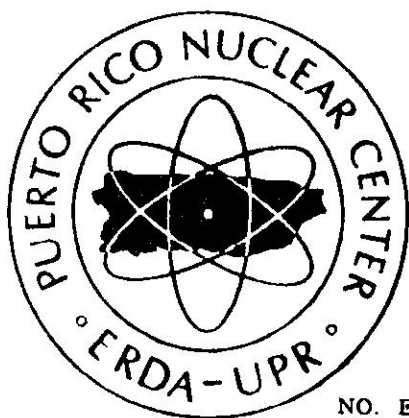# PUERTO RICO NUCLEAR CENTER

THE EFFECTS OF GAMMA IRRADIATION
ON DIFFERENT STAGES OF
FASCIOLA HEPATICA

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FASCIOLA HEPATICA

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TO MY WIFE, MY MOTHER AND FATHER

PREFACE

The sound practice of medicine requires a clear understanding of disease and its causative agents among which parasites are very prominent. Host-parasite relationships must be studied from all accessible approaches in order to formulate control and treatment measures.

This thesis work has been conducted as part of a broader effort of the _Fasciola hepatica_ Program of Puerto Rico to study, understand and attempt to control the disease in Puerto Rico. The application of nuclear techniques to various morphological, biochemical and physiological aspects of the problem are emphasized. The research work was carried out at the Human Ecology Division of the Puerto Rico Nuclear Center, Rio Piedras, Puerto Rico. The Puerto Rico Nuclear Center is operated by the University of Puerto Rico under Contract E-(40-1)-1833 with the Energy Research and Development Administration.

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Introduction

Fascioliasis or hepatic distomatosis is one of the most widespread liver diseases throughout the world. It is caused by a parasitic worm of the class TREMATODA of the phylum PLATYHELMINTHES. This flatworm is commonly known as "liver-fluke" in the United States and Europe; "babosa del higado" in Cuba; "cucaracha del higado" in Puerto Rico; "saguaype" in Argentina and Chile; "pirihuin" in Chile; "yuta" in North Chile and it is taxonomically classified as *fasciola* *hepatica*. The adult trematode is a common parasite of warm blooded animals especially cattle, sheep, and goats. It has also been found in many other animals including marsupials, rodents, pigs, horses, carnivores, primates, and in the rabbit, its natural host.

Life *Cycle.* Eggs are deposited by the adult fluke of *Fasciola* *hepatica* in the bile ducts and are expelled in the feces of the infected animal. These eggs develop and hatch in humid places into motile microscopic larvae which constitute the intermediate infective stage of the parasite. These larvae, called miracidia, migrate through the water until they find a specific snail that will serve as an intermediate host, where they develop and metamorphose into a sporocyst, rediae, at times daughter rediae, and cercariae. The intermediate hosts consist of many species of amphibious snails, of which *Lymnaea* *cubensis* and *Lymnaea* *columella* are the two known

intermediate hosts in Puerto Rico (Hoffman, 1930; Van Volkenberg, 1934, 1939; Van der Schalie, 1948). The cercariae emerge from the snails and encyst on grasses, watercress, bark, or soil as metacercariae. The metacercaria is the effective stage for the warm blooded animal or definitive host. These encysted larvae passively find their way into the definitive host via the oral route with the ingested vegetation. Once the metacercariae have been ingested, excystation begins within two hours in the small intestine (Dawes, 1961). Active excystation of viable F. hepatica metacercariae has been induced in vitro (Wright, 1927; Susuki, 1931; Hughes, 1959; Wikerhauser, 1960; Dixon, 1966). Two processes of excystation have been described: (i) a passive excystation, related to the digestion of the cyst wall by the host enzymes and the release of the young flukes, and (ii) an active process in which the metacercaria responds to stimuli of appropriate factors in the gut of the host and actively breaks out of the cyst wall (Dixon, 1966). The mechanism of emergence is not well understood. From observations of the sectioned cyst of F. hepatica, Dawes (1961, 1963b) concluded that the young worms emerge from the cyst through a hole made by the oral sucker of the parasite.

Soon after excystation, the metacercariae begin to penetrate the small intestine wall. Young flukes have been found in the abdominal cavity by various authors (Dawes, 1962a; Kendall and Parfitt, 1962). Juvenile flukes have been found in the abdominal cavity of mice less than 24 hours after infection (Dawes, 1962a). After this,

most of the young worms penetrate and start to migrate through the liver. The time required to reach the liver and move through its parenchyma varies in different host species. Studies on this matter reveal that juvenile flukes begin to penetrate the liver of mice 48 hours after infection (Urquhart, 1956; Dawes, 1961b) while 90 hours are required in sheep (Kendall and Parfitt, 1962). The phenomenom by which the young flukes find the liver has been attributed to a chemotaxis (Sinclair, 1967). Finally the parasites lodge in the biliary system where oviposition is initiated, thus starting a new cycle. The precise time during which the young fluke migrates through the liver substance is unknown. Parasites have been found in the bile ducts of experimental albino rats, 28 days after infection (Thorpe, 1963).

Epidemiology. F. hepatica is endemic across the whole continents of America, Europe, Australia, and the Hawaiian Islands. It was first officially reported in Puerto Rico by Van Volkenberg (1939), but its occurrence was observed as early as 1911 (Ashford and Igaravidez) and 1928 (Van Volkenberg). In Puerto Rico the epidemiological situation varies in the different regions where fascioliasis is most prevalent. Rivera-Anaya and Martinez de Jesus (1952) were the first to tabulate percentages of infected cows in Puerto Rico, based on a slaughterhouse survey. They found as high as 35 percent of infected livers in the west-central zone of the Island during 1948-49. The extent of liver fluke infection of dairy cows has been

studied in two subtropical ecological zones of Puerto Rico (Dorado and Jayuya) by Chiriboga et al. (1970, 1972) who reported an average total infection rate as high as 86 percent. In a more recent survey Chiriboga et al. (1975a) found that between 20 to 40 percent of bovine livers were condemned in the slaughterhouses of the Island during the years 1973 and 1974.

The intermediate snail hosts, L. cubensis and L. columella have also been studied in relation to their rate of infestation (De Leon et al., 1972). A rate of infestation as high as 27 percent was reported in the Dorado area where L. cubensis predominated among the positive specimens.

According to Brown (1969) human infections have been reported in Cuba, Southern France, Great Britain, and Algeria. It has also been reported in the Hawaiian Islands (Alicata, 1953; Stemmermann, 1953); Rhodesia (Perry, 1972); and Belgium (Vanbreuseghem et al., 1962). Bendezu, (1969, 1973) reported an incidence of infection as high as 60 percent in children in a Peruvian village. Rodriguez-Molina (1938) and Chiriboga (1975b) have reported isolated cases of human infection in Puerto Rico. Man can be infected by ingesting vegetation, mainly watercress contaminated with metacercariae or simply by drinking water containing floating metacercaria. In abnormal hosts, such as man, the parasites are usually found in extra-hepatic locations such as the lungs, and under the skin. (Neghme and Ossandon, 1943).

Economic Impact. The economical loss caused by fascioliasis throughout the world is high (Caballero, 1963; Bendezu, 1970: Honer, 1970: Alvarez, 1971). Losses have been reported through condemnation of livers in Puerto Rico amounting to at least $500,000.00 annually, not including losses from milk production, low quality and quantity of meat yields, (Chiriboga, et al., 1975).

Pathology and Symptomatology. This helminth is of vital importance in respect to human health, as the hepatic distomatosis caused by it constitutes a parasitic zoonosis, and is transmissible from animal to man. The pathology and symptomatology in fascioliasis will depend primarily upon the number of metacercariae which are ingested and their infectivity. It has been shown that the infectivity of the metacercariae depends on the environmental temperature during the development of the larvae in the snail, besides the climatic conditions to which they are exposed after encystment (Davtyan, 1956; Boray, 1963).

The disease is characterized by various lesions in the hepatic tissue which can be present in two consecutive stages; the first one is related to the erratic migrations of the young adults through the liver parenchyma and the second phase related to their dwelling in the biliary system. The main pathology of this disease is an acute hepatitis which degenerates into a permanent fibrosis. This occurs during the acute or first stage. The localization of the parasites in the biliary tract provokes inflammatory, adenomatous, and fibrotic

changes of this system (Brown, 1969) as a result of the pressure, toxic metabolic products, and feeding habits of the worms. This results in a parenchymal atrophy and periportal cirrhosis (Brown, 1969). Cirrhosis due to hepatic fascioliasis has been discussed as a result of infarcts produced by the flukes, chronic cholangitis, hyperplasia, and granulomatous lesions (Urquhart, 1956). Anaemia and hypoproteinaemia have been reported as common features of fascioliasis (Maclean et al., 1968).

The histopathology of the liver of laboratory experimental animals has been studied by several workers (Dawes, 1961a; Thorpe, 1965a, 1965b; Ross et al., 1966). Traumatic hepatitis and minute hemorrhagic spots were reported by Sinclair as the first evidence that the young flukes have reached the liver. Sinclair (1967) has also described the lesion as "discrete tracts with flukes surrounded by a layer of compressed parenchymal cells some of which were necrotic". Dawes (1961a) found masses of neutrofils in the wall and lumen of the debris-filled spaces left by the migratory route of the flukes. Fibrinous peritonitis has been described by Sinclair (1967) as prominent in the later stages of F. hepatica infection.

Severe headache, chills, fever, urticaria, a stabbing substernal pain, and right upper quadrant pain were reported by Brown (1969) as the first signs in human fascioliasis. This author also described the later stages of human infection as characterized by an enlarged tender liver, jaundice, digestive disturbances, diarrhea, and anemia. Other features present in human fascioliasis are malaise, pruritus,

eosinophilia, an increase in serum gamma globulin and pyrexia (Sagar, 1962; Ashton et al., 1970). Neurological syndrome by fascioliasis has been reported (Aubertin et al., 1966).

Biochemical tracers for hepatic mal-function by fascioliasis. Studies on the development and detection of F. hepatica metacercariae activity have been reported using physiological, biochemical, and histochemical methods (Connolly and Downey, 1968; Campbell and Barry, 1970). Biochemical parameters such as serum glutamic-oxaloacetic transaminase (SGOT) and serum glutamic-pyruvic transaminase (SGPT) have been studied (Thorpe, 1965a; Connolly and Downey, 1968; Campbell and Barry, 1970; Bundensen and Janssens, 1971). It has been suggested by Wrobleski and LaDue (1956), that SGPT might be a more specific index of liver cell damage than SGOT because of the former's selective concentration in hepatic tissue. Moreover, Connolly and Downey (1968), and Bundensen and Janssens (1971) reported that an increase in SGPT might be used as a means of assessing the activity of these damaging helminths.

Biochemical studies of F. hepatica. The molecular structure of various species of parasites has been studied by Von Brand (1966). Weinland and Von Brand (1926) found that 58 percent of the dry weight of F. hepatica adults is protein. Besides the structural proteins of the parasites, proteins with enzymatic activity have been studied that possibly have a role in the mechanism of liver

tissue penetration or migration by the developing parasite
(Abderhalden and Heise, 1909; Flury and Leeb, 1926; Pennoit-De
Cooman and Grembergen, 1942; Halton, 1963; Thorsell and Bjorkman,
1965; Howell, 1966; Howell, 1973). A significant and specific
proteolytic activity in the caecum of the adult fluke has been
demonstrated by Halton (1963) and Howell (1973).

In addition, it has been established by using fluorescent
antibody techniques that the adult worm possesses antigenic sites
on the caecal lining, and along the excretory ducts (Thorpe, 1965;
Movsesijan and Cuperlovic, 1970; Movsesijan, 1971). In 1972,
Mulligan et al. and Cuperlovic et al., reported independent studies
dealing with the isolation of an exoprotein with antigenic activity
and the ability of the adult parasite to incorporate ^{75}Selenium-
labeled methionine in this exoantigen.

Immunology. There appears to be some disagreement on the success
of attempts to produce a resistance to infection by the injection
of homogenized whole flukes. Kerr and Petkovich (1935), Shibanai
et al. (1956), and Ershov (1959) claimed to have demonstrated an
induced immunity, while others (Healy 1955; Hughes, 1962b, 1963;
Dawes, 1963c) were unable to confirm their findings. In some cases
(Ershov, 1959) acquired immunity was induced by the injection of an
antigen comprising a polysacharide-albumen complex antigen, but
later, Hughes (1963) failed to induce immunity when he repeated the
experiment with rabbits. Moreover, Urquhart, et al., (1954) employing

protein extract antigens could only retard the development of the challenge inoculum, but no differences were found with controls in the number of parasites nor in the liver lesions.

Several works have been reported on the viability of the infective stage of various species of animal parasites in response to changes in physical factors including ionizing radiation (Silverman, 1954; Mikacic, 1956; Meyers, 1957; Villella et al., 1961; Hsu et al., 1962; Smithers, 1962; Martinez-Silva et al., 1969; Sturrock and Upatham, 1973). Recently, interest has been centered on the induction of immunity to fascioliasis in experimental animals by inoculating them with metacercariae whose viability has been partially damaged by ionizing radiation (Thorpe and Broome, 1962; Chiriboga et al., 1971; Corba et al., 1971). In the latter experiments, rats that were inoculated three times with gamma-irradiated (2500 rads, cobalt 60) metacercariae at weekly intervals showed complete protection against a challenge infection. These experiments add importance to the generalized concept that attenuated infections caused by partially damaged flukes are far more efficient in producing immunity than their biologically inert antigens.

Control Methods. The control methods are aimed at the disruption of the parasite life cycle at any one of its various stages. They include the reduction of vector snail population by eliminating their semiaquatic habitats through proper drainage of pastures and improved farming practices, by the application of chemical molluscicides and

by biological competition or predation. At the mammalian level, adequate sanitation and chemotherapy have been employed. Although a considerable amount of effort has been exerted in all these areas, the overall progress has been slow.

Low leveled pasture lands have always been a problem because of their poor natural drainage. Here small pools are formed surrounded by marshy areas that offer an ideal environment for lymneid snails. Cattle can be restrained from entering these areas by fencing, but often times this type of land may constitute the best grazing fields in the farm. Draining by means of ditches is difficult and costly to start and to maintain. In many farms snail foci are man made by improperly designed drinking troughs. In spite of these difficulties improved farming practices should reduce snail populations and eliminate dense breeding foci.

The use of molluscicides to control the snail-mediated diseases has been and will continue to be one of the most useful tools in this respect. However, added to their inherent low efficiency in the control of aquatic snails, their efficiency is almost nil in treating amphibious snails due to the ability of the latter molluscs to escape the toxicant-treated waters. Some of the most commonly used chemicals have been copper sulphate, sodium pentachlorophenate, and copper pentachlorophenate.

Another practice to eliminate the snails makes use of adverse biological competition with other snails or predation by sciomycid flies (Berg 1964; Neff 1964; Knutson et al., 1967). Another

snail-killing agent has been studied in South America by Bendezu
(1970). This is an annelid, <u>Chaetogaster lymnaei</u>, which feeds on
<u>F. hepatica</u> eggs and miracidia. The annelid lodges and dies in the
intrashell spaces and kills the snail by fomenting an overwhelming
protozoan growth (Ruiz, 1951; Khalil 1961; Bendezu, 1970; Bendezu
et al., 1973).

Chemotherapy in sheep and cattle has been practiced in various
countries (Pearson and Boray, 1961; Koopman et al., 1964; Ueno
et al., 1964; Kruyt and Van der Steen, 1969; Arru and Deiana, 1970;
Campbell et al., 1970; Chowaniec et al., 1970b). One of the drugs
commonly used is hexachlorophene, but it has the disadvantage that
it only kills the adult parasites in the bile ducts of the infected
animal and not the young ones in their migratory path through the
liver parenchyma (Kendall and Sinclair, 1969). This is a critical
disadvantage since cattle are continuously being reinfected.
According to Swiezawska et al., (1967), Brown (1969), and Ashton
et al., (1970) human infection has been treated successfully with
dichlorophenol (Bithionol). Dubarry et al., (1968), state that
emetine camphosulphonate intravenously is very effective in human
fascioliasis.

Lately, emphasis has been placed on the production of atten-
uated vaccines by exposing the infective metacercariae to ionizing
radiation. In this regard various authors have reported the
reduction of pathogenicity after a vaccination course (Wikerhauser,

1961; Hughes, 1962, 1963; Movsesijan and Cuperlovic, 1970). This subject is more amply discussed under "Immunology".

Statement of the Proposed Research

This work is a portion of a broader experimental approach to the production of a suitable vaccine against F. hepatica. Several workers have attempted to induce immunity in different mammalian hosts but no conclusive protection has been achieved. The attempts have included the injection of the secretions and excretions of living flukes, dead parasite material, and the oral inoculation of attenuated metacercariae. Recently, Chiriboga et al. (1971), demonstrated in rats an almost complete protection against a normal F. hepatica challenge infection by inoculating these animals with three doses of metacercariae irradiated with 2.5 kilorads gamma-radiation. Pertinently, this prompted some inquiries, such as what is the optimum time for storage of immunogenic doses of F. hepatica metacercariae; what is the host-liver damage caused by them; and finally, what is the effect of gamma irradiation on the antigens excreted by the adult worm The elucidation of these questions is the scope of this dissertation.

Biological Studies. Up to the present time, several authors have discussed various aspects of the biological characteristics of Fasciola hepatica metacercariae (Davis, 1954; Wikerhauser, 1960; Dawes, 1963b; Boray, 1964; Dixon, 1964, 1965, 1966; Bénex, 1966; Chowaniec and Markiewicz, 1970a). However, no mention has been found in the literature of the biological characteristics of the irradiated metacercariae, particularly what is the stability of the

immunogenic preparation, i. e. how does the viability or potency of the vaccine deteriorates with storage. Correlation between viability in vitro and various levels of irradiation will be followed up to 14 days post radiation. Changes of viability vs. age of the metacercariae will also be considered. The viability will be tested by the method of Wikerhauser (1960) which appraises the excystation capacity.

Pathophysiology Studies. The pathophysiology of various naturally and experimentally infected animals has been studied by several workers (Dawes, 1961a; Thorpe, 1965a, 1965b; Sinclair, 1967; Connolly and Downey, 1968; Campbell and Barry, 1970; Bundensen and Janssens, 1971). However, host liver damage as a result of the development of the immunogenic inoculum has not been disclosed in the available literature. Nor a specific radiation dose that would provide complete protection without producing any hepatic damage has been stated. Correlation between liver damage and different doses of gamma-irradiated metacercariae will be studied up to 5 weeks after the initial infection. Liver damage assessment will be done by liver function studies in terms of serum glutamic pyruvic transaminase (SGPT) levels following the technique of Wrobleski and LaDue (1956). Histopathology studies at different stages of the infection will also be described.

Biochemical Studies. The general consensus, after the work of several investigators and our group, is that a good protection against F. hepatica infection is strongly related to the presence of a live, metabolically active parasite within the host (Lang, 1968, 1974; Lang and Dronen, 1972; Eriksen and Flagstad, 1974). As a first approach to determine what is the mechanism through which this protection is acquired, the exoproteins secreted by the young and adult fluke will be studied. Evidence involving these exoprotein has already been reported (Cuperlovic et al., 1972; Mulligan et al., 1972). It has also been reported that the subdermal implantation of mature flukes confers protection to mice (Eriksen and Flagstad, 1974). However, there is not conclusive evidence to indicate the mechanism through which exoproteins can induce immunity. For this reason it was decided to study and characterize the parasite exoproteins as to molecular size distribution and serologic reactivity, and to investigae the changes in the exoprotein secretion of irradiated parasites.

EXPERIMENTAL

I. MATERIALS AND METHODS

A. Effects of gamma radiation on the viability of _Fasciola hepatica_ metacercariae _in vitro_

Metacercariae: Miracidia were obtained from eggs collected from the gall bladder of naturally infected cows immediately after slaughter. The eggs were held in distilled water at room temperature in the dark. Under these conditions bacterial growth was kept to a minimum and the eggs matured after 12 days. Hatching was suppressed by darkness and occurred shortly after the eggs were placed in the light. One hundred (100) snails were exposed "en masse" to miracidia. When the infection was mature after 28-30 days, the snails were placed in water glasses (10 per glass) that were filled with water and covered with the bottom of a plastic Petri dish allowing no air spaces between water surface and Petri dish. After 15 minutes the emerging cercariae started to encyst on the Petri dish. The dish was then removed from the glass, and a wet filter paper was placed inside the dish top. The dish was sealed and stored in the refrigerator at 4°C until used for the viability tests, the age of the metacercariae being equivalent to the number of days in storage.

Irradiation: The metacercariae were divided in five (5) groups. The first group was not irradiated, but the second, third, fourth,

and fifth groups were exposed to 1.5, 2.5, 3.5, and 5.0 Kr respectively. A rate of 228 rads per minute at a distance of 50 cm. from a cobalt 60 source was used. The metacercariae were exposed while still attached to the dish surface. No water was added in order to avoid the effect of free radicals elicited during irradiation. After irradiation they were stored in the refrigerator at 4°C until used. The metacercariae used in these experiments were between 30 and 90 days of age.

<u>Viability</u> <u>Test</u>: The viability of the metacercariae was determined by the excystation method of Wikerhauser (1960) modified in the PRNC laboratory. Five metacercariae from each radiation group were placed in each of 20 wells or depressions in a block of optically clear plastic. Ten of these wells served as controls and instead of the digestion mixtures they received distilled water. The other 10 were exposed to the following digestion process. Ten drops of an acid digestion mixture, pH 1.08, consisting of 0.5 g pepsin (activity 1:3,000 U.S.P.), 0.8 g NaCl, and 100 ml N/20 HCl added to the 10 test wells containing the metacercariae. This was followed by incubation at 38°C for 2 to 3 hours. The digestion mixture was then removed and the excess acidity diluted out with distilled water. After this, the metacercariae were rinsed with trypsin solution and ten drops added of an alkaline digestion mixture, pH 8.0, containing 0.4 g trypsin (activity 1:250 U.S.P.), 0.8 g NaCl, 1.0 g $NaHCO_3$ in 100 ml distilled water and 20% of fresh

or prefrozen ox bile. The preparations then received a second incubation period of up to 48 hours at 38°C.

Microscopic observation at 40X magnification was performed starting 15 minutes after immersion and continued every half hour until excystation occurred. The viability was measured by the complete emergence of the juvenile fluke within 7 hours of incubation and by the number of empty cysts after 24 and 48 hours. For the purpose of this appraisal, movement of the fluke within the cyst without excystment was not considered a proof of viability.

B. Effects of gamma radiation on the viability of *Fasciola hepatica* metacercariae *in vivo*

Metacercariae: The metacercariae for this experiment were obtained as described in experiment A.

Rats: Thirty-six (36) out-bred Mai-Wistar female rats obtained from Microbiological Associates were used in the experiment. The rats were randomly divided in five groups of which the first one had thirteen animals, the second, third and fourth groups had six animals each, and the fifth consisted of five animals. The first group served as experimental control and was infected with 20 unirradiated metacercariae. The second, third and fourth groups were infected with metacercariae exposed to 1.5, 2.5, and 5.0 Kr respectively. The fifth group served as uninfected controls.

Irradiation: A cobalt 60 source was used for the irradiation. The metacercariae were irradiated with 1.5, 2.5, and 5.0 Kr at a rate of 56.02 rads per minute at 1 meter from the source. The metacercariae were irradiated without water in order to avoid the free radicals effect.

Inoculation: For the inoculation, the rats were anesthetized lightly in an ether chamber. The metacercariae were counted in a microtiter plate, under a stereo microscope. Twenty (20) metacercariae were recovered from the plate and inoculated by mouth into the rat stomach using a small bore, fire-polished Pasteur pipette as suggested by Lammler (1959) and Thorpe (1963).

Serology: Blood for the collection of serum to be used in the SGPT determination was obtained by aseptic cardiac puncture. For this, the rats were deeply anesthetized in an ether chamber. The thorax was cleaned with 70% ethyl alcohol and 0.5 to 2.0 ml blood was drawn with sterile 2cc disposable syringes and 5/8 in. long, 25 gauge needles. The blood was transferred very carefully to centrifuge tubes and allowed to clot overnight in the refrigerator. Following centrifugation, the cell-free clear sera were transferred to separate sterile vials and the hemolyzed ones discarded. The sera were immediately used or otherwise frozen.

SGPT Determination: Serum glutamic pyruvic transaminase (SGPT) was measured by the method of Wrobleski and LaDue (1956) modified in our

laboratory. This method measures the SGPT activity spectrophotometrically. The SGPT was determined at 18, 22, 26, 30 and 34 days after infection. For the determination, 0.05 to 0.1 ml of clear rat serum, 0.67 ml of 0.1 M phosphate buffer (pH 7.4), 0.18 ml of 0.2 M 1-alanine in buffer (pH 7.4), 0.07 ml of B-Nicotinamide Adenine Dinucleotide, reduced form (β-NADH) (1 mg/ml), and 0.03 ml of a solution of purified lactic dehydrogenase (8,000 units/1.0 ml) were mixed and placed in a cuvette having a 1 cm path. The blank contained all reactants listed except NADH. At the end of 15 minutes, 0.01 M α-ketoglutarate in buffer pH 7.4 was added. The absorbancy curve was recorded at a wavelength of 340 mμ for 5 minutes and the rate of decrease in optical density after one minute taken as the level of glutamic pyruvic transaminase activity of the serum. The reaction was measured in a Beckman DU spectrophotometer at a temperature between 23°C and 25°C and recorded at a chart speed of 1.25 cms/min. Serum activity is expressed in absorbancy units per ml of serum (Worthington, 1972). Because we reduced approximately 1/3 the reaction volume described by Worthington (1972), the SGPT activity was obtained by using a conversion factor of 0.3571. One absorbancy unit is a decrease in absorbancy of 0.001 per minute at 25°C. One International unit is the oxidation of one micromole of NADH per minute at 25°C.

Histopathology of Liver Tissue: A total of 27 liver sections were obtained from rats of the different infection groups. Thirteen of the animals were killed at weekly intervals; the remaining 14 died accidentally during the progress of the investigation. At autopsy the liver of each rat was examined and the lesions noted. Blocks of liver removed for histopathologic study were fixed in 10 percent formalin. After fixation, the tissues were dehydrated and embedded. Five-micron sections were stained with Harris Hematoxylin Eosin Y following the technique of the U. S. Armed Forces Institute of Pathology (1960). The sections were described under two headings: (i) livers from rats infected 2-7 weeks and (ii) livers from rats infected for 8-12 weeks. The changes observed in the livers were described together as they differ in degree only.

C. Effects of gamma radiation on the exoprotein synthesis of Fasciola hepatica

Experiment 1 - Separation and characterization of Fasciola hepatica exoproteins

Parasites: Fifty (50) young and adult alive flukes were removed from the bile ducts of infected cows immediately after slaughter.

Incubation: The parasites were immediately placed in 25 ml of a warm (37°C) Hedon-Fleig balanced salt sterile solution (Dawes, 1954) without glucose and incubated for one hour at 37°C to allow them to excrete their intestinal contents. After this, the flukes were

rinsed 4 times with fresh medium and further incubated for 6 hours in a medium containing 25 ml of Hedon-Fleig solution with glucose and 5uCi/ml of ^{75}Se-l-methionine. An antibiotic-antifungal mixture was added so that 1 ml of the final medium contained 1 μg of Fungizone and 100 IU and 100 μg of Potassium Penicillin G and Streptomycin Sulfate respectively.

Fasciola hepatica Exoprotein Separation: The separation of F. hepatica exoprotein was carried out as described by Cuperlovic and Movsesijan (1972) and modified in our laboratory. After the period of incubation, the medium was dialyzed for 2 days against 0.15 M NaCl pH 7.0, changing the solution 20 times. The medium was then centrifuged in a Sorvall centrifuge-RC2 at 12,100 g for 30 minutes at 4°C to eliminate debris, fecal excretion, etc. After centrifugation the supernatant was separated and lyophilized in a freeze dryer and redissolved in 2 ml of distilled water. The medium was then filtered through a Sephadex G-100 column (Pharmacia K 15/30), and eluted with 0.15 M NaCl pH 7.0. The optical density of the elutant was automatically recorded by a LKB 8300 Uvicord II (UV-Absorptiometer) at 280 mμ and registered by a LKB 6530 Recorder (Flat-Bed). The fractions were collected with an automatic LKB 7000 Ultro Rac Fraction Collector and then counted in a Keleket gamma well monitor, preset count 100, preset time 1.0, 1700 volts. After filtration and counting the fractions in peak A and peak B were pooled.

Each of the obtained pools (Peak A and Peak B) of each group was analyzed to determine first, the proportion of the precipitable labeled fraction due to proteins (TCA test); second, the protein contents of the pools (Sutherland et al., 1949); third, the antigenic activity of the fractions (Tarr Assay); and fourth, the homogeneity of pool A and B (Disc electrophoresis).

a. <u>Trichloroacetic Precipitation</u>. This test was followed routinely. For the determination, 0.1 ml of each pool of protein fraction was counted in a gamma well monitor as described above. After this, 0.1 ml of protein, 0.1 ml 10% TCA, and 0.1 ml of 1.0% albumin were mixed. The samples were allowed to stand 15 minutes at room temperature followed by centrifugation in a Sorval centrifuge-RC2 at $1475 Xg$ for 10 minutes. The supernate was collected and 0.1 ml counted in the gamma monitor.

b. <u>Protein Determination</u>. The protein content of each pool of fractions was measured by the method of Sutherland et al. (1949). For this, 0.5 ml of protein solution was added to 5.0 ml of a dilute alkaline copper solution (prepared twice weekly by adding 1 ml of 2% $CuSO_4 \cdot 5H_2O$ and 1 ml of 4% sodium tartrate to 100 ml of a 4% Na_2CO_3 solution). The mixture was incubated 15 minutes at 45°C, at which time 0.5 ml of phenol reagent (Folin-Ciocalteau) diluted 1:3 with water was added. After inversion, the tubes were allowed to stand 15 minutes at room temperature before being read in a Beckman DU spectrophotometer at wavelength 660 mμ. The standard used was .025% albumin.

c. <u>Farr Assay</u> (modified by Dr. R. A. Brown, PRNC). This method measures the magnitude of antigen-antibody reaction where one of the reactants is radioactively tagged. In these experiments, aliquots of peaks A and B of the parasite exoproteins were used. These were eluted from Sephadex 100 and had been previously labeled with radioselenium as described above. The assay was carried out by mixing 20   of normal or immune rat serum with 5   exoprotein solution in 1.0 ml phosphate-buffered saline (PBS). Following incubation periods of 2 hours at room temperature and 24 hours at 4°C each mixture was precipitated with a saturated solution of ammonium sulfate and centrifuged for 15 minutes at 1475 Xg and 5°C. The supernatant was then carefully separated from the precipitate and its radioactivity determined in a Picker scintillation counter for 10 minutes with a background of 94 counts per minute. The precipitate was similarly counted after 3 washings with PBS.

d. <u>Disc Electrophoresis</u>. As a means of understanding the specific pattern of <u>F</u>. <u>hepatica</u> proteins, each experimental control pool of fractions was analyzed by the Disc Electrophoresis Test using acrylamide crosslinked gels as described by Canalco Co. (1968). The standard gels (7%) were used which cover proteins ranging from 10^4 to 10^6 M. W.

Experiment 2.   Effect of gamma radiation on the exoprotein synthesis of <u>Fasciola</u> <u>hepatica</u>.

Parasites: A total of one hundred (100) young and adult alive flukes were removed from the bile ducts of infected cows immediately after slaughter, and randomly divided in four groups of twenty-five each.

Irradiation: The first group of worms served as control, but the second, third and fourth received 1.5, 2.5, and 5.0 Kr respectively. A rate of 171.76 rads per minute at a distance of 50 cm from a cobalt 60 source was used. The flukes were exposed in a test tube without water in order to minimize the effect of free radicals elicited during irradiation.

Incubation: The incubation of each group of parasites was performed as described in experiment 1. In order to follow the progress of the protein synthesis, 0.2 ml of each medium was taken out every hour up to five (5) hours of incubation.

Trichloroacetic Precipitation: After the incubation period, 0.5 ml of 10% TCA, and 0.8 ml of 1% albumin were mixed and allowed to stand 15 minutes at room temperature. The samples were then centrifuged in a Sorvall centrifuge RC-2 at 1475Xg for 10 minutes. Each precipitate was washed five (5) times with 5% TCA, and counted in the gamma monitor.

D.   Statistical Evaluation of the Data.   The viability of unirra-
diated and irradiated metacercariae was compared on the basis of
two variables, time of storage and dose of radiation, by the Z test
of significance for the 95% confidence interval.

Serum glutamic pyruvic transaminase (SGPT) in rats infected
with unirradiated and irradiated F. hepatica metacercariae was
statistically compared by the T-test.

II. RESULTS

A. Effects of gamma-irradiation on the viability of _Fasciola hepatica_ metacercariae _in vitro_

The excystation process of _F. hepatica_ metacercariae was appraised following the digestion method of Wikerhauser (1960) and the findings statiscally evaluated by the Z test. Under the action of the pepsin solution, partial digestion of the outer layer of the cysts and a rotatory motion of their larvae were observed after two (2) hours of treatment (figure 2).

Table 1 and figure 3 represent the percentage excystment for each group of irradiated metacercariae at 0.7, and 14 days after exposure. The excystation of the unirradiated metacercariae was never higher than 54%. A significant difference in excystation percentage was observed immediately after radiation between the unirradiated group (54%) and the groups of metacercariae exposed to 1.5, 2.5, and 3.5 Kr for which the excystation rates were 75, 75, and 83% respectively. However, the in vitro viability of the 5 Kr group (68%) did not deviate significantly from the normal. There was only a slight difference in the viability rates of the 4 groups of irradiated cysts.

On the 7th post-radiation day the 4 irradiated groups showed a greatly reduced viability from the initial values, (75 to 28, 75 to 50, 83 to 12, and 68 to 22% in the 1.5, 2.5, 3.5, and 5.0 Kr respectively), but no significant differences in viability was

observed in the unirradiated metacercariae (47%) when compared with the 0-day (54%). Likewise, the viability observed in the unirradiated metacercariae (47%) and those irradiated with 2.5 Kr gamma-radiation (50%) was almost the same. However, the differences between the normal and the 1.5, 3.5, and 5.0 Kr metacercariae were quite ample and significant (47 against 28, 12 and 22% respectively).

At the 14-day interval, the viability of the unirradiated metacercariae did not diverge significantly from that observed on the 0 and 7th day. The 1.5 and 2.5 Kr groups recovered considerably (65 and 70%) by a slight difference. However, the cysts exposed to 3.5 and 5.0 Kr suffered a large reduction in viability (31 and 32%) when compared both with that of the unirradiated group (52%) and with their own 0-day value (83 and 68%). These two groups (3.5 and 5.0 Kr) also recovered from their extremely low 7-day viability rate but to a lesser extent than the 1.5 and 2.5 Kr groups.

Table 2 shows the effect of age of the metacercariae (number of days in storage at 4°C) on their excystation capacity. Although the values obtained ranged from a low 29% on the 69th day to a high of 81 on the first day, the mean viability from the 1st to the 69th day was 52%. This mean does not differ substantially from the mean obtained with 4 different experiments with 90-day old metacercariae (55%), nor from that of two experiments with 92-day old cysts (48%). The mean for these last 6 experiments with 90 and 92-day old cysts is 52%. The Z test of significance indicates that

the excystation potential does not change with this type of treatment for up to 92 days. Nevertheless, a significant decrease was observed in cysts stored for 104 days or longer, in which the mean viability of cysts stored for 104, 129, and 130 days was 28%.

B. Effects of gamma-irradiation on the viability of Fasciola hepatica metacercariae in vivo

1. Serum Glutamic Pyruvic Transaminase Activity

The transaminase levels found in the sera of rats infected with irradiated and unirradiated Fasciola hepatica are compared in tables 3 to 7. The deaths shown by the tables were all classified as "bleeding accidents".

Serum GPT values in uninfected rats were relatively constant throughout the 34 days of the experiment (table 3 and figure 5). The levels ranged from 13 to 35 with a mean of 22.5 and a standard deviation of $\pm$ 6.7.

In rats infected with unirradiated F. hepatica, i. e., the unmodified infection, there was only a slight elevation of SGPT activity up to 22 days post inoculation, but subsequently enzyme values rose rapidly and reached a maximum 15 fold increase on the 30th day post infection. Enzyme levels then fell very rapidly and reached near normal values by the 34th day post inoculation (table 4 and figure 5).

SGPT activity in the serum of rats infected with metacercariae irradiated with 1.5 Kr rose very rapidly reaching a maximum 28 times the initial mean within 30 days (table 5 and figure 6). In this group the enzyme levels remained elevated up to the 34th day when the mean was 17 times the baseline mean. A T-test between the SGPT values of this group and those of the unmodified infection showed significance at 18, 22, 26, and 34 days post infection (table 8).

Rats infected with 2.5 Kr gamma-irradiated metacercariae showed a 5-fold increase in the SGPT levels by the 18th day remaining relatively constant thereafter up to the 34th day of infection when the mean value showed a slight increase to 6 times the baseline value (table 6 and figure 7). SGPT concentration showed significant elevations at 18, 30, and 34 days of infection (table 8).

Table 7 shows the changes in SGPT levels in rats infected with 5.0 Kr gamma-irradiated metacercariae. The transaminase activity rose to 3.8 times of the baseline value on the 18th day post-infection and then tapered down to 2.5 times after the 22nd day of the experiment (figure 7). The T-test for this group and the normal infection group showed a significant difference at 1, 22, 30 and 34 days (table 8).

2. Pathology

Infected livers are described under two headings: (i) livers from rats infected for 2 to 7 weeks; and (ii) livers from rats infected for 8 to 12 weeks.

Livers from 2 to 7 weeks infection

Gross Pathology

Normal infection. Cream coloured or pink subcapsular lesions were observed at 3 weeks after infection in rats inoculated with unirradiated metacercariae. These appeared as small areas (1-2 mm in diameter) or as irregular streaks (2 mm wide) on the infero-parietal border of the liver parenchyma. The lesions were most predominant in the left lobe. Severe hepatic damage scattered through the parenchyma was observed between the 3rd and 6th weeks of infection. Few necrotic areas were observed on the edges of the liver lobes. Parasites measuring 2 mm wide by 5 mm long were found attached to the surface of livers.

Infections with irradiated Fasciola hepatica. Between the 3rd and 6th weeks, rats infected with 1.5 Kr gamma-irradiated flukes showed severe hepatic damage involving almost 2/3 of the parenchyma. Long filiform tracts located immediately below the Glisson capsule were observed. Upon sectioning the liver of rats infected with 1.5 Kr metacercariae, young motile flukes measuring 1.5 to 2 mm wide by 2 to 4 mm long were observed embedded in the parenchyma. Moderate lobular enlargement with moderate parenchymal involvement was observed in one of the 1.5 Kr rats 5 weeks post-infection.

No macroscopic lesions were observed in the liver of rats infected with 5.0 Kr irradiated F. hepatica metacercariae.

The mail bile ducts of all animals, whether infected with unirradiated or irradiated metacercariae, appeared normal throughout this observation period.

Microscopic findings

Normal infection. The microscopic examination of liver sections from rats 4 weeks after infection with unirradiated metacercariae revealed small peripheric necrotic areas (figure 8). These are the spaces left by the young flukes in their path through the liver parenchyma. These spaces were filled with cell debris, mononuclear cells, polymorphonuclear eosinophils, macrophages and red cells. Polymorphonuclear cells with clearly delineated eosinophilic cytoplasm predominated. Loose connective tissue was found around this area. Atrophied cells with pyknotic nuclei were also some of the features of the liver tissue immediately adjacent to the fluke's wake. In areas adjacent to the healing tract diffuse infiltration was observed in the portal tract with neoformation of interlobular bile ducts (figure 8, 9, and 10). In some sections, inflammation and edema of the hepatic artery was observed (figure 9).

Infection with irradiated Fasciola hepatica. Infiltration of the portal tract, edema of the artery, and extensive necrotic areas were the outstanding features of the liver pathology in rats infected with

1.5 Kr gamma-irradiated metacercariae four weeks after infection (figure 11). A young parasite migrating through the parenchyma was also found at five (5) weeks of infection (figure 12). Migrating flukes lay in a mixture of macerated liver cells and blood surrounded by a heavy cellular infiltration (figure 12).

Sections of liver infected with 2.5 Kr gamma-irradiated F. hepatica showed vacated healing tracts at five (5) weeks post infection (figure 13). These areas presented a necrotic contracted central core with peripheral infiltration. Eosinophilic infiltration was also prominent in the portal tract (figure 14).

The liver of rats infected with 5.0 Kr gamma-irradiated metacercariae showed very mild damage characterized by a light diffuse infiltration in the portal tract (figure 15).

Livers from 8 to 12 weeks infection

Gross Pathology

No "localized phase" of migration was observed between the 8th and 12th week after infection.

Normal infection. At the 12th week after inoculation the hepatic parenchyma showed no recent tracts, but patchy fibrosis was observed in one or two lobes, particularly in the left one.

Infection with irradiated F. hepatica. A corrugated appearance as a result of fibrosis was observed on the liver surface at the edge of

the lobes, particularly in the liver of rats infected with 1.5 and 2.5 Kr gamma-irradiated cysts. Large extrahepatic bile ducts were abnormally prominent and contained adult F. hepatica, 6-9 mm wide by 9-11 mm long, in the livers of rats infected with 1.5 and 2.5 Kr gamma-irradiated flukes. The walls of the ducts were not thickened and there was never any evidence of calcification.

Microscopic findings

Normal infection. Unmodified liver infections showed heavy cellular infiltration in the portal tract at 4 weeks post infection (figure 16). Red blood cells were scattered through the parenchyma which suggest a mild hemorrhage. Patches of fibrous tissue could be observed at the periphery of older tracts.

Infection with irradiated F. hepatica. Slight interlobular and intralobular infiltration was observed in the group of rats infected with 1.5 and 2.5 Kr metacercariae. However, healed tracts with complete reabsorption of the central necrotic core were found in livers infected with 2.5 Kr gamma-irradiated F. hepatica. Atrophied cells and biliary ducts neoformation were other features of this infection. Very few small foci of infiltration were observed in livers infected with 5.0 Kr gamma-irradiated flukes. Otherwise, the microscopic picture was that of a normal liver.

C. Effects of gamma-irradiation on the exoprotein synthesis of
Fasciola hepatica

Experiment 1 - Separation and characterization of F. hepatica
exoprotein

Adult Fasciola hepatica worms incubated in Hedon-Fleig
solution released to the medium a sizeable amount of proteins.
After Sephadex G-100 filtration, two separate peaks (A and B) were
obtained (figure 17). When the worms were incubated in the presence
of ^{75}Se-Methionine it could be observed that this radiolabeled
aminoacid had been metabolically incorporated in the released
neoproteins. This is evidenced by the perfect correlation between
the absorbancy curve at 280 mu and the radioactivity curve.
Furthermore, by means of the TCA test, it was demonstrated that
^{75}Se-Methionine had been incorporated in the neoproteins synthetized
by the liver fluke. Table 9 shows the results obtained before and
after the precipitation. Treatment with TCA elicited the proportion
of newly synthetized proteins in peak A (96%) and in peak B (92%).

Positive binding of these proteins with the antibodies present
in hyperimmune Fasciola rat resum was demonstrated with the Farr
Assay. The percent of binding in peak A (19.4%) was low when
compared with 51.2% binding in peak B. It was further found that
peak A is a mixture of four electrophoretically different proteins
and peak B is a mixture of three proteins (figure 17).

Experiment 2 - Effects of gamma-irradiation on the exoprotein
synthesis of _Fasciola hepatica_

Table 10 and figure 18 show the quantity of labeled proteins synthetized by the adult _F. hepatica_ after 1 hour of incubation, and precipitated with TCA. No significant differences in the quantity of protein synthesis were observed between unirradiated parasites and those irradiated with 1.5, 2.5, and 5.0 Kr. After one hour of incubation the 1.5 and 2.5 Kr flukes showed an increase of 24 and 13% labeled protein synthesis when compared with the normal parasites. Those exposed to 5.0 Kr, however, showed a decrease of 12%. Further incubation resulted in an overall decrease in radiolabeled protein including the unirradiated control group.

III. DISCUSSION

A. Effects of gamma-irradiation on the viability of _Fasciola hepatica_ metacercariae _in vitro_

(1) Viability vs. Age

In previous reports by several authors (Wikerhauser, 1960; Dixon, 1964, 1966) on studies of the various physiological aspects of the excystation process of the _F. hepatica_ metacercariae, the age of the cyst has not been mentioned. In our studies it was considered that this factor could have some bearing on successful excystation together with other factors such as temperature and humidity. The effect of age, equivalent to days in storage at approximately 4°C, was assessed keeping the temperature and humidity factors constant, but varying the age factor. The viability of normal (unirradiated) metacercariae was determined by following the excystation behavior up to 130 days of storage before they were used in the ionizing radiation experiments.

Our findings demonstrate that storage at 4°C up to 92 days did not affect the in vitro excystation mechanisms of normal meta-cercariae. They tend to confirm the generalized concept that _F. hepatica_ cysts are quite stable in their natural habitat and that if they are used within this storage limit the normal variation in viability is acceptable for the subsequent radiation experiments. Defining "P_1" as the proportion of successful excystations between

1 and 69 days and "P_2" as the proportion of excystations between 90 and 92 days, we have found no difference between the two groups since both were 52%. However, storage for 104 days and over resulted in a "P_3" value of 28%, a marked difference from P_1 and P_2.

(2) Viability as affected by ionizing radiation and by storage post exposure

All the investigations conducted by others heretofore in search of a radiation-attenuated vaccine for F. hepatica have considered the effect of such inoculum only immediately after the radiation exposure (Thorpe and Broome, 1962: Chiriboga et al., 1971; Corba et al., 1971). However, it is obvious that the stability of a vaccine is one of its most critical characteristics. Therefore, a preparation that would loose its activity after a few days of storage would have very little practical applicability. In our investigation we have studied this stability up to 14 days, thus covering the vaccination period of 14 days employed by Chiriboga et al. (1971) for their 3 inoculations at weekly intervals.

As expected, the viability of the normal, unirradiated meta-cercariae remained stable during the 14 days of observation as evidenced by the unvariable excystation rate. Irradiated and normal metacercariae revealed two well-defined distributions when tested immediately after the radiation exposure. One of them is the excystation rate of the normal (unirradiated) cysts and the other

includes the rates exhibited by the metacercariae exposed to 1.5, 2.5, and 3.5 Kr. A third group, the one irradiated with 5.0 Kr, is difficult to fit in any of the other two since it does not bear a significant statistical difference from any of them.

The increase in the excystation rate observed immediately after radiation in the 1.5, 2.5, and 3.5 Kr metacercariae leads us to suspect that rather than producing deleterious effects on the excystation mechanisms of the metacercariae, these particular radiation doses activated them. This consideration, however, does not preclude the possibility of any damage caused by the radiation to the cyst or to the emerging larva which could be manifested in other ways.

The striking reduction in the excystation rate observed on the 7th day post-radiation in all the irradiated groups of meta-cercariae was possibly due to an external environmental factor to which the irradiated cysts were more susceptible than the normal unirradiated cysts. A fact pointing to this possibility is the apparent "recovery" of the viability to near 0-day rates in the 1.5 and 2.5 Kr groups on the 14th day post-radiation. On the other hand, this drastic drop in viability cannot be ascribed directly to either the time or to the radiation variable or to a time-radiation con-comitant effect since these variables did not impinge so drastically on the 7-day excystation of the normal metacercariae.

In regard to the 14-day observation it is obvious that neither time nor radiation exerted significant damaging effects on the excysta-tion of the normal and the 1.5, and 2.5 Kr groups since the rates for

these groups fell so close to the 0-day values. Although in these 3 groups there was a slight decrease in excystation rate, the excystation "activation" effect seen on the 0-day was still apparent in the 1.5 and 2.5 Kr metacercariae. The most prominent reduction in excystation rate that can be unquestionably ascribed to both the time and radiation variables is that exhibited on the 14th day by the groups exposed to 3.5 and 5.0 Kr.

It can be inferred from the above data that radiation doses lower than 2.5 Kr do not impair the excystation mechanism of F. hepatica metacercariae when this process is measured in vitro up to 14 days post radiation. It is thus demonstrated that in the 3-innoculations immunization scheme of Chiriboga et al (1971) at least the excystation mechanism of the metacercariae is preserved and that the attenuation produced by the ionizing radiation must be attributed to an injury manifested in the excysted fluke.

B. Effects of gamma-irradiation on the viability of Fasciola hepatica metacercariae in vivo

1. Serum Glutamic Pyruvic Transaminase Activity (SGPT)

Our investigation corroborates the work of Bundensen and Janssens (1971) of assessing the degree of liver damage caused by the intra-hepatica migration of Fasciola hepatica by determining the SGPT levels released by the infected animal liver. In mice experimentally infected with F. hepatica, Bundensen and Janssens (1971) found a maximum SGPT activity level coinciding with maximum tissue damage on the

28th day of infection. The enzyme pattern that we found in rats is consistent with the results of these authors, the maximum SGPT activity found by us appearing on the 30th day of infection (figure 5).

SGPT values of rats infected with 1.5 Kr-irradiated metacercariae do not return to the normal level as quickly as those of animals inoculated with unirradiated metacercariae (figure 6). This was probably due to a delay in the parasite journey through the liver.

There is a good correlation between radiation doses over 1.5 Kr and the enzyme levels released (figure 7). The decrease in SGPT observed in rats infected with 2.5 and 5.0 Kr-irradiated metacercariae can not be ascribed to other than radiation damage, but as mentioned above, rather than excystation impairment, the deleterious or attenuation effect may be on the chemotactile capacity or on the exoprotein excretion of the excysted flukes. The injury caused to the parasite by these doses was of such a magnitude that it possibly did not interfere with its initial invasion of the liver, but apparently was enough to slow it down giving the organ ample time to organize a local defense, or simply hindered the complete development of the flukes before they reached the liver.

2. Pathology

The gross hepatic lesions observed in the rats infected with normal (unirradiated)metacercariae followed the pattern already

described by various authors in this type of infection (Urquhart, 1956; Dawes, 1961b; Thorpe, 1965a, 1965b; Dow et al., 1966). There was, however, an outstanding feature not previously mentioned by others in discussing the gross pathology of fascioliasis, namely, the appearance of long filiform tracts on the liver surface of rats infected with 1.5 Kr-irradiated metacercariae. This tract, which was located immediately below the Glisson capsule, gives the impression of a parasite that has lost its sense of orientation towards its final localization in the intrahepatic biliary ducts.

The microscopic picture was also similar to detailed investigations of the histopathology of _Fasciola_ _hepatica_ normal infections which have been reported in rabbits (Urquhart, 1956), mice (Dawes, 1963a), rats (Thorpe, 1965b). and in sheep (Dow et al., 1968).

A very good correlation was found between the SGPT levels obtained and the extent of microscopic liver damage observed. However, some of the animals showed a tendency to present hepatic lesions very early in the infection. These lesions were characterized by interlobular fibrosis in which the extent of the damage could not be correlated with the radiation exposure of the inoculum. Worth of mention is the evidence of recent lesions and massive necrosis of the hepatic tissue in an animal infected with 1.5 Kr-irradiated metacercariae (Figure 11). This rat died on its 30th day of infection which coincided with the highest SGPT level recorded (table 5). The massive necrosis observed in this animal was probably the result of the occlusion of a large blood vessel by a developing parasite.

There is evidence in the experiments described above of qualitative and quantitative differences in the pathogenicity of metacercariae exposed to different radiation doses. The SGPT levels in the groups of rats given 2.5 and 5.0 Kr-irradiated metacercariae were quite consistent with the mild degree of pathology observed.

It is evident that the immunizing inoculum employed by Chiriboga et al. (1971) and Corba et al. (1971), consisting of metacercariae exposed to 2.5 Kr ionizing radiation, although conferring protection to the animals. is still capable of producing damage even if relatively mild. Also, some of the parasites developed into mature, though abnormal flukes.

Our results suggest that it will be necessary to try other dosing in order to learn if immunity can be obtained in a single or multiple dose that will not cause serious hepatic lesions. Further studies in which the immunizing inoculum consists of metacercariae exposed to 5.0 Kr gamma-radiation are recommended.

C. Effects of gamma-irradiation on the exoprotein synthesis of <u>Fasciola</u> <u>hepatica</u>

Experiment 1 - Purification and characterization of <u>F</u>. <u>hepatica</u> exoprotein

Previous immunization attempts using irradiated-metacercariae have shown adequate protection in experimental hosts (Chiriboga et al., 1971; Corba et al., 1971). It has also been reported that the subdermal implantation of mature flukes confers protection to

mice (Eriksen and Flagstad, 1974). Exoproteins with antigenic activity synthetized in the cecum of adult worms have been detected and described by Cuperlovic and Movsesijan (1972).

These exoproteins, which are probably the proteolytic and cytolytic enzymes employed by the metabolically active parasite in penetrating large masses of tissue and in procuring its nutritional requirements, are of fundamental importance in the study of the immune response of the definitive hosts of F. hepatica.

Our investigations corroborate the work of Cuperlovic and Movsesijan (1972). However, we have demonstrated that each of the two proteins described by these authors as the "antigens active during the infection" is actually a mixture of several electro-phoretically separable proteins. The higher percent of specific antibody binding found in peak B indicates that one or more of these proteins is much more serologically reactive than peak A. Future studies should be aimed at determining how do the individual pro-teins in peaks A and B rank in serological reactivity as well as in antigenicity and immunogenicity.

Experiment 2 - Effects of gamma-irradiation on the exoprotein synthesis of Fasciola hepatica

It has been reported that X-irradiation of the cysts of F. hepatica caused destruction and elimination of the epithelial cell lining of the cecum, and a suppression of the development of the genital organs (Movsesijan et al., 1967). These facts suggest the

possibility of suppressing the exoproteins synthesis in the cecum by irradiating the adult flukes, in comparing the immunogenic activity of proteins excreted by unirradiated parasites and those irradiated with gamma-radiation. Preliminary results on this subject showed no variation on protein synthesis after irradiation of the adult flukes with 1.5, and 2.5 Kr. However, it appears that with 5.0 Kr a mild suppression of protein synthesis can be obtained (figure 18).

After an hour of incubation the amount of labeled protein in the solution diminished substantially meaning that probably no more protein synthesis took place due to the in vitro deterioration of the parasites or to digestion of the neoformed proteins by proteolytic enzymes present in the solution.

SUMMARY AND CONCLUSIONS

Alterations in the viability of normal _Fasciola_ _hepatica_ metacercariae as a result of time of storage at 4°C were studied by the method of Wikerhauser (1960). A significant decrease in viability was observed only after one hundred days of storage.

The _in vitro_ viability of normal metacercariae was compared with that of metacercariae that were exposed to increasing radiation doses. Two plainly opposite effects were observed: a) the activation of the excystation process by the lower radiation doses (1.5 and 2.5 Kr), an effect that persisted up to the 14th day post-radiation and b) the impairment of the process evidenced on the 14th day in the metacercariae exposed to 3.5 and 5.0 Kr.

Correlation between liver damage and the increasing radiation doses to which the metacercariae were exposed was studied up to 34 days after the oral inoculation in rats. Hepatic parenchymal destruction was measured in terms of serum glutamic pyruvic transaminase (SGPT) levels. It was found that rats infected with 1.5 Kr-irradiated metacercariae showed an increase over the normal infection in the levels of SGPT. This increase has been tentatively associated with the inability of the developing worms to readily find the liver ducts. Metacercariae irradiated with 2.5 and 5.0 Kr produced only a slight elevation of SGPT. A very good correlation was found between the levels of SGPT and the macroscopic and microscopic lesions of rat livers infected with unirradiated and irradiated worms.

Adult $\underline{F}$. hepatica incubated in Hedon-Fleig solution for 6 hours, released to the medium many types of proteins. Some of these proteins are neoformed judging by the incorporation of ^{75}Se-methionine, a radioactive aminoacid. These proteins were purified first by using Sephadex G-100 followed by gel electrophoresis. In the graph of the Sephadex elution curve two peaks were found (peaks "A" and "B"). Peak B specifically precipitates with Fasciola-positive rat serum, but not with normal rat serum. In addition, by using the Farr method of ammonium sulfate precipitation a high specific binding was observed. Each peak obtained was found to be a mixture of proteins. The acrylamide electrophoresis pattern showed 4 bands in peak A and 3 bands in peak B. Adult parasites irradiated with 1.5 and 2.5 Kr exhibited an increase in protein synthesis contrasting with a slight decrease in the parasites irradiated with 5.0 Kr.

From the experiments described above, it can be inferred that:

1) Metacercariae excystation is not hindered appreciably by storing at 4°C for less than 3 months. However, a large reduction is observed in metacercariae stored for longer than 3 months.

2) Radiation doses of up to 2.5 Kr do not impair metacercarial excystation within a period of 2 weeks post-irradiation.

3) Inocula of 2.5 Kr-irradiated metacercariae are capable of excystation in rats, some of the flukes reaching the liver and very few completing the normal trajectory through its parenchymal but never developing into normal adults.

4)   Protein synthesis by adult flukes is not significantly affected by radiation doses as high as 5.0 Kr.

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VI - TABLES

TABLE 1

IN VITRO VIABILITY OF FASCIOLA HEPATICA METACERCARIAE AT WEEKLY INTERVALS
AFTER EXPOSED TO VARIOUS DOSES OF GAMMA IRRADIATION

Radiation Exposure (Rads)	Days Post Radiation					
	0		7		14	
	Viable Observed	% Viability	Viable Observed	% Viability	Viable Observed	% Viability
0	27/50	54	14/30	47	25/48	52
1500	31/41	75	12/43	28	31/48	65
2500	31/41	75	19/38	50	35/50	70
3500	31/37	83	6/52	12	16/51	31
5000	29/43	68	10/47	22	16/50	32

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TABLE 2

AGE vs. VIABILITY OF UNIRRADIATED F. HEPATICA METACERCARIAE

Age (Days)	Viable/Total	% Viability	Mean % Viability
1	35/43	81	
30	14/30	47	
68	32/50	64	52
69	22/76	29	
90	28/50	56	
90	38/50	76	
90	26/50	52	
90	17/50	34	52
92	21/50	42	
92	27/50	54	
104	14/66	21	
129	16/50	32	28
130	15/46	33	

TABLE 3

SGPT LEVELS IN NORMAL RATS AT VARIOUS INTERVALS

Animal No.	Days of Bleeding						
	0	18	22	26	30	34	
1	34.97	21.95	11.38	18.70	21.14	15.45	
2	28.46	33.34	13.01	21.95	21.95	34.15	
3	26.02	17.89	15.45	19.52	30.90	32.52	
4	21.95	25.20	13.82	13.82	21.14	Died	
5	23.58	26.84	19.52	21.95	Died	--	
Mean	27.00	25.04	14.64	19.19	23.78	27.37	
± St. Dev.	5.09	5.76	3.10	3.33	4.76	10.36	

Test Mean N_{27} = 22.47 ± 6.73

TABLE 4

SGPT LEVELS IN RATS INFECTED WITH UNIRRADIATED METACERCARIAE
AT VARIOUS INTERVALS AFTER INFECTION

Animal No.	Days Post Infection					
	0	18	22	26	30	34
1-1	7.32	24.40	97.58	24.40	139.05	--
2	21.14	40.66	81.32	60.99	69.94	32.53
3	18.70	Died	--	--	--	--
4	22.36	26.84	Died	--	--	--
5	14.23	24.40	69.94	97.58	69.12	44.73
6	6.51	26.84	69.94	40.66	487.92	44.73
7	30.49	15.45	24.39	40.66	121.98	14.23
8	13.42	34.56	48.79	162.64	Died	--
9	14.23	13.42	60.99	Died	--	--
10	8.27	*	39.23	53.50	385.20	15.34
11	18.12	*	89.17	Died	--	--
12	9.85	*	78.47	85.60	249.67	--
13	11.82	*	82.03	71.33	256.80	21.40
Mean	15.11	25.82	67.44	70.82	222.46	28.83
± St.Dev.	6.90	8.98	22.27	41.44	152.46	13.93

* Data not available

TABLE 5

SGPT LEVELS IN RATS INFECTED WITH IRRADIATED METACERCARIAE
(1500 rads) AT VARIOUS INTERVALS AFTER INFECTION

Animal No.	Days Post Infection					
	0	18	22	26	30	34
2-1	13.42	105.71	121.98	325.28	348.86	487.92
2	22.36	81.32	121.98	195.16	1219.80	Died
3	15.45	60.99	121.98	195.16	325.28	243.96
4	10.57	8.13	97.58	Died	--	--
5	24.39	Died	--	--	--	--
6	23.58	37.40	121.98	243.96	195.16	195.16
Mean	18.30	58.71	117.10	239.89	522.28	309.01
± St. Dev.	5.88	37.87	10.91	61.41	469.90	156.85

TABLE 6

SGPT LEVELS IN RATS INFECTED WITH IRRADIATED METACERCARIAE
(2500 rads) AT VARIOUS INTERVALS AFTER INFECTION

Animal No.	Days Post Infection						
	0	18	22	26	30	34
3- 1	10.67	138.24	88.64	53.67	69.12	37.40
2	9.75	162.64	97.58	116.29	97.58	146.37
3	14.23	57.73	48.79	43.91	37.40	53.67
4	15.45	--	--	--	--	--
5	26.02	48.79	88.64	89.45	121.98	195.16
6	30.90	43.91	60.99	60.99	48.79	81.32
Mean	17.84	90.26	76.93	72.86	74.97	102.78
+ St. Dev.	8.64	55.83	20.90	29.62	34.83	66.30

TABLE 7

SGPT LEVELS IN RATS INFECTED WITH IRRADIATED METACERCARIAE (5000 rads) AT VARIOUS INTERVALS AFTER INFECTION

Animal No.	Days Post Infection					
	0	18	22	26	30	34
4-1	10.57	40.60	65.05	97.58	54.48	40.66
2	13.42	43.91	32.52	44.72	48.79	44.72
3	6.51	121.98	32.52	60.99	37.40	37.40
4	10.57	92.70	37.40	37.40	54.48	37.40
5	40.66	32.52	37.40	26.02	32.52	48.79
6	26.84	81.32	48.79	32.52	37.40	44.72
Mean	18.10	68.84	42.28	49.87	44.18	42.28
± St. Dev.	13.08	35.46	12.64	26.27	9.61	4.57

TABLE 8

STATISTICAL COMPARISON OF SGPT LEVELS IN RATS INFECTED WITH
UNIRRADIATED AND IRRADIATED METACERCARIAE

Days Post Infection	Unirradiated Mean ± S.D.	1500 Rads			2500 Rads			5000 Rads		
		Mean ± S.D.	Δ*	S	Mean ± S.D.	Δ	S	Mean ± S.D.	Δ	S
0	15.11 ± 6.90	18.30 ± 5.88	3.19	N.S.**	17.84 ± 8.64	2.73	N.S.	18.10 ± 13.08	2.99	N.S.
18	25.82 ± 8.98	58.71 ± 37.87	32.89	p ≤ .05	90.26 ± 55.83	64.44	p ≤ .01	68.84 ± 35.46	43.02	p ≤ .01
22	67.44 ± 22.27	117.10 ± 10.91	49.66	p ≤ .001	76.93 ± 20.90	9.49	N.S.	42.28 ± 12.64	-25.16	p ≤ .05
26	70.82 ± 41.44	239.89 ± 61.41	169.07	p ≤ .001	72.86 ± 29.62	2.04	N.S.	49.87 ± 26.27	-20.95	N.S.
30	222.46 ± 152.46	522.28 ± 469.90	229.82	N.S.	74.97 ± 34.83	-147.49	p ≤ .01	44.18 ± 9.61	-178.30	p ≤ .02
34	28.83 ± 13.93	309.01 ± 156.85	280.18	p ≤ .01	102.78 ± 66.30	73.95	p ≤ .05	42.28 ± 4.57	13.45	p ≤ .05

Δ * = Deviation from the normal infection mean

NS** = Not significant

TABLE 9

QUALITATIVE DETERMINATION OF FASCIOLA HEPATICA

PRECIPITABLE LABELED PROTEIN

Fraction	CPM*	CPM After TCA** Precipitation	Percent Precipitated Protein
Peak A	3,093	133	96
Peak B	2,272	178	92

* Counts per minute per 0.1 ml of the whole fraction

** Counts per minute per 0.1 ml of supernatant

TABLE 10

^{75}SE-METHIONINE INCORPORATED IN PROTEINS BY IRRADIATED

FASCIOLA HEPATICA WORMS

Radiation Dose	Counts/minute/mg	
	CPM*	% of Normal
0	4,277	100
1.5	5,303	124
2.5	4,824	113
5.0	3,759	88

* Counts per minute per mg. F. hepatica (dry weight) after

   1 hr of incubation

VII - FIGURES

Fig. 1.   Intact _Fasciola_ _hepatica_ metacercariae attached to a plastic
petri dish.   (X 75)

Fig. 2.   Activated _Fasciola_ _hepatica_ metacercariae starting to rupture
a 56-day old cyst after in vitro treatment with pepsin and
trypsin following the method of Wikerhauser (1960)   (X 150)

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Figure 3

Effects of gamma-irradiation on
the viability of *Fasciola hepatica* metacercariae
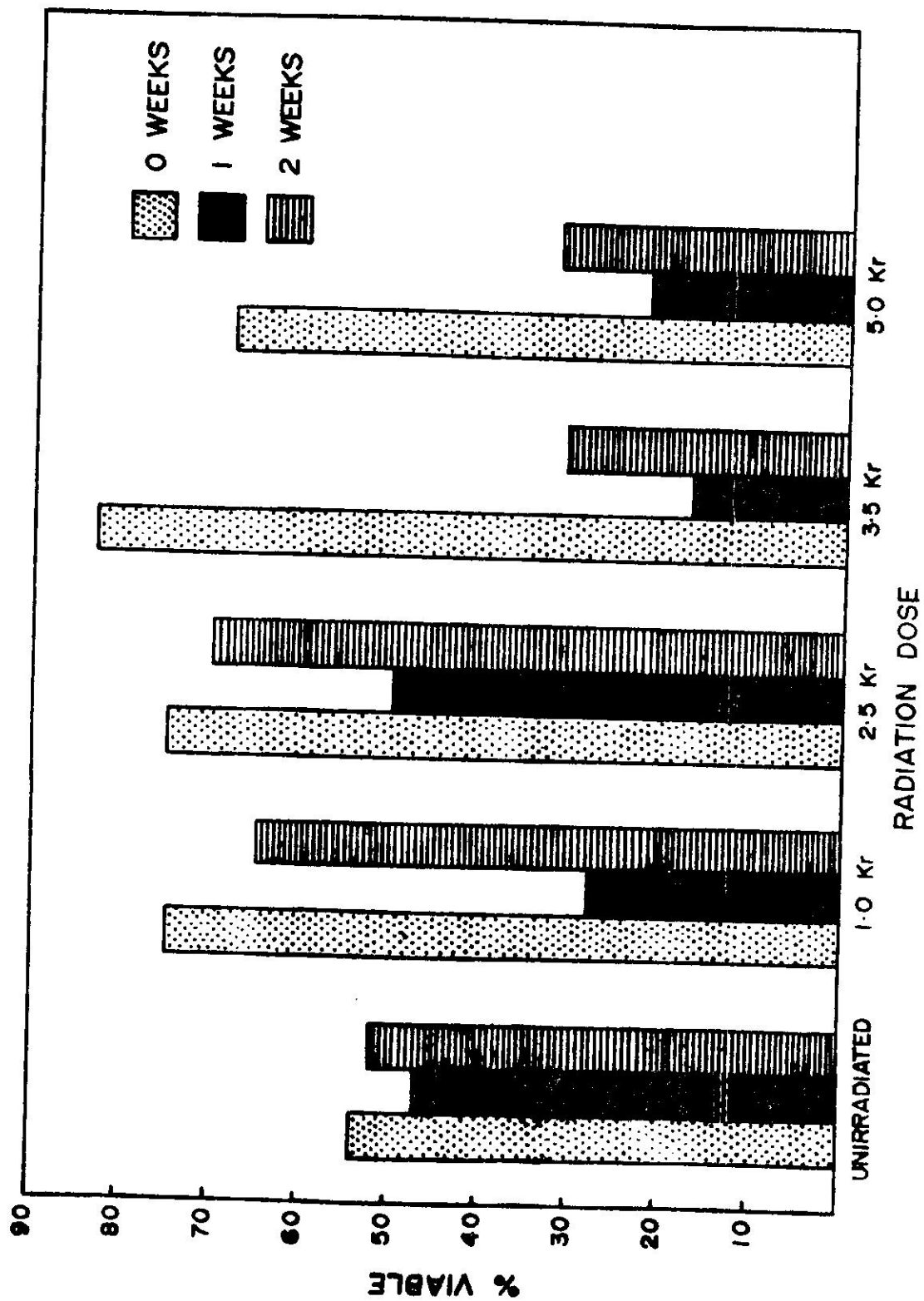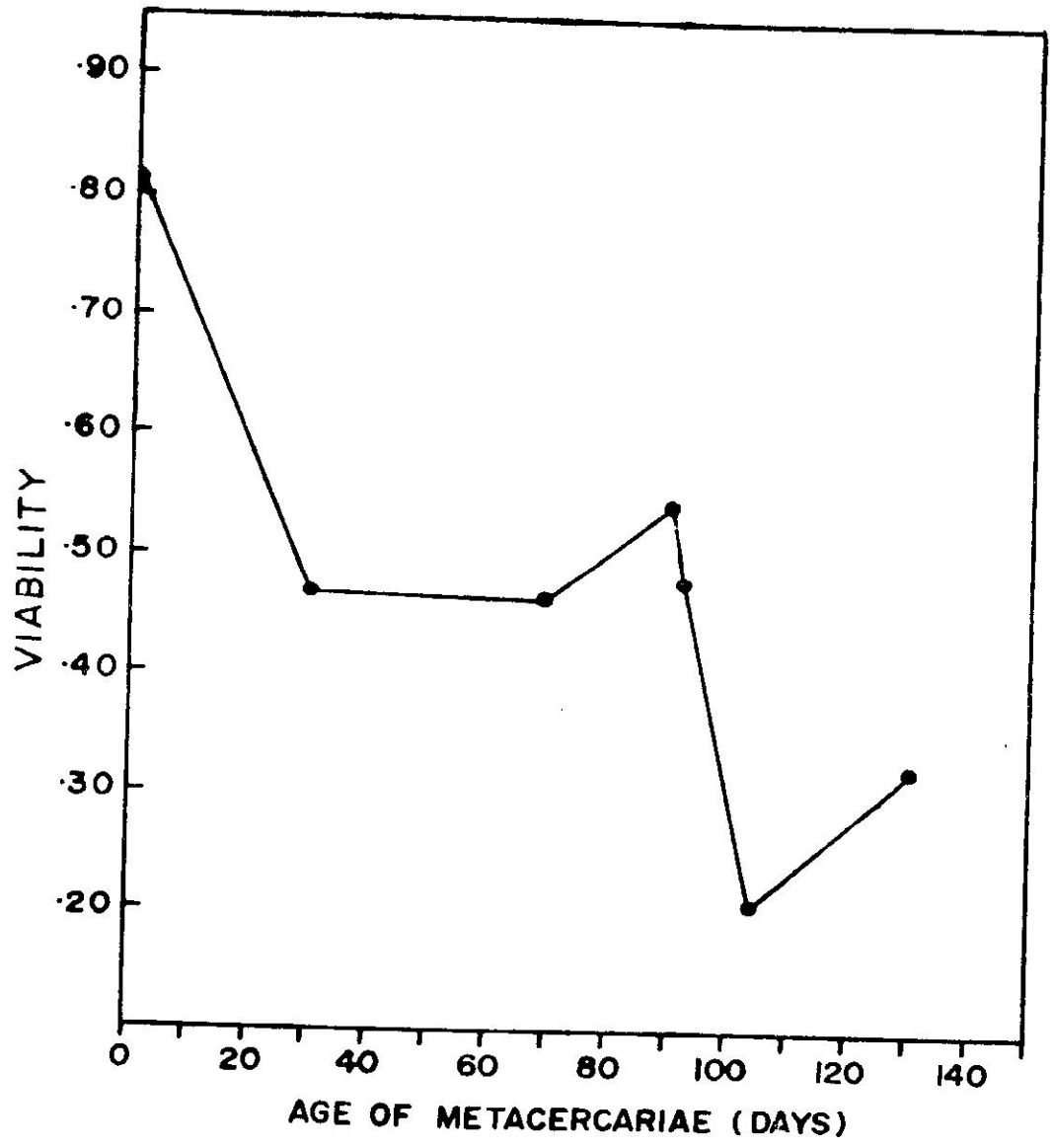
Figure 4

Age vs. viability of _F. hepatica_ metacercariae
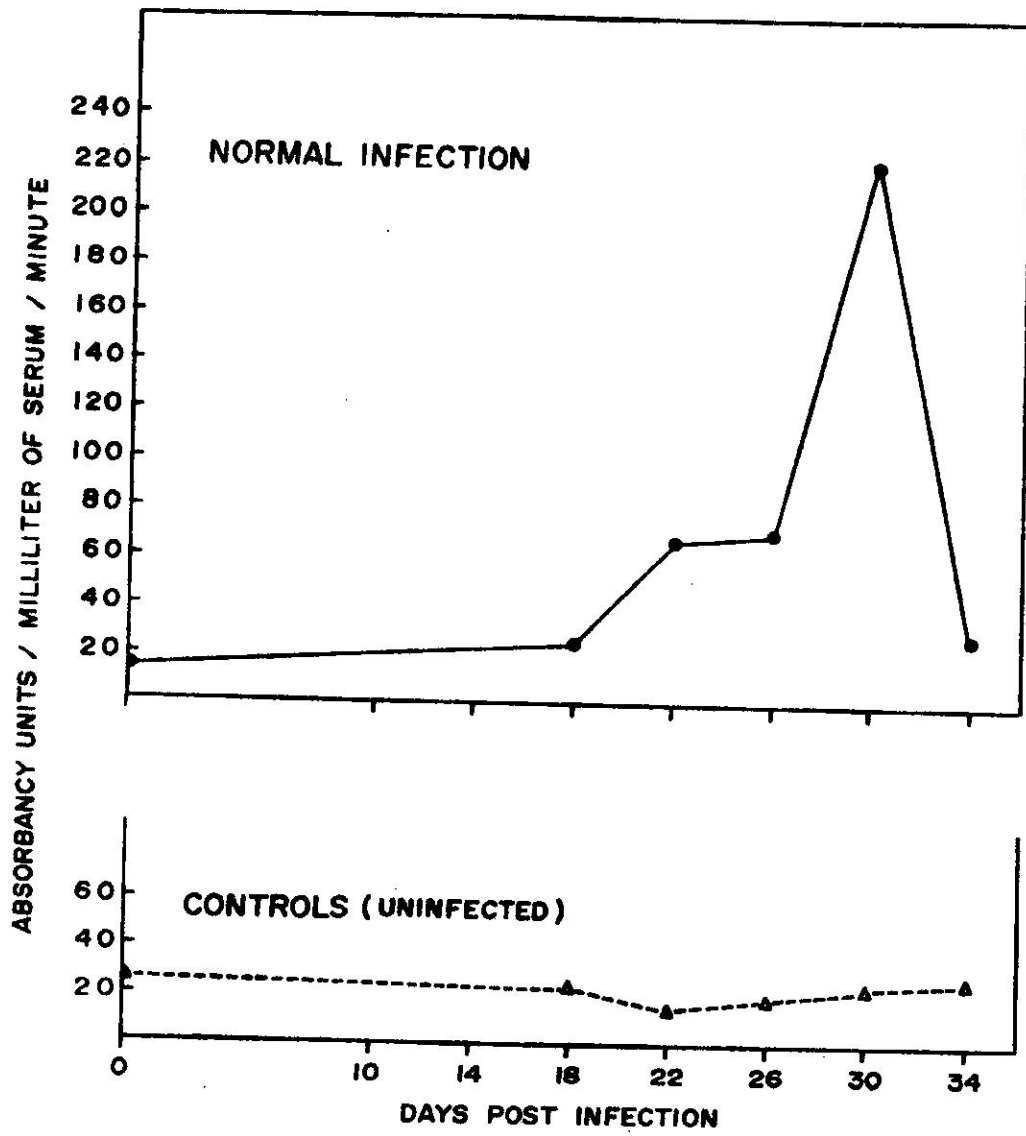
Figure 5

SGPT Levels In Uninfected Rats and Rats Infected
with Normal Metacercariae

ABSORBANCY UNITS / MILLILITER OF SERUM / MINUTE

540
520
500
480
460
440
420
400
380
360
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20

1500 RADS

2500 RADS

5000 RADS

NORMAL INFECTION

CONTROLS (UNINFECTED)

0   10   14   18   22   26   30   34

DAYS POST INFECTION
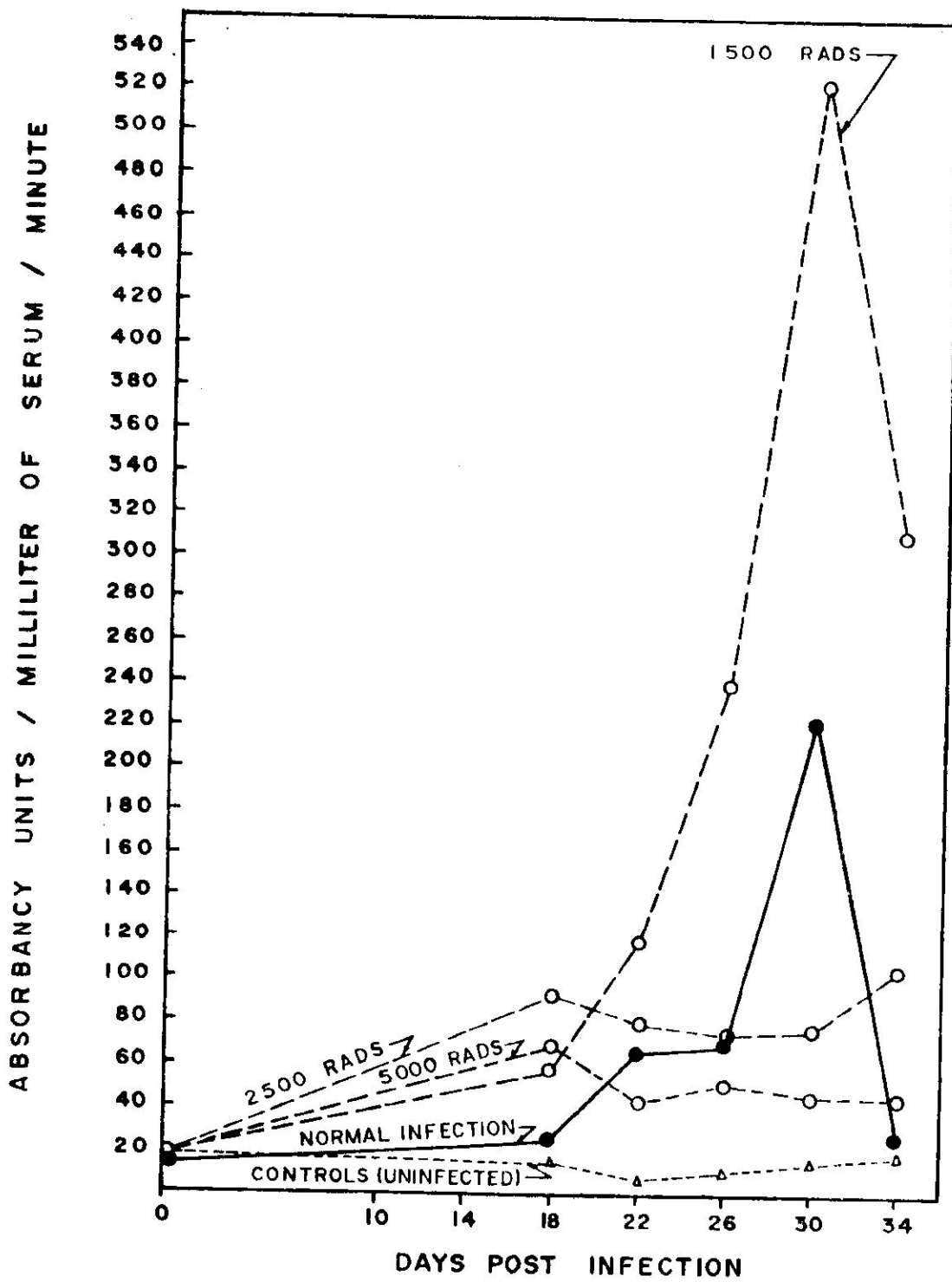
Figure 6

SGPT Levels in Rats Infected with
Gamma-irradiated F. hepatica Metacercariae
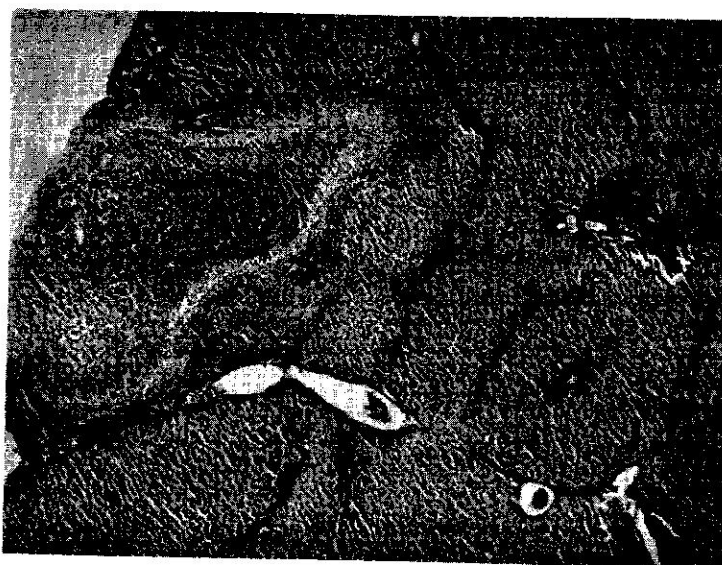
Fig. 7. Recent fluke-tract in liver parenchyma of normal infection
         (26 days post infection) filled with mononuclear, poly-
         morphonuclear eosinophilic cells, lymphocytes, cell debris
         and red cells, surrounded by fibrin and a band of loose
         connective tissue (Rat 1-2; 60.99 units of SGPT).   (X 30)
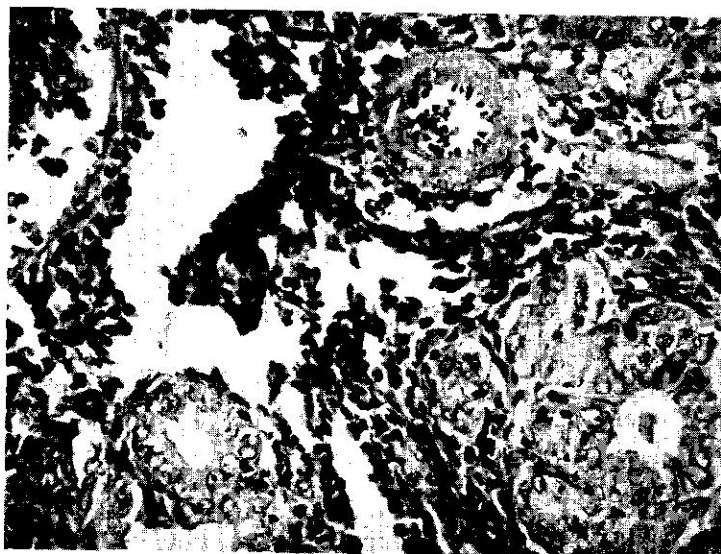
Fig. 8. Portal tract showing diffuse infiltration, edema of the artery
         and neoformation of biliary ducts in normal infection 30 days
         after inoculation (Rat 1-1; 139.05 units of SGPT).   (X 300)
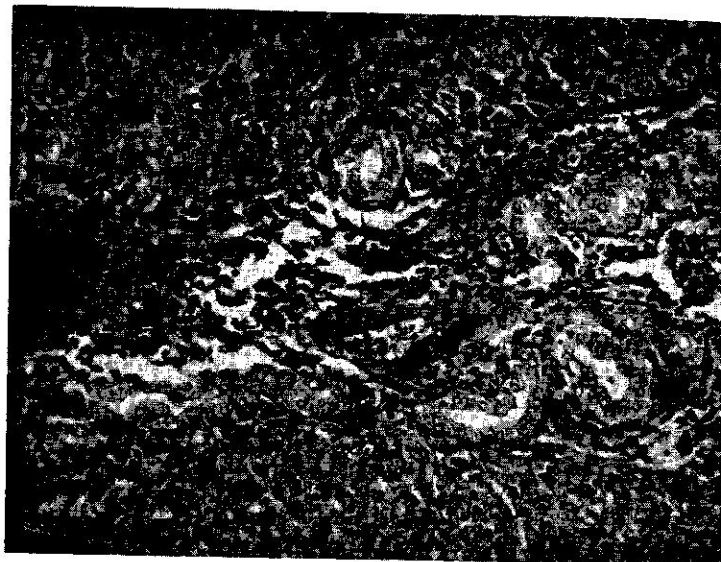
Fig. 9. Infiltration of the portal tract in normal infection with
neoformation of biliary ducts 30 days post infection (Rat
1-1; 139.05 units of SGPT).   (X 150)
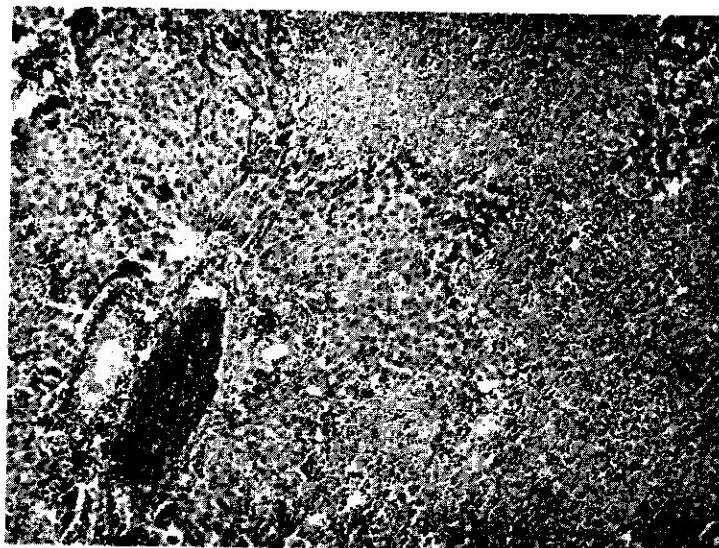
Fig. 10. Extense necrotic areas with portal infiltration in liver of
rat (2-2) infected with 1.5 Kr gamma-irradiated metacerca-
riae 30 days post infection (1219.80 units of SGPT).   (X 75)

Fig. 11. Migrating fluke irradiated with 1.5 Kr gamma-irradiation embedded in liver parenchyma (Rat No. 2-2, 30th day post-infection). (X 75)
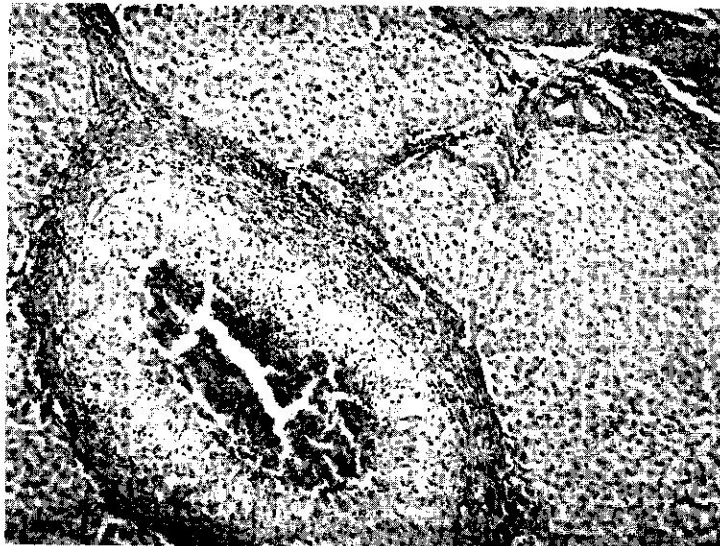
Fig. 12. Healing fluke in liver or rat (Rat 3-2) infected with 2.5 Kr gamma-irradiated metacercariae 36 days post infection (146.37 units of SGPT). (X 75)

Fig. 13. Eosinophilic infiltration in liver of rat (3-2) infected with
2.5 Kr gamma-irradiated metacercariae 34 days post infection
(146.37 units of SGPT). (X 750)
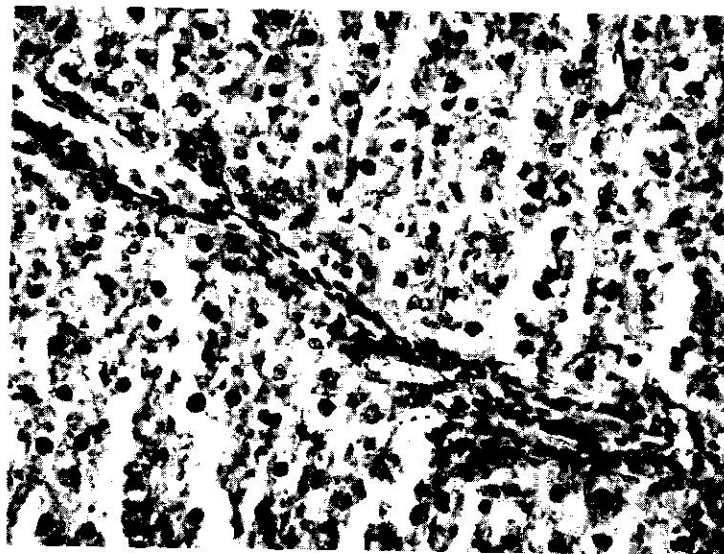
Fig. 14. Infiltration and proliferation of fibroblasts in portal
tract of rat (4-1) infected with 5.4 Kr gamma-irradiated
metacercariae 34 days post infection. (X 300)
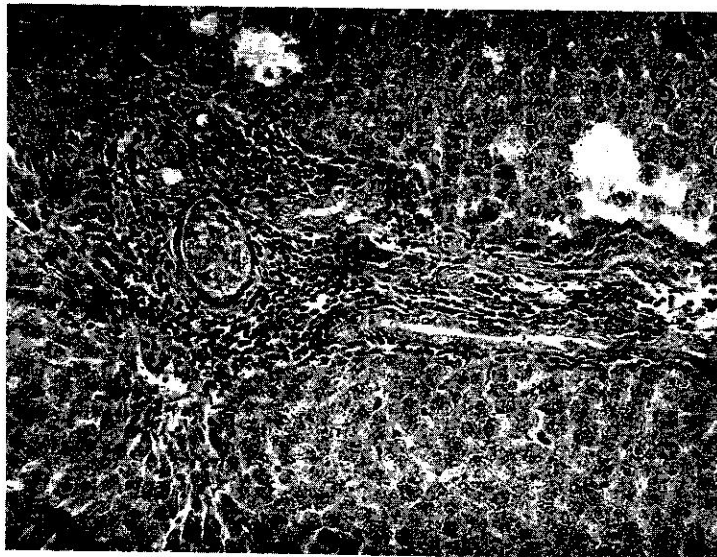
Fig. 15. Heavy infiltration of portal tract 84 days (12 weeks) post infection showing a deposited _Fasciola hepatica_ egg (Rat 1-6). (X 150)
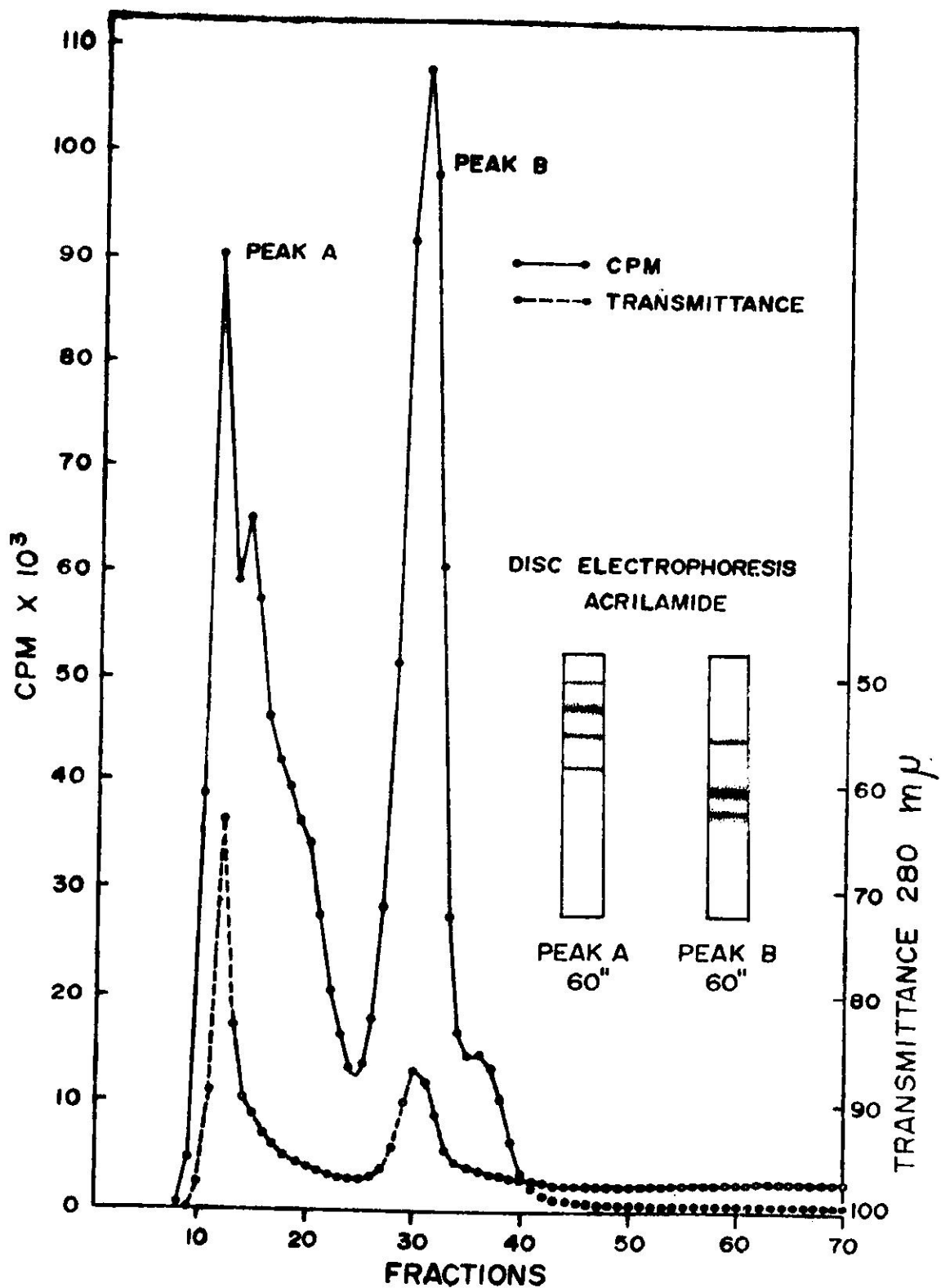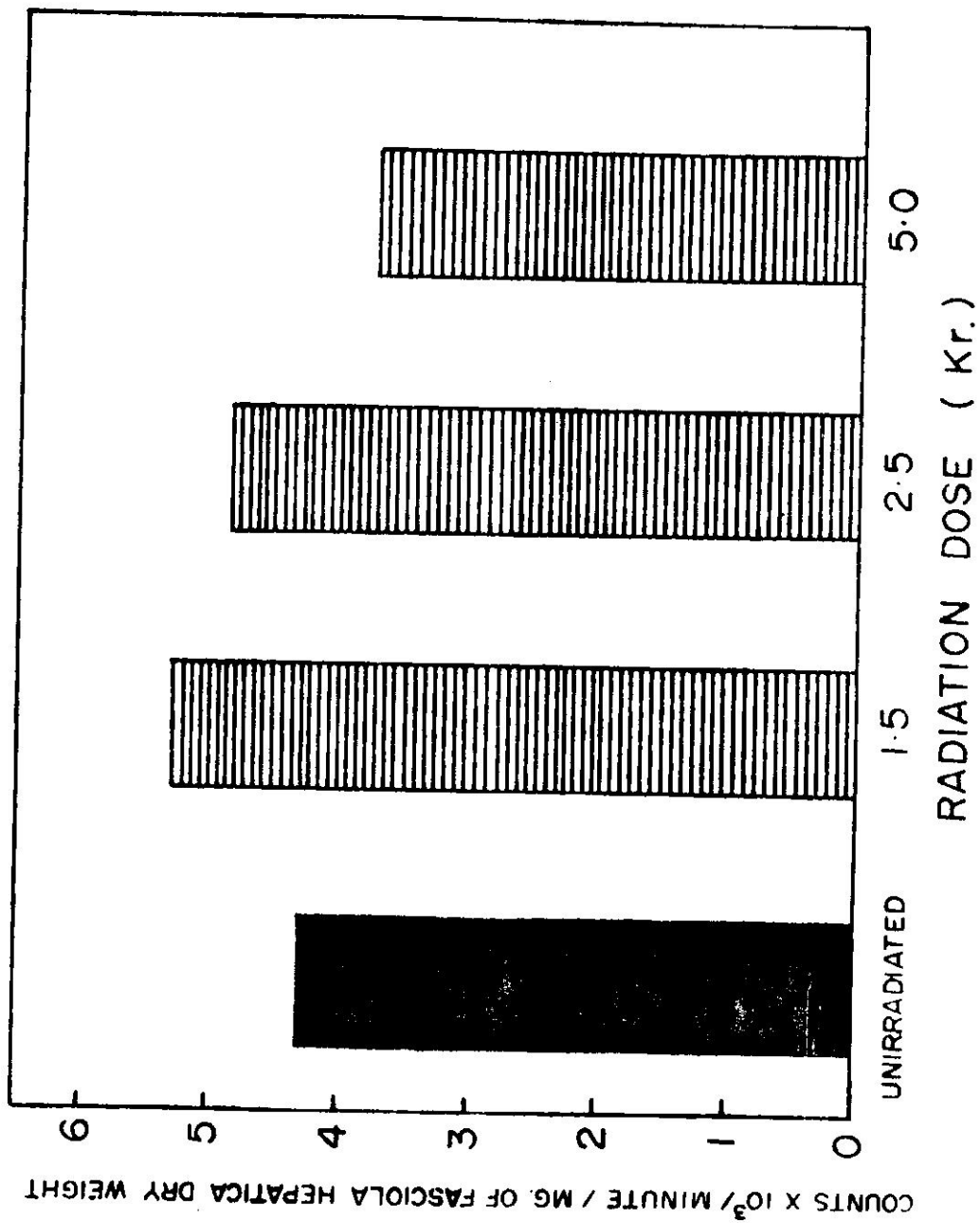
Figure 17

Isolation of Fasciola hepatica

Exoproteins Labeled With ^{75}Se-Methionine

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Figure 18

Effects of Gamma-Irradiation on the
Exoprotein Synthesis of Adult Liver Fluke

RADIATION DOSE (Kr.)

COUNTS X 10³/ MINUTE / MG. OF FASCIOLA HEPATICA DRY WEIGHT