THE PENETRATION PROTEASE OF THE CERCARIAE OF SCHISTOSOMA MANSONI

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ABSTRACT

This thesis is concerned with a study of the proteases released by the cercariae of Schistosoma mansoni while penetrating mammalian skin. The proteases present in secretions collected from the preacetabular glands of cercariae were shown to be active against ^{125}I-labelled fibrin but not against undenatured ^{3}H-collagen. A sensitive solid phase radioenzyme assay, with ^{125}I-fibrin as the substrate, was used to show that the cercarial protease could be totally inhibited by serine protease inhibitors such as diisopropylfluorophosphate or phenylmethylsulfonyl fluoride, but not by the sulfhydryl reagents iodoacetamide or p-chloromercuribenzoate. Typical trypsin inhibitors such as soy bean trypsin inhibitor, trasylol or benzamidine inhibited the enzyme to a lesser degree. The active-site labels, TLCK, TPCK and AcAAAACK of trypsin, chymotrypsin and elastase respectively had no effect.

Calcium and magnesium stimulated protease activity at concentrations below 0,5 mM but inhibited at higher concentrations, whereas EDTA had no effect. The pH optimum of the protease lay between pH 9,0 and 9,5. From these studies, I have concluded that the major cercarial penetration protease is an alkaline serine protease with trypsin-like specificity, but not acting via the same mechanism.

A technique was developed for examining cercarial proteases in polyacrylamide gels containing SDS and copoly-

merized gelatin substrate. Bands of proteolytic activity could be detected by negative staining. This method was used to show that cercarial secretions contained one major protease with a molecular weight of 35 000 and that crude enzyme preparations are readily contaminated with bacterial proteases.

Partial purification of the major cercarial protease was achieved by cation exchange chromatography.

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CONTENTS

INTRODUCTION

The life cycle of many parasites requires rapid morphological and physiological adaptations to environmental change. The schistosome is no exception to this general rule and it is now well established that during its life span this organism encounters three different econiches - snail tissue, fresh water and the tissues of the definitive host. It is during its passage between intermediate and definitive hosts, i.e. during the miracidial and cercarial stages, that the parasite is most vulnerable, and it is essential for survival of the species that the fresh water phases be terminated as rapidly as possible. Numerous studies have indicated that miracidia (1, 8) and cercariae (3, 4) survive in fresh water and retain their infectivity for limited periods measurable in hours. The processes involved in the expedient passage from water to host are, therefore, of relevance to the spread of schistosomiasis, for if these could be retarded or inhibited some measure of protection might be afforded to man. In this thesis I report the results of my attempts to characterize the proteolytic enzymes believed to be involved in cercarial penetration of the skin of the host.

Research on schistosomal penetration mechanisms dates back to the beginning of this century. While studying the life cycle of Schistosoma japonicum Miyagawa et al (5) noted that entry of the cercariae was through healthy skin of animals. This was later verified by Miyairi (3) and Leiper (6) who also noticed that penetration did not occur

through pores or hair follicles, but directly through the stratum corneum.

Cort (7) observed the presence of five pairs of "large unicellular cephalic glands" in the cercariae of Schistosoma japonicum and Faust (8) suggested that these glands contained "proteolytic ferments, which are poured out through the duct openings and digest away host tissue". The same was suggested for Schistosoma mansoni cercariae by Faust (9) who showed, with histochemical techniques, that the 5 pairs of glands comprised 2 pairs of anterior and 3 pairs of posterior cephalic glands. In addition one head gland and one escape gland could be seen in cercariae present in snail tissue. Since the secretions of the escape gland were expelled during emergence from the snail, and the contents of the head gland could still be demonstrated in the schistosomule, it was deduced that the cephalic glands were involved in the penetration of human skin. The cephalic glands were later termed pre- and post-acetabular glands by Stirewalt and Kruidenier (10).

Histochemical techniques showed that these glands were indeed of two types, and probably served different functions.

The post-acetabular glands contained a "mucigen" that stained strongly with periodic acid-Schiff reagent and other proteoglycan stains. After emission this material swelled by absorption of water to assume mucoid characteristics (11, 12, 13 14). It was suggested that this secretion promoted the adhesion of cercariae to the skin (12, 10, 15) and facilitated penetration of the horny layer by further hydration and swelling within the substance of the epidermis leading to mechanical

disarticulation of the squamous cells (15). These functions were ascribed on the basis of observations that the oral and ventral suckers were surrounded by a mass of gland secretion during attachment (12) and the packets of this secretion could be identified between the squamous cells ahead of the cercariae.

The preacetabular gland contents, on the other hand, did not give histochemical reactions characteristic of proteoglycans (16, 13) but stained strongly with Alizarin Red S, a dye that detects calcium (10). Microscopic, ultrastructural and histochemical studies had provided evidence to suggest that these glands contain proteolytic enzymes that aid cercarial penetration. In the first instance it was noted that the epidermal basement membrane and the ground substance between cells were altered histochemically (17) and ultrastructurally (18, 15) around penetrating cercariae and granules of preacetabular gland contents could be identified at the oral end of the cercariae by microscopic observation. At the same time, cercariae lost the ability to be stained with Alizarin Red S (10, 19, 20).

The search for the active penetration enzymes, presumed to be present in preacetabular glands, began when Levine et al (21) demonstrated hyaluronidase activity in extracts of cercariae. Since then extracts of homogenates of live and frozen cercariae have been shown to be active against a large variety of polypeptide substrates, such as Azocoll and gelatin (22, 23, 24); denatured hemoglobin (25); elastin (26, 27); keratin (28); casein (26, 24) and a variety of peptides and

synthetic substrates (29). The fact that none of these activities could be detected in extracts of schistosomules provided further evidence for the presence of a penetration enzyme (25).

It was also observed that cercariae, when incubated on a gelatin film, secreted an enzyme that dissolved the gelatin around them (17). Stirewalt (30) was able to localize this gelatinase activity in the preacetabular glands, by placing sections of live cercariae on gelatin films. She also demonstrated that secretion could be induced by application of skin lipid to these films. The fractions in the skin lipid were later identified as containing unsaturated fatty acids (31, 32). The collected secretions showed the same multiple proteolytic specificities as extracts of cercariae (28, 33).

Attempts to determine the number of proteases in preacetabular gland secretion or to characterize these enzymes have been largely unsuccessful. It is however evident that a number of proteases might be involved. Gazzinelli et al (26) found three peaks of proteolytic activity after DEAE Sephadex chromatography. Dresden and Asch (24) reported 5 peaks probably with different specificities and Campbell et al (33) found two peaks on Sephadex G75. These investigators also assigned a tentative molecular weight of 27 000 - 29 000 to the main proteolytic fraction. From inhibitor and substrate studies, it was deduced that the cercarial penetration enzyme is an alkaline serine protease, which is different from trypsin and chymotrypsin (29) and is not a collagenase (28).

I became interested in this field by the idea that there might be a possibility to protect against infection by an immune response of the animal to these proteases. For this purpose I made an attempt to further elucidate the biological functions of the enzyme and to purify it in view of obtaining a pure antigen. These attempts however led to a number of difficulties, one of these being the minute amounts of enzyme that can be obtained from a large batch of cercariae. This compelled me to develop an electrophoresis method, by which small amounts of these enzymes can be detected. By this method I was able to determine the molecular weights of the two enzymes present in my preparation. An important factor probably often overlooked by other investigators is the contamination of the enzyme by external bacterial proteases. The electrophoresis technique was also used to develop methods of obtaining uncontaminated preparations of the enzyme. Inhibitor and substrate studies showed the enzymes to be alkaline serine proteases in agreement with previously published work, although a number of observations, which will be emphasized in my work, were at variance. Attempts to purify the enzymes mainly failed because of lack of material, although a very sensitive assay was used for the detection of the proteases.

The thesis comprises five chapters. The maintenance of the schistosome life cycle in the laboratory is described in Chapter 1.In the second chapter assays for the measurement of cercarial proteases are described. The third chapter gives a technique by which proteases in cercarial secretions could be examined by SDS-polyacrylamide gel

electrophoresis. The collection of protease containing secretions from cercariae is discussed in Chapter 4. In Chapter 5 I describe attempts to characterize proteases in cercarial secretions to achieve their partial purification by ion exchange chromatography.

CHAPTER 1

THE MAINTENANCE OF THE LIFE CYCLE OF SCHISTOSOMA MANSONI IN THE LABORATORY.

The success with which Schistosoma species can be maintained in the laboratory is largely dictated by the nature of the molluscan vector that is used for this purpose. The oriental species of Oncomelania and the African Bulinus, intermediate hosts of Schistosoma japonicum and Schistosoma haematobium respectively, are both semiaquatic snails and methods for rearing these in aquaterraria have been described (48, 49, 50). Generally speaking, purely aquatic snails, such as most of the vectors of Schistosoma mansoni, are easier to accommodate and present fewer difficulties. As I did not intend to study the life cycle of the parasite, I decided to establish a colony of albino, Australorbis glabratus to serve as intermediate hosts for the Puerto Rican strain of Schistosoma mansoni. This aquatic strain of snails proved easy to breed and provided large numbers of cercariae for subsequent experiments. The strain was derived originally from a cross between an albino Brazilian strain and a pigmented Puerto Rican strain (51).

The life cycle in this laboratory was started with thirty infected snails, that were generously supplied by Dr. S.R. Smithers of the National Institute for Medical Research, London. All of the techniques that I used were adopted, with minor modifications, from his laboratory.

Maintenance and breeding of infected snails.

Adult breeding snails were kept in 15 litre plastic aquaria, with air bubbling through a charcoal filter and temperature control set at 28°C. Each aquarium was fitted with a hammock of 100 mesh nylon net material immersed in the water to a depth slightly above the bottom of the tank. The hammock, supported by a plastic coated wire frame was secured at the top of the tank. The nylon net served as a coarse filter which allowed debris to pass through and settle on the bottom of the tank while the snails were retained in the hammock. The tanks were cleaned once a week by aspirating the debris from the bottom and adjusting the volume with filtered tap water that had been standing in 200 litre plastic drums for a week. The charcoal filters and nylon nets were changed every six weeks.

The snails were fed once a day on pieces of head lettuce, which had been boiled and air-dried. In addition, a spatula of a mixture of 10 parts of wheat germ flakes to one part of $CaCO_3$ was sprinkled on top of the water 2-3 times a day. Old lettuce which had sunk to the bottom of the tanks, was removed to avoid fouling of the water.

Strips of polythene sheeting, floating on the surface, provided effective egg depositories. Once a week, 4-5 egg masses were were transferred to one of seven clean tanks equipped with a 200 mesh net hammock (1.1), where they were allowed to hatch. When the small snails reached the age of seven weeks (i.e. when the tank had to be used for a new batch of eggs) they were either used immediately for infection or transferred to the larger tanks. By this method

Figure 1.1: Photograph showing a glass tank used for hatching of snail eggs and rearing the snails. It is equipped with a 200 mesh net hammock and a charcoal water filter. The snails that can be seen inside the net are approximately 7 weeks old.

of rotation, the approximate age of the snails could be determined before infection.

Infected snails were kept in plastic tanks in a dark room where the temperature could be adjusted to 28°C. Each tank was occupied by 15 - 25 snails in about 5 litres of water. The snails were taken out twice a week for shedding of cercariae. At the same time the tanks were cleaned with Biocide, thoroughly rinsed and refilled with filtered water. Feeding was the same as for the breeders.

The carefully controlled schedule of feeding and cleaning ensured that the snails were free of any other parasites but cercariae. It is well known, that other snail parasites exist, some of which may feed on young sporocysts developing in the snail and thus reduce the yield of cercariae (52). As soon as any external parasites were detected in a snail batch, the snails were either discarded or treated with 1% urethane.

Snails fed exclusively on dried lettuce developed circumscribed shell lesions such as those shown in Fig. 1.2. A healthy snail is shown in Fig. 1.3. By supplementing the basic diet with the mixture of wheat germ and $CaCO_3$, these lesions could be prevented. Tropical fish food (Tetra Fin Goldfish Food) proved to be an ineffective and unsatisfactory dietary supplement.

Recovery of cercariae from infected snails

Cercariae were collected every third or fourth day from each batch of snails having the same exposure date. The snails were washed in deionized water and then placed in a

Figure 1.2: Photograph showing a snail which had developed circumscribed shell lesions, due to inadequate feeding.

Figure 1.3: A healthy snail fed on dried lettuce, wheat germ and $CaCO_3$.

volume of 100 ml/50 snails of dechlorinated tap water containing 200 units Penicillin and 200 µg/ml Streptomycin in a glass beaker. They were illuminated by bright light from a 250W bulb for 2 hours, during which time the cercariae were shed into the surrounding water. The snail debris was removed by pouring the suspension through a metal sieve into a measuring cylinder. After they had been immobilized with Lugol's iodine, the cercariae were counted in 200 µl aliquots of the suspension. The total number of cercariae emerged was calculated from the total volume of suspension.

Snails started to shed cercariae 4½ - 5 weeks after infection. The yield of cercariae per snail generally rose to reach a maximum and then declined. The useful shedding life of the snails was approximately 10 weeks. The life-span of the heavily infected snails was limited. Graphs depicting snail survival and average cercarial yield per snail as a function of time are presented in Fig. 1.4.

Batches were discarded, when the number of cercariae shed per snail declined. The addition of antibiotics had no effect on either snails or cercariae.

The infection of mice with cercariae

Schistosome ova were obtained from an outbred strain of Ha(ICR) albino mice infected with cercariae according to the following procedure. The abdomens of 15 - 20 g male mice were shaved and the animals were anaesthetized with an intra-peritoneal injection of 0,3 ml of a 1/10 saline dilution of veterinary Sagatal (60 mg/ml). The animals were then placed on their backs between wooden strips fixed to a baseboard.

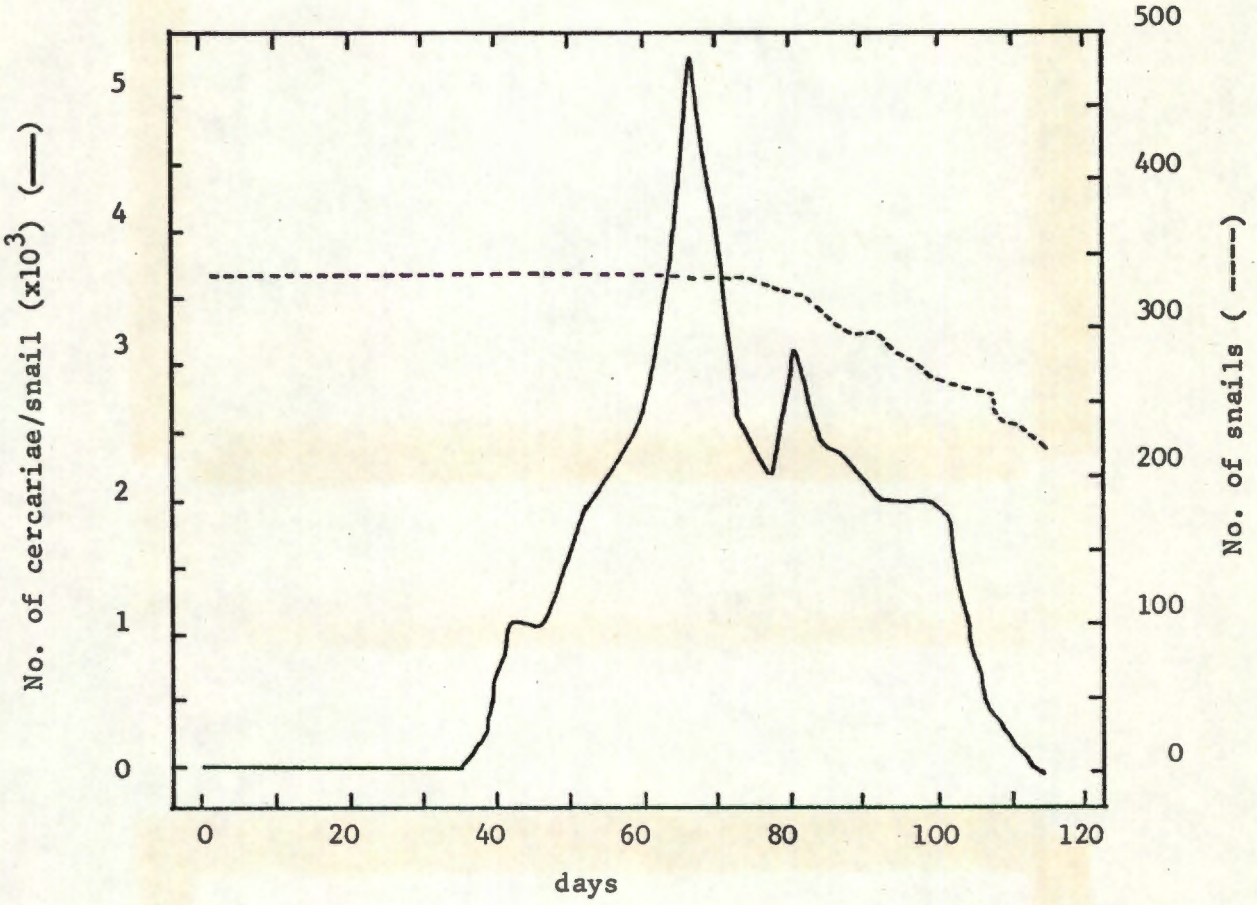


Figure 1.4 Snail survival and cercarial yield

Graph showing snail survival and average cercarial yield per snail as a function of time. The snails were infected on day 0 and cercariae were shed twice a week.

Nickle plated brass rings with an internal diameter of 1,3 cm, a height of 1 cm and weighing 12 g were positioned on the abdomens, thus forming a well into which the cercarial suspension could be pipetted. These rings held a maximum volume of 1,2 ml. A suitable volume of the suspension containing 150 cercariae was pipetted into the rings and left there for 10 minutes, which allowed adequate time for the cercariae to penetrate. The mice usually recovered from the anaesthetic within one hour.

Smithers et al (53) emphasized that the method and dose of anaesthetic given was critical. If the dose was too low the animals struggled during exposure and dislodged the ring. A dose that was too high led to an undesirably high anaesthetic mortality.

It is of interest to note that on one occasion the abdominal skin of the mice was treated with a soap solution to facilitate shaving. This resulted in a very poor infection, due possibly to the removal of surface skin lipids believed to serve as a stimulus for cercarial penetration (32).

Recovery of schistosome eggs from mouse intestine

After 7 weeks of infection the mice were killed with an overdose of Sagatal by injecting them intraperitoneally with 0,3 ml of the undiluted anaesthetic. The small intestines from the duodenum to the caecum were removed and immersed in 50 ml ice-cold PBS in a glass beaker. The intestines were minced with fine scissors and homogenzied with an Ultra Turrex homogenizer. A further 50 ml volume of PBS containing 10 mg of Trypsin (Difco certified 1:250) was added, and the suspension

was incubated for 2 hours at 37°C with slow shaking. The homogenate was then poured through a sieve (mesh 80, 180µm) and rinsed with saline to remove any gross debris. After centrifugation of the suspension at 500 rpm for 2 minutes in a bench top centrifuge, the eggs settled in a dark pellet at the bottom of the centrifuge tube. They were washed at least five times by resuspension in saline, recentrifugation and removal of the supernatant.

The eggs could be stored in sterile Tyrode's solution at 4°C for at least 7 days. Typical schistosome eggs are shown in Fig. 1.5.

Hatching of miracidia

After having been washed thoroughly in water, the schistosome eggs were placed into a glass beaker containing about 100 ml of aged tap water. They were illuminated from above with a 250W bulb, which caused the miracidia to swim to the surface of the water, after hatching. After 15 minutes, when the eggs had settled to the bottom of the beaker, the supernatant containing the miracidia could easily be poured off into a measuring cylinder. Miracidia in small aliquots of the suspension were counted under a dissecting microscope, and the volume in the measuring cylinder adjusted, such that 0,5 ml contained 10 - 15 miracidia.

Infection of snails with miracidia

Five to seven-week old snails were infected individually with 10 - 15 miracidia. Each snail was placed in a McCartney bottle containing 10 - 12 ml aged tap water and 0,5 ml of the miracidial suspension was added. Miracidia

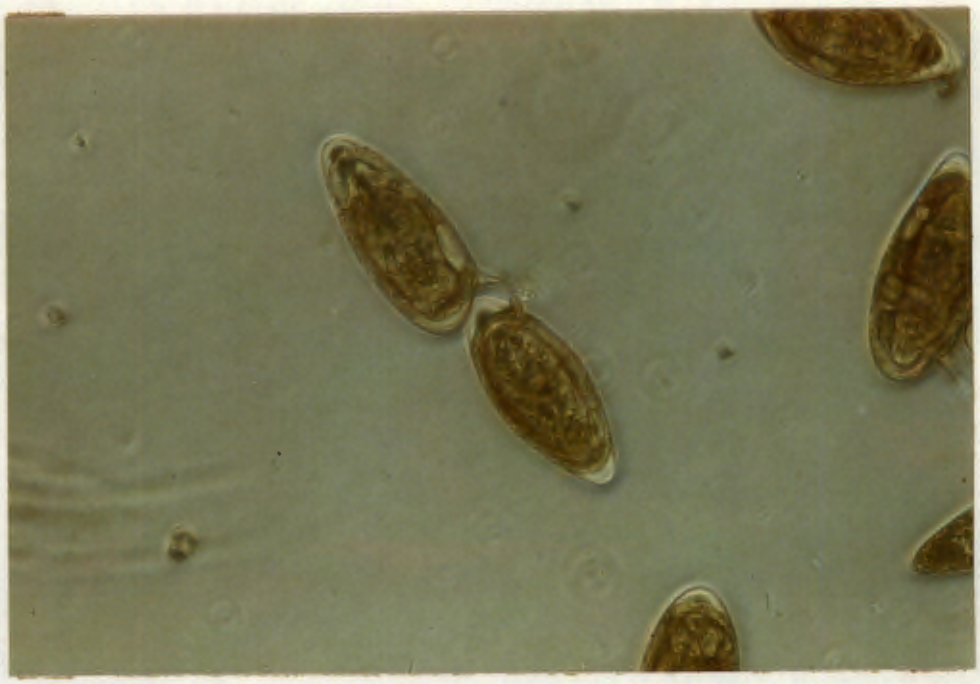


Figure 1.5: Schistosome eggs recovered from mouse intestines. Note the lateral spine characteristic of S. mansoni eggs.

were used as soon as possible after hatching. Infection was allowed to continue overnight. The next morning the snails were transferred to plastic tanks and kept in the dark room.

As my main intention was to obtain as many cercariae in a batch as possible the snails were usually heavily infected and started to die after two months.

CHAPTER 2

MEASUREMENT OF CERCARIAL PROTEASES

In this chapter I present the assay procedures used for the detection and quantitation of proteolytic enzymes present in cercarial secretion. For the most part, these enzymes were collected by incubating a concentrated suspension of freshly-shed cercariae in 2,5% glucose at 37°C for 2 hrs. This procedure involved release of the proteases into the incubating medium which caused them to be separated from cercariae by centrifugation. Deviations from this procedure are discussed in the text. The variables involved in the collection of protease-containing secretions from cercariae are discussed in greater detail in Chapter 4.

Proteolytic enzymes present in the secretions and in extracts of cercariae have been demonstrated with a variety of substrates. A summary of the substrates and methods that have been used for the detection of enzyme activity is presented in Table 2.1. As is evident from this synopsis, most workers have attempted to define the natural specificity of cercarial proteases by choosing substrates such as collagen, elastin or chondromucoprotein PPL that are normal constituents of skin. These were used either in a partially purified form or as the crude material.

Assays have usually been based upon the measurement of amino acids and peptides released during substrate hydrolysis. This was determined by direct spectrophotometric

TABLE 2.1 SUMMARY OF THE SUBSTRATES AND METHODS USED FOR THE DETECTION OF ENZYME ACTIVITY IN SECRETIONS AND EXTRACTS OF CERCARIAE

SUBSTRATE	ENZYME PREPARATION	ASSAY	REFERENCE
Azocoll	Extract of lyophilized cercariae	Absorbance of 540nm of supernatant	22,64
"	Homogenate of live cercariae	Qualitative observation of dye release	34
"	Extract of sonicated cercariae	Absorbance of 540 nm of supernatant	24
"	Ammonium sulphate fraction of extracted sonicated cercariae	"	62
"	Cercarial secretion	"	33,37
Azocartilage	Extract of lyophilized cercariae	"	22
Casein	"	Absorbance at 280 nm (Kunitz)	26,24
Elastin	"	Lowry protein determination of supernatant	27,26,24
Elastin-Orcein	"	Absorbance at 590 nm of supernatant	27, 24
Gelatin	Extract of sonicated cercariae	^{14}C-labelled peptide release	24
"	Cercarial secretion	Measurement of lysis area on film	23,30
Haemoglobin	Extract of lyophilized cercariae	Tyrosine release (Anson)	22,25
Chondro mucoprotein (PPL)	Extract of sonicated cercariae	Reduction in viscosity	24
Keratin sheets from foot cast removals	Cercarial secretions	Ninhydrin reactive peptides in supernatant	28

measurement at 280 nm of the supernatant after protein precipitation; or colorimetrically with the Folin-Ciocalteau reagent by the method of Lowry and Randall (35). The use of dye-coupled substrates such as Azocoll and elastin-orcein had the advantage that the products of hydrolysis could be measured directly after separation of insoluble residual substrate and no secondary colour reactions had to be employed to increase the sensitivity. Because of its simplicity the assay with Azocoll as the substrate, has been favoured for the routine measurement of the proteolytic activity of the cercarial enzyme. This substrate, however, lacks specificity since it is not only degraded by collagenase, but also by trypsin (36).

Two other widely used protease substrates, haemoglobin and casein, have also been used to determine the effects of buffers, pH and inhibitors on the activity of the cercarial enzyme (25, 26).

It is difficult, from the published data, to decide which of these assays provides the most sensitive or satisfactory system for the characterization of cercarial proteases since no one has compared them in the same experiment, and assay conditions used by different authors varied considerably. One might be tempted to select, as the most sensitive assay, that which gave reliable results with the least amount of enzyme as estimated on the basis of the number of cercariae from which the enzyme was obtained. Stirewalt (37), however, has shown that the amount of enzyme obtained from batches of cercariae shed from the same group of snails on different days was variable, and suggested that the production of enzymes

in the preacetabular glands of cercariae depended on the physiological conditions of the snail host. Furthermore, the total protein in enzyme solutions obtained from a constant number of cercariae bore no relationship to the enzyme activity and therefore could not be used as a basis for comparison.

My choice of a suitable enzyme assay was dictated by several factors. Since the amount of enzyme that could be obtained was limited by the number of snails that could be maintained, a highly sensitive assay was desirable. It is generally accepted that enzyme assays involving radioactively labelled substrates are considerably more sensitive than conventional methods.

It was also desirable to minimise the total reaction volume of the assay since dilution of the enzyme into large volumes might well have had a deleterious effect on its stability when prolonged incubation was required for quantitating low levels of activity. In spectrophotometric assays the minimum reaction volume is determined by the size of the optical cell whereas reaction volumes in radioactive assays are limited only by the accuracy of pipetting small aliquots.

Since collagen is a prominent constituent of dermal tissues it seemed both obvious and appropriate to examine cercarial secretions for collagenolytic activity. Although the results with Azocoll would suggest that cercarial collagenases do exist, this is an unsatisfactory substrate inasmuch as its collagen component is denatured and hence susceptible to hydrolysis catalysed by trypsin-like enzymes. I therefore developed a collagenase assay using undenatured (i.e. trypsin-

resistant) guinea pig skin collagen labelled in vivo with [2-^{14}C] -glycine. As I shall describe later, cercarial secretions appeared to be devoid of collagenolytic activity.

In my search for an alternative substrate, it so happened that a radioenzyme assay using ^{125}I-fibrin was in routine use in this laboratory and preliminary experiments showed that the cercarial enzyme was capable of degrading this material. The insolubility of the fibrin substrate was advantageous since no secondary precipitation steps for separating the substrate from the hydrolysis products was necessary. In the assay, which I will describe, the substrate was deposited as an insoluble layer at the bottoms of wells in a plastic tray. After addition of the enzyme solution to the wells the time course of the reaction could be followed by withdrawing, at different time points, aliquots of the reaction mixture containing solubilized radioactive fibrin degradation products. Radioactivity in these aliquots could be measured without further preparation or the need for clarification - a prerequisite when optical methods are used.

The ^{125}I-fibrin assay proved to be extremely sensitive and reproducible for the detection and quantitation of proteolytic activity in cercarial secretions. I have been able, with this technique, to obtain satisfactory elution profiles of proteolytic activity from chromatography column fractions that contained undetectable amounts of protein and it has enabled me to characterize the enzyme in several biochemical respects.

In this section I shall describe the ^{14}C-collagen and ^{125}I-fibrin assays and the results that I have obtained.

Collagenase Assay

Guinea pig skin collagen, labelled *in vivo* with ^{14}C-glycine and purified by the method of Gross (38, 39) was used as the substrate. Aliquots of the collagen solution were gelled as even layers covering the bottoms of wells in multiwell Linbro tissue-culture plates (Flow Laboratories Ltd., Irvine KA12 8NB, Scotland). Enzyme solutions were added to the wells, and the reaction was followed by monitoring the release of solubilized ^{14}C as a function of time.

Preparation of ^{14}C-collagen

A guinea pig (250g) received two intraperitoneal injections of 100 µCi of [2-^{14}C] -glycine (specific activity: 114 mCi/mmol) dissolved in 2 ml of 0,9% NaCl. The injections were given 20 hrs apart and the animal was killed 22 hrs after the second injection. After shaving, the skin was removed from the animal and cleared of subcutaneous fat and areolar tissue by scraping with a scalpel blade. All subsequent steps were performed at 4°C.

The corium was minced in a meat grinder and extracted by shaking for 16 hrs in 2 volumes of 0,45M NaCl. The suspension was then centrifuged at 40 000g for 1 hr, and the supernatant was filtered through seven layers of surgical gauze and then through a porosity 2, pyrex sintered glass filter. The clear filtrate was dialysed against four changes of one litre volumes of 0,45M NaCl over 2 days.

The concentration of NaCl in the solution was brought to 16% (w/v) by the slow addition with stirring, of

solid salt and the solution was left to stand for 6 hours. The white precipitate that formed was removed by centrifugation (7000xg; 30 min) and dissolved in distilled water such that the concentration of NaCl was approximately 0,4M. The resulting turbid solution was dialysed against sodium phosphate buffer, 0,067M, pH 7,60 for 12 hours with 3 changes. A small amount of insoluble material that formed after dialysis was removed by centrifugation at 1000g for 30 min, and the collagen was reprecipitated by adjusting the NaCl concentration to 16%. Reprecipitation and dissolution in phosphate buffer were repeated three times.

A final solution of 40 ml was obtained; this contained 1,2 mg/ml protein (as estimated by the Biuret reaction against a gelatin standard) with a specific activity of 50 000 dpm ^{14}C/mg. A 0,5 ml sample of this solution formed a gel within 1 hour at 37°C.

Preparation of ^{14}C-collagen-coated Linbro trays

The 24 wells of a plastic Linbro tray were precoated by adding 100 µl of a 0,1% nonradioactive collagen solution to each well and drying at 45°C for 48 hrs. This coat of dried collagen allowed better adherence of the radioactive collagen gel to the bottom of the well. The undiluted ^{14}C-collagen solution was added in 250 µl volumes, containing 0,3 mg protein, to each well and allowed to form a gel at 37°C for 3 hours. The gels were washed gently with 1 ml of 0,05M glycine-NaOH pH 8,8 and used immediately. The total radioactivity in each well could be calculated from aliquots of the radioactive solution counted before addition.

In a preliminary experiment, the amount of radioactivity released spontaneously from the gels was measured. A volume of 100 µl 0,05M glycine-NaOH, pH 8,8 was added to duplicate wells and left for 10 minutes. The total solution was withdrawn and the radioactivity measured. This was repeated three times for each well. The radioactivity present in each sample did not exceed that of a background sample containing fresh buffer only.

Assay

In a typical assay, the enzyme solutions were added in a total volume of 500 µl to duplicate wells and 100 µl were withdrawn after different periods of incubation at 37°C. The 100 µl aliquots were placed into 5 ml Instagel and counted in a scintillation counter (Packard). Duplicate background wells containing buffer only were included for each time point. The average background counts were subtracted from the corresponding average enzyme sample counts at each time point to obtain the true counts per minute present in the 100 µl aliquot. The total cpm released in the wells was then calculated from the total volume present at a particular time point, and the aliquot volume. The cumulative percentage of total radioactivity released at each time point could be obtained by dividing by the total radioactivity initially added to each well.

The calculations can be expressed in short as follows:

$$\text{Total cpm}_t = \frac{(\text{cpm}_t - \text{cpm}_{bt}) \times V_t}{vt}$$

$$\% \text{ radioactivity released} = \frac{\text{Total cpm}_t}{\text{Total cpm in well at } t_o}$$

where cpm_{bt} = background cpm at time t

cpm_t = sample cpm at time t

V_t = total volume in well at time t

vt = aliquot volume counted at time t

(Note that V_t was reduced at each time point by the cumulative sum of aliquot volumes withdrawn at previous time points).

This assay system was used to examine the cercarial secretions for collagenolytic activity. Collagenase derived from Clostridium histolyticum was assayed simultaneously to establish the validity of the method; trypsin controls were included to detect lack of specificity of the substrate.

Results

The results of a typical experiment are given in Table 2.2 and summarized graphically in Figs. 2.1 - 2.4. In all cases the initial reaction volumes in the wells was 500 µl. Trypsin and collagenase were dissolved in 0,02M Tris-HCl pH 7,5 containing 10mM $CaCl_2$. Cercarial secretions were incubated in 0,05M glycine-NaOH pH 8,5.

The series of progress curves in Fig. 2.1 show that over a prolonged incubation time (21 hr) only the lower concentrations of collagenase (0,2 µg/ml) gave radioactivity release which was linear with time. Collagenase concentrations

TABLE 2.2. HYDROLYSIS OF ^{14}C-COLLAGEN BY COLLAGENASE, TRYPSIN AND CERCARIAL SECRETION

ENZYME	CONCENTRATION (µg Protein/ml)	CUMULATIVE % TOTAL RADIOACTIVITY RELEASED†			
		1 hr	2 hr	3 hr	21 hr
Collagenase	20	24,58	39,92	46,24	51,01
	10	17,61	36,62	41,09	49,02
	2	8,81	21,09	25,07	45,92
	1	3,77	11,08	15,51	40,24
	0,2	0,66	3,37	4,50	30,44
Trypsin	500	1,79	3,71	2,50	4,45
Cercarial secretion	500	0,47	0,28	0,14	2,14
None	-	1,41	2,28	3,49	4,85

†Each well contained 0,3 mg (15 000 cpm of gelled ^{14}C-collagen; radioactivity solubilized by hydrolysis is expressed as cumulative % of the total initial radioactivity as described in the text.

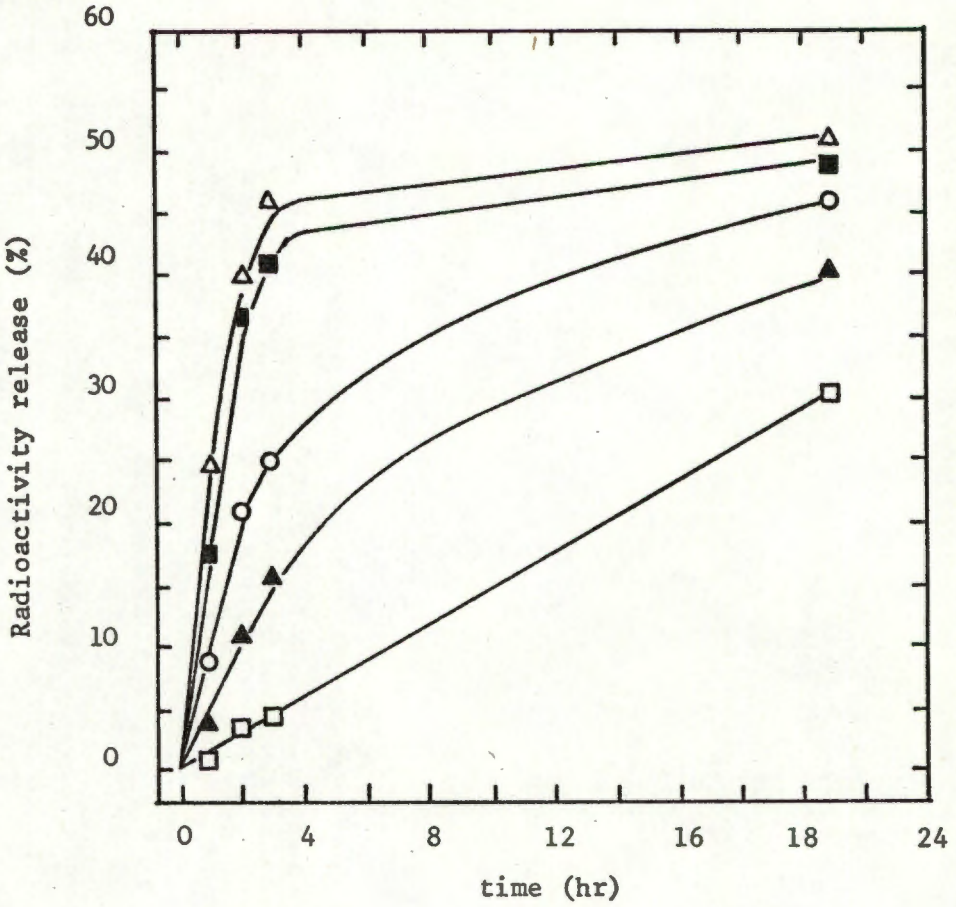


Figure 2.1 Hydrolysis of ^{14}C-collagen by collagenase

The reaction mixture in a final volume of 500 µl contained 0,02M Tris-HCl pH 7,5, 10 mM $CaCl_2$ and 20 µg/ml (△), 10 µg/ml (■), 2 µg/ml (○) 1 mg/ml (▲) and 0,2 µg/ml (□) of collagenase. Samples of 100 µl were withdrawn after 1, 2, 3 and 21 hours, and the radioactivity released was measured.

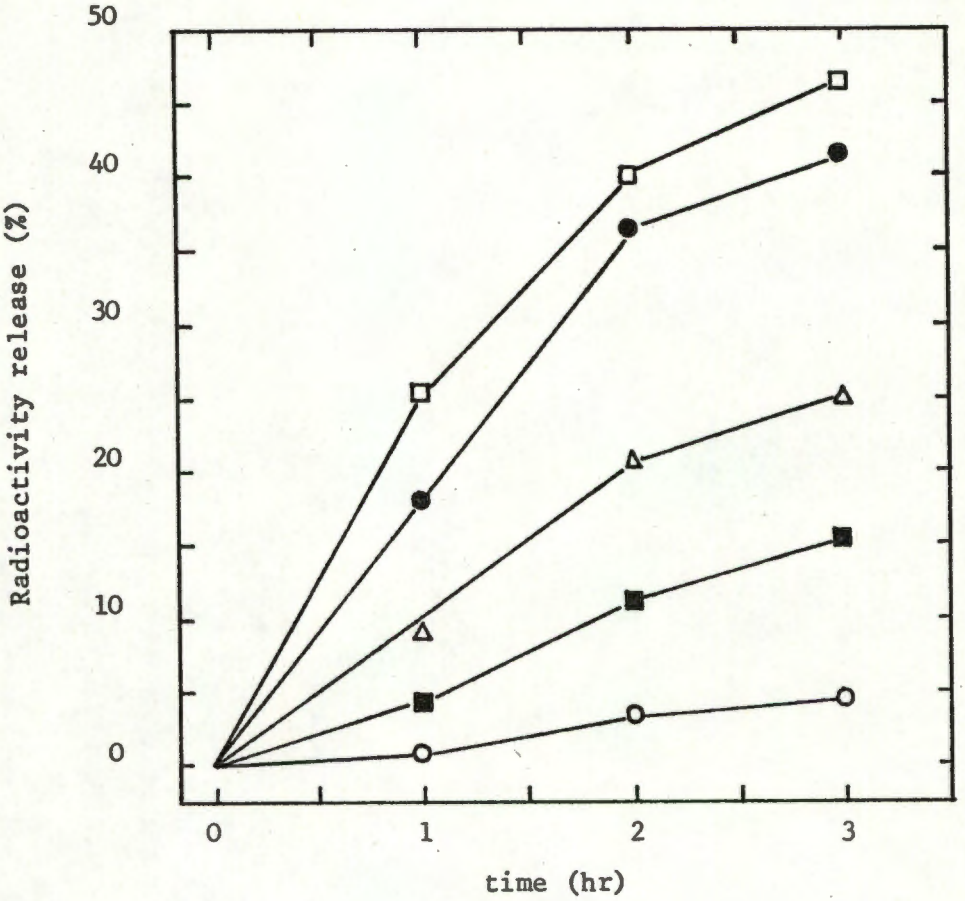


Figure 2.2 Hydrolysis of ^{14}C-collagen by collagenase

The reaction mixture in a final volume of 500 µl contained 0,02M Tris-HCl pH 7,5, 10 mM $CaCl_2$ and 20 µg/ml (□), 10 µg/ml (●), 2 µg/ml (△) 1 µg/ml (■), and 0,2 µg/ml (○) of collagenase. Samples of 100 µl were withdrawn after 1, 2 and 3 hours, and the radioactivity measured.

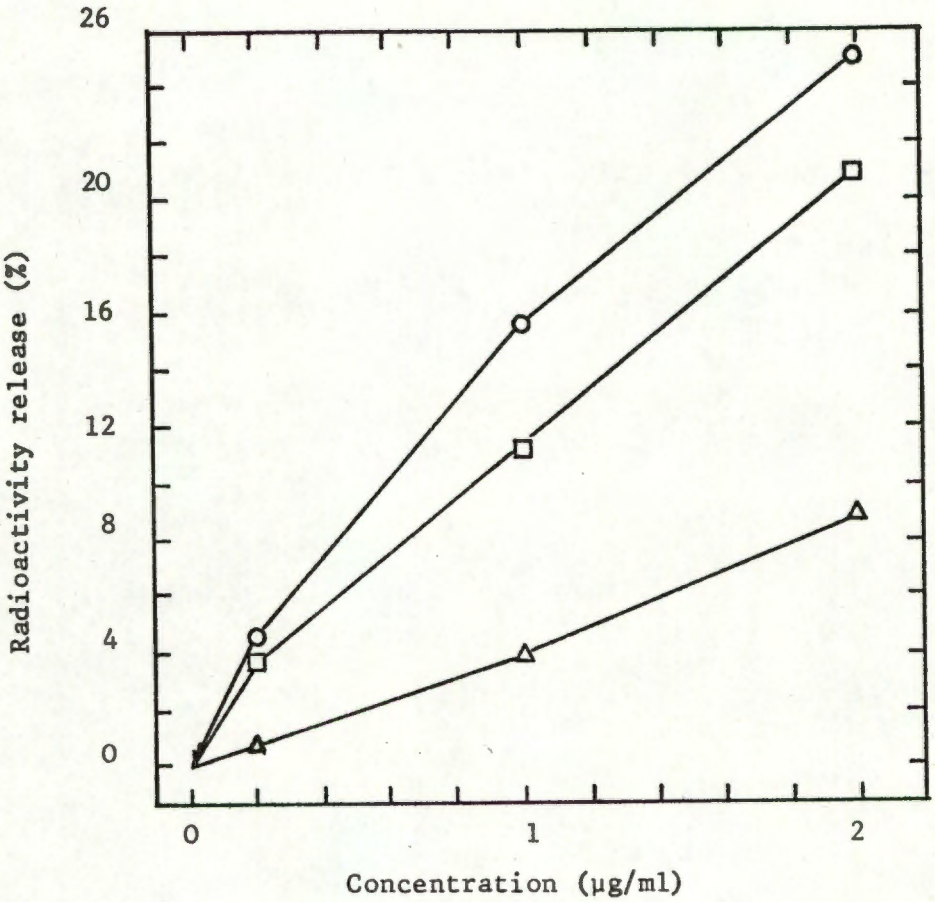


Figure 2.3 Hydrolysis of ^{14}C-collagen by collagenase

Replot of Fig. 2.1 showing the dependence of collagen hydrolysis on collagenase concentration after 1 hour (△), 2 hr (□) and 3 hr (O) incubation.

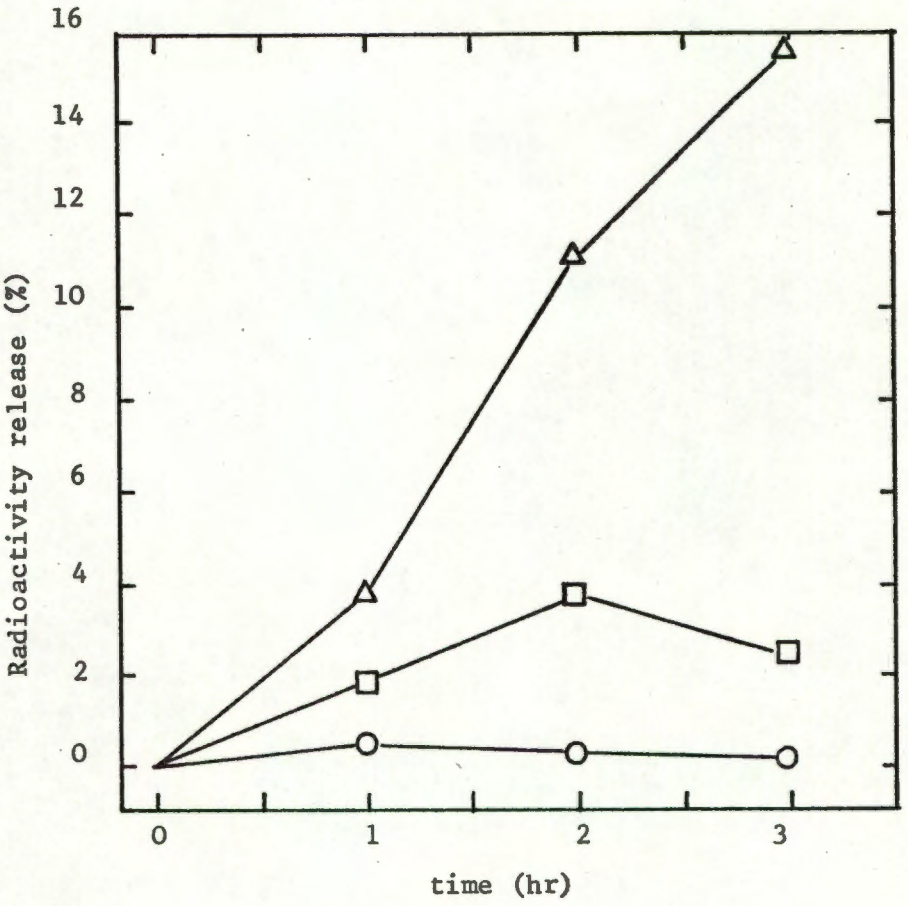


Figure 2.4 Hydrolysis of ^{14}C-collagen by collagenase, trypsin and cercarial secretion.

The reaction mixture in a final volume of 500 µl contained either 1 µg/ml collagenase in 0,02M Tris HCl pH 7,5 (Δ), 500 µg/ml trypsin in 0,02M Tris HCl pH 7,5 (□) or cercarial secretion at a protein concentration of 500 µg/ml in 0,05M Glycine-NaOH, pH 8,5 (O). Aliquots of 100 µl were withdrawn after 1, 2 and 3 hours and the radioactivity measured.

up to 2 µg/ml gave results that were linear with time for short time periods (Fig. 2.2).

A linear relationship between collagenase concentration and release of ^{14}C-labelled degradation products was only observed at the 1 and 2 hr time point below 2 µg/ml. If longer incubation times were to be employed, the enzyme concentrations used should be reduced to less than 0,2 µg/ml. In Fig. 2.4, the activity of trypsin (500 µg/ml) and cercarial secretions (500 µg protein/ml) on the substrate are compared to that of 1 µg/ml collagenase. The results indicate that cercarial secretions were devoid of collagenolytic activity as measured by this procedure. This is in agreement with the observations of others (24, 34). The minimal amount of radioactivity released by trypsin showed that nonspecific proteolysis in this assay was negligible.

Discussion

True collagenases are restricted, in their specificity, to hydrolysis of undenatured collagen, and, in particular, the polyproline portion of the collagen helix. I have already mentioned that Azocoll does not represent a true collagenase substrate. Various non-protein substrates have been synthesized but these are often hydrolysed by nonspecific peptidases in crude preparations of a particular collagenase. For these reasons I have used native collagen to test for collagen activity in cercarial secretions.

Enzymatic hydrolysis of native collagen has been measured by various methods. Some have involved observations of changes in such physical properties of soluble collagen

as viscosity or opacity (38, 39). In others, dissolution of insoluble collagen was measured by examining the supernatant for biuret- phenol- or ninhydrin-reactive peptides after separation from the substrate. The most sensitive assay involved degradation of radioactive insoluble collagen as described by Nagai et al (39). In this method the enzyme was added to a suspension of insoluble collagen fibres in a test tube and the release of radioactive peptides were measured after centrifugation. The method I have devised was based on the same principle and proved capable of detecting less than 0,2 µg of collagenase.

^{125}I-FIBRIN ASSAY

The method used was adapted from that devised by Unkeless et al (40, 41) for the measurement of plasminogen-dependent fibrinolytic activity released by cells in culture. Preliminary experiments showed that fibrinolysis by cercarial proteases was direct and showed neither enhancement by, nor a requirement for, plasminogen.

The principle of the assay was essentially similar to that described for the ^{14}C-collagen assay. Purified bovine fibrinogen was iodinated with ^{125}I, diluted with non-radioactive fibrinogen and added to the wells of a Linbro plate, where it was allowed to dry. Before the assay was performed, the fibrinogen in the wells was converted to fibrin by the addition of foetal calf serum which contained enough thrombin for this purpose. It is probable that crosslinking of the fibrin strands also occurred under these conditions owing to the presence of Factor XIII in the serum. The cercarial enzyme solutions, in appropriate buffers, were added to the wells and aliquots were withdrawn at different time points for radioassay of the released fibrin degradation peptides.

Purification of fibrinogen

Commercially obtained bovine fibrinogen (Fraction I F-4000, Sigma Chemical Company, St. Louis MO 63178, U.S.A.) was further purified by precipitation as described by Laki (42) and freed of plasminogen by the method of Mosesson (43). The latter method exploits the differential solubility of fibrinogen and plasminogen in the presence of lysine.

A solution of 2g of bovine fibrinogen in 100 ml of 0,1M potassium phosphate buffer pH 6,7 was brought to 200 ml with distilled water. After standing overnight at 4°C the solution was centrifuged at 1000g for 15 minutes and the pellet was discarded. The fibrinogen was precipitated by the addition of ammonium sulphate to a final concentration of 25% saturation, collected by centrifugation and redissolved in 50ml of 0,6M NaCl. The pH was adjusted to 7,4 with dilute NH_4OH. After dialysis against three changes of 0,6M NaCl, the fibrinogen concentration was adjusted to 10mg/ml with 0,6M NaCl. A volume of 0,12M lysine-HCL in 0,005M sodium phsophate buffer, pH 7,0 was then added to the solution such that the final concentration of lysine was 0,02M. The solution was brought to 0°C in a salt/ice bath and any precipitate that formed was discarded. The fibrinogen was then precipitated by the addition of ice-cold ethanol to a final concentration of 7%. The precipitate was collected by centrifugation (1500g; 10 min), redissolved in 0,6M NaCl and adjusted to a protein concentration of 10mg/ml. Ethanol precipitation in the presence of lysine was repeated once. The final precipitate was dissolved in 0,6M NaCl, dialysed against phosphate buffered saline containing 10^6 U/l of penicillin and 0,2g/l streptomycin. The protein concentration was adjusted to 10 mg/ml and the final solution was stored frozen in 2 ml aliquots at -80°C.

Fibrinogen concentrations were determined spectrophotometrically by taking $E^{1\%}_{280nm} = 1,3$.

Preparation of ^{125}I-fibrinogen

The purified fibrinogen was labelled with ^{125}I using the iodine monochloride method of Helmkamp et al (44).

A stock solution of ICl, 2,0M in NaCl, 0,02M in KCl and 1,0M in HCl was kept at 4°C until use. The exact molarity of the ICl in solution was 0,0174M as determined by titration with a standardized thiosulfate solution. Before use the stock solution was diluted with 2M NaCl and stored on ice. Usually 242,4 nmoles of ICl in a volume of 0,8 ml was used for iodinating 20 mg (60,6 nmoles) of fibrinogen.

Borate buffer was prepared by adjusting a solution of 0,32M NaCl and 0,40M H_3BO_3 to pH 7,65 with 2M NaOH. Fibrinogen was prepared for iodination by adding 2 ml of this buffer to 2 ml of purified fibrinogen solution containing 20 mg protein.

Carrier-free $Na^{125}I$ (10mCi; 17,5 mCi/µg) in dilute NaOH was diluted to 1 ml with borate buffer in a pyrex tube. To this was added 0,3 ml of a 0,6% Na_2SO_3 solution in distilled water to destroy any peroxides present. After standing at room temperature for 15 minutes, the solution was placed in a boiling water bath and aerated with moist air for 15 minutes to oxidize the excess sulfite to sulfate. After cooling to room temperature, the 0,8 ml ICl solution was rapidly added and the mixture was squirted into the fibrinogen solution. The reaction mixture was then passed through a 2 ml column of Dowex AG1-X4 in the Cl^- form previously treated with nonradioactive fibrinogen to block nonspecific binding sites.

The ^{125}I-fibrinogen solution was collected as the

non-adsorbed radioactive fraction and dialysed against three changes of phosphate buffered saline containing antibiotics. It was then passed through a 0,45 µ millipore filter and stored at 4°C in small portions.

The efficiency of the iodination procedure was usually approximately 50% giving an average of 2 atoms of iodine/molecule of fibrinogen.

Preparation of ^{125}I-fibrin-coated Linbro plates

The 24 wells of a Linbro plate were coated with 10 µg/cm^2 of ^{125}I-fibrinogen by adding, to each well, 200 µl of a solution adjusted to contain 30 µg of fibrinogen and approximately 100 000 cpm. The plates were dried at 37°C for 72 hours and stored at room temperature. Before use the fibrinogen was converted to fibrin by the addition of 1 ml of a 5% solution of foetal calf serum in minimal essential medium to each well and incubation at 37°C for 2 hours. After incubation the wells were washed three times with 1 ml volumes of the buffer to be used in the enzyme assay.

^{125}I-fibrin plate assay for measuring cercarial protease activity

Preliminary experiments showed that maximal activity of the cercarial proteases was obtained in the presence of 0,05M glycine-NaOH buffer, pH 8,8. Enzyme solutions (usually in 5% glucose - cf Chapter 4) were therefore added to assay wells and diluted with an equal volume of 0,1M glycine-NaOH buffer. Total reaction volumes varied from 0,2 to 1,0 ml, depending upon experimental circumstances.

The charged plates were then incubated at 37°C. After different time intervals aliquots were withdrawn from the wells for radioassay in a Packard gamma spectrometer.

All samples were assayed in duplicate and controls containing buffer only or buffer with water from infected snails after harvesting of cercariae were included.

Cumulative radioactivity released by enzyme samples was calculated as the percentage of the total radioactivity released by 300 µl of 0,2% trypsin in 0,1M Tris-HCl buffer, pH 7,4. The calculations were otherwise similar to those used for the collagenase assay (page 26).

RESULTS

Since the ^{125}I-fibrin substrate was present in the assay mixture as a solid-phase cross-linked polymer coated on to a plastic surface, the kinetics of proteolysis were somewhat complex. The following experiments were therefore performed to define optimal conditions for valid comparative assays.

(i) A freshly shed suspension of cercariae was counted and dispensed into 4 separate tubes such that each tube contained, in the final volume of 2 ml, 240 000, 160 000, 80 000 and 40 000 organisms respectively. Enzyme release was induced by the addition of 0,5 ml 25% glucose and incubation at 37°C for 2 hours. The organisms were removed by centrifugation and 100 µl aliquots of the supernatant fluid were assayed in duplicate in the ^{125}I-fibrin assay. Each well contained, in addition to the 100 µl of enzyme solution, 200 µl of 0,1M glycine-NaOH buffer, pH 8,8.

Cumulative release of radioactivity was measured as a function of time by counting 25 µl aliquots of the reaction volume after 15, 30, 45, 60, 90 and 150 minutes of incubation at 37°C.

The results are summarized graphically in Fig. 2.5 and indicate (a) that the cumulative release of radioactivity was directly related to the concentration of enzyme in the wells (i.e. the number of cercariae from which the protease was obtained); (b) that the reaction was linear with time until approximately 40% of the trypsinizable fibrin had been solubilized. This linearity was evident for 150 min when samples from 40 000 and 80 000 cercariae were assayed; (c) when higher concentrations of enzyme were added to the wells, available sites for hydrolysis became limited after approximately 60% of the trypsinizable fibrin had been digested. At this point a plateau was reached and the reaction proceeded at a negligible rate.

(ii) To ascertain the lower limit of the assay system for reliable enzyme detection, enzyme was collected from 10 000 cercariae into a volume of 1,5 ml. Doubling dilutions of this solution were made in 5% glucose and 500 µl aliquots of these dilutions were added to assay wells with 500 µl of glycine-NaOH buffer. Aliquots (100 µl) of the reaction mixture were assayed for radioactivity at hourly time points for 6 hours.

The results are shown graphically in Fig. 2.6 and show that, after a very short lag period, the cumulative release of radioactivity proceeded linearly with time for 6 hrs in all cases. Furthermore, cumulative fibrinolysis

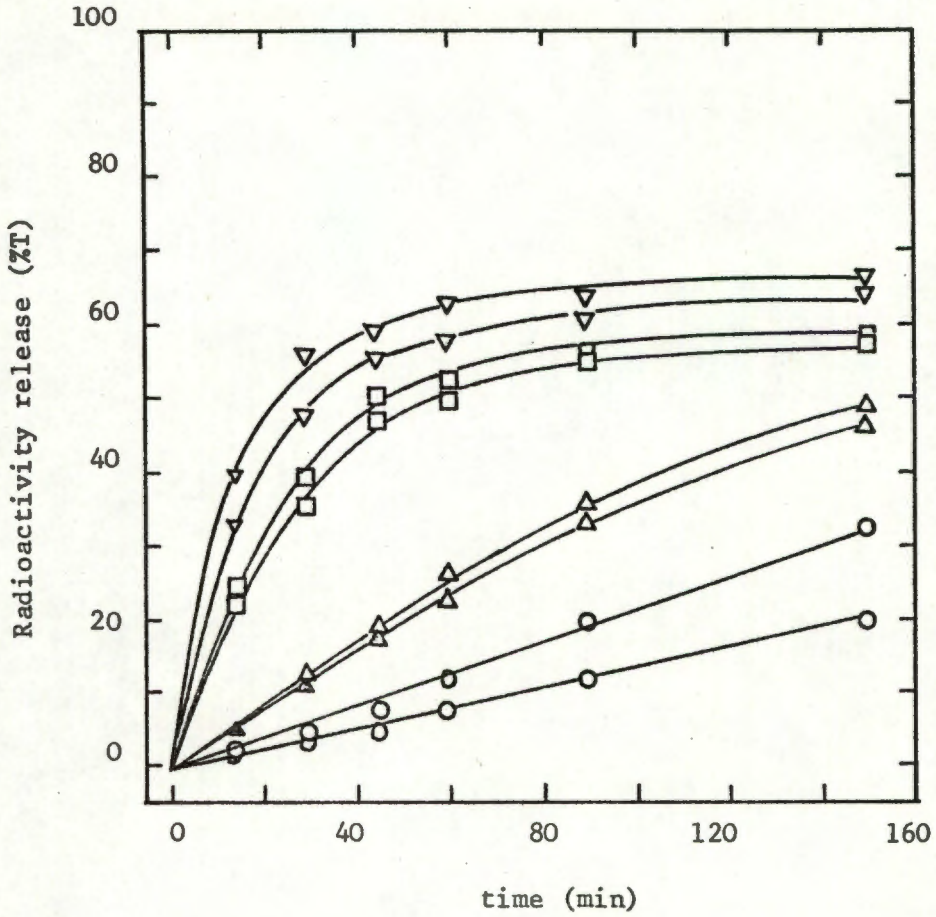


Figure 2.5 Hydrolysis of ^{125}I-fibrin by cercarial secretions.

Graphs showing rate of fibrinolysis by 100 µl of cercarial secretion collected from 240 000 (▽), 160 000 (□), 80 000 (△) and 40 000 (○) organisms. The total volume in each well was 300 µl and 25 µl aliquots were withdrawn after different time intervals.

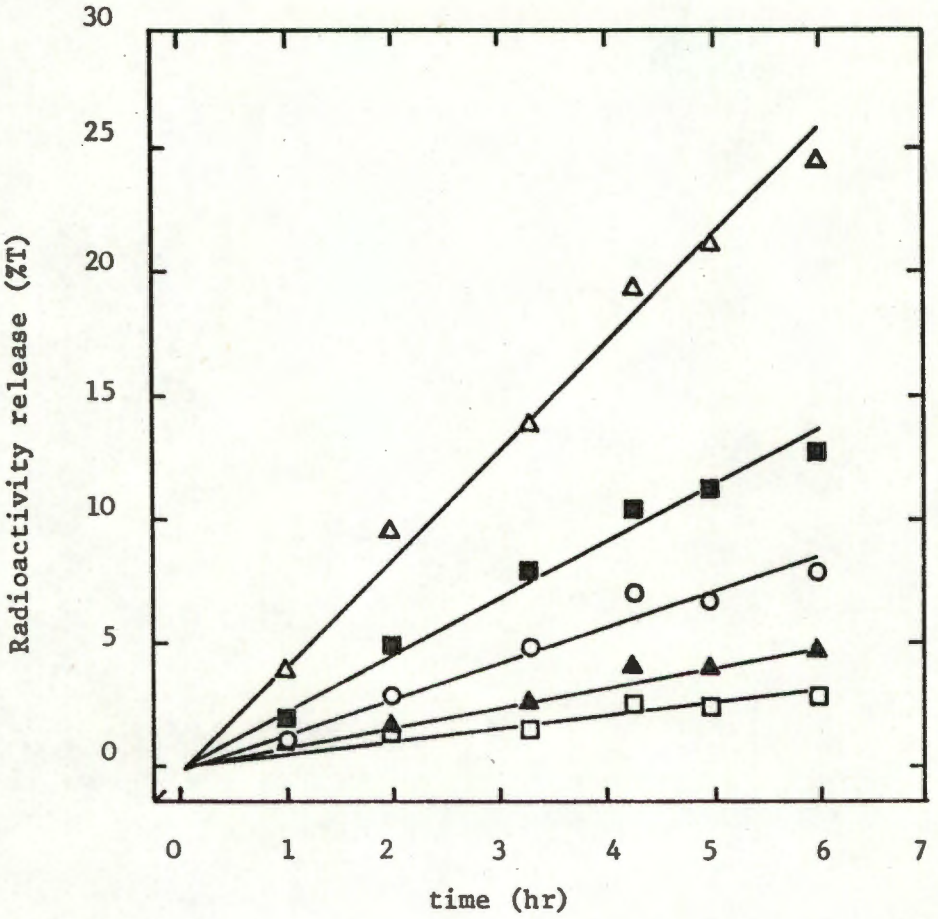


Figure 2.6 Hydrolysis of ^{125}I-fibrin by dilutions of cercarial secretions.

Plots of radioactivity released vs. time for the following dilutions of a cercarial secretion sample: 1/1 (△), 1/2 (■), 1/4 (○), 1/8 (▲) and 1/16 (□). Aliquots of 500 µl of the dilutions were added to 500 µl Gly-NaOH, pH 8,80 and 100 µl withdrawn at hourly intervals for 6 hours.

was linearly related to the enzyme concentration (Fig. 2.7). When the rates of fibrinolysis were calculated from the slopes of the least mean squares regression line for cumulative release of radioactivity upon time (i.e. Δ% Trypsinisable radioactivity/Δt) and these rates were plotted as a function of enzyme concentration, excellent linearity was observed (Fig. 2.8).

DISCUSSION

The results of these experiments showed that cercarial proteases were capable of catalysing the hydrolysis of fibrin and that, with certain experimental constraints, the hydrolysis of radioactive fibrin could be used to form the basis for a sensitive assay for these enzymes that was linear with time and enzyme concentration.

As is apparent from Fig. 2.5, the proteolytic capacity of the cercarial enzyme was limited to approximately 60% of the available substrate, whereas trypsin was able to solubilize effectively 100% of the cross-linked fibrin polymer. Although I have been unable to investigate this observation in any detail, it has been a constant feature of my experience with this system and suggests that the action of the cercarial enzyme is restricted to those regions of the fibrin matrix that are not responsible for retaining it in an insoluble or plastic-adherent form. In this respect it differs from trypsin.

The practical effect of this apparent restriction has been to impose the experimental constraint upon the assay system that the reaction rate, for any given enzyme

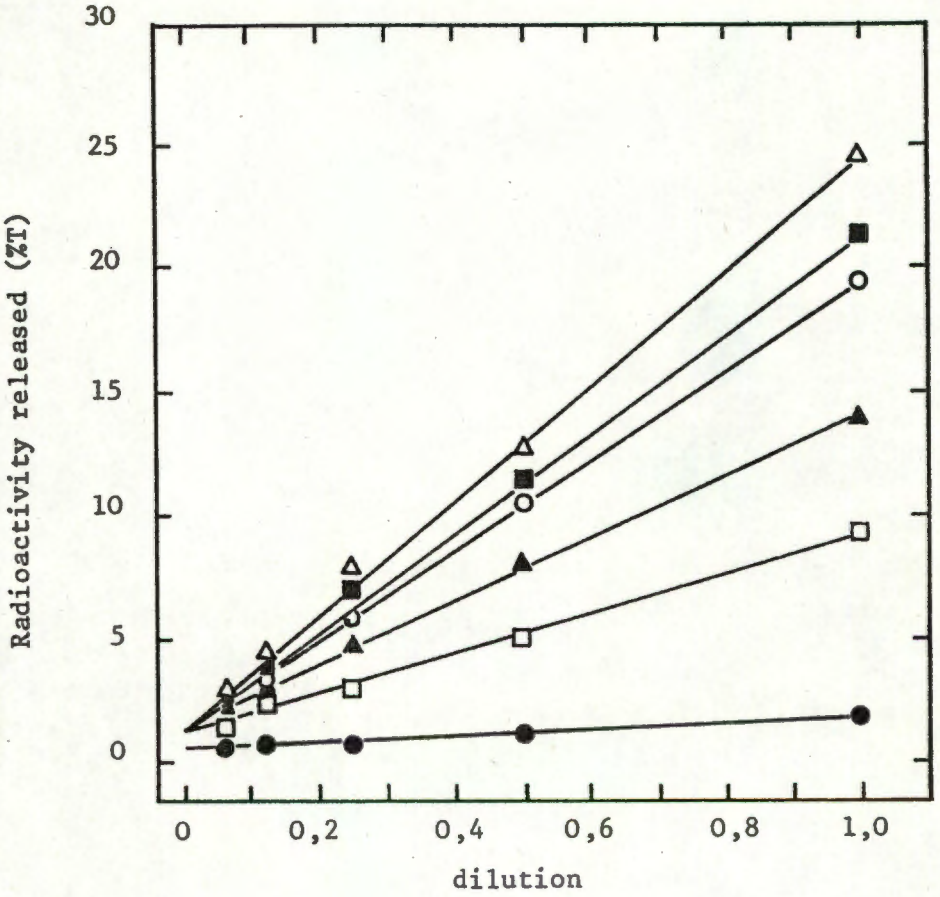


Figure 2.7 Linearity of fibrinolysis with dilution of cercarial secretion.

The percentage radioactivity released after 1 hr (●) 2 hr (□) 3 hr (▲) 4 hr (○) 5 hr (■) and 6 hr (△) incubation is plotted against the dilution of the cercarial secretion. Conditions of the assay were the same as in Fig. 2.6.

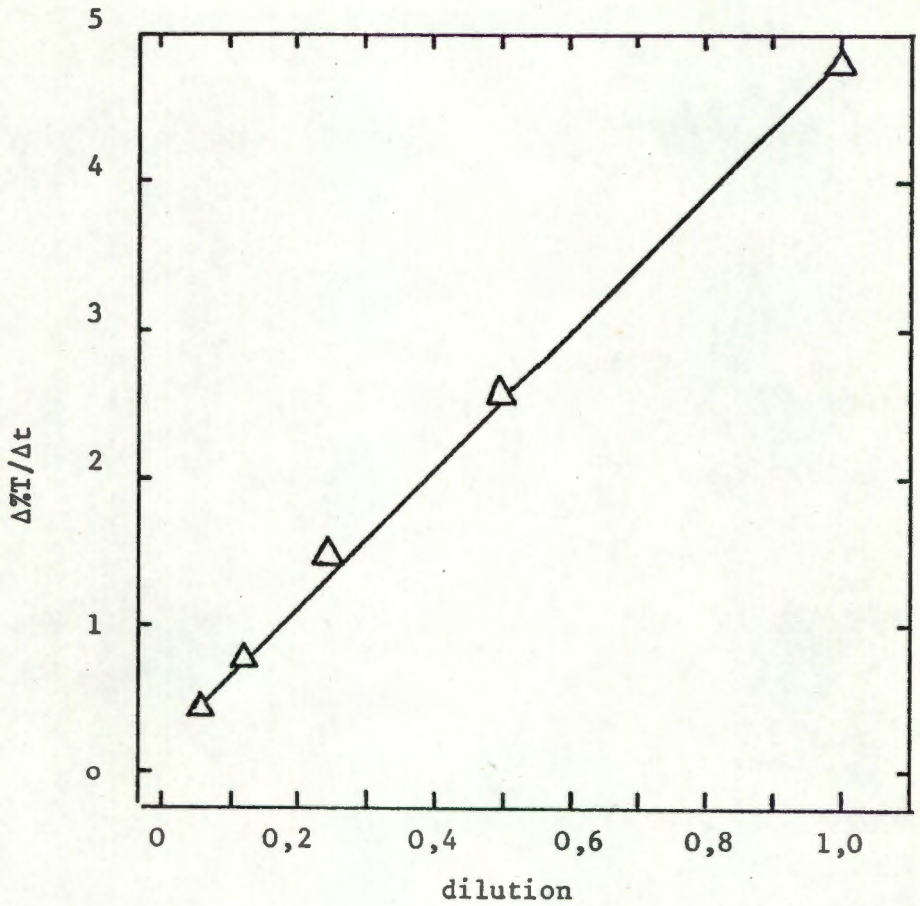


Figure 2.8 Linearity of the rate of fibrinolysis with enzyme dilution.

The rates of fibrinolysis calculated from the slopes of the least mean squares regression lines for cumulative release of radioactivity upon time are plotted as a function of enzyme dilution. The data of Figure 2.6 were used (ΔZT/Δt - Rate of percentage trypsinable radioactivity released).

sample, should be measured over a period of time that does not give proteolysis in excess of 50% of the total trypsin-soluble radioactivity. Provided this constraint was observed, quantitatively reliable, linear relationships between velocity and amount of enzyme could be obtained.

With low levels of enzyme activity an initial lag period was observed during which the reaction rate was ostensibly slower. I am, once again, unable to offer an entirely satisfactory explanation for this kinetic phenomenon but it could be accommodated by taking samples at several time points and using only the linear portion of time versus cumulative release curve for calculating the reaction rate.

As I gained experience with the assay system I found that I was able to obtain uniformly satisfactory results by the use of a protocol in which an amount of enzyme released by 80 000 to 100 000 cercariae per 2,5 ml suspension was assayed in a final reaction volume of 300 µl. Aliquots taken at time points between 15 min and 2 hr gave useful linear results.

With this standard protocol I have arbitrarily defined one unit of enzyme activity as that amount that would solubilize 1% of the trypsin-soluble fibrin in 1 hour.

CHAPTER 3

CHARACTERIZATION OF PROTEASES PRESENT IN CERCARIAL SECRETION SAMPLES BY ELECTROPHORESIS IN POLYACRYLAMIDE GELS CONTAINING SODIUM DODECYL SULPHATE.

In attempting to define the proteolytic activity of any complex biological system, it is desirable to establish, at an early stage, the number of proteolytic enzymes present in that system. This information is required to characterize the system in biochemical terms, to serve as a guide for purification, and to provide the basis for further investigation of the relationships that exist between different molecular species of protease if more than one is present.

The number of proteases present in cercariae is uncertain, and the literature on the subject is conflicting. In most cases, different molecular forms of cercarial proteases have been defined by chromatographic procedures using gel filtration or ion exchange techniques and the results obtained have tended to vary with the experimental conditions used to obtain and study the enzymes (33). Gazzinelli et al (26), who reported three peaks of proteolytic activity with DEAE Sephadex; and Dresden and Asch (24) who reported five peaks with Sephadex G150, both used whole cercarial extracts which may have contained non-secretory proteases.

Campbell et al (33) observed only two peaks of activity with Sephadex G75 chromatography of collected

secretions from cercariae. In their hands, calibrated molecular exclusion chromatography indicated an approximate molecular weight of 29 000 daltons for the main proteolytic component.

The molecular characterization of cercarial proteases has not been straightforward owing to a number of factors. In the first instance, analytical column chromatography of impure preparations require conditions that preserve enzyme activity. These conditions, particularly when low total protein concentrations are involved, may lead to enzyme aggregation or to the formation of coacervates between the enzyme and other proteins present, with the result that several peaks of proteolytic activity may appear in the column effluent to give a spurious impression of several enzyme species. Furthermore, molecular weight estimates based upon analytical gel exclusion chromatography are notoriously liable to errors resulting from electrostatic or hydrophobic protein-gel interactions. These can be counteracted by the use of high ionic strength buffers or chaotropic solutes, but, in the case of cercarial proteases, enzyme activity is inhibited under these conditions.

In the second instance, conventional procedures for molecular weight determination such as analytical ultracentrifugation or electrophoresis in polyacrylamide gels containing dodecyl sulphate (SDS), require pure enzyme in amounts that allow its confident detection as a protein. In the case of cercarial proteases, this ideal is techni-

cally and logistically very difficult to achieve. A large commitment of laboratory and animal house resources provides cercariae in limited numbers and these, in turn, provide very small quantities of crude enzyme to serve as source material for the purification.

A solution to this problem was suggested by a recent publication by Granelli-Piperno and Reich (45) showing that the inhibition of serine-proteases by SDS was reversible; that activity of enzymes electrophoresed in SDS-polyacrylamide gels could be restored by removal of the SDS with non-ionic detergents; and that relative electrophoretic mobilities of enzyme bands could be measured definitively by contact zymography on layers of agar containing a fibrin substrate.

Since mobility relative to markers of known molecular weights under the dissociating conditions of SDS-gel electrophoresis provides reliable estimates of protein molecular weights (46) application of the following approach seemed appropriate. The cercarial proteins would be electrophoresed in SDS-polyacrylamide gels, the SDS would then be removed by incubating with Triton X-100 and the position of the enzymatically active bands detected by zymography.

Several techniques for zymographic detection of enzyme activity in the gels were available to me:

(i) the gel could be sliced and each sequential slice assayed for enzyme activity.

(ii) the gel could be incubated with a chromogenic substrate and zones of enzyme activity identified

as coloured bands.

(iii) the polyacrylamide slab gel could be placed in contact with a layer of agar containing fibrin and the zones of activity detected by clearing of the opaque fibrin layer or by fixation and staining of the fibrin-agar slab for clear zones of proteolysis against a stained background.

(iv) the protein substrate could be incorporated into the running gel at the time of polymerization of the polyacrylamide. After SDS removal, incubation of the polyacrylamide slab followed by staining would detect bands of proteolysis as protein-depleted clear zones against a stained background. This approach was suggested by a paper by Hochstrasser and Schorn (47) who used this detection procedure for proteinases in polyacrylamide gels run without SDS.

This general approach proved very successful and I was able to develop a useful procedure for the characterization of cercarial proteases by electrophoresis in polyacrylamide slab gels containing SDS and gelatin. Copolymerization of the gelatin into the polyacrylamide proved more satisfactory than the fibrin-agar overlay procedure.

Using this technique I have been able to identify two major proteolytic molecular species in cercarial harvest water. The first, with a molecular weight of 35 000 daltons appeared to be a true cercarial protease. The second, with a molecular weight of 22 000 daltons proved to be a bacterial

protease, derived from micro-organisms contaminating the cercarial preparations.

Fibrin overlay technique

The method used was a modification of that described by Granelli-Piperno and Reich (45). Samples of cercarial secretions and molecular weight marker proteins were electrophoresed at 4°C in a 5-16% polyacrylamide gradient slab gel containing 0,1% SDS. After electrophoresis, the track containing the marker proteins was cut from the rest of the gel and fixed and stained in 0,1% Coomassie blue in methanol: acetic acid:water (30:10:60).

The SDS was removed from the remainder of the slab by incubation at room temperature for 1 hour in 2,5% aqueous Triton X-100, followed by brief sequential washes in water and 0,1M glycine-NaOH, pH 8,8. Excess liquid was drained from the slab after which it was layered on a 0,5mm thick indicator gel of 1% agar containing 2,6 mg/ml of freshly clotted fibrin in glycine-NaOH buffer. The polyacrylamide/ fibrin-agar gel assembly was then incubated at 37°C in a humid environment and examined periodically under dark ground illumination for clear dark zones of fibrinolysis against the opaque, white background of undigested fibrin.

After 12 hours of incubation, no bands of proteolysis could be seen, so the fibrin-agar indicator layer was fixed and stained with 0,1% Amido black in methanol: acetic acid:water (30:10:60).

After destaining, faint bands of partial proteolysis were visible, the most prominent corresponding to a protease with an apparent molecular weight of 35 000 (Fig. 3.1 and Fig. 3.2).

Numerous attempts were made to improve the quality of the results obtained with fibrin-agar zymography, including the addition of plasminogen to the fibrin agar layer; prolongation of the incubation time; substitution of agarose for agar; and the use of different buffer systems for the indicator gel. The proteolytic bands remained barely visible, however, and this indicator gel substrate was finally abandoned.

Gelatin-gel electrophoresis

Although ^{125}I-fibrin proved a useful substrate for the radio-enzymatic assay (cf Chapter 2), this protein gave unsatisfactory results when used as an indicator in agar gels.

Gelatin appeared to be a suitable alternative for a number of reasons. It is well established that cercarial proteases are able to digest gelatin both in tube assays using a radioactive substrate (24) and in thin films (23,33). This protein is susceptible to hydrolysis by a wide variety of proteases and should, therefore, serve as a general substrate whose usefulness as an indicator would not be restricted by the specificity of any enzymes present in cercarial secretions. Finally, Hochstrasser and Schorn (47) had shown that gelatin, when copolymerized into polyacrylamide gels, did not migrate out of the gel during electrophoresis and could thus serve as an *in situ* substrate

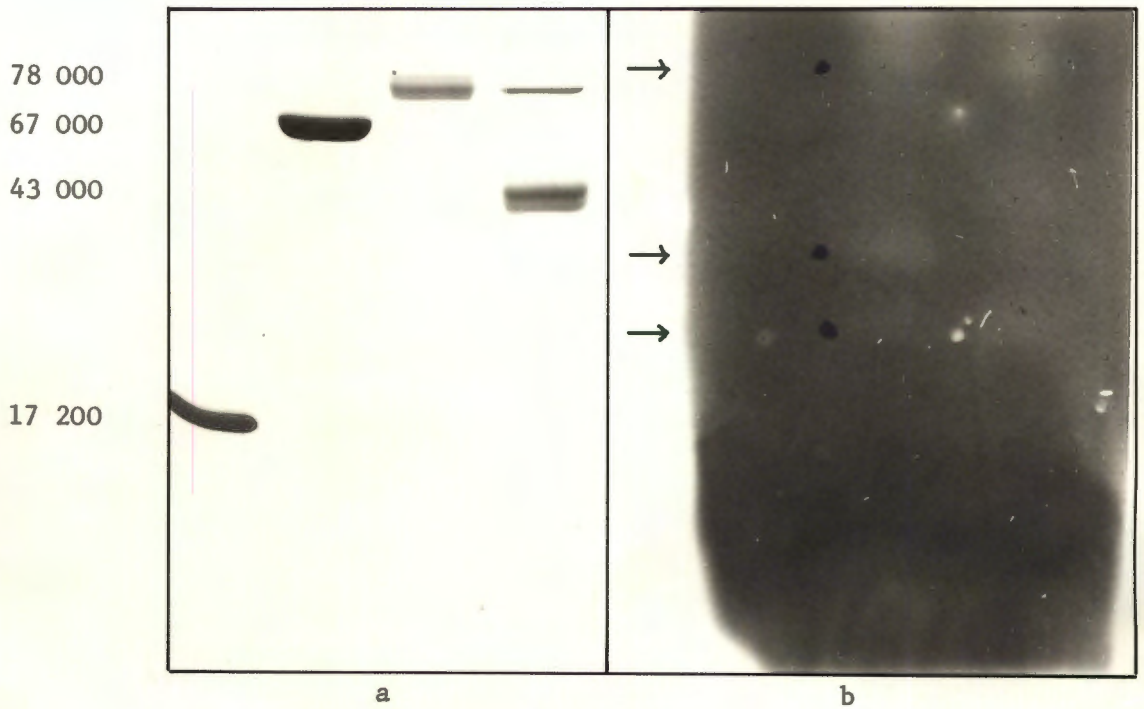


Figure 3.1 Analysis of cercarial secretions by the fibrin overlay technique.

Samples of cercarial secretions and molecular weight marker proteins were electrophoresed in a 5-16% gradient polyacrylamide slab gel containing 0,1% SDS. The track containing the marker proteins was cut from the rest of the gel and stained. The remainder of the gel was layered on a fibrin agar slab for detection of proteases.
(a) The gel contained the following marker proteins: myoglobin, bovine serum albumin, lactoperoxidase and ovalbumin.
(b) Fibrin overlay showing faint bands of lysis caused by proteases present in the overlayered polyacrylamide gel (arrows).

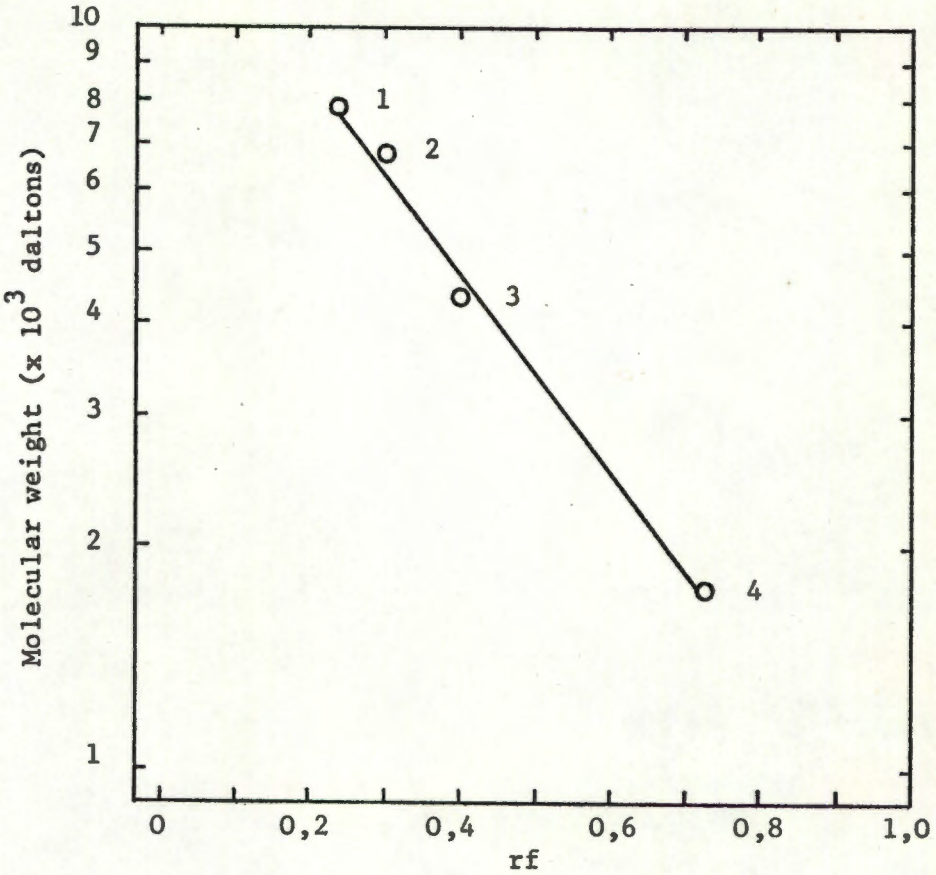


Figure 3.2 Estimation of the molecular weight of proteases in cercarial secretion by the fibrin overlay technique.

The figure shows a plot of the relative mobilities (rf) for each marker protein against the logarithm of their respective molecular weights:

1) lactoperoxidase 78 000, 2) BSA 67 000

3) ovalbumin 43 000, 4) myoglobin 17 200.

The following molecular weights for the proteases were obtained by interpolation:

1) 62 700 2) 35 000 3) 32 600 and 4) 20 400.

for electrophoresed protease bands. This information, taken together with the knowledge that enzymatic activity of electrophoresed protease bands in SDS-polyacrylamide gels could be restored by removal of the SDS with Triton X-100, suggested an experimental approach in which cercarial proteases would be electrophoresed in polyacrylamide gels containing SDS and copolymerized gelatin. The SDS would be removed and the gel incubated to allow localized hydrolysis of the gelatin substrate to proceed where bands had been concentrated by the electrophoretic process. After staining and destaining, the bands of proteolytic activity should be visible as clear zones against a stained background.

Preliminary experiments with this approach gave very encouraging results and led to the adoption of the following standard of experimental protocol.

Polyacrylamide resolving slab gels measuring 60 x 70 x 1 mm were cast so as to contain a uniform total polyacrylamide concentration of 10% or a linear concentration gradient from 6% to 15%. The solution of monomer contained acrylamide and bis-acrylamide in a ratio of 30:1; 0,1% gelatin and 0,1% SDS dissolved in 0,38M Tris-HCl buffer pH 8,8. Polymerization was initiated by the addition of 20 µl of 10% ammonium persulphate and 10 µl of NNN'N' tetramethyl ethylene diamine to 8 ml of the mixture. The resolving gel solution was pipetted into the electrophoresis chamber, carefully overlaid with 0,01% gelatin in water and allowed to polymerize at room temperature. When

polymerization was complete, a stacking gel containing sample wells was cast on top of the resolving gel. The stacking gel contained 3% polyacrylamide in ,125M Tris-HCl, pH 6,8; it did not contain gelatin.

When polymerization of the stacking gel was complete, samples were introduced into the sample wells and electrophoresed at 4°C at a constant current of 8 mA until the phenol red tracking dye had reached the bottom of the resolving gel (usually 3-4 hr). The reservoir buffer was 0,025M Tris, 0,192M glycine, 0,1% SDS pH 8,5.

After electrophoresis the gels were shaken gently in an aqueous solution of 2,5% Triton X-100 for 1 hr to remove the SDS. They were then incubated in 0,1M glycine-NaOH buffer, pH 8,8 at 37°C for 3 - 5 hr for localised proteolysis to take place. The gels were then fixed and stained with 0,1% Amido black in methanol:acetic acid:water (30:10:60). After destaining, concentrated zones of proteolytic activity could easily be seen as clear bands. Marker proteins were seen as darkly staining blue bands (Fig. 3.3).

The results of experiments performed during the development of this procedure may be summarized as follows:

(i) Gelatin could be satisfactorily copolymerized into resolving gels containing between 5% and 16% polyacrylamide. The mechanical properties of the resolving gels and their ability to serve as electrophoretic matrices for other proteins were not appreciably altered by the presence of the gelatin. A satisfactory linear relationship

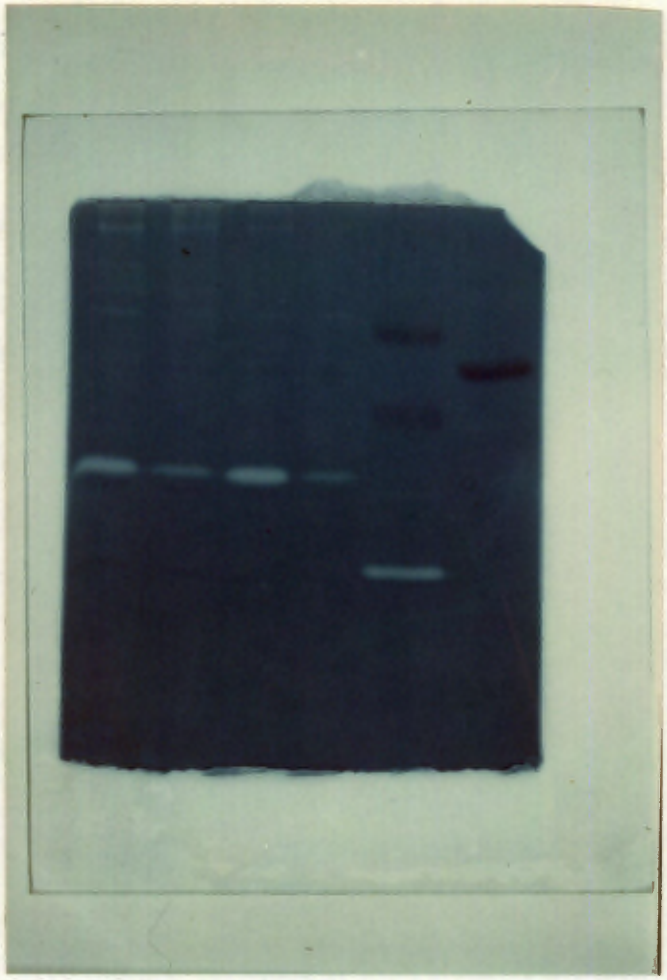


Figure 3.3 Gelatin gel electrophoresis of molecular weight marker proteins and cercarial secretions.

The figure shows a colour photograph of a gelatin gel. Aliquots of 40µl of different cercarial secretion samples (tracks 1 to 4 from L to R) and molecular weight marker proteins (track 5: lactoperoxidase and ovalbumin, track 6: bovine serum albumin) were electrophoresed on the same gel. Note the light bands caused by hydrolysis of the gelatin by proteases, as opposed to the dark blue staining bands of the marker proteins.

between electrophoretic mobility and logarithm of molecular weight was observed for five marker proteins. Although irrelevant, it is of interest to note that commercially obtained myoglobin, cytochrome C and ovalbumin were contaminated with a 21 000 M.W. Protease (Fig. 3.4, 3.5)

(ii) The gelatin did not migrate out of the gel during electrophoresis provided the final concentration of polyacrylamide was 5% or higher and provided the ratio of acrylamide to bisacrylamide was at least 30:1. When the usual ratio of 30:0,8 was used, the gelatin tended to electrophorese out of the matrix, particularly when the total polyacrylamide concentration was low. For this reason, gelatin was omitted from the stacking gel. Its inclusion gave rise to transverse bands of gelatin in the resolving gel.

(iii) Generally speaking, more satisfactory final zymograms were obtained when electrophoresis was performed at $4^{o}C$. Electrophoresis at room temperature frequently gave rise to "trails" of gelatin depletion in the sample tracks (Fig. 3.6). This phenomenon was not investigated in any detail and it may have represented "melting" of the gelatin in the gel matrix as a result of local heating at the ionic discontinuity produced by the difference in ionic strength between the sample buffer and the resolving gel buffer. Alternatively,

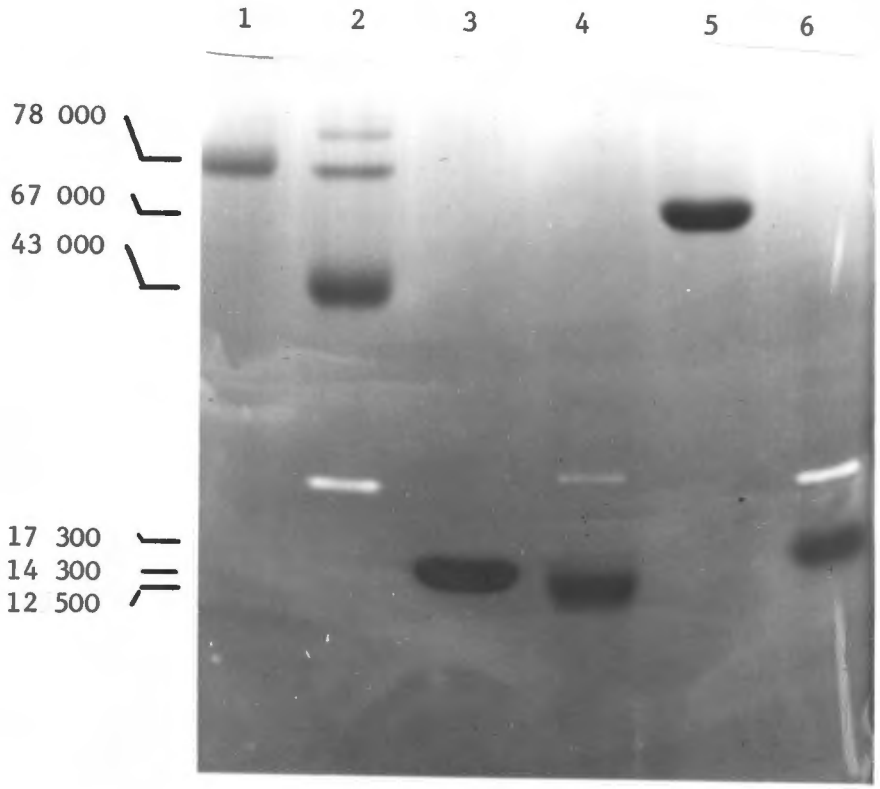


Figure 3.4 Gelatin gel electrophoresis of molecular weight marker proteins.

The following marker proteins were electrophoresed in a 6-15% gradient gelatin gel: track 1. lactoperoxidase (78 000) 2. ovalbumin (43 000) 3. lysozyme (14 300) 4. cytochrome C (12 500) 5. bovine serum albumin (67 000) 6. myoglobin (17 200).
Note the clear proteolytic bands present in tracks containing ovalbumin, cytochrome C and myoglobin.

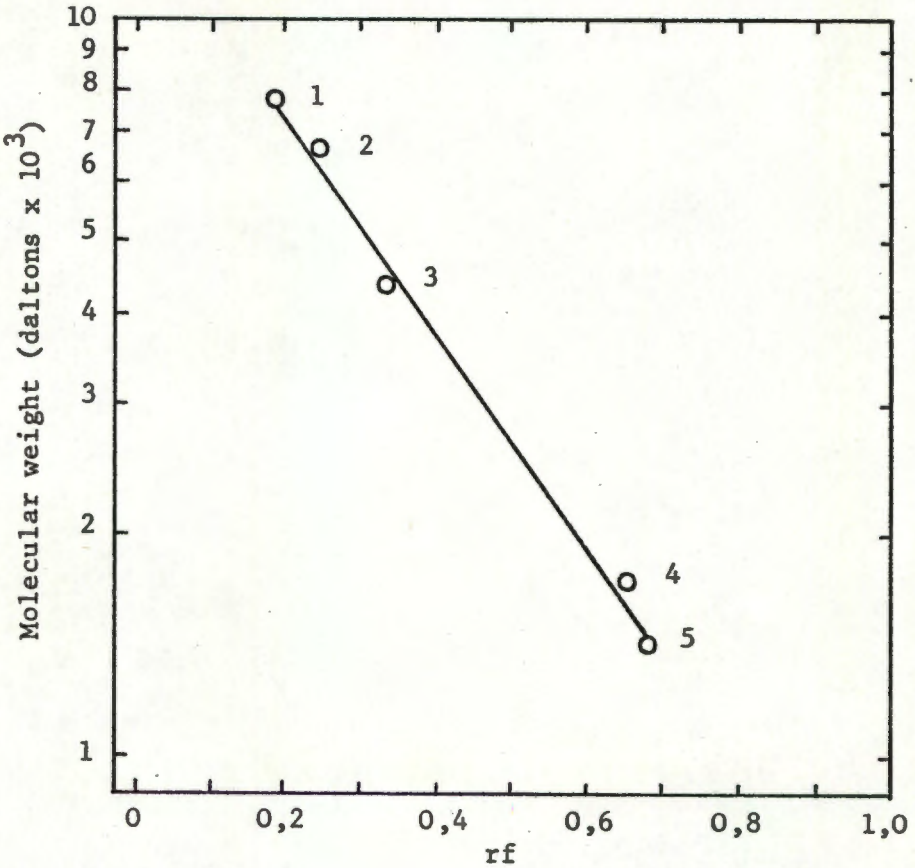


Figure 3.5 Relationship between electrophoretic mobility and logarithm of molecular weight as analysed on a gelatin gel.

An inverse relationship is obtained, when the relative electrophoretic mobilities of five marker proteins were plotted against the logarithms of their respective molecular weights. The marker proteins were:
1) lactoperoxidase (78 000) 2) bovine serum albumin (67 000) 3) ovalbumin (43 000) 4) myoglobin (17 200) 5) lysozyme (14 500) and 6) cytochrome C (12 500).
Note: Cytochrome C was not included in the graph since it migrated close to the ion front.
From the graph a molecular weight of 21 700 daltons was estimated for the contaminating protease band.

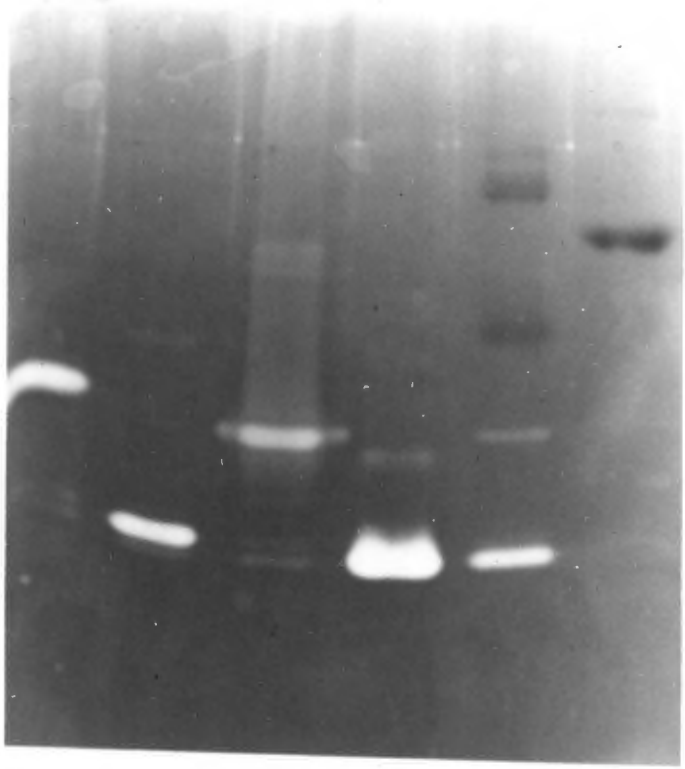


Figure 3.6 Gelatin gel

Gel showing "trails" of gelatin depletion in the first and third sample tracks.

the SDS concentration may not have been adequate to inhibit enzymatic activity completely with the result that at the higher temperatures, digestion of the substrate took place during electrophoretic migration.

(iv) A gelatin concentration of 0,1% at the time of copolymerization provided the most satisfactory final substrate concentration as judged by the intensity of the colour of the gel after fixation, staining with 0,1% Amido black and destaining. The contrast of this colour with clear proteolytic bands and darkly staining molecular weight reference proteins was good.

(v) To obtain adequate dissociation of enzyme aggregates before electrophoresis, it was necessary to incubate the samples in the presence of 2,5% SDS for 1 hr at 37°C before introducing them into the sample wells (Fig. 3.7). Samples (usually 10 µl - 50 µl) were pipetted into the sample wells after the reservoir buffer had been added. The density of the samples was increased by the addition of sucrose to a final concentration of 10%. Phenol red was added to the samples at a final concentration of 0,01% to serve as a tracking dye.

(vi) When enzyme containing electrophoretic tracks that had been freed of SDS were incubated in buffers of different composition and pH, the results obtained in Fig. 3.8 were obtained. As can be seen from

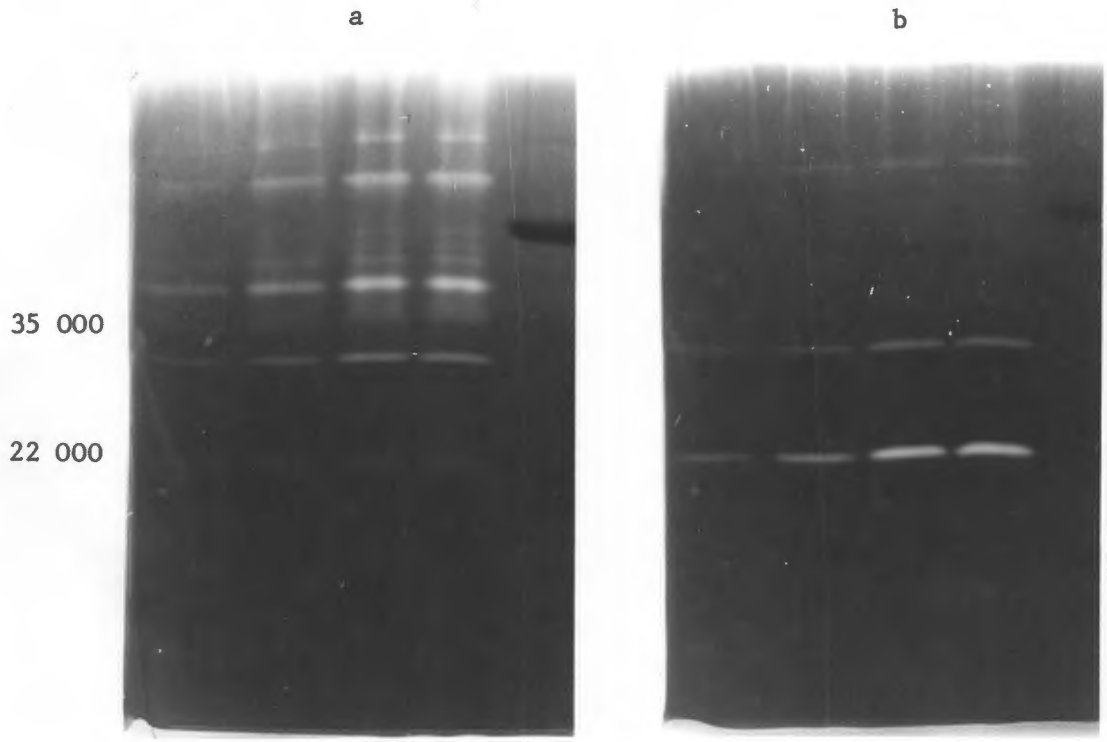


Figure 3.7 Effect of SDS and temperature on the dissociation of proteases to be electrophoresed in gelatin gels.

The figure shows 2 gelatin gels that were loaded with 20, 30, 40 and 50 µl of the same cercarial secretion samples. The samples in gel (a) were incubated at room temperature for 1 hr in the presence of 0,1% SDS, whereas those in gel (b) were incubated at 37°C for 1 hr in the presence of 2,5% SDS. Note the few distinct bands present in gel (b) as opposed to the multitude of bands present in gel (a).

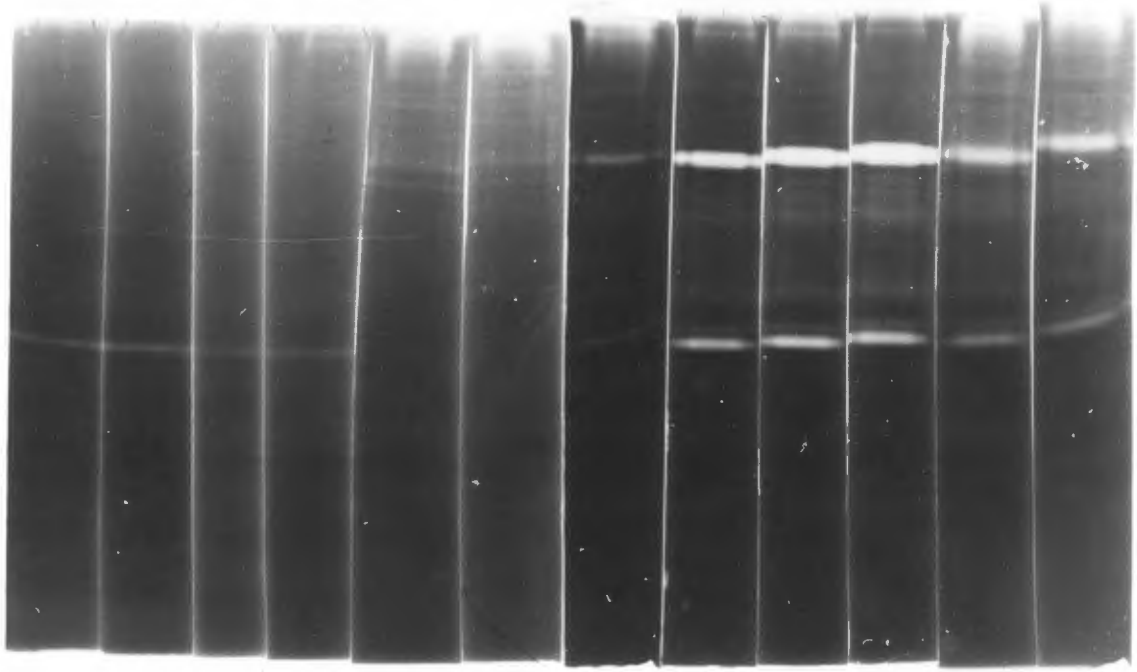


Figure 3.8 The effect of pH and buffer composition on the development of proteolytic bands in gelatin gels.

Electrophoretic tracks which had been loaded with 40 µl of the same cercarial secretion samples, were incubated at 37°C in 10 ml of the following buffers: HCl-glycine Γ/2 = 0,1, pH's 2,5, 3,5 (tracks 1,2) Na-acetate Γ/2 = 0,1, pH's 3,5, 4,5, 5,5 (tracks 3,4,5) Tris-HCl Γ/2 = 0,1, pH's 7,5, 8,5 (tracks 6,7) Glycine-NaOH Γ/2 = 0,1 pH's 8, 9, 9,5, 10,0, 10,4, 11,0 (tracks 8,9,10,11,12).
Note that the pH optimum for gelatinolysis lies between pH 8,5 and 10,5.

the photograph, distinctive bands of hydrolysis corresponding to a 35 000 M.W. enzyme were only observed in the tracks incubated in higher pH buffers. A pH optimum for this enzyme lay in the range 8,5 to 10,5.

Molecular weights of proteases present in cercarial secretion

Estimates for the molecular weights of proteases present in cercarial secretions were obtained from the SDS-gelatin gels by referring the relative electrophoretic mobilities of the bands of enzyme activity to the mobilities of molecular weight marker proteins according to the method of Maizel (46).

The results of six estimates of the molecular weights of proteases in cercarial secretions are summarized in tabular form in Table 3.1. An illustrative gelatin-SDS gel is shown in Fig. 3.9 together with a graph showing the method of analysis.

These results may be summarized as follows:

(i) A major proteolytic band was consistently observed in all specimens with an apparent molecular weight of 35 400 $\pm$ 2 800 daltons. This appeared to be a true cercarial protease.

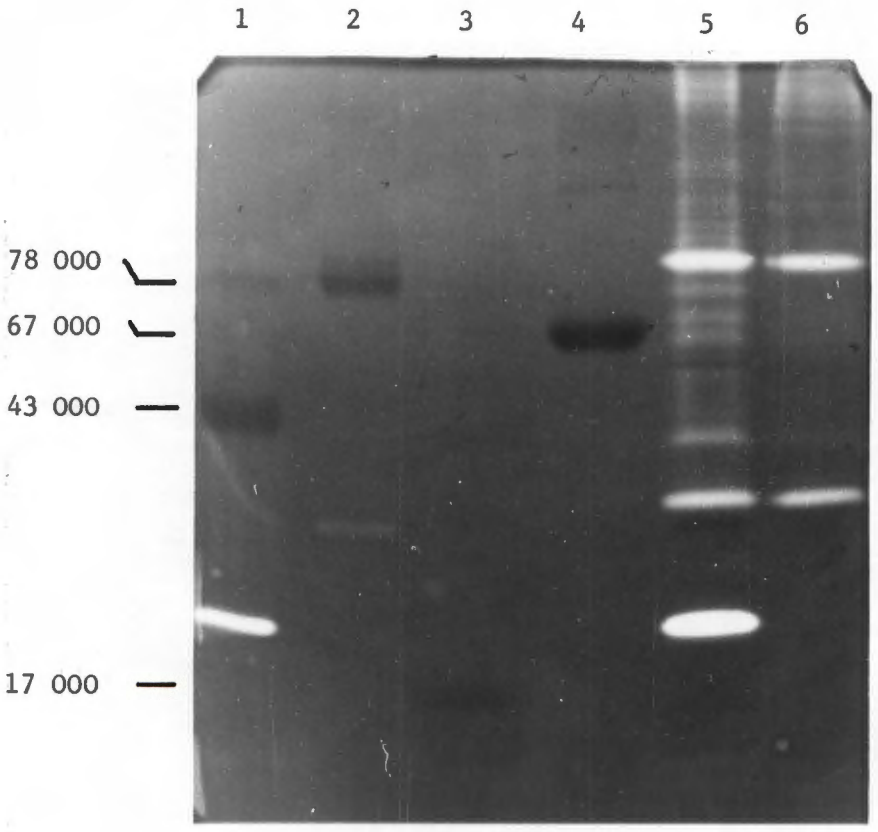
(ii) In many cases, proteolytic bands were observed corresponding to an enzyme with a molecular weight of 22 000 $\pm$ 1 400 daltons. This, as I shall discuss later, appeared to be a contaminating bacterial protease.

Table 3.1 Molecular weight estimates of proteases in cercarial secretions as obtained by gelatin gel electrophoresis.

Gel	Polyacrylamide concentration (%)	Molecular weight Band 1	Band 2	Band 3*
1	6-15	32 600	81 300	
2	6-15	35 700	86 900	21 100
3	4-16	37 000	81 900	23 400
		37 900	80 600	
4	6-15	35 100	83 700	22 000
		34 700	83 400	
5	4-15	35 500	83 200	21 800
		34 900	82 400	
6	6-16	35 200	83 500	21 700
		35 400	83 500	
	Mean	35 400 ± 2800		22 000 ± 1400

*Refer to figure 3.9 for identification of the bands.

FIGURE 3.9


1
2
3
4
5
6
78 000
67 000
43 000
17 000

(iii) On a few occasions a minor band with an apparent molecular weight of 83 000 daltons was seen. This may have been a precursor of the 35 500 M.W. enzyme, or a coacervate between the cercarial enzyme and some other protein. Its inconsistent presence as a quantitatively minor species precluded my performing the definitive experiemtns that would have distinguished between these two alternatives. It should be noted, however, that high molecular weight enzyme species were observed when preincubation at 37°C with SDS was not performed (Fig. 3.7) and it may be that the occasions in which I observed the 83 000 M.W. enzyme were those in which adequate dissociation of protein-protein interactions was not achieved.

CHAPTER 4

COLLECTION OF PROTEASE-CONTAINING SECRETIONS FROM CERCARIAE

Most previous attempts to purify the acetabular gland proteases have used cercarial homogenates as the starting material. The enzymes together with many irrelevant proteins were then freed from the insoluble debris by extraction or centrifugation to yield preparations containing a heterogeneous mixture of biological molecules necessitating many purification steps to obtain a reasonably pure preparation of the cercarial proteases. Furthermore, the presence of unknown non-secretory proteases would hinder the identification and characterization of the specific preacetabular gland enzymes. Most of these difficulties could be circumvented by the use, as the enzyme source, of acetabular gland secretions, rather than whole extracts of cercariae and well-defined procedures for the induction of enzyme release based upon an understanding of the mechanisms involved would clearly be of value in this regard.

Release of pre- and post-acetabular gland contents is one of the earliest events taking place during skin penetration and the transformation of cercariae into schistosomules. Available evidence indicates that, under natural circumstances, the major inducing stimulus for the initiation of this process is provided by host skin lipid (31, 32, 54), the most active stimulating constituent being 18-carbon chain fatty acids such as linoleic and linolenic acids. Austin and Stirewalt

(31) have suggested that skin lipid serves an a "contact" penetration stimulus, while the temperature of the object to be penetrated plays an important role as a "remote attractant".

These observations have led to the development of *in vitro* procedures for studying the transformation of cercariae to schistosomules and for the collection of preacetabular gland proteases. Several workers (31, 32, 54), for example, have observed morphological and behavioural changes, in cercariae exposed to plastic surfaces coated with skin sipids, that mimic in all respects the changes *in vivo* and *in vitro* when cercariae are exposed to skin. These changes, accompanied by the release of proteases into the medium, are very striking when observed microscopically.

Penetration behaviour is evident initially as a change from random, darting movements of the organism with sporadic, propulsive gyrations of the tail, to fixation of the body to the surface and violent, to-and-fro lashing movements of the tail that end in its separation from the body. For a while the tail-less body retains a rigid and immobile appearance. After a short while, however, the body becomes ostensibly less rigid and commences to move with undulating movements that have the purposive, coordinated character of directional crawling. At this stage the transformation into the schistosomule form is complete.

If freshly-shed cercariae are stained with a saturated aqueous solution of the vital dye Alizarin Red S, the contents of the preacetabular gland are clearly visible

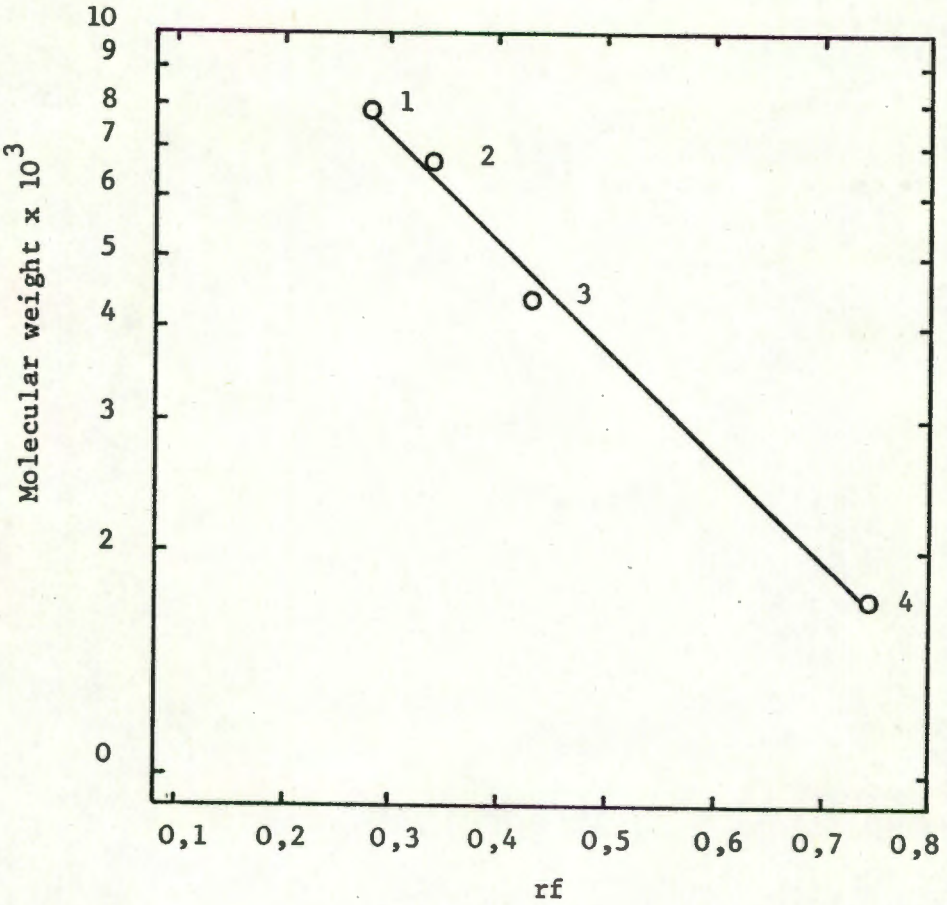


Figure 3.9 Molecular weight determination of cercarial proteases by gelatin gel electrophoresis.

Aliquots of 40 µl of two cercarial secretions were electrophoresed in a 6 - 15% polyacrylamide gradient gelatin gel. The following molecular weight marker proteins were electrophoresed in the same gel.
1) lactoperoxidase (78 000) 2) bovine serum albumin (67 000) 3) ovalbumin (43 000) and 4) myoglobin (17 200). From the plot of the relative electrophoretic mobilities (rf) of the markers versus the logarithm of their respective molecular weights, the following molecular weights for the cercarial proteases were obtained by interpolation: Band 1 35 100, 34 700, Band 2 83 700, 83 400.

owing to the affinity of the dye for the calcium present in high concentration in the secretions (10, 55). With transformation into the schistosomular form, the preacetabular glands empty and staining with Alizarin Red S is no longer visible. Thus three microscopical criteria may be used for monitoring transformation *in vitro* - tail loss, morphological change of the body and loss of Alizarin staining.

In vitro transformation of cercariae in the absence of any biological material which might stimulate a "natural" penetration response, was first achieved by Gazzinelli et al (56) by the simple expedient of centrifugation and brief incubation, either at room temperature or 30°C, of the packed organisms. Under these conditions gradual release of the preacetabular gland contents was noted. This process could be enhanced by prior separation of the tails from the cercarial bodies by subjecting the organisms to mechanical stress such as repeated passage through a 22-gauge needle (57) or vigorous agitation on a vortex mixer (58, 59). Subsequent incubation in appropriate media resulted in complete transformation of cercariae into schistosomules, with simultaneous evacuation of the pre- and post-acetabular glands.

The *in vitro* transformation procedures described by Ramalho-Pinto et al (58) formed the basis for the collection of cercarial secretions that I shall describe in this section. The steps followed included concentration of the cercarial suspension to obtain packed cercariae; agitation of these on a vortex mixer to separate tails from bodies; incubation of the organisms in 5% glucose and subsequent centrifugation. Supernatant medium was used as the enzyme source. It was

important throughout to eliminate, as far as possible, bacterial contamination as a source of non-cercarial proteases.

Standard conditions for the collection of cercarail secretions.

Cercariae were harvested from infected snails as described in Chapter 1, and separated from snail detritus by pouring the suspensions through a 80 mesh metal sieve into a measuring cylinder. The total number of cercariae present was determined by counting aliquots.

A flow diagram of the method for collecting cercarial secretions is presented in Fig. 4.1.

The dilute cercarial suspension was distributed into a number of 50 ml conical plastic centrifuge tubes and these were kept on ice for one hour. By cooling the suspension, cercarial motility was reduced and the organisms settled to the bottom of the tubes. They were concentrated into a fairly firm pellet by brief centrifugation (200xg; 10 sec). Samples of the supernatant water were saved to serve later as controls for the presence of bacterial proteases, and the remainder was aspirated. The packed cercariae were pooled into one of the 50 ml tubes by gentle swirling and decantation. Ice-cold, deionized water was added such that the concentration of the suspension was 5000 - 10 000 cercariae/ml. The concentrated suspension was then distributed into 15 ml conical graduated plastic centrifuge tubes such that each tube received approximately 100 000 cercariae. The cercariae were allowed to settle

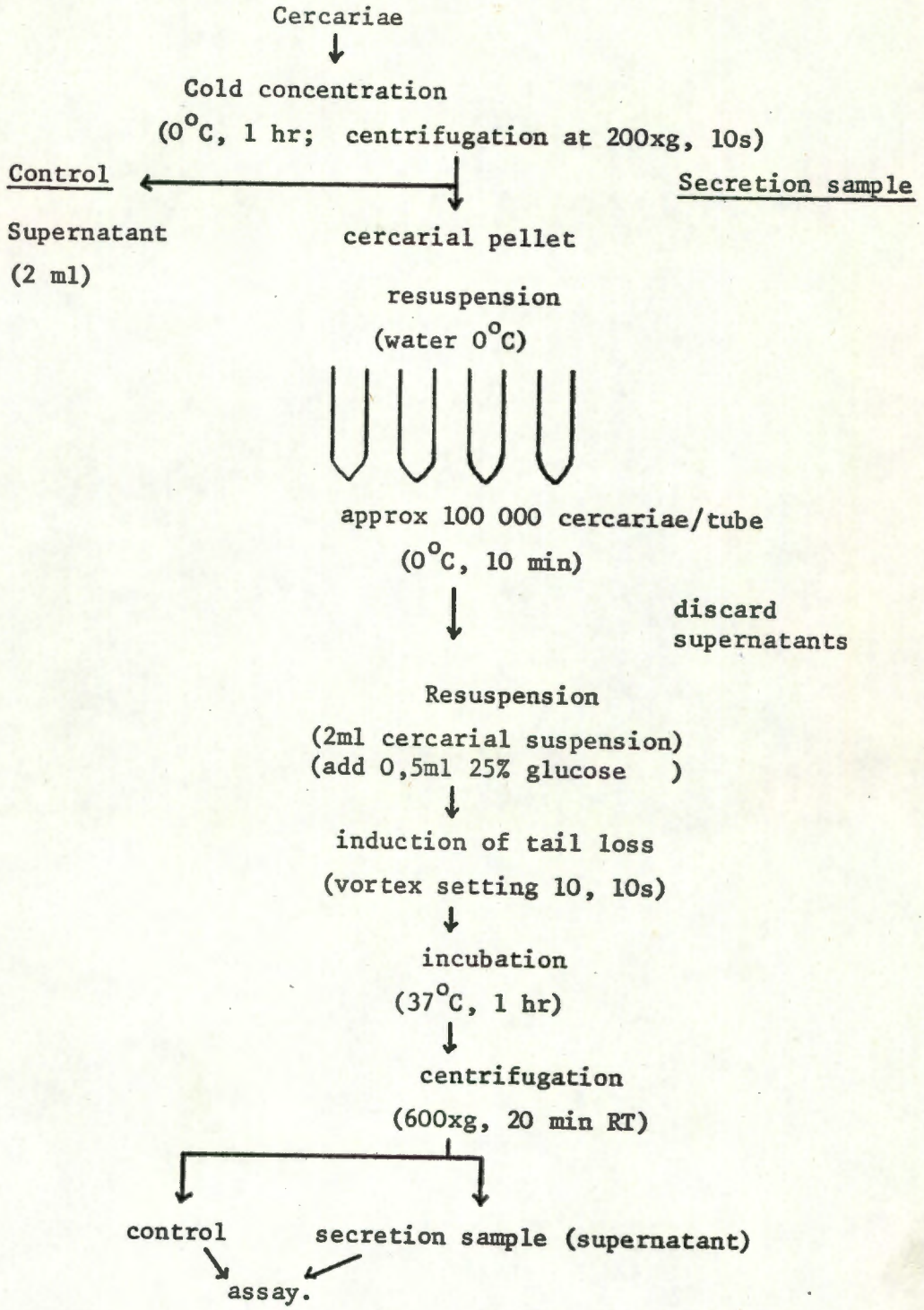


Figure 4.1 Flow diagram of the method for collecting cercarial secretions.

to the bottom of the tubes by keeping these on ice for 10 minutes. After removing the supernatant water to the 2 ml mark 0,5 ml of sterile, cold 25% glucose in water containing 200 u/ml Penicillin and 200 µg/ml Streptomycin was added. Control tubes containing 2 ml of the first supernatant obtained after concentration of the cercariae were treated in the same way. The contents of all tubes were then agitated on a vortex mixer for 10 seconds and then either centrifuged directly at 600xg for 30 minutes at room temperature or after incubation at 37°C for 1 hour. The protease-rich supernatants were stored in small portions at -20°C after millipore filtration. The cercarial pellets were also stored, either wet or lyophilized, at -20°C.

Proteolytic activity in the secretions was determined in the ^{125}I-fibrin plate assay described in Chapter 2. The protein concentration was measured by the method of Lowry (35).

Photographs of the typical appearances of the organisms obtained after the different steps in the procedure are shown in Figures 4.2 to 4.5. The preparations were stained with a saturated aqueous solution of Alizarin Red S. Live cercariae that had extruded their gland contents did not stain at all or only at the aboral end of the body, whereas dead cercariae appeared evenly brown.

After the initial cold concentration step, some cercariae had lost their tails, but all could still be stained with Alizarin. A typical organism is shown in Figure 4.2. After agitation on the vortex mixer, most of the cercariae

Figure 4.2 A cercaria

Typical appearance of a cercariae of S. mansoni after cold concentration and staining with Alizarin Red S. Note the reddish colour of the preacetabular glands.

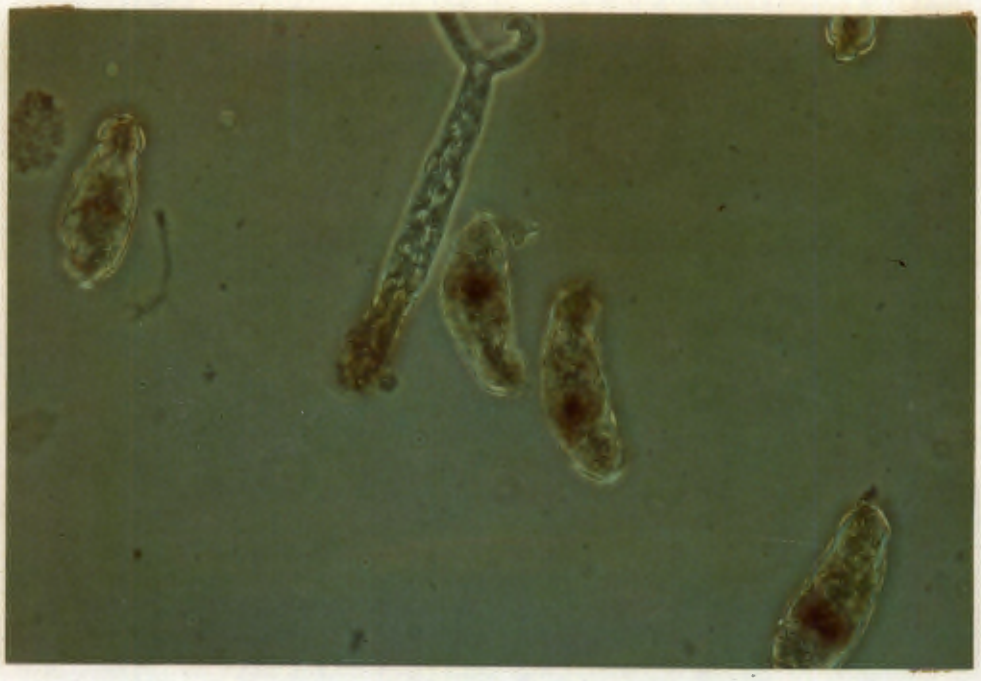


Figure 4.3 Cercarial bodies and tails

Tailless cercariae were obtained from concentrated cercariae by vigorous agitation on a vortex mixer. Note the well defined shape of the cercarial bodies, and the darkly staining preacetabular glands.

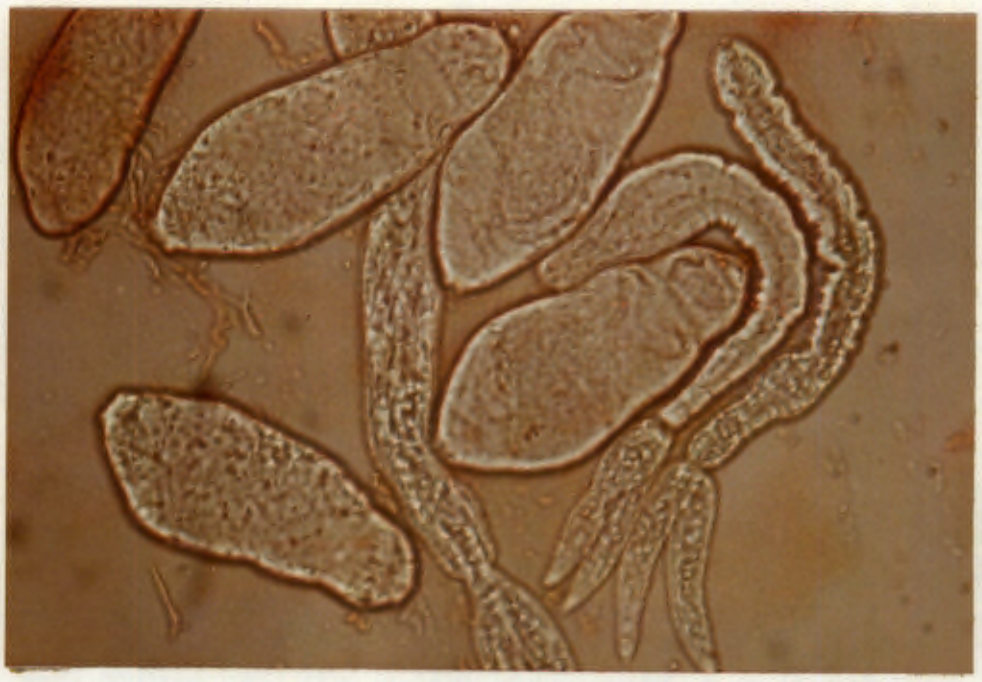


Figure 4.4 Cercarial bodies and tails

The photograph shows the appearance of cercarial bodies after centrifugation at 600xg for 20 min. Note the well defined shape of the cercarial bodies, and the absence of red dye in the region of the preacetabular glands. These had been evacuated during centrifugation.

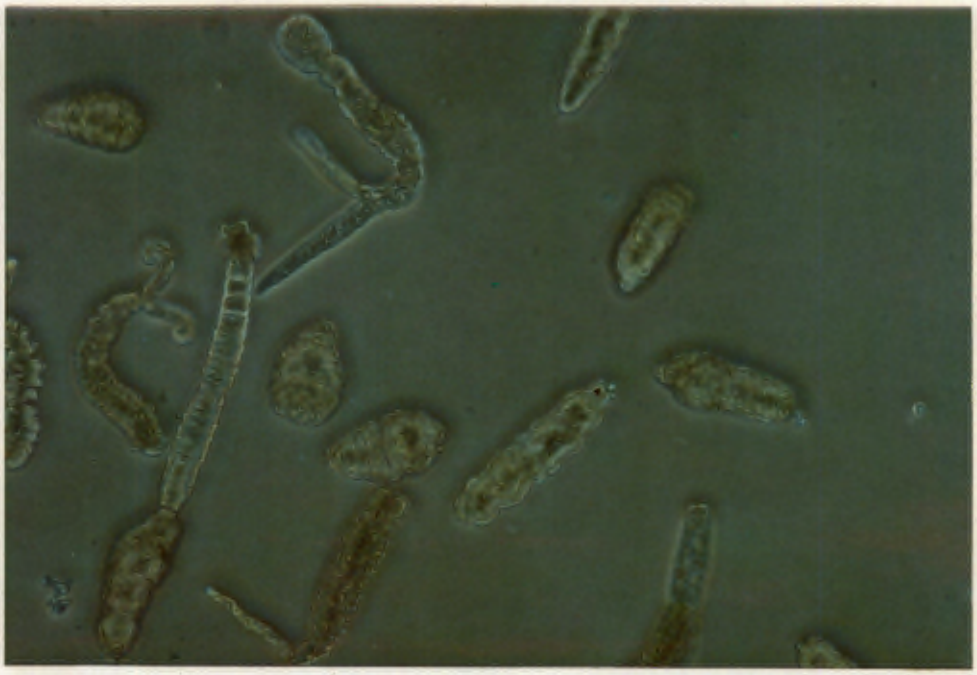


Figure 4.5 Schistosomules

Schistosomules were obtained by incubation of packed cercariae in the presence of 5% glucose at 37°C for 1 hr. Note the flaccid, wormlike appearance characteristic of schistosomules.

had lost their tails and a small porportion was seen in the process of evacuating their glands (Fig. 4.3). After centrifugation at 600xg most of the organisms had evacuated their preacetabular glands but many retained the characteristic rigid shape of the cercarial body (Fig. 4.4). When the organisms were incubated in the presence of 5% glucose for 1 hr, most had the flaccid, characteristic appearance of schistosomules (Fig. 4.5).

During the handling of the cercarial suspensions, I encountered a number of difficulties which had to be overcome. I noticed for instance,that cercariae adhered firmly to glass and to each other after the initial cold concentration step. The same was noticed by Ramalho-Pinto et al (58) with cercariae packed by centrifugation in iced water. In their case the organisms seemed to be clumped together by a mucoid material which could be stained with Hale's dialysed iron or the PAS technique and probably originated from the postacetabular glands. Aggregation of organisms did not occur if they were centrifuged in 0,14M NaCl, Hanks balanced salt solution (HBSS) or 5% glucose. The clumps that formed after centrifugation from water, could be dispersed by NaCl or HBSS but not by water or glucose.

I found that, by using plasticware instead of glassware throughout the procedure, sticking of cercariae to the walls of measuring cylinders and tubes could be avoided. It was necessary, however, to use water or glucose for the centrifugation step, because, as I shall describe later, inferior yields of enzyme activity were obtained with other media.

Instead of concentrating cercariae by immobilization and settling in the cold, they could also be collected by filtration on an 8 µm cellulose-acetate filter. This method, however, was not entirely satisfactory since the filters became clogged easily unless the cercarial suspension was vigorously stirred. Clumping of the cercariae, although less than with cold concentration, was also observed with this technique.

In most cases secretions from approximately 100 000 cercariae were collected in a volume of 2,5 ml. The proteolytic activity of the resultant solution could be measured directly in the fibrin assay under the standard conditions described in Chapter 2. When more cercariae were allowed to shed their enzyme into the same volume, dilutions were necessary in order to obtain linear progress curves in the assay. From these and the following experiments, optimal conditions for the collection of protease containing secretions from cercariae could be defined.

(i) Effect of incubation medium on the yield of cercarial proteases.

The enzymatic activity of secretions obtained from the same number of cercariae incubated in different media was investigated in an experiment in which a volume of 1 ml of different media containing antibiotics was added to 0,2 ml water containing approximately 13 000 cercariae. The following media and incubation conditions were tested in duplicate:

5% glucose in water; distilled water with incubation at 37°C and at 22°C; 0,85% NaCl; Hanks Balanced Salt Solution; and Eagle's minimal essential medium. All tubes (except the two containing water as the medium) were incubated at 37°C for 90 minutes. After incubation, the tubes were centrifuged at 200xg rpm for 10 seconds and the supernatants were assayed on fibrin plates as described in Chapter 1. The different media used for incubation and the results obtained are summarized in Table 4.1 and Fig. 4.6.

As is evident from these data, incubation in 5% glucose gave the highest yield of enzyme activity in the supernatant. There are two obvious explanations for the apparent superiority of 5% glucose over other incubation media in this regard. Firstly, as I describe in a later section, enzyme activity is directly affected by ionic strength and other assay conditions and the salts and amino acids present in the other media may have inhibited in the assay. Secondly, clumping of the organisms was most prominent in the glucose medium. This effect in itself may have provoked more rapid and complete evacuation of the acetabular gland contents. Since I did not have an available specific assay for the enzyme as a protein, I am unable to produce data in support of one or other of these two alternatives. For practical purposes, however, 5% glucose was adopted as the most suitable medium for enzyme release.

Table 4.1 Effect of incubation medium on the yield of cercarial proteases.

Sample	Medium	Activity* (%T/hr)	Activity corrected (%T/hr)
Cercariae	Glucose 1%	6,19	6,09 ± 0,08
	H_2O (37°C)	3,56	3,50 ± 0,05
	H_2O (22°C)	3,25	3,10 ± 0,12
	NaCl 0,85%	2,76	2,75 ± 0,02
	Hanks balanced salt solution	0,71	0,58 ± 0,07
	Minimal essential medium (MEM)	0,46	0,30 ± 0,02
Control	Glucose 5%	0,10	-
	H_2O (37°C)	0,05	-
	H_2O (22°C)	0,15	-
	NaCl 0,85%	0,00	-
	Hanks balanced salt solution	0,13	-
	MEM	0,16	-

*The activity is expressed as the cumulative percentage of the total radioactivity released per hour in the assay, 0,2 ml of the supernatant from the cercariae was added to 0,8 ml of 0,1M glycine-NaOH pH 8,80 to wells in a linbro plate. Aliquots of 0,2 ml were withdrawn for counting at 3, 5 and 12 hour intervals.

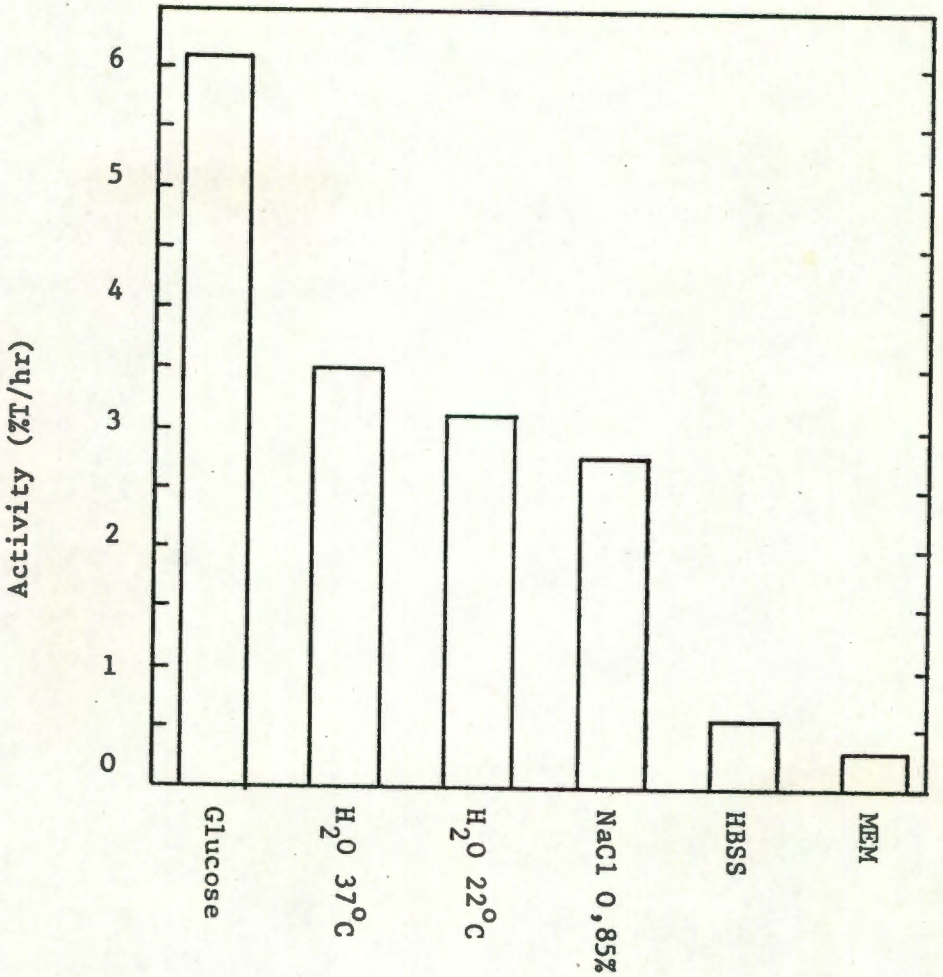


Figure 4.6 Effect of incubation medium on the yield of cercarial proteases

Cercariae were incubated in the different media shown in the figure. The supernatants obtained after centrifugation were measured for proteolytic activity in the fibrin assay.

(ii) The effect of centrifugation on the release of enzyme from cercariae.

Two experiments were carried out to see whether mere centrifugation of cercariae in glucose would induce them to release their enzyme or whether incubation at elevated temperatures is essential. In the first experiment, an equal number of cercariae in the same volume were centrifuged immediately after addition of medium, or after incubation in the medium for one and two hours. In the second experiment, packed cercariae were incubated for an equal time period, and the surrounding medium was separated either by centrifugation or by passing the suspension through a millipore filter or by both.

Experiment 1: A volume of 1 ml of 5% glucose in 0,1M glycine-NaOH, pH 8,8 was added to each of six tubes containing 200 µl of suspension containing 3000 cercariae. The tubes were incubated for 0, 1 and 2 hours at 37°C, after which they were centrifuged at 3000 rpm for 30 minutes at room temperature. Aliquots of 0,5 ml of the supernatants were assayed in the fibrin assay. Controls containing water from the original cercarial suspension were treated in the same way. The results expressed in total radioactivity released per hour are shown in Table 4.2.

Experiment 2: A volume of 6 ml of 5% glucose was added to centrifuge tube containing 20 000 cercariae in a 1 ml suspension. The contents of the tube was agitated on a vortex mixer and incubated at 37°C for 2 hours. After thorough mixing, 1 ml of the solution was passed through a 10 µm millipore filter, and another ml through a filter that had been pretreated with

Table 4.2 Effect of centrifugation on enzyme release

Time of incubation (hr)	Activity (%T/hr)
0	15.95 ± 0,21
1	19,81 ± 0,23
2	18,75 ± 0,19

0,1% bovine serum albumin. The remaining contents of the incubation tube was centrifuged at 2500 rpm for 30 minutes at room temperature. One ml of the supernatant was passed through a millipore filter, and another through a filter pre-treated with albumin. The remainder of the supernatant was used untreated.

The various solutions obtained were assayed by adding 0,5 ml of each into duplicate wells containing 0,5 ml 0,1M gly-NaOH pH 8,80. The results are shown in Table 4.3.

Most of the proteases are released from the cercariae by mere centrifugation at room temperature, as shown by the first experiment. The second experiment showed that the same amount of enzyme could be recovered in the supernatant, when this was separated from the cercariae by filtering rather than centrifugation after incubation. Loss of the enzyme on the millipore filter could be excluded, since all the activity could be recovered by filtering a supernatant obtained by centrifugation. Pretreatment of filters with albumin had no obvious effect on the recovery of enzyme.

Comparison of proteases present in extracts of sonicated cercariae, in secretions from skin lipid stimulated cercariae, and in "secretions" obtained from centrifuged cercariae.

Cercarial extraction : Cercariae were shed from snails in the usual way. They were concentrated and washed with deionized water on a 8 µm cellulose acetate filter. The concentrated suspension containing approximately 100 000 cercariae was

Table 4.3 Effect of centrifugation on the release of enzyme from cercariae.

Condition	Activity (%T/hr)
a. Centrifugation without millipore	17,71
b. Centrifugation with millipore (no albumin)	17,38
c. Centrifugation with millipore (with albumin)	17,52
d. Millipore only (no albumin)	17,14
e. Millipore only (with albumin)	17,64

Supernatants obtained from 20 000 cercariae in a total volume of 7 ml after 2 hours incubation were assayed in the fibrin assay. Activity is expressed as the cumulative percentage total radioactivity released per hour. Supernatants (d) and (e) were obtained by filtering 1 ml of the cercarial suspension through 10 µm millipore filters which had been either treated with albumin (e) or were untreated (d). Supernatant (a) was measured directly after centrifugation of the cercarial suspension, and supernatants (b) and (c) were the same as (a), but filtered through 10 µm millipore filters. In the assay 0,5 ml of the supernatants were added to 0,5 ml 0,1M glycine-NaOH pH 8,80 in Linbro wells and aliquots of 200 µl were withdrawn for counting after 1, 2, 3 and 4 hr incubation.

transferred to a graduated conical tube and kept on ice for ten minutes. Water was removed to the 2 ml mark, and 0,5 ml sterile 25% glucose in water containing antibiotics was added. After gentle resuspension, the cercariae were added to a 10 ml glass beaker and sonicated for 3 x 30 seconds with an MSE sonicator with a medium probe attachment. The amplitude setting was 4, and the pulse height meter indicated 8 - 9 microns. The beaker was kept on ice during sonication. The cercariae were extracted for 15 hours at 4°C into the surrounding medium and then centrifuged at 10 000 rpm for 30 minutes. The supernatant was used directly for analysis on a gelatin polyacrylamide gel.

Cercarial secretion obtained by stimulation by skin lipid.

The cercariae in a dilute suspension were concentrated as before by filtration to 6500 cercariae/ml. Cold concentration and centrifugation was avoided in order to prevent clumping of the cercariae. Secretions were then collected by the method of Campbell (33). Skin lipid, which had been removed from human forearms using cotton wool swabs soaked in ethanol was transferred to the bottoms of small plastic petri dishes (5cm x 1cm). The dishes were dried and approximately 13 000 cercariae in 2 ml water were added. The dishes were incubated at 37°C for 1 hour. Controls consisted of cercariae in dishes without skin lipid, or snail water in skin lipid coated dishes. After the incubation period, the cercariae were moved from the surrounding medium by filtration. The filtrate was kept for analysis.

Cercarial secretion obtained by centrifugation

A volume of 1 ml containing 13 000 cercariae was placed into a plastic conical tube and kept on ice for 1 hour. 200 µl of the supernatant was replaced by 200 µl 25% glucose, and the cercariae were induced to lose their tails by vortex agitation. The supernatant obtained after centrifugation at 3000 rpm for 30 minutes was analysed by electrophoresis.

Two 6 - 15% gradient polyacrylamide gels containing gelatin were prepared as described in Chapter 3. Preparation of samples for electrophoresis was as usual. They contained 1% SDS and glycerol and were incubated at 37°C for 30 min. The order and volumes for loading the gels are shown in Tables 4.4(a and b) and photographs of the gels are presented in Fig. 4.7 (a and b).

As is evident from these results, extracts of cercariae contained the same proteases (molecular weights approximately 35 000 and 83 000) as those observed in supernatants from centrifuged cercariae. Many other proteins, which could not be detected in the supernatants, were present as well. It is interesting to note that the 83 000 molecular weight protease was only detected in the cercarial extracts and not in the secretion. The secretions obtained from skin lipid-stimulated cercariae contained the 35 000 molecular weight protease exclusively.

Table 4.4 Gelatin polyacrylamide electrophoresis on extracts of sonicated cercariae and secretions from skin lipid stimulated and centrifuged cercariae.

a.

Well no.	Sample	Load (ml)
1	Extract of sonicated cercariae	20
2	Extract of sonicated cercariae	35
3	Supernatant from centrifuged cercariae	40
4	" (stored at -20°C)	40
5	Lactoperoxidase + ovalbumin	10 + 10
6	BSA + myoglobin	10 + 10

b.

Well no.	Sample	Load (ml)
1	Supernatant from centrifuged cercariae	50
2	Secretion (induced by skin lipid)	50
3	Control (skin lipid absent)	50
4	Control (snail water + skin lipid)	50
5	-	
6	Supernatant from centrifuged cercariae (-20°C)	35

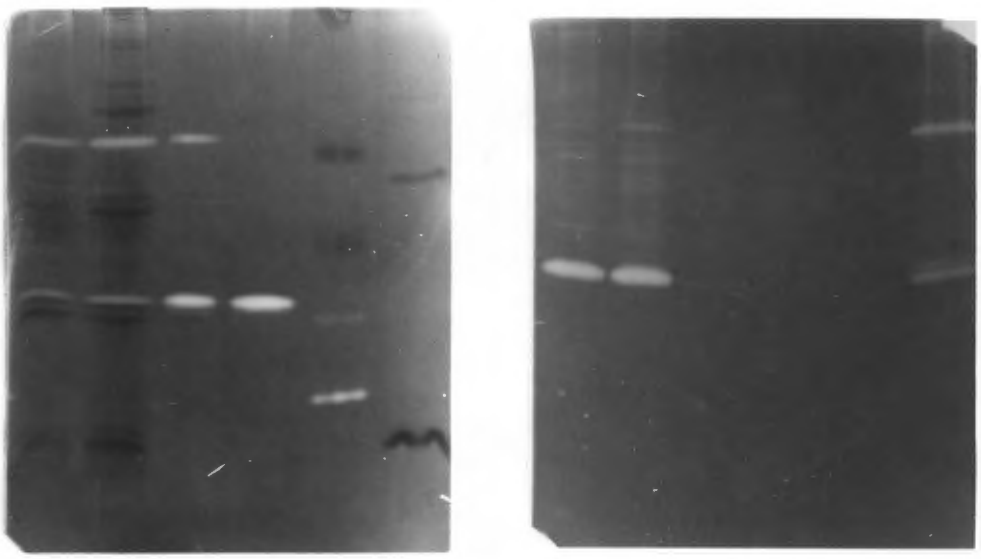


Figure 4.7 Gelatin polyacrylamide electrophoresis on extracts of sonicated cercariae, on secretions from skin lipid stimulated cercariae, and on "secretions" obtained from centrifuged cercariae.

The details for loading of the gels are given in Table 4.4.

The identification of contaminating bacterial proteases in samples of cercarial secretion.

One of the advantages that I derived from the gelatin-gel procedure for the electrophoretic analysis of cercarial proteases was the ability to identify the molecular species of proteases present and to correlate them with technical and experimental variables related to the source of the enzymes and the manner in which they were collected.

When the technique had been developed I noticed, quite fortuitously, the inconstant presence of a protease with an apparent molecular weight of approximately 22 000 daltons in cercarial harvest fluids, in chromatography column effluents and in solutions that did not contain cercarial products! These latter samples included buffers that had been stored for prolonged periods at room temperature and solutions of molecular weight marker proteins such as myoglobin, cytochrome C and ovalbumin. The molecular weight marker protein solutions were of particular interest, since, although the contaminating proteolytic enzymes they contained could be inhibited by serine protease inhibitors such as diisopropylfluorophosphonate (DFP), phenyl methyl sulphanyl fluoride (PMSF) and nitrophenyl-guanadinobenzoate (NPGB) they were resistant to iodoacetamide, parachloromercuribenzoate (PCMB) and to boiling in 2,5% SDS for 1 min - the procedure routinely employed to prepare these samples for electrophoresis. It seemed likely, from the circumstantial and biochemical evidence available, that this enzyme owed its presence to bacterial contamination.

On reflection it became obvious that I had been maintaining the snails under unsterile conditions and that I had taken no special precautions to minimize bacterial contamination during harvesting of the cercariae or during collection of the cercarial secretions. It was, therefore, likely that microorganisms had contributed to the proteolytic activity of the secretions that I had assumed were representative only of cercarial enzymes. To investigate this possibility I performed two experiments.

In the first experiment, proteases present in unsterile cercarial secretions were compared with those observed when measures were taken to diminish, as far as possible, bacterial contamination. These measures included washing the snails in three changes of sterile water before shedding; the addition of penicillin (200 u/ml) and streptomycin (200 µg/ml) to the shedding water and to the cercarial suspensions; sterile filtration of the cercarial secretion samples and the 5% glucose used for induction of cercarial secretion; and the autoclaving of all buffers and other heat stable reagents used for the electrophoresis.

As can be seen from the results presented in Fig. 4.8 the adoption of procedures for control of bacterial contamination did, indeed, eliminate several proteases, including the 22 000 M.W. species. Samples collected under these conditions contained only the 83 000 M.W. and 35 000 M.W.enzymes. Since these enzymes were also present in sonicates of extensively washed cercariae, I have concluded that these represent true cercarial proteases and that other proteases were probably bacterial in origin.

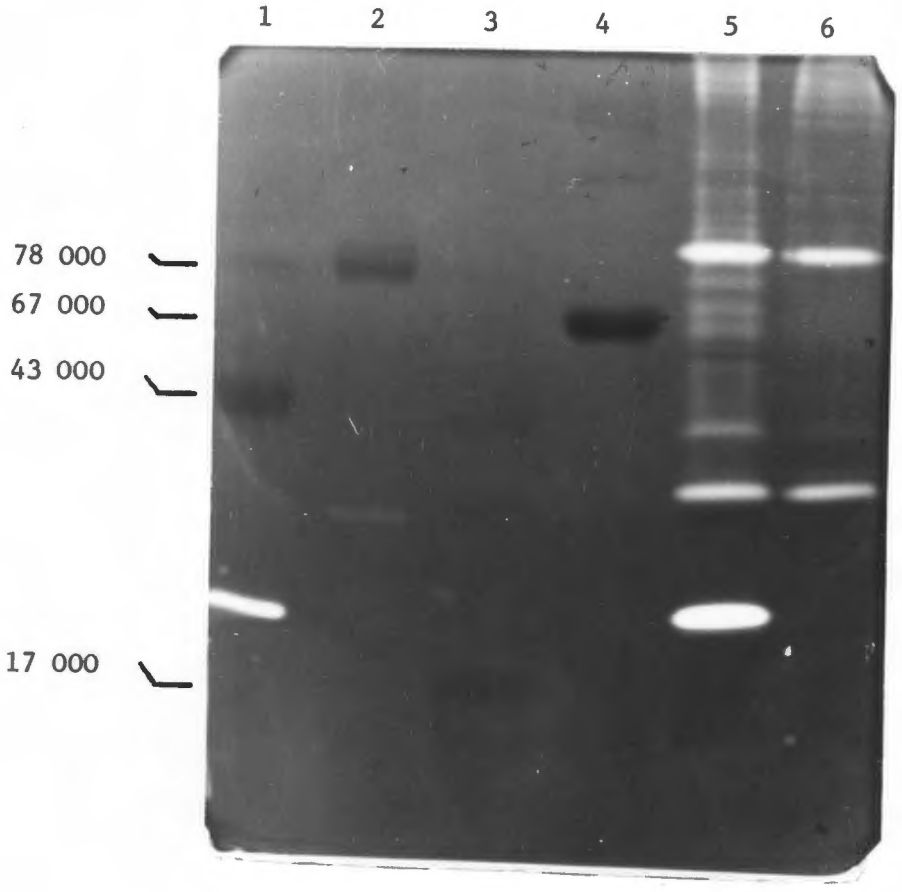


Figure 4.8 Gelatin polyacrylamide electrophoresis of cercarial secretion samples.

Aliquots of 40 µl of cercarial secretions collected under standard (track 6) and non sterile (track 5) conditions were electrophoresed in a 6-15% gelatin gel. Molecular weight marker proteins were run as usual.

In the second experiment I collected cercariae and cercarial secretions either under the standard or under relatively non-sterile conditions and examined the medium at various stages of the procedure for bacterial contamination. Bacteriological nutrient agar plates and tubes containing nutrient broth were seeded with 0,1 ml of the following solutions:

- water from tanks in which snails had been kept for one week;
- dechlorinated laboratory tap water;
- deionized water;
- the supernatant medium after concentration of the cercariae; and
- cercarial secretions from the same batch of cercariae.

In addition secretions that had been stored at -15°C were inoculated into tubes containing nutrient broth. The tubes and plates were incubated at 37°C for 24 hrs.

As can be seen from the results summarized in Table 4.5 the snail water, cercarial supernatant fluids and secretion samples were all infected when the non-sterile protocol was used. This infection could be eliminated by use of antibiotics, washing procedures and sterilization of buffers and suspending media.

Individual bacterial colonies from nutrient agar plates seeded with infected samples were transferred to nutrient broth and cultured for 24 hr at 37°C. The bacterial suspension was then cleared of organisms by centrifugation and samples

Table 4.5 Bacterial growth in nutrient medium inoculated with solutions obtained from various stages of shedding of cercariae and collection of secretions.

Condition	Sample	Bacterial growth+	Colonies* isolated
Non-sterile	Snail water	+++	Small white Large white Yellow
	Dechlorinated tap water	+	White
	Supernatant medium after cercarial concentration	++	White Yellow
	Secretion	+	White
	Secretion stored at -15°C	a. + b. + c. -	Small white White
Standard	Snail water	+	
	Deionized water	-	
	25% glucose solution	-	
	Supernatant medium after cercarial concentration	-	
	Secretion	-	

+Bacterial growth on nutrient agar was scored +++ for heavy growth, + for slight growth, - for absence of growth after incubation for 24 hrs at 37°C.

*Colonies isolated from the primary cultures, and inoculated into nutrient broth.

of the broth were analyzed for proteases by the gelatin-gel electrophoretic procedure. As can be seen from Fig. 4.9 (a and b) the bacterial isolated from contaminated specimens were capable of synthesizing and releasing proteolytic enzymes.

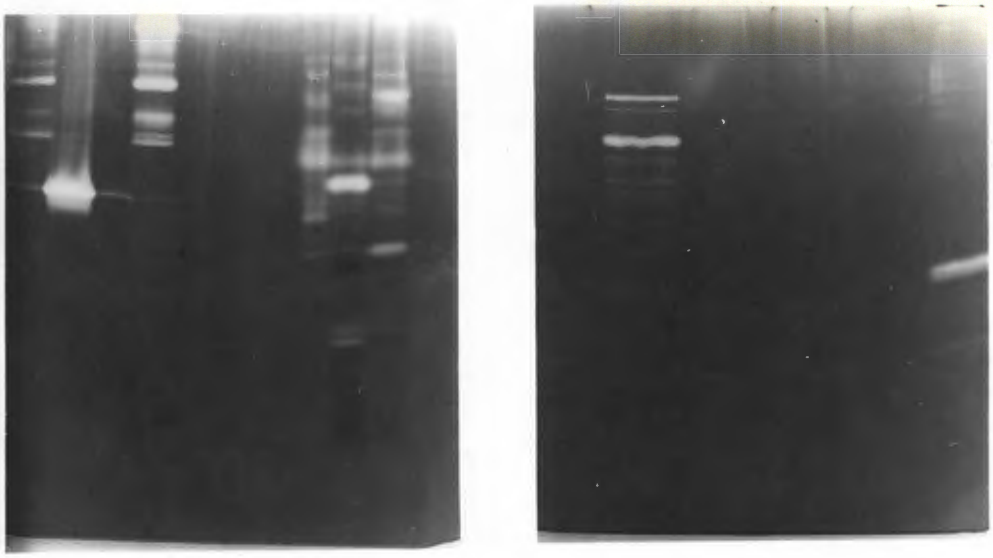
It is well known that a variety of microorganisms are able to release extracellular proteases into their surrounding medium. Many of these secreted enzymes, such as the subtilisins produced by Bacillus subtilis and related species, have been isolated and extensively characterized (60). In his review on the specificity of microbial proteinases, Morihara (60) mentioned a number of microorganisms including certain species of Aspergillus, Bacillus and Streptomyces, which produce alkaline serine proteases with molecular weights of 20 000. Morihara et al (61) isolated a strain of Streptomyces fradiae which produced 5 different proteases and 2 peptidases. The characteristics of one of these proteases (their fraction Ib) resembled the protease I detected with the gelatin-gel technique in that it was thermo-stable; it had a sedimentation coefficient of 2,88s (corresponding approximately to a molecular weight of 20 000); it had an alkaline pH optimum and could be inhibited by DFP. Another protease (their fraction III) showed a high elastolytic activity at alkaline pH. This is one of many examples where a single strain of a particular microbial species is able to produce a wide variety of proteases.

Considering the wide distribution of such microorganisms in nature, their presence in cercarial secretions was not surprising particularly since the tanks used for

FIGURE 4.9

Figure 4.9 Gelatin polyacrylamide electrophoresis of bacterial protease contaminants.

Gel a. Aliquots of 40 µl of nutrient broth inoculated with bacterial colonies isolated from solutions obtained from various stages of shedding of cercariae and harvesting of secretions under non sterile conditions, were electrophoresed in 6-15% gelatin polyacrylamide gels. The tracks contained: 1. yellow colonies from snail water; 2. large white colonies from snail water; 3. small white colonies from snail water; 4. yellow colonies from supernatant after cercarial concentrations; 5. white colonies from same as 4; 6. white colonies from cercarial secretions; 8. white colonies from dechlorinated tap water; 9 & 10. frozen cercarial secretion inoculated directly into nutrient broth.



a b

Gel b. Aliquots of 40 µl of nutrient broth inoculated with solutions obtained from various stages of cercarial shedding and harvesting of enzyme under standard conditions were electrophoresed in 5-16% gelatin polyacrylamide gels. The following samples were loaded: Track 1. broth inoculated with supernatant medium after cercarial concentration, 2. - snail water, 3. - 25% glucose solution, 4. - deionized water, 5. - cercarial secretion, 6. a sample of cercarial secretion showing the presence of the two cercarial proteases.

breeding and keeping infected snails provided an ideal environment for organisms to flourish.

The finding of bacterial proteases as contaminants of the cercarial preparations was important in two respects. Firstly, it emphasized the need for careful, clean work with precautions to keep bacterial growth to a minimum.

Secondly, this finding brings into question the validity of most published conclusions regarding the nature of cercarial proteases, since authors who have published on this subject appear to have been unaware of the possible presence of contaminating microbial enzymes.

CHAPTER 5

CHARACTERIZATION OF THE PROTEASES PRESENT IN CERCARIAL SECRETIONS

In this chapter I describe the results of my attempts to characterize the proteases present in cercarial secretions in terms of their susceptibility to inhibition or inactivation by physical and chemical means, their catalytic specificity and their requirements for maximal hydrolytic function.

As indicated in Chapters 3 and 4, collected cercarial secretions contained more than one proteolytic species when examined by electrophoresis in polyacrylamide gels containing SDS and gelatin. One of these proteases, with a molecular weight of 22 000 daltons, appeared to be contributed by bacterial contaminants. To avoid studying irrelevant enzymes, I used the quantitative fibrin plate radioenzymatic assay only with samples that were demonstrably free of the 22 000 M.W. enzyme. The results of inhibitor studies were confirmed by semi-quantitative zymographic assays in which the intensities of proteolytic bands in SDS-gelatin gels were scored visually after pre-electrophoretic exposure of the enzyme solutions to inhibitors or after incubation of gel tracks in inhibitors. With these procedures different susceptibilities of each proteolytic species to inhibitors could be assessed.

Determination of the pH optimum

The crude enzyme sample containing only the 35 000 molecular weight protease was dialysed against water, lyophilized and redissolved in water to a total protein concentration of 2 mg/ml. A volume of 25 µl was added to wells in an ^{125}I-fibrin plate containing 275 µl of the different buffers listed in Table 5.1. Buffer molarities were adjusted to give an approximate ionic strength ($\Gamma/2$) of 0,1, since enzyme activity is affected by ionic composition of the medium.

The ionic strength of each buffer solution was calculated according to the equation

$$\Gamma/2 = \frac{\Sigma C_i Z_i^2}{2}$$

where C_i is the concentration of each ionic species (i) present and Z_i is the charge on that species. The pKa of the buffer system was taken into account in calculating ionic strength. Activity coefficients of unity for the ionized species were assumed.

The results obtained for the pH experiments are summarized in Fig. 5.1 and Table 5.1.

As can be seen from these data, the apparent pH optimum for the proteolytic activity of the 35 000 M.W. enzyme lay between 9,0 and 9,5. This agrees with the observations of others (Table 5.2) inasmuch as alkaline pH optima for cercarial enzymes have usually been found.

Precise values given for the pH optima have varied, and a number of explanations for this variability can be suggested. In the first instance, different enzyme preparations

Table 5.1 List of buffers used for the determination of the pH optimum of the cercarial protease and the results obtained.

Buffer system	pH	Activity* (%T/hr)
HCl-glycine	2,52	0
	3,05	0
	3,50	0
	4.12	0
Acetic acid-Na acetate	3,50	0
	4,00	0
	4,50	0,06
	5,00	0,03
	5,50	0,38
Na K phosphate	6,00	0,32
	6,50	1,37
	7,00	2,75
HCl-Tris (hydroxymethyl-amino methane)	6,50	1,35
	7,01	2,19
	7,50	3,45
	8,01	4,64
Glycine-NaOH	8,51	12,08
	8,97	15,26
	9,51	15,59
	10,00	14,86
	10,40	14,34
	10,91	5,92

*Activity is expressed as the cumulative percentage radioactivity release per hour in the ^{125}I-fibrin assay.

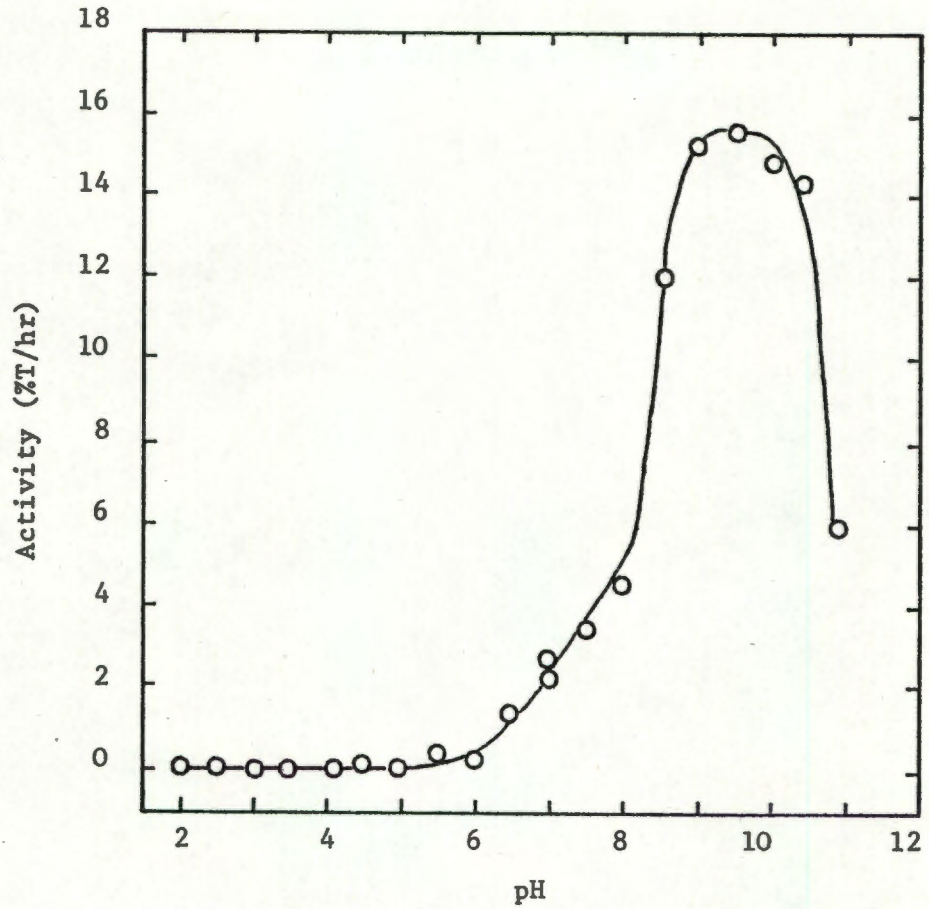


Figure 5.1 The effect of pH on the fibrinolytic activity of cercarial secretions.

The assay mixture contained 25 µl of a 2 mg/ml solution of cercarial secretion and 275 µl of the buffers listed in Table 5.1 with pH's ranging from 2 to 11. Aliquots of 50 µl were withdrawn after 30, 60 and 120 minutes. Activity is expressed as the cumulative percentage total radioactivity released per hour.

have been used with some reports referring to secretions and others to whole cercarial extracts. The number of proteases present and the possibility that these exhibit different pH optima has, generally, not been considered. The most critical reported study is that of Gazzinelli et al (26) who used partially purified protease, which was contaminated with only one additional band on cellulose acetate electrophoresis.

In the second instance, complex interactions between buffer anions, other salts and compounds present in the medium and the enzyme have effects upon the precise measurements of pH optima that lead to lack of reproducibility unless precisely identical experimental conditions are employed.

Ideally, the choice of buffers should be such that the variation of enzymatic activity is only measured as a function of pH and all secondary effects due to differences in ionic strength or other interactions are avoided. This is an important point to consider in this context, since the cercarial enzyme has been shown by Campbell et al (33) and Dresden and Edlin (62) to be extremely sensitive to ionic composition of the medium. This is well illustrated in an early experiment that I performed where I compared the activity of the cercarial enzyme in phosphate and glycine buffers of the same pH (8,9) but of varying concentrations. A plot of enzymatic activity against molarity (Fig. 5.2) showed a pronounced decrease in proteolytic activity with increasing molarity in the phosphate system that was less marked in the glycine

Table 5.2 pH optima of cercarial proteases as recorded in the literature

Protease preparation	Substrate	Buffer	pH opt.	Reference
Extract of lyophilized cercariae	Azocoll	0,1M phosphate buffer	7,5	22
DEAE cellulose fraction of extract of lyophilized cercariae	Casein	0,1M phosphate	7,9	26
	ATEE	0,05M phosphate	7,9	
Extract of lyophilized cercariae	Haemoglobin	Na phosphate	8,0-8,5	25
		Γ/2 0,1	8,3	
"	Azocoll	0,05M Tris maleate pH 5,5-7,8 0,05M glycine-NaOH pH 8,25-9,65	8,8	24
Cercarial secretion	Azocoll	0,1M phosphate	8,0	33
		0,05M glycine NaOH	8,5-8,8	
		0,05M Tris-phosphate	8,0-10,0	
Extract of lyophilized cercariae	Elastin	0,075M Na borate	8,1-8,3	27

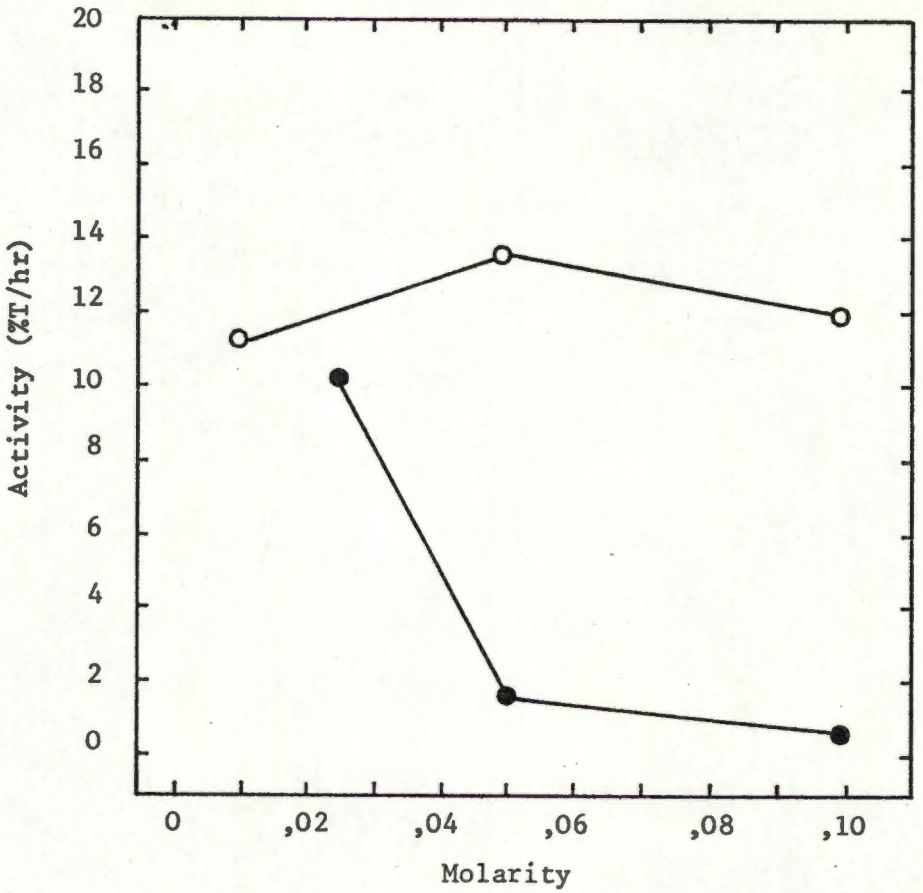


Figure 5.2 The effect of buffer concentration on the fibrinolytic activity of cercarial secretion.

The reaction mixture contained 150 µl of either sodium phosphate buffer pH 8,5 (●) or glycine-NaOH pH 8,5 (O) and 150 µl cercarial secretion. Aliquots of 50 µl were withdrawn after 30, 60 and 120 min. Enzyme activity is expressed as the cumulative percentage total radioactivity released.

system. This may have been due to inactivation of calcium ions in the phosphate system, since it has been shown by Dresden and Edlin (62) and by my own experience that enzyme activity is affected by the concentrations of divalent cations such as calcium and magnesium. Alternatively, the activity coefficients of the individual ions in the solutions may have been more important. Calculated values for ionic strengths of the phosphate and glycine buffers are given in Table 5.3.

Yet another variable which makes the comparison of the different pH optima difficult is the difference in substrates that have been used. In most of the enzyme assays for cercarial proteases complex protein molecules whose charge and conformation are themselves pH-dependent have been employed. This could in turn influence the binding to the enzyme and therefore the measured enzyme activity, and conclusions regarding possible mechanisms of proteolytic activity should include a consideration of the effects of pH on the Km. This problem is, to a certain extent, overcome by the use of simpler substrates. The well known bell-shaped pH titration curves of enzyme activity do not give an unequivocal indication of the effects of proton activity on the catalytic site. It has been shown, for example in the case of chymotrypsin, that the bell-shaped profile is the result of a combination of two sigmoid curves, where the one describes the pH-dependent substrate binding and the other the actual catalytic activity. In the case of the cercarial enzyme, which exhibits rather a high pH optimum, the descending part of the bell-shaped curve could also represent a reduction of enzymatic activity due to protein denaturation.

Table 5.3 The effect of ionic strength on the activity of cercarial secretions.

Buffer*	Molarity	Γ/2 Calculated	Activity(%T/hr)
Na phosphate	0,1	0,298	0,65
	0,05	0,149	1,61
	0,025	0,074	10,18
Glycine-NaOH	0,1	0,011	11,90
	0,05	0,006	13,59
	0,01	0,001	11,21

* All buffers were at pH 8,90.

Stability to pH

With a view to establishing a convenient pH working range for the purification of cercarial proteases by cation exchange chromatography, the ability of the enzymes to withstand exposure to conditions varying from pH 2 to pH 6 was investigated.

Glycine-HCl buffer ($\Gamma/2 = 0,1$) was used to prepare buffers ranging from pH 2 to 3,5 and sodium acetate-acetic acid ($\Gamma/2 = 0,1$) for those ranging from pH 4 to 6. Aliquots (100 µl) of a crude cercarial secretion sample were added to 100 µl of the various buffer solutions, and incubated at $4^{o}C$ and $22^{o}C$ for 4 and 20 hours. After incubation, the pH's of the mixtures were adjusted to approximately pH 8,8 by the addition of a predetermined volume of 0,1M NaOH.

After pH adjustment, the total volume in each tube was adjusted to 500 µl with 0,1M Gly-NaOH buffer, pH 8,8 and 300 µl from each tube was added to a separate well in a fibrin plate and assayed for proteolytic activity by the usual method. Zero-time controls consisted of a series of samples treated in the same way but neutralized immediately without incubation. Appropriate enzyme-free buffer controls were included; all had activities below 0,12% T/hr.

The results, presented in Fig. 5.3 showed that no appreciable denaturation of the proteases occurred at pH's above pH 4 for the temperatures and times tested. The activity, however, decreased rapidly at pH's below 3,5, presumably due to irreversible enzyme denaturation. The slight decline of activity seen at pH 6 was probably the result of autodigestion rather than denaturation.

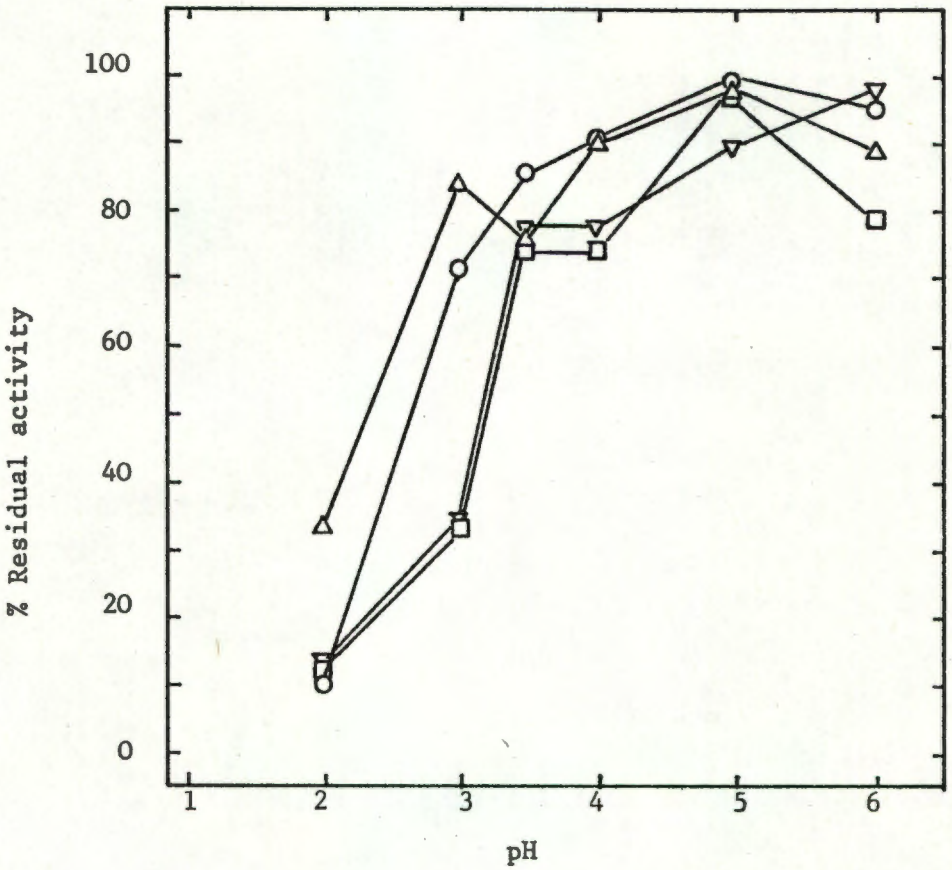


Figure 5.3 Stability of the cercarial protease to pH

Aliquots (100 µl) of a crude cercarial secretion sample were added to 100 µl of glycine HCl buffer (Γ/2 = 0,1 pH 2, 3, 3,5) and Na-acetate buffer (Γ/2 = 0,1, pH's 4, 5, 6). They were incubated at 20°C for 4 hrs (Δ) or 20 hr (□) or at 4°C for 4 hrs (▽) or 20 hrs (O). The percentage residual activity in the fibrin assay was calculated by dividing the activity of the test samples by the activity shown by zero time controls treated in the same way.

Thermostability

A lyophilized cercarial protease sample was dissolved in 0,1M Gly-NaOH pH 8,8 to a concentration of 2 mg protein/ml and incubated at various temperatures (-15°C to 56°C) for periods varying from 5 min to 48 hr. After the treatment the incubation mixtures were cooled on ice and assayed for residual activity in the fibrin assay. Sterile working conditions were preserved throughout.

As is evident from the results presented in Fig. 5.4, the protease was fairly stable at 4°C for 48 hours, but showed a pronounced decrease in activity after incubation at 37°C for 4 hours.

My results confirm and enlarge upon those reported in the literature. Campbell et al (33) observed a decrease in cercarial protease activity at a rate of 0,4%/hr at 5°C and 1,7%/hr at 35°C. I did not observe a linear decrease of activity as a function of time. However, by taking the percentage decrease after 48 hrs incubation and dividing this by 48, rates of inactivation of 0,2%/hr and 1,8%/hr at 4°C and 37°C respectively were observed. These agree with the results of Campbell et al.

The decrease in activity after incubation at 56°C (Fig. 5.5) was not as rapid as that observed by Lewert and Lee (22) who reported a 50% reduction after 30 min. These authors, together with Gazzinelli et al (26) and Campbell et al (33) also found that the protease(s) were completely inactive after incubation at 60°C for 10 - 15 minutes.

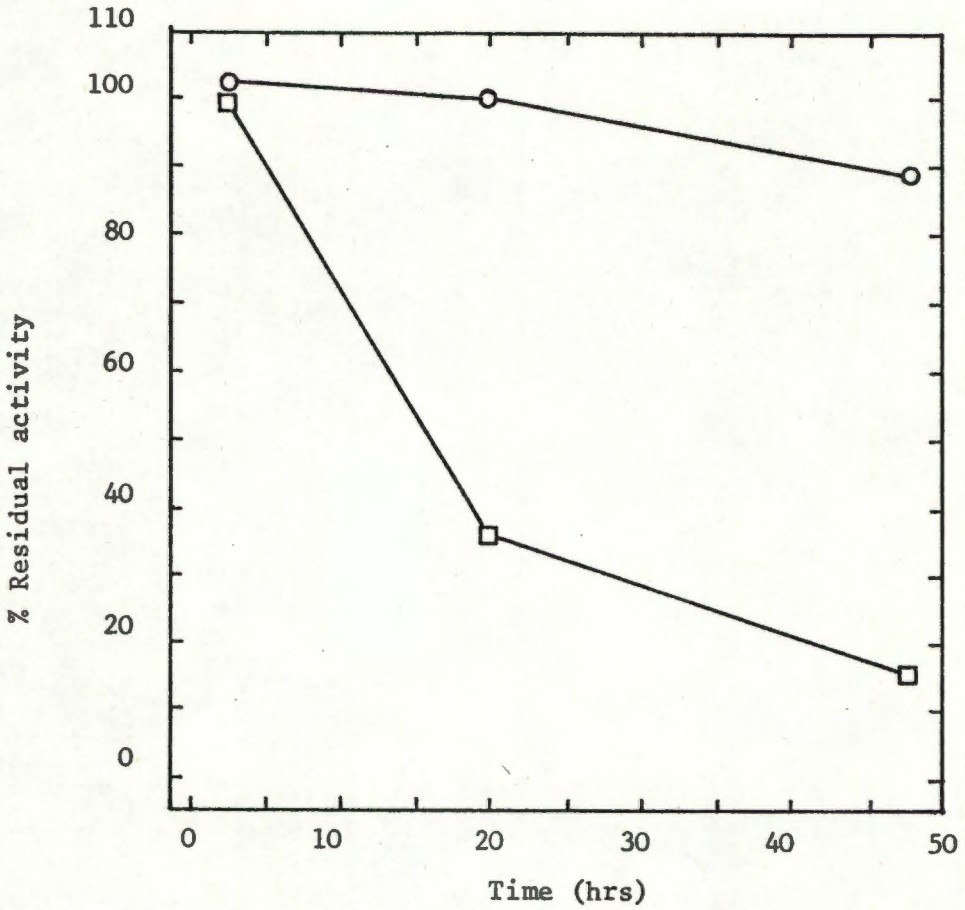


Figure 5.4 Thermostability of the cercarial protease

A cercarial protease sample dissolved in 0,1M glycine-NaOH pH 8,80 was incubated at 4°C (O) and 37°C (□) for 0 to 48 hrs. The residual activity was measured in the fibrin assay by adding 50 µl to 250 µl glycine-NaOH, pH 8,80 to each well. The results are expressed as the percentage residual activity as compared to a sample kept at -20°C for the same periods of time.

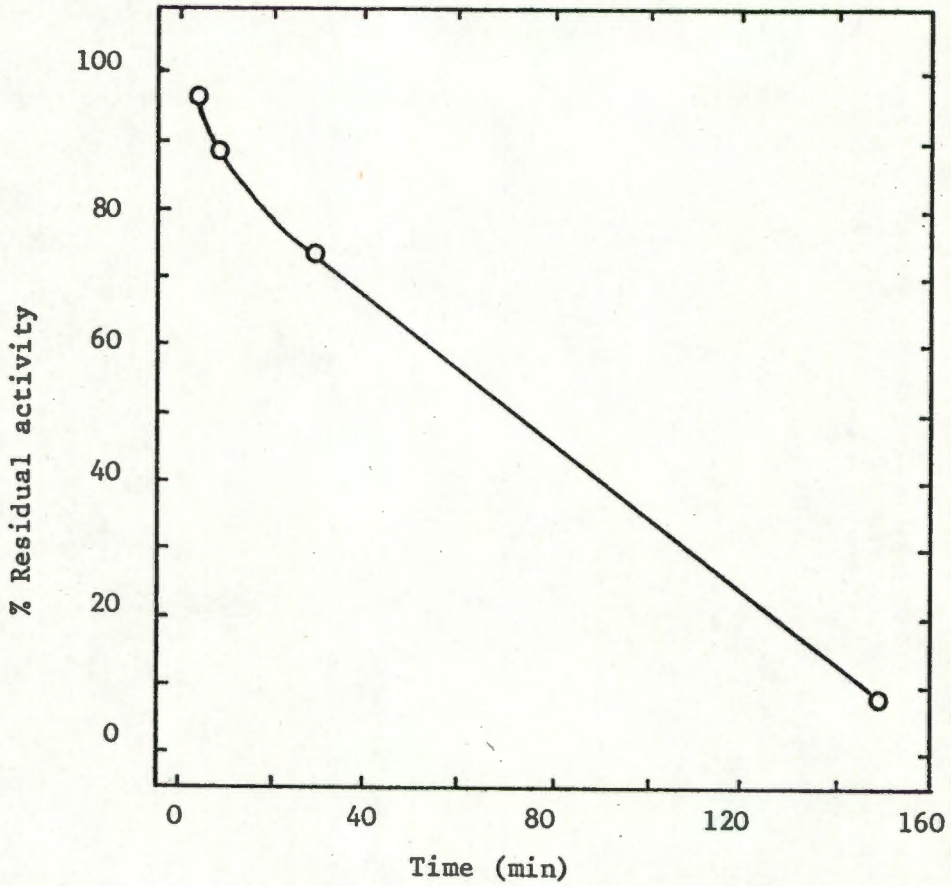


Figure 5.5 Thermostability of the cercarial protease

A cercarial protease sample was incubated at 56°C in 0,1M glycine-NaOH pH 8,80 and the residual activity measured in the fibrin assay.

Adsorptive and other losses

Proteases, like many other enzymes, are notoriously liable to irreversible loss of activity when kept at low concentration in the absence of other proteins or protective molecules such as detergents, glycerol or sucrose. These losses are variously ascribed to adsorption to glass or plastic surfaces, autodigestion, instability of tertiary structure if the binding or catalytic site is unoccupied or denaturation by complex intermolecular forces in the presence of a high activity of water. I accordingly performed the following experiments to see if addition of albumin, glycerol or sucrose to solutions of cercarial proteases would preserve enzyme activity during incubation at 37°C.

A volume of 100 µl cercarial secretion was added to 260 µl glycine-NaOH buffer, pH 8,8 in plastic tubes. To these were added 40 µl of bovine serum albumin solutions in glycine-NaOH at different concentrations such that the final concentrations of the protein were 1%, 0,1% and 0,01%. The tubes were incubated at 37°C for 0, 19 and 24 hours and the contents assayed for residual activity in the fibrin plate assay.

The results (Table 5.4 and Fig. 5.6) showed that the presence of albumin afforded protection against loss of activity to a degree that was related to the albumin concentration. Glycerol or sucrose failed to protect (results not shown).

Table 5.4 Protection of the cercarial protease by addition of bovine serum albumin

	BSA (%)	Time of incub. (hr)	%T/hr	% Total
*	0	0	21,79	100
	,01	0	23,76	109,8
	,10	0	23,61	109,3
	1,0	0	23,78	110,1
	0	19	18,71	85,5
	,01	19	20,19	93,0
	,10	19	20,57	95,0
	1,0	19	22,66	104,8
	0	24	6,52	28,2
	0,01	24	11.27	51,1
	0,10	24	11,99	54,7
	1,00	24	14,64	67,1

*100% activity control.

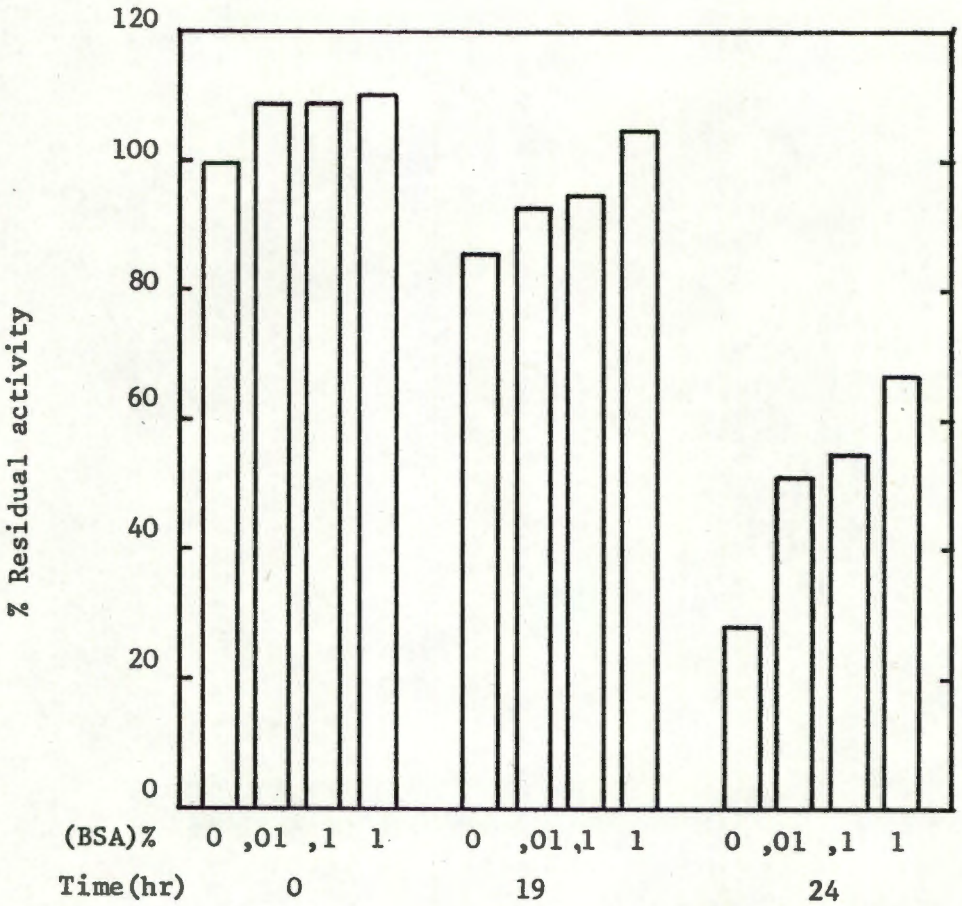


Figure 5.6 Protection of the cercarial protease by albumin

Aliquots of 100 µl of cercarial secretion were incubated in total volumes of 400 µl with concentrations of bovine serum albumin of 0,01%, 0,1% and 1% at 37°C for 0, 19 and 24 hours. The residual activity obtained in the fibrin assay was calculated from the activity shown by a sample that was frozen at -20°C without BSA

Effect of cations on proteolytic activity

Since the activity of many enzymes is affected by divalent cations, cercarial secretions were depleted of these by dialysis against 10mM EDTA in 0,05M glycine-NaOH pH 8,8, and then into the same buffer without EDTA. The activity of these depleted preparations was then assayed in the presence of various concentrations of $CaCl_2$, $MgCl_2$ and $ZnSO_4$. In addition, the effects of EDTA was measured directly.

The results obtained (Figs. 5.7 to 5.10) generally agree with those reported by Dresden and Edlin (62) in that low concentrations of Ca^{++} and Mg^{++} stimulated proteolytic activity whereas high concentrations inhibited. The threshold levels for Ca^{++} and Mg^{++} were 3 and 0,2 mM respectively whereas those reported by Dresden and Edlin were 8 and 12 mM respectively; these differences can be ascribed to differences in the enzyme assays used. $ZnSO_4$ on the other hand inhibited the activity at all concentrations tested, which agrees with results reported by Lewert and Lee (22) and Dresden and Edlin (62), who found that Fe^{++}, Cu^{++}, Ca^{++} and Mg^{++} all inhibited the enzyme at concentrations as low as 0,1 mM in the azocollytic assay.

On the other hand, EDTA at a concentration of 10 mM inhibited proteolytic activity in cercarial secretions by approximately 30%, whereas Dresden and Edlin (62) observed a slight stimulation at the same concentration. In another report, Dresden and Asch (24) observed no effect on the activity at a concentration of 5mM EDTA.

Except in the case of EDTA the effects of the various cations on proteolytic activity in vitro seemed to parallel

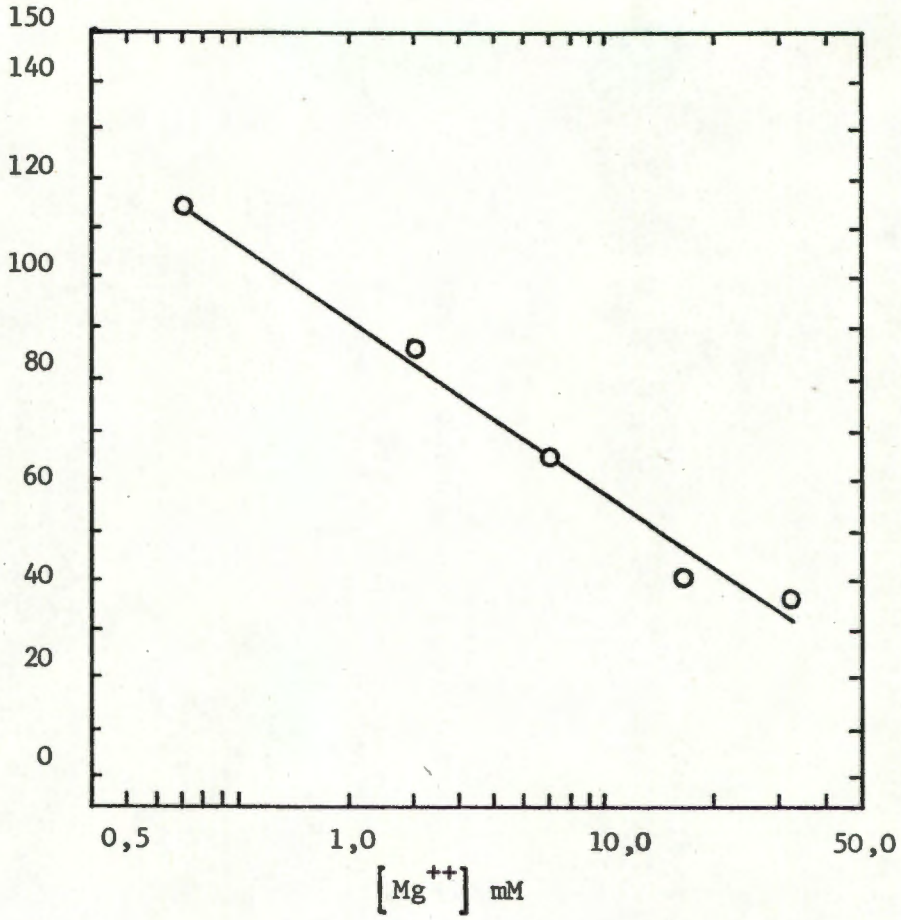


Figure 5.7 The effect of magnesium on the proteolytic activity of cercarial secretion.

A cercarial secretion sample was dialysed against 10 mM EDTA in 0,05M glycine-NaOH pH 8,80 and then into the same buffer without EDTA. Aliquots of 300 µl were assayed for fibrinolytic activity in the presence of various concentrations of $MgCl_2$. Aliquots of 50 µl were withdrawn after 30, 60 and 90 min incubation. The results are expressed as the percentage activity of the samples as compared to that of a sample without added ions.

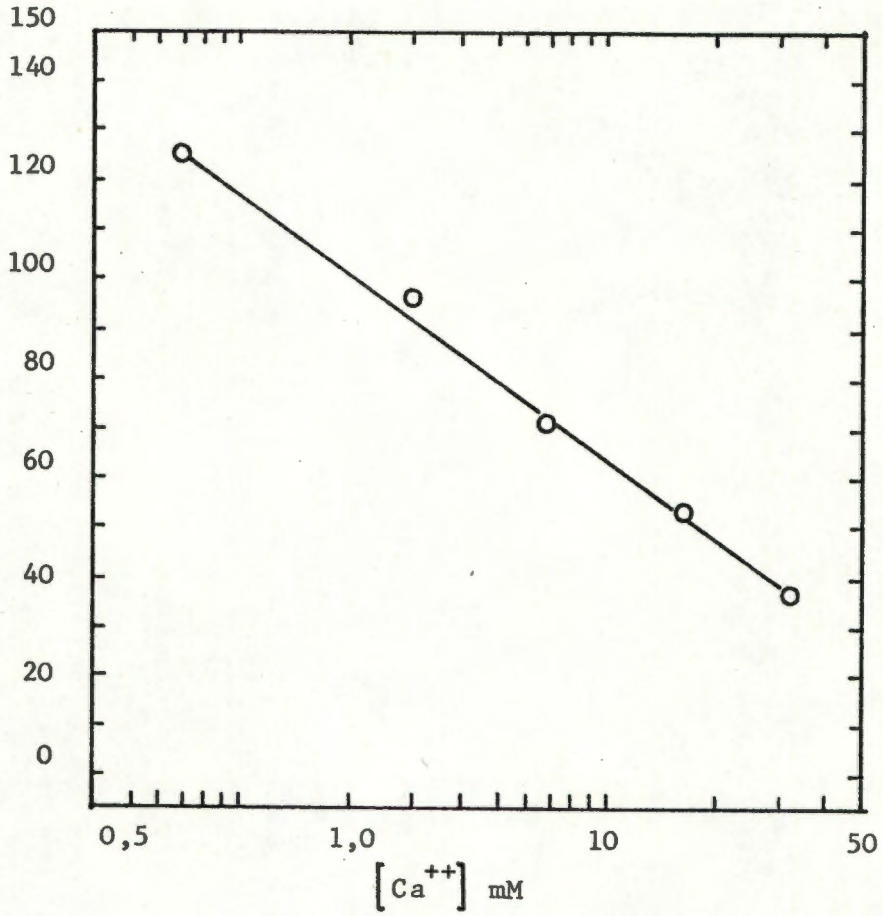


Figure 5.8 The effect of calcium on the proteolytic activity of cercarial secretion.

A cercarial secretion sample was dialysed against 10 mM EDTA in 0,05M glycine-NaOH pH 8,80 and then into the same buffer without EDTA. Aliquots of 300 µl were assayed for fibrinolytic activity in the presence of various concentrations of $CaCl_2$. Aliquots of 50 µl were withdrawn after 30, 60 and 90 min incubation. The results are expressed as the percentage activity of the samples as compared to that of a sample without added ions.

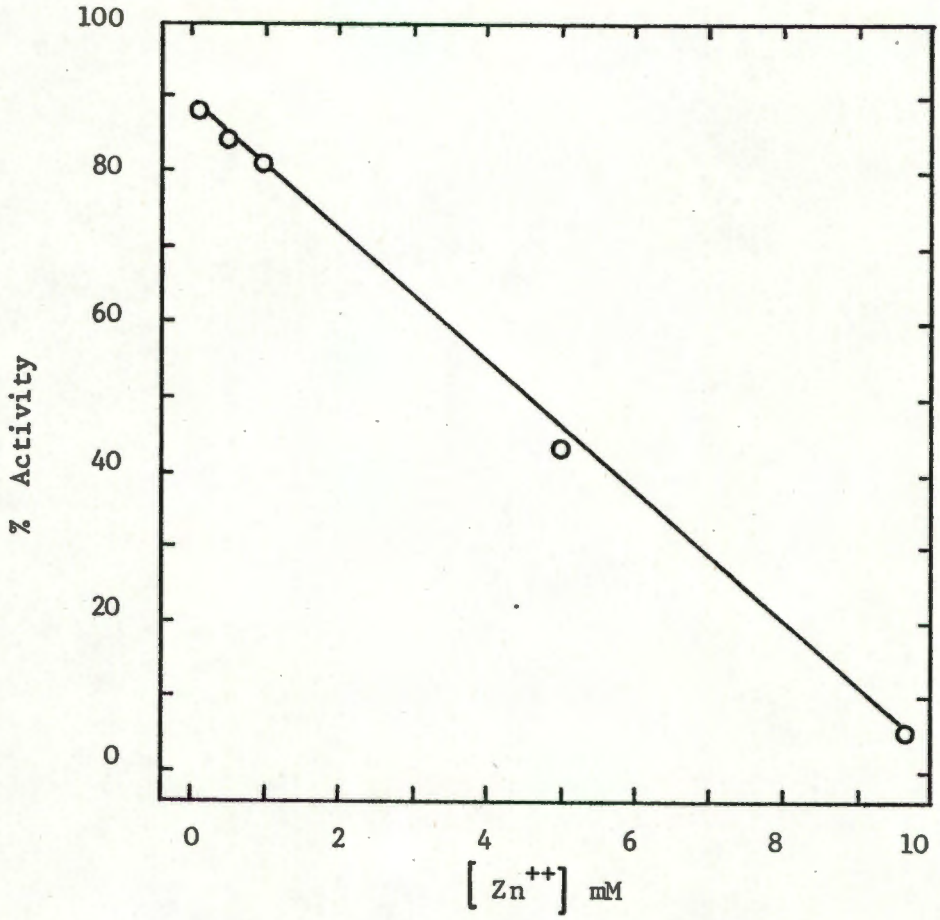


Figure 5.9 The effect of zinc on the proteolytic activity of cercarial secretion.

A cercarial secretion sample was dialysed against 10 mM EDTA in 0,05M glycine-NaOH pH 8,80 and then into the same buffer without EDTA. Aliquots of 300 µl were assayed for fibrinolytic activity in the presence of various concentrations of $ZnCl_2$. Aliquots of 50 µl were withdrawn after 30, 60 and 90 min incubation. The results are expressed as the percentage activity of the samples as compared to that of a sample without added ions.

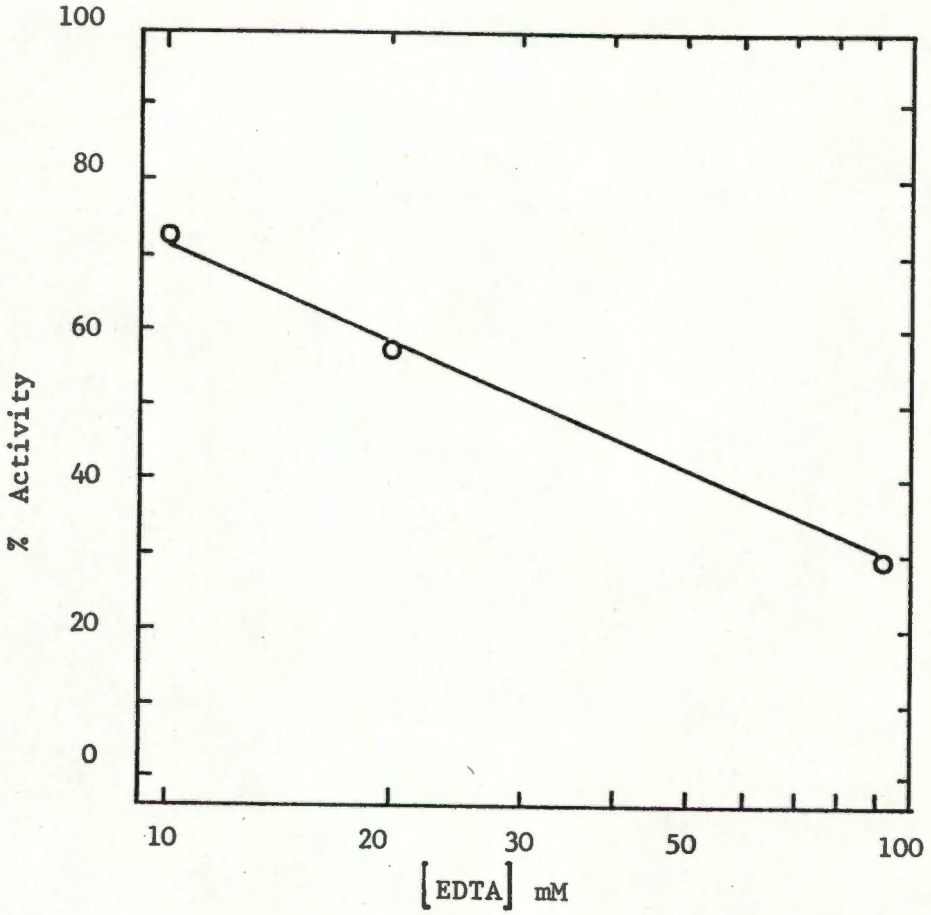


Figure 5.10 The effect of EDTA on the proteolytic activity of cercarial secretion

Aliquots of 300 µl of a cercarial secretion sample were assayed for fibrinolytic activity in the presence of 0,1M glycine-NaOH pH 8,80 containing various concentrations of EDTA. Aliquots of 50 µl were withdrawn after 30, 60 and 90 min incubation. The results are expressed as the percentage activity of the samples as compared to that of a sample where no EDTA had been added.

those observed on penetrating cercariae in vivo. Lewert, Hopkins and Mandlowitz (55) have shown that the infectivity of cercariae was stimulated by the presence of 1 mM Ca^{++} or Mg^{++} ions as measured by penetration of rat skin and subsequent worm burden. On the other hand, Zn^{++} and EDTA inhibited these processes. Inhibition of Evans blue spreading during penetration of rat skin by cercariae in the presence of 1 mM EDTA, Cu^{++} and Hg^{++} was also observed by Lewert and Lee (22). While Cu^{++} and Hg^{++} were actually cercaricidal, the presence of EDTA appeared to inhibit a vital process necessary for penetration.

From the effects of Ca^{++} in <u>in vitro</u> and <u>in vivo</u> experiments, and the fact that large amounts of Ca^{++} are present in the preacetabular glands of cercariae (10, 55, 63), Dresden and Edlin (62) have suggested that Ca^{++} might play an important role in regulating the penetration protease activity in the skin. In the gland Ca^{++} might act as a natural inhibitor, which becomes diluted during secretion, thereby activating the proteases.

Effect of protease inhibitors

To characterise the cercarial protease further I examined the effects of a variety of protease inhibitors with different specificities. The inhibitors and the abbreviations I use to describe them in the subsequent text were as follows:

STI	Soybean trypsin inhibitor (Worthington Biochem)
NPGB	Nitrophenylguanidinobenzoate (Sigma)
DFP	Diisopropylfluorophosphate (Sigma)
PMSF	Phenylmethylsulphonylfluoride (Schwarz Mann)
pCMB	p-chloromercuribenzoate (Sigma)
TLCK	Tosyl-lysine-chloromethylketone (Sigma)
TPCK	Tosyl-phenylalanine-chloromethylketone (Sigma)
AcAAAACK	Acetyl-$(alanyl)_4$-chloromethylketone*
	iodoacetamide (BDH)
	benzamidine (Sigma)
	Trasylol (Bayer)

*AcAAAACK was a gift from Dr. J. Powers of the Georgia Institute of Technology, Georgia.

The fibrinolytic assay and the gelatin-polyacrylamide electrophoresis technique were employed to define the effects of inhibitors on enzyme activity.

The activity of the cercarial protease at a final concentration of 25,3 µg protein/ml was measured in a standard ^{125}I-fibrin assay in the presence of 5 and 1 mM concentrations of the various inhibitors listed in Table 5.5.

Inhibitor-free controls consisted of enzyme samples assayed in 0,1M Gly-NaOH buffer pH 8,3, containing the same concentration of organic solvents used to dissolve the inhibitors. Enzyme-free controls, consisting of buffer and inhibitor solution only were included.

A more extensive protocol was used with the specific protease affinity labels, the chloro-methyl ketones. The cercarial protease was preincubated at 37°C in the presence of 1 mM TLCK (a trypsin inhibitor), 1 mM TPCK (a chymotrypsin inhibitor) or 0,3 mM AcAAAACK (an elastase inhibitor) for 0, 30, 60, 90 minutes in 0,1M Tris HCl pH 7,0. The total incubation volume was 300 µl and the final protein concentration was 66 µg/ml. Both TLCK and TPCK were therefore present in at least 500-fold molar excess, whereas AcAAAACK was present in at least 166-fold molar excess assuming a molecular weight for the enzyme of 35 000 daltons. The residual activity of the enzyme was then measured in the standard fibrin assay over a 1 hour period, after diluting each sample 1:1 with 0,1M Gly-NaOH pH 8,9. Appropriate inhibitor- and enzyme-free controls were included for each preincubation time period.

TABLE 5.5

Table 5.5 The effect of protease inhibitors on the protease in cercarial secretion.

Inhibitor	Mode of Action	Ref	Concentration	Solvent	% inhibition
	Naturally occurring inhibitors				
STI (Kunitz)		65	200mg/ml	Buffer	68,2
Trasylol	Polypeptide from bovine lung reversible inhibitor of trypsin, kallikrein, chymotrypsin, plasmin	66	10^4KIU/ml $3x10^3$ KIU/ml	Buffer	39,2 0
	Synthetic competitive inhibitor				
Benzamidine	of trypsin-like enzymes analogue of arginine	67	5mM 1mM	Buffer Buffer	33,9 15,8
	Substrate for trypsin-like enzymes				
nPGB	deacylation rates differ for different enzymes	68	10mM 1mM	5% DMSO	99,4 98,7

	Active site label for ser-enzymes				
DFP	Reaction with serine residue at active site of enzymes	68	5mM	5% isopropanol	58,8
			1mM		41,5
PMSF	Replacement of serine-OH with -SH in active centre	70	5mM	5% DMSO	95,1
			1mM		53,9
	Active site label for SH-enzymes				
pCMB	mercuration of SH groups	71	1mM	NaOH/Buffer	0*
Iodoacetamide	carboxymethylation of -SH and imidazole groups	72	5mM	Buffer	0
			1mM		0
	Affinity label, specific alkylation of a histidine residue at the active site of				
TLCK	Trypsin	73	1mM	3% Methanol	0†
TPCK	Chymotrypsin	74	1mM	3% Methanol	7,5
AcAAAACK	Elastase	75	0,3mM	3% Methanol	8,0

*pCMB was insoluble at 5mM and is hence taken at 1mM only.

†inhibition after 90 min preincubation.

The results of these inhibitor studies are presented in Table 5.5 where the inhibitors are grouped according to their specificities or presumed mode of action. The results show clearly that the cercarial protease was inhibited by DFP and PMSF and hence belongs to the group of proteases such as trypsin, chymotrypsin and elastase which have a reactive serine residue at the active site. The inhibition by other inhibitors such as STI, Trasylol, Benzamidine and NPGB indicate that the protease might be trypsin-like in its specificity although the enzyme differs from trypsin in its resistence to inhibition by TLCK. It also differed from chymotrypsin and elastase since no inhibition was found with their respective affinity labels TPCK and AcAAAACK. Iodoacetamide and pCMB had no effect on the protease at the lower concentrations, indicating that it does not belong to the group of proteases, such as papain or clostripain, which require a sulfhydryl group for activity.

As indicated in Chapter 2, cercarial secretion samples frequently contained more than one enzyme species as determined by SDS-gelatin gel electrophoresis. Since these different proteases may have differed in their susceptibility to inhibition by the various agents used, it was not possible to draw definitive conclusions from observations on the effects of inhibitors on crude samples of cercarial harvest water. This problem would not have arisen had pure samples of each enzyme species been available. Limited availability of the enzymes, however, precluded this ideal and I therefore resorted to the semiquantitative expedient of observing the effects of inhibitors on individual enzymes resolved by polyacrylamide

gel electrophoresis.

Aliquots (40 µl) of the same cercarial secretion sample containing the 35 000, the 82 000 and the additional 21 000 dalton protease bands were loaded into each of 6 wells of a polyacrylamide gel containing SDS and gelatin. After electrophoresis the gel was washed in Triton X-100 at 4°C, to remove the SDS without allowing proteolysis to proceed. The gel was then cut into six longitudinal strips, each strip corresponding to one electrophoretic track, and these were incubated individually in 10 ml 0,1M glycine-NaOH pH 8,3, containing various concentrations of the different inhibitors as indicated in Fig. 5.11. The gel strips were then incubated at 37°C with shaking for 3 hours with 3 changes of inhibitor solution during this period. They were then stained and destained as usual.

Two examples of gels showing the effect of iodoacetamide and PMSF at various concentrations on the three proteolytic bands are shown in Fig. 5.11. The overall results are given in Table 5.6. Only the effects of the inhibitors at 10 and 1 mM concentrations are shown. The intensities of the proteolytic bands were scored visually on an arbitrary scale varying from 0 - +++++. To compare the effects of the chloromethyl ketones on the activity of the cercarial proteases with their effects on trypsin and elastase, samples containing elastase (10 µg), chymotrypsin (1 µg), trypsin (1 µg) or cercarial proteases were incubated at room temperature in 0,01M Tris-acetate pH 7,0 for 12 hours in the presence of 50 µM TLCK, TPCK, AcAAAACK or in the absence of inhibitor. The samples

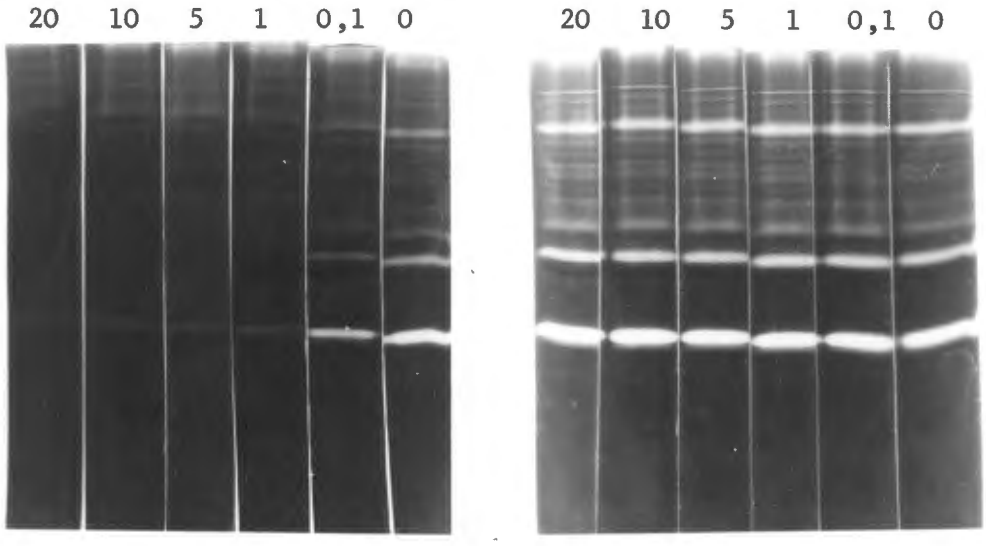


Figure 5.11 Effect of iodoacetamide and phenylmethylsulphonylfluoride on cercarial proteases as visualized in gelatin gels

Aliquots of 40 µl of a cercarial secretion sample were loaded into each of 6 wells of 2 gelatin gels. After electrophoresis the gels were washed in Triton X-100 at 4°C, and the gels were cut into 6 strips, each corresponding to one electrophoretic track. These were incubated at 37°C individually in 10 ml 0,1M Glycine-NaOH pH 8,3 containing 20, 10, 5, 1, 0,1 mM of inhibitor. The last track was incubated in the absence of any inhibitor. Gel (a) in the figure shows the effect of PMSF on the proteases, while Gel (b) shows that of iodoacetamide.

Table 5.6 Effect of various protease inhibitors on the activity of cercarial secretions in gelatin gels.

Inhibitor	Concentr.	Proteolytic Band*		
		1	2	3
STI	10µg/ml	0†	++	+++
	1µg/ml	+	+++	++++
Trasylol	$1x10^4$ KIU/ml	0	0	+
	$1x10^3$ KIU/ml	+	+	++
Benzamidine	10mM	0	+	++
	1 mM	0	++	++++
NPGB	10mM	0	0	0
	1mM	0	0	0
PMSF	10mM	0	0	+/0
	1mM	0	0	+
DFP	10mM	0	0	0
	1mM	+	++	+++
Iodoacetamide	10mM	+++	+++	++++
	1mM	+++	+++	++++

*The proteolytic bands are marked 1, 2 and 3 for the 82 000, 35 000 and 21 000 dalton bands shown by cercarial secretions.

pCMB precipitated on the gels and therefore could not be tested for inhibition.

† The intensities of the proteolytic bands are scored visually on an arbitrary scale ranging from 0 - ++++, where 0 means the absence of any clear lysis band.

were then analysed by SDS-gelatin gel electrophoresis for residual activity.

As can be seen from the results shown in Fig. 5.12 and 5.13, trypsin and elastase were inhibited by TLCK and AcAAAACK respectively, whereas the cercarial protease was unaffected by any of the inhibitors. These results correlated well with the results obtained with the ^{125}I-fibrin assay. It is interesting to note that the commercial elastase I used contained an additional enzyme band of similar molecular weight to trypsin which was inhibited by TLCK.

The results obtained with the gelatin-gel procedure indicated that all bands were inhibited by NPGB and PMSF to the greatest extent. DFP, Benzamidine and Trasylol inhibited to a lesser extent and STI least of all. Iodoacetamide had no effect on any of the bands. The inhibition of the third 21 000 band followed a similar pattern of inhibition to that shown by the other bands indicating that the presumed bacterial protease band might also be a trypsin-like serine protease.

Taken overall, the effects of the various inhibitors indicate that the cercarial enzyme is an alkaline serine protease. The cercarial proteases were strongly inhibited by PMSF and to a lesser degree by DFP, both being irreversible inhibitors of serine proteases. This is in agreement with the results reported by Gazzinelli et al (29) and Dresden and Asch (24). Gazzinelli et al (26) initially concluded that the cercarial protease is chymotrypsin-like in nature due to its ability to hydrolyse Nαacetyl-L-tyrosine ethyl ester (ATEE) and Nαacetyl-L-phenylalanyl-ethyl ester (APEE), both being substrates for

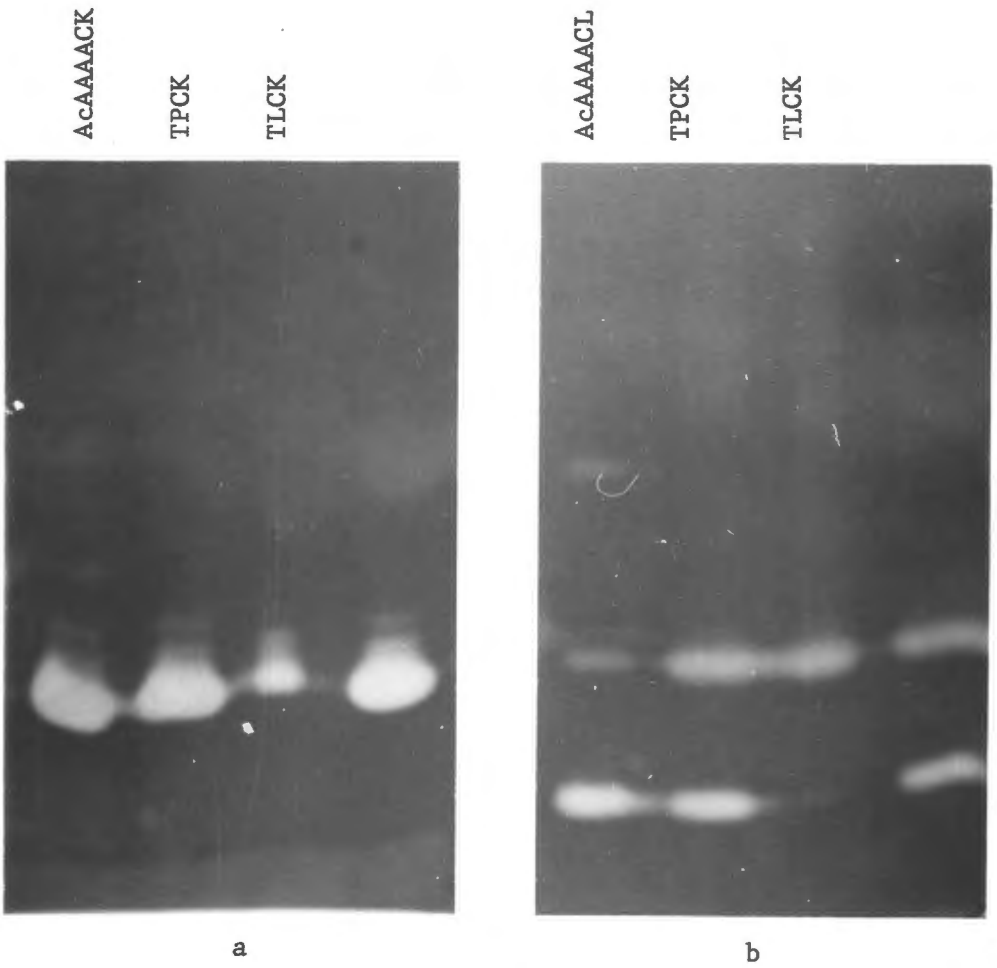


Figure 5.12 The effect of various chlormethylketones on the proteolytic activity of Trypsin and Elastase in gelatin gels.

Samples containing either trypsin (1 μg) or elastase (10 μg) were incubated in the presence of 50 μM of 3 chloromethyl-ketones for 12 hours, and then analysed on gelatin gels for residual activity. Gel (a) shows the effect of AcAAAACK, TPCK, TLCK on trypsin, wherease Gel (b) shows the effect on elastase. The last well in each gel contained the untreated enzymes.

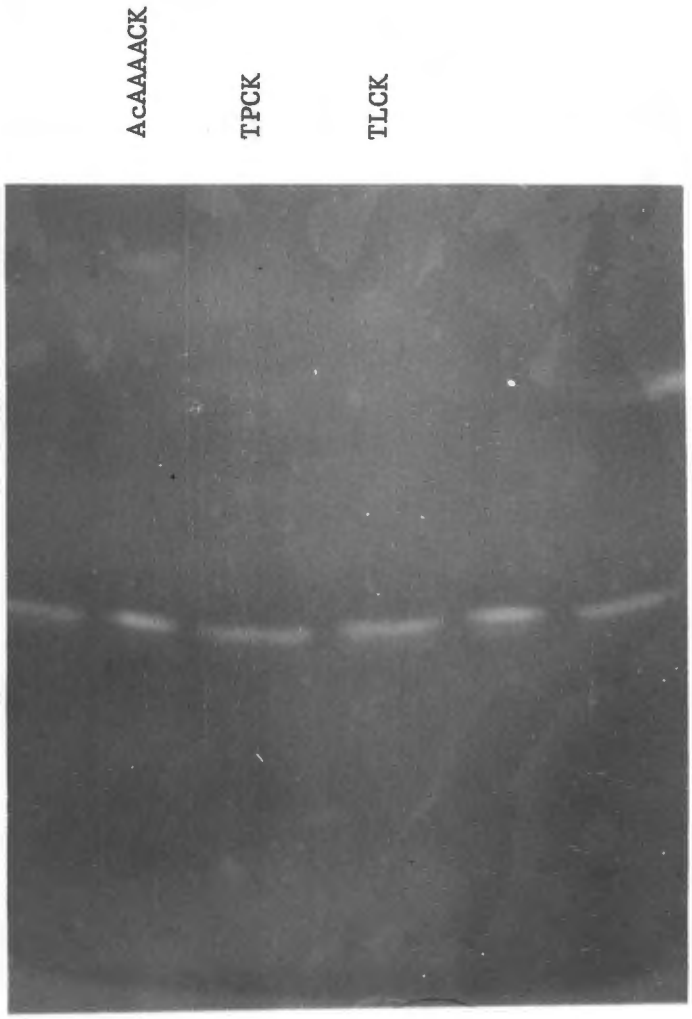


Figure 5.13 The effect of various chlormethylketones on the proteolytic activity of cercarial proteases in gelatin gels.

Samples of cercarial secretions (10 µl, 2mg/ml) were incubated in the presence of 50 µM of the chlormethylketones in a total volume of 80 µl at 37°C for 12 hours. Aliquots of 40 µl were analysed for residual activity in gelatin gels.

chymotrypsin. It was not able to hydrolyse typical trypsin substrates, such as Nαbenzoyl-L arginine ethyl ester (BAEE) and Nαbenzoyl-DL arginine p-nitroanilide. However, in a more recent report, they showed, in agreement with my findings, that neither TLCK nor TPCK inhibited the protease in any way (29). Once again, this is an indication that the cercarial enzyme belongs to the group of alkaline serine proteases since these are not inhibited by TLCK and TPCK and are specific against hydrophobic amino acid residues according to Morihara (60). Comparable proteases would be the subtilisins.

Another property of the protease is its ability to solubilize elastin, as shown by Gazzinelli, Ramalho Pinto and Pellegrino (26) and Dresden and Asch (24). The inability of AcAAAACK, a specific elastase inhibitor, to inhibit the cercarial enzyme, however, indicates that the protease does not operate by the same mechanism as does porcine pancreatic elastase.

The failure of pCMB and iodoacetamide to inhibit the cercarial protease at low concentration indicates that the enzyme does not belong to the group of proteases such as papain and chlostripain which have a reactive SH group at their active centres.

The inhibition caused by the naturally occurring inhibitors, STI and Trasylol, might suggest that the protease belongs to the group of trypsin-like proteases, such as trypsin, plasmin, thrombin and acrosin. This is further substantiated by the strong inhibition caused by NPGB and the weaker one caused by benzamidine, both being low molecular weight, syn-

thetic inhibitors of trypsin-like enzymes.

It is however premature to conclude that the cercarial protease is a trypsin-like enzyme on the basis of the inhibition caused by their inhibitors. Kinetic studies using synthetic substrates would be needed to identify clearly the specificity of the enzyme. In this respect, the inhibition caused by these trypsin-specific inhibitors might indicate that the enzyme I have analysed is different from that of Gazzinelli et al (26) who ascribed a chymotrypsin-like specificity to their protease on the basis of its hydrolysis of typical chymotrypsin substrates. As I have mentioned in Chapter 4, there is a danger of confusing microbial proteases with those of cercarial origin, and, since none of the published work to date makes mention of this difficulty, it is difficult to reconcile my observation with those in the literature in every respect.

Partial purification of the cercarial protease by cation exchange chromatography

Preliminary studies indicated that the protease(s) present in cercarial secretion samples were strongly bound to the cation exchanger, CM cellulose at acid pH. It, therefore, seemed appropriate to use this type of chromatography as a first purification step. With this procedure the protease could be partially separated from contaminating proteins and could be concentrated to a useful extent. I was, however, unable to obtain large enough quantities of the partially purified material for further studies or for more extensive purification since the amounts of enzyme available with the facilities at my disposal were inadequate.

In the following section, I shall describe the preliminary experiments performed to establish the conditions for the purification procedures that I describe subsequently.

Preliminary experiments

Whatman's DE52 cellulose was equilibrated with 1 mM Tris-HCl, pH 7,5 and added as a slurry to a set of 8 plastic tubes to give approximately 0,25 ml packed volume of anion exchanger per tube. The DEAE cellulose in duplicate tubes was equilibrated with 3 mM Tris-HCl buffer at pH 7,5; 8,0; 8,5 and 9,0 by repeated centrifugation and resuspension until the conductivity and pH of the supernatant was that of the equilibrating buffer. The cellulose was then pelleted, the supernatant removed and 0,5 ml of cercarial secretion sample diluted 10-fold with the corresponding buffer was added to

each tube. The tu-bes were tumbled at 4°C for 1 hour and centrifuged at 500g for 10 minutes. The supernatants were carefully removed and kept for measurement of enzymatic activity of the non-adsorbed enzyme protein. The matrices were washed twice with 1 ml aliquots of the corresponding starting buffers, and then twice with 1 ml aliquots of the starting buffers containing 0,1M NaCl, to elute any bound protein. The activity of each supernatant was measured using the fibrin assay by adding 50 µl aliquots to 250 µl of 0,1M glycine-HCl pH 8,8 to the wells. Controls consisting of the various buffers used in the experiment were included. Total enzyme activity controls consisted of the protease samples which had been diluted 1/10 in the various starting buffers but which had not been adsorbed to the matrix. These samples were kept at 4°C throughout the whole procedure.

The batchwise adsorption experiment using CM cellulose was performed in a similar fashion using 1 mM Tris HCl, pH 7,0 and 1 mM sodium acetate pH 4,0 for 1 hour, 2 hour and 3 hour periods and 0,1M NaCl in equilibrating buffer for elution.

As can be seen from the results presented in Table 5.7, 95 - 98% of the activity was removed by CM cellulose at pH 4,0, but recoveries of the adsorbed enzyme on subsequent elution with 0,1M NaCl were very low (4,5% - 6,6%). Somewhat better recoveries were obtained with CM cellulose at pH 7,0, and much better recoveries with DEAE cellulose at all pH's tested, but initial absorption to the resin was inferior, leaving 20% - 50% in the supernatant.

In an attempt to improve the yield from CM cellulose,

Table 5.7 Adsorption of the cercarial protease to anion- and cation exchangers.

a) Adsorption to DEAE- cellulose

pH	% nonadsorbed*	% adsorbed	% recovered
7,5	21,2	79,0	88,5
8,0	44,0	86,0	69,2
8,5	35,0	65,0	81,5
9,0	50,4	50,0	78,9

b) Adsorption to CM cellulose

pH	Time (hr)	% nonadsorbed	% adsorbed	%recovered
4,0	1	5,2	94,8	6,6
	2	2,4	97,6	4,8
	3	1,9	98,1	4,5
7,0	1	34,6	65,4	43,5
	2	31,7	69,3	39,8
	3	26,3	73,7	34,5

*The activity of each supernatant was measured using the fibrin assay. The results are expressed as the percentage activity exhibited by the supernatants as compared to the activity of a cercarial secretion sample that had not been in contact with the matrices.

the following experiment was performed. Two millilitres settled volume of CM cellulose equilibrated with 1 mM Na acetate pH 5,4, was packed into a 2 ml plastic syringe that had been plugged with glass wool and coated with 1% bovine serum albumin solution to block non-specific binding sites on the plastic.

One tenth volume of 0,01M Na acetate pH 5,4 was added to a volume of a cercarial secretion sample, and the pH was adjusted to 5,4. The sample was applied to the column in a 1 ml volume at a flow rate of 12 ml/hr. The column was washed with 10 ml of starting buffer and was then eluted with 5 ml steps of 0,01M NaCl and 1M NaCl in starting buffer. Fractions (0,5 ml) were collected at 4°C. Each fraction was adjusted to pH 8,0 by the addition of a predetermined volume of 0,1M Tris-HCl buffer pH 8,5 and enzyme activity in each fraction was assayed using the ^{125}I-fibrin assay procedure. Protein concentrations were too low to be measured accurately.

The elution profile from the column is shown in Fig. 5.14. All of the protease applied to the column was adsorbed and a major peak was eluted with 1M NaCl. A smaller peak was eluted with 0,1M NaCl. The recovery of activity from the column was only 16%, but this may have been an underestimate since activity in the fractions was measured in the presence of 1M NaCl, which is inhibitory for the protease.

The results of these and similar preliminary experiments led to the adoption of the following final system for CM cellulose chromatography in which cercarial proteases were adsorbed to the column in a buffer concentration of 10 mM

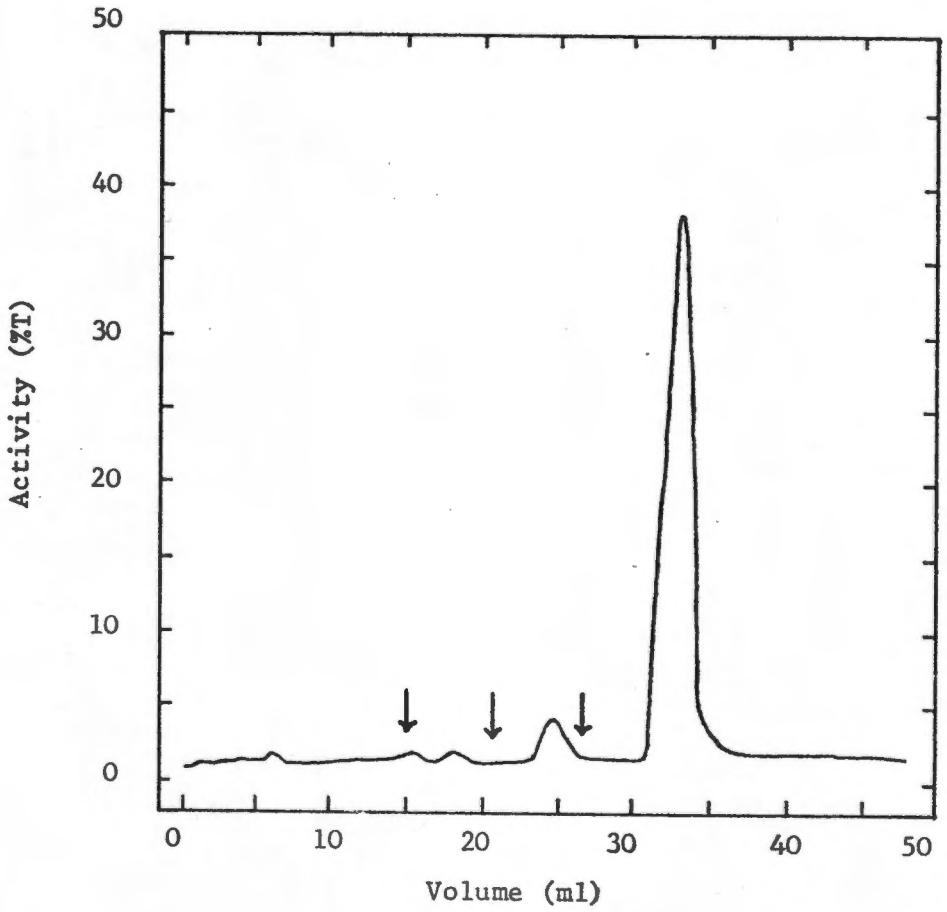


Figure 5.14 Partial purification of the protease in cercarial secretion by CM cellulose chromatography.

A cercarial secretion sample (1 ml) was chromatographed on a CM-cellulose column (2 ml) equilibrated in 0,01M Na acetate pH 5,4. The column was eluted with 5 ml steps (arrows) of 0,01M NaCl, 0,1M NaCl and 1M NaCl in starting buffer. Each fraction was assayed for fibrinolytic activity, and the results are expressed as the percentage total radioactivity released after 90 min incubation.

instead of 1 mM sodium acetate. Under these conditions it was less tightly bound and could be eluted with a NaCl gradient at a salt concentration less than 1 M.

Whatman's CM52 was regenerated by washing in acid and alkali and equilibrated with 0,01 M Na acetate pH 5,6 containing 0,01% NaN_3. The matrix was packed into a perspex column (140 mm x 15 mm) fitted with a porous polyethylene retaining disc.

The column and the tubing were coated with a 1% aqueous solution of bovine serum albumin followed by extensive washing with water before use. Packing was done under a flow rate of 22 ml/hr until a bed volume of 25 ml was reached. The column was washed with starting buffer, until the pH and conductivity of the effluent were the same as those of the equilibrating buffer. All subsequent operations were done at $4^{o}C$.

A large pool of cercarial secretions was dialysed against water and lyophilized. For the column run, 8,0 mg of the lyophilized material was weighed and dissolved in 2 ml of 0,01M Na acetate pH 5,6. Insoluble material was removed by centrifugation and the supernatant, containing 4,82 mg protein in 1,7 ml, was applied to the column. The column was run at a flow rate of 22 ml/hr and 2 ml fractions were collected. The column effluent was monitored for 256 nm absorbing material with an LKB Uvicord II.

After the sample had been applied, the column was washed with starting buffer until the tracing of UV-absorbed material had reached the baseline.

The column was then eluted with 150 ml (total volume) of a linear gradient of increasing NaCl concentration (0 - 1,0M) in the starting buffer. After the total volume of the gradient had run through the column, 1M NaCl in starting buffer was applied. Protein, enzyme activity and conductance elution profiles are shown in Fig. 5.15.

The fractions containing the highest activity or protein content were combined to give three pools comprising fractions 9 to 17, fractions 32 to 74, and the fractions 102 to 109. The activity in each pool was measured in the fibrin assay and compared with that in the original sample. The recovery of activity was greater than 100% as shown in Table 5.8. The protein content of the pools was too low for accurate direct measurement. The pools were therefore concentrated by dialysis against water, lyophilization and re-dissolved in a small volume of buffer. The results of protein determination in these samples are given in Table 5.8. Electrophoretic analysis of these concentrates is shown in Fig. 5.16 and 5.17.

As can be seen from Fig. 5.15, two protein peaks were observed, the first consisting of material that was not adsorbed to the CM cellulose, and the second which eluted with 1M NaCl. Most of the 256 nm absorbing material eluted in the first peak. A large peak of enzyme activity coincided with the first protein peak but most of the activity was eluted with the gradient in a very broad peak. No protein could be detected in this peak by absorbance measurement, and a very small amount could be recovered after lyophilization. The analysis of two of the fractions from this peak on the gelatin

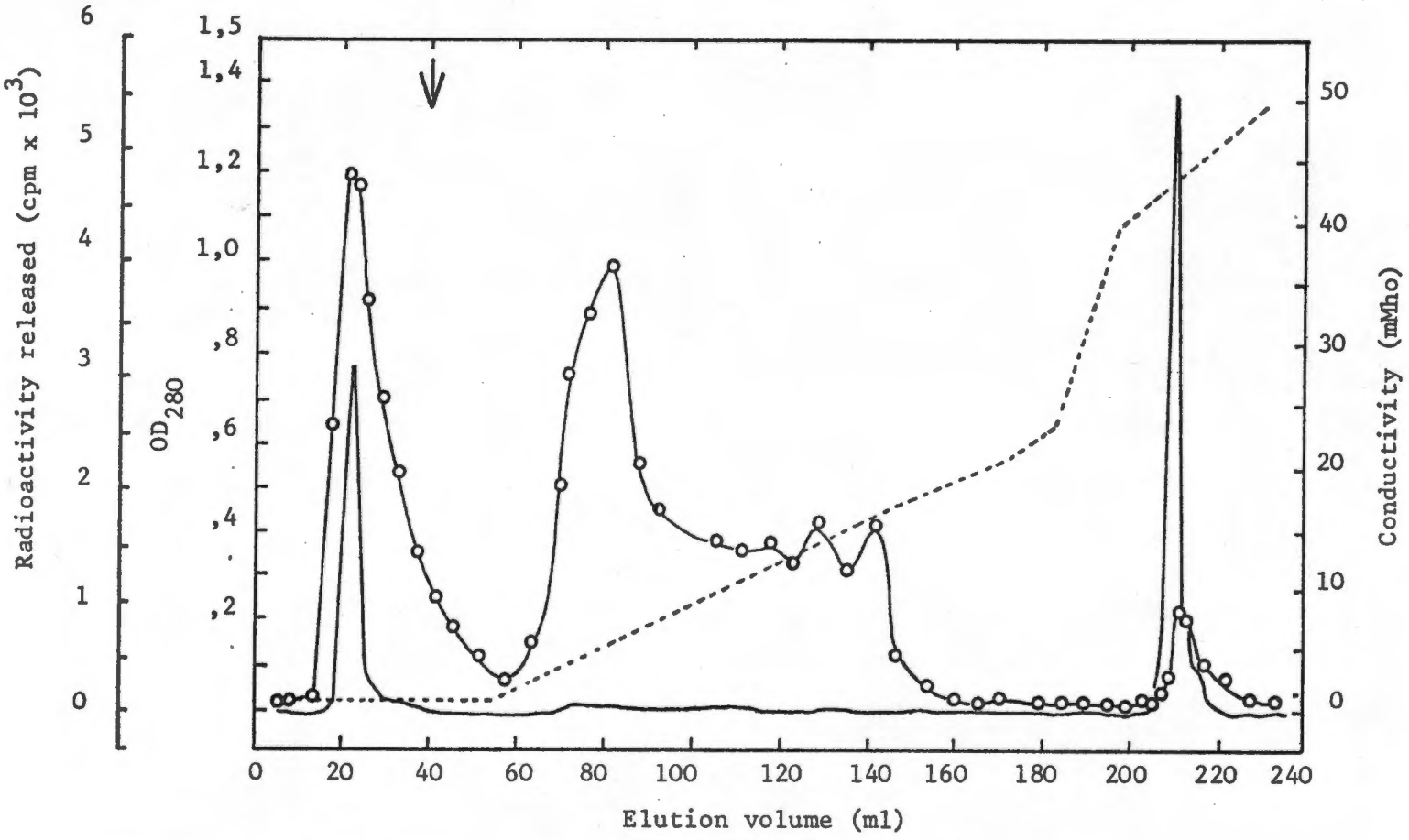


Figure 5.15 CM-cellulose chromatography on cercarial secretion

A cercarial secretion sample was chromatographed on a CM-cellulose column equilibrated in 0,01M Na acetate pH 5,6. The adsorbed protein was eluted with a linear gradient of increasing NaCl concentration (0 - 1,0M). The arrow marks the start of the gradient.

Table 5.8 Partial purification of the protease present in cercarial secretion by cation exchange chromatography.

Fractions	Volume (ml)	Protein (mg)	Total[1] Activity (%T/hr)	Specific[2] activity
Lyophilized cercarial secretion	1,7	4,821	5618	1165
Pool 9-17	19	1,200	1638	1365
Pool 32-74	88	(0,040)[3]	6767	-
Pool 102-109	17	1,230	39	32

1) The activity is expressed as the percentage radioactivity released per hour in the fibrin assay.

2) The specific activity is expressed as the total activity present in each fraction divided by the total mg protein

3) The values are given in brackets, since the protein concentration in this fraction was still very low for accurate measurement.

gel, showed that the major 35 000 dalton protease was eluted at this stage, whereas the first peak contained an additional protease of higher molecular weight. The third peak contained very little activity (Fig. 5.16).

From Table 5.8 it can be seen that more than 100% of the initial activity was recovered, which could be an indication that an inhibitor had been separated from the cercarial protease. Recovery of protein however, was only 50%. This may be attributed to losses of protein by adsorption to walls of the dialysis bags and the lyophilising vessels, and therefore does not reflect the true recovery from the column. Very good purification was achieved as judged by the specific activities of the initial cercarial secretion and the second pool and the relatively few bands observed when this pool was electrophoresed on SDS-polyacrylamide gels (Fig. 5.17).

From these results I conclude that cercarial secretions contain one major 35 000 dalton protease and, possibly, one minor species of higher molecular weight. Campbell et al (33) who fractionated cercarial secretions by chromatography on Sephadex G75, also obtained only one major protease with an apparent molecular weight of 27 000 daltons and a minor enzyme of lower molecular weight.

The number of proteases obtained by fractionation of cercarial secretions stands in contrast to the number obtained when whole cercarial extracts were used as starting material. Dresden and Asch (24) obtained 5 peaks of proteolytic activity after Sephadex G150 chromatography. They used casein, azocoll and gelatin as substrates, and found that

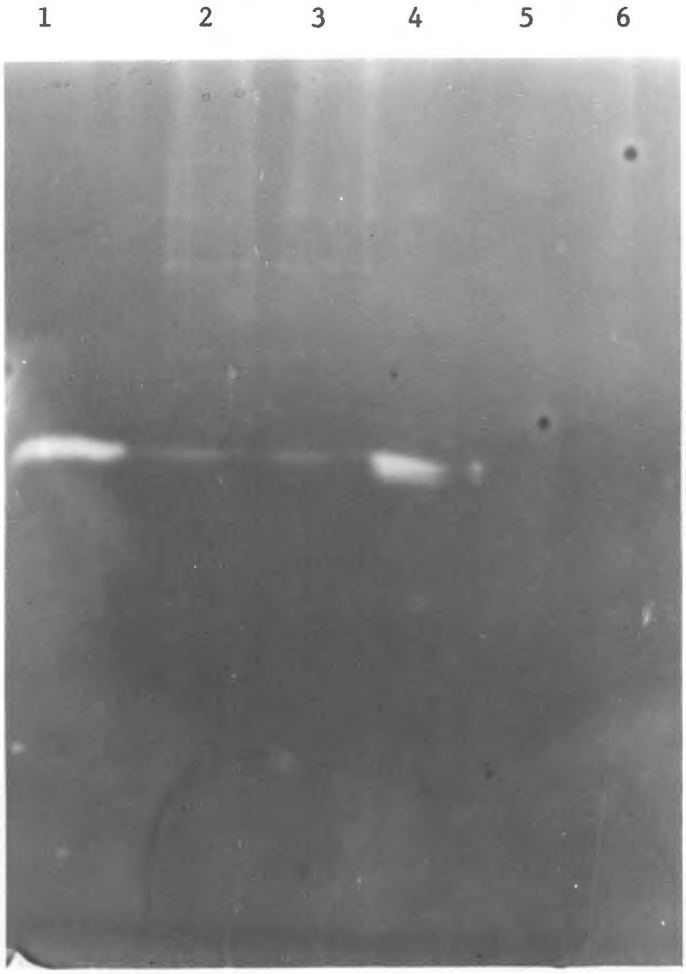


Figure 5.16 Electrophoretic analysis of fractions obtained by CM-cellulose chromatography of cercarial secretions on gelatin-polyacrylamide gels.

Aliquots of 5 µl of the concentrated, pooled fractions obtained by CM-cellulose chromatography were electrophoresed in a 6-15% SDS-gelatin gel. The sequence of loading was (1) cercarial secretion, (2) and (3) pool 9-17; (4) pool 32-74; (5) and (6) pool 102-109.

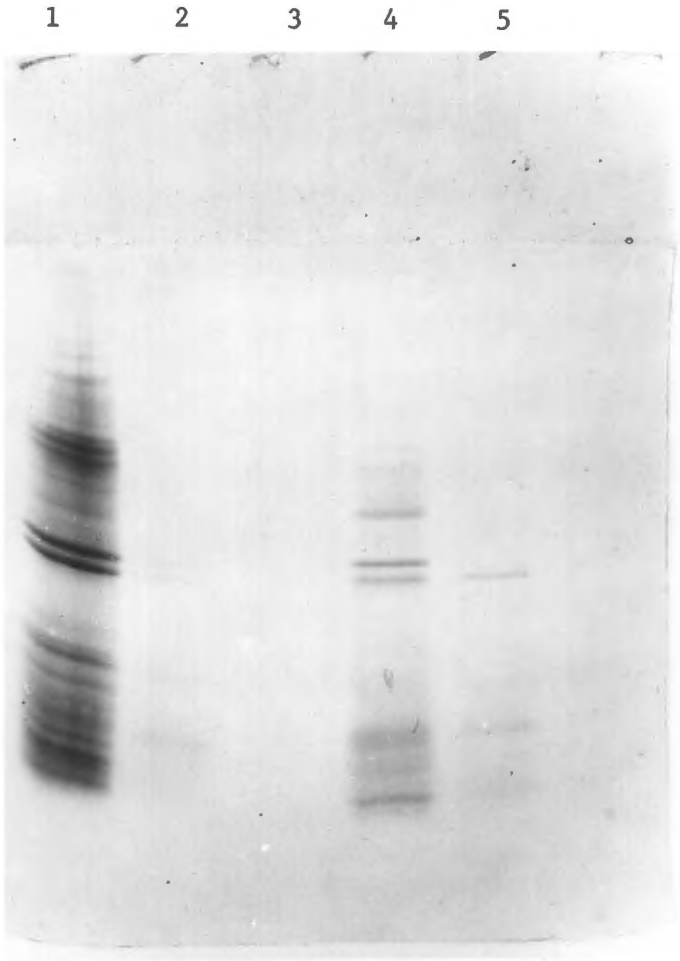


Figure 5.17 Electrophoretic analysis of fractions obtained by CM cellulose chromatography of cercarial secretions on SDS polyacrylamide gel.

Aliquots of 25 µl of the concentrated pooled fractions were electrophoresed in a 5-16% gradient SDS polyacrylamide gel. The sequence of loading was (1) cercarial secretion, (2) pool 9-17, (3) pool 32-74, (4) pool 102-109, (5) pool 32-74 after precipitation of 1 ml of concentrate by 10% TCA. The total precipitate taken up in 25 µl of 1% SDS and glycerol was loaded.

the different peaks hydrolysed the substrates at different rates. Therefore they concluded that a number of different proteolytic species are present in cercariae. Campbell et al (33) suggested that part of these enzymes could represent non-secretory proteases, since extracts of cercariae had been used as starting material. Also, only one Azocollytic species was found in cercarial secretions, and not three as reported by Dresden and Asch (24).

Three peaks of activity against casein, elastin and ATEE were obtained by Gazzinelli et al (26) who fractionated whole cercarial extracts on DEAE-Sephadex at pH 7,0. These workers concluded that there were at least two distinct proteolytic species present in cercariae, since the ratios of proteolytic to esterolytic activities of two peaks were different.

As I have indicated, however, these conclusions should be reviewed in the light of possible bacterial contamination of their samples.

APPENDIX

DETERMINATION OF PROTEIN CONCENTRATIONS

Three methods were used to determine the protein concentrations of solutions.

Determination by UV Absorbtion

This method was adapted from Layne (76) and was used to give approximate estimates of protein concentrations. The absorbance of a protein solution was measured at wavelengths of 280 nm and 260 nm and the protein concentration calculated according to the formula:

$$\text{Protein concentration in mg/ml} = 1{,}55\ OD_{280} - 0{,}76\ OD_{260}$$

Biuret reaction

The method was based on that of Weichselbaum (77) as modified by Dittelbrandt (78). The biuret reagents were made by dissolving 9,0 g of sodium potassium tartrate ($NaKC_4H_4O_6.4H_2O$) in 400 ml 0,2N NaOH. 3,0g of cupric sulphate ($CuSO_4.5H_2O$) and 5,0g potassium iodide (KI) were then added in succession and dissolved. The volume was made up to 1,0 litre with 0,2N NaOH.

Solutions of gelatin (Merck, Darmstadt) in 0,015 potassium phosphate buffer pH 7,7 were used to construct a standard curve that was linear over the range 0,1 to 5,0 mg/ml protein.

The Lowry Method

This procedure for the determination of protein concentrations was developed by Lowry, Rosebrough, Farr and Randall (35). In this method the intensity of colour development depends not only on the content of peptide bonds as in the biuret reaction, but also on the content of the aromatic amino acid residues present in proteins.

Solutions of bovine serum albumin in water were used to construct the standard curve.

As this method does not give a linear relationship between concentration and absorbance, the standard curve of OD_{750} vs µg BSA was constructed by fitting a second degree polynomial equation to the observed points. Concentrations of the unknowns could then be computed by interpolation using the parameters of the quadratic equation. A typical standard curve is shown in Fig. A.1.

Very dilute samples were concentrated by acid precipitation as follows. An equal volume of 20% (w/v) ice cold trichloracetic acid (TCA) was added to the sample and kept on ice for one hour. The tubes were centrifuged at 2500 rpm for 15 min and the pellet was washed with cold 10% TCA. After centrifugation, the supernatant acid was removed and the pellet dissolved in 0,1 ml 1M NaOH. This solution was then used for the protein determination with appropriate adjustment of the reagents and the standard solvents to allow for the NaOH in the sample.

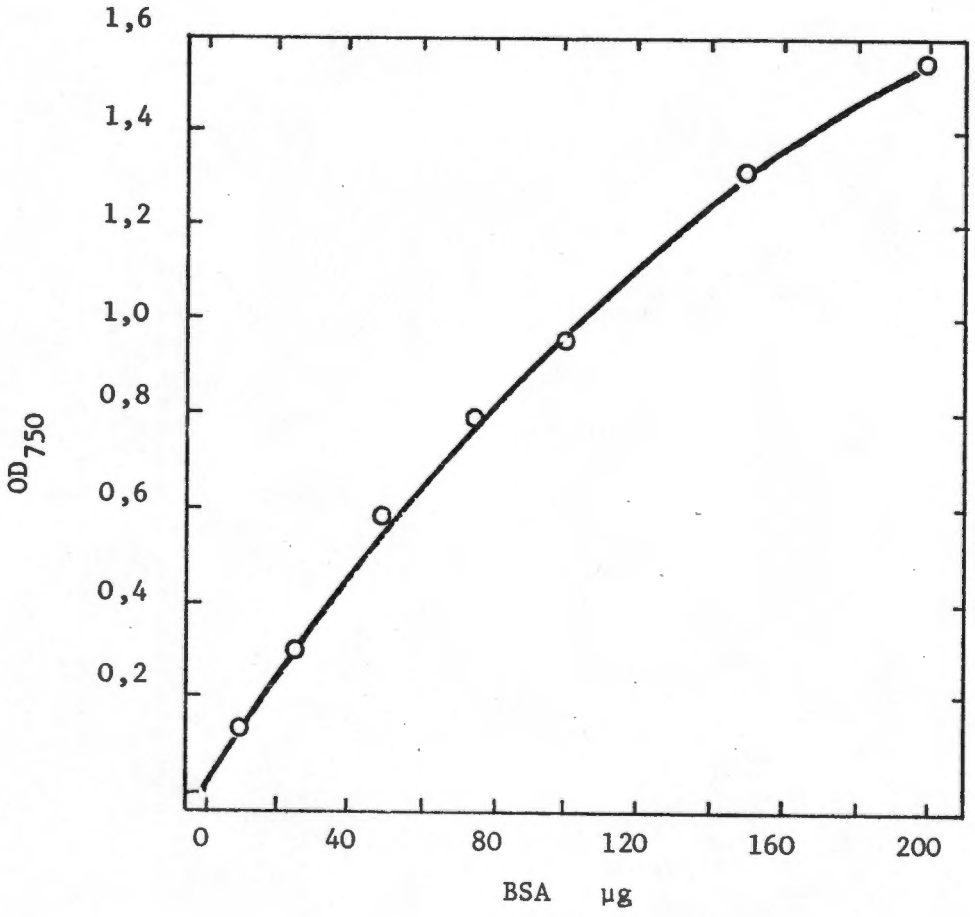


Figure A.1 Standard curve for protein determination by the method of Lowry.

The standard curve of OD_{750} vs µg BSA was constructed by fitting a second degree polynomial equation to the observed points.

SDS-polyacrylamide gel electrophoresis

Both the fibrin overlay system and the gelatin gel system were based on the method described below.

Stock solutions

Acrylamide-bis acrylamide stock (A-bis-A 30:0,8) (A-bis-A 30:1,0)

Both acrylamide and N,N'-methylene bis acrylamide were purchased from Eastman Kodak Company.

30g of acrylamide and 0,8g (or 1,0g for gelatin gels) bis acrylamide were dissolved in water to 100 ml. The solution was decolourized with activated charcoal and then filtered through Whatman's 542 paper. The solution was stored at 4°C.

Catalyst "TEMED"

N,N,N'N' tetramethylethylenediamine obtained from BDH Chemicals Ltd. was used as the undiluted liquid and stored at 4°C.

Initiator

1g Ammonium persulphate was added to 9 ml of water to give a 10% solution. The solution was kept at 4°C and used within a week.

10% SDS

10g sodium lauryl sulphate (BDH) was dissolved in water to 100ml and kept at 4°C. (For gelatin gels it was sterilized at 10 lb for 10 minutes).

Running gel buffer (RGB)

(1,5M Tris/HCl pH 8,8, 0,4% SDS)

18,17g Tris (hydroxymethyl)-aminomethane was dissolved in approximately 80 ml of water and the pH was brought to 8,8

with concentrated hydrochloric acid.

4,0 ml of 10% SDS was added and the solution made up to 100 ml with water. It was stored at 4°C (after sterilization at 10 lb for 10 min for gelatin gels).

Stacking gel buffer (SGB)

(0,5M Tris/HCl pH 6,8, 0,4% SDS).

6,057g Tris base was dissolved in approximately 80 ml of water. The pH was adjusted to 6,8 with concentrated HCl and 4,0 ml of 10% SDS was added. The volume was brought to 100 ml and the solution was kept at 4°C after sterilization for gelatin gels.

Reservoir buffer (RB)

(0,025M Tris, 0,192M glycine pH 8,5, 0,1% SDS)

3,03g Tris base, 14,41g glycine and 10 ml 10% SDS were made up to 1 litre with water. The solution was kept at 4°C (after sterilization for gelatin gels).

Sample buffer

The following reagents were added together shortly before use: 2,5 ml SGB, 1,0 ml glycerol, 1,5 ml water, 0,025ml 0,4% phenol red in water.

Stain A

Coomassie Brilliant Blue R250 (BDH)

1% in ethanol. This was diluted to 0,1% in Destain shortly before use.

Stain B

Amido Black 10B (Merck). 1% in 30% methanol. This was diluted to 0,1% in Destain shortly before use.

Destain

30 ml methanol and 10 ml glacial acetic acid were made up to 100 ml with water.

1% agar sealing mixture

1g agar (Difco) was dissolved in 100 ml water by boiling in a waterbath.

Procedure for slab gels

Usually 6 - 15% gradient gels with or without gelatin were employed. The different solutions for the stacking and resolving gels were made up according to Table A2.1. The initiator and catalyst were added to the solutions immediately before these were added to the electrophoresis chamber. All volumes are given in millilitre.

Two glass plates (10,0 x 8,0 cm; 8,5 x 8,0 cm) were cleaned thoroughly with detergent and ethanol. Two perspex spacers (1mm thick) were clamped between the plates along the side edges. The bottom edges of the plates were flush with each other and were sealed with a strip of parafilm. The "chamber" was placed in a vertical position. A "wick" of polyacrylamide at the bottom of the chamber was cast, by filling the chamber with the wick solution such that the bottom of the two spacers were just covered. When the wick had set, the parafilm sealer was removed and the chamber was clamped to the electrophoresis tank in a vertical position. The edges

Table A2.1 Solutionf for 6-15% gradient resolving gels*

	NORMAL				GELATIN GEL			
			Resolving gel				Resolving gel	
Solution	Wick 15%	Stacking gel 3%	6%	15%	Wick 15%	Stacking gel 3%	6%	15%
A-bis A(30 : 0,8	1,00	0,5	0,80	2,00				
A-bis A(30:1,0)					1,00	0,50	0,80	
Running gel buffer	0,50		1,00	1,00	0,50		1,00	
Stacking gel buffer		1,25				1,25		
Distilled water	0,50	3,25	2,20	0,50	0,50	3,25	1,80	0,10
Glycerol				0,50				0,50
1% gelatin							0,40	0,40
10% ammonium persulphate	0,020	0,025	0,01	0,01	0,020	0,025	0,01	0,01
TEMED	0,01	0,010	0,005	0,005	0,01	0,01	0,005	0,005
Total volume	2,03	5,035	4,015	4,015	2,03	5,035	4,015	4,015

*Volumes are given in ml.

near the spacers were sealed with molten agar. Then the gradient resolving gel was cast by using a pump. A volume of the 5% solution was delivered to the 16% solution via a tubing connected to the pump. At the same time, two volumes were withdrawn from the progressively diluted 16% solution through a tubing leading to the chamber via the same pump. When the 5 and 16% reservoirs were empty, the pump was stopped and the tubing removed from the chamber. The resolving gel was carefully overlaid with water using a pasteur pipette. This ensured the formation of a sharp flat surface at the top of the gel, and also blocked out atmospheric oxygen which could interfere with even polymerization. The gel usually had set after 30 minutes. The water was removed from the top of the gel and the stacking gel was cast with a six pronged comb in position. The assembly could be stored at 4°C overnight, covered with a moist tissue without any adverse effects on the subsequent electrophoresis run.

Before sample application, the upper reservoir was sealed with agar where the glass plates rested. Reservoir buffer was filled into the upper and lower reservoirs and the comb was carefully lifted from the stacking gel. The heavy samples were underlayered into the wells using a 5 µl microdispenser. The anode leading from a powerpack was connected to the lower reservoir and the cathode to the upper reservoir. Electrophoresis was carried out at 4°C with a constant current set at 8mA. When the voltage increased to more than 100V during the run, the current was reduced. Overheating of the gels and resultant abnormal curvature of protein bands was thus

avoided. When the phenol red marker band, which travels near the ion front, had reached the wick, the electrophoresis was stopped and the slab gel removed from the assembly for further processing.

Molecular weight determination using polyacrylamide gels.

These were determined by the method of Maizel (46). A series of marker proteins whose molecular weights are known were run on the same gel as the protein whose molecular weight was to be determined. The marker proteins were usually dissolved in PBS containing sodium azide to a concentration of 2 mg/ml. Before electrophoresis they were diluted 1:1 in sample buffer. 10% SDS was added to a final concentration of 1% and the mixtures were placed in a boiling water bath for 1 minute. The volumes loaded on the gel ranged from 5 - 10 µl and contained 5 - 10 µg protein. After staining of the gel, the relative mobilities (rf values) for each protein was determined by dividing the distance they had travelled into the gel by the distance the tracking dye had moved. These rf values of the marker proteins were then plotted against the logarithm of their respective molecular weights. A straight line with a negative slope should be obtained. The molecular weight of the unknown protein whose rf value is known can be read off the graph or calculated from the slope and the intercept on the y axis. The results obtained from a typical experiment are illustrated in the table and graph below.

Table A2.2 Molecular weight determination using SDS polyacrylamide gels.

Marker protein	Molecular weight	rf
Lactoperoxidase	78 000	0,282
bovine serum albumin	67 000	0,342
ovalbumin	43 000	0,434
myoglobin	17 200	0,748
unknown		0,523
		0,257

From Figure A2 a slope of -3,267 and an intercept of 2,964 was obtained. The molecular weights of the unknowns were estimated to be 35 100 and 83 700.

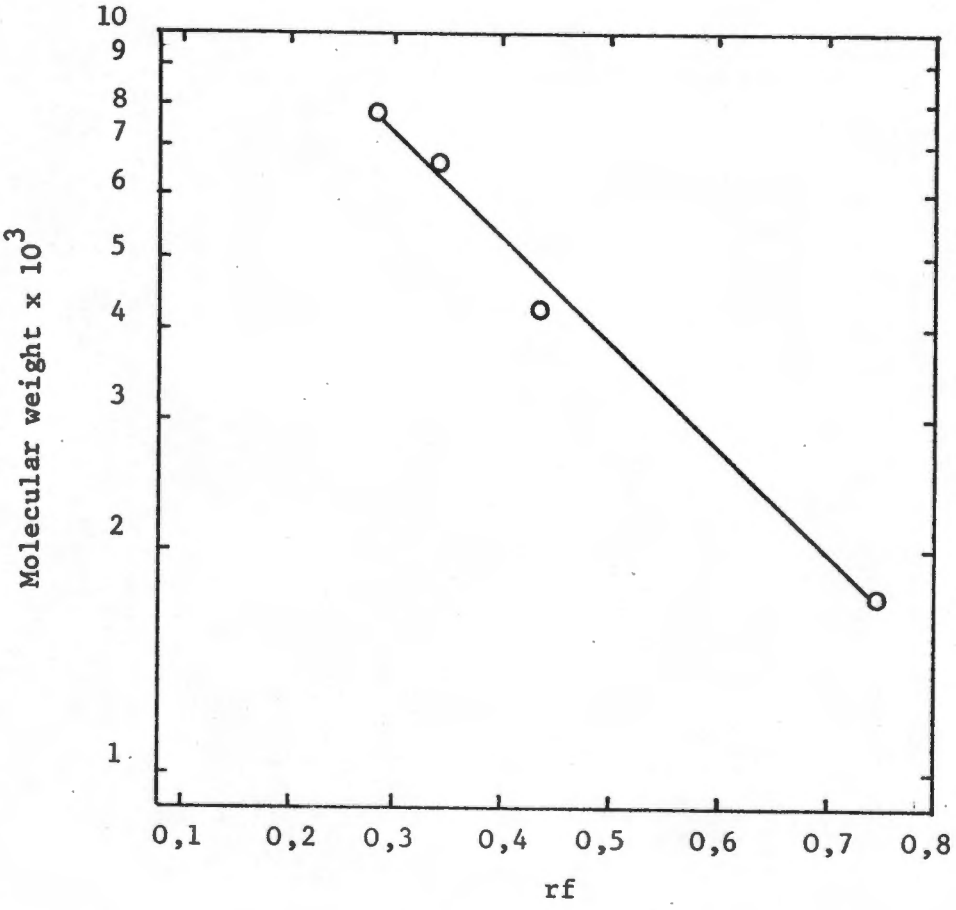


Figure A.2 Molecular weight determination using polyacrylamide gels.

Plot of rf values for various marker proteins against the logarithm of their respective molecular weights yields a straight line with a negative slope. The molecular weight of the unknown protein whose rf value is known can be read off the graph.

Preparation of ion exchange adsorbents

Both the anion exchanger, diethyl aminoethyl (DEAE) - cellulose, and the cation exchanger, carboxymethyl (CM)-cellulose used for the partial purification of the cercarial proteases were supplied by Whatman Ltd., Springfield Mill, Maidstone, Kent in a preswollen microgranular form as Whatman DE 52 and CM 52 respectively.

The matrix was stirred into a beaker containing the starting buffer and was left standing for 30 minutes. The dilute slurry was poured into a Büchner funnel with Whatman's 541 filter paper at the bottom, and the fluid was removed by suction. The moist cake was transferred to fresh buffer and the whole procedure was repeated 2-3 times. During the last standing period, the pH of the dilute slurry was adjusted to the pH of the starting buffer by adding either the basic or the acidic component of the buffer. Equilibration was continued for another 30 minutes and the matrix filtered again. The moist cake was then transferred to a measuring cylinder containing fresh starting buffer, and was allowed to settle. Any fines were removed together with the supernatant fluid, and the procedure repeated once. Usually a slurry of 25% matrix in buffer was prepared for packing the columns. Since I used only small columns, up to a total matrix volume of 14 ml, the slurry was simply added into the column with a pipette, while the outlet was opened such that the flow rate was approximately 30 ml/hr.

The column was further washed with starting buffer

until the effluent had the same pH and conductivity as the starting buffer.

In order to regenerate the ion exchangers after use, they were put through a cycle of washes as follows: 0,5M NaOH - 0,5M NaCl; water; 0,5M HCl; water; 0,5M NaOH - 0,5M NaCl; water; in the case of CM cellulose. DEAE cellulose was first washed in the acid, then alkali and acid again. After the last wash they were ready for re-equilibration in starting buffer.

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