit serum instead of antiserum for controls.

The following bovine antisera were used in the experiments: Z155, E675, E67 (from calves injected with antigen) and E674, E674 concentrated X5 and E668 (from orally dosed calves).

All antisera from orally dosed sheep and the rabbit were used and all antisera from rabbits that had been hyperimmunized by injection (see antiserum production).

For absorption studies, antiserum was incubated with an equal quantity of a heterologous antigen at 37$^{o}$C for 30 minutes. The resulting fluid was used as absorbed antiserum against homologous antigen.

# CHAPTER TEN

## THE FLUORESCENT ANTIBODY TEST

This test was done in different ways using strobila, metacestode or ova of T. saginatus as antigens. Homologous antiserum was added to the antigen, incubated and washed off. Fluorescein isothiocyanate (FITC, Nordic Immunological Laboratories), or rhodamine isothiocyanate (TRITC, Nordic Immunological Laboratories), conjugated to the specific serum antiglobulin, was then added and after incubation the antigen was washed and examined in a fluorescent microscope. An indirect technique was also used in which fluorescent antiglobulin was used against another antiglobulin, which in turn was specific for the antisera used.

### Antigen

T. saginatus ova were used unhatched, treated to disrupt the embryophore (but not necessarily resulting in hatching), as hatched onchospheres or sectioned in gravid proglottides. An 0.8 per cent sodium hypochlorite solution (80% "Milton", Richardson Merrel Ltd.) was used to disrupt the embryophores by soaking them in it up to five hours. Alternatively they were incubated in hatching solution A for 30 minutes or in solution A for about one and a half hours followed by solution B for about two and a half hours. These times were also

varied. Approximately ten µm cryostat sections of gravid proglottides prepared by the Pathology Department were used fresh, or kept in dry conditions at -20°C for a few days before use. Fixed sections, approximately five µm thick, were also prepared in paraffin wax and were dewaxed and rehydrated prior to the test.

Antisera

Bovine antisera E10, E674, E668, Z155 and rabbit antiserum to T. saginatus were used in tests against ova. Table 9 shows antisera used in tissue section tests.

Conjugated antisera

Goat anti-rabbit globulin conjugated with FITC was made. In addition, FITC and TRITC conjugated goat anti-rabbit globulin and FITC conjugated rabbit anti-bovine globulin were purchased from a commercial source (Nordic Immunological Laboratories).

The method used for conjugation was based on that described by Holborow & Johnson (1967) and Johnson & Holborow (1973). Conjugation was done at 4°C throughout.

One hundred ml goat anti-rabbit serum (GAR) was precipitated, dropwise, with continuous stirring, by 100 ml saturated ammonium sulphate solution. The pH was then adjusted to 7.8 with 4N sodium hydroxide. After 16 hours, the suspension was centrifuged at 1,500 g, and

the precipitate was dissolved in PBS pH 7.3 up to 100 ml and then reprecipitated with 50 ml saturated ammonium sulphate. The whole procedure was repeated three times. 0.15 M saline was used to redissolve the precipitate and transfer it for dialysis over three days against PBS pH 7.3. The solution was then centrifuged to remove denatured protein (1,500 g for ten minutes). The protein content was estimated by absorption spectrophotometry at 260 nm and 280 nm and checked by the Biuret method. The solution was then diluted to give a final protein concentration of ten mg per ml by the addition of 0.15 M saline and 0.5 M carbonate/bicarbonate buffer pH 9 in which FITC had been dissolved (one mg per ml). The buffer that was added constituted one-tenth of the final volume and a little over one mg FITC was added for each 100 mg of protein in the solution.

The FITC was added drop by drop whilst the solution was stirred continuously. Stirring was continued for 18 hours. Excess FITC was removed by dialysis overnight against PBS pH 7.3, and the solution was then dialysed overnight against 0.0175 M phosphate buffer preparatory to passing it through a column containing diethylaminoethyl (DEAE) cellulose (DE 52, Whatman LabSales Ltd.). Approximately one g DEAE-cellulose for every ten mg globulin was prepared according to the manufacturer's directions for the removal of fines, degassing and equilibration to 0.0175 M phosphate buffer pH 6.3. The

conjugate was then passed through the column using fractionating equipment (LKB Instruments Ltd.). The optical density of the effluent was monitored at 254 nm (Spectochrom F analyser, Gilson) and recorded on paper (6520-4 LKB Instruments Ltd.). Collections were made in test tubes so that fractions of the effluent corresponding to peaks shown on the paper could be collected separately.

By substituting the buffer, first by 0.0175 M phosphate pH 6.3 containing 0.123 M NaCl and then by 0.0175 M phosphate pH 6.3 containing 0.250 M NaCl, separate collections were made of peaks that were recorded, one being eluted by each of the substitute buffers. The collections were then dialysed against PBS pH 7.3, and the labelling ratio measured by optical extinction at 495 nm, which is the peak absorbance of FITC conjugated to protein and 280 nm, which is the tyrosine absorption peak. One peak collection had an optical extinction ratio of 0.9 and this was concentrated with polyethylene glycol to about the original volume. The conjugate was then divided into aliquots and stored at -20$^{o}$C. The specific antibody content was tested in the micro-gel diffusion test against normal rabbit serum. Yellow precipitation lines were visible at a titre of 1:32.

Test procedure using ova

a) Tube test

A suspension of 50 to 200 ova was washed in PBS pH

7.3 by lightly centrifuging, discarding the supernatant and resuspending in PBS pH 7.3. This washing procedure was repeated twice and the ova were finally suspended in 0.5 ml or one ml PBS. To this suspension was added 0.2 ml serum, or serum diluted with PBS and it was incubated at room temperature (about 20°C) usually for 30 minutes but sometimes up to 16 hours. The ova were then washed again twice and resuspended in PBS. Conjugated antiglobulin (0.1 ml) was added to the ova, incubated again and then washed.

The sediment of ova was then transferred to a slide for microscopical examination. A ring had previously been drawn in the centre of the slide, about 1.5 cm diameter, with a wax pencil to confine the drop. The drop was covered with a cover slip.

b) Slide test

Other methods were also tried: ova were dried onto a slide that was then bathed with the test fluids. Washing was by flooding repeatedly with PBS or by dipping slides into a dish to soak for periods up to two hours in changes of PBS. Slides were mounted with nine parts glycerol and one part saline pH 9.2 for microscopy.

c) Quenching of autofluorescence

Dilutions of Nile blue (G.T. Gurr) and Lugol's iodine were used to stain ova for ten minutes. The ova were washed twice to remove the stain before microscopy.

Test procedure using tissue sections

Cryostat sections had serum and conjugate placed directly over them. Incubation time was 30 minutes at 37°C. Washing was done by suspending the slides in a rack in a continuous flow tank of PBS which was kept stirred. Slides were sometimes counterstained with 1:100 Evan's blue in PBS for five minutes, washed and mounted as before.

A "sandwich" method was also tried using bovine antiserum, rabbit anti-bovine globulin and goat anti-rabbit globulin conjugate. The washed sections were covered with a drop of glycerol and a cover slip and examined microscopically.

Table 9 shows antisera, antiglobulins and conjugates used in these tests.

Table 9

Scheme of Immunofluorescent Experiments using T. saginatus Tissue Sections

| Antigen | Antiserum | Method of hyper-immunization | Anti-globulin | Conjugate |
|---|---|---|---|---|
| T. saginatus gravid proglottis sections | Rabbit | Injection | - | GAR FITC or TRITC |
| | Bovine | Injection | - | RAB FITC |
| | Bovine | Oral | - | RAB FITC |
| | Bovine | Injection | RAB | GAR FITC |
| | Bovine | Injection | RAB | GAR TRITC |

RAB = Rabbit anti-bovine
GAR = Goat anti-rabbit

Microscopical examination

Vickers equipment was used for fluorescent microscopy according to the manufacturer's instructions (Vickers Ltd.). An H.B.O. 200 mercury lamp was used, together with a B G 12 primary filter for excitation. Barrier filters OG 1 and GG 9 were used in light ground microscopy. With dark ground illumination OG 4 and GG 9 barrier filters were used. On one occasion a Leitz Orthoplan universal large field microscope with Ploem illumination was used with an H.B.O. 200 mercury vapour lamp, four mm B G 12 excitation filter and a K 510 or K 530 barrier filter.

# CHAPTER ELEVEN

## IMMUNOELECTROPHORESIS

Scheidegger's technique for micro-immunoelectrophoresis, with a few minor modifications, was used (Crowle, 1961). Single or multiple perspex templates were used.

### Antigens and antisera

Strobilate antigens from Cittotaenia, T. hydatigena, M. multiceps and T. saginatus were used in this test against both homologous and heterologous sera raised in rabbits.

### Single template method

In this method, single glass microscope slides (75 mm by 25 mm) were used. After thorough cleaning, the slides were coated with 0.2 per cent agar. Five layers of waterproof vinyl tape, about two mm in width, were laid along the long edges to act as a support for the template. Hot, one per cent agar (Purified agar, Code L.28, Oxoid Ltd.) or 0.9 per cent agarose (BDH Chemicals Ltd.) in buffer was pipetted onto the slides in between the tapes. A perspex template, the same size as the slide, with a central trough and a well at each side of it, was greased with silicone grease and laid on the hot agar to rest on the tapes. Dimensions of the trough were 5.5 cm x 1 mm; the wells had a diameter

of one mm and were located in the middle of the template, two mm either side of the trough.

Gel was cleared from the two wells by means of a hypodermic needle attached to a water suction pump and the wells were filled with antigen. The slide was set on the bridge of a Shandon U 77 tank (Shandon Southern Instruments Ltd.) which had previously been filled with buffer. Filter paper wicks (Whatman No. 1 paper, Whatman LabSales Ltd.) soaked in buffer were used to connect the electrical circuit from the buffer to the gel at each end of the slide. A sodium barbital buffer of 0.1 ionicity and pH 8.2 was used in most experiments (Crowle, 1961).

When the circuit was complete, a constant voltage was applied from a power supply (Vokam, SAE 2761, Shandon Southern Instruments Ltd.). Voltage was usually 150 V but other voltages (20, 50 or 100 V) were used in some experiments. In four experiments electrophoresis was done on duplicate or triplicate slides so that the effect of different periods of electrophoresis could be compared. Slides were submitted to electrophoresis for periods ranging from 25 minutes to 150 minutes.

After electrophoresis, the gel was scooped out of the trough, it was filled with antiserum and the slide was incubated for two to three days at room temperature in a moist chamber or until precipitation lines developed. The template was then removed, the grease cleaned off the agar and the agar stained in the same way as in the

micro-gel diffusion test.

Multiple trough template

This method was the same as that already described, but a glass slide 82 mm by 82 mm was prepared by levelling it and pipetting onto it 12 ml of molten agar made up with buffer to give a layer one mm thick. A template of the same size was temporarily held over the agar and up to eight troughs alternating with up to nine wells were cut into the agar using a scalpel blade and punch. A barbital acetate buffer of 0.05 ionicity and pH 8.6 was used (Luckins, 1975).

The template was then removed, the wells emptied of agar, filled with antigen and electrophoresis performed. After electrophoresis, the agar was removed from each trough and replaced by an equal quantity of antiserum. The slide was then incubated at $4^{o}C$ for 24 hours.

# CHAPTER TWELVE

## THE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The antigen coated tube test technique was used as described by Engvall & Perlmann (1972).

### Antigens

These were prepared from Cittotaenia, T. hydatigena, M. multiceps, T. pisiformis and T. saginatus strobila. Antigen was also prepared from one week old ova of T. hydatigena and T. ovis. In experiment E8, 300 ova of each species and, in experiment E9, about 5,000 ova of each species were prepared by crushing them with a pestle and mortar. They were used against both unabsorbed and absorbed antisera. Saline was used for controls.

### Antisera

Antisera were raised in rabbits and normal rabbit serum was used for controls.

From information gained in the gel diffusion test, heterologous antigens that gave the largest number of precipitation lines were used for absorption. An antiserum was absorbed by one or by two heterologous antigens. This was done by adding to it an equal volume of each antigen and incubating for 30 minutes at 37°C. Absorbed sera were usually used immediately, otherwise they were stored at -20°C.

Preparation of enzyme conjugate

As commercial supplies of enzyme conjugate were not available at first, horse-radish peroxidase was linked to goat anti-rabbit globulin. Most tests, however, were done with a commercial goat anti-rabbit IgG linked to horse-radish peroxidase (Nordic Immunological Laboratories). For the preparation of conjugate, it was first necessary to prepare rabbit IgG. This was then aggregated as an immunoadsorbent in order to extract pure goat anti-rabbit IgG which was then conjugated to horse-radish peroxidase.

a) Preparation of rabbit IgG

Precipitation of globulin with 33 per cent ammonium sulphate was done by the method described by Campbell et al (1964). Four hundred ml of normal rabbit serum was stirred continuously and precipitated by adding, drop by drop, 200 ml of saturated ammonium sulphate solution. The pH was adjusted to 7.8 by the addition of sodium hydroxide, stirred for three hours at room temperature and then centrifuged at 1,400 g for 30 minutes. The precipitate was dissolved in saline to 400 ml, reprecipitated, and the whole procedure repeated twice more. The precipitate was finally dissolved in 0.1 M phosphate buffered saline pH 6.8, made up to 200 ml and then dialysed against the same buffer for three days at $4^{o}C$ until the dialysate was free of ammonium sulphate. The dialysate was tested by acidifying a small aliquot with a drop of dilute hydrochloric acid and then adding a few

drops of two per cent barium chloride; a precipitate indicated the presence of sulphate ions. The globulin solution was then centrifuged at 1,400 g at 4°C for 30 minutes and stored at -20°C.

The protein content was checked spectrophotometrically and the solution passed through a DEAE-cellulose column (DE 52, Whatman) equilibrated to 0.01 M phosphate buffer pH 6.8. Immunoglobulins other than IgG are retained at this molarity (Fahey & Terry, 1974). It was then concentrated to one-third of its volume with polyethylene glycol. The specificity of the globulin was shown by immunoelectrophoresis against goat anti-rabbit serum using a template with four troughs and a barbital buffer of 0.1 ionicity and pH 8.2 (Crowle, 1961).

The solution was further concentrated to 50 ml, centrifuged at 1,400 g and the protein content re-estimated spectrophotometrically. Gel filtration then removed large and small molecular contaminants. Sephadex Superfine G 200 (Pharmacia Fine Chemicals A B) was swollen with eluant and packed into a column (26/100 Pharmacia Fine Chemicals) according to the manufacturer's instructions (Pharmacia Fine Chemicals, 1972). Homogeneity of the bed was checked by passing Blue Dextran 2000 (Pharmacia Fine Chemicals) through the column. The globulin solution was passed through the column in 0.1 M Tris/HCl (Tris-hydroxymethyl - methylamine, (BDH Chemicals Ltd.) pH 8.0 plus 1 M NaCl and 0.02 per cent sodium azide. An

automatic concentrator was included in the pumping circuit which concentrated the effluent five times (Amicon CEC 1 concentrator containing a Diaflo ultra filtration membrane UM 10 of molecular weight cut off >10,000, Amicon Corporation). Further concentration to 50 ml was made with polyethylene glycol and the protein content was re-estimated.

b) Preparation of immunoadsorbent

Insolubilization of the protein was done by the method described by Avrameas & Ternynck (1969). The IgG was dissolved in 0.1 M phosphate buffer pH 7.0 at 50 mg per ml. Whilst stirring at room temperature, one ml of a 2.5 per cent aqueous solution of glutaraldehyde (G1631, Grade IV, Sigma) was added dropwise for each five ml of solution. The gel that formed was allowed to stand for three hours at room temperature. Complete insolubilization was shown by the absence of protein in a supernatant obtained by centrifuging a small quantity of the insoluble material dispersed in a little water at 2,500 g for 15 minutes.

The insolubilized protein was then homogenized in a tissue grinder (BC/15, R.B. Radley & Co. Ltd.) and dispersed in the proportion of 500 mg to 200 ml 0.2 M phosphate buffer pH 7.3. This was centrifuged at 2,500 g for 15 minutes at 4°C. The homogenization and centrifugation procedures were repeated three times and the protein then suspended in 200 ml 0.1 M glycine/HCl buffer pH 2.8 and

recentrifuged. This procedure was repeated. The immunoadsorbent was finally washed by centrifugation in phosphate buffer until the supernatant had an optical density of zero at 280 nm.

c) Immunoadsorption and elution of goat anti-rabbit IgG

Ten ml goat anti-rabbit antiserum was stirred with 467 mg of insolubilized globulin at room temperature for 30 minutes and then centrifuged at 2,500 g for 15 minutes at 4°C. The precipitate was suspended in 40 ml PBS and washed by centrifugation and resuspension until the supernatant had an optical density of less than 0.04 at 280 nm. The adsorbed protein was eluted twice, each time using about four ml of 0.1 M glycine/HCl buffer pH 2.8. The immunoadsorbent was again washed with the buffer and then with several changes of PBS. It was then re-used to adsorb more antibodies. The protein content of each eluate was estimated and electrophoresis of each showed the presence of only specific antibodies against normal rabbit IgG. The total recovery amounted to 23.6 mg of protein. The pooled eluates were then concentrated with polyethylene glycol and lyphogel and tested against normal rabbit serum by micro-gel diffusion, giving a titre of 1:32.

d) Conjugation of goat anti-rabbit IgG to horse-radish peroxidase

A conjugation procedure was reported by Kawaoi & Nakane (1973) but details were not given. The procedure

was developed by Harrison (1976). Ten mg horse-radish peroxidase (HRPO, Type VI, Sigma) was dissolved in two ml 0.3 M sodium bicarbonate. 0.2 ml of one per cent fluoronitrobenzene in absolute ethanol was then added and the solution stirred for two hours at room temperature to block the $\alpha$ and $\epsilon_{\lambda}^{amino}$ groups of HRPO. This prevents self-coupling of peroxidase upon the subsequent formation of aldehyde groups. Two ml 0.05 M sodium metaperiodate in water was added and mixed in the dark overnight at $4^{o}C$ to oxidize the carbohydrate moiety. The next day, two ml 0.16 M ethanediol (AnalaR, BDH Chemicals Ltd.) in water was added to stop the oxidation reaction. The mixture was then dialysed against 0.01 M carbonate buffer pH 9.5. Ten mg specific goat anti-rabbit IgG was meanwhile dialysed against the same buffer. The IgG in two ml carbonate buffer was then allowed to react with the HRPO aldehyde for three hours at room temperature. The resulting conjugate was concentrated with polyethylene glycol to three ml and purified by fractionating on a G 200 Sephadex column using 0.1 M Tris/HCl pH 8.0 and 1.0 M NaCl. Only the first peak was kept, thus excluding unconjugated enzyme and protein. The selected fraction was dialysed against PBS pH 7.3 and concentrated to 0.5 mg per ml, including five per cent bovine serum albumen for stabilization and 0.02 per cent sodium azide to prevent bacterial contamination.

The conjugate was then tested with substrate and produced an immediate intense brown colour reaction. It was stored at 4°C.

e) Test procedure

The method for the tube test was described by Engvall & Perlmann (1972). Five cm by one cm disposable polystyrene tubes (Luckham Ltd.) were coated with antigen by placing in each one ml of a solution of antigen in 0.1 M sodium carbonate buffer pH 9.6 plus 0.02 per cent sodium azide and incubating them for one to three hours at 37°C and then at 4°C overnight.

The next day, the tubes were emptied and washed three times with 0.9 per cent NaCl containing 0.05 per cent polyoxyethylene sorbitan monolaurate ("Tween 20", Sigma). This was done by filling the tubes with the washing fluid and leaving them for two minutes before emptying. One ml of the rabbit antisera being studied, diluted with PBS containing 0.05 per cent Tween 20 and 0.02 per cent sodium azide, was then added to each tube. The Tween 20 was added to prevent non-specific adsorption. The tubes were incubated at room temperature (+20°C) for about six hours and then washed again.

HRPO conjugate was diluted with PBS/Tween, one ml was added to each tube and incubated at room temperature overnight. The next day, the tubes were washed again, one ml substrate was added to each tube and the colour reaction allowed to develop. The substrate used was described by

Ruitenberg et al (1975) and consisted of 80 mg of 5-amino salicyclic acid (Cambrian Chemicals Ltd.) dissolved in 100 ml hot distilled water and adjusted to pH 6.0 with 1 N sodium hydroxide. To nine ml of this solution, one ml of 0.05 per cent hydrogen peroxide solution was added and one ml of the final solution was delivered into each tube.

The reaction was stopped by adding 100 µl 4 M NaOH per tube, and the optical density was read at 449 nm. Substrate was used as a standard for optical density.

Duplicate tubes were usually included in the tests.

# CHAPTER THIRTEEN

## ENZYME ELECTROPHORESIS: PREPARATION OF EXTRACTS

### Strobila and cyst material

Tapeworm material was homogenized in various fluids in a proportion of 1:9 approximately. Small quantities (one or two proglottides) were homogenized with a pestle and mortar or in a one ml mini homogenizer (Jencons). Larger quantities were homogenized at maximum speed for eight minutes in two minute bursts at 4°C in a machine with a rotating cutter (Model 7700A MSE).

Ultrasonication for up to six minutes in bursts of one to two minutes at 4°C sometimes followed. Usually the homogenate was then centrifuged at 2,500 g for 30 minutes at 4°C and the supernatant frozen at -20°C. After thawing, it was ultracentrifuged at 100,000 g for 30 minutes at 4°C, and the supernatant stored at -20°C. Hydatid cyst fluid was centrifuged at 100,000 g and the supernatant was freeze dried.

Freeze drying was done using a dry ice-ethyl alcohol freezing mixture either in 0.5 ml quantities in two ml glass ampoules (Adelphi Tubes Ltd.) or in larger quantities in flasks using a Speedivac High Vacuum pump (Model 2SC20A, Edwards High Vacuum Ltd.).

In Nigeria, facilities for centrifugation at 4°C and for ultracentrifugation were not available and samples were filtered through a 0.22 μm filter instead (Millipore

(U.K.) Ltd.). Some difficulties were experienced due to blockage, but filtering was possible if the extract was allowed to stand at $+4^{o}C$ overnight and only the supernatant used.

Ova

Ova were collected and washed as described previously.

In Nigeria, sieves were not available and visible pieces of strobilate material were removed by forceps under a magnifying glass. Ova were then washed by repetitive centrifugation and discarding of supernatants.

Ova were finally centrifuged and as much supernatant fluid as possible was removed. The sediment was crushed, together with broken glass, with a pestle and mortar.

Enzyme solutions

Extracts were often prepared in a solution of 1mM each of EDTA (Ethylenediamine tetra acetic acid, ED2SS Sigma) dithiothreitol (D632 Sigma) and $\alpha$ amino-n-caproic acid (A2504 Sigma) in distilled water (EP solution). Some extracts were prepared in 2.4 M ammonium sulphate solution or in 2.6 M ammonium sulphate solution plus $10^{-4}$M magnesium acetate (M0631 Sigma).

Extracts for freeze drying were made up in distilled water, in normal saline and then dialysed against distilled water or in EP solution. Extracts for immediate use were made up in normal saline, water, or EP solution.

Extracts of host tissue taken from the same animals in which cysts were found were also made and preserved in

the same way for comparative studies.

Storage of extracts

Extracts were stored in several different ways: at +4°C, -20°C or in liquid nitrogen. For storage in liquid nitrogen 10µl quantities were pipetted into liquid nitrogen to form small beads. These were collected and stored in containers in liquid nitrogen.

Extracts in ammonium sulphate were stored at +4°C and freeze dried samples were stored at +4°C or at -20°C in a glass vacuum dessicator or in sealed ampoules.

Fractionation

A freeze dried extract of Taenia hydatigena strobilate material, which had been shown to be enzymically active, was reconstituted in PBS (160 mg in three ml). This was ultracentrifuged at 100,000 g for 30 minutes and then run through a G200 Sephadex column using PBS 7.3 at the rate of 18 ml per hour, monitored at 254 nm wavelength. Fractions were collected, concentrated with polyethylene glycol and freeze dried.

An approximate molecular weight range wac calculated for the fractions containing most enzymic activity by the formula:

$$K_{av} = \frac{Ve - Vo}{V_t - V_o}$$

where $V_e$ = eluted volume (fraction), $V_o$ = void volume, $V_t$ = total volume of column.

The molecular weight was obtained by applying the calculated $K_{av}$ to a graph giving the relationship between elution behaviour and molecular properties (Pharmacia Fine Chemicals AB, 1972).

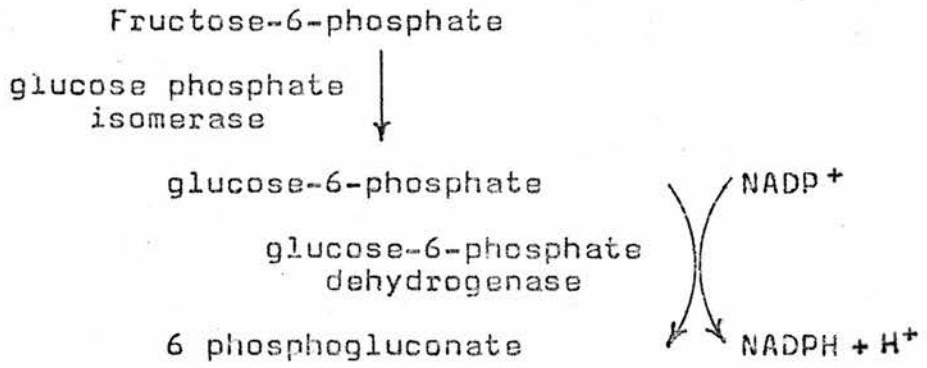
Detection of enzymes

0.025 ml of tapeworm extract or a $6mm^2$ piece of filter paper dipped in extract was added to 0.5 ml of developing solution incorporating a substrate linked to a tetrazolium dye system. This was incubated until a colour change was visible. The intensity of the colour change was sometimes measured in a spectrophotometer at 540 nm.

Enzyme assay

This was based on the absorbance of light by reduced nicotinamide adenine dinucleotides (NADH and NADPH) and the non-absorbance by the oxidised forms (NAD and NADP). Any dehydrogenase reaction in which NAD or NADP is reduced may therefore be measured by the increase in light absorbance at 366 nm wavelength (Bergmeyer, 1965). In order to measure the enzyme activity of a sample, a known amount was added to the appropriate substrate in the presence of the coenzyme NADP. Thus for glucose phosphate isomerase 2 ml of 0.15 M Tris/HCl buffer pH 8.0 was mixed with 0.3 ml 0.1 M $MgCl_2$, 0.1 ml NADP solution (ten mg per ml), 0.2 ml fructose-6-phosphate solution (ten mg per ml) and 10 U glucose-6-phosphate dehydrogenase. In the

presence of NADP, the glucose-6-phosphate dehydrogenase converted any glucose-6-phosphate, which was produced by the activity of the glucose phosphate isomerase on the fructose-6-phosphate, to 6 phosphogluconate:




To 0.8 ml of this solution at 28°C, 5 µl of the sample was added. The mixture was shaken and inserted in a spectrophotometer (SP1800 Ultraviolet, Pye Unicam). The change in light absorbance at 366 nm was recorded automatically by a recording attachment (AR25 Linear recorder, Pye Unicam) on the spectrophotometer. From the results, the enzyme activity present in the sample was calculated using the formula (Mattenheimer, 1976):

$$U/ml = \frac{\Delta A}{t \times E \times d} \times \frac{TV}{SV}$$

Where $\Delta A$ = change of absorbance
t = time in minutes
TV = total assay volume
SV = a sample volume
E = molar absorption coefficient at 366 nm (3·3)
d = distance of light path in cm

Extracts were diluted prior to use with ten per cent bovine serum albumin (A4378, Sigma) to bring their

concentration to 1 U/ml.

# CHAPTER FOURTEEN

## ENZYME ELECTROPHORESIS: SUPPORT MEDIA

The Millipore System (Millipore (U.K.) Ltd.)

The "Phoroslide" is made of cellulose acetate bonded for strength onto a hard flexible backing. Prebuffering, blotting and insertion of the Phoroslide into the specially designed buffer cell was done according to the manufacturer's instructions. The applicators were loaded by passing the grooved contact area through the sample four times. If the multiple cell containing four Phoroslides for comparing eight samples simultaneously was used, the special serum tray and dropper was employed. The duration of contact with the Phoroslide was two minutes, after which the applicators were removed and electrophoresis commenced. Electrophoresis was done at room temperature; buffers, voltages and times were varied.

Prepared slides of agarose ("Agaroslide", Millipore (U.K.) Ltd.) were also used with this apparatus in a similar way. These were used in the multiple cell and required no prebuffering. Samples were applied to the precut slots by a Pasteur pipette that had been drawn to a fine point.

Electrophoresis using a Shandon tank (Model U77, Shandon Southern Instruments Ltd.)

Several different media were used for enzyme electrophoresis.

a) Cellulose acetate

"Celagram" strips were used (Shandon Southern Instruments Ltd.) and the manufacturer's directions were followed for prebuffering and handling. Samples were applied by means of a multiple applicator (Shandon Southern Instruments Ltd.). This applicator was comb-like with ten six mm long and six mm wide "teeth" each separated by six mm and having a groove cut on the free end of each to hold the sample.

b) Polyacrylamide gel

The method described by McCarthy (1974) was used to make the gel. A mould for a 16 cm x nine cm and three mm thick gel was made with two sheets of glass separated at opposite short ends by three mm thick glass strips cemented onto one of the sheets. The mould was held together with bull-dog clips and made water-tight with petroleum jelly. One wide edge was sealed off with plasticine and the solution poured in carefully so as to avoid bubbling. When quite full, the last edge of the mould was also sealed off with plasticine.

The solution was made by adding five to ten g "Cyanogum 41" gelling agent (BDH Chemicals Ltd.) to 95 ml of buffer solution. When dissolved, 0.5 to one ml ten per cent w/v 3-dimethylaminopropionitrile (BDH Chemicals Ltd.), and one ml 10% w/v ammonium persulphate, both made up freshly in the same buffer, were added.

When set, the plasticine and cover sheet of glass were removed and a line of slots was cut in the gel three cm

from one long edge. The slots, eight mm wide and separated by six mm from each other, were cut with a piece of razor blade resting against a ruler.

Pieces of cellulose acetate paper ("Celagram", Shandon Southern Instruments Ltd.) of about eight mm x one mm were soaked in the tapeworm extract and inserted into the slots for electrophoresis.

c) Thick starch gel

The method used was based on that described by Smith (1968). The mould was made up of two sheets of glass and a six mm thick hollow rectangle of plastic. Two sizes were used: small (11 cm x 17 cm) and large (17.5 cm x 18 cm). The plastic mould was laid on one clean sheet of glass and the molten starch was poured into it until it was over-full. The other glass sheet, at least six mm thick, was laid on top of the plastic mould and pressed down, thus squeezing out excess starch. The gel was then allowed to solidify at room temperature before being stored at $4^{o}C$ and used within 24 hours.

The starch solution was made by adding ten per cent w/v hydrolysed starch ("Electrostarch", BDH Chemicals Ltd. or starch from Connaught Medical Research Laboratories or S4501, Sigma) to a weak buffer solution in a flask. This was stirred continuously whilst heating over a flame to boiling point. It was then degassed for 20 to 30 seconds before pouring into the mould. Sugar was sometimes added as it is supposed to improve zone resolution and handling (Shaw & Koen, 1968).

Slots were cut into the gel on a line six cm from one end in a similar way to that described for polyacrylamide gel. Care was taken not to cut right through the gel to the base plate. Samples were applied by soaking four mm x four mm pieces of filter paper (No. 1, Whatman) or cellulose acetate and inserting them into the slots. These inserts were usually left in situ but on some occasions they were removed during electrophoresis after 20 minutes or one hour.

d) Thin layer starch gel

The method described by Wraxall and Culliford (1968) was used. A mould was prepared by cementing strips of perspex slightly less than one mm thick and five mm wide along the edges of a glass sheet to form a rectangular well of 13.5 cm x 21.5 cm.

Starch gel was prepared (usually 11% w/v in weak buffer) and poured into the mould at one end. A scraper (SAE2557, Shandon Southern Instruments Ltd.) was used to push the starch over the whole area of the mould: excess starch spilled over the end. This made a gel of not more than one mm thickness. The gel was cooled at $4^{o}C$ until it turned milky (about ten minutes).

A slightly modified method of Kilgour and Godfrey (1973) was used for application of the samples. The gel was placed over a marked out sheet of paper so that the samples could be inserted on a line at the cathodal end exactly five cm from the wick. Samples were inserted by

first cutting the gel with slots by means of a comb-like instrument similar to a multiple applicator. Slots were about six mm long and had a space of about six mm in between each. Coloured embroidery cotton was cut to the length of a slot and teased out to its six component strands. One or two strands were then soaked in the sample, drained and inserted into each slot.

# CHAPTER FIFTEEN

## ENZYME ELECTROPHORESIS: PROCEDURE

Power for electrophoresis was delivered from a Vokam Power Pack with constant voltage and constant current settings (Shandon Southern Instruments Ltd.) that delivered up to 400 V. An LKB power supply with constant settings was also used which could deliver up to 2,000 V (2103, LKB Instruments Ltd.).

Either a constant voltage or a constant current setting was used for the duration of a run. The actual voltage across the gel was measured by a voltmeter with attached probes.

The manufacturer's instructions for electrophoresis using a Shandon tank Model U77 were followed. The tank was filled with a minimum of 600 ml buffer (300 ml each side). Fresh buffer solution was usually used for each run, otherwise the direction of the current flow was reversed for a second run.

Thick gel plates rested on the rods provided with the tank but thin gel plates rested on an insulated cooling platen, which in turn was housed on a perspex holder (modification to U77 tank SAE3232, Shandon Southern Instruments Ltd.).

When cellulose acetate or agarose gels were used, electrophoresis was done at room temperature. When thick starch gels were used the apparatus was housed in a cold room at $4^{o}C$. Thin starch gels were used at about $8^{o}C$, with

cooling by ice-cold water circulating through the metal platen on which they rested.

Thick starch gels were covered by thin plastic film ("Cling Film", Boots) to prevent drying during electrophoresis. Four layers of medical gauze were used as a wick for all gels in the Shandon apparatus except for thin starch gels. For these gauze and paper wicks were first tried but abandoned in favour of household sponges, 15 cm x ten cm x one cm. The distance between the wicks was constant and was 7.5 cm for polyacrylamide gels, 14 cm for thick starch gels and 15 cm for thin starch gels. The wicks were held in place by a glass sheet laid over them. The metal cooling platen was insulated underneath by a rubber pad lying on the perspex holder and on top by a sheet of 50 µm Melinex (Imperial Chemical Industries Ltd.).

Electrolyte solutions

The choice of electrolyte solution for use in the electrode tank and for making the gel depended upon the enzyme to be studied. Initially an electrolyte was used which had been reported in the literature as suitable for that particular enzyme, but eventually most electrolyte solutions were tried for each of the main enzymes. Most electrolytes were diluted for use in gel preparation. Discontinuous buffer systems were also tried in which the electrolyte solution used in the tank was quite different from that used for the preparation of the gel. In Table 10 all the buffers used for each enzyme are given.

Table 10

Electrolyte systems used for Electrophoresis

| Enzymes | Molarity range (electrode buffer) | pH range (electrode buffer) | Electrode buffer | Dilution for gel buffer, pH as for electrode buffer |
|---|---|---|---|---|
| GPI, G6PD, GDH (NADP and NAD) HK, AK, LDH, 6PGD, MDH (NADP and NAD) | 0.05M – 0.5M Tris | 6.0–8.9 | Tris/HCl | 1 in 5 to 1 in 25 |
| MDH (NADP and NAD) GPI, αNA esterase, 6PGD, ASAT, ALAT, GDH,LDH | 0.1M Tris 0.05M–0.1M barbitone | 7.6–8.8 | Tris/HC1/barbitone | 1 in 5 |
| GPI, GDH, G6PD, Alk. Phos., MDH, Acid Phos., ICD, PGM, HK, AK, ALD LDH, Peroxidase, ASAT, ALAT, Cholinesterase | 0.01M – 0.5M | 5.1–9.0 | Tris/citrate | 1 in 5 to 1 in 25 |
| AK, HK, LDH, MDH, GDH, GPI | 0.032M–0.1M phosphate | 6.2–8.0 | Phosphate/citrate | 1 in 5 |
| AK, GPI, MDH, 6PGD, GDH, (NAD and NADP) | 0.2M | 5.8–8.0 | Phosphate | 3 in 40 |
| MDH, LDH | 0.5 I (Dawson *et al*, 1969) | 4.0–8.2 | Phosphate/citrate/KC1 | 1 in 5 |
| GPI, MDH, LDH, ASAT, ALAT, Cholinesterase | 0.1755M–0.682M borate | 8.0–10.0 | Borate/NaOH | 1 in 10 |
| ALD | 0.5M Tris 0.0179M $Na_2EDTA$ 0.647M boric acid | 8.0 | Tris/versene/borate | 1 in 10 |
| SDH | 0.1M succinic acid 0.186M Tris | 6.0 | Succinic acid/Tris | 5 mM succinic acid 8.7 mM Tris |
| LDH, MDH | 0.1M Tris 0.05M–0.1M barbitone | 7.6–8.1 | Tris/HC1/barbitone | Normal saline or Phosphate/citrate pH 7.0 (as above) |
| MDH | 0.3M boric acid | 8.6 | Boric/lithium | 0.36M Tris/cit pH 8.5 |

A key to abbreviations is given in Appendix I

Markers

Stained proteins were used to measure the distance travelled under standard conditions of electrophoresis: ten per cent γ bovine globulin (BG-11, Sigma) was stained by adding to one ml a few drops of 0.04 per cent solution of bromphenol blue indicator (21006, BDH); haemolysed blood samples from an ox, a sheep and a rabbit were also used.

Gel staining methods

a) Phoroslides and Agaroslides

The method followed was that described in Millipore Application Manual AM301 (Millipore (U.K.) Ltd.) except that different developing solutions were used. The slides were dipped, cellulose acetate side downwards in a developing solution for 15 seconds. Excess fluid was dabbed off and the slide was placed in a moist chamber at 37°C until visible bands developed. Phoroslides were fixed in five per cent acetic acid for ten minutes, blotted and dried. The dried slides were placed slowly in ethanol, left for five minutes and then transferred to a clearing solution (ethyl alcohol and glacial acetic acid, 30:70) for one minute and dried.

Agaroslides were dipped in a developing solution for 15 seconds and then incubated until bands were visible. They were then fixed in 60 per cent ethyl alcohol in deionized water.

b) Cellulose acetate

Sheets other than Phoroslides were dipped in developing solution, incubated in the same way and dried.

c) Thick starch gels

After electrophoresis the gel was sometimes sliced horizontally into two or three sheets, three mm or two mm thick respectively. This was done with either a large sharp carving knife or a slicer mechanism similar to that made by Shandon (SAE-2568, Shandon Southern Instruments Ltd.). In the latter, the gel, still in position on the glass plate, was pushed along a board across which a thin wire was stretched. The wire was adjusted to slice the gel horizontally as the gel was pushed past.

The sheets of gel were then floated off the plate into a basin of water and from there each was transferred to a plastic sandwich box, slightly larger than the gel, and drained. The gel was submerged in developing solution and incubated at 37$^{o}$C until bands developed, after which the solution was poured off and the gel kept moist until the result was recorded.

d) Thin layer starch gels

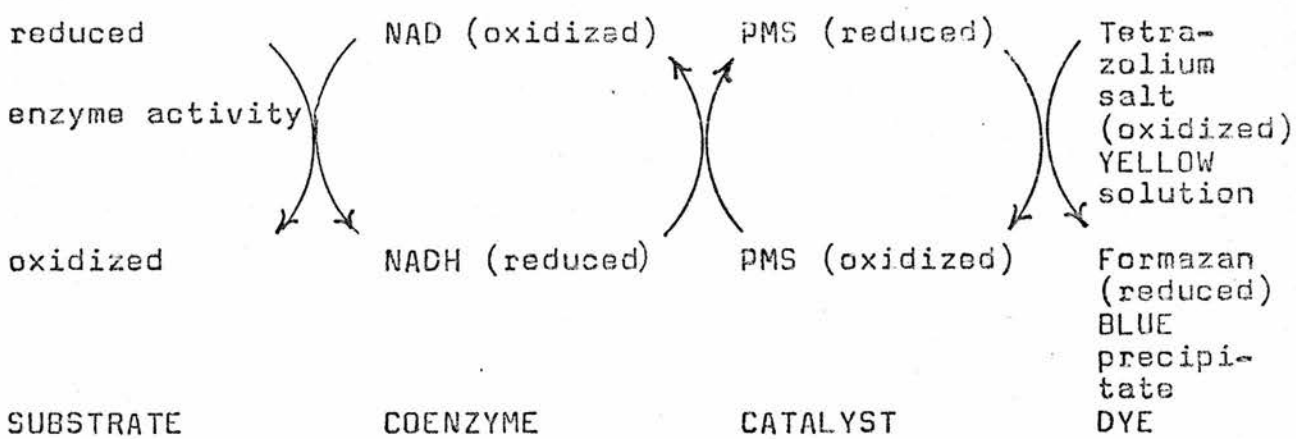
An open perspex square of internal dimensions 12 cm x 12 cm was placed over the middle of the gel to confine the developing solution which was poured into it. The gel was then incubated at 37$^{o}$C until bands developed.

# CHAPTER SIXTEEN

## DEVELOPING SOLUTIONS AND RECORDING RESULTS

### Developing solutions

Basic recipes were compiled by Shaw & Prasad (1970) and many of the formulas used were modifications of these. Enzyme activity with the appropriate substrate was linked to a dye system so that the position of an enzyme in the gel could be seen. This system was first used by Markert & Møller (1959) and is based on hydrogen transfer:




NAD = Nicotinamide adenine dinucleotide

PMS = Phenazine methosulphate

Recipes for different enzymes are given in Table 11.

Table 11

Developing Solutions for Staining Specific Enzymes in Gels

| Enzyme | Buffer | Substrate, Coenzyme and other ingredients |
|---|---|---|
| Acid phosphatase EC 3.1.3.2 Shaw & Prasad (1970) | 100 ml 0.05 M sodium acetate pH 5.0 | 100 mg sodium α-naphthyl acid phosphate, 100 mg Black K salt |
| Aconitase EC 4.2.1.3 Shaw & Prasad (1970) | 8.0 ml 0.5 M phosphate pH 8.0 | Solution A (25 mg NADP, 30 mg NBT or MTT, 8 ml 0.1 M cis-aconitate pH 7.5, 24 ml water). Solution B (3 ml water, 2 ml Solution A + 6 U isocitrate dehydrogenase warmed to 37°C). Place starch gel in Solution B then pour rest of Solution A + 40 ml water + 2 mg PMS on top |
| Adenylate kinase EC 2.7.4.3 Carter (1973) | 30 ml 0.1 M Tris/HCl pH 7.4 | 5 mg NADP, 20 mg ADP, 40 mg Glucose, 6 U G6PD, 20 U HK, 20 mg $MgCl_2$, 6 mg MTT. 0.5 mg PMS added just before use |
| L-alanine: 2 oxoglutarate aminotransferase EC 2.6.1.2 (modification of ASAT recipe) | 0.1 M phosphate pH 7.0, 100 ml | 532 mg L-alanine, 73 mg α ketoglutaric acid, 50 mg pyridoxal phosphate. Add 200 mg fast blue BB salt just before use |

| Enzyme | Buffer | Substrate, Coenzymes and other ingredients |
|---|---|---|
| Aldolase EC 4.1.2.7<br>Shaw & Prasad (1970) | 0.5 M Tris/HCl<br>pH 7.1, 10 ml | 50 mg NAD, 545 mg $Na_4$ fructose 1, 6 diphosphate. $5H_2O$, 100 U glyceraldehyde 3PD, 30 mg NBT or MTT, 150 mg $Na_2HAsO_4$. $7H_2O$, 90 ml water. 2 mg PMS added just before use |
| Alkaline phosphatase<br>EC 3.1.3.1<br>Shaw & Prasad (1970) | 100 ml<br>distilled<br>water | 50 mg $\beta$-naphthyl sodium phosphate, 50 mg fast blue RR, 123 mg magnesium sulphate. $7H_2O$. |
| Alkaline phosphatase<br>EC 3.1.3.1<br>Engvall & Perlmann (1972) | 1 ml 0.05 M<br>sodium<br>carbonate<br>pH 9.8 | 1 mg/ml $\beta$-nitrophenylphosphate and $MgCl_2$ $10^{-3}$ M/ml buffer |
| L-aspartate: 2 oxogluterate<br>aminotransferase EC 2.6.1.1<br>Shaw & Prasad (1970) | 0.1 M<br>phosphate<br>pH 7.0, 100 ml | 532 mg L-aspartic acid, 73 mg $\alpha$ ketoglutaric acid, 50 mg pyridoxal phosphate. Add 200 mg fast blue BB salt just before use |
| Cholinesterase EC 3.1.1.8<br>Boehringer Mannheim<br>GmbH Diagnostica<br>Ellman et al (1961) | 52mM<br>phosphate<br>0.26 mM<br>5,5'-dithiobis<br>(nitro-benzoic)<br>acid, 3 ml | Acetylthiocholine iodide 156 mM, 1 ml |
| $\alpha$-naphthyl acetate esterase<br>Carter (1974) | 50 ml 0.1 M<br>sodium<br>phosphate<br>pH 7.4 | 30 mg Na $\alpha$-naphthyl acetate dissolved in a little acetone, 30 mg fast blue RR |

| Enzyme | Buffer | Substrate, Coenzymes and other ingredients |
|---|---|---|
| Fumarase EC 4.2.1.2<br>Shaw & Prasad (1970) | 0.1 M phosphate pH 7.1, 20 ml | 80 mg NAD, 30 mg NBT or MTT, 770 mg K fumarate, 200 U MDH, 80 ml water. Add 1 mg PMS just before use |
| Glucose phosphate isomerase EC 5.3.1.9<br>Carter (1974) | 50 ml 0.1 M Tris/HCl pH 8.0 | 50 mg D-fructose-6-phosphate, 5 mg NADP, 6 mg MTT, 0.01 ml G6PD (Sigma type X1), 20 mg $MgCl_2$. Add 0.5 mg PMS just before use |
| Glucose-6-phosphate dehydrogenase EC 1.1.1.49<br>Carter (1974) | 50 ml 0.1 M Tris/HCl pH 7.0 | 100 mg glucose-6-phosphate, 5 mg NADP, 6 mg MTT, 40 mg $MgCl_2$. Add 0.5 mg PMS just before use |
| Glutamate dehydrogenase EC 1.4.1.2<br>Carter (1974) | 50 ml 0.1 M Tris/HCl pH 7.5 | 1 ml 7% sodium glutamate pH 7.5 (by adding NaOH), 7 mg NAD or 5 mg NADP, 6 mg MTT, 0.5 mg PMS added just before use |
| Hexokinase EC 2.7.1.1<br>Carter (1973) | 30 ml 0.1 M Tris/HCl pH 7.4 | 5 mg NADP, 100 mg ATP, 500 mg glucose, 6 U G6PD, 5 mg KCN, 20 mg $MgCl_2$, 6 mg MTT. Add 0.5 mg PMS just before use |
| Isocitrate dehydrogenase EC 1.1.1.42<br>Shaw & Prasad (1970) | 85 ml water | 20 mg NADP, 20 mg NBT or MTT, 0.1 M $Na_3$ isocitrate. $H_2O$, 20 ml 10% $MnCl_2$. $4H_2O$, add 3 mg PMS just before use |

| Enzyme | Buffer | Substrate, Coenzyme and other ingredients |
|---|---|---|
| Lactate dehydrogenase<br>EC 1.1.1.27<br>Carter (1974) | 50 ml 0.1 M<br>Tris/HCl<br>pH 8.0 | 200 mg lithium lactate, 7 mg NAD, 6 mg MTT, 0.5 mg PMS added just before incubation |
| Malate dehydrogenase<br>(or Malic enzyme)<br>EC 1.1.1.37 (or<br>EC 1.1.1.40)<br>Carter (1974) | 50 ml 0.1 M<br>Tris/HCl<br>pH 8.0-8.5 | 5 ml 10% malic acid solution pH 7.5 with NaOH, 7 mg NAD (or NADP), 6 mg MTT, 0.5 mg PMS added just before incubation |
| Peroxidase EC 1.11.1.7<br>Ruitenberg, Steerenberg & Brosi (1975) | - | 80 mg 5-amino salicylic acid dissolved in 100 ml hot distilled water, adjusted to pH 6 with NaOH. To each 9 ml add 1 ml 0.05% $H_2O_2$ |
| Phosphoglucomutase<br>EC 2.7.5.1<br>Shaw & Prasad (1970) | 10 ml 0.5 M<br>Tris/HC l<br>pH 7.1 | 10 mg NADP, 200 mg or 600 mg $NA_2$ glucose 1-phosphate. $4H_2O$, 200 mg $MgCl_2$. $6H_2O$, 80 U G6PD, 20 mg NBT or MTT, 90 ml water |
| 6-phosphogluconate<br>dehydrogenase<br>EC 1.1.1.44<br>Carter (1974) | 50 ml 0.1 M<br>Tris/HCl<br>pH 7.0 | 50 mg 6-phosphogluconate, 5 mg NADP, 6 mg MTT, 0.5 mg PMS added just before incubation |

| Enzyme | Buffer | Substrate, Coenzymes and other ingredients |
|---|---|---|
| Succinate dehydrogenase Shaw & Koen (1968) | 100 ml 0.5 M phosphate pH 7.0 (included in recipe) | Solution A (13.5 mg $Na_2$ succinate. $6H_2O$, 25 ml 0.5 M $K_2HPO_4$ anhydrous, 65 ml $H_2O$). Solution B (27.0 mg $Na_2$ succinate. $6H_2O$, 35 mg MBT or MTT, 50 mg ATP, 10 ml 0.1 M $Na_2EDTA$, 100 ml 0.5 M phosphate pH 7.0), 80 mg PMS. Incubate in A for 15 min. then pour off and incubate in B |

Abbreviations are explained in Appendix I.

All chemicals were supplied by Sigma Chemical Co. Ltd. except the following:

From BDH Chemicals Ltd.:

Acetone AnalaR
D-glucose AnalaR
Magnesium chloride. $6H_2O$, AnalaR
di-potassium hydrogen orthophosphate
Sodium hydroxide
Sodium dihydrogen orthophosphate
di-sodium hydrogen orthophosphate dihydrati
Tris (hydroxymethyl) methylamine

From M & B Ltd.:

Hydrochloric acid

From Cambrian Chemicals Ltd.:

5-amino salicylic acid

When using the thin layer starch gel method the following modifications were made:

Ingredients for developing solutions were prepared as stock solutions and stored at $4^{o}C$.

Agar was included in the formulation and this solution was poured over the gel to set, after which it was incubated at $37^{o}C$ in the dark.

About 150 mg agar was heated in ten ml water or buffer until it melted. Meantime the rest of the ingredients except PMS were mixed into the remainder of the buffer. When the agar solution cooled to $55^{o}C$, the PMS was added to the other ingredients, the molten agar was poured in, mixed and the whole tipped onto the gel within the frame. About 20 ml altogether was required to cover the gel.

Formulations for this method were modifications of recipes obtained from Boid (1974) and those shown in Table 11. They were made partly from the following stock solutions, which were stored at $4^{o}C$:
NADP 10 mg/ml, sodium glutamate 7% pH 7.5 with NaOH, NAD 10 mg/ml, D-fructose-6-phosphate 10 mg/ml, sodium malate 0.1M pH 7.5 with NaOH, 0.1M $MgCl_2$ and MTT 2 mg/ml. Details are shown in Table 12.

MTT was used in all solutions except those used with cellulose acetate for which NBT was substituted. NBT was also sometimes used with polyacrylamide gel.

Table 12

Formulations used for thin layer starch gels

| Enzyme | Buffer | Substrate, Coenzymes and other ingredients | Diluent for 150 mg agar (Code L28. Oxoid Ltd.) |
|---|---|---|---|
| Glucose phosphate isomerase | 0.4M Tris/HCl pH 8.0 2.4 ml | 7.6 ml $H_2O$, 1.0 ml NADP, 1 ml F-6-P, 10U G6PD, 2 ml MTT, 2 ml $MgCl_2$, 0.5 mg PMS added just before use | 10 ml water |
| Glutamate dehydrogenase | 0.4M Tris/HCl pH 8.0 10 ml | 1.5 ml sodium glutamate, 2 ml NAD, or NADP, 2.5 ml MTT, 0.5 mg PMS added just before use | 15 ml 0.4M Tris/HCl pH 8.0 |
| Malate dehydrogenase (NADP) | 0.4M Tris/HCl pH 8, 2.4 ml | 7.6 ml $H_2O$, 2 ml NADP, 0.8 ml malate, 2.5 ml MTT 2 ml $MgCl_2$, 0.5 mg PMS added just before use | 10 ml water |
| Malate dehydrogenase (NAD) | | As above but 2 ml NAD instead of NADP | |
| Malate dehydrogenase (NAD) (alternative recipe) | | 10 ml $H_2O$, 5 ml malate, 0.7 ml NAD, 3 ml MTT, 0.5 mg PMS added just before use | 10 ml 0.2M Tris/HCl pH 8.0 |
| Hexokinase | 0.1M Tris/HCl pH 7.4 10 ml | 500 mg glucose, 1 ml $MgCl_2$, 100 mg ATP, 0.5 ml NADP, 3 ml MTT, 6U G6PD, 5 mg KCN, 0.5 mg PMS added just before use | 10 ml 0.1M Tris/HCl pH 7.4 |
| Adenylate kinase | 0.1M Tris/HCl pH 7.4 10 ml | 40 mg glucose, 0.5 ml NADP, 20 mg ADP, 6U G6PD, 20U HK, 1 ml $MgCl_2$, 3 ml MTT, 0.5 mg PMS added just before use | 10 ml Tris/HCl pH 7.4 |
| 6-phospho-gluconate dehydrogenase | 0.4M Tris/HCl pH 8.0 5 ml | 5 ml $H_2O$, 50 mg 6PG, 0.5 ml NADP, 3 ml MTT, 0.5 mg PMS added just before use | 10 ml water |

Abbreviations are explained in Appendix I

Recording results

A densitometer ("Phoroscope", Millipore (U.K.) Ltd.) was used for recording the bands on a Phoroslide. This was fitted with a 580 nm filter to enable a blue colour to be recorded. It was operated according to the manufacturer's instructions to align the pattern on the screen. A trace was then made automatically onto paper.

Recording of band patterns in starch gels and polyacrylamide gels was made by tracing with an indelible felt tip pen on polyester film (Type 5, 50 µm Melinex, ICI). Photographic recordings of some gels were also made. For photography thick gels and polyacrylamide gels were lit by reflected light but transmitted light was used for thin starch gels, which were laid on top of a light box. Photography was done by the photographic unit at the Veterinary Field Station.

# RESULTS

# CHAPTER SEVENTEEN

## DIFFERENTIATION BY STAINING AND SEROLOGY

### Staining

The most satisfactory method was to stain sections of gravid proglottides by the hot Ziehl-Neelsen method. Seven taeniid worms were examined: Taenia hydatigena, M. multiceps, T. ovis, T. pisiformis, T. saginatus, T. solium and H. taeniaeformis. The embryophore of all these species was acid and alcohol fast in contrast to the onchosphere and other tissue. These results were consistent and confirmed by staining two specimens of each species on several occasions. Sections were also given to two technicians, one in the Pathology Department and one in the Centre for Tropical Veterinary Medicine M.Sc. laboratory, both with experience in staining, for further confirmation.

Figs. 1 and 2 show stained sections of T. solium and T. saginatus respectively.

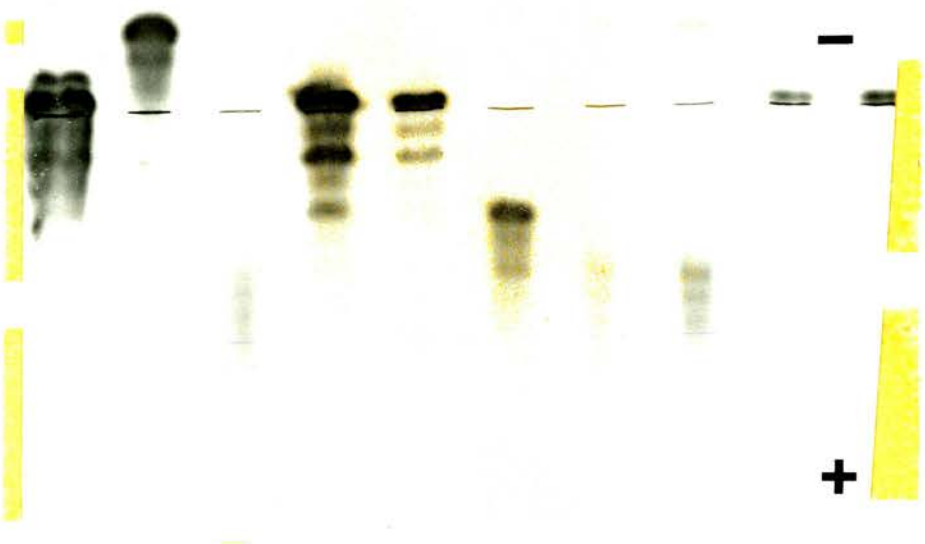
The other staining methods were not satisfactory for the following reasons: ova in test tubes stained poorly due to dilution of the staining fluids and many were lost during the washing process; recovery of ova from the Millipore filter method was also poor and ova were unevenly stained; ova fixed to a glass slide by evaporation appeared very dark and it was difficult to see any colours.

Sections were stained by the Ziehl-Neelsen method. Embryophores are stained red (acid and alcohol fast) and are seen sectioned in different planes; some are almost whole whilst others have been broken by the microtome and the embryophoral blocks displaced. Onchospheres and strobila tissue are counter-stained blue.

(X 240)

Figure 18

Strain variation in *E. granulosus* (glucose phosphate isomerase)




From left to right:

| | |
|---|---|
| Ox | *E. granulosus* germinative epithelium (Glasgow) |
| | *E. granulosus* germinative epithelium (Glasgow) |
| Horse | *E. granulosus* hydatid fluid and sand (Belgium) |
| | *E. granulosus* germinative epithelium (Belgium) |
| Sheep | *E. granulosus* germinative epithelium (Glasgow) |
| Ox | *E. granulosus* germinative epithelium (Wales) |
| | *E. granulosus* germinative epithelium (Glasgow) |
| Horse | *E. granulosus* hydatid fluid (London) |
| Horse liver | |
| Ox liver | |

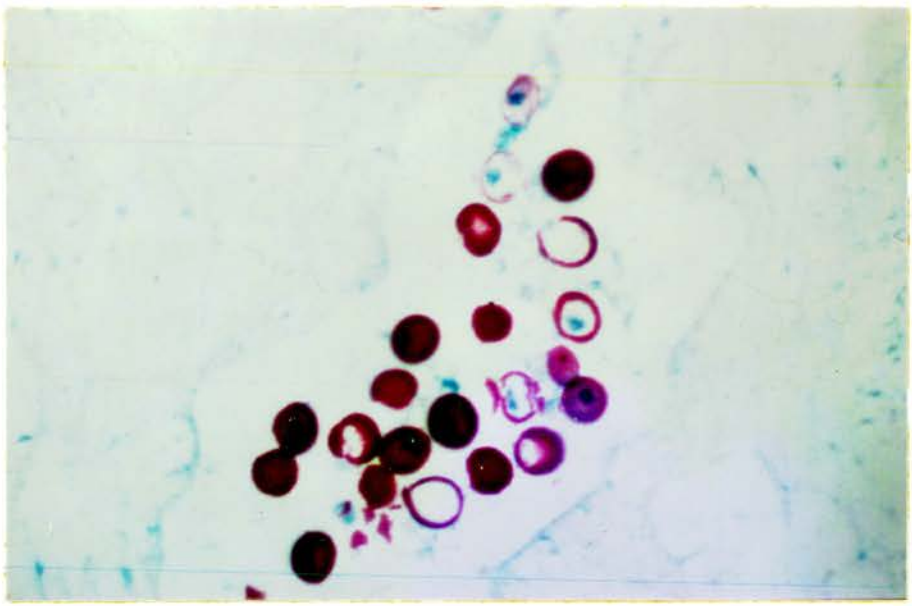


Figure 1
*T. solium* gravid proglottis

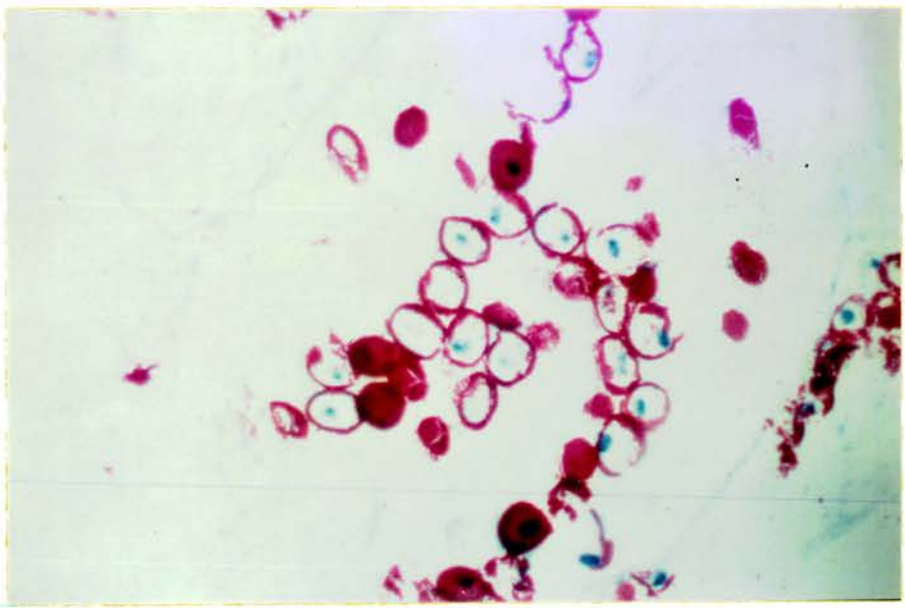


Figure 2
*T. saginatus* gravid proglottis

Ova did not stain deeply enough by the cold method described by Brygoo et al (1959).

Circum-oval precipitation test

Untreated ova were used in five experiments (OP 1 to 5) against bovine and rabbit antisera. Precipitates of both types were seen in all slides including the controls although higher scores were allotted to those on slides treated with antisera. It was difficult, however, to distinguish between precipitate, yolk sac material attached to the embryophore and other particulate matter present.

Ova were treated in further experiments by different methods designed to clean the ova or to disrupt the embryophore and so allow a more intimate contact with onchospheral material. Ova incubated in pepsin and hydrochloric acid (solution A) for half to one hour still had remnants of the yolk sac adhering. In two experiments (OP 6 and OP 7) precipitates were seen with antisera and with normal sera. In another experiment (OP 8) treatment of ova with 0.2% sodium hypochloride solution (20% Milton, Richardson Merrel Ltd.) resulted in loss of the embryophore and the disappearance of most of the onchospheres. This was partly due to the washing process but the onchospheres appeared misshapen after contact with the Milton.

Finally, a test (OP 9) was done on hatched onchospheres that were still in their membranes. Both bovine

antiserum and rabbit antiserum were used but no precipitates were seen.

Micro agar diffusion precipitation test

In a preliminary experiment (IP 1) T. saginatus antigen was tested against antisera from the rabbit and the calves that had been hyperimmunized by intramuscular injection of strobilate material and compared to serum from the calves that had been given repeated oral doses of ova. The results are tabulated in Table 13.

Table 13

Immunoprecipitation between T. saginatus antigen and antisera raised by oral dosing of calves or by intramuscular injection of strobilate material

| | Experiment IP 1 | |
|---|---|---|
| Antiserum | Method of hyper-immunization | Maximum number of lines |
| 1 Rabbit | Intramuscular | 5 |
| 3 Calves | Intramuscular | 4 |
| 2 Calves | Oral | Nil |

It appeared from this result that there was no reaction between strobilate antigen and antiserum from the calves hyperimmunized by oral dosing of ova. Antisera from orally infected animals were included in other experiments and results are summarised in Table 14.

Table 14

Immunoprecipitation between antigens and homologous antisera from animals infected orally

| Experiment | Antigen | Donor of Homologous Antiserum | Maximum No. of lines |
|---|---|---|---|
| IP 2 | *T. saginatus* | Calf (concentrated X5) | 1 |
| IP 3 | *T. pisiformis* | Rabbit | Nil |
| | *T. ovis* | Sheep No. 1 | Nil |
| | | Sheep No. 2 | Nil |
| | | Sheep No. 3 | Nil |
| | | Sheep No. 4 | Nil |
| | *M. multiceps* | Sheep No. 5 | Nil |
| IP 4 | *M. multiceps* | Sheep No. 5 | Nil |
| IP 5 | *M. multiceps* | Sheep No. 5 | Nil |

These results largely confirmed the low level of reaction in the gel diffusion test between strobilate antigen and antisera from animals that had been immunised by oral dosing with ova.

Antigen from *T. saginatus* ova was then compared to antigen from *T. saginatus* strobila in experiment IP 2. Concentrated antiserum from the calf that had been hyperimmunized by oral dosing was also included. Results are given in Table 15.

Table 15

A comparison by immunoprecipitation between strobilate and oral antigens of T. saginatus

Experiment IP 2

| Source of Antigen | Antiserum | Method of hyperimmunization | Maximum No. of lines |
|---|---|---|---|
| Strobila | Rabbit | Intramuscular | 2 strong |
| | Calf (X 5) | Oral | 1 strong |
| Ova | Rabbit | Intramuscular | Nil |
| | Calf (X 5) | Oral | Nil |

It was evident from the results shown in Tables 13, 14 and 15 that this test could not be reliably used to detect antibodies in animals immunized by oral dosing of ova. Antigens from strobilate material of Cittotaenia, T. hydatigena and M. multiceps were tested against homologous antigens in experiments IP 3 and IP 4. Results are seen in Tables 16 and 17.

Table 16

Immunoprecipitation between antigens from different tapeworm species and homologous antisera

| Experiment IP 3 | |
|---|---|
| Antigen | Maximum No. of lines |
| Cittotaenia | 7 |
| T. hydatigena | 6 |
| M. multiceps | 6 |
| T. pisiformis | 7 |

Table 17

Immunoprecipitation between antigens from different tapeworm species and homologous rabbit antisera

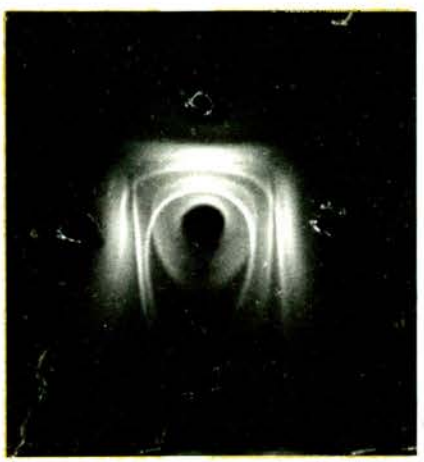
| Experiment IP 4 | |
|---|---|
| Antigen | Maximum No. of lines |
| Cittotaenia | 6 |
| T. hydatigena | 7 |
| M. multiceps | 8 |
| T. pisiformis | 5 |
| T. saginatus | 4 |

Fig. 3 shows the lines produced between M. multiceps antigen and its homologous antiserum.

Experiments IP 3 and IP 4 showed that there were strong reactions in all the systems. Cross reaction

Figure 3

Immunoprecipitation lines between *M. multiceps* antigen and homologous antiserum



| | |
|---|---|
| Centre well: | antiserum |
| Top well: | antigen, neat |
| Right well: | antigen, 1:2 |
| Left well: | antigen, 1:4 |
| Bottom well: | saline control |

studies were then made in experiments IP 6, IP 7 and IP 8. Each antigen was used against its homologous rabbit antiserum and against each of the heterologous rabbit antisera. The results are shown in Table 18.

Table 18

Immunoprecipitation between antigens from different tapeworm species and homologous and heterologous antisera

| Experiments IP 6, IP 7 and IP 8 | | |
|---|---|---|
| Antigen | Tapeworm used to raise antiserum | Maximum No. of lines |
| Cittotaenia | Cittotaenia | *4 |
| | T. hydatigena | 1 (weak) |
| | M. multiceps | 1 |
| | T. pisiformis | 1 |
| | T. saginatus | 1 |
| T. hydatigena | Cittotaenia | 4 (weak) |
| | T. hydatigena | *6 (strong) |
| | M. multiceps | 5 (strong) |
| | T. pisiformis | 3 |
| | T. saginatus | 7 |
| M. multiceps | Cittotaenia | 2 (weak) |
| | T. hydatigena | 5 (strong) |
| | M. multiceps | *6 (strong) |
| | T. pisiformis | 4 |
| | T. saginatus | 5 |
| T. pisiformis | Cittotaenia | 3 (weak) |
| | T. hydatigena | 5 (strong) |
| | M. multiceps | 4 (strong) |
| | T. pisiformis | *5 |
| | T. saginatus | 5 |
| T. saginatus | Cittotaenia | 1 (weak) |
| | T. hydatigena | 4 (strong) |
| | M. multiceps | 3 (strong) |
| | T. pisiformis | 2 |
| | T. saginatus | *4 (strong |

* homologous systems

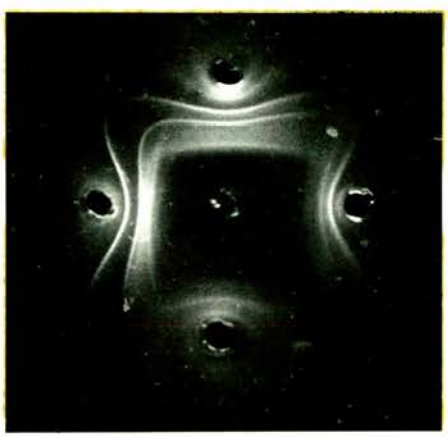
Fig. 4 shows cross reactions between antigen and heterologous antisera in experiment IP 7.

There were considerable cross-reactions between taeniid species, but less between Cittotaenia and the taeniid worms.

Each antiserum was then absorbed with the heterologous antigen with which it had given the strongest reaction in experiments IP 6 and IP 7. Thus:
Cittotaenia antiserum was absorbed with T. pisiformis antigen
T. hydatigena antiserum was absorbed with M. multiceps antigen
M. multiceps antiserum was absorbed with T. hydatigena antigen
T. pisiformis antiserum was absorbed with T. hydatigena antigen
T. saginatus antiserum was absorbed with T. hydatigena antigen

Figure 4

Immunoprecipitation between *M. multiceps* antigen and heterologous antisera



| | |
|---|---|
| Centre well: | *M. multiceps* antigen |
| Top well: | *T. hydatigena* antiserum |
| Right well: | *T. pisiformis* antiserum |
| Bottom well: | *Cittotaenia* antiserum |
| Left well: | *T. saginatus* antiserum |

Table 19

Immunoprecipitation lines between antigens of different tapeworm species and absorbed antisera

| Experiments IP 8 and IP 9 | | |
|---|---|---|
| Antigen | Tapeworm used to raise antiserum | Maximum No. of lines |
| Cittotaenia | Cittotaenia | *4 |
| | T. hydatigena | 0 |
| | M. multiceps | 0 |
| | T. pisiformis | 0 |
| | T. saginatus | 0 |
| T. hydatigena | Cittotaenia | 0 |
| | T. hydatigena | *4 |
| | M. multiceps | 0 |
| | T. pisiformis | 0 |
| | T. saginatus | 0 |
| M. multiceps | Cittotaenia | 0 |
| | T. hydatigena | 0 |
| | M. multiceps | *2 |
| | T. pisiformis | 0 |
| | T. saginatus | 0 |
| T. pisiformis | Cittotaenia | 0 |
| | T. hydatigena | 2 |
| | M. multiceps | 0 |
| | T. pisiformis | *0 |
| | T. saginatus | 0 |
| T. saginatus | Cittotaenia | 0 |
| | T. hydatigena | 1 |
| | M. multiceps | 0 |
| | T. pisiformis | 2 (weak) |
| | T. saginatus | *1 |

* homologous system

Strong precipitation lines were seen between all antigens and unabsorbed homologous antisera. Precipitation lines between antigens and unabsorbed heterologous antisera were comparable to the results of Experiments IP 6 and IP 7

and for convenience of presentation these results were included in Table 18.

Absorption appeared to remove non-specific reaction in most, but not all, systems.

Fig. 5 shows precipitation between an absorbed antiserum and its homologous antigen.

The fluorescent antibody test

a) Ova

Autofluorescence of the embryophore was very strong whether light ground or dark ground illumination was used. Lugol's iodine or Nile blue at 1:10 dilution was not successful in quenching this. Stronger solutions of Lugol's iodine had some effect in reducing autofluorescence, but it was felt that this could eliminate specific fluorescence as well and its use was discontinued.

After ova had been bathed in antiserum and conjugate, some faint fluorescent globules were seen on the embryophore. This was not seen in ova bathed in normal serum and conjugate but the globules were not seen in all tests or on all ova.

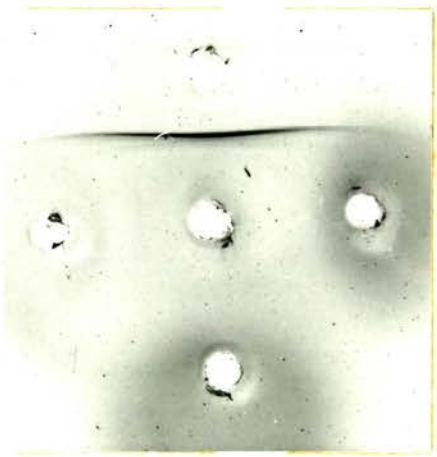
Treatment of ova with 80 per cent Milton for up to five hours resulted in the rupture of the embryophore of some but did not improve the results.

In the tube test eggs were retained as a sediment in a little fluid and it was therefore difficult to gauge dilutions properly. This was not the case with the slide test.

Figure 5

Immunoprecipitation between antigens and *Cittotaenia* antiserum that had been absorbed with a heterologous antigen

(Photograph of a stained slide)



| | |
|---|---|
| Centre well: | *Cittotaenia* antiserum absorbed with *T. pisiformis* antigen |
| Top well: | *Cittotaenia* antigen |
| Right well: | *M. multiceps* antigen |
| Bottom well: | *T. hydatigena* antigen |
| Left well | *T. pisiformis* antigen |

Ova that were submitted to various periods in digestion fluids hatched readily but few ova became activated; most were still retained in the onchospheral membrane.

Many ova were lost during washing in the digestion process or in the test. Although embryophoral blocks were seen, onchospheres were difficult to find under the fluorescent microscope, in spite of enclosing the sediment within a waxed circle.

Intense fluorescence around the onchospheral membrane was sometimes seen. This was found to be related to how thoroughly the onchospheres had been washed. Autofluorescence of the hooklets was always present. In some experiments an increase in intensity of fluorescence was detected in ova that had been exposed to antiserum compared to the controls but this was not consistent.

b) Sections

Results of these experiments were also inconclusive. In gravid sections embryophores fluoresced brightly and it was difficult to detect any difference in colour of the onchospheral membrane. Fluorescence was more intense when neat sera were used in the "sandwich" technique using FITC. TRITC did not produce any fluorescence.

Immunoelectrophoresis

The microimmunoelectrophoresis template method on microscope slides was used in most of the experiments as this was found to be the most sensitive. Lines were more clearly seen with agarose than with agar.

T. pisiformis and T. hydatigena and their homologous antisera were found to precipitate the strongest lines and were used in all the experiments. Concentrating the antisera improved the visibility of the lines. Up to six arcs were seen with antisera concentrated 12 times, but arcs were not always clear. No arc peculiar to any particular antigen/antibody system was detected.

Enzyme-linked immunosorbent assay (ELISA)

This test was used with antigens of different tapeworm species against homologous and heterologous antisera and also against homologous antisera that had been absorbed with one or more heterologous antigens.

The immunoprecipitation gel test results determined the choice of antigen for absorption. Antiserum to T. hydatigena was absorbed with M. multiceps antigen and all other antisera were absorbed with T. hydatigena.

M. multiceps antigen at one, five and ten µg/ml initially was used to determine the optimal concentration of antigen for the test. The results can be seen in Table 20.

Table 20

The determination of antigen concentration suitable for the ELISA test

| Experiment E 1 | | | | |
|---|---|---|---|---|
| M. multiceps Antigen (ug) | | Serum dilution | Conjugate (1 in 500) | Reading at 449 nm |
| Saline | Antiserum | 1:50 | + | 0 |
| Saline | | 1:100 | + | 0 |
| 10 | | 1:50 | + | 1.6 |
| 5 | | 1:50 | + | 1.6 |
| 1 | | 1:50 | + | 0.12 |
| 10 | | 1:100 | + | 0.92 |
| 5 | | 1:100 | + | 0.84 |
| 1 | | 1:100 | + | 0.1 |
| 10 | Normal rabbit serum | All 1:50 | - | 0 |
| 5 | | | - | 0 |
| 1 | | | - | 0 |
| Saline | | | - | 0 |
| 10 | | | + | 0 |
| 5 | | | + | 0 |
| 1 | | | + | 0 |
| Saline | | | + | 0 |

In all subsequent tests 5 /ug/ml was chosen as the antigen concentration.

Each antigen was tested against homologous, heterologous and absorbed homologous antisera. The results can

be seen in Tables 21 to 28.

Table 21 shows the reactions between antigens and homologous or heterologous antisera. There were cross reactions between all antisera with usually a slightly stronger reaction in homologous systems than in heterologous systems.

Table 21

Reactions between antigens and homologous and heterologous antisera

| Antigen | Antiserum (1:100) | Readings at 449 nm Experiment E 3 | |
|---|---|---|---|
| T. hydatigena | T. hydatigena | *0.6 | 0.56 |
| | M. multiceps | 0.6 | 0.52 |
| | T. pisiformis | 0.27 | 0.25 |
| | T. saginatus | 0.56 | 0.54 |
| | Cittotaenia | 0.25 | 0.28 |
| M. multiceps | T. hydatigena | 0.48 | 0.47 |
| | M. multiceps | *0.62 | 0.53 |
| | T. pisiformis | 0.18 | 0.26 |
| | T. saginatus | 0.55 | 0.56 |
| | Cittotaenia | 0.27 | 0.28 |
| T. pisiformis | T. hydatigena | 0.56 | - |
| | M. multiceps | 0.35 | - |
| | T. pisiformis | *0.25 | - |
| | T. saginatus | 0.5 | - |
| | Cittotaenia | 0.22 | - |
| T. saginatus | T. hydatigena | 0.52 | 0.56 |
| | M. multiceps | 0.51 | 0.52 |
| | T. pisiformis | 0.1 | 0.11 |
| | T. saginatus | *0.56 | 0.59 |
| | Cittotaenia | 0.18 | 0.14 |
| Cittotaenia | T. hydatigena | 0.2 | - |
| | M. multiceps | 0.24 | - |
| | T. pisiformis | 0.07 | - |
| | T. saginatus | 0.29 | - |
| | Cittotaenia | *0.43 | - |

* homologous systems

Table 22 shows the results obtained when homologous, heterologous and absorbed heterologous antisera were tested against M. multiceps antigen.

Table 22

Reactions between M. multiceps antigen and homologous, absorbed homologous and heterologous antisera

| Antigen | Antiserum | Concentration | Absorption antiserum: antigen | Readings at 449 nm | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Experiments: | | | | |
| | | | | E 1 | E 2 | | | E 3 |
| M. multiceps | M. multiceps | 1:50 | - | 1.6 | - | - | - | - |
| | | 1:100 | - | 0.84 | 0.83 | 0.85 | 0.62 | 0.53 |
| | | 1:50 | 1.1 | 0.27 | - | - | - | - |
| | | 1:100 | 1:1 | 0.22 | 0.22 | 0.24 | 0.18 | 0.16 |
| | | 1:100 | 1:2 | - | 0.19 | 0.24 | 0.15 | 0.18 |
| | | 1:100 | 2:1 | - | 0.2 | 0.2 | 0.11 | 0.1 |
| | T. hydatigena | 1:100 | - | - | 0.76 | 0.8 | 0.48 | 0.47 |
| | T. pisiformis | 1:100 | - | - | 0.46 | 0.46 | 0.18 | 0.26 |
| | T. saginatus | 1:100 | - | - | 0.8 | 0.86 | 0.55 | 0.56 |
| | Cittotaenia | 1:100 | - | - | 0.48 | 0.45 | 0.27 | 0.28 |
| | Normal rabbit serum | 1:100 | .. | 0 | 0 | 0 | 0 | 0 |
| Saline | All sera | 1:100 | - | 0 | 0 | 0 | 0 | 0 |

These results show that M. multiceps antiserum absorbed in the ratio of 1:1, 1:2 or 2:1 with T. hydatigena antigen still retained activity against M. multiceps antigen. As there did not appear to be any difference in absorption between the ratios used, the intermediate ratio of 1:1 was chosen for absorption in other experiments.

Different antigens were then tested against absorbed homologous and heterologous antisera. Antisera were absorbed in a 1:1 proportion with heterologous antigen. The results are shown in Table 23.

Table 23

Reactions between different antigens and absorbed antisera

| Antigen | Absorbed antiserum (1:100) | Readings at 449 nm Experiment E 4 | |
|---|---|---|---|
| T. hydatigena | T. hydatigena | *0.61 | 0.65 |
| | M. multiceps | 0.035 | 0.04 |
| | T. pisiformis | 0 | 0 |
| | T. saginatus | 0.07 | 0.07 |
| M. multiceps | T. hydatigena | 0.21 | 0.22 |
| | M. multiceps | *0.3 | - |
| | T. pisiformis | 0 | 0 |
| | T. saginatus | 0.24 | 0.24 |
| T. pisiformis | T. hydatigena | 0.47 | 0.51 |
| | M. multiceps | 0.07 | 0.08 |
| | T. pisiformis | *0.14 | 0.11 |
| | T. saginatus | 0.06 | 0.08 |
| T. saginatus | T. hydatigena | 0.16 | 0.175 |
| | M. multiceps | 0.09 | 0.1 |
| | T. pisiformis | 0 | 0 |
| | T. saginatus | *0.39 | 0.39 |

*homologous systems

It was evident from these results that absorption was not complete. T. hydatigena antiserum was then absorbed in different proportions with M. multiceps antigen (1:1 or 1:3 respectively) or successively with two antigens, M. multiceps and T. pisiformis, both in a 1:1 proportion with the antiserum. This absorbed antiserum was tested against different antigens. The results are seen in Table 24.

Table 24

Reactions between different antigens and T. hydatigena antiserum absorbed in different ways

| Antigen | Readings at 449 nm: Experiment E 5 | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | T. hydatigena antiserum (1:100) | | | | | | | |
| | Not absorbed | | Absorbed with M. multiceps 1:1 | | Absorbed with M. multiceps 1:3 | | Absorbed with M. multiceps 1:1 and T. pisiformis 1:1 | |
| T. hydatigena | 0.41 | 0.44 | 0.265 | 0.265 | 0.25 | 0.23 | 0.16 | 0.16 |
| M. multiceps | 0.38 | 0.37 | 0.06 | 0.06 | 0.01 | 0 | 0 | 0 |
| T. pisiformis | 0.38 | 0.39 | 0.22 | 0.23 | 0.15 | 0.15 | 0.03 | 0 |
| T. saginatus | 0.15 | 0.14 | 0.14 | 0.14 | 0.02 | 0.02 | 0 | 0.01 |

Absorption appeared to be adequate when two antigens were used to absorb an antiserum. Other antisera were therefore absorbed in the same way. The results are seen in Table 25.

Table 25

Reactions between different antigens and antisera absorbed by two heterologous antigens

| Antigen | Absorbed antiserum (1:100) | Readings at 449 nm | | | |
|---|---|---|---|---|---|
| | | Experiment E 6 | | Experiment E 7 | |
| T. hydatigena | T. hydatigena | 0.13 | 0.14 | 0.06 | 0.09* |
| | M. multiceps | 0.03 | 0.01 | 0 | 0 |
| | T. pisiformis | 0 | 0 | 0 | 0 |
| | T. saginatus | 0 | 0 | 0 | 0 |
| M. multiceps | T. hydatigena | 0 | - | 0 | 0 |
| | M. multiceps | 0.07 | - | 0.04 | 0.04* |
| | T. pisiformis | 0 | - | 0 | 0 |
| | T. saginatus | 0.04 | - | 0.02 | 0.02 |
| T. pisiformis | T. hydatigena | 0.01 | - | 0 | 0 |
| | M. multiceps | 0.01 | - | 0 | 0 |
| | T. pisiformis | 0.02 | - | 0.03 | 0.03* |
| | T. saginatus | 0.01 | - | 0 | - |
| T. saginatus | T. hydatigena | 0.02 | - | 0 | 0.01 |
| | M. multiceps | 0.02 | - | 0.01 | 0 |
| | T. pisiformis | 0 | - | 0 | 0 |
| | T. saginatus | 0.04 | - | 0.1 | 0.11* |

* homologous systems

Again the absorption appeared adequate but reactions to absorbed homologous antisera were poor.

The results of experiments using egg antigens are seen in Table 26.

Table 26

Reactions between antigens prepared from ova and absorbed antisera

| Antiserum (1:100) | Unabsorbed antiserum | | | | | | | | Absorbed antiserum | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *T. ovis* ova antigen | | | | *T. hydatigena* ova antigen | | | | *T. ovis* ova antigen | | | | *T. hydatigena* ova antigen | | | |
| Experiment: | E 8 | | E 9 | | E 8 | | E 9 | | E 8 | | E 9 | | E 8 | | E 9 | |
| *T. hydatigena* | 0.03 | - | 0 | 0 | 0.03 | 0.03 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 |
| *M. multiceps* | 0.02 | 0.01 | 0 | 0 | 0.03 | 0.02 | 0 | 0 | 0.01 | 0.01 | 0 | 0 | 0 | 0.01 | 0 | 0 |
| *T. pisiformis* | 0.02 | 0.02 | 0 | 0 | 0.02 | 0.02 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.02 | 0 | 0 |
| *T. saginatus* | 0.03 | - | 0 | 0 | 0.02 | 0.02 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Very low reactions were obtained in Experiment E 8 and none in E 9 between the egg antigens and unabsorbed sera, whilst virtually no reactions occurred against absorbed antisera in either experiment.

# CHAPTER EIGHTEEN

## ENZYME ELECTROPHORESIS: PHYSICAL EFFECTS

### Support media

a) Cellulose acetate

This support required no preparation, was easy to work with and a larger number of samples could be compared in one run, but the method was abandoned as enzyme banding and definition were very poor.

b) Agaroslides

These were preferable to Phoroslides as staining was seen more clearly. Enzyme banding, however, was very poor. This method was also abandoned mainly due to the high cost of the slides.

c) Phoroslides

Enzyme location was possible but banding was very poor. The advantage of these slides was that an attempt could be made to quantify results by the use of a densitometer.

d) Polyacrylamide gels

Seven per cent gels were the most satisfactory, being economical and yet reasonably strong. These gels had the advantage of translucency, thus enabling location of enzymes more easily than with starch gels. They were also easier to handle than starch gels. Banding, however, was poor and they were abandoned in favour of starch gel.

e) Starch gels

Much of the initial work was done using thick gels. The starch concentration varied from 8.8 per cent to 20 per cent. Definition improved with higher starch content but the gels became too friable at a high concentration and ten per cent was found to be both adequate and easy to work with. Different commercial brands of starch were tried. Although the handling qualities differed, the results were not altered by the brand. The addition of ten per cent sucrose to the gel did not appear helpful. Gel thickness was varied from two mm to six mm, the latter being adopted as standard. Some gels of 18 x 17.5 cm in size were tried but most gels were 17 x 11 cm as this size was more convenient for handling after electrophoresis.

A higher starch content was chosen for thin layer gels as they did not have to be handled: 11 per cent was used routinely.

Thin layer gels gave superior results to thick layer gels and all later work was done with the former. An example of each is seen in Fig. 6.

Sample application

With samples soaked on filter paper, difficulties were encountered due to softening and tearing of the paper during insertion into the pre-cut slots. Cellulose acetate film was found to be more suitable. The inserts were left in the gel throughout electrophoresis as their removal 20 minutes to one hour after the start of electrophoresis did not improve results.

Figure 6

Zymogram of glucose phosphate isomerase on a thick and a thin starch gel

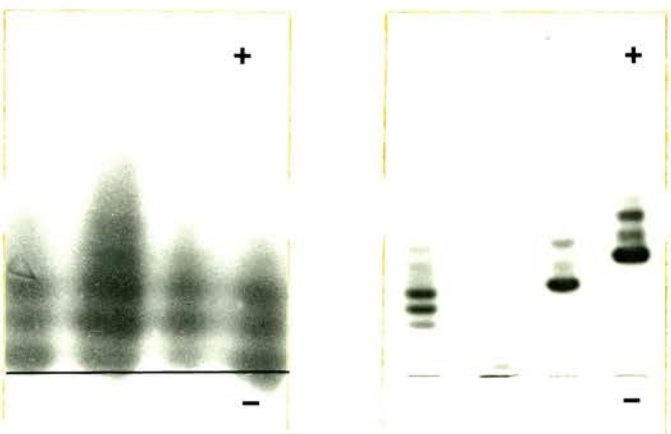



Plate 1
Thick gel (photograph by reflected light)

Plate 2
Thin gel (photograph by transmitted light)

Cooling

Electrophoresis of thick gels was done in a cold room at 4°C. It was probable that the temperature rose under the safety hood of the apparatus during electrophoresis and as a result the gel over-heated in parts with burning at the contact point between wick and gel on some occasions. The acquisition of a cooling platen coincided with the change from using thick gels to using thin layer gels. Cooling by means of a single platen lying underneath the gel was adequate, when the water circulating through it was kept at about 8°C.

Preparation of extracts

Extracts were sometimes prepared merely by homogenization of tissue in some water, normal saline or EP solution with a tissue grinder or pestle and mortar. These gave a clear zymogram pattern provided they were not too concentrated. Ultrasonication tended to heat samples and was later omitted from the procedure. Extracts destined for preservation and storage were usually ultracentrifuged.

Because of the absence of facilities in Nigeria, extracts were not centrifuged there. Instead, they were allowed to stand overnight at 4°C and the supernatant was sucked off for use. A comparison was made between filtered and unfiltered supernatants. No difference in definition of enzyme patterns could be detected between the two and filtering was discontinued.

Figure 7

Electrophoresis of *T. saginatus* and *T. solium* extracts in different buffers.
Zymogram of glucose phosphate isomerase

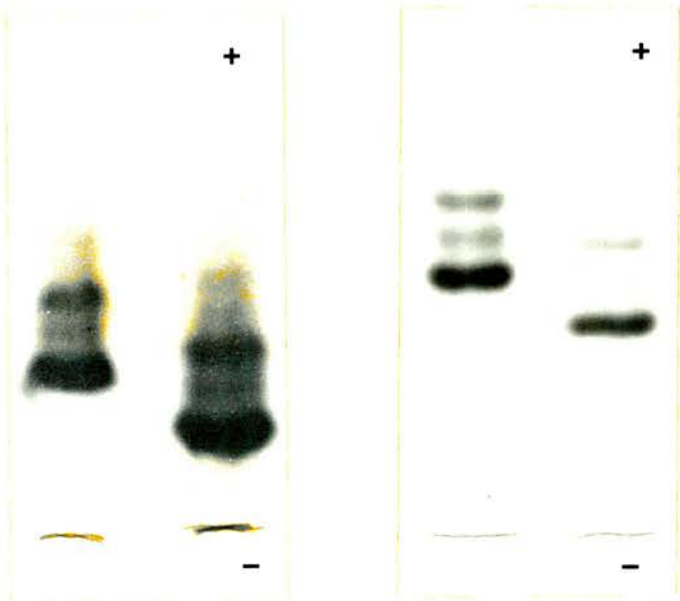



Plate 1
0.5M Tris/HCl pH 8.0
(gel buffer diluted 1:5)

Left: *T. saginatus*
Right: *T. solium*

Plate 2
0.2M phosphate pH 7.4
(gel buffer 0.015 M phosphate pH 7.4)

Left: *T. saginatus*
Right: *T. solium*

Both thin gels were run for three hours at 330 volts

# CHAPTER NINETEEN

# ENZYMES FOUND IN TAPEWORMS

## Tube tests for enzymes

At first extracts were tested in tubes for the presence of a particular enzyme before electrophoresis. Results of such a test for the presence of malate dehydrogenase are seen in Table 27.

Table 27

The presence of malate dehydrogenase in some tapeworm extracts

| Tapeworm species | Dilution of extract with water | Reading at 540 nm |
|---|---|---|
| Moniezia sp | 1:1 | 0.17 |
| | neat | 0.33 |
| | concentrated x 3 | 0.36 |
| T. saginatus | 1:1 | 0.09 |
| | neat | 0.13 |
| | concentrated x 6 | 0.16 |
| T. hydatigena strobila | 1.32 | 0.001 |
| | 1:16 | 0.02 |
| | 1.8 | 0.04 |
| | 1.4 | 0.09 |
| | 1.2 | 0.16 |
| | neat | 0.41 |
| Saline control | 0 | 0 |

This test was repeated for glucose-6-phosphate dehydrogenase, 6 phosphogluconate dehydrogenase, lactate dehydrogenase, glutamate dehydrogenase and $\alpha$-naphthyl acetate esterase. Colour changes occurred in very few

samples but indicated some glucose-6-phosphate dehydrogenase activity in Moniezia, glutamate dehydrogenase activity in T. hydatigena and T. saginatus, some lactate dehydrogenase activity in Moniezia, T. hydatigena and E. granulosus and glucose phosphate isomerase activity in Moniezia, T. hydatigena cyst, E. granulosus and T. saginatus.

Subsequently these same extracts were tested again after starch gel electrophoresis. Enzyme activity shown by the enzyme in the test tube was not always apparent in the gel and therefore tube tests were not continued. All extracts were thereafter tested directly by electrophoresis.

Enzymes found in tapeworm extracts

Table 28 shows tapeworm enzymes that were seen in zymograms.

Of the enzymes tested, glucose phosphate isomerase was found to be the most useful as it was present in all specimens and showed clear differences between species. Other enzymes which were useful were malate dehydrogenase, glutamate dehydrogenase, adenylate kinase and hexokinase. All these enzymes were present to some degree in all tapeworm species and some differences in mobility were seen. Other enzymes were difficult to detect, were not present in all species or showed no generic or specific differences and were not studied further.

Table 28

Enzymes in Tapeworms

| Enzyme | *E. granulosus* | *T. hydatigena* | | *M. multiceps* | | *T. ovis* | *T. pisiformis* | *T. saginatus* | | *T. solium* | | *H. taeniaformis* | | Species differences shown |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | cyst | adult | cyst | adult | cyst | adult | adult | adult | cyst | adult | cyst | adult | cyst | |
| ALAT | | NIL | NIL | | | | | NIL | | | | | | |
| ASAT | | + | NIL | | | | | NIL | | | | | | NO |
| Acid phos. | | + | NIL | | | | | NIL | | | | | | NO |
| Alk. phos. | | + | + | | | | | NIL | | | | | | NO |
| Aldolase | | + | + | | | | + | | | | | | | |
| Aconitase | | NIL | NIL | | | | | NIL | | | | | | |
| Adenylate kinase | ++ | ++ | | ++ | | ++ | ++ | ++ | | ++ | | ++ | | YES |
| ·NA esterase | NIL | NIL | NIL | | | | | NIL | | NIL | | | | |
| Esterase (non specific) | | NIL | NIL | | | | | NIL | | | | | | |
| Cholinesterase | | NIL | NIL | | | | | NIL | | | | | | |
| Fumarase | | + | | + | | | + | | | | | | | NO |
| GDH(NAD) | + | ++ | | ++ | | ++ | ++ | ++ | | + | | + | | YES |
| GHD(NADP) | | + | | + | | | + | + | | | | + | | YES |
| G-6-PD | | + | ++ | | | | | NIL | | | | | | NO |
| GPI | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | YES |
| Hexokinase | + | ++ | | ++ | | ++ | ++ | ++ | | ++ | | ++ | | YES |
| ICD | | NIL | NIL | | | | | NIL | | | | | | NO |
| LDH | + | + | ++ | | | | | + | | NIL | | | | NO |
| MDH(NAD) | ++ | ++ | ++ | ++ | | ++ | ++ | ++ | | ++ | | ++ | | YES |
| MDH(NADP) | | + | + | | | | | + | | + | | | | YES |
| Peroxidase | | NIL | NIL | | | | | NIL | | | | | | NO |
| PGM | | + | + | + | | | + | + | | | | | | NO |
| 6PGD | + | + | | | | | + | | | | | | | NO |
| SDH | | + | ++ | | | | | + | | | | | | NO |

+++ Strong bands
++ Strong bands seen on some occasions
+ faint bands

A key to abbreviations is given in Appendix 1

# CHAPTER TWENTY

## ENZYME ELECTROPHORESIS: VARIATIONS IN TECHNIQUE

### Voltage and current

In a series of studies using different voltages it was found that a constant setting of 110 V (reading across gel about 6.5 V/cm) gave the best results when thick gels were run overnight. This usually gave a reading of 14 mA when 0.1 M Tris/citrate buffer pH 7.5 was used and it was later found that setting the current at a constant 14 mA gave a more consistent result than a constant voltage setting.

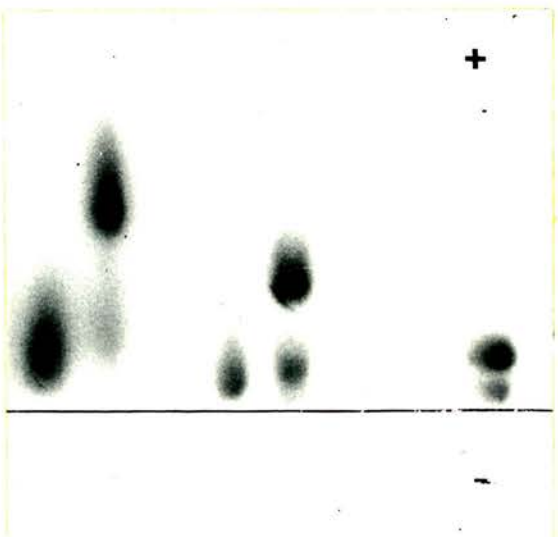
In the thin layer technique, the best results were obtained with a constant 330 V measured across the gel using 0.2 M phosphate buffer pH 7.4. This usually gave an ammeter reading of 22 mA if the gel was sufficiently thin. A reading greater than this was a sign that the gel was too thick.

### Time

The effect of varying the time of the electrophoretic run, under constant conditions, can be seen in Fig. 8. (MDH) Lengthening the period of electrophoresis increased the movement of enzymes. It did not necessarily improve definition. With thick gels comparable results could be obtained by a shorter period of electrophoresis if the voltage was increased, provided the product of time and voltage remained about the same. Even with a thick gel, electrophoresis could be completed in two hours, but the quality

Figure 8

The effect of time on electrophoresis




From right to left:

| | |
|---|---|
| Sheep's heart extract<br>*T. hydatigena* extract | After two hours |
| Sheep's heart extract<br>*T. hydatigena* extract | After four hours |
| Sheep's heart extract<br>*T. hydatigena* extract | After six hours |

Electrode buffer: 0.341 borate/NaOH pH 9.0
Gel buffer: 0.034 M borate/NaOH pH 9.0
150 volts (power pack reading). Thick gel

of the gel was not always as good.

pH

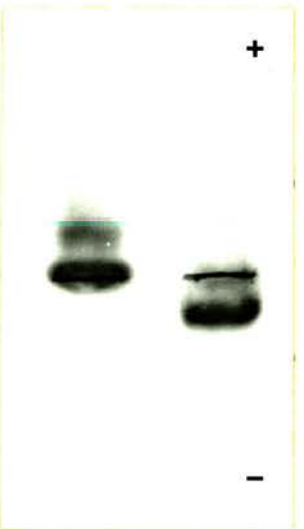
The effect of pH was observed when comparing gels using a buffer of constant ionic strength of 0.5 (Dawson et al, 1969). Electrophoresis was run at pH 4, 5.4, 6.2, 7.2 and 8.2 and a borate/NaOH buffer was used for pH 9 and pH 10. Gels were developed for malate dehydrogenase and lactate dehydrogenase and showed malate dehydrogenase to have an isoelectric point between pH 6.2 and 7.2. No development occurred at pH 4. There was very little lactate dehydrogenase activity. Electrophoresis of all the gels was at a constant 80 V over 16½ hours.

Movement towards the anode increased with higher pH. The effect of pH was used later to help distinguish between T. saginatus and T. solium. Extracts of these two worms showed differences in glucose phosphate isomerase in thin layer gels. In order to enhance this difference the pH was lowered to around the isoelectric point so that the enzyme of the one tapeworm would move anodally, and that of the other, cathodally.

In a 0.2 M phosphate buffer pH 7.4, isoenzymes of both tapeworms moved anodally but those of T. saginatus moved more rapidly. The pH was then lowered to 5.9 until the main bands of T. solium moved cathodally and those of T. saginatus anodally. Fig. 9 shows the results. It was found, however, that very small variations in pH could alter the direction of movement.

Figure 9

Glucose phosphate isomerase isoenzymes of *T. saginatus* and *T. solium* at pH 5.9




The black cotton thread marks the point of application. The main band of *T. saginatus* (left) has moved anodally and that of *T. solium* (right), cathodally.

Electrode buffer: 0.2M phosphate pH 5.9
Gel buffer: 0.015M phosphate pH 5.9
330 volts across a thin gel for 3 hours.

Buffer concentration

Thick gels made with 0.034 M borate/NaOH pH 9 buffer were held at a constant 80 V over 16½ hours, using three different borate/NaOH electrode buffers pH 9: 0.1755 M, 0.341 M and 0.682 M borate. Increase in buffer concentration caused an increase in mobility of malate dehydrogenase but not necessarily better separation of bands.

However, this effect was not consistent with all enzymes as was shown when several enzymes were run in thin gels using three different buffer concentrations. With hexokinase, glucose phosphate isomerase and glutamate dehydrogenase mobility was increased in the higher concentration but the reverse was the case with adenylate kinase. This is shown in Fig. 10.

Storage and preservation of extracts

The activity of glucose phosphate isomerase in T. pisiformis material that was preserved in several ways is shown in Table 29.

Figure 10

The effect of buffer concentration on the movement of adenylate kinase

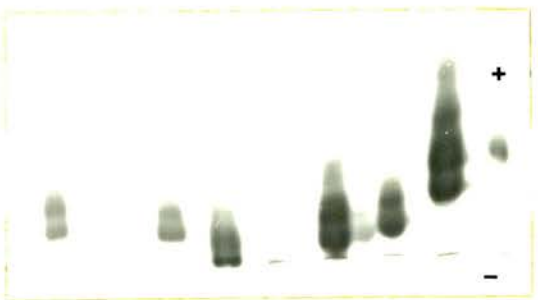



Plate 1. Electrode buffer: 0.1 M Tris/HCl pH 8.0
From left to right: *T. hydatigena*, blank, *T. ovis, E. granulosus*, blank, *T. saginatus, T. solium, M. multiceps, Dipylidium caninum*

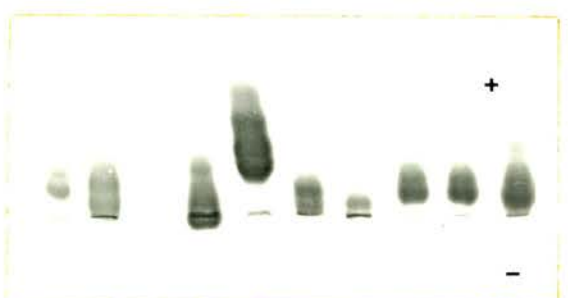



Plate 2. Electrode buffer: 0.5 M Tris/HCl pH 8.0
From left to right: *T. ovis, H. taeniaeformis*, blank, *T. pisiformis, M. multiceps, T. hydatigena, E. granulosus, T. ovis, T. solium, T. saginatus*

Buffer for both gels was 1:5 dilution of electrode buffer
330 volts across a thin gel for three hours

Table 29

Glucose phosphate isomerase activity in samples of T. pisiformis one month after preparation in different ways

| Extract diluent | Preservation | Temperature during storage | Enzyme activity after 1 month, U/mg protein |
|---|---|---|---|
| Water | Freeze dried powder* | +5°C | 54.5 |
| EP solution | solution | -196°C | 47.3 |
| EP solution | solution | +5°C | 39.8 |
| 2.4 M $(NH_4)_2$ $SO_4$ | solution | +5°C | 8.8 |

*Reconstituted in distilled water

Enzyme activity did not necessarily indicate preservation of electrophoretic characteristics as enzyme activity was sometimes strong when zymogram bands were altered or blurred. Table 30 shows the effect of preservation and storage on the bands.

Table 30

The effect of preservation and storage on electrophoretic quality of glucose phosphate isomerase enzyme

| Extract diluent | Storage temperature | Length of storage (days) | Storage as Extract (E) or whole worm (W) | Enzyme activity | Banding |
|---|---|---|---|---|---|
| Saline | Fresh | - | - | +++ | +++ |
| | +20°C | 8 | W | + | + |
| | +4°C | 13 | W | ++ | ++ |
| | -20°C | 540 | W | +++ | +++ |
| EP solution | +20°C | 8 | E | Nil | Nil |
| | +5°C | 60 | E | +++ | +++ |
| | +5°C | 90 | E | ++ | ++ |
| | +5°C | 150 | E | + | + |
| | -20°C | 60 | E | +++ | +++ |
| | -20°C | 90 | E | ++ | ++ |
| | -20°C | 210 | E | ++ | ++ |
| | -196°C | >365 | E | +++ | +++ |
| EP solution + 0.2% betapropriolactone | +37°C | 30 min | E | Nil | Nil |
| 2.4 M $(NH_4)_2 SO_4$ | +20°C | 8 | E | ++ | ++(warped) |
| | +5°C | 180 | E | + | +(warped) |
| Freeze dried: | | | | | |
| Water | +5°C | 600 | E | + | - |
| EP solution | +5°C | >365 | E | +++ | +++ |

2.6 M ammonium sulphate solution containing 0.0001 M magnesium acetate was also used but appeared to have no advantages over 2.4 M ammonium sulphate.

When ammonium sulphate was used as a preservative, enzyme activity was retained but there were fewer bands and they became warped or blurred. This can be seen in Fig. 11.

Dilution of extracts

Diluting the samples resulted in fainter bands, those furthest from the point of application being most affected. As dilution increased so the lighter bands furthest from the cotton thread disappeared. This effect is shown in Fig. 12. To allow comparison of samples of equivalent enzymic strength, they were diluted as indicated by the assay results.

Extract fractionation

There were two major peaks when an extract of T. hydatigena was passed through a Sephadex G200 column and the effluent monitored at 254 nm. Six fractions were taken of the effluent including each of the two major peaks, one at the beginning (F1) and one at the end (F6). A drop of each fraction was added to about one ml of glucose phosphate isomerase developing solution. An immediate colour reaction was seen with fractions F2 and F3 and a weak reaction with F1 after one hour. The fractions were freeze dried.

Figure 11

The effect of preservation and storage on band patterns of glucose phosphate isomerase

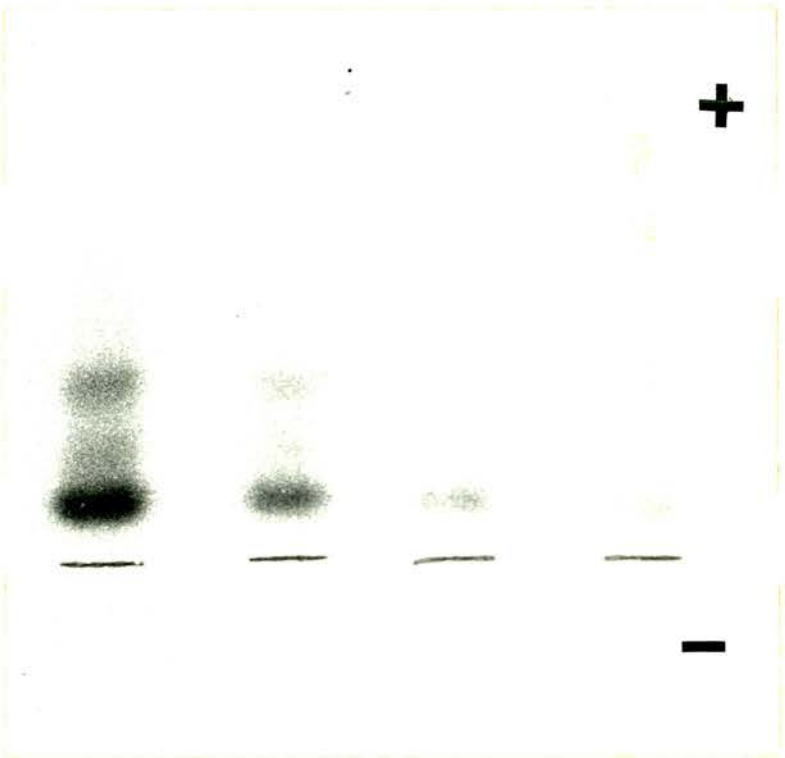



The left half of the plate shows *H. taeniaeformis* and the right half *T. ovis*.
From left to right:
Extracts in EP solution, stored in liquid nitrogen
Extracts in ammonium sulphate solution, stored at 4°C
Fresh extracts
Extracts in EP solution, stored at 4°C
Extracts in EP solution, stored at -20°C
All extracts were at least three months old
Clear bands can be seen in fresh specimens and in those preserved in nitrogen. There is loss of banding and enzymic activity in extracts preserved at 4°C and in *T. ovis* preserved at -20°C. This extract was five months old; the equivalent *H. taeniaeformis* extract was three months old

Electrode buffer: 0.2M phosphate pH 7.4
Gel buffer: 0.015M phosphate pH 7.4
330 volts across a thin gel for three hours

Figure 12

The effect of sample dilution on enzyme bands




Doubling dilutions of a *T. pisiformis* extract

Electrode buffer: 0.2M phosphate pH 7.4
Gel buffer: 0.015M phosphate pH 7.4
330 volts across a thin gel for three hours

Electrophoresis was carried out with reconstituted fractionated material on several occasions. Strong glucose phosphate isomerase activity was seen in fractions F2 and F3 but there was some activity in all fractions when concentrated about five times. All fractions showed identical gel mobility and banding and this was indistinguishable from that seen with unfractionated T. hydatigena extract. Malate dehydrogenase activity similarly showed no differences.

The approximate molecular weight range of fractions F2 and F3 was between 75,000 and 180,000.

# CHAPTER TWENTY ONE

## SPECIFIC ENZYMES IN TAPEWORM DIFFERENTIATION

### Adenylate kinase (AK)

Various buffers were used but a similar pattern was seen with each. Tris/HCl buffers were found to give most satisfactory results. Fig. 10 shows gels run in 0.1 M and 0.5 M Tris/HCl pH 8.0.

Table 31 gives a summary of the results on gels run in Tris/HCl buffer, pH 8.0. These show both generic and specific differences.

Table 31

Adenylate kinase. Relative position of enzyme forms from different tapeworm species

| | -1 | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|---|---|
| Avitellina centripunctata | | | | | | | | | | X |
| Helictometra giardi | | | | | X | X | | | | |
| Moniezia benedeni | | | | X | X | | | | | |
| Raillietina sp. | | X | | X | | | | | | |
| E. granulosus | | | X | X | X | | | | | |
| T. hydatigena | | | X | X | X | | | | | |
| M. multiceps | | | | | X | X | X | X | X | |
| T. ovis | | | | X | X | X | | | | |
| T. pisiformis | X | | X | X | X | | | | | |
| T. saginatus | | | | X | X | X | X | | | |
| T. solium | | | | X | X | X | X | | | |
| H. taeniaeformis | | | X | X | X | X | X | X | | |
| | -ve | +ve | | | | | | | | |

O = position of insertion

The difference between T. solium and T. saginatus was most obvious at a molarity of 0.1 M.

Glutamate dehydrogenase (GDH)

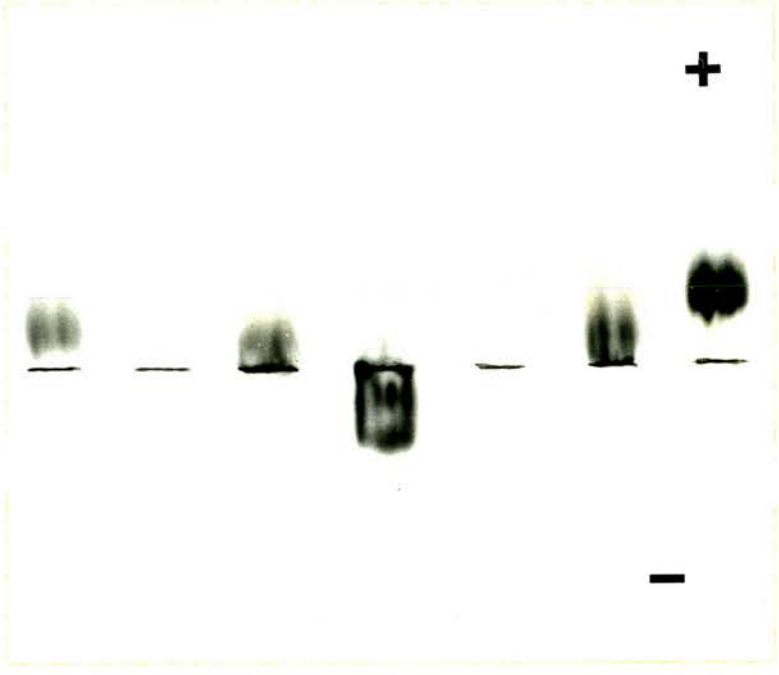
This enzyme was not seen easily or clearly on the zymograms but obvious differences between tapeworm species could be detected. Most success was achieved with 0.2 M

phosphate buffer pH 7.4, but 0.1 M Tris/HCl pH 8.0 was also suitable (see Fig. 13). NAD dependent enzyme was dominant, but there was slight NADP dependent enzyme activity.

Table 32 is a summary of the results using 0.2 M phosphate pH 7.4 electrode buffer. Other tapeworms examined included Dipylidium caninum from a cat and Hymenolepis diminuta from a rat, both of which showed characteristic patterns quite different from the taeniid worms. D. caninum iso-enzymes moved more rapidly cathodally in 0.1 M Tris/HCl pH 8.0, while those of H. diminuta moved more rapidly anodally than any of the taeniid worms. There was only slight activity in the E. granulosus and T. ovis samples and the location could not be accurately determined. In 0.1 M Tris/HCl pH 8.0 buffer, T. ovis appeared to have the same mobility as T. hydatigena.

Figure 13

Glutamate dehydrogenase. Zymogram of some taeniid tapeworm extracts




From left to right: *T. hydatigena*, blank, *E. granulosus* (horse), *M. multiceps*, blank, *T. solium*, *T. saginatus*

Electrode buffer: 0.5M Tris/HCl pH 8.0
Gel buffer: electrode buffer diluted 1:5
330 volts across a thin gel for three hours

Table 32

Glutamate dehydrogenase. Relative position of enzyme forms from different tapeworm species. Electrode buffer 0.2 M phosphate pH 7.4 330 V for 3 h

| | -4 | -3 | -2 | -1 | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A. centripunctata | X | X | X | X | | | | | | | | |
| H. qiardi | | | | | | | | | X | | | |
| T. hydatigena | | | | | | | X | | | | | |
| M. multiceps | | | | X | | X | | | | | | |
| T. pisiformis | | | | | | X | | | | | | |
| T. saginatus | | | | | | | | X | | | | |
| T. solium | | | | | | X | | | | | | |
| H. taeniaeformis | | | | | | | | | | | X | |
| | | | | -ve | | +ve | | | | | | |

0 = position of insertion

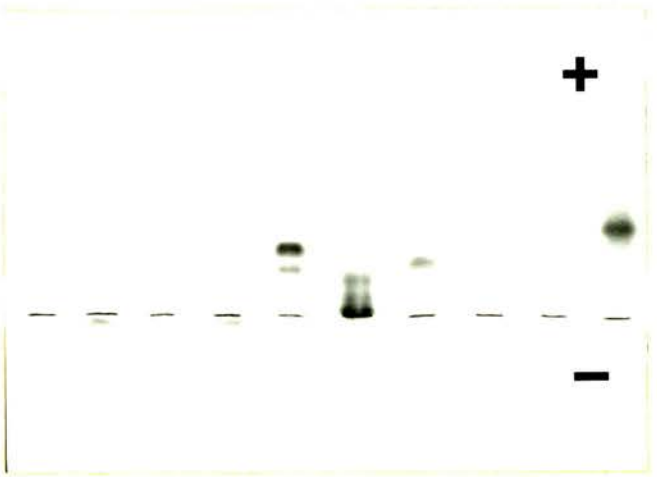
Hexokinase (HK)

Clear species differences were seen on zymograms, but the extracts had to be fresh. Tris/HCl buffer pH 8.0 was the most successful. Fig. 14 shows a zymogram.

Table 33 is a summary of the results of gels run with a Tris/HCl buffer. Results on gels run with other buffers were comparable. An electrode buffer of 0.5 M Tris gave the clearest bands, but at this high molarity the gel sometimes melted at the wick interface and the duration of electrophoresis had to be curtailed.

Figure 14

Hexokinase. Zymogram of some taeniid tapeworm extracts




From left to right: *T. solum, T. saginatus, T. solium, T. saginatus, E. granulosus* (horse) *M. multiceps, T. ovis*, blank, blank, *T. hydatigena*

Electrode buffer: 0.5M Tris/HCl pH 8.0
Gel buffer: electrode buffer diluted 1:5
330 volts across a thin gel for three hours

Table 33

Hexokinase. Relative position of enzyme forms from different tapeworm species. Electrode buffer 0.5 M Tris/HCl pH 8.0 330 V for 3 h or less

| | -1 | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| E. granulosus (sheep) | | | | | | X | X | | | | | | | | | | | | | |
| T. hydatigena | | | | | | | X | X | X | X | X | | | | | | | | | |
| M. multiceps | ?X | | X | X | X | | | | | | | | | | | | | | | |
| T. ovis | | | | | | | X | | | | | | | | | | | | | |
| T. pisiformis | | | | | | X | X | X | | | | | | | | | | | | |
| T. saginatus | X | | X | X | | | | | | | | | | X | X | | | | | |
| T. solium | | | | X | X | | | | | | | | | | | | | | | X |
| H. taeniaeformis | | | | | | | X | X | X | | | | | | | | | | | |
| | -ve | +ve | | | | | | | | | | | | | | | | | | |

0 = position of insertion. ? = occasionally seen.

Malate dehydrogenase (MDH)

Enzyme activity was strong in all the tapeworms examined. Both NAD and NADP dependent enzymes were present, but enzymic activity of the former was stronger and no differences in band position were seen between the two.

Many buffers were tried and the conditions of electrophoresis varied in order to obtain clear banding. On thick gels with 0.05 M Tris/HCl buffer pH 8.0 at a high voltage (215 V to 320 V) for a short period (three hours) numerous bands were seen on a few occasions. Ten bands were seen with T. hydatigena, five with M. multiceps and four with T. pisiformis. Unfortunately this banding could not be reproduced and adequate comparisons could not be made. All taeniid species were tested but very little, if any, difference in mobility was seen between them, although there were obvious ordinal differences. Results are summarised in Table 34 for Tris/HCl/barbitone pH 8.5 buffer and in Table 38 for phosphate/citrate pH 7.0 buffer.

Table 34

Malate dehydrogenase. Relative positions of enzyme forms from different tapeworm species. Electrode buffer Tris/HCl/barbitone pH 8.5 diluted 1:5 for gel. Thick gel. 100 V for 16.5 h

| | -2 | -1 | 0 | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|---|---|
| E. granulosus | | | | X | | | | |
| T. hydatigena | | | | X | | | | |
| T. hydatigena cyst | | | | X | | | | X |
| T. saginatus | | | | X | | | | |
| T. solium | | | | X | | | | |
| Cittotaenia sp. | | | | | X | | | |
| Hymenolepis diminuta | | | | X | | X | | |
| Moniezia expansa | | X | | | · | | X | |
| | | -ve | | +ve | | | | |

0 = position of insertion

Glucose phosphate isomerase (GPI)

This enzyme was also present in all tapeworm extracts that were tested. It retained its activity well in storage. Differences in mobility were clearly seen between most of the taeniid species and even greater differences were seen between tapeworm species from other orders. With this enzyme alone it was possible to differentiate most of the taeniid species that were available. Banding was usually clear. Extracts were usually assayed and diluted as necessary to equivalent enzymic activity. Fig. 15 shows

Figure 15

Glucose phosphate isomerase. Zymogram showing taeniid tapeworm extracts




From left to right: *T. hydatigena, T. ovis, E. granulosus* (horse), *T. saginatus, T. solium, M. multiceps, H. taeniaeformis, T. pisiformis, T. saginatus* and *T. solium*. Enzyme activity of each sample was approximately 1 U $ml^{-1}$

Electrode buffer: 0.2 M phosphate pH 7.4
Gel buffer: 0.015 M phosphate pH 7.4
330 volts across a thin gel for three hours

all the taeniid species tested.

Many species of tapeworm were tested both in Nigeria and in the United Kingdom. A summary of the results obtained by comparing the zymograms is given in Table 35. Although T. ovis and T. saginatus are shown to have identical positions, T. ovis was seen on some gels to be slightly slower moving than T. saginatus.

Table 35

Glucose phosphate isomerase. Relative positions of enzyme forms from different tapeworm species. Electrode buffer 0.2 M phosphate pH 7.4, gel buffer 0.015 M phosphate pH 7.4 330 V for 3 h

| | -3 | -2 | -1 | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| E. granulosus (horse) | | | | | | | | | | | | | | | | X | | X | | X | | X | | | | | | | |
| T. hydatigena | | | | | | | | | | | | X | | X | | X | | X | | X | | X | | X | | | | | |
| M. multiceps | | | | | | | | | | | | | | | | | | | | | | | X | | X | | X | | X |
| T. ovis | | | | | | | | | | | | | | | X | | X | | X | | X | | | | | | | | |
| T. pisiformis | | | | | X | | X | | X | | X | | X | | X | | X | | | | | | | | | | | | |
| T. saginatus | | | | | | | | | | | | | | | X | | X | | X | | X | | X | | | | | | |
| T. solium | | | | | | | | | | | | | X | | X | | X | | X | | X | | | | | | | | |
| H. taeniaeformis | | | | | | X | | X | | X | | X | | X | | X | | X | | | | | | | | | | | |
| A. perfoliata | | | | | | | | | | | | | X | | X | | X | | X | | | | | | | | | | |
| A. centripunctata | | | | | | | | | | | | | | | | X | | X | | | | | | | | | | | |
| Cittotaenia | | | | | | | | | | | | | X | | X | | X | | X | | | | | | | | | | |
| D. caninum (cat) | X | X | X | | X | | X | | | | | | | | | | | | | | | | | | | | | | |
| D. caninum (dog) | | | X | | X | | X | | | | | | | | | | | | | | | | | | | | | | |
| H. giardi | | | | | | | X | | X | | X | | | | | | | | | | | | | | | | | | |
| M. expansa | | | | | | | | | | | | | | | | | | X | | X | | X | | | | | | | |
| M. benedeni | | | | | | | | | | | | | X | | X | | X | | | | | | | | | | | | |
| Camel meat | | X | | | X | | | | X | | | | X | | | | | | | | | | | | | | | | |
| Ox liver | | | | | X | | X | | X | | X | | X | | | | | | | | | | | | | | | | |
| Pig muscle | | X | X | | X | | X | | X | | | | | | | | | | | | | | | | | | | | |
| Sheep liver | | | | | X | | X | | X | | X | | X | | | | | | | | | | | | | | | | |
| Mouse muscle | | X | X | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Horse liver | X | X | X | | | | | | | | | | | | | | | | | | | | | | | | | | |

-ve +ve

0 = position of insertion

# CHAPTER TWENTY TWO

## ENZYME ELECTROPHORESIS: VARIATION WITHIN SPECIES

### Effect of anthelmintics

Table 2 shows the range of anthelmintics used in Nigeria to obtain tapeworm specimens. Specimens of all canine taeniids were obtained with and without the use of arecoline hydrobromide. Zymograms of GPI were made with specimens of the same species obtained by each of the anthelmintics used and also with those obtained post-mortem or by spontaneous discharge. No differences were found.

### Extracts from different parts of the tapeworm

a) Strobila

T. hydatigena immature, mature and gravid segments were extracted separately and compared for AK and GPI. No differences could be detected (see Fig. 16).

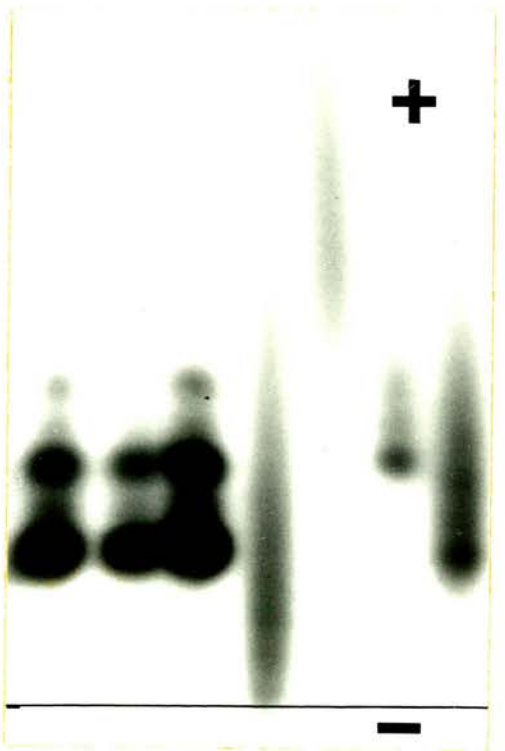
b) Ova

Ova of T. saginatus, T. ovis, T. pisiformis and H. taeniaeformis were used. Few remained whole after crushing with a pestle and mortar. Extracts were usually made from 1,000 to 2,000 ova.

In Nigeria, GPI bands were seen in an extract from 1,320 ova of T. hydatigena. These bands were identical to those in extracts of strobilate tissue, but when sieving was employed in their preparation in the United Kingdom, no bands were seen in any of the four species even when an

Figure 16

Glucose phosphate isomerase in different parts of a *T. hydatigena* tapeworm




From left to right: *T. hydatigena* gravid proglottis, *T. hydatigena* mature proglottis, *T. hydatigena* immature proglottis, *T. pisiformis, M. multiceps, Moniezia expansa, T. hydatigena* (whole worm)

Electrode buffer: 0.1 M Tris/citrate pH 7.5
Gel buffer: electrode buffer diluted 1:5
110 volts (power pack reading) across a thick gel for 16.5 hours

extract was made from 32,000 ova of T. ovis.

c) Larval cysts

T. hydatigena, M. multiceps, T. pisiformis, T. saginatus, T. solium and H. taeniaeformis strobilate extracts were compared to homologous whole larval extracts. Larval extracts of hydatid cysts were prepared from hydatid sand, germinative epithelium and cyst fluid separately and extracts from T. hydatigena cysts were prepared from scolices and cyst wall separately. When comparisons were made between larval tissue and strobilate tissue, extracts of host tissue from the organ in which the cyst was found were also included in the zymogram.

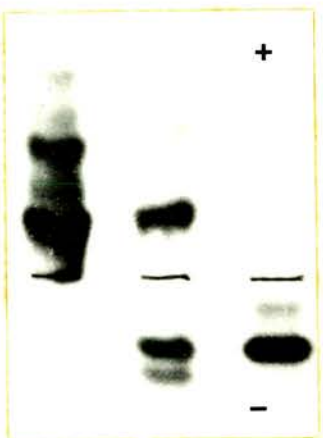
All larval and homologous strobilate tissues showed identical GPI bands in zymograms. Hydatid fluid had little parasite enzyme activity and all larval extracts had host tissue enzymes present in them as well. The positions of the enzymes in host tissue extracts are shown in Table 35. Fig. 17 shows a zymogram comparing larval cyst and strobilate material.

Strain variation

It was not possible to compare many worms originating from different areas of the world. H. taeniaeformis originating from an experimental strain in Belgium was compared with the same species found post-mortem in Scottish cats; T. saginatus strobila from Nigeria and Kenya were compared with one another and with T. saginatus

Figure 17

*H. taeniaeformis* strobila and larval cyst. A comparison of extracts




From left to right: strobila, cyst and mouse muscle

Electrode buffer: 0.2M phosphate pH 7.4
Gel buffer: 0.015M phosphate pH 7.4
330 volts across a thin gel for three hours

cysts from cattle experimentally infected in Scotland by ova obtained from worms in Kenya mixed with ova from the London School of Tropical Medicine patients.

Extracts examined in Nigeria could also be compared indirectly with those in the United Kingdom. Many samples of strobila of T. saginatus and T. solium were imported into the United Kingdom after being tested in Nigeria against worms of animal origin. The same samples were then tested against worms of animal origin in the United Kingdom.

No differences were found between any adult specimens from different hosts even if the hosts originated from different countries.

Echinococcus cysts were also collected from a variety of hosts, in Belgium, Nigeria and the United Kingdom. Fig. 18 shows differences that were seen in mobility between extracts of cysts derived from sheep and horses. Those from cattle were similar to those from sheep. Table 36 shows the relationship of the strains found in sheep, cattle, horses and camels.

Table 36

Echinococcus granulosus. Relative positions of glucose phosphate isomerase forms in extracts from cysts derived from different hosts

| Host | Number of animals | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Camel | 2 | | | | | | | | X | X | X | |
| Horse | 7 | | | | | | | X | X | X | X | X |
| Ox | 6 | | | | | X | X | X | X | X | | |
| Sheep | 3 | | | | | X | X | X | X | X | | |
| | | -ve | +ve | | | | | | | | | |

0 = position of insertion

# CHAPTER TWENTY THREE

## ENZYME ELECTROPHORESIS: STANDARDS

a) Enzymes

Sheep's heart muscle was extracted in the same manner and under the same conditions as tapeworm material and was used in early work with thick gels as a standard with which to compare the mobility of tapeworm enzymes. This was discontinued and various other preparations were tried including Sigma commercial enzyme preparations (an ammonium sulphate suspension of MDH from beef heart, an ammonium sulphate suspension of GPI from yeast and a freeze dried preparation of GPI from rabbit muscle).

The iso-enzymes in these preparations had very different mobilities from those of tapeworms. Yeast GPI moved anodally very rapidly in 0.2 M phosphate pH 7.4 buffer and the bands were warped. Banding was clearer at a lower pH. Under the same conditions rabbit muscle GPI reconstituted in EP solution did not give clear banding and mobility was mostly cathodal.

b) Markers

Stained proteins were used to measure the distance travelled under standard conditions of electrophoresis. Bromphenol blue stained ten per cent bovine globulin moved too fast. Haemoglobin from an ox, a rabbit and a sheep was then tried. Under the optimal conditions of electrophoresis for GPI in tapeworms, sheep haemoglobin moved approximately 1.5 cm anodally (electrode buffer 0.2 M

phosphate pH 7.4; gel buffer 0.015 M phosphate pH 7.4; 330 V across thin gel for 3 h). Sheep GPI was present in the haemoglobin and moved anodally at a similar rate to that of tapeworms. Rabbit haemoglobin and its GPI did not move much from the point of application. Ox haemoglobin and its GPI moved very fast and was located at the end of the gel after the run. Sheep haemoglobin was therefore the most satisfactory marker of the haemoglobins tested.

In addition to the above, tissue extracts of hosts were always included as a control when larval cyst extracts were studied.

Measurement of zymogram traces

A study was made on thin layer starch gel zymograms run under similar conditions (electrode buffer 0.2 M phosphate pH 7.4; gel buffer 0.015 M phosphate pH 7.4; 330 V for 3 h). The distance travelled from the cotton thread by the nearest band was measured for T. hydatigena, T. saginatus and T. solium. The results are shown in Table 37.

Table 37

The distance travelled (mm) from the point of application by the slowest band of glucose phosphate isomerase under identical electrophoretic conditions

| Zymogram | T. hydatigena | T. solium | T. saginatus |
|---|---|---|---|
| 1 | 15 | 17 | 22 |
| 2 | 14 | 15 | 19 |
| 3 | 7 | 14 | 17 |
| 4 | 12 | 19 | 23 |
| 5 | 9 | 15 | 19 |
| 6 | - | 18 | 22 |
| 7 | 10 | 20 | 25 |
| 8 | 9 | 16 | 22 |
| 9 | 9 | 15 | 20 |

The relative positions of each species was constant, but the distances moved by the first bands varied considerably and overlapped between species in different zymograms. A constant voltage setting of 330 V was used in all these runs. The current, however, differed from gel to gel, ranging from 22 mA to 38 mA.

Densitometry

Zymograms were run on Millipore Phoroslides which were then cleared and used for measuring enzyme mobility in a densitometer. During the clearing process the stain which

developed when using MTT faded considerably or disappeared. It was found that the use of NBT instead of MTT prevented this. A blue line was drawn with a ball-point pen three cm from the cathode end of the slide. This showed as a peak on the screen. Paper recordings were made of the screen pattern to include this line together with the enzyme band pattern.

Measurements were made from this line to three points on the enzyme band: the beginning of the ascent, a mid-point in the sharpest rise and the first major peak.

The Tris/HCl/barbitone electrolyte recommended for electrophoresis of lactate dehydrogenase in this equipment (XE 2100042, Millipore (U.K.) Ltd.) or 0.1 M Tris/Citrate pH 7.4 were mainly used and variations in voltage from 25 V to 200 V were made. Variations in the period of electrophoresis were adjusted to suit these voltages - from 18 minutes to two hours.

As banding was not clearly defined, the peaks were not sharp. Measurements to half way up the first major peak or to the summit of the peak gave results by which the species could be compared but this method was less accurate and more inconsistent than visual appraisal. Using the same buffer and the same electrophoretic conditions, no two Phoroslides gave the same measurements for the same extract and only obvious differences could be detected between species - i.e. between a very fast moving iso-enzyme and a very slow moving one.

# CHAPTER TWENTY FOUR

## ENZYME ELECTROPHORESIS: SPECIES CHARACTERISTICS

Table 38 gives a summary of the locations of the multiple enzyme forms of the different tapeworm species. Differentiation of the taeniid worms was achieved by comparing the position of the band nearest the point of application. It was thus possible with GPI alone to differentiate most of the species that were examined.

The horse strain of *E. granulosus* was studied most and the relative position of the other strains is known to differ (see Fig. 18). The band positions with the horse strain had close similarities to those of *T. ovis*.

*T. hydatigena* had medium GPI mobility in contrast to *T. pisiformis* which was the slowest, and to *M. multiceps*, which was the fastest. *H. taeniaeformis* had a very similar location to *T. pisiformis* except with glutamate dehydrogenase. In both cases the glutamate dehydrogenase activity was weak but extract from these two worms showed very different mobilities. *T. ovis* and *T. saginatus* were the only two taeniid worms that were more or less identical although *T. ovis* was slightly slower on some zymograms.

*T. saginatus* and *T. solium* also had a similar GPI location. The mobility difference could be enhanced by the use of 0.5 M Tris/HCl pH 8.0 buffer. Differentiation of these human taeniids was the subject of a published paper (Le Riche & Sewell, 1977. See Appendix II).

HK multiple forms showed many specific differences. After GPI, this enzyme was the most useful as it was also fairly easy to detect. M. multiceps HK has cathodal mobility in contrast to the fast anodal mobility of the GPI forms. T. ovis and T. saginatus had very different HK mobilities.

Unrelated tapeworm species of other orders usually had very different zymogram patterns and could not be confused with taeniid species. There also appeared to be species differences within these orders.

Table 38

Distribution of electrophoretic forms of enzymes in taeniid extracts. Relative positions of bands numbered from the point of application. Minus signs denote cathodal movement.

| | Adenylate kinase (Tris/HCl pH 8.0) | Glutamate dehydrogenase (0.2 M phos. pH 7.4) | Hexokinase (0.5 M Tris/HCl pH 8.0) | Malate dehydrogenase (0.05 M phos/ cit pH 7.0) | Glucose phosphate isomerase (0.2 M phos. pH 7.4) |
|---|---|---|---|---|---|
| E. granulosus (horse) | 1,2,3 | | 4,5 | 2,3 | 12,14,16,18 |
| T. hydatigena | 1,2,3 | 2 | 5,6,7,8,9 | -1,1,2,3 | 8,10,12,14,16,18,20 |
| M. multiceps | 3,4,5,6,7 | -1,1 | -1,1,2,3 | 1,2 | 19,21,23,25 |
| T. ovis | 2,3,4 | | 5 | -1,1 | 11,13,15,17 |
| T. pisiformis | -1,1,2,3 | 1 | 4,5,6 | -1,1,2 | 1,3,5,7,9,11,13 |
| T. saginatus | 1,2,3,4,5 | 3 | -1,1,2,12,13 | -1,1,2 | 11,13,15,17,19 |
| T. solium | 2,3,4,5 | 1 | 1,2,18 | -1,1 | 9,11,13,15,17 |
| H. taeniaeformis | 1,2,3,4,5,6 | 5 | 5,6,7 | -1,1 | 2,4,6,8,10,12,14 |
| A. perfoliata | | | | | 9,11,13,15 |
| A. centripunctata | 8 | -5,-4,-3,-2,-1 | | -1,1 | 12,14 |
| Cittotaenia | | | | | 9,11,13,15 |
| D. caninum (cat) | | | | | -3,-2,-1,1,3 |
| D. caninum (dog) | | | | | -1,1,3 |
| H. giardi | 3,4 | 4 | | 6,7,8 | 3,5,7 |
| M. expansa | | | | | 14,16,18 |
| M. benedeni | 2,3 | | | 4,5,6,7,8 | 9,11,13 |

# DISCUSSION

# CHAPTER TWENTY FIVE

## MATERIAL

### Introduction

A variety of different techniques has been utilized in the search for reliable and practical methods of identifying taeniid worms. These techniques have included those used in clinical medicine to diagnose specific diseases such as echinococcosis and cysticercosis and also others that have not previously been applied to helminthological diagnosis. Although sophisticated and expensive apparatus has sometimes been used in this work, an attempt has been made to simplify tests wherever possible to enable their use in small laboratories in developing countries.

### Material

Much of the initial serology was done before fresh material from different worm species became available and was mostly confined to work with ova. However, much of the findings from this work were later confirmed by more sensitive tests using fresh material.

No difficulties were experienced with infecting definitive hosts except with T. ovis. Very few viable cysts were collected from sheep on the first occasions and these cysts were punctured during dissection. Worms did not develop in dogs that received them. Although it was optimistic to expect infection of dogs from such a small dose, it would in any case appear from Sutton's work

that, unlike T. hydatigena and H. taeniaeformis, cysts of T. ovis are not infective if they have been dissected from their surrounding capsule or if they have been damaged in any way (Sutton, 1974).

The regime of corticosteroid treatment was recommended in a personal communication by Heath (1976) as a method of minimizing the development of immunity in sheep to infection, and a similar method has been reported recently in the literature by Sutton (1976). In addition to dexamethasone, Sutton also used betamethasone and found that the former increased survival rate of the cysts whereas the latter increased the infection rate. Only dexamethasone was used in this work but it gave very good results.

One dog retained T. ovis infection for more than 14 months. Sweatman & Henshall (1962) reported the longevity of T. ovis infection in the dog to be from one month to more than nine and a half months, but Gregory (1976) found that infection could last nearly four years. The longevity of infection in the dog was confirmed by the present observation. Infection with other taeniid worms was also of long duration.

It was not possible to collect all taeniid species. M. serialis, M. serialis brauni and E. multilocularis were notable omissions.

# CHAPTER TWENTY SIX

## DIFFERENTIATION BY STAINING AND SEROLOGICAL TESTS

### Staining

Capron & Rose (1962) first reported a difference in staining by the Ziehl-Neelsen method between *T. saginatus* and *T. solium*. They found the embryophores of *T. saginatus* were acid and alcohol fast and so stained red, whereas those of *T. solium* did not. This finding has not been confirmed. Taeniid worms of seven different species were stained by the method described by Capron & Rose and also by the usual method with heating. All embryophores were definitely acid and alcohol fast, including those of *T. solium*, and this method could therefore not be used for differentiation.

### Serological tests

The circum-oval precipitation test has been found useful in the diagnosis of schistosomiasis in clinical medicine. Electron microscope studies showed that precipitates were associated with defective portions of the egg shell (Sakumoto *et al*, 1972). The shell of the schistosome egg is quite different in construction from the robust and thick embryophore of the taeniid ovum and defects allowing intimate contact between the onchosphere, which is also protected by its membrane, and antisera bathing the embryophore may not br so common. Observations have been recorded by several workers on the effects of antiserum on

taeniid onchospheres or onchospheral blocks, but reports of precipitates in or around intact ova have only been observed with Hymenolepis nana, which has quite differently constructed ova (Hyneman & Welsh, 1959). Even with Hymenolepis observations of precipitates have been contradictory, with false positive and false negative results.

A further difficulty arose because the attachment of vitelline material to the outside of the ovum obscured observations. This material may be more obvious on ova teased out of proglottides than on naturally released ova. Attempts to clean ova with Milton were unsuccessful due to the deleterious effects of the chemical on them.

Silverman (1955) first hatched taeniid ova and observed the effect of immune sera on "activated" onchospheres. Unfortunately the adjectives "hatched" and "activated" are often not clearly defined in the literature when applied to taeniid ova. Silverman observed precipitates on onchospheres that had been liberated from their membranes and also inside membranes of those that had not. Chen (1950) did not observe any precipitates in onchospheres which were not liberated from their membranes.

Silverman's observations could not be confirmed in the work reported here. The process of hatching and activating ova is lengthy and very wasteful. Care has to be taken to keep the fragile onchospheres warm once they have hatched. For practical purposes the identification of unhatched ova is desirable as these may be too few to pass through the hatching procedure.

Identification of ova by the fluorescent antibody test was also attempted. The FITC conjugate which was made in the laboratory had a labelling ratio of 0.9. The optimum is 1.0, and the conjugate was therefore considered quite satisfactory. Other purchased conjugates were also used.

Autofluorescence of the embryophore was so strong that it seemed likely to obscure specific fluorescence and attempts to quench autofluorescence were not successful. In spite of this, success has been reported using taeniid ova in this test by Euzéby & Dubra (1970), Calamel & Soulé (1972) and Machnicka (1973) but, as with the circum-oval precipitation test, the results of the different workers were contradictory. These workers all experienced difficulties due to autofluorescence, or due to non-specific fluorescence with negative sera. Calamel & Soulé used hatched onchospheres in very large numbers in tube tests and in frozen sections: ten million embryos/ml were used. They found that bright fluorescence of the onchospheral membrane contrasting with the unstained interior of the embryo was characteristic of specific fluorescence. Machnicka, on the other hand, reported fluorescence of the onchospheral membrane and hooks when they were bathed in normal sera. She considered fluorescence of the embryo cells to be characteristic of specificity although fluorescence of the onchospheral membrane also increased in a specific reaction.

Euzéby & Dubra sometimes failed to obtain fluorescence with immune sera and observed cross reactions with Dicrocoelium dendriticum. Calamel & Soulé did not see cross reactions with D. dendriticum but did between taeniid worms.

Fluorescence of the onchospheral membrane was seen in experiments reported here. Although at first this was thought to be specific, it was later concluded that it was related to adherence of fluorescein conjugate to the membrane through improper washing before microscopy. It is possible that with better filters, which were not available, some of the autofluorescence could have been cut out, but a test which requires such large numbers of onchospheres is, in any case, impractical.

Searching for onchospheres by dark ground illumination was very difficult; hatching and activating was time consuming and wasteful. The sectioning of gravid proglottides would be the most satisfactory technique if autofluorescence could be reduced.

This test however is not only very subjective but has been shown to be of poor specificity by other workers and it did not seem to be worth while developing it further.

The micro agar diffusion precipitation technique was, in contrast, quite useful. The tests were fairly easy to perform and gave clear-cut results. This technique has been used by Enyenyi (1970) to compare some tapeworm and other antigens and by Gallie & Sewell (1974a,b) to trace the

immune response of calves to T. saginatus infection.

In experiments IP 1 and IP 2 (see Tables 13 and 14), the orally dosed animals lacked a strong immune response to strobilate antigen. Gallie & Sewell have demonstrated an immunoprecipitation response in orally dosed calves. This response was stronger in some individuals than in others, but was much weaker in orally dosed calves than in calves immunized with strobilate antigen by intra-muscular injection (Gallie & Sewell 1974a,b; 1976). Harrison (1977) also obtained a weak reaction with antisera from orally dosed calves and concentrating the sera five times was necessary before lines could be seen.

In most of the tests antigen was made from homogenized strobila or cyst material. This antigen was identical to that used to hyperimmunize the animals by intra-muscular injection. A stronger response in the test when this antiserum was used was therefore more likely than when antisera from orally infected animals was used. Soluble antigens from homogenized strobilate material may be very different from the excretory and secretory products of infecting organisms which are experienced by the host after oral infection. Nevertheless, the use of strobilate antigens for immunizing animals and for subsequent tests may be a useful tool in the identification of cestode material.

The use of ova as antigen (see Table 15) also gave no reaction in tests against antisera from orally infected

animals. This may merely indicate the weakness of the system, which could have been due to a poor yield of soluble antigenic material from the ova, or due to the lack of precipitins in it. On the other hand, the immune response in the host may be against excretory and secretory products of the invading organism rather than the soluble antigens of which it is composed.

Lines between homologous systems were clear and strong but cross reactions with other species were also strong, though less so between Cittotaenia and the taeniid worms. Cittotaenia belongs to a different order, Anaplocephalidea, and appears to share fewer antigens with the taeniid species.

Absorption of antisera was successful in removing much of the cross reaction, yet leaving, in most cases, specific reaction. Further absorption studies were continued using the more sensitive ELISA technique.

Immunoelectrophoresis has been shown to provide a specific test for hydatid disease in man because of the presence of the characteristic arc 5. Material was not available for testing Echinococcus, but the large number of lines (up to 23) illustrated in the paper by Capron et al (1968) were not obtained. It is possible that their method of preparing antigen by lyophilisation was superior although details of antigen concentration are not given in the paper. The technique uses large amounts of sera and, since Capron et al, who had previously described the specificity of arc 5 for hydatid disease (Capron et al, 1967), had not noted

any specific lines for T. saginatus (23 lines), T. pisiformis (17 lines), T. solium (22 lines) or T. hydatigena (17 lines), this test was abandoned. Recent work has shown that the arc 5 antigen is present not only in E. granulosus but in E. multilocularis (Yarzábal et al, 1977, and in T. hydatigena cyst fluid as well (Varela Díaz et al, 1977). Previous studies had often been made with sera from human patients and therefore cross reactions with other worm species were not encountered. The specificity of the test for echinocccosis therefore appears to be in doubt.

Absorption studies were pursued using the ELISA technique. As in the micro agar diffusion precipitation test, absorption of antiserum with one closely related cestode extract did not absorb out all non-specific reaction. Absorption with two closely related extracts removed most of the cross reacting antibodies but the reaction to homologous antigen was then also poor. The spectrophotometer readings were very low but it was felt that results had been masked to a great extent because the colour faded after the addition of sodium hydroxide solution to stop the enzyme reaction. Perhaps because of this, the technique was too insensitive to record a reaction from egg antigen.

Alkaline phosphatase was used by Engvall & Perlmann (1972) in their work with the ELISA test. This enzyme is expensive to use, hence the choice of horse-radish peroxidase in this work.

In a report on the ELISA technique by an informal consultation group (WHO, 1975), the possibility of the substrate 5-amino salicylic acid being a carcinogen was noted and a continued search for an alternative was suggested. The HRPO test therefore requires further development with regard to the substrate, and, in preliminary studies, O-phenylenediamine has recently been found more satisfactory (Voller, 1977). This substrate which changes to an orange colour appears to be more stable and more sensitive. A better method of enzyme inhibition is in any case desirable with 5-amino salicylic acid substrate.

The conjugate for this procedure was at first prepared in the laboratory, but it did not retain activity on storage. Later details released by Nakane (1975) showed that it is necessary to stabilize the conjugate by the addition of sodium borohydride. This instability was first observed early in the work and a commercial conjugate was used thereafter, so the results were not affected by this omission.

# CHAPTER TWENTY SEVEN

## ENZYME ELECTROPHORESIS: TECHNIQUES

Enzyme electrophoresis was found to offer better possibilities than serology for making a clear-cut differentiation between taeniid worms and effort was concentrated in this field.

Of the supports used, the thin layer starch gels appeared to give superior results. The decision to change to this technique, however, coincided with the purchase of improved electrophoretic equipment with a cooling platen. Although electrophoresis in thick gels had been done in the cold room at 4°C, the protective plastic hood incorporating the safety switch that covered the gel prevented the circulation of the cool air and allowed heat to build up inside. Because of this, and also because of an inadequate power supply at that time, low voltages over a long period generally had to be used.

The apparent advantage of being able to slice a thick gel and develop the slices simultaneously in different substrates was outweighed by the handling difficulties in doing so, which often resulted in damage to all the slices. Successful results were not achieved with cellulose acetate, but this may have been due to the brand used. Successful electrophoresis on cellulose acetate has been reported by Meera Khan (1971) and van Someren *et al* (1974) but both

used a particular brand that is reputed to have special properties.

In the preparation and storage of enzymes it was important to distinguish between the preservation of zymogram characteristics of the enzyme or merely its enzymic activity. Improper preservation could result in retention of enzymic activity, as measured by assay, but loss of the characteristic banding patterns. This loss may occur due to alteration of the molecular conformation of the enzyme, as has been observed with human glucose phosphate isomerase by Detter et al (1968).

Extracts were prepared for immediate use by merely homogenizing the material in the solution of enzyme preservatives, using the supernatant for electrophoresis. A ten per cent w/v homogenate was usually satisfactory and dilutions of this could be tested in the same gel if necessary. Particulate matter did not seem to cause "drag", especially if the supernatant was removed after leaving the extract to stand overnight. Although ultracentrifugation was considered desirable, especially if the extract was to be stored, centrifugation of samples was not essential.

Homogenization of specimens released sufficient soluble enzymes. Ultrasonication was liable to heat the homogenate and did not appear to improve the yield.

Some form of cold storage was necessary to preserve enzyme activity, although the incorporation of ammonium

sulphate, which crystallizes the enzyme (Bergmeyer, 1965), preserved enzyme activity best at room temperature. Unfortunately this procedure caused warping effects on the bands which made it difficult to compare specimens.

The transport of animal tissues across international borders is tightly controlled in order to prevent the spread of disease. $\beta$ -proprio-lactone, a sterilizing agent commonly used, unfortunately destroyed enzyme activity. Binary ethyleneimine, a new virus inactivator that does not denature serum proteins (Bahnemann, 1976), may prove useful if it is accepted internationally as a sterilizing agent.

It was at first thought desirable to assay the enzyme activity of the extracts so that their concentration could be adjusted to allow comparison of samples of similar activity, but it was found that assay was not always helpful or necessary. When samples from cysts were assayed, for instance, it was not possible to discriminate between host and parasite enzyme; strong enzymic activity did not necessarily indicate the presence of parasite enzymes. In any case, it was found that dilution of extracts did not distort the enzyme pattern, merely causing the disappearance of the weaker, distal and faster moving bands. Accordingly, when the position of the proximal slow moving band was used as the criterion, samples of widely differing activity could be compared. Crude samples could therefore be included in a run if immediate identification was required.

GPI and MDH preserved and stored well and dilution of extracts was usually necessary in order to obtain clear

banding. The concentration of other enzymes in the extracts was not so high and they did not store as well either. Better results with them were usually obtained if extracts were used neat.

T. hydatigena extract was fractionated as it was thought that GPI activity might be derived from iso-enzymes of different molecular sizes. If so, it should have been possible to separate the bands, but this did not appear to be the case as most of the enzyme was concentrated in two small adjacent fractions. The molecular weight of GPI from yeast is 145,000 (Bergmeyer, 1965) and lies within the range of these two fractions. Enzyme activity in the other fractions was probably due to tailing of the samples.

Testing extracts with substrate in test tubes for enzyme activity was not found to be a necessary preliminary to electrophoresis and was extravagant with developing solution. A positive result in a test tube did not indicate whether an enzyme would be seen after electrophoresis on a gel or whether it would show multi-molecular forms. Walkey & Fairbairn (1973) were also unable to demonstrate the presence of enzyme after electrophoresis. They were unable to detect lactate dehydrogenase iso-enzymes although they had shown, by other methods, that these were present in Hymenolepis diminuta. It was therefore found more expedient to test extracts directly by electrophoresis. Those enzymes that could be seen in the gel were selected for further study and five were found to show multi-molecular variation that was of value in differentiating tapeworms.

# CHAPTER TWENTY EIGHT

## ENZYME ELECTROPHORESIS: RESULTS

### Enzymes showing species characteristics

Multiple molecular enzyme forms have previously been recognized in tapeworm extracts. Walkey & Fairbairn (1973) examined the electrophoretic forms of lactate dehydrogenase in *Hymenolepis diminuta* and found two forms. Carter & Fairbairn (1975) reported five pyruvate kinase iso-enzymes in the same species but no comparisons were made with other species. Lee *et al* (1976), on the other hand, found both inter and intra-specific differences in esterase activity in *Echinococcus* species by iso-electric focussing.

In the present study, the five enzymes found to have multi-molecular forms that showed specific differences in tapeworms were GDH, AK, MDH, HK and GPI. GDH was not always present in sufficient concentration to show up in the gel or else it showed up faintly, but it was seen in extracts from all the taeniid worms on one occasion or another. Waitz (1963) failed to detect this enzyme in *H. taeniaeformis* and Cornish & Bryant (1975) failed to find it in *Moniezia expansa*. There would appear to be some difficulty in gel penetration by this enzyme (Wilkinson, 1970).

The presence of AK in tapeworms has not previously been noted. Its value in differentiating species is not high. Although specific differences were seen, the patterns

were rather similar to GPI.

The anomaly shown in Fig. 10 where mobility was faster in low rather than high concentration of a similar buffer is inexplicable but requires further confirmation. It is of no significance to the present study.

Two forms of MDH are known to exist in mammalian tissues, one being of mitochondrial and one of cell sap origin, and it has been suggested that the soluble cell sap form may be a complex of the mitochondrial form with a lipoprotein (Sophianopoulos & Vestling, 1960).

In a study of enzymes in Haemonchus contortus and Ascaris suum, Rhodes et al (1970) showed MDH to be present in soluble tissue extract, but failed to get clear banding with electrophoresis on starch gel or ion agar. With iso-electric focussing in polyacrylamide gels, however, they found many discrete bands: up to 11 bands were seen in A. suum, but only three in swine serum. Shirley & Lee (1977), on the other hand, were unable to separate MDH of Eimeria species into bands by iso-electric focussing, although they succeeded with lactate dehydrogenase. The work of Sophianopoulos & Vestling (1960) and Rhodes et al (1970) shows that some nematode enzymes may have many more multi-molecular forms than the same enzymes in mammals.

Zee & Zinkham (1975) also studied MDH in ascarid worms by electrophoresis. They showed that three groups of worms had different patterns: Ascaridia columbae, A. dissimilis and A. galli resembled one another; Toxascaris leonina was

similar to Toxocara canis and T. cati whereas Ascaris suum and A. lumbricoides were identical although they did not find as many bands as Rhodes et al (1970).

In the present work, numerous bands of MDH were seen on a few occasions, although these patterns could not always be reproduced. Iso-electric focussing would probably separate them and enable comparisons to be made. From the zymogram patterns that were produced it was nevertheless possible to distinguish between taeniid and non-taeniid worms, but identical mobility of taeniid enzyme prevented species differentiation.

MDH may have been an unfortunate choice for the study of species differences by Zee and Zinkham in ascarid worms; a study of another enzyme such as GPI might have been more rewarding in differentiating all the species. MDH has nevertheless been found useful in distinguishing between trypanosomes in the NADP-dependent form ("malic enzyme") (Bagster & Parr, 1973) and between Plasmodia (Carter, 1973), although doubt was expressed about Carter's conclusions due to confusion with host enzyme (Killick-Kendrick, 1974).

HK has been described in Moniezia expansa (Behm & Bryant, 1975b) and Echinococcus granulosus (Agosin & Aravena, 1959). In the present study, it had strong enzymic activity in fresh extracts and was present in all the taeniid worms. The pattern was quite different from that of GPI and the former enzyme was therefore found to be quite useful.

However, GPI was the most useful of the five enzymes for differentiating the taeniid worms, as each species had a characteristic pattern. The enzyme was stable under several conditions of preservation and storage.

GPI was first reported to be present in multiple forms in yeast (Nakagawa & Noltman, 1967). Carter & Parr (1967) identified heterogeneity of the enzyme in animal tissue and were the first to use a visible staining reaction. Since then, this enzyme has been studied in many organisms in which it is known to be present in multiple forms. It has been used to identify Plasmodia (Carter, 1970; Carter & McGregor, 1973; and Carter & Voller, 1973) and Entamoeba (Rieves & Bischoff, 1968) and variations within populations have been detected with it in other species, such as man (Detter et al, 1968; Fitch et al, 1968) and yeasts (Nakagawa & Noltman, 1967). It has also been used to identify strains of moths and butterflies (Jelnes, 1974; 1975). Detter et al and Fitch et al, working independently, concluded that GPI was dimeric in structure in man.

In tapeworms GPI has been shown to be present in H. taeniaeformis (Waitz, 1963) and E. granulosus (Vessel & Abdolrasulnia, 1976). Up to seven bands were seen in the present work in some tapeworm extracts. The origin of these bands is not known. Zee & Zinkham (1968) found that the three forms of soluble MDH in ascarid worms were each a different protein although they all had similar molecular

weights. These forms were not due to conformational changes as had been postulated by Thorne et al (1963) in their work with mitrochondrial and supernatant forms of MDH in pig's heart, but were determined by two genetic loci.

Presumably, the enzyme bands seen in extracts in the present work are also genetically controlled because they were consistently present no matter what solvent was used or whether they were preserved or fresh extracts, but their nature would need to be confirmed by further biochemical studies.

As the band characteristics appear to be common to each species, it is likely that the iso-enzymes are controlled by multiple gene loci. Such iso-enzymes result from mutations that alter amino acid sequences in the polypeptide chains thus forming iso-enzymes of similar activity but slightly different construction. Individual variation, which was not seen in the present work, is more likely to result due to the occurrence of multiple alleles. Hybridization between these two types may form additional bands (Moss & Butterworth, 1974; Harris & Hopkinson, 1976).

Secondary alteration of molecular structure can occur as a result of various effects upon the enzyme. It can occur as a result of treatment during extraction or storage causing alteration in configuration of the molecule. It is possible, but not likely, that this would account for the bands as differently prepared and preserved extracts were identical. Loss of banding which occurred in some

old samples, however, may have been due to this phenomenon.

No attempt was made to determine the origin of the bands or to study the nature of the iso-enzymes in the tapeworms. Only the mobility of the proximal band of glucose phosphate isomerase was used as the criterion for comparison between tapeworm species. The origin of the other bands was therefore of incidental interest only as they were not used in identification.

Measurement of mobility

Quantitative assessment of these findings by measurements in a densitometer or by measuring the mobility of bands on tracings was not successful. Measurement of Phoroslides in the densitometer failed to give consistent results because of the imprecise banding. The staining quality of the thin starch gels was good and the banding clear but variation occurred from gel to gel even under similar electrophoretic conditions.

Although electrophoretic conditions were intended to be the same with respect to gel quality, voltage, time and buffers, it is obvious that there must have been some variable effects because current flow was not identical during each run. This variation may have been caused by small differences in the thickness of the gel or differences in the temperature of the gel during electrophoresis. This variation made it impossible to use measurements of mobility as a standard for comparison between tapeworm

species or between tapeworm specimens and markers under the electrophoretic conditions that prevailed.

Comparison with other standard commercial enzymes offered no advantages. These enzymes often behaved very differently from those of tapeworms in respect to mobility and direction of movement and thus comparisons were more difficult than between tapeworm species. Multiple forms of GPI have been reported in many species and interspecies variation is common, occurring even in yeasts (Nakagawa & Noltmann, 1967). Since such variation occurs, there would not appear to be any advantage in their use as standards as their reliability may be questioned. Commercial enzyme preparations, in any case, are often available as crystallized suspensions in ammonium sulphate solution which is not suitable for electrophoresis. Direct comparison between tapeworm species was the most reliable and satisfactory way to differentiate them.

# CHAPTER TWENTY NINE

## DIFFERENTIATION OF TAPEWORMS

### Extrinsic effects

The possibility of extrinsic factors affecting the enzyme patterns was considered. A record was kept of the anthelmintic used for expelling each worm specimen. The pharmacological action of many anthelmintics is not fully understood and enzyme inhibition is a possibility. There appeared to be no effect on the zymogram pattern when extracts from worms expelled by different anthelmintics were compared to those derived from spontaneously expelled worms. The inefficiency of anthelmintic treatment for human tapeworms in Nigeria, as judged by the low recovery of scolices, was, incidentally, remarkable. Incorrect dosing was probably the reason for this.

Inclusion of host enzymes in parasite extracts is a problem associated with the identification of protozoan parasites. This is less likely to occur with lumen dwelling helminths. Muthukrishnan (1975), however, reported the absorption of horse-radish peroxidase through the tegument of *T. hydatigena in vitro*. Reid (1973) also reported and investigated the adsorption of pancreatic $\alpha$-amylase onto the cuticle by *Hymenolepis diminuta*. When testing for tapeworm enzymes, this possibility should be borne in mind as host enzyme may complicate the zymogram patterns and give false results. The adult worm and the metacestode

cyst are both permeable to many large molecules (Wardle et al, 1974; Pappas & Read, 1975). Hustead & Williams (1977) have recently demonstrated the ability of tapeworm cysts to absorb, both in vitro and in vivo, host immunoglobulins. Host immunoglobulins were detected by them in H. taeniaeformis, T. crassiceps and E. granulosus cysts. In the present work, host enzymes were found in extracts from various cysts. These enzymes may have been introduced from the surface of the cyst during processing but, in any case, all the host enzymes included in this study were clearly different in zymogram location and pattern from worm enzymes. They could be readily distinguished by comparison with enzymes in extracts from the host tissue surrounding the cyst.

Tissue differences

Enzymes have been widely used as a sensitive indication of basic changes during differentiation of organisms (Masters & Holmes, 1975). The amino acid sequence on various globin polypeptides gives evidence of ancestry and both convergence and divergence in protein evolution has been recognised.

Markert & Møller (1959) pointed out differences that may be encountered between embryonic and adult tissues. Embryonic tissues may have a different spectrum of isoenzymes from adult tissues and, during the course of development, the relative quantities of these may vary.

This may reflect the state of differentiation of the cells in each tissue.

Yamao (1952b), Erasmus (1956a), Waitz (1963), Cheah (1967), Walkey & Fairbairn (1973) and Carter & Fairbairn (1975) reported differences in the distribution of enzymes in various developmental stages of tapeworms.

Erasmus (1956a) studied acid and alkaline phosphatase levels and found the acid enzyme to predominate in the larva of T. pisiformis and the alkaline enzyme to predominate in the adult worms but he failed to find any activity in the scolex. Waitz (1963) found various enzymes common to both larva and adult H. taeniaeformis, but glyceraldehyde dehydrogenase, although present in larval extracts, was not found in the adult. Walkey & Fairbairn (1975), working with H. diminuta, found evidence supporting the existence of two forms of lactate dehydrogenase; one in ova and cysticercoids and the other in various parts of the adult strobila.

No quantitative measurements of enzyme activity were attempted in this work, but extracts from both ova and larval cysts were compared with strobila material by enzyme electrophoresis. Although enzymes were found in the cysts, the enzyme activity was not strong and this may indicate differences in enzyme concentration between larval and strobilate tissue.

On some occasions enzymes were seen in extract prepared from ova, but it is likely that the enzymes were

in fact derived from small pieces of strobila that had been homogenized with the ova. After more careful filtering, no enzymes were found in homogenates from large numbers of ova. The reason for this may be either that the particular enzymes tested do not occur in ova or that they occur in low concentrations that were not detectable in the gel after electrophoresis. To be of practical value, sufficient enzyme must be detectable in small numbers of ova such as those found in faecal, soil or vegetable samples. Abdussalam et al (1968) pointed out the need for a practical method of differentiating taeniid ova and it is unfortunate that none of the methods met with success in this respect.

Differences in enzyme distribution within the strobila have also been reported but these were mainly quantitative (Walkey & Fairbairn, 1973). When extracts were made in bulk during the present study, they were usually prepared from immature, mature and gravid proglottides mixed together. In the two enzymes studied, no differences were seen in zymograms in the distribution within the strobila although no attempt was made to quantify enzyme activity.

Differences between species

The more distantly one species of organism is related to another, the greater are the differences in protein structure. This protein polymorphism is the result of natural selection exerting its effect on the various amino

acid substitutions in the polypeptide chains that result from mutation. The substitution of one amino acid near the active site of an enzyme can result in iso-enzyme variation. Yoshida (1967) showed, for instance, that the substitution of asparagine for aspartic acid at the active site resulted in a structural difference in glucose-6-phosphate dehydrogenase that could be detected as a variant by enzyme electrophoresis.

Such differences in enzyme structure may also lead to an alteration in substrate specificity or to a different response to inhibitors, activators or other modulators of activity (Masters & Holmes, 1975), but will only be detected by electrophoresis if there is a net change in protein charge (Hopkinson, 1970).

Inter-species differences between tapeworm enzymes have previously been reported by Waitz & Schardein, 1964, 1965). They examined sections of Hymenolepis nana, H. diminuta, Hydatigena taeniaeformis and Dipylidium caninum and found differences in location and intensity of activity with alkaline phosphatase, amylophosphorylase, esterase and cholinesterase between the species. Enzyme activity was not actually measured but merely judged by visual appraisal of colour change at the site of activity after sections had been incubated in a substrate linked to a dye.

In the present comparative work with tapeworm extracts, both closely related and distantly related species were included. Inter-specific differences were more obvious

with some enzymes than with others, but inter-ordinal differences were very obvious and seen with all the five enzymes. This was especially so with MDH, in which there were no obvious differences amongst the taeniid worms (see Tables 34 and 38), but very clear differences between these and worms belonging to other orders. This therefore tends to confirm the broad morphological classification of these tapeworms.

Inter-species differences were seen not only among the taeniid worms but also in other groups as well. Moniezia expansa and M. benedeni appeared to have different GPI forms although those two species were not compared directly with each other.

Amongst the taeniid worms, M. multiceps differed most from the other species. Some species appeared to have very similar zymogram characteristics. Thus H. taeniaeformis and T. pisiformis were very similar as were T. saginatus and T. ovis. Strains of Echinococcus also had similarities with other worms, such as the horse strain of E. granulosus and T. ovis. Echinococcus is not likely to be confused morphologically however.

H. taeniaeformis and T. pisiformis do not generally occur in the same host but T. pisiformis has occasionally been reported in the cat as well as in the dog (Abduladze, 1970). Glutamate dehydrogenase is difficult to see in gels but H. taeniaeformis and T. pisiformis can be clearly

distinguished using this enzyme.

T. solium and T. saginatus are easier to distinguish as the difference in GPI location is more obvious. This method was used during the Nigerian survey to differentiate samples and was found satisfactory. The difference was more obvious using a Tris/HCl buffer and the fast moving bands of HK had very different mobilities for these two species. T. saginatus and T. ovis do not occur in the same host but can be separated by their HK patterns.

Lowering the pH until the slower moving specimen progressed cathodally while the other still moved anodally to the point of application was considered as a possible method of separating two closely similar species. This method would have been useful for identifying a single specimen at a particular pH by its direction of mobility without recourse to relating this mobility to that of the other species. Unfortunately, however, at the iso-electric point very slight changes in pH will result in different directional movement. Slightly imperfect pH meters or some slight alteration in the pH of the buffer would therefore give a false result and thus the method was found to be unreliable.

Strain variation

Differences in GPI patterns were seen within the E. granulosus species. Seven horses from Belgium, England and Scotland had an identical pattern which was different from that of six cattle and three sheep from

Scotland and Wales. A third strain may be present in Nigerian camels. This confirms differences within the Echinococcus genus that have already been recognized by morphological and other biological studies. Smyth (1977) and Thompson (1977) reported differences in growth and maturation between British horse and sheep strains. Lee et al (1976) found differences in non-specific esterase patterns after iso-electric focussing, not only between E. granulosus and E. multilocularis, but also between sheep and horse strains of E. granulosus.

Whether these differences should constitute strain or sub-specific differences is a matter for morphologists to decide. It was very interesting in this respect to see a difference in GPI pattern between Dipylidium caninum found in the dog and the same species found in the cat (see Table 35). This difference requires confirmation by further study of more specimens but, if it is consistent, then it may be another example of strain difference in tapeworms.

# CHAPTER THIRTY

## SPECIES VARIATION AND CLASSIFICATION

### Interaction between host and parasite

Many hypotheses have been devised to explain the genetic interaction between parasite and host (Clark, 1976). It has been suggested that polymorphism in the host would be stimulated by antigenic mimicry by the parasite and also that convergent molecular evolution among different parasite species adapted to the same host could also occur (Damian, 1964). The ability of parasites to vary their phenotypic structure enables them to attack resistant hosts and this dynamic interaction between host and parasite stimulates polymorphism in both.

Some trypanosomes have a considerable capacity for antigenic variation. During an infection antigenic change can occur every few days thus rendering the host's immune response ineffective. There appears to be no limit to the number of antigens produced and some may be reproduced during an infection or many may be produced at the same time. Certain antigens may develop at a particular stage of infection and there may be a relatively ordered sequence of development in each infection (Gray & Luckins, 1976).

These antigens are not the result of mutation and selection as they revert to a "basic antigen" in the vector,

but are due to the ability of the trypanosome to vary surface antigens by the production of a range of glycoproteins in the surface coat, which is lost in the vector (Vickerman, 1974).

This phenomenon does not appear to exist with T. vivax as reversion to a basic antigen does not occur. This trypanosome has the ability to bind host serum proteins to its surface, thus possibly disguising itself (Vickerman, 1974).

The ability of cestode forms to absorb host proteins and to adsorb host enzymes onto the surface has already been mentioned. These phenomena and antigenic mimicry may complicate serological studies.

Evidence of interaction between host and parasite due to mutational changes may be detected by enzyme electrophoresis. For instance, hosts resistant to Plasmodium falciparum have a deficiency of glucose-6-phosphate dehydrogenase which adversely alters the environment for the parasite (Damian, 1964).

Since differences in enzyme structure occur independently of one another and, since there are several thousand gene loci implicated in enzyme synthesis, the chances are that each individual's enzyme constitution is unique (Hopkinson, 1970). This probably applies to all living organisms as enzyme polymorphism has been found in many species.

It is surprising that no differences were found between individual tapeworms, especially as in a few instances worms of the same species, but originating from different parts of the world, were compared. Tapeworms are hermaphrodites and sexual reproduction between worms is not known to occur (Wardle & McLeod, 1968). Divergence of characteristics would therefore seem to be less likely but Smyth (1977) postulated that a population of double recessives could develop very quickly in Echinococcus because of asexual reproduction in the hydatid cyst, where, if a recessive were present, it would replicate itself rapidly. If the characteristics of the recessive were more suitable to a new host species, the production of a new strain would then occur.

With the stable environment offered by a host species to which the tapeworm is already well adapted, divergence of strains is presumably unlikely to occur. Asexual reproduction, however, is not common to all the taeniid worms as it usually occurs only with those having a hydatid or a coenurus metacestode stage, although it has also been reported in vitro with T. pisiformis (Opuni, 1970). It is possible therefore that those taeniids that have cysticerci as larval forms may not be so adaptable.

Any mutants that occur would first develop from the egg through larval forms and if the mutation were not advantageous the larval form would not survive. Individual variation in adult tapeworms may therefore be rarely seen

if the worm is in its normal host, which has fed on its natural prey, the intermediate host.

Speciation occurs as a result of a population being split into at least two parts followed by sufficient genetic change in each sub-population to prevent inter-breeding. Each sub-population must have developed sufficient differences in niche preference to survive competition from another sub-population if or when it has to co-exist with it. This probably applies to speciation of all living organisms, though other methods of speciation applying to self fertilizing hermaphrodites may have been overlooked (Mayr, 1963).

Inglis (1965) pointed out that the presence of one species of nematode in a host is not necessarily indicative of it being the only specific parasite for that particular niche. One set of conditions, for instance the nutrition or health of the host, may favour one species whereas another set may favour another. One species in a niche may also merely indicate that it arrived first thereby excluding others.

This appears to be somewhat contradictory to the theory of speciation unless it can be regarded as a transient stage in the fight for dominance between species occupying the same niche.

The development of strains of Echinococcus into separate species would depend on complete separation of sub-populations

according to Mayr's theory. Sweatman & Williams (1963) thought that E. granulosus borealis was an indigenous Canadian sub-species whereas E. granulosus canadensis was introduced into Canada from Norway. Rausch (1967) refuted the establishment of separate sub-species as they occur in common hosts in the same geographical region. He argued that any genetic differences there may have been would have disappeared in the resulting common gene pool. This argument is difficult to understand unless one believes there is sexual reproduction between tapeworms.

Rausch considered the fox to be an unlikely natural definitive host for E. granulosus, and one can speculate on the origin of the horse and sheep strains in the United Kingdom. The strains may have developed in separate sylvatic cycles long ago, both eventually becoming adapted to the dog as the definitive host, or they could have developed rapidly in more recent times in different areas of the country where they adapted to a horse-dog or a sheep-dog cycle.

If both strains appear to occupy the same niche in the dog, they must be in direct competition with one another. If no sexual reproduction occurs between separate worms, then it is presumably possible for diversification between strains to proceed in the same host, but this could be frustrated by competition for the same niche. On the other hand, the gut environment of a dog

being fed on horse flesh may favour the survival of the equine strain of Echinococcus, whereas the gut environment of a dog being fed other meat may favour the ovine strain.

Classification

Tapeworms are well adapted to their natural definitive hosts and rarely cause serious clinical disease but it would appear that they retain an ability to diversify rapidly if necessary. The taeniid worms have each adapted themselves to a slightly different niche with regard to host species or tissue preference and, although very similar as a group antigenically and in many other ways, they are each distinguished by some morphological or biochemical characteristics. This is reflected, as this work has shown, in enzyme variation, some of which can be detected by electrophoresis. This enzyme variation can be used as a means of distinguishing individual species and strains. Biochemical classification may not necessarily agree with that based on morphological features but nevertheless it appears an alternative, perhaps less subjective, basis on which to distinguish groups of organisms.

There is much controversy on the classification of taeniid worms: whether Taeniarhynchus should be separated generically from Taenia; whether all the taeniid worms should be grouped under one generic name, excluding Echinococcus, or how Echinococcus should be sub-divided.

The work presented in this thesis offers some biochemical evidence for grouping the taeniid worms together on the one hand and for separating the species on the other, as each had its characteristic zymogram patterns. Within the group, Multiceps was at one extreme of the range of GPI patterns and T. pisiformis and H. taeniaeformis were at the other. With MDH and HK the positions were altered.

Differences in GPI patterns were as marked between E. granulosus strains as between the taeniid species.

Whether the generic divisions of Wardle et al are justified or whether it is justified to separate E. granulosus into sub-species remains a matter for taxonomists to decide but further study of iso-enzyme variation between all the tapeworm species would help to elucidate the relationships between them.

# BIBLIOGRAPHY

## BIBLIOGRAPHY

ABDUSSALAM, M., ACHA, P.N., AGOSIN, M., BLOOD, D.B., GEMMELL, M.A., KAGAN, I.G., KAPLAN, M.M., RAUSCH, R.L., SCHWABBE, C.W., SMYTH, J.D. & YAMASHITA, J. (1968) - "Research needs in echinococcosis (hydatidosis)" Bulletin of the World Health Organization 39: 101-113.

ABDULADZE, K.I. (1970) - "Essentials of Cestodology. Volume 4. Taeniata of Animals and Man and Diseases Caused by Them" edited by K.I. Skrjabin. English translation by A. Isseroff. 1st edition. U.S. Department of Agriculture.

AGOSIN, M. & ARAVENA, L. (1959) - "Studies on metabolism of E. granulosus. III. Glycolysis with special reference to hexokinase and related glycolytic enzymes" Biochimica et Biophysica Acta 34: 90-102.

AGOSIN, M. & ARAVENA, L. (1960) - "Studies on the metabolism of Echinococcus granulosus. IV. Enzymes of the pentose phosphate pathway" Experimental Parasitology 10: 28-38.

ANDERSON, R.I. (1963) - "VII. Current and potential value of immunodiagnostic tests employing whole organisms" in "Immunodiagnosis of Helminth Infections". Proceedings of the international panel workshop, University of Maryland Nov. 19-20, 1962. American Journal of Hygiene monographic series No. 22: 97-112.

ARME, C. (1966) - "Histochemical and biochemical studies on some enzymes of Ligula Intestinalis (Cestoda: Pseudophyllidea)" Journal of Parasitology 52: 63-68.

ANON. (1967) - "Joint FAO/WHO Expert Committee on Zoonoses. Third Report" Food and Agriculture Organization of the United Nations, Rome, Italy.

ANON.(1976) - "The National Hydatids Council 16th annual report" edited by C.J. Guild. Ministry of Agriculture and Fisheries, P.O. Box 2298, Wellington, New Zealand.

AVRAMEAS, S. & TERNYNCK, T. (1969) - "The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents" Immunochemistry 6: 53-66.

ARUNDEL, J.A. (1972) - "A review of cysticercosis of sheep and cattle in Australia" Australian Veterinary Journal 48: 140-155.

BAGSTER, I.A. & PARR, C.W. (1973) - "Trypanosome identification by electrophoresis of soluble enzymes" Nature 244: 364-366.

BAHNEMANN, H.G. (1976) - Inactivation of viruses in serum with binary ethyleneimine" Journal of Clinical Microbiology 3: 209-210.

BARRETT, J. (1975) - "The occurrence and intracellular distribution of nucleosidediphosphate kinase in parasitic helminths" Journal of Parasitology 61: 545-546.

BEARD, T.C. (1973) - "Observations for Icelanders on hydatids and precautions against them" Australian Veterinary Journal 49: 395-401.

BEGG, N.C. (1961) - "The campaign against hydatid disease: an experiment in social medicine" New Zealand Medical Journal 60: 229-234.

BEHM, C.A. & BRYANT, C. (1975a) - "Studies of regulatory mechanism in Moniezia expansa: general considerations" International Journal for Parasitology 5: 209-217.

BEHM, C.A. & BRYANT, C. (1975b) - "Studies of regulatory mechanism in Moniezia expansa: the role of phospho-fructokinase (with a note on pyruvate kinase)" International Journal for Parasitology 5: 339-346.

BEHM, C.A. & BRYANT, C. (1975c) - "Studies of regulatory mechanism in Moniezia expansa: the role of phosphoenolpyruvate carboxykinase" International Journal for Parasitology 5: 347-354.

BELTRAN-H., F. & GOMEZ-PRIEGO, Y.A. (1973) - "Evaluación de los contraimmunoelectrophoresis (CIEF) para la detección de anticueropos en la cisticercosis experimental y humana" Antioquia Medica 23: 472-473.

BENEX, J. (1974) - Diagnostic Immunologique des Parasitoses à Protozoaires et Helminthes 1st edition. Maloine S.A. Éditeur, Paris: 121-143.

BERGMEYER, H.U. (1965) - "Methods of Enzymatic Analysis" 2nd edition. Academic Press, New York and London: 993-994.

BEVERIDGE, I. & GREGORY, G.G. (1976) - "The identification of Taenia species from Australian carnivores" Australian Veterinary Journal 52: 369-373.

BIAGI, F. & TAY, J. (1958) - "A precipitation reaction for the diagnosis of cysticercosis" American Journal of Tropical Medicine and Hygiene 7: 63-65.

BIAGI, F.F. & PIÑA, A.P. (1964) - "Presence of antigens in calcareous corpuscles of Cysticercus" Revista do Instituto de Medecina Tropical de São Paulo 6: 114-116.

BIGUET, J., CAPRON, A., TRAN VAN KY, P. & d'HAUSSY, R. (1962) - "Étude immunoélectrophorétique comparée des antigènes de divers helminthes" Compte rendu hebdomadaire des séances de L'Académie d'agriculture de France 25: 3600-3602.

BLUNDELL-HASELL, S.K. (1971) Ph.D. Thesis, University of Otago, Dunedin, New Zealand, cited by Gemmell & Macnamara (1972): 249.

BOGDANOV, V.R. (1974) - "Histochemical studies of dehydrogenases in the integument of the cestode Hydatigera taeniaeformis" abstract in Helminthological Abstracts (1975) 44: 210.

BOID, R. (1974) - personal communication.

BOUT, D., DUGIMONT, J.C., FARAG, H. & CAPRON, A. (1975) - "Le diagnostic immunoenzymologique des affections parasitaires. II. Immunoenzymologie quantitative (ELISA)" Lille Médicale 20: 561-566.

BRYGOO, E.R., CAPRON, A. & RANDRIAMALALA, J.C. (1959) - "Sur quelque méthodes de coloration sélective des coques d'oeufs d'helminthes parasites de l'homme" Bulletin de la Société de Pathologie Exotique 52: 655-664.

BRYGOO, E.R. & RANDRIAMALALA, J.C. (1959) - "Différence de colorabilité au Ziehl entre les oeufs de Schistosoma mansoni et ceux de Schistosoma haematobium" Bulletin de la Société de Pathologie Exotique 52: 26.

CALAMEL, M. & SOULÉ, C. (1972) - "Choice and preparation of an antigen for the diagnosis of bovine cysticercosis by immunofluorescence" Revue de Médicine Vétérinaire 123: 1105-1104.

CAMPBELL, D.H., GARVEY, J.S., CREMER, N.E. & SUSSDORF, D.H. (1964) - "Methods in Immunology" 1st edition. W.A. Benjamin, Inc., New York and Amsterdam: 118-120.

CANN, J.R. & GOAD, W.B. (1968) - "Two or more electrophoretic zones from a single macromolecule" in "Multiple Molecular Forms of Enzymes" edited by E.M. Weyer. 1st edition. Annals of the New York Academy of Sciences New York 151: 638-649.

CAPRON, A., BIGUET, J., VERNES, A. & AFCHAIN, D. (1968) - "Structure antigénique des helminthes. Aspects immunologiques des relations hôte-parasite" Pathologie Biologie 16: 121-138.

CAPRON, A. & ROSE, F. (1962) - "The comparison of helminth eggs. II. Acid-alcohol fastness in cestodes. Differences in staining by Ziehl of the embryophores of T. saginata and T. solium" Bulletin de la Société de Pathologie Exotique 55: 765-767.

CAPRON, A., VERNES, A. & BIGUET, J. (1967) - "Le diagnostic immunophoretique de l'hidatidose" in "Le Kyste Hydatique du Foie" Journées Lyonnaises d'hydatidologie 1966. SIMEP, Lyon, France: 27-40.

CAPRON, A., WATTRE, P., CAPRON, M. & LEFEBVRE, M.N. (1972) "Le diagnostic biologique moderne des parasitoses humaines" La Revue du Practicien 22: 3661-3674.

CAPRON, A., YARZÁBAL, L., VERNES, A. & FRUIT, J. (1970) - "Le diagnostic immunologique de l'échinococcose humaine" Pathologie Biologie 18: 357-365.

CARTER, C.E. & FAIRBAIRN, D. (1975) - "Multienzymic nature of pyruvate kinase during development of Hymenolepis diminuta (Cestoda)" Journal of Experimental Zoology 194: 439-448.

CARTER, N.D. & PARR, C.W. (1967) - "Isoenzymes of phosphoglucose isomerase in mice" Nature 216: 511.

CARTER, R. (1970) - "Enzyme variation in Plasmodium berghei" Transactions of the Royal Society of Tropical Medicine and Hygiene 64: 401-406.

CARTER, R. (1973) - "Enzyme variation in Plasmodium berghei and Plasmodium vinckei" Parasitology 66: 297-307.

CARTER, R. (1974) - personal communication.

CARTER, R. & McGREGOR, I.A. (1973) - "Enzyme variation in Plasmodium falciparum in the Gambia" Transactions of the Royal Society of Tropical Medicine and Hygiene 67: 830-837.

CARTER, R. & VOLLER, A. (1973) - "Enzyme typing of malaria parasites" British Medical Journal 1: 149.

CASTAGNARI, L. & SORICE, F. (1971) - "L'immunoprecipitazione elettroforetica (crossed-over electrophoresis) nella diagnosi dell'idatidosi umana. Nota II" Bollettino dell'Instituto Sieroterapico Milanese 50: 99-106.

CHEAH, K.S. (1967) - "Histochemical and spectrophotometric demonstration of peroxidase in Moniezia expansa (Cestoda)" Comparative Biochemistry and Physiology 21: 351-355.

CHEN, H.T. (1950) - "The in vitro action of rat immune serum on the larvae of T. taeniaeformis" Journal of Infectious Diseases 86: 205.

CHOWDHURY, A.B. (1955) - "Histological and histochemical observations on Taenia saginata" Bulletin of the Calcutta School of Tropical Medicine 3: 143:144.

CLARK, B. (1976) - "The ecological genetics of host-parasite relationships" in "Genetic Aspects of Host-parasite Relationships" edited by A.E.R. Taylor and R. Muller. 1st edition. Blackwell Scientific Publications, Oxford 14: 87-103.

COLTORTI, E.A. & VARELA DÍAZ, V.M. (1974) - "Echinococcus granulosus: penetration of macromolecules and their localization in the parasite membranes of cysts" Experimental Parasitology 35: 225-231.

COMAN, B.J. (1973) - "Helminth parasites of the fox (Vulpes vulpes) in Victoria" Australian Veterinary Journal 49: 378-384.

CORNISH, R.A. & BRYANT, C. (1975) - "Studies of regulatory mechanism in Moniezia expansa: glutamate, and the absence of the γ-aminobutyrate pathway" International Journal for Parasitology 5: 355-362.

COUDERT, J., AMBROISE-THOMAS, P., DESPEIGNES, J., CADI-SOUSSI, M. & KIEN TRUONG, T. (1966) - "Diagnostic sérologique de l'échinococcose alvéolaire par immuno-fluorescence" Bulletin de la Société de Pathologie Exotique 59: 859-865.

COUDERT, J., AMBROISE-THOMAS, P., TRUONG THAI, K. & POTHIER, M.A. (1967) - "Premiers résultats concernant le diagnostic sérologique du kyste hydatique par une nouvelle technique d'immuno-fluorescence sur lames" Bulletin de la Société de Pathologie Exotique 60: 555-563.

CROWLE, A.J. (1961) - "Immunodiffusion" 1st edition. Academic Press, London.

DAMIAN, R.T. (1964) - "Molecular mimicry: antigen sharing by parasite and host and its consequences" The American Naturalist 98: 129-149.

DAOC, ARNAULT, J.P., PETITHORY, J. & BRUMPT, L. (1972) - "L'immunofluorescence indirecte sur coupes de T. solium dans le diagnostic de la cysticercose: Résultats obtenus chez 35 maladies" La Nouvelle Presse Médicale 1: 2049-2050.

DAUGHERTY, J.W. (1955) - "Intermediary protein metabolism in helminths. III. The l-amino acid oxidase in Hymenolepis diminuta and some effects of changes in host physiology" Experimental Parasitology 4: 455-463.

DAWSON, R.M.C., ELLIOT, D.C., ELLIOT, W.H. & JONES, K.M. (1969) - "Data for Biochemical Research" 2nd edition. Clarendon Press, Oxford: 501.

DE LEY, J. & VERCRUYSSE, R. (1955) - "Glucose-6-phosphate and gluconate-6-phosphate dehydrogenase in worms" Biochimica et Biophysica Acta 16: 615-616.

DENHAM, D.A., RIDLEY, D.S. & VOLLER, A. (1971) - "Immunodiagnosis of parasitic diseases" Practitioner 207: 191-196.

DENT, C.H.R. & KELLY, J.D. (1974) - "Cestode parasites of the fox in the central tablelands of New South Wales" Australian Veterinary Journal 50: 176-177.

DE PITA, R.V., VELEZ-PRATT, G.V., & BIAGI, F.F. (1965) - "Immunofluorescence detectable antigens in calcareous corpuscles of Cysticercus cellulosae" Revista de la Facultade de Medicina México 7: 379-383.

DETTER, J.C., WAYS, P.O., GIBLETT, E.R., BAUGHAN, M.A., HOPKINSON, D.A., POVEY, S. & HARRIS, H. (1968) - "Inherited variation in human phosphohexose isomerase" Annals of Human Genetics, London 31: 329-338.

DOBSON, C. (1972) - "Immune response to gastrointestinal helminths" in "Immunity to Animal Parasites" edited by E.J.L. Soulsby. 1st edition. Academic Press, London and New York: 191-216.

DRURY, R.A.B. & WALLINGTON, E.A. (1967) - Carleton's Histological Technique". 4th edition. Oxford University Press, New York and Toronto: 334-335.

ELLMAN, G.L., COURTNEY, K.D., ANDRES, V. & FEATHERSTONE, R.M. (1961) - "A new and rapid colorimetric determination of acetylcholinesterase activity" Biochemical Pharmacology 7: 88-95.

ENYENIHI, U.K. (1970) - "Immunodiagnosis of infections in bovine and humans" 1969/70 report on the WHO/FAO Cysticercosis-Taeniasis Project. University of Ibadan, Ibadan, Nigeria.

ENGVALL, E. & PERLMANN, P. (1972) - "Enzyme-linked immunosorbent assay, ELISA" Journal of Immunology 109: 129-135.

ENIGK, K. & STICINSKY, E. (1957) - "Über die Bohrdrüsen der Onkosphäre von Davainea proglottina (Cestoda)" Zeitschrift für Parasitenkunde 18: 48-54.

ERASMUS, D.A. (1956a) - "Studies on phosphatase systems of cestodes. 1. Studies on Taenia pisiformis (cysticercus and adult)" Parasitology 46: 70-80.

ERASMUS, D.A. (1956b) - "Studies on phosphatase systems of cestodes. II. Studies on Cysticercus tenuicollis and Moniezia expansa (adult)" Parasitology 46: 81-90.

ESCH, G.W. & SELF, J.T. (1965) - "A critical study of the taxonomy of Taenia pisiformis Bloch, 1780; Multiceps multiceps (Leske, 1780); and Hydatigera taeniaeformis Batsch, 1786" Journal of Parasitology 51: 932-937.

EUZÉBY, J. & DUBRA, M. (1970) - "Diagnostic "ante mortem" de la cysticercose musculaire bovine (ladrerie) à Cysticercus bovis par la méthode de l'immuno-fluorescence" Bulletin de la Société des Sciences Vétérinaires et de Médecine Comparée de Lyon 72: 507-516.

FAHEY, J.L. & TERRY, E.W. (1974) - "Ion exchange chromatography and gel diffusion" in "Handbook of Experimental Immunology" edited by D.M. Weir. 2nd edition. Blackwell Scientific Publications, Edinburgh: 8.1-8.23.

FARAG, H., BOUT, D. & CAPRON, A. (1975) - "Specific immunodiagnosis of human hydatidosis by the enzyme linked immunosorbent assay (ELISA)" Biomedicine 23: 276-278.

FEFEVERE, S. (1952) - "Sur la phosphatase alcaline chez les cestodes" Bulletin de l'Institut Royal des Sciences Naturelles de Belgique 27: 1-4.

FIFE, E.H. (1971) - "Advances in methodology for immunodiagnosis of parasitic diseases" Experimental Parasitology 30: 132-163.

FITCH, L.I., PARR, C.W. & WELCH, S.G. (1968) - "Phosphoglucose isomerase variation in Man" Proceedings of the Biochemical Society 110: 56P.

FOSTER, W.D. (1965) - "A History of Parasitology". 1st edition. E. and S. Livingstone Ltd., Edinburgh and London.

GALLIE, G.J. & SEWELL, M.M.H. (1970) - "A technique for hatching Taenia saginata eggs" Veterinary Record 86: 749.

GALLIE, G.J. & SEWELL, M.M.H. (1974a) - "The serological response of three month old calves to infection with Taenia saginata (Cysticercus bovis) and their resistance to reinfection" Tropical Animal Health and Production 6: 163-171.

GALLIE, G.J. & SEWELL, M.M.H. (1974b) - "The serological response of calves infected neonatally with Taenia saginata (Cysticercus bovis)" Tropical Animal Health and Production 6: 173-177.

GALLIE, G.J. & SEWELL, M.M.H. (1976) - "Experimental immunisation of six-month old calves against infection with the cysticercus stage of Taenia saginata" Tropical Animal Health and Production 8: 233-242.

GEMMELL, M.A. (1973) - "Hydatid disease in New Zealand. The first 100 years (1873 to 1972)" in "Recent advances in hydatid disease". Proceedings of the Hamilton Medical Veterinary Association symposium, October 28th-29th, 1973, Hamilton, Victoria, Australia: 54-61.

GEMMELL, M.A. & JOHNSTON, P.D. (1977) - "Experimental epidemiology of hydatidosis and cysticercosis" in "Advances in Parasitology" edited by B. Dawes. 1st edition. Academic Press, London 15: 312-385.

GEMMELL, M.A. & MACNAMARA, F.N. (1972) - "Immune response to tissue parasites. II. Cestodes" in "Immunity to Animal Parasites" edited by E.J.L. Soulsby. 1st edition. Academic Press, London and New York: 235-272.

GEMMELL, M.A. & SOULSBY, E.J.L. (1968) - "The development of acquired immunity to tapeworms and progress towards active immunization, with special reference to Echinococcus spp." Bulletin of the World Health Organization 39: 45-55.

GIUNCHI, G. (1960) - "Stato attuale della diagnosi biologica dell'idatidosi" Il Progresso Medico 16: 701-711.

GNEZDILOV, V.G. (1957) - "The golden hamster (Mesocricetus auratus Waterhouse) as a potential definitive host of the tapeworm Taenia solium" cited by A.J.M. Verster (1971).

GOEZE, J.A.E. (1782) - "Versuch einer Naturgeschichte der Eingeweidewürmer thierischer Körper". Printed by P.A. Pope, Blankenburg, Germany: 42 and 278.

GRAY, A.R. & LUCKINS, A.G. (1976) - "Antigenic variation in salivarian trypanosomes" in "Biology of Kinetoplastida" edited by W.H.R. Lumsden and D.A. Evans. 1st edition. Volume 1. Academic Press, London: 493-542.

GREGORY, G.G. (1976) - "Fecundity and proglottid release of T. ovis and T. hydatigena" Australian Veterinary Journal 52: 277-279.

GUISANTES, J.A., YARZÁBAL, L.A., VARELA-DÍAZ, V.M., RICARDES, M.I. & COLTORTI, E.A. (1975) - "Standardization of the immunoelectrophoresis test with whole and purified hydatid cyst fluid antigen for the diagnosis of human hydatidosis" Revista do Instituto de Medicina Tropical de São Paulo 17: 69-74.

HALL, M.C. (1919) - "The adult taenioid cestodes of dogs and cats, and of related carnivores of North America" Proceedings of the United States National Museum (1920) 55: 1-94.

HARRIS, H. & HOPKINSON, D.A. (1976) - "Handbook of Enzyme Electrophoresis in Human Genetics" 1st edition. North-Holland Publishing Company, Oxford.

HARRISON, L.J.S. (1976) - personal communication.

HARRISON, L.J.S. (1977) - personal communication.

HATCH, C. & SMYTH, J.D. (1975) - "Attempted infection of sheep with Echinococcus granulosus equinus" Research in Veterinary Science 19: 340.

HEATH, D.D. (1970) Ph.D. Thesis, Australian National University, Canberra, cited by Gemmell & Macnamara (1972): 249.

HEATH, D.D. (1976) - personal communication.

HEYNEMAN, D. & VOGE, M. (1960) - "Succinic dehydrogenase activity in cysticercoids of Hymenolepis (Cestoda: Hymenolepididae) measured by tetrazolium technique" Experimental Parasitology 9: 14-17.

HEYNEMAN, D. & WELSH, J. (1959) - "Action of homologous antiserum in vitro against life cycle stages of Hymenolepis nana, the dwarf mouse tapeworm" Experimental Parasitology 8: 119-129.

HILES, I. (1926) - "Serological studies on hydatid" Journal of Helminthology 4: 143-178.

HOEPPLI, R. (1956) - "The knowledge of Parasites and Parasitic infections from ancient times to the 17th century" Experimental Parasitology 5: 398-419.

HOLBOROW, E.J. & JOHNSON, G.D. (1967) - "Immunofluorescence" in "Handbook of Experimental Immunology" edited by D.M. Weir. 1st edition. Blackwell Scientific Publications, Oxford and Edinburgh: 571-596.

HOPKINSON, D.A. (1970) - "A systematic approach to the study of enzyme variation in man" in "Modern Trends in Human Genetics - 1" edited by A.E.H. Emery. Butterworths, London: 42-67.

HUSTEAD, S.T. & WILLIAMS, J.F. (1977) - "Permeability studies on taeniid metacestodes: 1. Uptake of proteins by larval stages of Taenia taeniaeformis, T. crassiceps, and Echinococcus granulosus" The Journal of Parasitology 63: 314-321.

INGLIS, W.G. (1965) - "Patterns of evolution in parasitic nematodes" in "Evolution of Parasites". Third symposium of the British Society for Parasitology edited by A.E.R. Taylor. 1st edition. Blackwell Scientific Publications, Oxford: 79-124.

JACKSON, P.J. & ARUNDEL, J.H. (1971) - "The incidence of tapeworms in rural dogs in Victoria" Australian Veterinary Journal 47: 46-53.

JELNES, E.J. (1974) - "Genetics of three isoenzyme systems in Aricia artaxerxes F. (Lep.Rhophalocera)" Hereditas 76: 79-82.

JELNES, E.J. (1975) - "Electrophoretic studies on two sibling species Thera variata and Thera obeliscata (Lepidoptera, Geometridae) with special reference to phosphoglucomutase and phosphoglucose isomerase" Hereditas 79: 67-72.

JOHNSON, G.D. & HOLBOROW, E.J. (1973) - "Immunofluorescence" in "Handbook of Experimental Immunology" edited by D.M. Weir. 2nd edition. Blackwell Scientific Publications, Oxford and Edinburgh: 18.1-18.2.

KAGAN, I.G. (1968) - "A review of serological tests for the diagnosis of hydatid disease" Bulletin of the World Health Organization 39: 25-37.

KAGAN, I.G. (1974) - "Advances in the immunodiagnosis of parasitic infections" Zeitschrift für Parasitenkunde 45: 163-195.

KAGAN, I.G. & NORIMAN, L. (1974) - "Serodiagnosis of parasitic diseases" in "Manual of Clinical Microbiology" edited by E.H. Lennette, E.H. Spalding and J.P. Truant. 2nd edition. American Society for Microbiology, Washington D.C., U.S.A.

KAGAN, I.G. & PELLEGRINO, J. (1961) - "A critical review of immunological methods for the diagnosis of bilharziasis" Bulletin of the World Health Organization 25: 611-674.

KAMALOVA, A.G. (1953) - "Comparative study of eggs of Taeniarhynchus saginatus and Taenia solium" Raboty po Gel'minthologii K 75-Letiiu Akademia K.I. Skrjabina. Akad Nauk, Moscow, U.S.S.R.: 278-285.

KAPLAN, N.O. (1968) - "Nature of multiple molecular forms of enzymes" in "Multiple Molecular Forms of Enzymes" edited by E.M. Weyer. 1st edition. Annals of the New York Academy of Sciences, New York 151: 638-649.

KAWAOI, A. & NAKANE, P.K. (1973) - "An improved method of conjugation of peroxidase with proteins" Federation Proceedings 32: 840.

KENT, J.F. (1963) - "VI. Current and potential value of immunodiagnostic tests employing suitable antigens" American Journal of Hygiene Monographic Series No. 22, University of Maryland, U.S.A.: 68-96.

KILEJIAN, A., SCHINAZI, L.A. & SCHWABBE, C.W. (1961) - "Host-parasite relationships in echinococcosis. V. Histochemical observations on Echinococcus granulosus" Journal of Parasitology 47: 181-185.

KILGOUR, V., GARDENER, P.J., GODFREY, D.G. & PETERS, W. (1974) - "Demonstration of electrophoretic variation of two aminotransferases in Leishmania" Annals of Tropical Medicine and Parasitology 68: 245-246.

KILGOUR, V. & GODFREY, D.G. (1973) - "Species characteristic isoenzymes of two aminotransferases in Trypanosomes" Nature New Biology 244: 69-70.

KILLICK-KENDRICK, R. (1974) - "Parasitic protozoa of the blood of rodents: a revision of Plasmodium berghei" Parasitology 69: 225-237.

KLOETZEL, K. (1959) - "A reação de precipitação periovular na esquistossomose. II. Correlação aos dados clínicos" Revista do Instituto de Medecina Tropical de São Paulo 1: 129-137.

KONOVALOVA, L.M. (1973) - "Development and application of new methods for serodiagnosis of cysticerciasis in man" Meditsinskaya Parazitologiya i parazitarnye Boleznie 42: 536-542.

LEE, D.L., ROTHMAN, A.H. & SENTURIA, J.B. (1963) - "Esterases in Hymenolepis and Hydatigera" Experimental Parasitology 14: 285-295.

LEE, D.L. & TATCHELL, R.J. (1964) - "Studies on the tapeworm Anaplocephala perfoliata (Goeze, 1782)" Parasitology 54: 467-479.

LEE, D.L., THOMPSON, R.C.A. & DAVIES, C.A. (1976) - cited by Smyth (1977).

LEIBY, P.D. & KRITSKY, D.C. (1972) - "Echinococcus multilocularis: a possible domestic life cycle in central North America and its public health implications" Journal of Parasitology 58: 1213-1215.

LE RICHE, P.D. & SEWELL, M.M.H. (1977) - "Differentiation of Taenia saginata and Taenia solium by enzyme electrophoresis" Transactions of the Royal Society of Tropical Medicine and Hygiene 71: 327-328.

LICHTENBERG, F. & LINDENBERG, M. (1954) - "An alcohol-acid-fast substance in eggs of Schistosoma mansoni" American Journal of Tropical Medicine 3: 1066-1076.

LUCKINS, A.G. (1975) - personal communication.

MACHNICKA, B. (1973) - "The indirect immunofluorescence test with Taenia saginata onchospheres to diagnose Cysticercus bovis infection in calves" Bulletin de l'Académie Polonaise des Sciences Biologiques" 21: 743-746.

McCARTHY, D.H. (1974) - "Zone electrophoresis of microbial proteins" Medical Laboratory Technology 31: 287-291.

MARKERT, C.L. & MØLLER, F. (1959) - "Multiple forms of enzymes: tissue, ontogenetic, and species specific patterns" Biochemistry 45: 753-763.

MASTERS, C.J. & HOLMES, R.S. (1974) - "Isoenzymes, Multiple Enzyme Forms, and Phylogeny" edited by C. Lowenstein. 1st edition. Academic Press, London 5: 109-195.

MASTERS, C.J. & HOLMES, R.S. (1975) - "Frontiers of Biology. Volume 42. Haemoglobin, Isoenzymes and Tissue Differentiation" edited by A. Neuberger and E.L. Tatum. 1st edition. North-Holland Publishing Company, Oxford.

MATTENHEIMER, H. (1976) - "The Theory of Enzyme Tests" Boehringer Mannheim GmbH, Diagnostica, West Germany.

MAYR, E. (1963) - "Animal Species and Evolution". 1st edition. The Belknap Press of Harvard University Press. Oxford University Press, London: 424-515.

MEERA KHAN, P. (1971) - "Enzyme electrophoresis on cellulose acetate gel: zymogram patterns in man-mouse and man-chinese hamster somatic cell hybrids" Archives of Biochemistry and Biophysics 145: 470-483.

MELDRUM, G.K. & McCONNELL, J.D. (1968) - "The control of hydatid disease in Tasmania" Australian Veterinary Journal 44: 212-217.

MILLER, L.H. & BROWN, H.W. (1969) - "The serologic diagnosis of parasitic infections in medical practice" Annals of Internal Medicine 71: 983-992.

MORRIS, N., PROCTOR, E.M. & ELSDON-DEW, R. (1968) - "A physico-chemical approach to the serological diagnosis of cysticercosis" Journal of the South African Veterinary Medical Association 39: 41-43.

MOSS, D.W. & BUTTERWORTH, P.J. (1974) - "Enzymology and Medicine" 1st edition. Pitman Medical Publishing Company Ltd., London.

MOVESIJAN, M. & MLADENOVIĆ, Ž. (1971) - "The possibility of using different developmental stages of Echinococcus granulosus for detection of the specific antibodies against the parasite" Veterinarski Glasnik 25: 159-163.

MULLER, R. (1975) - "Worms and Disease. A Manual of Medical Helminthology" 1st edition. William Heinemann Medical Books Ltd., London.

MUTHUKRISHNAN, S. (1975) - "Peroxidase uptake through the tegument of the cestode Taenia hydatigena" Experimentia 31: 1036-1037.

NAKAGAWA, Y. & NOLTMAN, E.A. (1967) - "Multiple forms of yeast phosphoglucose isomerase. 1. Resolution of the crystalline enzyme into three isoenzymes" Journal of Biological Chemistry 242: 4782-4788.

NAKANE, P.K. (1975) - "Recent progress in the peroxidase-labelled antibody method" Annals of the New York Academy of Sciences 254: 203-211.

NEWSOME, J.(1958) - "Species specific serological tests for bilharzia" Annals of Tropical Medicine and Parasitology 52: 82-92.

OLIVIER, L.J.(1974) - "The economis of human parasitic infections" Zeitschrift für Parasitenkunde 45: 197-210.

OLIVER-GONZÁLEZ, J.(1954) - "Anti-egg precipitins in the serum of humans infected with Schistosoma mansoni" Journal of Infectious Diseases 95: 86-91.

OLIVER-GONZÁLEZ, J., BAUMAN, P.M. & BENENSON, A.S. (1955a) - "Immunological aspects of infections with Schistosoma mansoni" American Journal of Tropical Medicine and Hygiene 4: 443-452.

OLIVER-GONZÁLEZ, J., BAUMAN, P.M. & BENENSON, A.S. (1955b) - " "Species specificity of the anti-egg precipitin in Schistosome serums" Journal of Infectious Diseases 96: 95-100.

OPUNI, E.K. (1970) - "Asexual multiplication in Cysticercus pisiformis (Cestoda)" Journal of Helminthology 54: 321-322.

PANAITESCO, D. (1965) - "Contribution à l'étude de la fluorescence primaire du kyste hydatique" Archives Roumaines de Pathologie Expérimentale et de Microbiologie 24: 459-466.

PAPPAS, P.W. & READ, C.P. (1975) - "Membrane transport in helminth parasites: a review" Experimental Parasitology 37: 469-530.

PARKER, R.D. & MacINNIS, A.J. (1977) - "Hymenolepis diminuta: isolation, purification and reconstruction in vitro of a cell-free system for protein synthesis" Experimental Parasitology 41: 2-16.

PAULUZZI, S. (1970) - "The diagnosis of human hydatid disease by the fluorescent antibody test". Proceedings of the second international congress of parasitology 6-12 Sept. 1970. Journal of Parasitology 56: 260-261.

PAWLOWSKI, Z.& SCHULTZ, M.G. (1972) - "Taeniasis and cysticercosis (Taenia saginata)" in "Advances in Parasitology" edited by B. Dawes 10: 269-343. Academic Press, London and New York.

PÉREZ FONTANA, V. (1968) - "Unidad o dualidad de la Taenia echinococcus" Helminthologia 9: 467-489.

PHARMACIA FINE CHEMICALS AB (1972) - "Sephadex. Gel filtration in Theory and Practice" Pharmacia Fine Chemicals, Box 175, S-751 04 Uppsala 1, Sweden.

PHIFER, K. (1958) - "Aldolase in the larval form of Taenia crassiceps" Experimental Parasitology 7: 269-275.

POLYDOROU (1977) - "The anti-echinococcus campaign in Cyprus" Tropical Animal Health and Production 9: 141-146.

POZZNOLI, R., COSTANZI, G., DEIANA, S. & TAMBURINI, G. (1965) - "Localizzazione degli antigeni nella cisti di Echinococcus granulosus. Studio isto-immunologico nel della idatidosi umana" Rivista di Parassitologia 26: 85-94.

PROCTOR, E.M. (1972) - "Identification of Tapeworms" South African Medical Journal 46: 234-238.

PULLAR, E.M. (1946) - "A survey of Victorian canine and vulpine parasites" Australian Veterinary Journal 22: 12-21.

PYLKKÖ, O.O. (1956a) "Cholinesterase in Diphyllobothrium latum and Taenia saginata" Annales Medicinae Experimentalis et Biologiae Fenniae (Helsinki) 34: 328-334.

PYLKKÖ, C.O. (1956b) - "Cholinesterase in Diphyllobothrium latum and Taenia saginata" Annales Medicinae Experimentalis et Biologiae Fenniae (Helsinki) 34; Supplement 8: 1-81.

QUILICI, M., ASSADOURIAN, Y. & RANQUE, P. (1971) - "Le diagnostic immunologique de l'hydatidose" Médicine Tropicale 31: 207-213.

RAUSCH, R.L. (1967) - "A consideration of infraspecific categories in the genus Echinococcus Rudolphi, 1801 (Cestoda: Taeniidae)" The Journal of Parasitology 53: 484-491.

RAUSCH, R.L. (1968) - "Taxonomic characters in the genus Echinococcus (Cestoda: Taeniidae)" Bulletin of the World Health Organization 39: 1-4.

RAUSCH, R.L. (1975) - "Taeniidae" in "Disease Transmitted from Animals to Man" edited by W.T. Hubbert, W.F. McCulloch and P.R. Schnurrenberger. 6th edition. C.C. Thomas, Springfield, Illinois, U.S.A.: 678-707.

RAUSCH, R.L. & NELSON, G.S. (1963) - "A review of the genus Echinococcus Rudolphi, 1801" Annals of Tropical Medicine and Parasitology 57: 127-135.

READ, C.P. (1949) - "Preliminary studies on the intermediary metabolism of the cestode Hymenolepis diminuta" Journal of Parasitology 35: Supplement 26-27.

READ, C.P. (1973) - "Contact digestion in tapeworms" Journal of Parasitology 59: 672-677.

REEVES, R.E. & BISCHOFF, J.M. (1968) - "Classification of Entamoeba species by means of electrophoretic properties of amoebal enzymes" Journal of Parasitology 54: 594-600.

RHODES, M.B., FERGUSON, D.L. & MARSH, C.L. (1970) - "Haemonchus contortus: Enzymes. 1. Isoelectric focusing of malate dehydrogenase in sucrose and in polyacrylamide gels" Experimental Parasitology 28: 194-209.

ROGERS, W.P. (1947) - "Histological distribution of alkaline phosphatase in helminth parasites" Nature 159: 374-375.

ROTHMAN, A.H. (1966) - "Ultrastructural studies of enzyme activity in the cestode cuticle" Experimental Parasitology 19: 332-338.

ROTHMAN, A.H. & LEE, D.L. (1963) - "Histochemical demonstration of dehydrogenase activity in the cuticle of cestodes" Experimental Parasitology 14: 333-336.

RUITENBERG, E.J., STEERENBERG, P.A. & BROSI, B.J.M. (1975) - "Micro-system for the application of ELISA (Enzyme-Linked Immunosorbent Assay) in the serodiagnosis of Trichinella spiralis infections" Medikon Nederland 4: 30-31.

RYDZEWSKI, A.K., CHISHOLM, E.S. & KAGAN, I.G. (1975) - "Comparison of serologic tests for human cysticercosis by indirect haemagglutination, indirect immunofluorescent antibody and agar gel precipitin tests" Journal of Parasitology 61: 154-155.

SAKUMOTO, D., TONGU, Y., SUGURI, S., ITANO, D., INATOMI, S., TANAKA, H. & MATSUDA, H. (1972) - "Ultrastructural observation of the circumoval precipitation of Schistosoma japonicum" in "Research in Filariasis and Schistosomiasis" edited by M. Yokogawa. 1st edition. University Park Press, London. 2: 199-209.

SCHARDEIN, J.L. & WAITZ, J.A. (1965) - "Histochemical studies of esterases in the cuticle and nerve cords of four cyclophyllidean cestodes" The Journal of Parasitology 51: 356-363.

SCHILLER, E.L. (1967) - "Progress and problems in the immunodiagnosis of helminth infections" Advances in Clinical Chemistry 9: 43-68.

SCHWABBE, C.W. (1959) - "Host-parasite relationships in Echinococcus. I. Observations on the permeability of the hydatid cyst wall" American Journal of Tropical Medicine and Hygiene 8: 20-27.

SCHWABBE, C.W. (1968) - "Epidemiology of echinococcosis" Bulletin of the World Health Organization 39: 131-135.

SCHWABBE, C.W. (1969) - "Veterinary Medicine and Human Health" 2nd edition. Bailière, Tindall and Cassel, London.

SEDON, H.R. (1967) - "Diseases of Domestic Animals in Australia. Part I. Helminth Infestations" 2nd edition revised by H.E. Albiston. Service Publication No. 5, Department of Health, Commonwealth of Australia.

SHAW, C.R. & PRASAD, R. (1970) - "Starch gel electrophoresis of enzymes - a compilation of recipes" Biochemical Genetics 4: 277-320.

SHAW, C.R. & KOEN, A.L. (1968) - "Starch gel zone electrophoresis of enzymes" in "Chromatographic and Electrophoretic Techniques" edited by I. Smith. 2nd edition. Volume 2. William Heinemann Medical Books Ltd., London: 325-364.

SHIRLEY, M.W. & LEE, D.L. (1977) - "Isoelectric focusing of coccidial enzymes" Journal of Parasitology 63: 390-392.

SHUMAKOVICH, E.E. (1958) - "Problems and prospects of the control of coenuriasis in the U.S.S.R." cited by Abduladze (1970).

SILVERMAN, P.H. (1955) - "A technique for studying the in vitro effect of serum on activated taeniid hexacanth embryos" Nature 76: 598.

SLAIS, J. (1967) - "The morphology of Cysticercus racemosus and the determination of the Cysticercus species" Folia Parasitologica (Praha) 14: 27-34.

SLAIS, J. (1970) - "The Morphology and Pathogenicity of the Bladder Worm. Cysticercus cellulosae and Cysticercus bovis". Translated by E. Kalinová. Published by Dr. W. Junk N.V., The Hague, Netherlands.

SMORODINZEW, I.A. & BÈBÈCHINE, K.V. (1936) - "Les protéinases des ténias" Bulletin de la Société de Chimie Biologique 18: 1095-1105.

SMITH, I. (1968) - "Techniques of starch gel electrophoresis" in "Chromatographic and Electrophoretic Techniques" edited by I. Smith. 2nd edition. Volume 2. William Heinemann Medical Books Ltd., London: 217-238.

SMYTH, J.D. (1967) - "Studies on tapeworm physiology. XI. In vitro cultivation of Echinococcus granulosus from the protoscolex to the strobilate stage" Parasitology 57: 111-133.

SMYTH, J.D. (1969) - "The Physiology of Cestodes". 1st edition. Oliver and Boyd, Edinburgh.

SMYTH, J.D. (1977) - "Strain differences in Echinococcus granulosus with special reference to the status of equine hydatidosis in the United Kingdom" Transactions of the Royal Society of Tropical Medicine and Hygiene 71: 93-108.

SMYTH, J.D. & SMYTH, M.M. (1964) - "Natural and experimental hosts of E. granulosus and E. multilocularis, with comments on the genetics of speciation in the genus Echinococcus" Parasitology 54: 493-514.

SOPHIANOPOULOS, A.J. & VESTLING, C.S. (1960) - "Nature of the two forms of malic dehydrogenase from rat liver" Biochemica et Biophysica Acta 45: 400-402.

SORICE, F., CASTAGNARI, L. & TOLU, A. (1966) - "La diagnosi biologica dell'idatidosi umana" Giornale di Malattie Infettive e Parassitarie 18: 192-228.

SOULÉ, C., CALAMEL, M., CHEVRIER, L. & PANTALEON, J. (1971) - "La cysticercose bovine expérimentale: aspects parasitologique immunologique et hématologique" Recueil de Médicine Vétérinaire de l'École d'Alfort 147: 1247-1257.

SOUSA, O.E. & THATCHER, V.E. (1969) - "Observations on the life-cycle of Echinococcus oligarthrus (Diesing, 1863) in the Republic of Panama" Annals of Tropical Medicine and Parasitology 63: 165-175.

STEIGER, R., KRASSNER, S.M. & JENNI, L. (1974) - "Comparison of specific and relative alanine and aspartate amino-transferases of Trypanosoma brucei subgroup trypanosomes" Acta Tropica 31: 202-218.

STILES and HASSELL (1912) - "Cestoda and Cestodaria" Hygienic Laboratory Bulletin No. 85, Government Printing Office, Washington: 399 and 428.

SUTTON, R.J. (1974) - "Observations on experimental infections of dogs with Taenia ovis" Research in Veterinary Science 17: 200-213.

SUTTON, R.J. (1976) - "The effect of corticosteroids on the cysticerci of Taenia ovis in sheep" Research in Veterinary Science 21: 354-355.

SVIRIDENKO-STEPANKOVSKAYA, L.P., KONOVALOVA, L.M., POLETAEVA, O.G. & SOKOLOVSKAYA, O.M. (1972) - "Detection of group antigens in echinococcal fluid and reconstituted lyophilized extracts of Cysticercus cellulosae, C. bovis, Taenia saginata and Ascaris suum" Medidsinskaya Parazitologiya: Parazitarnie Boleznie. U.S.S.R. 41: 131-135.

SWEATMAN, G.K. & HENSHALL, T.C. (1962) - "The comparative biology and morphology of Taenia ovis and Taenia krabbei, with observations on the development of T. ovis in domestic sheep" Canadian Journal of Zoology 49: 1287-1311.

SWEATMAN, G.K. & WILLIAMS, R.J. (1963) - "Comparative studies on the biology and morphology of Echinococcus granulosus from domestic livestock, moose and reindeer" Parasitology 53: 339-390.

TEMPLETON, A.C. (1968) - "Human coenurus infection. A report of 14 cases from Uganda" Transactions of the Royal Society for Tropical Medicine and Hygiene 62: 251-255.

TEMPLETON, A.C. (1971) - "Anatomical and geographical location of human coenurus infection" Tropical Geographical Medicine 23: 105-108.

TERRY, R.J. (1968) - "Application of immunology to helminth diseases" in "Immunity to Parasites", 6th symposium of the British Society for Parasitology edited by A.E.R. Taylor. 1st edition. Blackwell Scientific Publications, Edinburgh: 91-103.

THATCHER, V.E. (1970) - "Studies on neotropical echinococcosis in Panama and Colombia" Journal of Parasitology 56: 342.

THATCHER, V.E. & SOUSA, O.E. (1966) - "Echinococcus oligarthrus Diesing, 1863, in Panama and a comparison with a recent human hydatid" Annals of Tropical Medicine and Parasitology 60: 405-416.

THOMPSON, G.A. (1970) - "The anthelmintic value of arecoline hydrobromide and bunamidine hydrochloride for use against Echinococcus in the field". FAO/WHO Inter-regional seminar on control of echinococcosis (hydatidosis), Ramos Mejia (Buenos Aires), 14-19 September, 1970. World Health Organization, Geneva.

THOMPSON, R.C.A. (1977) - "Growth, segmentation and maturation of the British horse and sheep strains of Echinococcus granulosus in dogs" International Journal for Parasitology 7: 281-285.

THOMPSON, R.C.A. & SMYTH, J.D. (1976) - "Attempted infection of the rhesus monkey (Macaca mulatta) with the British horse strain of Echinococcus granulosus" Journal of Helminthology 50: 175-177.

THORNE, C.J.R., GROSSMAN, L.I. & KAPLAN, N.O. (1963) - "Starch gel electrophoresis of malate dehydrogenase" Biochimica et Biophysica Acta 73: 193-203.

TORRES, J.M., GUISANTES, J., ALVAREZ, I. & YARZÁBAL, L.A. (1973) - "Contribution de la electrosineresis al diagnostico immunologico de la hidatidosis" Boletin de la Oficina Sanitaria Panamericana 75: 119-126.

VANNI, V. & RADICE, J.C. (1952) - "Estructura del quiste hidatico con microscopia fluorescente" La Prensa Medica Argentina 39: 1132-1135.

van SOMEREN, H., van HENEGOUWEN, H.B., LOS, W., WURZER-FIGURELLI, E., DOPPERT, B., VERVLOET, M. & KHAN, P.M. (1974) - "Enzyme electrophoresis on cellulose acetate gel. II. Zymogram patterns in man-Chinese hamster somatic cell hybrids" Humangenetik 25: 189-201.

VARELA-DIÁZ, V.M., COLTORTI, E.A., RICKARD, M.D. & TORRES, J.M. (1977) - "Comparative antigenic characterization of Echinococcus granulosus and Taenia hydatigena cyst fluids by immunoelectrophoresis" Research in Veterinary Science 23: 213-216.

VERSTER, A.J.M. (1965a) - "Review of Echinococcus species in South Africa" Onderstepoort Journal of Veterinary Research 32: 7-118.

VERSTER, A.J.M. (1965b) - "Taenia solium Lin.(1758) in the Chacma baboon, Papio ursinus (Kerr, 1792)" Journal of the South African Veterinary Medical Association 36: 580.

VERSTER, A.J.M. (1967) - "Redescription of Taenia solium LINNAEUS, 1758 and Taenia saginata GOEZE, 1782" Zeitschrift für Parasitenkunde 29: 313-328.

VERSTER, A.J.M. (1969) - "A taxonomic revision of the genus Taenia Linnaeus, 1758 S. STR." Onderstepoort Journal of Veterinary Research 36: 3-58.

VERSTER, A.J.M. (1971) - "Preliminary report of the golden hamster as a definitive host of Taenia solium Linnaeus, 1758 and Taenia saginata Goeze, 1782" Onderstepoort Journal of Veterinary Research 38: 63-64.

VESSEL, M. & ABDOLRASULNIA, R. (1976) - "Partial purification and properties of ovine liver Echinococcus granulosus protoscolices phosphoglucose isomerase" Clinica Chimica Acta 68: 59-65.

VESSEL, E.S. & BEARN, A.G. (1957) - "Localization of lactate dehydrogenase activity in serum fractions" Proceedings of the Society for Experimental Biology and Medicine 94: 96-99.

VICKERMAN, K. (1974) - "Antigenic variation in African trypanosomes" in "Parasites in the Immunized Host: Mechanisms of Survival" Ciba Foundation Symposium 25 (new series). 1st edition. Associated Scientific Publishers, London: 54-80.

VOLLER, A. (1977) - personal communication.

VOLLER, A., BARTLETT, A. & BIDWELL, D.E. (1976) - "Enzyme immunoassays for parasitic disease" Transactions of the Royal Society for Tropical Medicine and Hygiene 70: 98-106.

von BRAND, T. (1966) - "Biochemistry of Parasites" 1st edition. Academic Press Inc. (London) Ltd.

von BRAND, T. (1974) - "The biochemistry of parasites" Zeitschrift für Parasitenkunde 45: 109-124.

WAITZ, J.A. (1963) - "Glycolytic enzymes of the cestode Hydatigera taeniaeformis" Journal of Parasitology 49: 285-293.

WAITZ, J.A. & SCHARDEIN, J.L. (1964) - "Histochemical studies of four cyclophyllidean cestodes" Journal of Parasitology 50: 271-277.

WARBURG, O. & CHRISTIAN, W. (1941) - cited by Dawson et al (1969): 625-626.

WARDLE, R.A. & McLEOD, J.A. (1968) - "The Zoology of Tapeworms". Facsimile of 1st edition. Hafner Publishing Co., Inc., New York.

WARDLE, R.A., McLEOD, J.A. & RADINOVSKY, S. (1974) - "Advances in the Zoology of Tapeworms, 1950-1970" 1st edition. University of Minnesota Press, Minneapolis, U.S.A.

WALKEY, M. & FAIRBAIRN, D. (1973) - "L(+)-lactate dehydrogenases from Hymenolepis diminuta (Cestoda)" Journal of Experimental Zoology 183: 365-374.

WEINMANN, C.J. (1966) - "Immunity mechanisms in cestode infections" in "Biology of Parasites" edited by E.J.L. Soulsby. 1st edition. Academic Press Inc. (London) Ltd., London: 301-320.

WERTHEIM, G., ZELEDON, R. & READ, C.P. (1960) - "Transaminases in tapeworms" Journal of Parasitology 46: 497-499.

WHO (1975) - "The enzyme-linked immunosorbent assay (ELISA)" Report of an informal consultation on the enzyme-linked immunosorbent assay (ELISA) 28-31 October, 1975, World Health Organization, Geneva.

WIELAND, T. & PFLEIDERER, G. (1957) - "Nachweis der heterogenität von Milchsäure-dehydrogasen verschiedenen ursprungs durch Trägerelektrophorese" Biochemische Zeitschrift 329: 112-116.

WILKINSON, J.H. (1970) - "Isoenzymes". 2nd edition. Chapman and Hall Ltd., London.

WILLIAMS, R.J. & SWEATMAN, G.K. (1963) - "On the transmission, biology and morphology of Echinococcus granulosus equinus, a new subspecies of hydatid tapeworm in horses in Great Britain" Parasitology 53: 391-407.

WITENBURG, G.G. (1964) - "Cestodiases" in "Zoonoses" edited by J. van der Hoeden. 1st edition. Elsevier Publishing Company, London: 649-707.

WRAXALL, B.G.D. & CULLIFORD, B.J. (1968) - "A thin-layer starch gel method for enzyme typing of bloodstains" Journal of the Forensic Science Society 8: 81-82.

YAMAGUTI, S. (1959) - "Systema Helminthum. Volume 2. The Cestodes of Vertebrates". 1st edition. Interscience Publishers Ltd., London.

YAMAO, Y. (1952a) - "Histochemical studies on endoparasites. VII. Distribution of the glycero-mono-phosphatases in the tissues of cestodes, Anaplocephala perfoliata, Anaplocephala magna, Moniezia expansa, Moniezia benedeni and Taenia taeniaeformis" Dobutsugaku Zasshi 61: 254-260.

YAMAO, Y. (1952b) - "Histochemical studies on parasites. VIII. Distribution of the glycero-mono-phosphatases in various tissues of larvae of cestodes, Cysticercus bovis, Echinococcus cysticus fertilis and Cysticercus fasciolaris" Dobutsugaku Zasshi 61: 290-294.

YARZÁBAL, L.A., BOUT, D.T., NAQUIRA, F.R. & CAPRON, A.R. (1977) - "Further observations on the specificity of antigen 5 of Echinococcus granulosus" The Journal of Parasitology 63: 495-499.

YOSHIDA, A. (1967) - "A single amino acid substitution (asparganine to aspartic acid) between normal (B+) and the common negro variant (A+) of human glucose-6-phosphate dehydrogenase" Proceedings of the National Academy of Sciences 57: 835-840.

ZEE, D.S. & ZINKHAM, W.H. (1975) - "Demonstration of parasitism in tissues by electrophoretic definition of parasitic enzymes in host tissues" Journal of Paediatrics 86: 408-410.

ŽĎÁRSKÁ, Z. (1973) - "On the histochemistry of the tegument of Cysticercus bovis" Folia Parasitologica (Praha) 20: 307-318.

# APPENDIX I

# APPENDIX I

## A key to abbreviations

| | |
|---|---|
| E-ACA: | E-amino-n-caproic acid |
| Acid phos.: | acid phosphatase |
| ADP: | adenosine 5'-diphosphate |
| AK: | adenylate kinase |
| ALAT: | alanine aminotransferase |
| ALD: | aldolase |
| Alk. phos.: | alkaline phosphatase |
| ASAT: | aspartate aminotransferase |
| ATP: | adenosine 5'-triphosphate |
| DEAE cellulose: | diethylaminoethyl cellulose |
| DTT: | dithiothreitol |
| EDTA: | ethylenediamine tetraacetic acid |
| EP solution: | enzyme preservative solution (see page 78) |
| FITC: | fluorescein isothiocyanate |
| GAR: | goat anti-rabbit |
| GDH: | glutamate dehydrogenase |
| Glyceraldehyde 3PD | glyceraldehyde-3-phosphate dehydrogenase |
| G6PD: | glucose-6-phosphate dehydrogenase |
| GPI: | glucose-6-phosphate isomerase |
| HCl: | hydrochloric acid |
| HK: | hexokinase |
| ICD: | isocitrate dehydrogenase |
| KCN: | potassium cyanide |
| $K_2HPO_4$: | potassium phosphate |

| | |
|---|---|
| LDH: | lactate dehydrogenase |
| MDH: | malate dehydrogenase |
| $MgCl_2$: | magnesium chloride |
| MTT: | 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide |
| NAD: | nicotinamide adenine dinucleotide phosphate |
| NADH: | reduced form of NAD |
| NADP: | nicotinamide adenine dinucleotide phosphate |
| NADPH: | reduced form of NADP |
| $\alpha$NA esterase: | $\alpha$-naphthyl acetate esterase |
| $Na_2HAsO_4$: | sodium arsenate |
| NaOH: | sodium hydroxide |
| NBT: | nitro blue tetrazolium |
| $(NH_4)_2SO_4$: | ammonium sulphate |
| PBS: | phosphate buffered normal saline |
| 6PG: | 6-phosphogluconate (tri-sodium salt) |
| 6PGD: | 6-phosphogluconate dehydrogenase |
| PGM: | phosphoglucomutase |
| RAB: | rabbit anti-bovine |
| SDH: | succinic dehydrogenase |
| Tris: | tris-(hydroxymethyl)-aminomethane |
| TRITC: | tetramethylrhodamine isothiocyanate |

# APPENDIX II

TRANSACTIONS OF THE ROYAL SOCIETY OF TROPICAL MEDICINE AND HYGIENE, VOL. 71, NO. 4, 1977.

# Differentiation of *Taenia saginata* and *Taenia solium* by enzyme electrophoresis

P. D. LE RICHE AND M. M. H. SEWELL
*University of Edinburgh, Centre for Tropical Veterinary Medicine, Easter Bush, Roslin, Midlothian, Scotland, U.K.*

**Summary**

Forty-four *Taenia saginata* and seven *T. solium* specimens were collected in Nigeria. Extracts of these worms and of their metacestodes were compared by enzyme electrophoresis. The mobility of glucose phosphate isomerase was consistently faster with *T. saginata* than with *T. solium*. Extracts of the strobilate and cysticercus forms of the same species gave identical results. It was thus possible to distinguish clearly between material of either species.

## Introduction

*T. solium* is the more important of the two taeniid worms found in man, because of the danger of human cysticercosis and, according to VERSTER (1967), it is possibly more common than is usually supposed. Inaccurate identification is one of the probable reasons for underestimating its prevalence. Although *T. solium* and *T. saginata* can be distinguished by various morphological characteristics of the scolex and the mature proglottides, it is difficult to differentiate the gravid proglottides usually found in the faeces. The number of branches of the uterus, which was formerly the characteristic used to distinguish gravid proglottides, is not reliable because there is considerable variation (VERSTER, 1967). Furthermore, it is not possible to distinguish between the ova of these two species (KAMALOVA, 1953).

Electrophoresis shows up small structural differences in iso-enzymes which permit biochemical classification of species and sometimes even of strains within species. This procedure has been used as a tool to identify some parasites of vertebrates, for example trypanosomes (STEIGER *et al.*, 1974; KILGOUR & GODFREY, 1973; BAGSTER & PARR, 1973), *Leishmania* (KILGOUR *et al.*, 1974), *Plasmodium* (CARTER, 1970, 1973), *Entamoeba* (REEVES & BISCHOFF, 1968) and ascarid worms (ZEE & ZINKHAM, 1975). This report suggests that enzyme electrophoresis may also be used to distinguish taeniid material of human origin.

## Materials and methods

During the course of a recent small survey in Nigeria, 44 specimens of *T. saginata* and seven of *T. solium* were collected. Nine *T. saginata* and two *T. solium* scoleces were present. The material was either obtained following treatment of the patients with male fern, mepacrine hydrochloride or niclosamide or was passed spontaneously. The larval cysticerci of *T. solium* were also obtained from pork in Nigeria and those of *T. saginata* from calves experimentally infected in the U.K. with eggs obtained from Kenya. Fresh material was prepared for immediate use by homogenizing it in ten volumes of physiological saline. Other preparations were homogenized in water containing 1 mM of each of the enzyme stabilizers E.D.T.A., dithiothreitol and ε-aminocaproic acid (KILGOUR & GODFREY, 1973), which were then stored at +5°C or –20°C. Additional preparations were made in the same way from whole worms stored in saline and antibiotics at –20°C (100 units Penicillin G, 100 mg dihydrostreptomycin and 100 units nystatin per ml saline). Centrifugation at 100,000 *g* for 30 minutes at +4°C of some samples gave no difference in the electrophoretic results. Other samples were allowed to sediment overnight at 4°C, only the supernatant being retained for analysis.

Electrophoresis by the thin-layer starch gel method (WRAXALL & CULLIFORD, 1968) was used, as modified by KILGOUR & GODFREY (1973). Glucose phosphate isomerase (E.C. No. 5, 3, 1, 9) was located by reduction of tetrazolium salt to give its blue formazan insoluble precipitate at the site of enzyme activity in the gel (DELORENZO & RUDDLE, 1968). 20 ml of the solution containing the reagents and 1·4% agar, was allowed to solidify over the gel and the whole then incubated at 37°C until development of bands occurred.

## Results

Up to seven equidistant bands were seen with each of the two tapeworm species, but fewer bands were more usually seen. Extracts from all tapeworms of one species were identical in mobility but the number of bands detected was determined by the activity of the enzyme in the specimen. No differences attributable to the method of collection or preparation of the specimens were seen. Other methods of preservation may result in loss of banding even in enzymically active samples but the overall difference in mobility remains. With both *T. solium* and *T. saginata* extracts prepared from either strobila or cysticerci had identical mobility.

At pH 7·4, the band nearest the point of application was the strongest band and was visible if any enzyme activity was present. The mobility of this band only was used as the criterion for comparison. Differences in mobility between the two species can be seen in Fig. 1.

## Discussion

Enzyme electrophoresis of glucose phosphate isomerase appears to distinguish between *T. saginata* and *T. solium* clearly and consistently, provided the material is preserved correctly. It therefore has advantages over identification based on morphological features, which may vary or be difficult to see, and it may be used to identify small fragments of segments found in stool specimens.

Further studies will be required to ascertain whether these species derived from other parts of the world show

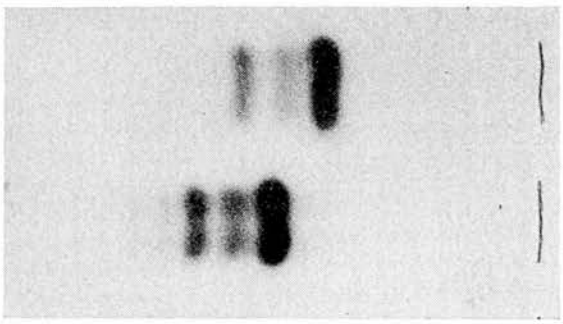


Fig. 1. Isoenzymes of glucose phosphate isomerase at pH 7·4. On the top *T. solium* and on the bottom *T. saginata*. Sample contained in 8 mm lengths of cotton thread seen at the origin. Movement of the bands from thread towards anode. Enzyme activity of each sample approximately 1 U ml $^{-1}$. Voltage 330V across a 15 cm gel of 1 mm thickness. Current 20 mA. Time 3 hours. Gel buffer 0·015M phosphate pH 7·4. Electrode buffer 0·2M phosphate pH 7·4.

the same electrophoretic mobilities and whether differences can be shown between the taeniid worms of canine origin.

## References


Bagster, I. A. & Parr, C. W. (1973). Trypanosome identification by electrophoresis of soluble enzymes. *Nature*, **244,** 364–366.

Carter, R. (1970). Enzyme variation in *Plasmodium berghei*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **64,** 401–406.

Carter, R. (1973). Enzyme variation in *Plasmodium berghei* and *Plasmodium vinckei*. *Parasitology*, **66,** 297–307.

Delorenzo, R. J. & Ruddle, F. H. (1969). Genetic control of two electrophoretic variants of glucophosphate isomerase in the mouse (*Mus musculus*). *Biochemical Genetics*, **3,** 151–162.

Kamalova, A. G. (1953). Comparative studies of eggs of *Taeniarhynchus saginata* and *Taenia solium*. *Raboty po Gel'mintologii k 75-letiyu Akademika K.I. Skryabina*, 1st edit. Moscow, Akad Nauk SSSR, pp. 278–285.

Kilgour, V. & Godfrey, D. G. (1973). Species-characteristic isoenzymes of two aminotransferases in trypanosomes. *Nature New Biology*, **244,** 69–70.

Kilgour, V., Gardener, P. J., Godfrey, D. G. & Peters, W. (1974). Demonstration of electrophoretic variation of two aminotransferases in *Leishmania*. *Annals of Tropical Medicine and Parasitology*, **68,** 245–246.

Reeves, R. E. & Bischoff, J. M. (1968). Classification of *Entamoeba* species by means of electrophoretic properties of amebal enzymes. *Journal of Parasitology*, **54,** 594–600.

Steiger, R., Krassner, S. M. & Jenni, L. (1974). Comparison of specific and relative alanine and aspartate aminotransferases of *Trypanosoma brucei* subgroup trypanosomes. *Acta tropica*, **31,** 202–218.

Verster, A. (1967). Redescription of *Taenia solium* Linnaeus, 1758 and *Taenia saginata* Goeze, 1782. *Zeitschrift für Parasitenkunde*, **29,** 313–328.

Wraxall, B. G. D. & Culliford, B. J. (1968). A thin-layer starch gel method for enzyme typing of bloodstains. *Journal of the Forensic Science Society*, **8,** 81–82.

Zee, D. S. & Zinkham, W. H. (1975). Demonstration of parasitism in tissues by electrophoretic definition of parasite enzymes in host tissues. *Journal of Pediatrics*, **86,** 408–410.


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# APPENDIX III

## APPENDIX III

### Names and addresses of commercial companies

Adelphi Tubes Ltd., Duncan Terrace, London N1 8BZ

Ambersil Ltd., Whitney Road, Daneshill, Basingstoke, Hants.

Amicon Corporation, Lexington, Massachusetts, U.S.A.

BDH Chemicals Ltd., Poole BH12 4NN

Boots, 101 Princes Street, Edinburgh 2

Burroughs Wellcome & Co. (The Wellcome Foundation Ltd.), Berkhamstead, Herts.

Cambrian Chemicals Ltd., Suffolk House, George Street, Croydon CR9 3QL

Ciba-Geigy A G, Basel, Switzerland.

Connaught Medical Research Laboratories, University of Toronto, Canada.

Difco Laboratories, P.O. Box 148, Central Avenue, East Molesey KT8 0SE

Edwards High Vacuum Ltd. (subsidiary of MSE).

Gelman Instrument Co. (Gelman Hawksley Ltd.), 1 Leeke Street, London, WC1K 9JA

Gilson, Villiers-le-bel, 95400 Aznouville-lès-Gonese, France.

G.T. Gurr (subsidiary of Searle Scientific Services).

Imperial Chemical Industries Ltd., Dumfries factory, Drungans, Dumfries.

Janssen Pharmaceutica, 2340 Beerse, Belgium.

Jencons (Scientific) Ltd., Mark Road, Hemel Hempstead, Herts.

R.A. Lamb, 6 Sunbeam Road, North Acton, London, NW10 6JL

LKB Instruments Ltd., 232 Addington Road, South Croydon, Surrey CR2 8YD

Luckham Ltd., Victoria Gardens, Burgess Hill, Sussex RH15 9QN

May & Baker Ltd., Dagenham RM10 7XS

Merck, Sharp & Dohme Ltd., Hertford Road, Hoddesdon, Herts., EN11 9BU

Millipore (U.K.) Ltd., Abbey Road, London NW10 7SP

MSE Scientific Instruments, Manor Royal, Crawley, Sussex RH10 2QQ

Nordic Immunological Laboratories Ltd., 60 King Street, Maidenhead, Berks. SL6 1EQ

Oxoid Ltd., London SE1 9HF

Pharmacia Fine Chemicals AB, Box 175, S-751 04, Uppsala 1, Sweden.

Pye Unicam Ltd., York Street, Cambridge CB1 2PX

R.B. Radley & Co. Ltd., London Road, Sawbridgeworth, Herts., CM21 9JH

Richardson Merrel Ltd., 20 Saville Row, London W1X 2AN

The Scientific Instrument Centre Ltd., 1 Leeke Street, London WC1K 9JA

Searle Scientific Services, P.O. Box 53, Lane End Road, High Wycombe, Bucks.

Shandon Southern Instruments Ltd., Lysons Avenue, Ashvale, Aldershot, Hants. GU12 5QF

Sigma Chemicals Co. Ltd., Norbiton Station Yard, Kingston-upon-Thames, Surrey KT2 7BH

Vickers Ltd., Vickers Instruments, Haxby Road, York YO3 7SD

Whatman LabSales Ltd., Springfield Mill, Maidstone, Kent ME14 2LE