Isotopes and Radiation in Parasitology III

PROCEEDINGS OF A RESEARCH CO-ORDINATION MEETING
KABETE, 22–26 NOVEMBER 1971
ORGANIZED BY THE
JOINT FAO/IAEA DIVISION OF ATOMIC ENERGY
IN FOOD AND AGRICULTURE

INTERNATIONAL ATOMIC ENERGY AGENCY, VIENNA, 1973

PANEL PROCEEDINGS SERIES

ISOTOPES AND RADIATION IN PARASITOLOGY III

PROCEEDINGS OF THE RESEARCH CO-ORDINATION MEETING
ON THE USE OF ISOTOPES AND RADIATION IN CONTROL OF
PARASITIC AND ASSOCIATED DISEASES IN DOMESTIC ANIMALS
ORGANIZED BY THE JOINT FAO/IAEA DIVISION OF ATOMIC ENERGY
IN FOOD AND AGRICULTURE
AND HELD IN KABETE, KENYA,
FROM 22 TO 26 NOVEMBER 1971

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FOREWORD

A continuously growing demand for high quality protein of animal origin has called for rapid intensification of livestock production and full exploitation of the existing animal wealth, particularly in the developing world. Many efforts have been made to increase animal productivity by better breeding standards, improved management systems, and more appropriate and economic feeding techniques. It is obvious, however, that only animals free from parasitic and other infective diseases can fully respond to better management and realize their capacity for growth and production. Prevention and treatment of parasitic diseases therefore form an integral part of all attempts to provide more and better food of animal origin.

It has now become well established that nuclear techniques are most useful or even essential for solving a number of problems related to animal parasitism. Radiation-attenuation by gamma or X-rays of infective parasitic stages is used to obtain safe and effective vaccines. As further examples, radioisotopes are used as tracers in studies on the aetiology and pathophysiology of parasitic diseases, and are applied in experiments to elucidate host-parasite relationships as well as the mechanisms of protective immunological processes, and are useful for diagnostic procedures. To study these promising methods of nuclear application, a co-ordinated research program, "The use of isotopes and radiation in studies of the aetiology, effects and control of parasitic diseases in domestic animals", was initiated by the Joint FAO/IAEA Division of Atomic Energy in Food and Agriculture in 1966. As the title indicates, it has been the aim of this program to further the study of, and to stimulate and co-ordinate the use of nuclear methods in animal parasitology projects in various countries. Since it was felt that the apparent similarity between many human and animal parasites would not justify a strict species barrier, the program has not been limited to animal parasitism in a strict sense. In particular the inclusion in this program of some protozoal diseases of man has proved to be extremely useful and stimulating.

The first Research Co-ordination Meeting took place in Vienna from 31 July to 4 August 1967, and the second meeting was held in Vienna from 2 to 6 June 1969. The papers and recommendations of these meetings were published by the IAEA in the Panel Proceedings Series under the title "Isotopes and Radiation in Parasitology" in 1968 and 1970. The present publication contains the contributions to the third meeting within this program, held from 22 to 26 November 1971 in Kabete, Nairobi, Kenya.

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HELMINTHIC DISEASES

ISOTOPIC METHODS IN THE STUDY OF IMMUNITY TO GASTRO-INTESTINAL HELMINTHS

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Abstract

ISOTOPIC METHODS IN THE STUDY OF IMMUNITY TO GASTRO-INTESTINAL HELMINTHS.

The uptake by Nippostrongylus brasiliensis of ^{32}P-labelled inorganic phosphate and ^{75}Se-labelled seleno-methionine from the host's tissue fluids was measured at different stages of the infection. It was found that such uptake declined rapidly from day 7 of the infection onward. Worms showing lowered uptake were able to "recover" when transferred surgically to a fresh rat, the extent of recovery depending on the length of sojourn in the original host. The results imply a significant interference with the parasites' metabolism several days before expulsion occurs.

INTRODUCTION

There are essentially two methods of approach to the immunological control of helminthic diseases. One can make a frontal attack in the sense of trying to develop vaccines against individual diseases. This approach, while demanding some knowledge of the particular host-helminth interaction, need not necessarily await a complete understanding of the immune mechanisms involved. The other method of attack is from the fundamental point of view and is aimed at understanding as completely as possible the precise mechanisms involved in the various manifestations of immunity which can be observed in many host-parasite systems. If one could separate out and characterize the antigens responsible for the stimulation of protective immunity and completely understand the mode of action of humoral and cellular elements on the parasite, it might be possible to develop techniques to enhance some of these vital phenomena and so develop practical methods of immunization against the worms concerned. It would seem logical in the present state of our knowledge that both approaches to the immunological control of helminthic disease should be prosecuted with energy.

The attention which has been given in recent years to the study of Nippostrongylus brasiliensis infection in the rat arises from the realization that it should be possible to arrive at a fairly complete understanding of the immune expulsion mechanisms in this simple host-parasite system. In many respects the system would appear to be ideal for this purpose. The host animal is cheap and large experimental groups are easily achieved; the adult rat develops a reliable immunity which is characterized by the expulsion of worms and a strong resistance to re-infection; the pre-patent period is short which means that many experiments can be carried out within a year; worms can be surgically transferred from one host to

another with reasonable facility. It is generally thought that the immunological mechanisms involved in the "rat-Nippostrongylus" system should apply, with perhaps some degree of modification, to other intestinal helminths.

Although a considerable amount of work has been carried out on this problem [1 - 4], our understanding still falls far short of the ideal outlined above. It seems likely that a disproportionate amount of energy has gone into describing changes taking place in the host at the expense of the study of changes in the parasite. Ultimately the description of immune expulsion must be given in terms of functional changes in the parasite. A description is given below of some experiments in which an attempt is made to follow how changes in the physiology of the worm relate to the development of immunity in the host. A preliminary account of some of these experiments has been given elsewhere [5].

UPTAKE BY N. brasiliensis OF ^{32}P-PHOSPHATE AND ^{75}Se-SELENOMETHIONINE FROM THE HOST

Nippostrongylus brasiliensis depends on the uptake of metabolites from the host's tissue fluids, rather than from digesta in the intestine [6]. The following experiment was designed to see whether any change took place in the uptake of metabolites from the host tissue fluids during the development of immunity. Female hooded Lister rats weighing approximately 150 g and infected with approximately 4000 N. brasiliensis larvae each were injected either with carrier-free ^{32}P sodium-dihydrogen-orthophosphate (20μCi per 150 g body weight) or with ^{75}Se-methionine (7 μCi per 150 g body weight). Separate groups of 5 - 6 rats were injected daily from day 6 to day 13 of the infection. The rats were killed at a standard time after injection (5 h after ^{32}P, 4 h after ^{75}Se) and parasites recovered as described previously [5]. In most of the experiments worms were

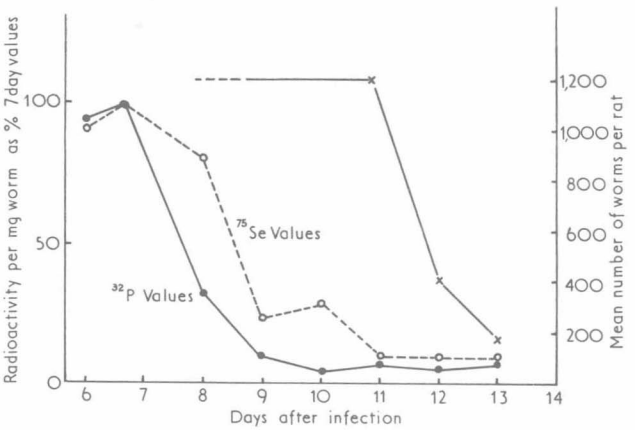

FIG. 1. Uptake by N. brasiliensis of ^{32}P-labelled phosphate and ^{75}Se-labelled selenomethionine at different stages of the infection.

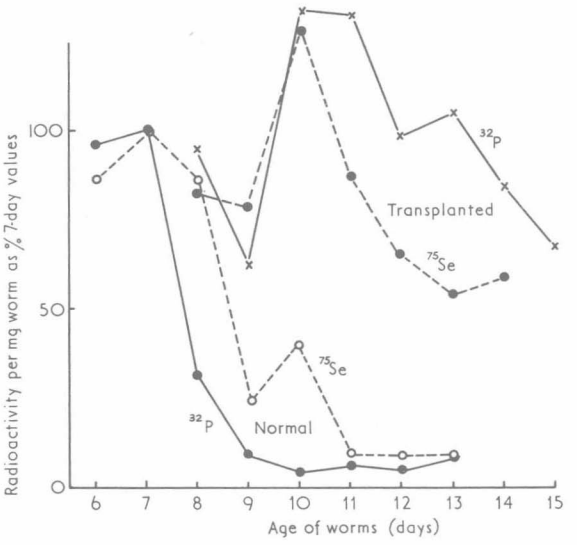

FIG. 2. Uptake of ^{32}P-labelled phosphate and ^{75}Se-labelled selenomethionine by "normal" and "transplanted" N. brasiliensis at different stages of the infection.

surface-dried and their weights recorded. Radioactivity measurements of ^{32}P were carried out by the Cerenkov counting method [7] and in the ^{75}Se experiments in a gamma-ray spectrometer.[1] The results were expressed as radioactivity per unit weight of worm and these are shown plotted against time after infection in Fig. 1. Figure 1 also shows how the worm population varies with time, i. e. the worm expulsion curve. The most striking feature of the present results is the rapid drop in the transfer of both metabolites from host to parasite several days before any worm expulsion occurs. If this depressed uptake of phosphate and selenomethionine by the parasites is immunological in origin it implies that the parasite is under some immunological pressure at a much earlier stage in the infection than has previously been thought.

UPTAKE OF ^{32}P-LABELLED PHOSPHATE AND ^{75}Se-LABELLED SELENOMETHIONINE BY "NORMAL" WORMS AND "TRANSPLANTED" WORMS AT DIFFERENT STAGES OF INFECTION

It has been recognized for some time that N. brasiliensis adults can successfully be transferred from one host to another. Indeed the most striking evidence for the immunological basis of expulsion is the fact that parasites removed from the original host when expulsion is imminent or under way get a new lease of life when introduced into the small intestine

[1] Nuclear Chicago Ltd.

TABLE I. PERCENTAGE ESTABLISHMENT IN THE NEW HOST OF WORMS TRANSPLANTED AT DIFFERENT STAGES OF THE INFECTION

Age of infection at transplant (d)	6	7	8	9	10	11	12
% Establishment	83	85	60	77	51	44	51

of "clean" rats. The previous experiment was therefore repeated on worms allowed to remain in the primary host ("normal worms") and with comparable numbers transferred to clean rats ("transplanted worms"). The results of this experiment are summarized in Fig. 2. It is clear from the curves in Fig. 2 that transplanted worms do experience a restoration of metabolic activity; indeed the day 10 - 11 worms show a supranormal uptake of ^{32}P-labelled phosphate.

An important feature of the uptake results on transplanted worms is the fairly rapid fall in both the ^{32}P and ^{75}Se graphs between day 10 and day 14. This would imply that these worms have suffered some degree of irreversible damage in the primary host which becomes more marked the longer they stay in the primary host. In this connection it is interesting to compare these results with the numbers in Table I which show how the fall in "take" changes according to the age of the primary infection.

CONTROL EXPERIMENTS

It is theoretically possible, if somewhat improbable, that the increased uptake of the labelled metabolites by transplanted worms could in fact be due to a post-surgical elevation of plasma cortisone. It is known that cortisone administration can interfere with the immune expulsion of N. brasiliensis [8]. To cover this point experiments were carried out with rats treated with cortisone (0.2 mg of betamethazone on days 5, 7, 9 of the infection) and also on "sham-operated" rats. The results of such an experiment are shown in Table II. Two points emerge clearly from these results: firstly, by the administration of cortisone the uptake of phosphate by the parasites remains elevated at day 11. This is completely in line with the effect that cortisone has on the prolongation of the infection; secondly there is no evidence that sham-operation has in any way parallelled this effect. The results in Table II were obtained using ^{32}P-labelled phosphate. Similar findings have arisen from the ^{75}Se experiment.

FACTORS RESPONSIBLE FOR REDUCED METABOLITE "UPTAKES"

At present it is not possible to be categorical about the explanation for the reductions in uptake of phosphate and selenomethionine by the parasites which occur during the development of immunity. These effects could be due to a specific interference by the host's humoral or cellular defence mechanisms with certain metabolic pathways in the parasite.

TABLE II. ^{32}P UPTAKE BY WORMS IN "SHAM-OPERATED" AND CORTISONE-TREATED RATS

Day of experiment	Counts per minute per mg of worm		
	Normal infection	Sham operated	Cortisone treated
6	104.8		
7	110.2		150.8 (4)
8	35.4		
9	10.6	22.0 (4)[a]	154.2 (4)
10	4.3	17.6 (6)	
11	8.4	7.9 (6)	97.8 (4)
12	5.8		
13	9.4		

[a] Figures in parentheses show the numbers of rats involved in each case. Analyses were carried out on bulked samples from each group. The mean uptake figures for the normal infection in the previous experiment are included for comparison.

On the other hand the same effects could arise from a more general relatively non-specific metabolic impairment, e. g. if the local anaphylactic reaction in the gut were responsible for displacing the parasite from its privileged position in the paramucosal region to a less salubrious environment towards the lumen of the gut. This might be particularly important in relation to changes in oxygen tension in the environment.

An attempt was made recently to investigate such a possibility by comparing the immune elimination of Nippostrongylus from rats kept in a normal atmosphere with that of groups kept for one to two days at the critical time in an atmosphere of pure oxygen [9]. It was thought that the increase in dissolved oxygen in the tissue fluids generally resulting from the 100% O_2 environment might partly ameliorate the effects on the worms of some displacement away from the region of high p_{O_2} in the paramucosa, and that this in turn might delay their elimination. The results were somewhat equivocal but in general exposure to 100% oxygen did appear to permit the parasites a slightly longer sojourn in the host. Interpretation of the results was rendered difficult however by the possible involvement of oxygen toxicity and its well-known effects on the adrenal cortex. It is possible that the oxygen exposure did nothing more than raise the level of blood cortisone in the rats of the treated group.

It seemed that changes in the uptake of labelled metabolites from the host might provide a more sensitive index to use in the study of the oxygen effect than actual elimination of worms. An experiment was carried out in which control rats were maintained in air and the experimental groups exposed for 4 h only to 100% oxygen. It was hoped that in this way possible oxygen toxicity would be limited. Labelled selenomethionine was injected into both groups of animals and after the standard time worms recovered and assayed for radioactivity. The results of this experiment are shown in Table III. At days 8, 9, 10 and 11 of the infection the oxygen group did in fact show significantly increased uptake. This would support the hypothesis that the depressed uptake observed at this

TABLE III. UPTAKE OF ^{75}Se-SELENOMETHIONINE BY N. brasiliensis IN CONTROL RATS KEPT IN AIR AND IN RATS KEPT IN 100% O_2 DURING THE 4-h UPTAKE PERIOD

Day of infection	Counts per minute per mg of worm	
	Control group	Oxygen group
7	30.3	17.0
8	7.7	12.7
9	4.5	6.0
10	4.2	8.9
11	4.3	7.5

time is due to partial anaerobiasis resulting possibly from a simple displacement of the worms. The experiment will need to be repeated and to include measurements of blood cortisone to eliminate the possibility of an oxygen toxicity effect resulting from even the 4-h exposure to 100% O_2.

No explanation can be offered at present for the day 7 result which is somewhat anomolous, except that in all experiments so far measurements made on day 7 often seem out of line with neighbouring measurements.

DISCUSSION

Over the last five years ideas about the expulsion mechanism in N. brasiliensis have been dominated by what one might call the "macromolecular leak" hypothesis. This idea arose from the demonstration of a local anaphylactic reaction in the gut of rats infected with N. brasiliensis [10, 11]. The increased capillary permeability associated with such a reaction seemed to provide a possible way in which circulating antibody could come in contact with the parasites in significant quantities. Strong supporting evidence was provided by the demonstration of the occurrence of fairly high titres of reagins in the serum of the rats infected with N. brasiliensis [12], and work with labelled PVP and ^{51}Cr plasma protein showing that the gut permeability to macromolecules reached a peak around the time of expulsion. This suggested a two-stage mechanism; stage 1 being a local anaphylaxitic reaction with a consequent increase in gut permeability which would then permit antiworm antibody to escape into the gut and in some manner bring about stage 2, i. e. the elimination of the worms. It seems now that this hypothesis will require modification.

Recent studies on the parasite itself have demonstrated that structural changes and changes in infectivity on transfer can be detected some time before elimination [13, 14] and the experiments reported here show that functional changes in the parasite are observable from day 7 onwards, i. e. a full four days before the onset of expulsion. It has been suggested recently [15] that the order of events described in the original leak hypothesis may in reality be reversed, and that the major leak reaction (days 11 - 14) is associated with a comparatively non-specific expulsion of worms which have already suffered serious immunological damage. The

physiological experiments described here would be in line with such a hypothesis.

A study of the effects of host immunity on parasite physiology is fraught with a number of difficulties and pitfalls. In the past parasites have been maintained in vitro and attempts have been made to observe changes in metabolism associated with the addition of immune serum or immune cells to the medium. The dangers inherent in such an approach are fairly serious. The in vitro environment is likely to be sufficiently abnormal for worm metabolism to be seriously interfered with and many misleading ideas about normal worm metabolism have indeed arisen as a result of in vitro studies [16]. To follow the metabolism of the parasite in its host is again not easy, but we feel that the system described here has something to commend it. By the use of a suitable range of labelled metabolites one should be able to get information about many aspects of the metabolism of parasites in their natural environment, and the changes which take place with developing immunity in the host. Interpretation of results will, however, always present difficulties. For example, if a parasite is suffering relatively non-specific damage, e.g. as a result of the formation of a precipitate at some important body orifice or by some change in its immediate environment, one must expect consequential metabolic alterations and it will always be a problem to decide whether such alterations are primary or secondary.

REFERENCES

[1] MULLIGAN, W., "Immunity to intestinal helminths: the self-cure reaction", Immunity to Parasites, Sixth Symposium of the British Society for Parasitology (TAYLOR, A.E.R., Ed.), Blackwell Scientific Publications, Oxford (1968).

[2] JARRETT, E.E.E., URQUHART, G.M., "The immune response to nematode infections", Tropical Medicine 4, Academic Press, New York (1971).

[3] OGILVIE, B.M., JONES, V.E., Nippostrongylus brasiliensis: a review of immunity and the host/parasite relationship in the rat, Expl parasit. 29 (1971) 138.

[4] MURRAY, M., JARRETT, W.F.H., JENNINGS, F.W., Mast cells and macromolecular leak in intestinal immunological reactions, Immunology 21 (1971) 17.

[5] HENNEY, R.W., Mac LEAN, J.M., MULLIGAN, W., The effect of host immunity on the metabolism of Nippostrongylus brasiliensis, Immunology 21 (1971) 711.

[6] ROGERS, W.P., LAZARUS, M., The uptake of radioactive phosphorus from the host tissues and fluids by nematode parasites, Parasitology 39 (1949) 245.

[7] PARKER, R.P., ELRICK, R.H., The assay of β-emitting radioisotopes using Cerenkov counting, Int. J. appl. Radiat. Isotopes 17 (1966) 361.

[8] OGILVIE, B.M., Use of cortisone derivatives to inhibit resistance to Nippostrongylus brasiliensis and to study the fate of parasites in resistant hosts, Parasitology 55 (1965) 723.

[9] MULLIGAN, W., JARRETT, E.E.E., URQUHART, G.M., The expulsion mechanism in Nippostrongylus brasiliensis infection, Parasitology 59 (1969) 16P.

[10] MULLIGAN, W., URQUHART, G.M., JENNINGS, F.W., NEILSON, J.T.M., Immunological studies on Nippostrongylus brasiliensis infection in the rat: the "self-cure" phenomenon, Expl parasit. 16 (1965) 341.

[11] URQUHART, G.M., MULLIGAN, W., EADIE, R.M., JENNINGS, F.W., Immunological studies on Nippostrongylus brasiliensis infection in the rat: the role of local anaphylaxis. Expl parasit. 17 (1965) 210.

[12] OGILVIE, B.M., JONES, V.E., Passive protection with cells or antiserum against Nippostrongylus brasiliensis in the rat, Parasitology 58 (1968) 939.

[13] LEE, D.L., Changes in adult Nippostrongylus brasiliensis during the development of immunity to this nematode in rats: 1. Changes in ultrastructure, Parasitology 59 (1969) 29.
[14] OGILVIE, B.M., HOCKLEY, D.J., Effects of immunity on Nippostrongylus brasiliensis adult worms: reversible and irreversible changes in infectivity, reproduction and morphology, J. Parasit. 54 (1968) 1073.
[15] JONES, V.E., OGILVIE, B.M., Protective immunity to N. brasiliensis: the sequence of events which expels worms from the rat intestine, Immunology 20 (1971) 549.
[16] SMITH, M.H., Do intestinal parasites require oxygen?, Nature, Lond. 223 (1969) 1129.

ANTIGENIC ANALYSIS OF Fasciola hepatica: EXTRACTION AND FRACTIONATION*

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Abstract

ANTIGENIC ANALYSIS OF Fasciola hepatica: EXTRACTION AND FRACTIONATION.

Antigenic material from adult Fasciola hepatica, caecal content of adult parasites and bile of infected sheep was separated and analysed. The studies were undertaken to isolate antigenic components which were immunologically active during the infection of rabbits with F. hepatica. In each of the three materials, the host serum proteins were identified using sheep serum antibodies. It was proven by disc electrophoresis that purification of the caecal content antigens was the most successful procedure.

INTRODUCTION

The complex structure of many parasites stimulates more detailed investigations on the localization of the site of antigen production, and of the sites of antibodies binding in the parasite [1, 2]. Investigations directed towards the isolation and characterization of antigens in parasites have shown that it is possible to differentiate between several antigens for diagnosis or immunoprophylaxis [3-7].

The immunological activity of somatic and metabolic antigenic material of Fasciola hepatica has not been studied exhaustively. Different methods for extraction and isolation of antigenic material from this parasite have been used to obtain purified antigens needed for immunodiagnosis [8-11]. Some recent investigations on antigen structure of F. hepatica showed that in the extract of this parasite at least 15 antigen components were present [12].

Examination of some lipoproteins [13, 14], polysaccharides [15-17] and protein components by column chromatography [18-20] enabled more detailed analyses to be made of the active antigen components during the infection of animals with F. hepatica. Special attention was paid to the analysis of extracts of adult stages of F. hepatica, bile of infected animals and caeca contents of liver flukes.

MATERIALS AND METHODS

Antigen sources

Antigenic material was obtained from lambs artificially infected with metacercariae of F. hepatica. Three months after infection the animals were sacrificed and the antigenic material separated as follows.

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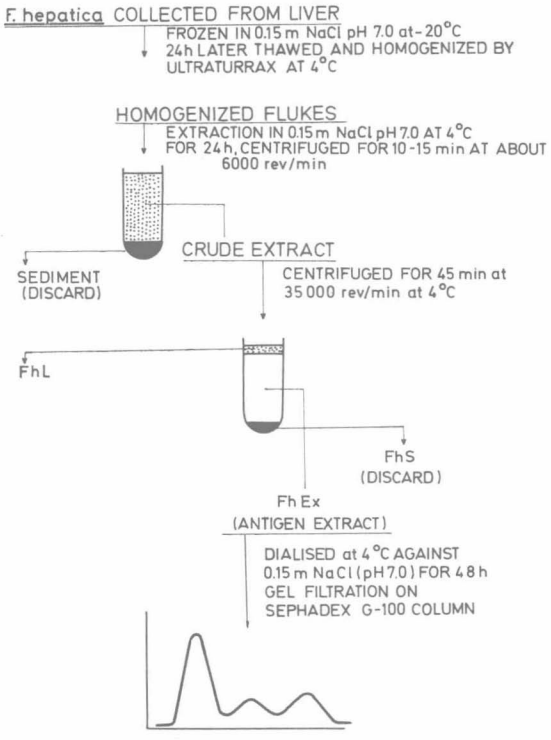

FIG. 1. Fractionation of antigenic material from F. hepatica.

An extract of the adult stage of F. hepatica was obtained after the removal of bile and caecal contents by rinsing the parasites in 0.15 M NaCl, pH 7. The washed parasites were frozen in 0.15 M NaCl at -20°C. Twenty-four hours later the parasites were brought to room temperature and homogenized in Ultraturrax and Teflon homogenizers. The homogenates were kept at 4°-6°C for 24 h. Large particles were sedimented by centrifugation for 10 - 15 min at 6000 rev/min at 4°C. The supernatant was centrifuged again at 35 000 rev/min for 45 min at 4°C, after which three layers had formed in the test tube. The sediment (FhS) was composed of fine particles; the middle layer (FhEX) represented 90% of the whole homogenate, and the thin surface layer (FhL) was probably of lipid origin. In further examinations the middle homogenate layer (FhEX) was used as an antigen extract of F. hepatica (Fig. 1).

Bile from the infected lambs was collected from the d. hepaticus and v. fellea and then centrifuged at 6000 rev/min for 15 min to remove all particulate material and F. hepatica eggs. The resulting supernatant was centrifuged at 35 000 rev/min for 45 min at 4°C, and for further investigations the middle layer, formed after the centrifugation, was used as antigen (FhB).