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Biochemical aspects of fascioliasis in domestic and experimental animals.

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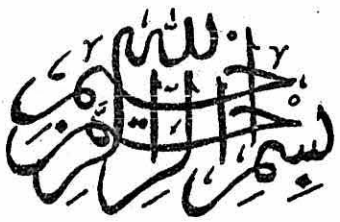

IN THE NAME OF ALLAH, THE BENEFICENT, THE MERCIFUL

BIOCHEMICAL ASPECTS OF FASCIOLIASIS IN DOMESTIC AND EXPERIMENTAL ANIMALS

Thesis submitted to the University of Wales

by

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SUMMARY

Sheep, cattle, rats and guinea pigs were experimentally infected with F. hepatica. During infection plasma and tissue ascorbic acid and plasma cholesterol levels decreased whereas the plasma low-density lipoproteins increased. Glucose levels remained unchanged. Disturbance of iron metabolism occurred as evidenced by the decrease in plasma and liver iron concentrations and the increase in the iron-binding capacity and these were related to the changes in ascorbic acid levels.

Supplementation of ascorbic acid to infected rats and guinea pigs was not effective in reducing fluke burdens. However, infected guinea pigs given a low vitamin C dose showed earlier symptoms of illness and mortality.

The phagocytic and bactericidal capacities of polymorphonuclear leukocytes of infected sheep and guinea pigs increased compared to controls. The effect of two dose levels of vitamin C on these activities was investigated in guinea pigs.

The plasma proteins, glycoproteins and lipoproteins were investigated in controls and fluke-infected sheep and cattle. A hyperproteinaemia, due to increased α- and γ-globulins was a feature of the prepatent period, and a progressive hypoalbuminaemia developed during the course of infection. Changes in glycoproteins were expressed as elevated protein-bound hexoses, hexosamine, fucose, seromucoid and sialic acid. The plasma α-lipoproteins decreased while the β-lipoproteins increased after exposure.

Collagenolytic and elastinolytic activities were demonstrated in F. hepatica metacercariae, immature flukes, and adult flukes. Hyaluronidase-like activity was also detected in mature flukes. These findings were discussed in relation to the penetrating mechanisms and migration of these parasites. Plasma of infected sheep inhibited the activities of Cl. histolyticum collagenase and testicular hyaluronidase.

CONTENTS

The fact that fascioliasis is a PARASITIC ZOONOSIS makes the study of this disease more interesting.

Man can be infected by the accidental ingestion of metacercariae and develop symptoms of liver damage. In areas where raw liver is habitually eaten, immature flukes may cause a sever laryngopharyngitis which could be fatal. This condition was described a long time ago in the residents of Lebanon and Syria under the name " the HALZOUN disease" (Khouri, A., 1905. Le halzoun. Arch. Parasitol., 9 , 78 - 94). A similar condition was also reported in Western Sudan, known locally as " MARRARA " disease (Salman, H. E. and Mahdi, Y. The Marrara syndrome at Abu Deleig. Sudan Med. J., 1, 1-4). In Sudan, Marrara is a dish composed of raw liver, rumen, omasum, reticulum and lung; sometimes spleen, kidney and trachea are added. These are thoroughly washed with water and mixed with onion, lemon, salt, chillies and bile.

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Fascioliasis is a cosmopolitan parasitic disease which affects a wide range of animal hosts and occasionally man. F. hepatica and F. gigantica are commonly incriminated in the causation of serious disease in sheep, cattle and goats in many parts of the world; the former parasite is prevalent in the temperate regions and the latter predominates in the tropical and subtropical zones. In economic terms, Fasciola infections may cause loss of revenue through partial condemnations of infected livers or total condemnations of whole carcasses which become severely emaciated by infection. In the field, outbreaks of acute fascioliasis in sheep and cattle may result in high mortalities among livestock, while the chronic infection could be associated with inappetance and decreased food utilization, low weight gains, poor milk production, poor fleece yield in the case of sheep, and still-birth or at least low birth weights. (See Dargie, 1973).

Fasciola species can cause varying degrees of liver damage seen as haemorrhages and hepatic fibrosis due to the action of the migrating immature flukes, and as hyperplastic cholangitis caused by adult parasites in the bile ducts (Dawes, 1963; Dow, Ross and Todd, 1968; Sinclair, 1972, Rushton and Murray, 1977). The liver is a central organ for carbohydrate, protein and lipid metabolism, as well as the main site for conjugation and detoxification processes. Damage to this organ, as occurs in fascioliasis, can cause many metabolic disturbances which could be fatal to the animal. The liver is

the main source of the plasma proteins and the latter may change markedly in fluke infection; these changes are often expressed as hypoalbuminaemia, initial hyperglobulinaemia and general hypoproteinaemia in advanced cases (Furmaga and Gundlach, 1967; Dargie, 1973). Although changes in plasma proteins have been investigated by many authors, little seems to have been reported on the changes which occur in plasma lipoproteins and particularly glycoproteins. The plasma lipoproteins are concerned with lipid transport, and the various lipid classes they carry are necessary for many cellular and membrane functions. Similarly glycoprotein components perform a wide range of functions and they include transport proteins, enzyme inhibitors, coagulation factors and antibodies (Gottschalk, 1972; Rodhradska, Rodhradsky and Andrasina, 1976). It is felt that investigations on the changes occurring in plasma glycoproteins and lipoproteins could reveal important metabolic changes which could be of value in understanding more about the pathogenesis of this important disease, and some aspects of these plasma proteins have been dealt with in this study.

Current literature attributes many important functions to ascorbic acid. The vitamin is reported to play an important role in preserving the structural integrity of tissues, in collagen formation, in wound healing and tissue repair, in iron and other mineral metabolism, in lipid metabolism, and in the functional activity of phagocytes, besides many other functions (Wilson, 1974, 1975; Barnes, 1975; Kotze, 1975; Charlton and Bothwell, 1976; Turley, West and Horton, 1976;

Kim, 1977). Deficiency of vitamin C could therefore lead to serious metabolic and functional disturbances. Disturbances in lipid and iron metabolism and reduction in the phagocytic activity of blood neutrophils have been reported in fascioliasis, besides the extensive hepatic damage and fibrosis which are the main features of infection. It seems possible that ascorbic acid deficiency might be involved in causing these disturbances. Information on ascorbic acid status in fluke-infected animals is apparently lacking and to the best of the author's knowledge no previous reports have ever associated the symptoms or lesions of fascioliasis with vitamin C deficiency or otherwise. Similarly, no reports seem to be available on the effect of ascorbic acid supplementation on the course of fluke infection. In the following study changes in plasma and tissue ascorbic acid are investigated in fluke-infected animals and these are discussed in relation to those changes occurring in other parameters such as plasma lipids and plasma iron concentrations. The effect of vitamin C supplementation to fluke-infected rats and guinea pigs was also investigated.

Fasciola species pass through different stages of development and use snail intermediate hosts to complete a cycle. It is generally accepted that the parasites penetrate the hosts' tissues mechanically but there is evidence that enzymatic action may be involved. Howell (1962) demonstrated collagenolytic activity in immature F. hepatica, which was almost absent from mature flukes and he suggested that different stages of the parasite may secrete different enzymes. In addition to collagenase, more information about other fluke

enzymes which could be directly involved in tissue breakdown i.e. elastase and hyaluronidase is needed; such information may explain much about the penetrating mechanism of flukes, especially in repeated infections. Pure preparations of these enzymes could be used to produce anti-enzymes which might prove to be effective in retarding the migration of flukes.

Review of the literature shows that F. hepatica is the most investigated fluke species. The same parasite is also used in this study. In the Sudan, the author's homeland, F. gigantica is the sole parasite causing fascioliasis, a disease which is of prime economic importance in that country; at meat inspection about 45% and 6.7% of the total number of condemned cattle and sheep livers respectively, is attributed to fascioliasis (Gameel, 1975). It is hoped that the information already available on F. hepatica and that which could, hopefully be revealed by this study may be fruitfully utilized in conducting future studies with F. gigantica. Many experiments which have already been carried out on F. hepatica could be repeated using F. gigantica in the hope that some light might be shed on the differences which may exist in the reactions and syndromes of animals infected by these two related species of liver flukes.

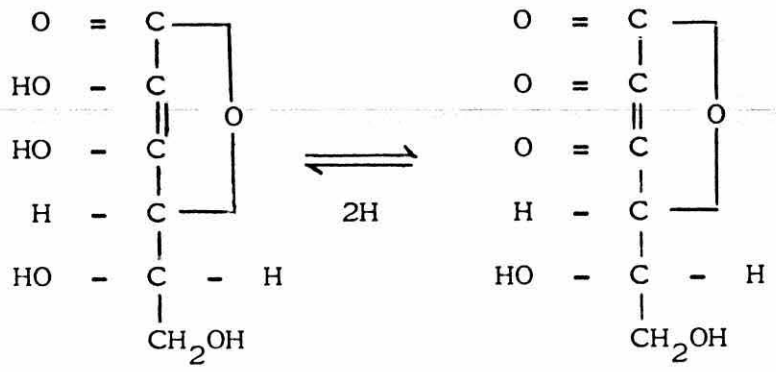
PART A

ASCORBIC ACID AND ITS METABOLIC INTER-RELATIONSHIPS

INTRODUCTION:

Ascorbic acid or vitamin 'C' is the first adjunct whose deficiency was recognised as the cause of a disease, Scurvy. The disease which is associated with increased vascular permeability, oedema, intestinal haemorrhages, bleeding into the skin and deeper tissues and a fulminating gingivitis, was a common disorder among seamen on long voyages and could be prevented and cured by eating fresh fruits and vegetables. The antiscorbutic vitamin, ascorbic acid, was isolated in 1928 and identified and synthesized in 1933 (Szent-Gyorgyi, 1928; Szent-Gyorgyi and Haworth, 1933; Haworth, 1933; Reichstein, Grussner and Oppenhauer, 1933).

Ascorbic acid is a powerful reducing agent, giving up two hydrogen atoms to become dehydroascorbic acid; this oxidation is reversible in the animal body (Bell, Davidson and Emslie-Smith, 1972).



L-ascorbic acid L-dehydroascorbic acid

L-ascorbic acid and L-dehydroascorbic acid are the only known naturally occurring biologically active forms.

The vitamin is synthesized by certain moulds, fungi, the higher plants and by animals except the guinea pig, some flying mammals and primates including man (Chatterjee, Majumder, Nandi and Subramanian, 1975). The biosynthetic pathway is as follows:

D-glucose ⟶ D-glucuronic acid ⟶ L-gulonic acid ⟶ L-gulono-gamma lactone ⟶ L-ascorbic acid

(Chatterjee, 1970; Gupta, Choudhary and Chatterjee, 1973). The conversion of L-gulono-gamma lactone to L-ascorbic acid is catalysed by the enzyme L-gulono-lactone oxidase which is lacking in animals incapable of synthesizing the vitamin (Gupta et al., 1973).

Among fruits and vegetables, rose hips, blackcurrant, leafy vegetables i.e. cabbage, paprika, Guava and citrus fruits are especially rich in vitamin C. In animals, ascorbic acid is present in all body tissues and fluids. The adrenals, pituitary, thymus, ovary, testes, corpus luteum and the eye lens are rich sources; liver, spleen, kidney, heart and lung possess relatively lower concentrations (Olliver, 1954; Bell et al., 1972).

METABOLIC INTERACTIONS OF ASCORBIC ACID:

Since its discovery, ascorbic acid has been subjected to extensive investigations and yet its exact mode of action remains largely unknown (Cameron, 1976). A summary of the basic cellular mechanism of ascorbic acid is given by Wilson (1974). He states that the effect of vitamin 'C' could be related to the physico-chemical properties of the ascorbate system (ascorbic acid and dehydroascorbic acid) which is involved in oxidation-reduction reactions, complex formation and hydrogen bonding. It also acts as a buffering system in the cell by acting as a hydrogen donor and acceptor. Indirectly it enhances adrenaline formation through the cyclic AMP system and prevents its oxidative degeneration. It also inhibits phosphodiesterase hydrolytic breakdown of cyclic AMP and enhances the action of and protects cyclic G.M.P.; in tissues both cyclic AMP and GMP potentiate many enzymatic activities associated with exocrine secretion, hormone secretion and action and increased membrane permeability. Ascorbic acid also plays a role in the metabolism of tyrosine and phenylalanine.

Ascorbic acid and connective tissue:

Perhaps the most important physiological function of ascorbic acid is its role in maintaining the integrity of the body's viscoelastic system. Ascorbic acid catalyzes collagen formation (Gould, 1958; Bavetta, O'Day and Bekhor, 1961), and enhances the synthesis of micropoly-saccharides (Kofoed and Robertson, 1966) and amino acids (Kim, 1977). The vitamin is reported to act as a cofactor

which participates with other factors in collagen and elastin hydroxyproline and hydroxylysin synthesis (Barnes, 1975; Barnes, Constable, Morton and Kodicek, 1970). Hence lack of ascorbic acid leads to impaired collagen formation and consequently to defective wound healing, occurrence of fractures, haemorrhages, impaired bone formation and delayed tissue repair (Priest, 1970; Veen-Baigent, Ten Cate, Bright-See and Rao, 1975; Belfield, 1976). Various osteodystrophies have been attributed to vitamin C deficiency in animals (Meier, Clark, Schnelle and Will, 1957; Holmes, 1962; Schimke, Seidel, Grunbaum and Barnert, 1967; Fortmeyer and Lade, 1976) and man (Charlton and Bothwell, 1976).

Ascorbic acid and cholestrol:

The relationship between ascorbic acid and cholestrol has long been recognised (Myasnikova, 1947). Increase in total tissue and plasma cholestrol was observed in guinea pigs deficient in the vitamin (Banerjee and Ghash, 1960; Ginter, Bolek, Kopec, Ovecka and Cerey, 1967; Ginter, Babala and Cerven, 1969; Ginter, Nemec, Cerven and Mikus, 1973; Ginter, 1976) and studies using both 4-^{14}C and 26-^{14}C cholestrol showed that the above finding was due to impaired cholestrol conversion into bile acids (Ginter et al., 1973; Ginter, Cerven, Nemec and Mikus, 1971; Ginter, 1973; Ginter, Nemec and Bobek, 1972; Guchhait, Guha and Ganguli, 1963). Some studies, however, revealed no change in plasma cholestrol in scorbutic guinea pigs (Banerjee and Singh, 1958; Ginter, Bilistics and Cerven, 1965), monkies

(Banerjee and Bandyopadhyay, 1965; Banerjee and Bal, 1959) or man (Bronte-Stewart, Roberts and Well, 1963; Hodges, Hood, Canham, Sauberlich and Baker, 1971). In fact decreased cholestrol levels were reported in the latter two species.

The formation of cholic acid from cholestrol requires the introduction of hydroxyl groups at positions 7 and 12 of the steroid nucleus and oxidation and shortening of the side chain, which is preceded by a hydroxylation reaction at C-26 (Hughes, 1976). In virtue of the role of ascorbic acid as a hydroxylating agent in many metabolic processes (Barnes and Kodicek, 1972) it is appealing to ascribe the hypercholestrolemia observed in scorbutic guinea pigs to defective hydroxylation (Hughes, 1976). Ascorbic acid deficiency might indirectly influence the integrity of the thiol group obligatory in the 7 - hydroxylase system (the rate limiting reaction in the conversion of cholesterol to bile acids), or produce changes in the microsomal cytochrome P-450 needed for electron transport in the conversion processes (Hughes, 1976).

Ascorbic acid and mineral metabolism:

Many reports describe the effect of ascorbic acid on the metabolism of various minerals in the body. Exogenous ascorbic acid was reported to cause increased calcium and phosphorus excretion in chickens (Thornton, 1968; Thornton and Omdahl, 1969) and this was suggested to be due to inhibition of intestinal absorption accompanied by increased excretion and reduced tissue retention (Thornton, 1970).

Selenium and/or vitamin E deficiency causes muscular dystrophy in animals. The malady has been associated with reduced levels of ascorbic acid in serum (Brown, Sweeny, George, Stanley and Moran, 1974) and livers (Brown, Partridge, Sharp and Young, 1971a; Brown, Sharp, Young and Van Dreumel, 1971b) caused by the probable interference with the de novo ascorbate synthesis. Supplementation of vitamin C with diet ameliorates the condition. Studies in the chick indicates that ascorbic acid promotes the absorption and subsequent utilization of selenium (Comb and Scott, 1974; Comb and Pesti, 1976).

The relationship between ascorbic acid and copper deficiency or toxicity has also been investigated. The intensity of the copper deficiency symptoms and lesions (i.e. reduced growth, anaemia, osteoporosis, alopecia etc.) in the chick and duck was found to be aggravated by dietary ascorbic acid and thus resulting in high mortality rates (Carlton and Henderson, 1964, 1965; Hill and Starcher, 1965; Hunt, Carlton and Newberne, 1970). The high mortality rate in chickens was partly due to the occurrence of aneurysms and subsequent rupture of the aorta and major blood vessels. This was explained by the observation that copper deficient birds supplemented with ascorbic acid had lower aorta elastin content than those with uncomplicated copper deficiency (Hill and Starcher, 1965). The above observation was substantiated by the finding that the activity of the copper-containing enzyme, amine oxidase, which may participate in the formation of elastin cross links, in the aorta is

inhibited by ascorbic acid (Bird, Savage and O'Dell, 1966). Moreover, copper deficiency is known to inhibit the synthesis of the enzyme cytochrome oxidase (Hunt et al., 1970) and hence the myocardial necrosis and calcification associated with copper deficiency could be related to reduced activity of that enzyme (Gallagher, 1964). Ascorbic acid accentuates the effect of copper deficiency on the cytochrome oxidase production.

Contrary to the unfavourable effects of vitamin C in the case of copper deficiency, the vitamin has a beneficial role in conditions of copper toxicity. It has been shown that addition of 250 ppm. of copper to the basic ration of pigs causes a severe microcytic, hypochromic anaemia accompanied by lowering of plasma and liver iron concentrations (Gipp, Pond, Tasker, Van Campen, Krook and Visek, 1973). The likely reason for this seems to be an impairment in iron absorption and utilization (Gipp et al., 1973; Gipp, Pond, Kallfelz, Tasker, Van Campen, Krook and Visek, 1974). Ascorbic acid reduces copper absorption and retention and consequently prevents the onset of the indirect iron deficiency symptoms (Van Campen and Gross, 1968; Gipp et al., 1974).

Ascorbic acid plays an important role in iron metabolism. By its capacity as a reducing agent ascorbic acid reduces the dietary ferric iron to the ferrous form which is more soluble and preferentially absorbed in the alkaline conditions of the small intestine; and hence is more readily

combined with transferrin and incorporated into haeme (Hopping and Ruliffson, 1966; Wintrobe, 1967; Kaneko, 1970a; O'Dell and Campbell, 1971). It has been reported that the vitamin increases iron absorption by the formation of soluble ascorbic acid-iron chelates (Conrad and Schade, 1968).

Vitamin 'C' is well absorbed in the mucosal cells of the intestine and can function by promoting ferritin synthesis (Loewus and Fineberg, 1959), and mediating iron release from it (Shorr, 1956). It has also been shown that incubation of transferrin with liver tissue in the presence of ascorbic acid and ATP results in transfer of transferrin-bound iron to ferritin (Mazur et al., 1960). Hopping and Ruliffson (1966) investigated iron transport using everted gut pouches in rats and concluded that: a) iron transport has a direct relation with ascorbate concentration in the medium and b) the vitamin helps the retention of the absorbed iron by the gut tissue. Furthermore, they showed that more iron is absorbed from $FeSO_4$ solution in in vivo legated intestinal loops when ascorbic acid and/or glutathione were added.

Storage iron in ferritin and haemosiderin can readily be mobilized for haemoglobin synthesis at the time of need (Moore, 1961). The iron in ferritin is mostly in the ferric state but the metal apparently enters and leaves the ferritin molecule in the reduced form (Mazur et al., 1955); again ascorbic acid may play the main role in the reduction process.

Charlton and Bothwell (1976) stated that in scurvy, the administration of ascorbic acid greatly and promptly improved the plasma iron concentrations. They quoted enough evidence to support the hypothesis that vitamin C deficiency leads to a defective reticuloendothelial cell function and consequently to disordered iron release into the plasma.

The overlapping distribution of ascorbic acid and iron in the suprarenal glands of the mouse and rat, mouse ovary, healing wounds in rabbits (Nissim, 1952) and in the trematoda Fasciola hepatica (Pantelouris and Hale, 1962) may further support the close relation between the two.

Stress conditions:

Ascorbic acid may have advantageous effects in stressful conditions such as injuries, infection, colds, pregnancy and lactation (Goldsmith, 1961; Chatterjee et al., 1967) A relation between stress, histamine release and ascorbic acid seems to exist. Thus, Nandi et al (1974) demonstrated that in stressful situations in rats and guinea pigs the histamine formation and release was increased and that administration of large doses of ascorbic acid markedly decreased the urinary histamine levels denoting in vivo histamine detoxification. Furthermore, Subramanian et al. (1973) showed that autooxidation of ascorbic acid in the presence of histamine resulted in the rupture of the imidazole ring with consequent inactivation of histamine and suggested that the role of vitamin C in stress conditions is the detoxification of the excess histamine produced.

Advantage of the above idea has been taken to relate the capillary degeneration and haemorrhages seen in scorbutic conditions to high levels of histamine; the latter is known for its vasodilating effects on capillaries which results in hyperaemia and increased capillary permeability (Catterjee et al., 1975).

Ascorbic acid and toxicity:

The protective action of vitamin C against the toxicity of some chemical compounds and heavy metals has been established. Ascorbic acid is found to be protective against toxicity of organochlorine agents such as DDT and lindane. These compounds are known inducers of hepatic microsomal enzymes, which include parts of the glucuronic acid and ascorbic acid biosynthetic pathways, and their administration stimulates ascorbic acid output in rats and D-gulonic acid in guinea pigs. The capacity of bio-degradation of these organochlorines is greatly reduced in ascorbic acid deficient guinea pigs (Street and Cladwick, 1975).

Vitamin C is also found protective against the hepatotoxicity of nitroso derivatives formed by the reaction of nitrites with naturally-occurring or synthetic secondary or tertiary amines (Lijinsky and Epstein, 1970; Kamm et al., 1973). Sodium nitrite administered together with aminopyrine in rats resulted in hepatic necrosis and a rise in alanine aminotransferase activity which was attributed to the formation of dimethylnitrosamine in the stomach (Lijinsky and Greenblatt, 1972). This adverse

effect was prevented by the simultaneous administration of ascorbic acid or sodium ascorbate (Kamm et al., 1973). Similarly the hepatic tumours induced by the above-mentioned compounds could be prevented by ascorbic acid administration (Chan and Fong, 1977).

How ascorbic acid acts is not fully known but in vitro studies showed that the vitamin interferes with nitrosamine formation by competing for available nitrate (Mirvish et al, 1972).

An example of heavy metal toxicity is that of cadmium. Toxicity in the Japanese quail is manifested by growth depression, severe hypochromic anaemia, hypogonadism, decreased bone ash and salient histopathological changes in the oesophagus, duodenum, adrenals and bone marrow. Small amounts of ascorbic acid alleviated most of the above effects (Fox, 1975).

Ascorbic acid and the common cold:

In humans, plasma, leukocyte and tissue ascorbic acid levels are greatly diminished during common cold episodes and their concentrations can be replenished by the supplementation of vitamin C (Hume and Weyers, 1973). Again the manner by which ascorbic acid acts against cold symptoms is not fully understood. However, the vitamin may act in the following ways:-

i) by reducing the severity of the catarrhal inflammation in the upper respiratory tract by virtue of its mucolytic activity (Briggs, 1973).

ii) by indirectly improving oxygenation by increasing the pulmonary volume (Zuskin et al., 1973).

iii) by activating the leukocytic immunological mechanisms, antibody production and phagocytic abilities (Collingham and Mills, 1943; Cate et al., 1964; Pereira et al., 1972).

iv) by enhancing interferon production (Lewin, 1974).

v) by maintaining the integrity of membranes and the amorphous intercellular and interfibrillary ground substance (Cameron, 1976) and hence checks the spread of the invading organism and

vi) by enhancing the healing of damaged tissue.

The paper of Wilson (1975) can be consulted for further information on this subject.

Ascorbic acid and cancer:

Reduced plasma and leukocyte ascorbic acid concentrations are observed in cancer patients. Neoplastic tissues, on the other hand, have high levels of vitamin C indicating that tumour tissue actively takes up the plasma ascorbic acid. The vitamin C levels should be adequately maintained in cancer patients for the proper functioning of the body's immunological defensive mechanisms and for the formation of the physiological hyaluronidase inhibitor which checks tissue proliferation (see Wilson, 1975; Cameron, 1976).

In conditions where tissue proliferation is an essential feature i.e. cancer, the cells first secrete hyaluronidase to depolymarise the surrounding tissues and

allow unrestricted cell division within the depolymerised area (Cameron and Pauling, 1973). The restraint guarding the release of hyaluronidase is the so-called physiological hyaluronidase inhibitor which bears a close chemical resemblance to the glycosaminoglycans of connective tissue and requires ascorbic acid for its formation (Newman et al., 1955; Cameron and Pauling, 1974).

There is a close structural similarity between ascorbic acid and glucuronic acid; the latter is a fundamental building block in all the glycosaminoglycan substrates of hyaluronidase. This relationships led to the suggestion that the physiological hyaluronidase inhibitor is a glycosaminoglycan residue in which some or all the glucuronic acid molecules are replaced by ascorbic acid (Cameron, 1976).

Ascorbic acid and phagocytic function:

The possible role of vitamin C in phagocytosis has been indicated by the early findings of Busing (1942) and Collingham and Mills (1943) who observed impaired phagocytosis in donors low in vitamin C. Nungester and Ames (1948) further found that the polymorphonuclear leukocytes harvested from peritoneal exudates of vitamin C-deficient guinea pigs were more fragile and had lower phagocytic activity than those of normal animals. On the other hand, Ganguly et al (1975) reported that macrophages of scorbutic guinea pigs showed a significant reduction in area of migration but a slight increase in phagocytic activity.

More recent reports indicate that the interaction between ascorbic acid and hydrogen peroxide *in vitro* generates a potent antibacterial mechanism active against gram-negative organisms. This mechanism causes bacterial death and lysis in the presence of lysozyme (Miller, 1969). Since ascorbic acid is normally present in leukocytes (Stephens and Hawley, 1936) and hydrogen peroxide is produced in phagocytes during engulfment and degranulation (Repine *et al.*, 1978), such a reaction may also occur in *in vivo* phagocytosis.

In humans, abnormal function of the polymorphonuclear leukocytes, brought about by impaired chemotaxis and delayed delivery of lysosomal enzymes into phagosomes, is found in the Chediak-Higashi syndrome; a disorder characterised by pigmentary dilution, increased susceptibility to pyogenic infections and the presence of abnormal giant granules in leukocytes and other cells. Treatment with oral doses of vitamin C increased leukocyte chemotaxis, promoted the release of the enzyme beta glucuronidase (a measure of the capacity for degranulation) and improved the bactericidal capacity of leukocytes (Boxer *et al.*, 1976). Ascorbic acid has also been reported to potentiate chemotaxis *in vitro* in normal leukocytes (Sandler *et al.*, 1975).

Although the above account proposes an important role for ascorbic acid to play in normal phagocytosis and bacterial killing, some reports may argue against that conclusion. Thus McCall *et al.* (1971), investigating the effect of ascorbic acid on the bactericidal mechanisms of

neutrophils, reported that ascorbic acid in certain concentrations inhibited both iodination of ingested zymozan and the aldehyde reaction. Despite the inhibition of these two important bactericidal mechanisms the clearance of Staphylococcus aureus and Escherichia coli was normal in intact leukocytes. They concluded that phagocytes possess other means of killing bacteria and that ascorbic acid may indirectly enhance their bactericidal activity. Moreover, Stankova et al. (1975) found that neutrophils obtained from scorbutic guinea pigs had a comparable capacity to produce hydrogen peroxide and kill Staphylococcus aureus as those obtained from normal animals, suggesting that ascorbate does not have a significant influence in hydrogen peroxide production and bacterial killing. However, the same authors (Stankova et al., 1975) suggested that the ascorbate may preserve the integrity of leukocytes by protecting their cell constituents from denaturation by oxidants and free radicles produced during phagocytosis.

It appears that the literature on ascorbic acid deficiency or supplementation, especially in laboratory animals, is voluminous. However, little seems to have been done on the effect of various diseases on ascorbic acid metabolism, especially with respect to fascioliasis, in large domestic animals.

Changes in blood and tissue ascorbic acid levels have been investigated in lambs (De Mille et al., 1972) and pigs (Brown et al., 1971a and b) with nutritional muscular dystrophy. No profound effect on ascorbic acid metabolism was

established in lambs but reduced liver levels were encountered in pigs. Plasma ascorbic acid content was found to be markedly reduced in calves with acute and subacute bronchopneumonia (Jagos et al., 1977) and those with enteropathies (Smirnov, 1962; Salageanu et al., 1971). Sheep with experimental eccinococcosis were also reported to have low vitamin C in the liver, spleen and lungs (Aminzhanov, 1975).

There are few reports which describe the histochemical distribution of tissue vitamin C in fascioliasis. These include the investigation of Ziolo (1960) who found low ascorbic acid and cholesterol content in the zona fasciculari and zona reticularis of the adrenal glands of infected sheep and that of Kadziolka (1962) who observed reduced ascorbic acid and glycogen levels in the livers of infected cattle. However, information about changes in ascorbic acid content in plasma, leukocytes or other tissues seems to be lacking.

The liver seems to be the main site for ascorbic acid synthesis in mammals. Chatterjee et al. (1975) presented a wide list of animals and their ability to synthesise ascorbic acid in their tissues from L-Gulono-1-4-Lactone, this is reproduced overleaf.

Ascorbic Acid Synthesis from L-Gulono-1,4-Lactone in Microsomal Fractions from Tissues of Different Species of Animals.

Animal	Ascorbic acid synthesized (ug/mg protein/hr)	
	Kidney	Liver
Insects	-	-
Invertebrates	-	-
Fishes	-	-
Amphibians		
Toad (*Bufo melanostictus*)	144 ± 10	-
Frog (*Rana tigrina*)	115 ± 10	-
Reptiles		
Turtle (*Lissemys punctata*)	98 ± 8	-
Bloodsucker (*Caloter versicolor*)	50 ± 5	-
House lizard (*Hemidactylus flaviviridis*)	46 ± 6	-
Common Indian Monitor (*Varanus monitor*)	32 ± 4	-
Angani (*Mabuya carinata*)	25 ± 4	-
Snake (*Natrix piscator*)	18 ± 2	-
Tortoise (*Testudo elegans*)	14 ± 2	-
Mammals		
Goat	-	68 ± 6
Cow	-	50 ± 6
Sheep	-	43 ± 4
Rat	-	39 ± 4
Mouse	-	35 ± 4
Squirrel	-	30 ± 4
Gerbil	-	26 ± 4
Rabbit	-	23 ± 2
Cat	-	5 ± 1
Dog	-	5 ± 1
Guinea pig	-	-

/Contd....

/Contd....

Animal	Ascorbic acid synthesized (ug/mg protein/hr)	
	Kidney	Liver
Flying mammals		
Indian fruit bat (Pteropus medius)	-	-
Indian pipistrel (Vesperugo abramus)	-	-
Primates		
Monkey (Macaca mulatta)	-	-
Man	-	-

At the same time the liver is the main organ involved in fascioliasis. The parasite causes tremendous damage to this organ by the migrating juvenile parasites as well as by adults in the bile ducts (Dow et al., 1968; Rushton and Murray, 1977). The damage is expected to cause impairment of ascorbic acid synthesis. The need for the vitamin for the various cellular functions and the disorders connected with its deficiency have been mentioned earlier.

The present study was undertaken to a) establish whether ascorbic acid metabolism is affected in F. hepatica infection and b) to disclose any other metabolic disorders that are known to result from ascorbic acid imbalance.

GENERAL

MATERIALS AND METHODS

Animals:

Cattle, sheep and rats were used in this study. Details about these animals and their treatment will be given under the appropriate section.

Fasciola hepatica Metacercariae:

Metacercariae (cysts) were supplied by other laboratories. The metacercariae were inspected under the microscope and counted under the low power. For cattle and sheep infection, the required number of cysts was put in a small gelatin capsule, capped and enclosed in another bigger capsule. The cysts were administered to cows by holding out the tongue by one hand and pushing the capsule into the oesophagus with the other. In sheep the capsules were administered with the aid of a small gun passed through a wooden gag. For rats, two methods were used:

a) The rats were held firmly by the scruff and the right number of metacercariae, contained in a small drop of water, administered using a siliconized Pasteur pipette.

b) The animals were similarly held and the right number of cysts, adhering to a small piece of cellophane was administered at the back of the tongue using a blunt forceps. Both methods were satisfactory but the second one was preferred because the counting and administration of the cysts was relatively easier.

Blood collection and separation of plasma:

Blood was drawn from sheep and cows weekly by jugular venipuncture into 10 ml heparinized vaccutainer tubes. The plasma was separated by centrifuging the blood at 2500 r.p.m. and samples showing visible haemolysis were discarded.

Plasma ascorbic acid determination:

This was done according to the method of Denson and Bowers, 1961.

Reagents:

1. Vitamin C Reagent:

   100 volumes 2.2% 2-4 dinitrophenyl hydrazine in 10N H_2SO_4,

   5 volumes 5% thiourea,

   5 volumes 0.6% copper sulphate ($cuSO_4.5H_2O$).

2. 5% w/v trichloroacetic acid AR. (TCA)

3. 65% H_2SO_4.

Procedure:

To 1 ml plasma 2 ml of 5% trichloroacetic acid were added and the mixture centrifuged at 3000 r.p.m. for 15 minutes. 1 ml of the supernatant was taken and 0.3 ml of the vitamin C reagent added. The tubes were then incubated for 4 hours in a 37°C water bath, cooled in ice water, 1.5 ml of 65% H_2SO_4 added, and the extinctions were read in a Pye Unicam SP8000 Ultraviolet Recording Spectrophotometer at

520 nm against a reagent blank (1 ml TCA treated similarly). The readings were transformed into ug ascorbic acid/ml from standard calibration curves made at frequent intervals; standards used contained 0.25; 0.5; 0.75; 1.5; 2; 3.1; 6.3; 10; 12.5 ug ascorbic acid/ml.

Calculations:

Reading (ug/ml) x 3 x 100 = ug/100 ml plasma.

Tissue ascorbic acid determination:

A known weight of tissue (liver, spleen, kidney, heart) was homogenized in 10 ml of 5% metaphosphoric acid using a glass homogenizer. After centrifugation at 3000 r.p.m. for 15 min., 1 ml of the supernatant was used for ascorbic acid determination as described above. As the metaphosphoric acid precipitates the proteins the estimation could be directly performed on the 1 ml supernatant fractions.

Plasma glucose:

The method described by Trinder (1969) was utilized.

Reagents:

1. Protein precipitant:

   20 g sodium tungstate, 20 g anhydrous disodium hydrogen phosphate and 18 g sodium chloride were dissolved in 1500 ml of water. The pH was adjusted to 3.0 with HCl and 2 g of phenol were then added and the solution made to 1 litre.

2. Colour reagent:

10 g disodium hydrogen phosphate, 1 g sodium azide and 0.3 g 4-aminophenazone were dissolved in 1 litre of water. 10 ml fermcozyme 653 AM and 10 ml of 0.1% peroxidase were used.

3. Standard:

200 mg D-glucose dissolved in 100 ml saturated benzoic acid.

Procedure:

To 0.1 ml of plasma, standard or water blank 6.0 ml of the protein precipitant were added and the mixture centrifuged at 2000 r.p.m. for 15 min. 2 ml of the supernatant were then withdrawn and 4 ml of the colour reagent added. The mixture was incubated in a 37^{o} water bath for 15 min and the colour developed measured at 515 nm against the blank in the Pye Unicam Spectrophotometer.

Total plasma cholesterol:

The method used is that described by Grafnetter et al. (1967).

Reagents:

1. Cholesterol reagent:

80 ml of glacial acetic acid was mixed with 110 ml acetic anhydride and the mixture was thoroughly cooled in the refrigerator. 40 ml of cold H_2SO_4 was then added very slowly, a

little at a time, 6 g of anhydrous sodium sulphate added and the final mixture stored at $4^{o}C$.

2. Standard:

Cholesterol standard solution 1 mg/ml glacial acetic acid (Sigma Co. Ltd.) was used.

Procedure:

To 0.1 ml plasma and standard, 4.0 ml of the cholesterol reagent were added, mixed and the colour developed measured at 615 nm.

Plasma low density lipoproteins (L.D.L.):

The test was performed using BDH assay set for L.D.L. and the procedure of the manufacturers was followed.

Reagents:

These were readily provided and consisted of:

1. Calcium-veronal reagent
2. Dextran sulphate reagent
3. Stock turbidity solution.

Procedure:

Plasma samples, controls and reagent blanks were prepared in 15 x 150 mm test tubes as shown in the table overleaf.

	Test	Control	Blank
Calcium veronal reagent	4.6 ml	4.8 ml	4.8 ml
Dextran sulphate reagent	0.2 ml	-	0.2 ml
Plasma	0.2 ml	0.2 ml	-

All tubes were allowed to stand for 20 min at room temperature and then mixed by inverting twice. The spectrophotometer (Pye Unicam) was zeroed with the blank and the optical density of the test and controls read at 680 nm. The control was then subtracted from the test and the low-density lipoprotein concentrations found by reference to a calibration curve, made from different dilutions of the turbidity standards, according to the following table:

Tube No.	Turbidity Standard (ml)	Distilled water (ml)	Equivalent to lipoprotein (mg/100 ml)
1	0.5	4.5	100
2	1.0	4.0	200
3	2.0	3.0	400
4	3.0	2.0	600
5	4.0	1.0	800
6	5.0	-	1000

Plasma Iron and iron-binding capacity:

The methods of Ramsay (1957a,b) were strictly followed.

Preparation of the glassware:

Test tubes with ground glass necks and stoppers were used. These were scrupulously cleaned by soaking in soap overnight, rinsing with distilled water then soaking again in concentrated H_2SO_4 for 24 hrs. They were washed thoroughly with distilled water and then again left in 5N HCl for 12 hrs. They were finally rinsed several times in distilled water, dried and kept in cellophane bags until required.

Reagents:

Analar grade chemicals were used.

1. 0.2% 2-2'-dipyridyl:

   0.2 g 2-2'-dipyridyl in 100 ml 3 percent acetic acid (v/v).

2. 0.2M sodium sulphite:

   2.52 g of anhydrous sodium sulphite dissolved in 100 ml of water. This was prepared freshly every week.

3. Chloroform.

4. Magnesium carbonate "light" for adsorption.

5. Ferric chloride stock solution:

   14.5 mg of ferric chloride ($FeCl_3$) dissolved in 100 ml of 0.5N HCl.

6. Ferric chloride working solution:

   Dilute 1 ml of the ferric chloride stock solution to 100 ml with distilled water. This solution will contain 5 ug iron per ml of 0.005N HCl.

7. Stock standard solution, 100 µg iron per ml:

   0.498 g of ferrous sulphate ($FeSO_4,7H_2O$) dissolved in water and 1 ml concentrated H_2SO_4 added. The solution is made to 1 litre.

8. Working standard, 3 µg iron/ml:

   3 ml of the stock solution are diluted to 100 ml with water.

Procedure:

A) Plasma iron:

1 ml of plasma is mixed with 1 ml of water in a test tube and 0.5 ml of each of the 0.2M sodium sulphate and 0.2% dipyridyl solutions were added. The tubes were stoppered, and heated in a boiling water bath for 5 min, cooled and then 1 ml chloroform added. They were again stoppered, shaken vigorously in a Vortex mixer for 30 seconds, centrifuged for 5 min at 300 r.p.m. and the supernatant read at 520 nm in a Pye Unicam spectrophotometer. 2 ml of water, used as blank, and 1 ml of the working standard was treated similarly.

Calculation:

$$\frac{\text{Reading of test}}{\text{Reading of standard}} \times 300 = \text{µg iron per 100 ml plasma.}$$

B) Plasma iron-binding capacity:

2 ml of ferric chloride solution were added to 1 ml of plasma. After standing for 5 min 200 mg of magnesium

carbonate were added. The tubes were frequently and vigorously shaken for 40 min, centrifuged and 2 ml of the supernatant were pipetted off for iron determination as described above.

Calculation:

$$\frac{\text{Reading of test}}{\text{Reading of standard}} \times 450 = \mu g \text{ iron per 100 ml plasma}$$

Liver iron and copper:

These were determined by atomic absorption spectrophotometry. The method used for the preparation of samples was principally that of Thompson and Blanchflower (1971).

Reagents:

1. Nitric-perchloric acid mixture 4:1 v/v using Analar grade.
2. 5% HCl.

Standards:

i) Stock iron standard:

4.84 g $FeCl_3.6H_{20}$ were dissolved into 200 ml H_2O and then diluted to 1 litre. The solution was kept in a darkened polythene bottle.

ii) Stock copper standard:

3.7980 g copper nitrate $Cu(NO_3)_2.3H_2O$ were dissolved into 250 ml deionized water and then diluted to 1 litre and stored in a polythene bottle.

Procedure:

0.5 g freeze-dried liver samples were weighed in scrupulously clean digestion tubes (25 ml capacity), 10 ml of the nitric-perchloric acid mixture were then added together with a few antibumping granules and 2-4 drops of octan-1-ol to prevent frothing. The tubes were heated on a heating rack at about 100°C till the tissues were digested. Then the excess acid was evaporated at a temperature of about 200°C. The tubes were cooled and the resultant white precipitate dissolved in 20 ml of 5% HCl. Iron and copper were measured in the clear solution thus obtained using a Pye Unicam atomic absorption spectrophotometer with hollow cathode iron and copper lamps. Standards containing 0.5; 1.0; 2.5; 5 and 10 ug/ml of either iron or copper were incorporated.

All glassware was successively washed in detergent, rinsed, dried, soaked in concentrated H_2SO_4 for 24 hrs., then rinsed in deionized water and dried.

Liver glycogen and total protein determination:

A) Glycogen:

The method of Morris (1948) was used as follows:-

Reagents:

1. 30% potassium hydroxide (KOH)
2. absolute ethanol.
3. Anthrone reagent: 200 mg anthrone dissolved in 100 ml concentrated H_2SO_4 (sp. gr. 1.84), cooled and used immediately.
4. Glucose standard: 20 mg dissolved in 1 ml H_2O.

Procedure:

To 10-15 mg liver samples, weighed using a torsion balance, 5 ml of 30% KOH were added, heated in a boiling water bath for 10 min and then cooled. 1 ml of this solution was taken and 2 ml absolute ethanol added and the mixture again heated in a boiling water bath up to the boiling point of ethanol (78°C) while being stirred continuously with a glass rod. The precipitated glycogen was centrifuged down at 3500 r.p.m. for 15 min and the precipitate dissolved in 1 ml H_2O. The precipitation procedure was repeated with a further 2 ml ethanol. The re-precipitated glycogen was dissolved in 5 ml H_2O. To 1 ml of this glycogen solution, 2 ml anthrone reagent were added in an ice bath. Again the samples were heated in a boiling water bath for 10 min and then the absorbance was read at 660 nm against the blank. 1 ml standard glucose solution and 1 ml water blank were treated similarly.

B) Total proteins:

The method of Lowry, Rosenbrough, et al. (1951) was utilized as follows:

Reagents:

1. 1N sodium hydroxide (NaOH): 40 g.NaOH in 1 litre H_2O.
2. 2% sodium carbonate (Na_2CO_3).
3. 0.5% copper sulphate ($Cu.SO_4.5H_2O$) in 1% aqueous solution of sodium potassium tartrate.
4. Folin's phenolic reagent.
5. Standard:

   A stock standard solution was prepared by

dissolving 288 mg crystalline albumin in 25 ml H_2O. Working standard was prepared by diluting the stock standard 1:10 with H_2O.

6. Blank: 0.5 ml 1N NaOH.

Procedure:

20-25 mg liver tissue were weighed using a torsion balance. 4 ml 1N NaOH were added and warmed up to 80°C till the tissue dissolved. 0.5 ml of this solution was taken and 5 ml of a mixture of 0.5% $CuSO_4$ and 2% Na_2CO_3 (1:50) were added. After standing for 10 min 0.5 ml of the Folin reagent were added, the mixture stirred and the absorbance read after 30 min at 500 nm in a Pye Unicam spectrophotometer.

Histology and Histochemistry:

Tissues for histology and histochemistry were fixed in 10% postformaldehyde, processed through an automatic tissue processor, embedded in paraffin and sections 5 u thick were prepared using a sliding Cambridge microtome. Haematoxylin and eosin stain was used for routine histopathology and consecutive sections were stained for the detection of ferric and ferrous iron.

A) Ferric iron:

The Prussian blue stain was used. (See Humeson, 1967).

Reagents:

1. 2% potassium ferrocyanide
2. 2% HCl
3. 1% neutral red stain.

Procedure:

Sections were put in xylene for 40 min then passed through xylene/absolute alcohol (1:1 v/v); absolute alcohol; 90% alcohol and 70% alcohol for 1 min each. Slides were then transferred to a freshly prepared solution of equal parts of 2% potassium ferrocyanide and 2% HCl for 30 min., washed in distilled water and counter-stained in 1% neutral red for 15 min. After washing with distilled water, sections were dehydrated through the various grades of alcohol, cleaned in xylene and mounted.

Ferrous iron:

The Turnbull method was used. (See Humason, 1967).

Reagents:

1. 20% potassium ferrocyanide
2. 1% HCl
3. 10% ammonium sulphide solution (AR)
4. 1% neutral red.

Procedure:

Sections were brought to water as described above. They were then put in the ammonium sulphide solution for 2 hrs., washed quickly with distilled water and stained in a freshly prepared solution of equal parts of 20% potassium ferrocyanide and 1% HCl. After 15 min, the sections were washed in distilled water, counter-stained in neutral red for 15 min, washed again, dehydrated and mounted.

Haematoxylin and Eosin stain:

The method used was described in Humason (1967).

Reagents:

1. Harris haematoxylin (25 ml 10% haematoxylin in absolute alcohol; 1.25 g mercuric oxide; 500 ml 10% aqueous aluminium potassium sulphate; 20 ml glacial acetic acid)
2. Eosin: 2.5 g Eosin yellowish made up to 250 ml with distilled water
3. Acid alcohol: 1% concentrated HCl in 70% ethanol
4. Scotts tap water substitute: consisting of 3.5 g sodium carbonate and 20 g magnesium sulphate made to 1 litre with distilled water.

Procedure:

The sections were brought to water as described previously. They were then stained in haematoxylin for 4 min, rinsed in running tap water, blued in Scotts substitute for 30 seconds, differentiated in 1% acid alcohol, blued again in the Scotts substitute and then counter-stained in eosin 1-2 min. After rinsing in tap water the sections were dehydrated, cleared and mounted.

Statistical Analysis:

Analysis of variance and the student 't'-test of significance were performed according to Parker (1975) and Spiegel (1972) respectively.

Practical Note:

a) Blood collection and the determination of the various plasma constituents were carried out at the same time every week.

b) Experimental rats were usually killed in the morning between 9 and 10 a.m. Homogenization of tissues for ascorbic acid determination started immediately after the animals were killed and their organs weighed. Assays for liver total proteins and glycogen were usually performed within 2 hrs after killing the rats.

EXPERIMENTAL I

Animals:

Twelve male Welsh mountain sheep, 9 months of age were used. They were housed in individual pens within the Department of Biochemistry and Soil Science, U.C.N.W., Bangor. The animals were free of fluke infection as judged by the faecal examination results. Each sheep was drenched with 15ml Thiobenzole and 7.5ml flukanide and left for an acclimatization period of one month before the start of preinfection studies. Body weight was recorded weekly.

Feeding:

A constant daily ration consisting of 400g each of hay and nuts (168 cattle nuts; 12% protein, 3.5% oil and 15% fibre; BOCM Silcock Co.) was given to each animal; one half of the ration was given in the morning and the other half given in the evening, water was provided *ad libitum*.

Experimental design:

Animals were divided into 3 groups (A, B and C) of 4 sheep each according to body weight. Sheep of group B (Nos. 4, 5, 10 and 12) and group C (Nos. 6, 7, 9 and 11) were orally infected each with 268 *F. hepatica* metacarcariae as previously described. Fourteen days later, sheep of group C received another similar dose of metacarcariae, while animals of group A (Nos. 1, 2, 3 and 8) remained as uninfected controls. Surviving animals were killed 36 weeks after the first infection.

Blood collection started 4 weeks before infection and continued for 14 weeks afterwards. Plasma ascorbic acid, glucose, cholestrol and L.D.L. were determined as described in the general methods.

Results

A) Body weight:

The mean weekly liveweight gains are presented in Table 1 and Figure 1.

It can be seen from Figure 1 and Table 1 that the infected animals had more or less similar liveweight gains to the controls during the pre-infection period and the first 7 weeks after infection. By this time the weight gains were as follows: 25.04 $\pm$ 6.93 for controls (group A); 25.29 $\pm$ 8.16 for group B sheep (single infection) and 28.13 $\pm$ 9.21 for group C animals (re-infected). The differences were not statistically significant ($p > 0.05$) as shown by the analysis of variance.

From the 8th week of infection and until the end of the observations, controls showed higher liveweight gains than infected animals, and by the last week these were: 25.45 $\pm$ 5.07 for controls; 18.17 $\pm$ 3.96 for group B sheep and 18.61 $\pm$ 3.24 for group C animals. Analysis of variance for the data obtained in the last 13 weeks revealed the differences between control and infected groups were just below significance at $p = 0.05$.

Table 1 Means ($\bar{x}$) and Standard Deviations (SD) of the weekly liveweight gain in experimental sheep.

Weeks of infection	Group A (Controls)		Group B (Single infection)		Group C (Reinfected)	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
-3	5.64	4.74	4.07	2.91	1.73	1.73
-2	7.55	5.54	8.52	2.04	6.24	3.91
-1	11.90	4.59	13.81	3.57	11.63	5.53
0	15.13	4.95	17.15	5.46	14.47	4.55
1	10.93	8.67	11.70	6.22	10.36	6.67
2	16.40	7.43	14.20	5.77	19.00	8.41
3	22.72	8.90	21.40	5.10	21.99	5.73
5	25.70	9.50	26.42	6.17	25.04	8.41
6	30.65	8.94	31.98	7.94	30.35	10.19
7	30.68	10.78	29.36	9.21	29.88	10.10
8	34.05	8.78	29.48	10.17	31.30	9.58
9	35.18	10.76	33.56	11.73	34.07	11.68
10	44.12	11.31	39.46	14.46	40.41	13.05
11	42.68	12.29	35.41	8.07	37.37	11.69
12	41.60	11.47	40.49	10.28	37.53	11.67
13	42.96	15.61	38.08	12.95	36.42	10.76
14	41.75	14.79	38.31	12.89	36.95	10.40
15	48.87	14.83	45.13	9.19	43.96	10.90
16	52.95	15.98	49.30	12.91	46.23	13.30
17	51.46	14.67	43.79	11.94	46.22	12.19
18	58.48	17.45	50.15	9.90	50.51	12.99
19	52.06	17.39	45.76	13.96	40.87	14.04
20	56.13	15.78	47.53	5.57	48.48	11.62

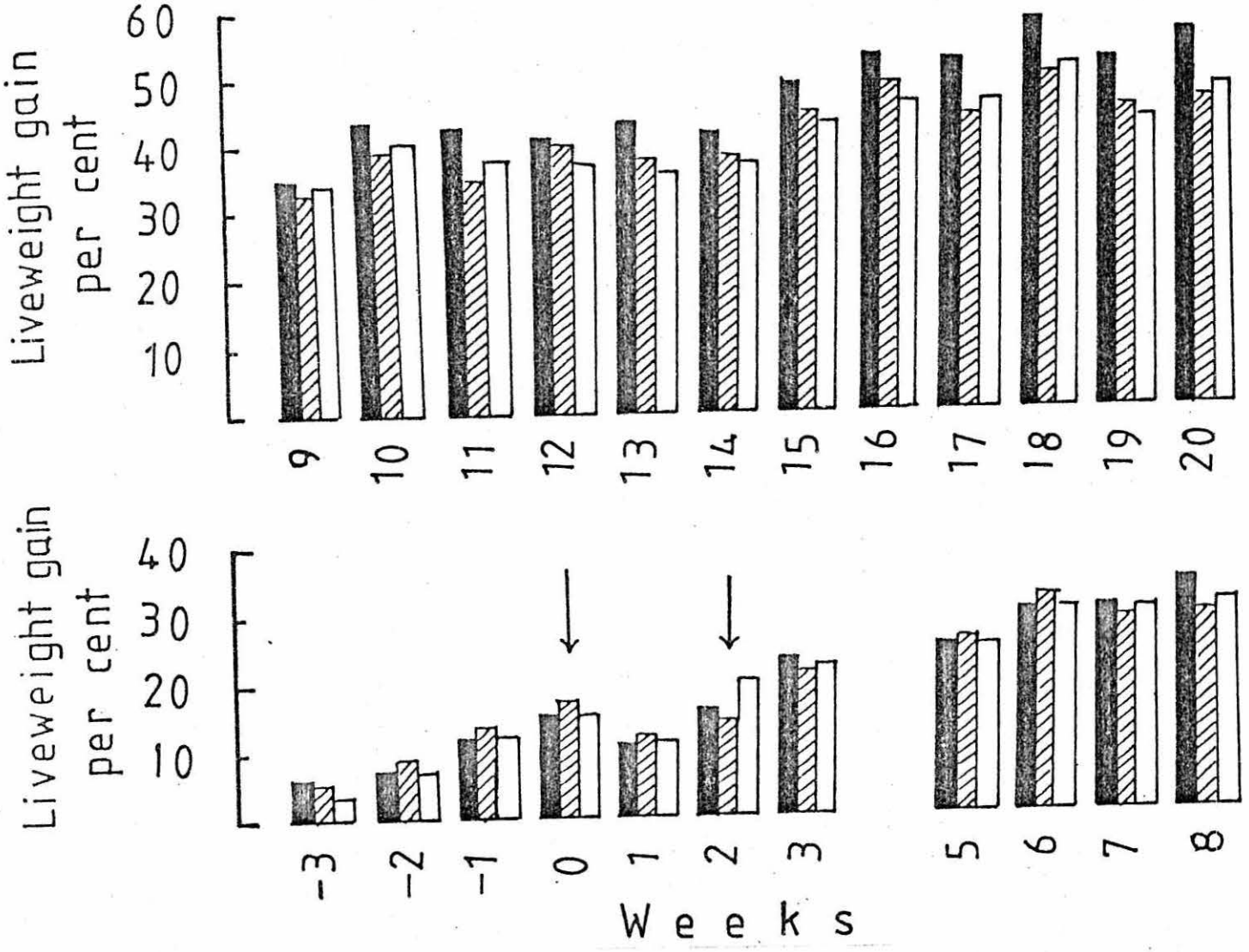


Fig. 1. Avarage weekly liveweights in experimental sheep:
group A (controls); group B. (single infection);
group C. (re-infected). ↑ = infection.

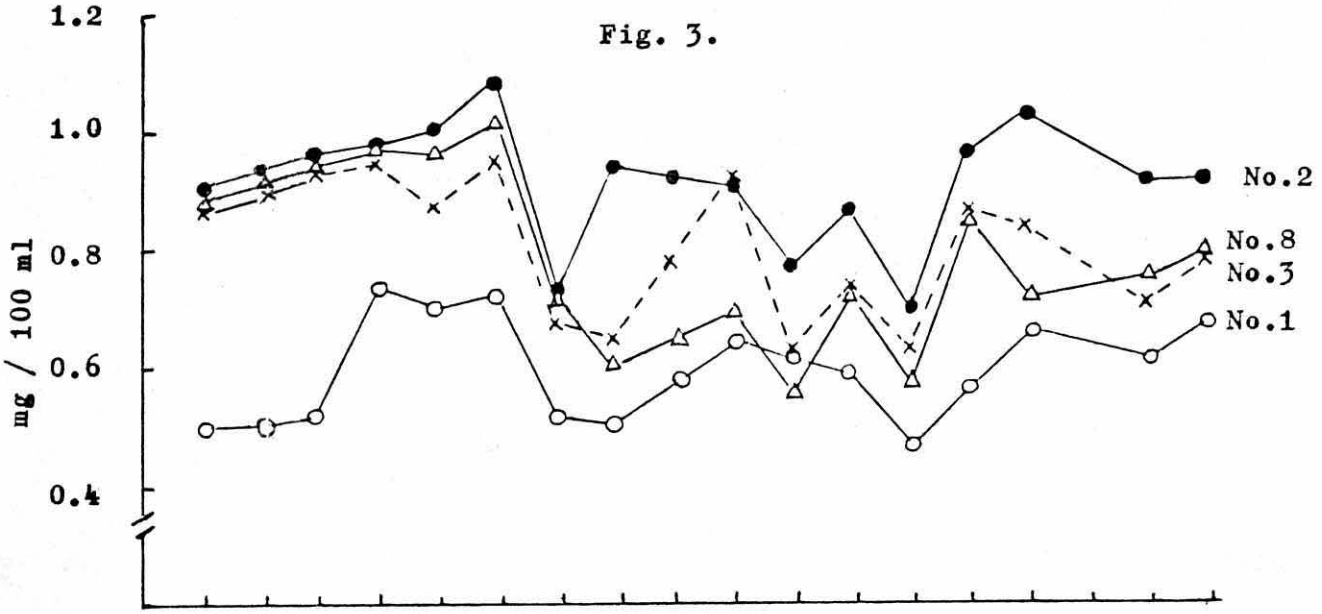
B) Plasma ascorbic acid:

Means and standard deviations of the plasma ascorbic acid values for each animal group are given in Table II and Figure 2. Individual values are presented in Figures 3-5.

Table II Means ($\bar{x}$) and standard deviations (SD) of plasma ascorbic acid levels (mg/100ml) in experimental sheep.

Weeks of infection	Group A (Controls)		Group B (Single infection)		Group C (Reinfected)	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
-3	0.784	0.164	0.920	0.086	0.895	0.060
-2	0.812	0.173	0.943	0.084	0.933	0.072
-1	0.840	0.182	0.967	0.083	0.971	0.084
0	0.907	0.100	0.920	0.113	0.997	0.123
1	0.885	0.115	0.926	0.094	0.915	0.105
2	0.941	0.135	0.784	0.113	0.877	0.151
3	0.655	0.089	0.373	0.115	0.412	0.111
4	0.670	0.167	0.634	0.178	0.679	0.157
5	0.731	0.150	0.579	0.135	0.598	0.161
6	0.791	0.133	0.525	0.091	0.517	0.166
7	0.635	0.080	0.446	0.080	0.412	0.078
8	0.731	0.101	0.416	0.064	0.446	0.112
9	0.589	0.083	0.247	0.086	0.319	0.051
10	0.817	0.147	0.472	0.040	0.487	0.152
11	0.810	0.137	0.521	0.080	0.499	0.129
12	-	-	-	-	-	-
13	0.746	0.109	0.572	0.060	0.510	0.178
14	0.799	0.083	0.551	0.050	0.450	0.177

It can be seen from Table II and Figures 2-5 that infected sheep in both groups B and C had a similar plasma ascorbic acid picture. The levels remained fairly constant



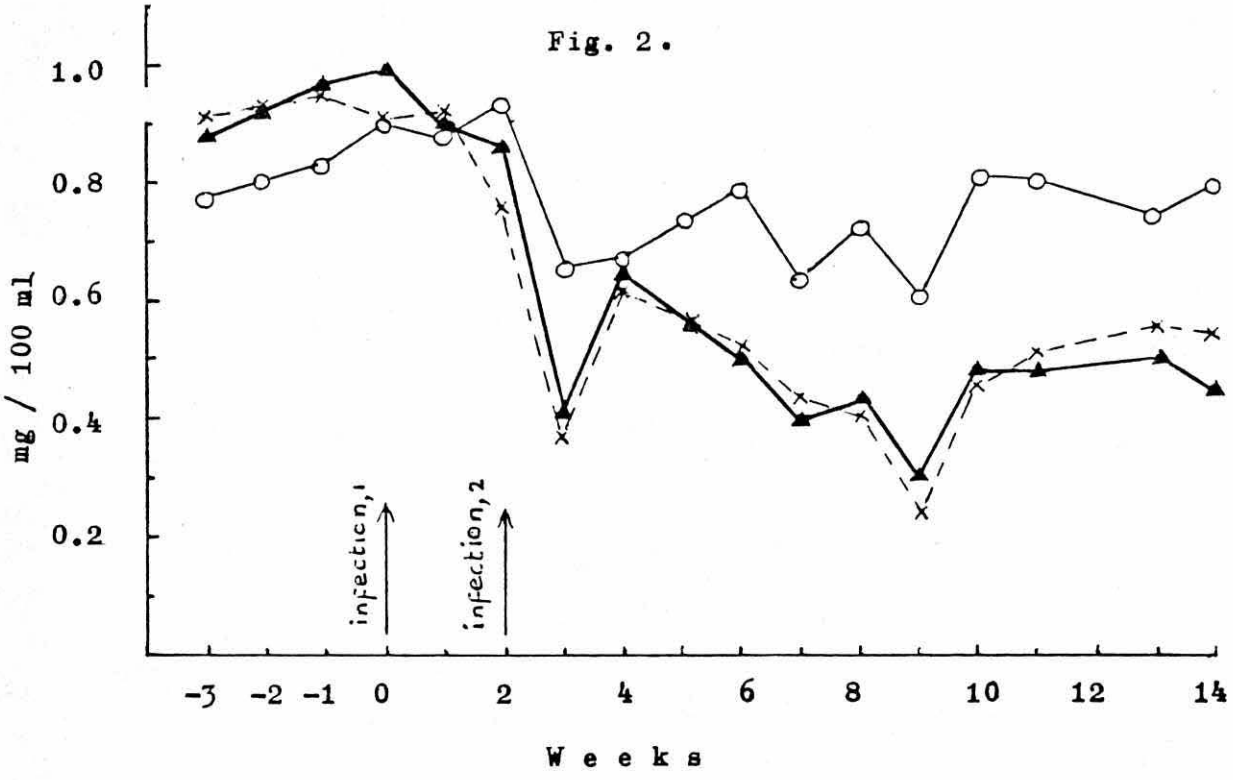
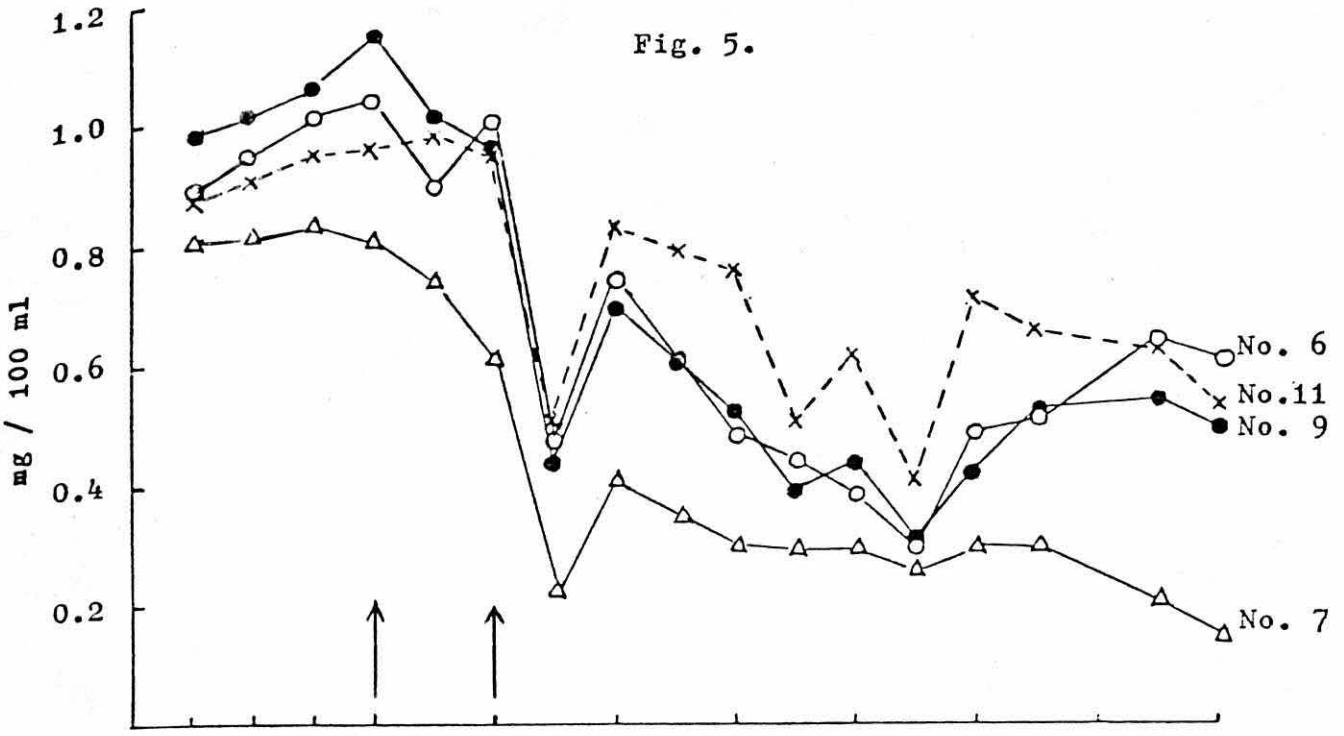


Fig.2. Mean plasma ascorbic acid. o—o group A (controls);
x--x group B (single infection; ▲—▲ group C (re-infected).

Fig. 3. Plasma ascorbic acid in group A animals (controls).



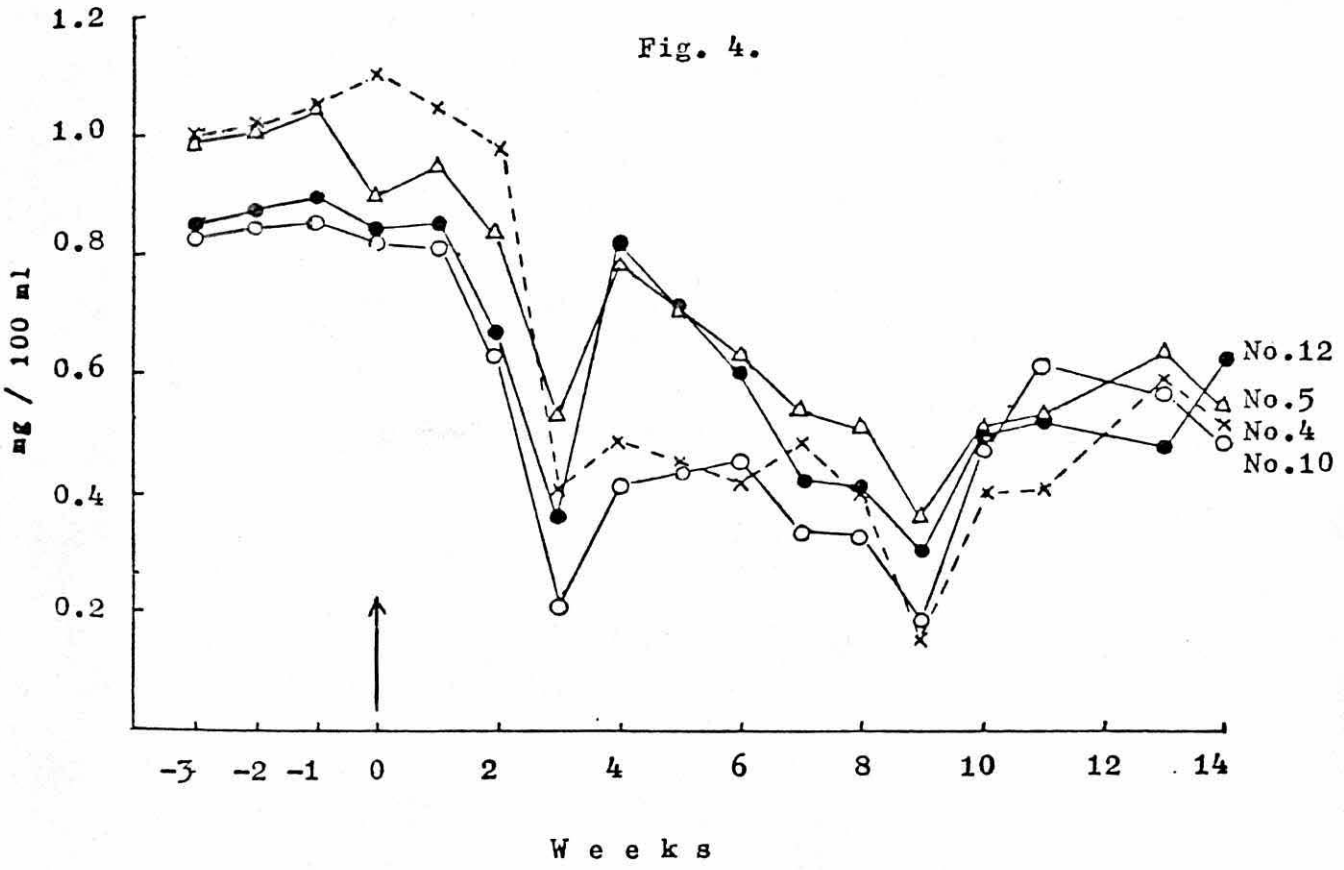


Fig. 4. Plasma ascorbic acid in group B animals (single infection)

Fig. 5. Plasma ascorbic acid in group C animals (re-infected).

↑ = infection.

during the preinfection period and the first week of infection but dropped in the second week and were reduced to about 30-50% of the preinfection values by the 3rd week post-infection. After showing a slight rise at week 4, the plasma ascorbic acid contents again progressively declined and reached a minimum by the 9th week. A slight increase was observed at week 10 but no significant changes were noticed during the last 4 weeks of infection; in animals of group B (single infection), however, the plasma ascorbic acid levels has a slight tendency to rise.

Control animals, on the other hand, showed an increase in plasma ascorbic acid content during the first 6 weeks of the experimental period. The levels dropped by the 7th week, fluctuated till the 13th week, increased to nearly initial values by the 14th week and remained fairly constant for the remainder of the observation period.

Analysis of variance was done for the values obtained during the preinfection period; the prepatent period (1-8 weeks of infection) and the patent period (8-14 weeks post-infection). No significant differences were observed between the three groups for the preinfection period but infected animals had significantly lower plasma ascorbic acid values than controls during the prepatent and patent periods ($p < 0.01$).

C) Plasma Glucose

The mean weekly values for plasma glucose in control and infected sheep can be seen in Table III and Fig. 6. The respective individual values are presented in Figs. 7-9.

Table III Mean ($\bar{x}$) and standard deviations (SD) of the plasma glucose levels (mg/100ml) in experimental sheep.

Weeks of infection	Group A (Controls)		Group B (Single infection)		Group C (Reinfected)	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
-3	46.8	8.2	44.7	3.5	50.3	5.9
-2	50.4	12.1	51.2	11.8	54.2	6.5
-1	55.1	11.7	60.4	4.1	55.5	1.0
0	61.9	8.7	66.0	5.7	67.1	5.3
1	52.7	5.4	53.3	8.1	53.7	3.7
2	49.6	4.7	45.4	6.7	47.8	4.9
3	62.9	6.3	59.6	4.4	60.4	6.8
4	59.5	6.4	62.0	3.2	59.1	2.8
5	58.4	5.3	59.1	5.3	58.3	2.8
6	57.3	4.2	56.3	7.5	57.6	2.8
7	56.8	10.8	47.5	5.5	48.5	7.6
8	64.6	3.5	55.7	11.9	59.4	3.6
9	62.5	7.1	58.1	3.8	57.4	5.4
10	60.6	5.5	58.8	4.2	50.6	6.0
11	56.8	4.6	56.6	3.5	55.8	5.7
12	-	-	-	-	-	-
13	50.0	9.1	51.6	6.7	50.3	5.9
14	59.9	2.8	59.2	5.9	56.1	6.9

Generally, the variations in the plasma glucose levels were similar in controls and infected sheep. The values increased and decreased showing some sort of periodicity which occurred about every 5 weeks. Analysis of variance revealed no significant differences between control and infected groups

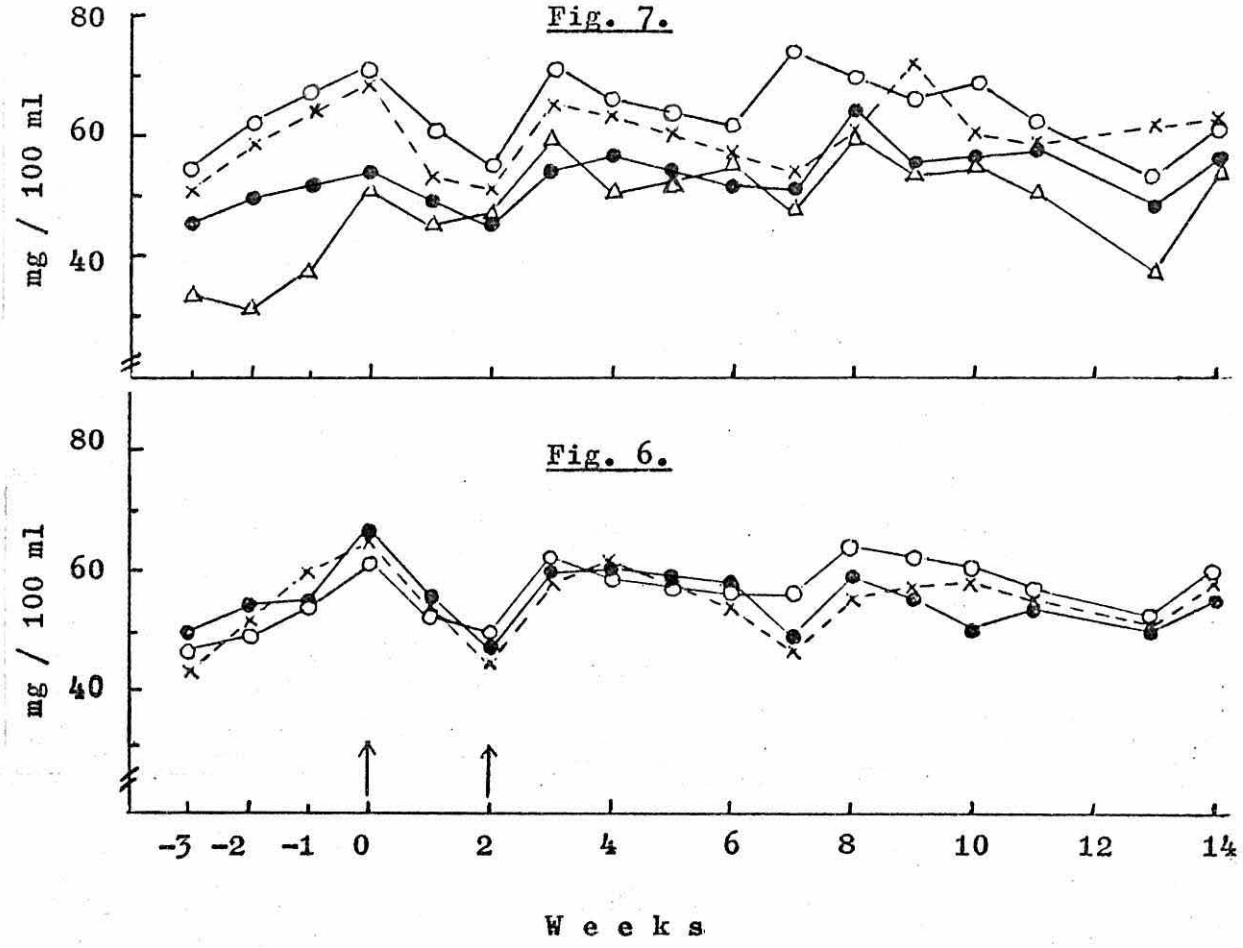


Fig. 6. Mean plasma glucose. o——o group A (controls); •——• group B (single infection); x——x group C (reinfected). ↑ =infection

Fig. 7. Plasma glucose in group A animals (controls). o——o No. 1; •——• No. 2; x——x No. 3; Δ——Δ No. 4.

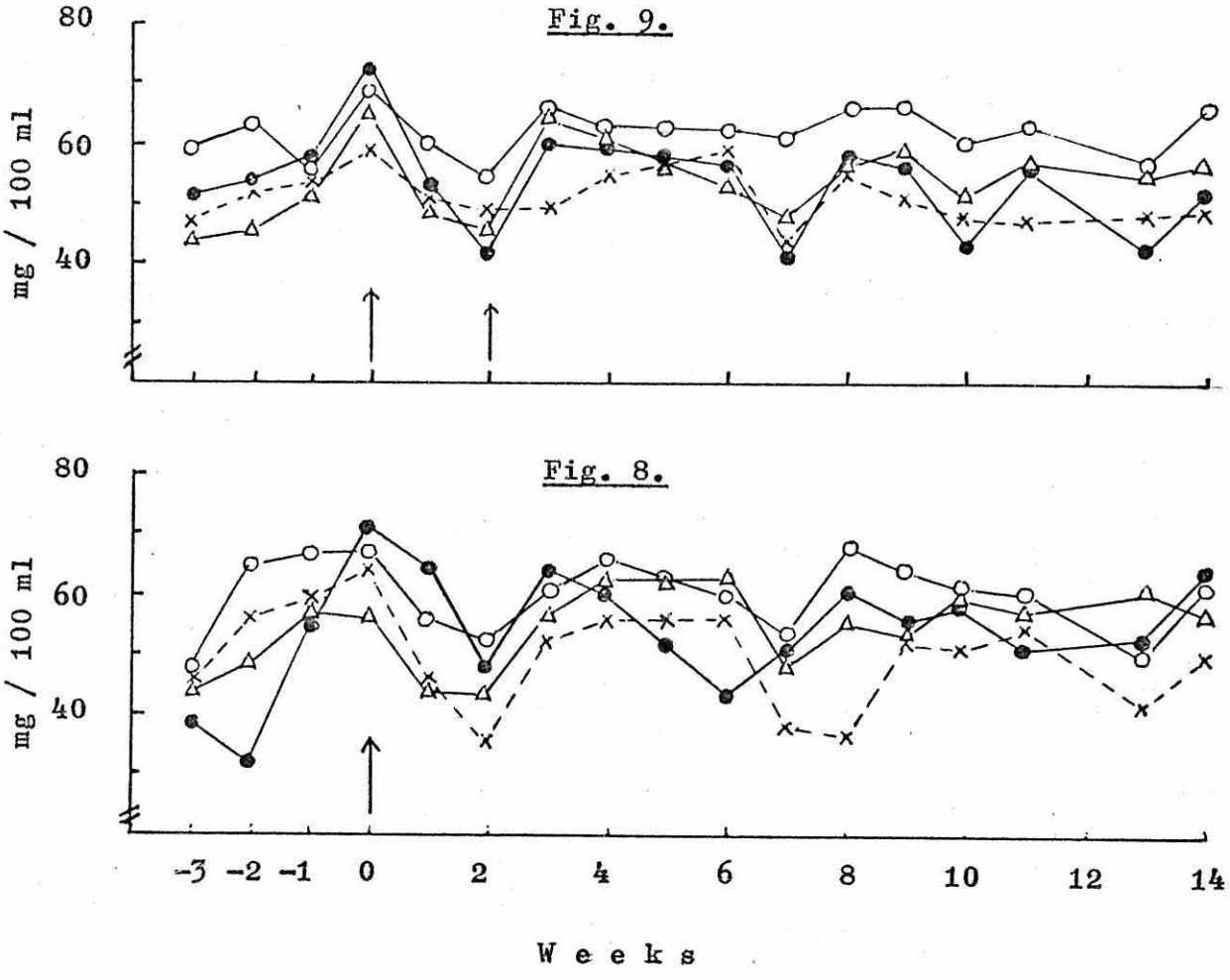


Fig. 8. Plasma glucose in group B animals (single infection).
o—o No. 4; •—• No. 5; ×—× No. 10; Δ—Δ No.12.

Fig. 9. Plasma glucose in group C animals (re-infected). o—o No.6;
•—• No. 7; ×—× No. 9; Δ—Δ No. 11. ↑ = infection.

($p > 0.05$) neither in the preinfection period or in the post-infection period.

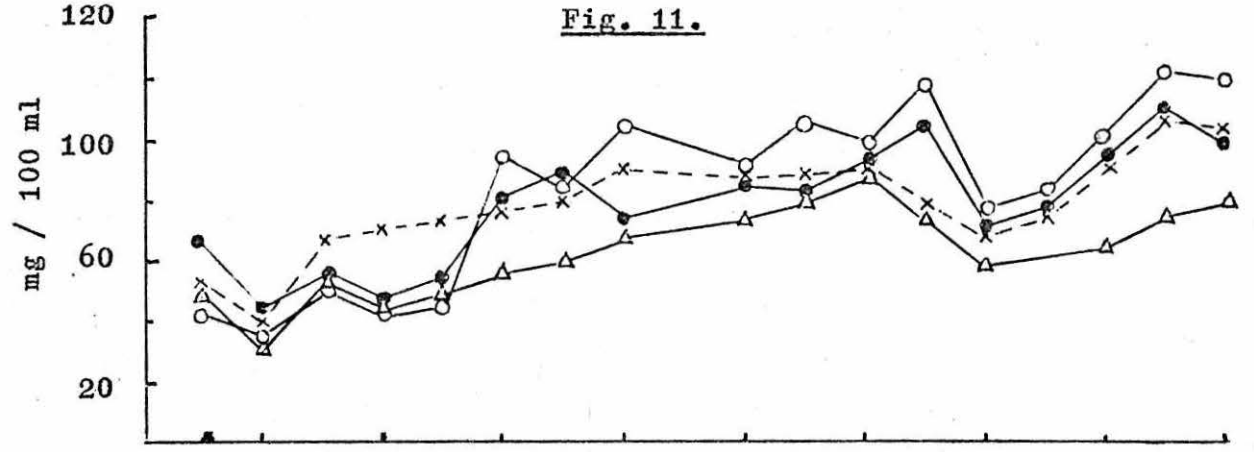
D) Plasma Cholestrol:

The mean weekly plasma cholestrol values in control and infected sheep are presented in Table IV and Fig. 10. Individual values within each group are plotted in Figs. 11-13.

Table IV Means ($\bar{x}$) and standard deviations of plasma cholestrol values (mg %) in experimental sheep.

Weeks of infection	Group A (Controls)		Group B (Single infection)		Group C (Reinfected)	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
-3	55.1	9.6	38.3	8.3	46.0	9.9
-2	41.5	3.5	33.6	3.8	42.7	13.3
-1	59.1	5.8	58.7	4.5	54.5	14.1
0	54.7	10.3	55.4	12.1	44.9	14.1
1	56.5	12.0	43.2	3.1	47.2	15.3
2	80.0	14.5	73.6	11.1	67.4	17.2
3	81.1	12.3	67.2	5.9	51.8	9.4
4	85.4	15.1	77.5	17.6	77.9	28.9
5						
6	87.1	7.2	80.5	5.7	75.9	2.3
7	92.1	10.1	68.4	4.3	59.0	13.2
8	95.8	4.3	83.4	12.9	76.3	7.5
9	95.5	18.8	69.5	26.0	74.2	8.2
10	71.2	7.2	54.9	6.8	53.0	2.6
11	80.2	2.3	65.2	3.0	65.5	8.0
12	93.4	10.3	70.4	17.9	62.5	5.8
13	106.7	19.4	84.3	18.5	84.5	12.6
14	102.7	14.2	87.2	4.4	86.0	12.4

As can be seen, the general pattern of the weekly variations in plasma cholestrol levels were not very different between the controls and infected sheep. In all animals the



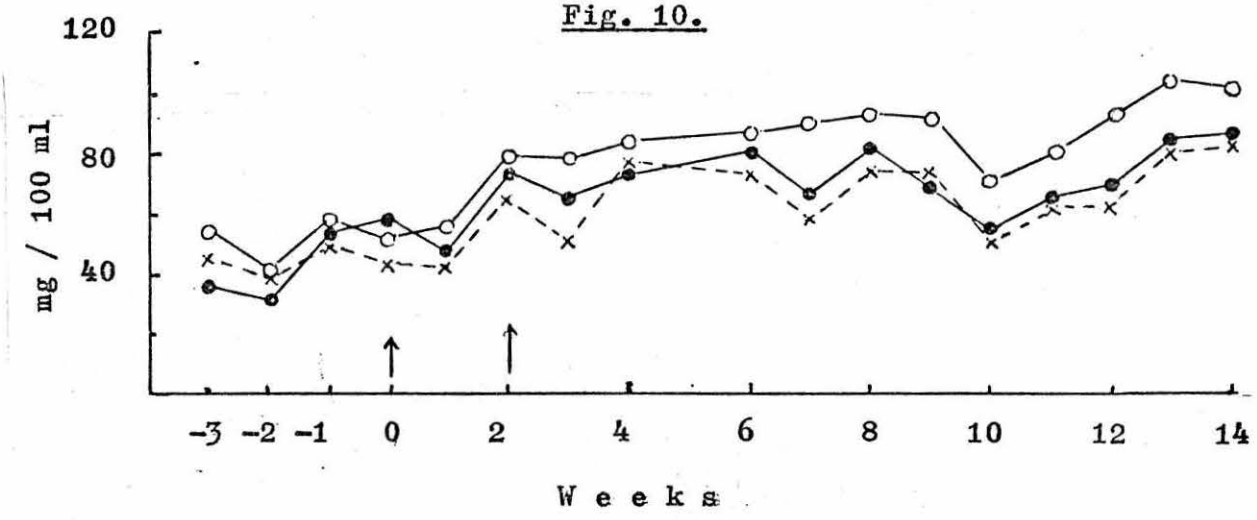


Fig. 10. Mean plasma cholesterol; o——o group A (controls); ●——● group B (single infection); ×——× group C (re-infected). ↑ = infection.

Fig. 11. Plasma cholesterol in group A animals (controls). o——o No. 1; ●——● No. 2; ×——× No. 3; Δ——Δ No. 8.

Fig. 13.

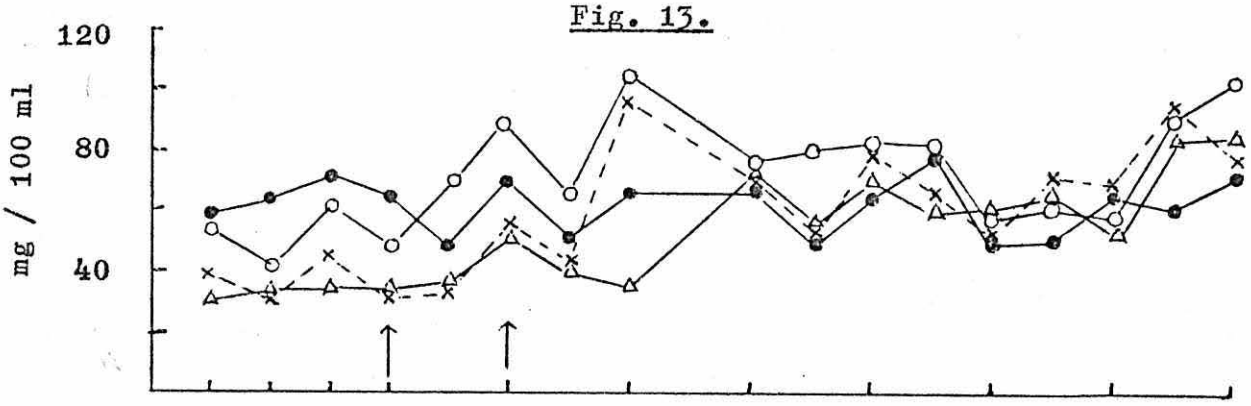


Fig. 12.

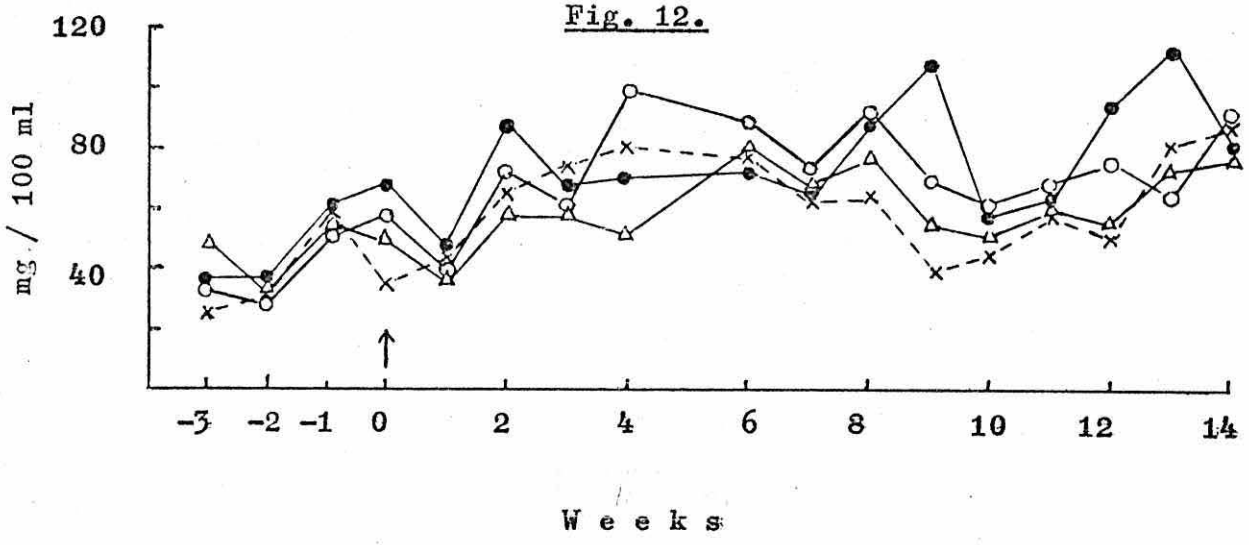


Fig. 12. Plasma cholesterol in group B animals (single infection). o—o No. 4; ●—● No. 5; ×—× No. 10; Δ—Δ No. 12.

Fig. 13. Plasma cholesterol in group C animals (re-infected). o—o No. 6; ●—● No. 7; ×—× No. 9; Δ—Δ No. 11; ↑ = infection.

values increased till about the 8th-9th week of infection, dropped temporarily at week 10 and again increased till the end of the experiment. Analysis of variance for plasma cholestrol values during the preinfection, prepatent and patent periods revealed the following:

a) The differences between controls and infected sheep were not significant ($p > 0.05$) during the preinfection period.

b) Infected animals had significantly ($p < 0.01$) lower plasma cholestrol levels than controls during the prepatent and patent periods.

c) The differences between the two infected groups were not statistically sound.

E) Plasma L.D.L.:

Means of the plasma L.D.L. values in control and infected animals are shown in Table V and Fig. 14. Individual values within each group are presented in Figs. 15-17.

Fig. 15.

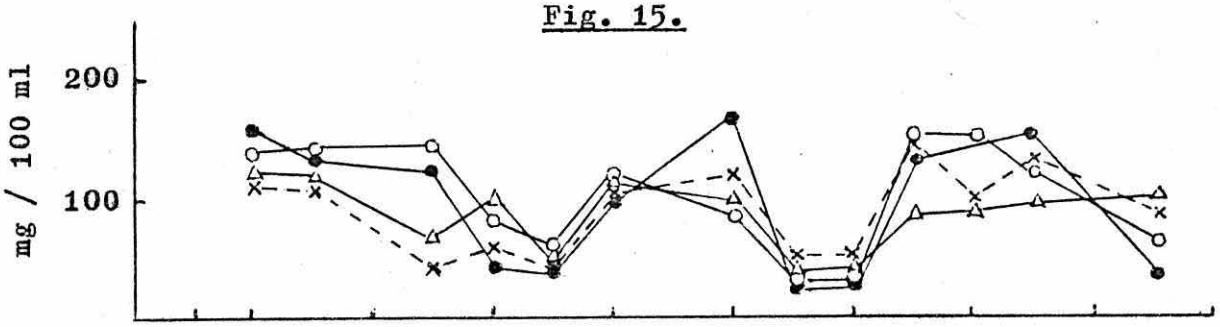


Fig. 14.

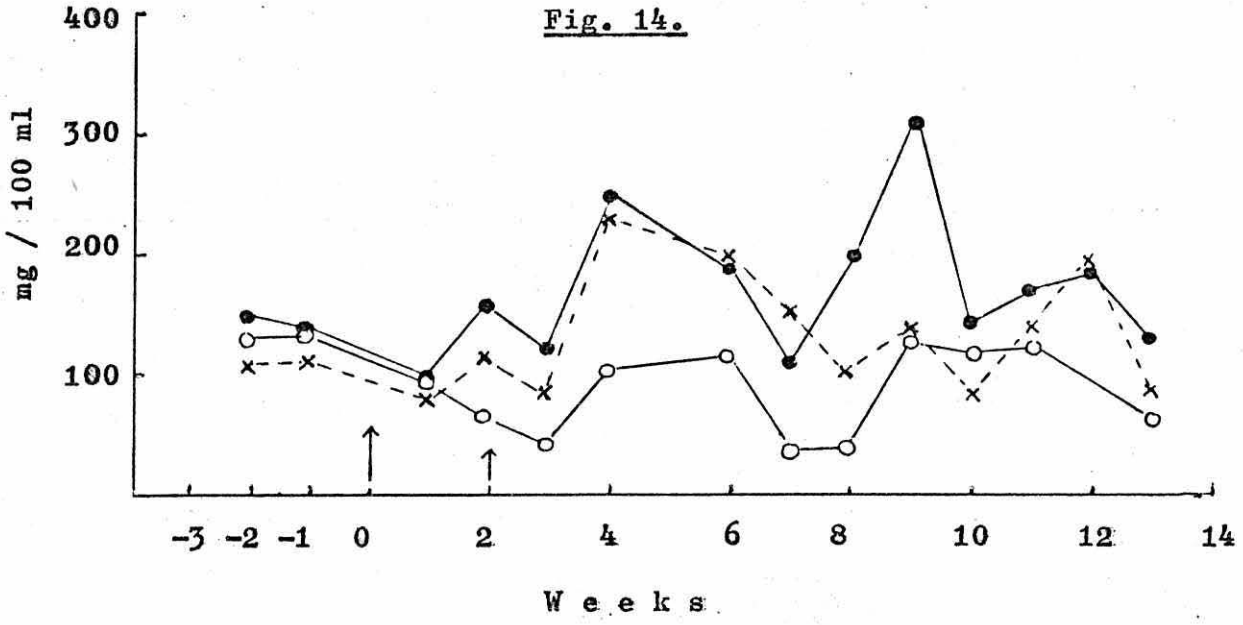
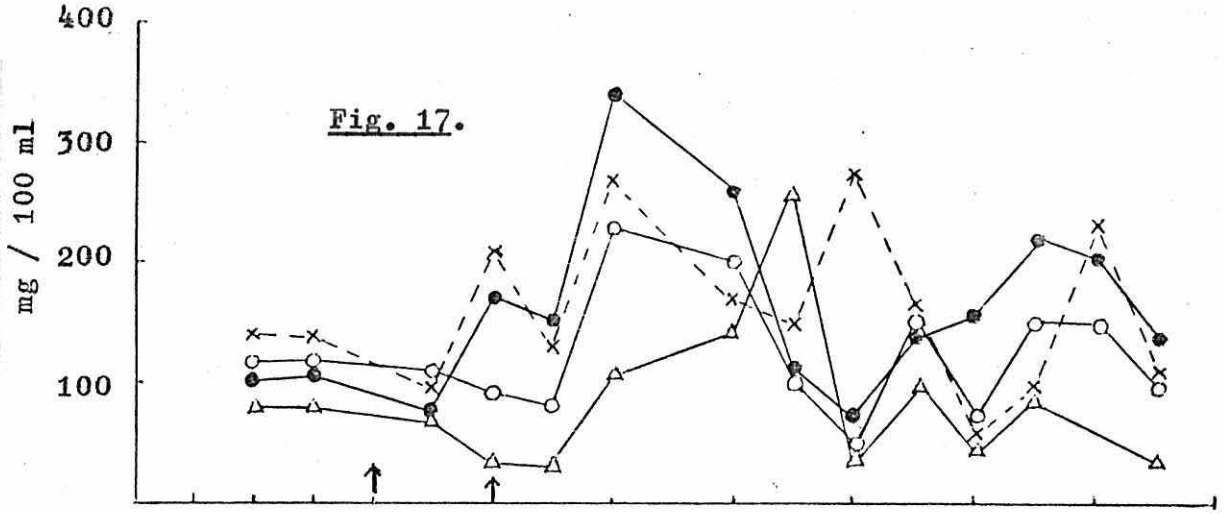


Fig. 14. Mean plasma Low-density Lipoproteins; o—o group A (controls); ●—● group B (single infection); ×——× group C (re-infected). ↑ = infection.

Fig. 15. Plasma Low-density Lipoproteins in group A animals (controls). o—o No. 1; ●—● No. 2; ×--× No. 3; Δ—Δ No. 8.



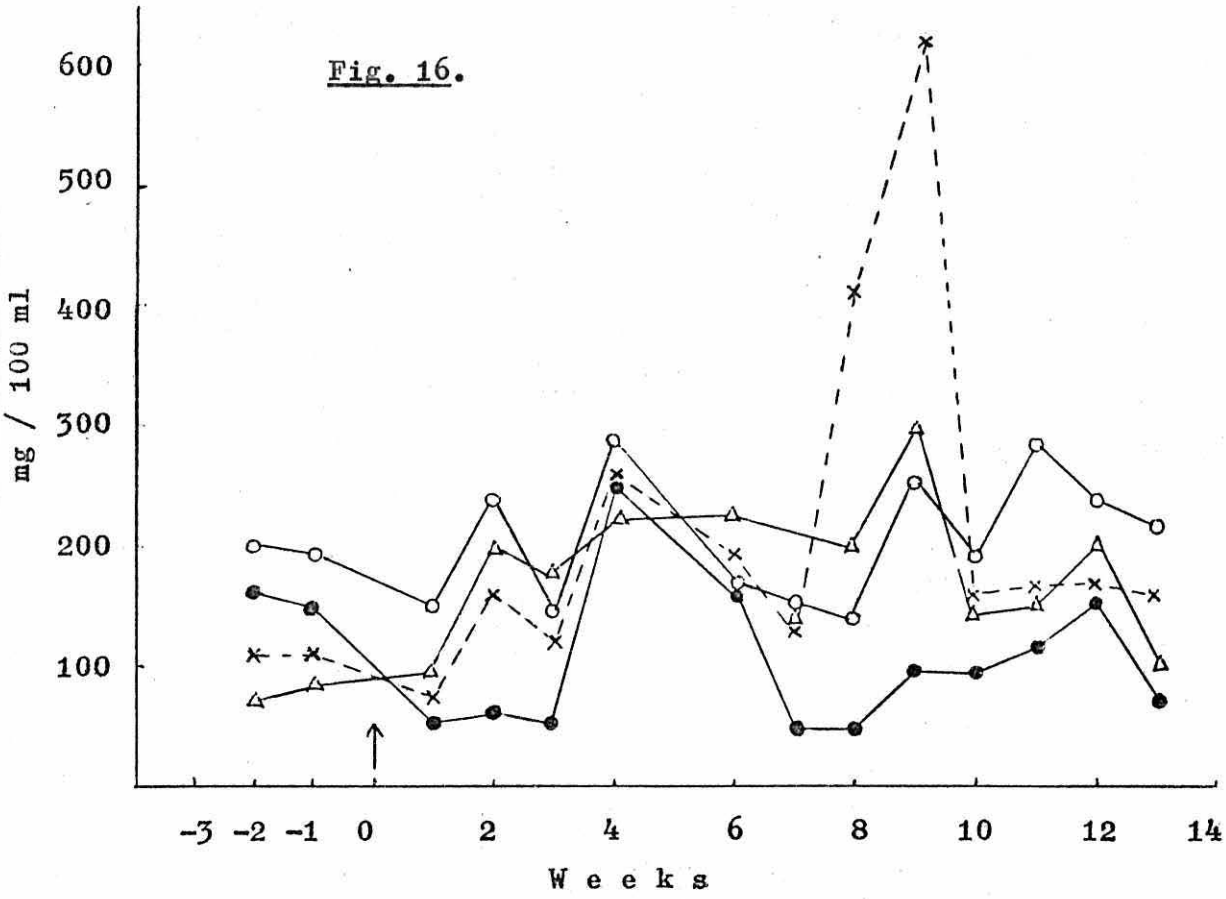


Fig. 16. Plasma Low-density Lipoproteins in group B animals (single infection). o—o No. 4; ⊙—⊙ No. 5; x--x No. 10; △—△ No. 12.

Fig. 17. Plasma Low-density Lipoproteins in group C animals (re-infected). o—o No.6; ⊙—⊙ No. 7; x--x No. 9; △—△ No. 11. ↑ = infection.

Table V Means ($\bar{x}$) and standard deviations (SD) of the plasma L.D.L. (mg/100ml) in experimental sheep.

Weeks of Infection	Group A (Controls)		Group B (Single infection)		Group C (Reinfected)	
	$\bar{x}$ ±	SD	$\bar{x}$ ±	SD	$\bar{x}$ ±	SD
-3	-	-	-	-	-	-
-2	133.2	15.2	135.7	48.3	111.5	21.5
-1	131.9	13.8	133.2	40.3	112.0	23.2
0	-	-	-	-	-	-
1	95.0	42.0	93.2	36.5	92.0	18.3
2	73.0	21.1	165.7	65.7	126.7	69.0
3	43.8	6.5	123.2	48.0	85.0	36.4
4	110.8	5.9	253.7	23.3	236.2	85.4
5	-	-	-	-	-	-
6	119.2	32.5	191.2	24.6	195.0	43.1
7	35.5	9.5	117.5	40.1	155.0	63.3
8	35.5	9.5	201.2	132.5	103.7	100.0
9	132.5	25.1	321.2	193.1	137.5	24.9
10	120.7	26.8	146.2	34.5	82.5	42.5
11	128.7	21.6	175.7	50.8	139.5	52.7
12	-	-	192.5	32.9	196.7	35.2
13	68.7	27.9	138.7	58.1	88.7	37.8

It can be seen from the figures that control animals showed a periodicity in the lipoprotein picture with alternating high and low levels occurring at fairly constant intervals, 4-5 weeks.

Figure 14 shows that infected animals of group B (single infection) sustained high plasma L.D.L. levels between the 3rd and 7th week of infection. The values dropped to preinfection levels at week 7, increased sharply to another peak by week 9, declined again and remained slightly higher than initial values for the rest of the observation period.

Reinfected animals showed a similar picture to those of Group B except that the plasma lipoprotein content was maintained low between the 7th and 10th weeks of infection but again increased to a smaller peak by the 12th week.

Analysis of variance revealed the following:

(1) The differences in the plasma L.D.L. values between controls and infected animals were not significant in the preinfection period.

(2) In the prepatent period (1-8 weeks P.I.) the plasma lipoproteins were significantly lower ($p < 0.01$) in controls than in sheep of either infected groups. The differences between the 2 infected groups themselves were not significant.

(3) In the patent period (8-14 weeks P.I.) the plasma lipoprotein content of infected group B was significantly higher ($p < 0.01$) than those of either the controls or reinfected animals.

F. Fluke recovery:

The number of flukes recovered from infected animals is recorded in Table Va. Fewer flukes were recovered from reinfected animals (Group C) and the percentage recovery was significantly lower ($P < 0.01$) than in Group B animals (single infection). No flukes were found in control animals.

Table Va. The number of adult F. hepatica recovered from livers of infected sheep.

Group	Sheep No.	No. of metacercariae given	No. of adult flukes	Per cent recovery
A controls	1	0	0	0
	2	0	0	0
	3	0	0	0
	8	0	0	0
B single infection	4	268	57	21.3
	5	"	85	31.4
	10	"	96	35.8
	12	"	107	39.8
C reinfected	6	536	83	15.5
	7	"	46	8.5
	9	"	115	21.5
	11	"	24	4.5

$t = 4.4^{**}$

** significant at $P < 0.01$.

EXPERIMENTAL II

Animals:

a) Six female, fluke free Welsh mountain sheep 1-2 years of age were randomly selected from a flock of sheep reared at the departmental field station at Pen-y-Ffridd, Bangor. They were brought inside and kept together in a large pen and were fed on hay *ad libitum* and supplemented with cattle nuts. They were numbered from 1 to 6.

b) In addition, two cows were used; one (No. B) was a Friesian, 7 years old and the other (No. R) was a Jersey, 5 years old. Both cows were previously used for bracken experiments. The cows were treated similarly to sheep.

Infection:

Each sheep was dosed with 518 *F. hepatica* metacercariae per os, while each cow was infected with 1880 cysts orally as described in the general methods.

Sampling:

Blood samples were collected weekly (fortnightly during the last 10 weeks) and the plasma separated. This started 2 weeks before infection and continued for 23 weeks after in the case of sheep and for 15 weeks in the case of the two cows. Plasma ascorbic acid content, plasma iron and the iron-binding capacity were determined as described earlier.

Results:

A) Plasma ascorbic acid:

Sheep:

Means of the weekly plasma ascorbic acid content can be seen in Table VI and Fig. 18. Values obtained for each sheep are separately presented in Figs. 19-24.

Table VI Means ($\bar{x}$) and standard deviations (SD) of the plasma ascorbic acid content (mg/100ml) in sheep infected with F. hepatica.

Weeks of Infection	$\bar{x}$	±	SD	Weeks of Infection	$\bar{x}$	±	SD
-2	0.938		0.094	11	0.406		0.161
-1	0.953		0.118	12	0.707		0.088
0	0.993		0.112	13	0.817		0.156
1	0.957		0.092	14	-		-
2	0.741		0.128	15	0.742		0.139
3	1.096		0.151	16	-		-
4	0.830		0.129	17	0.745		0.138
5	0.900		0.176	18	-		-
6	0.717		0.137	19	0.630		0.107
7	0.735		0.150	20	-		-
8	0.687		0.119	21	0.611		0.094
9	0.720		0.127	22	-		-
10	0.432		0.077	23	0.537		0.111

The figures show that sheep nos. 3, 4, 5 and 6 had a similar plasma ascorbic acid picture. The plasma vitamin C declined in the second week of infection, increased by the 3rd week and again decreased by the 4th week. The levels then

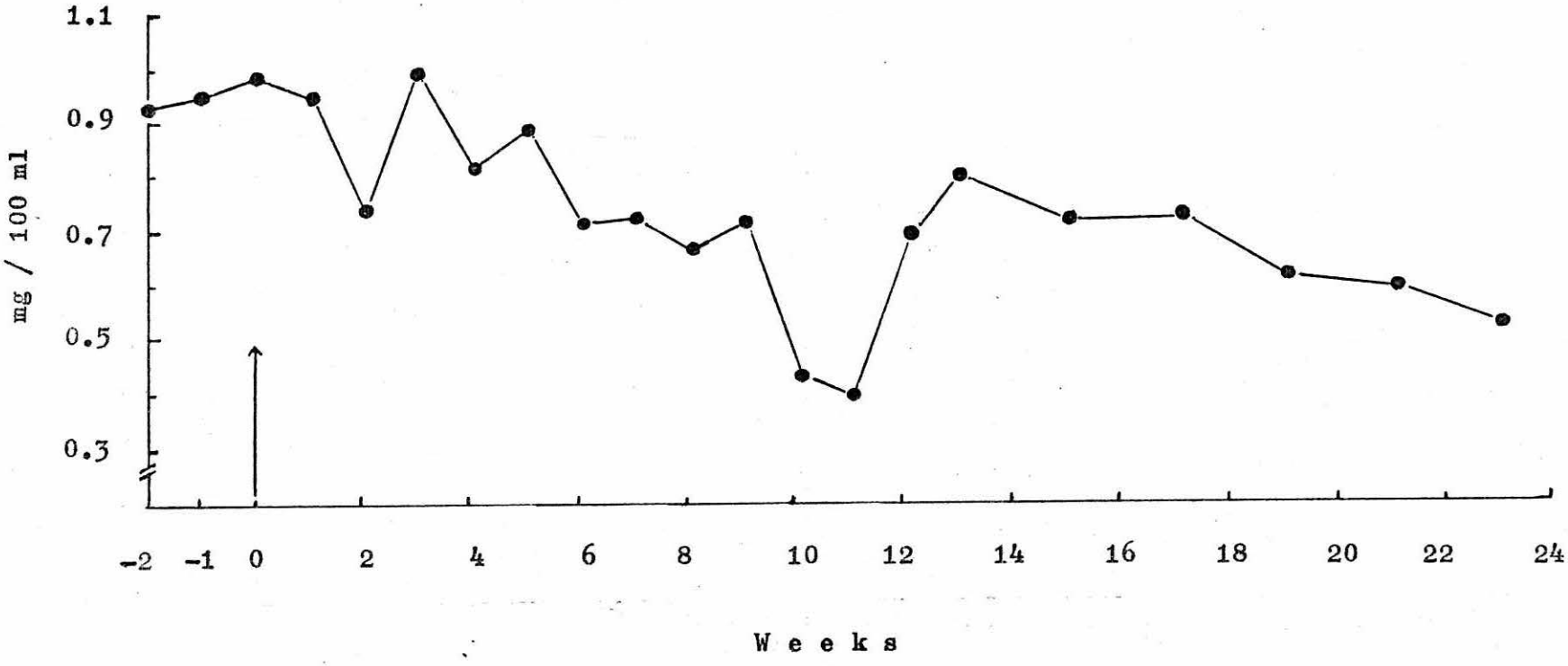
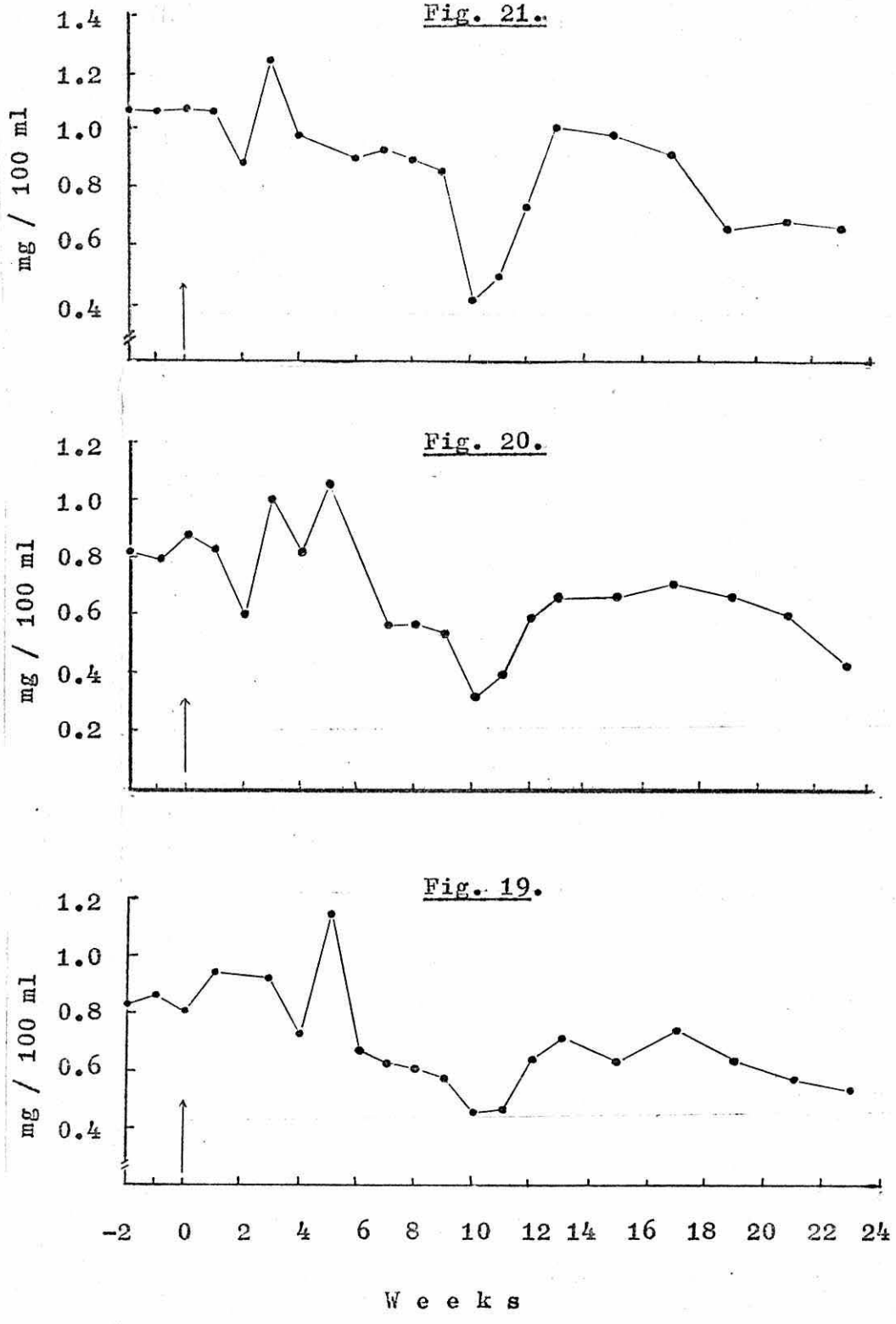
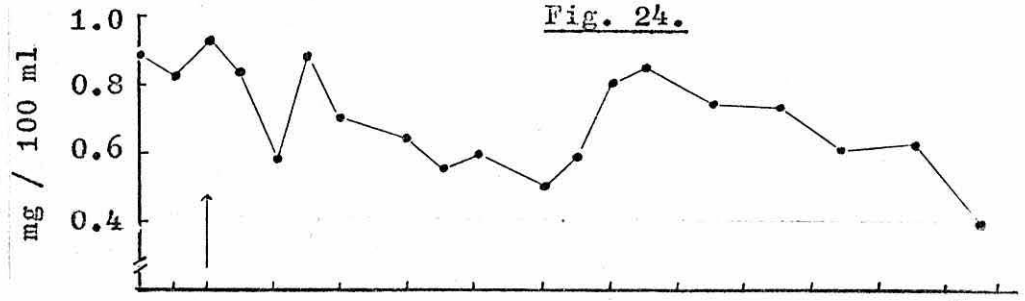
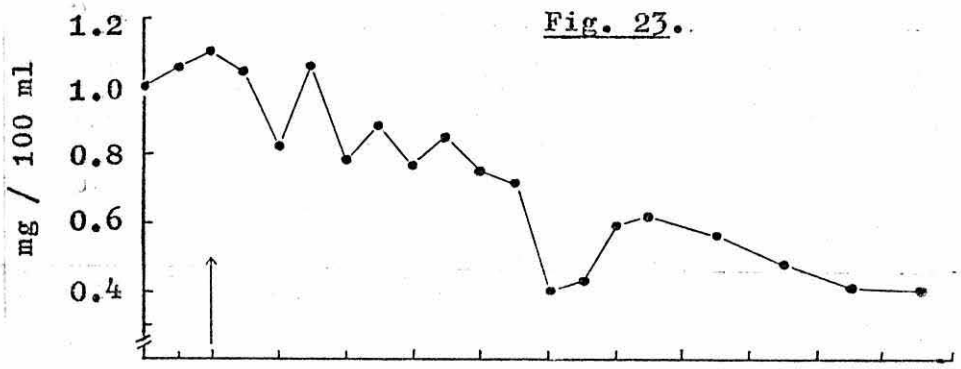


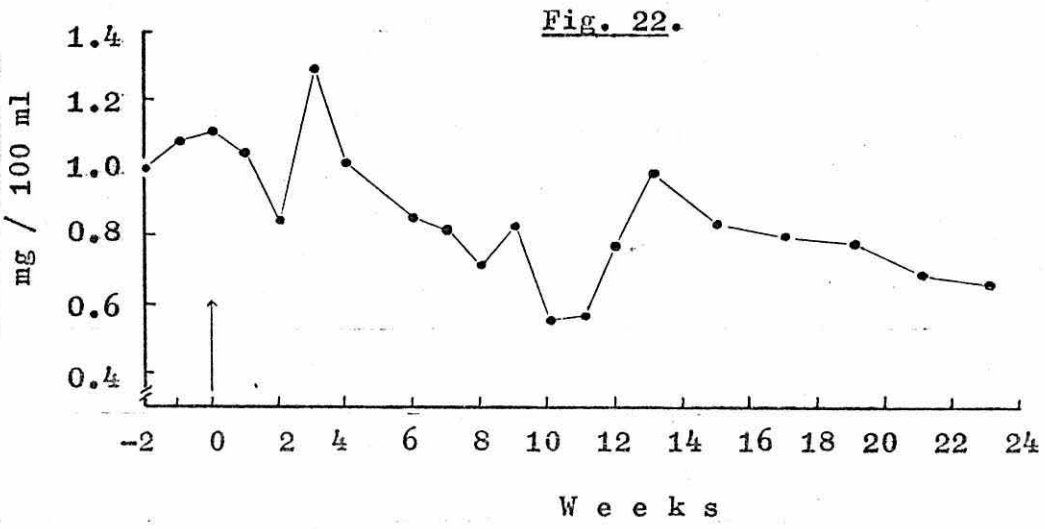
Fig. 18. Mean plasma ascorbic acid in 6 experimental sheep (1-6) each infected with 518 *F. hepatica* metacercariae. ↑ = infection.



Figs. 19; 20; & 21. Plasma ascorbic acid in sheep Nos. 1; 2; & 3, each infected with 518 F. hepatica cysts. ↑ = infection







Figs. 22; 23; & 24. Plasma ascorbic acid in sheep Nos. 4; 5; & 6, each infected with 518 F. hepatica metacercariae.

↑ = infection.

steadily declined reaching a minimum by the 10th week of infection. From the 10th week the plasma levels were increased and by the 12th week attained almost preinfection values in sheep nos. 3, 4 and 6 but remained low in animal no. 5. From the 12th week onwards the levels steadily decreased until the end of the experiment.

Sheep nos. 1 and 2 showed slightly different plasma ascorbic acid pictures. The former maintained slightly higher levels than preinfection values during the first three weeks following exposure. This was followed by a drop at the 4th week and a sharp rise at week 5. From the 6th week onwards the values progressively declined, reaching a minimum by the 10th week of infection. Then the plasma ascorbic acid increased and approached preinfection values by the 12th week, and remained almost similar till the 16th week. The levels then declined until the end of the observation.

In sheep no. 2, the plasma ascorbic acid content decreased at week 2 of infection, increased to above initial levels by the 3rd week, declined to preinfection values by the 4th week and was again elevated by the 5th week of exposure. Afterwards, the levels progressively declined reaching a minimum by the 10th week. From the 10th week onwards the changes were very similar to those observed for sheep no. 1.

Cows:

The weekly plasma ascorbic acid content in cows no. R and B are presented in Table VII and Fig. 25.

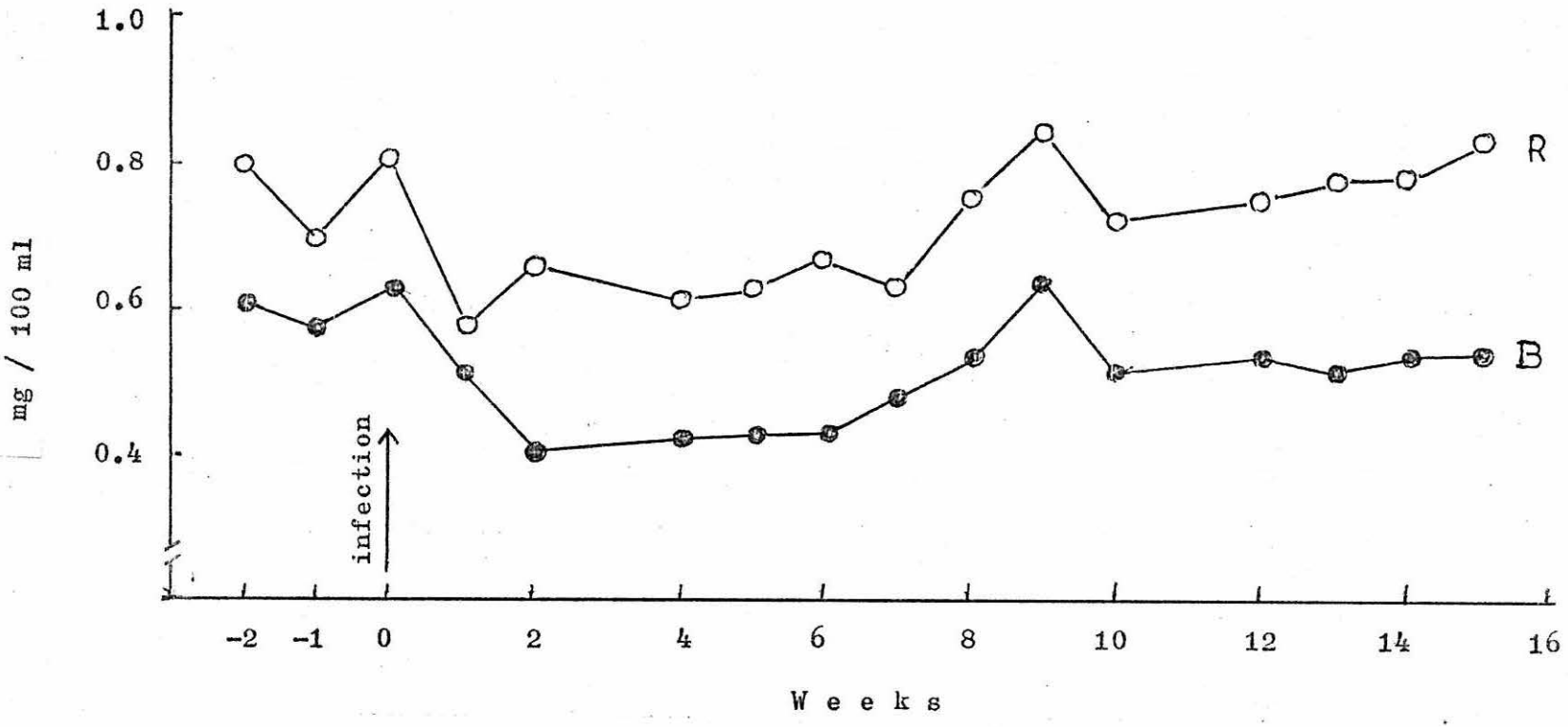


Fig. 25. Plasma ascorbic acid in cows A and B, each infected with 1880 F. hepatica cysts

In cow no. R the plasma ascorbic acid levels dropped slightly at the first week of infection and then remained fairly constant till the 7th week. This was followed by a marked rise at weeks 8 and 9. The levels decreased at week 10 and thereafter increased very gradually till the end of the observation. Cow no. B, on the other hand, had an initial drop in plasma vitamin C by the first and second weeks of infection. The values were maintained constant until the 6th week. From the 7th week to the end of the experiment the changes were nearly identical with those reported for the former cow (no. R).

Table VII The weekly plasma ascorbic acid content (mg/100ml) in experimental cows nos. R and B.

Weeks of Infection	Plasma ascorbic acid		Weeks of Infection	Plasma Ascorbic acid	
	Cow R	Cow B		Cow R	Cow B
-2	0.800	0.620	7	0.630	0.480
-1	0.690	0.570	8	0.750	0.525
0	0.810	0.630	9	0.840	0.645
1	0.570	0.510	10	0.720	0.510
2	0.660	0.405	11	-	-
3	-	-	12	0.750	0.540
4	0.615	0.420	13	0.780	0.510
5	0.630	0.435	14	0.780	0.540
6	0.675	0.435	15	0.840	0.540

B) Plasma iron and Iron-binding capacity:

Sheep:

The mean weekly plasma iron, iron-binding capacity and the percent saturation are given in Table VIII and Fig. 26. Individual values are also presented in Figs. 27-32.

i) Plasma iron:

The changes in plasma iron concentrations following infection were basically similar. The general pattern was as follows:

a) Between the 3rd and 7th weeks of infection the levels were increased to above preinfection values.
b) From the 7th week the plasma iron declined and minimum values were reached by the 10th week.
c) Thereafter the iron concentrations varied from animal to animal but were generally increased; at one time or another between the 11th and 19th week preinfection levels were reached and even exceeded in most animals.
d) Terminal values were very low.

ii) Plasma Iron-binding Capacity:

The plasma iron-binding capacity exhibited wide variations. The general pattern could be summarised into the following points:

a) In most animals the levels decreased by the second week of infection, gradually increased till about the 6th week and again declined till the 8th week.

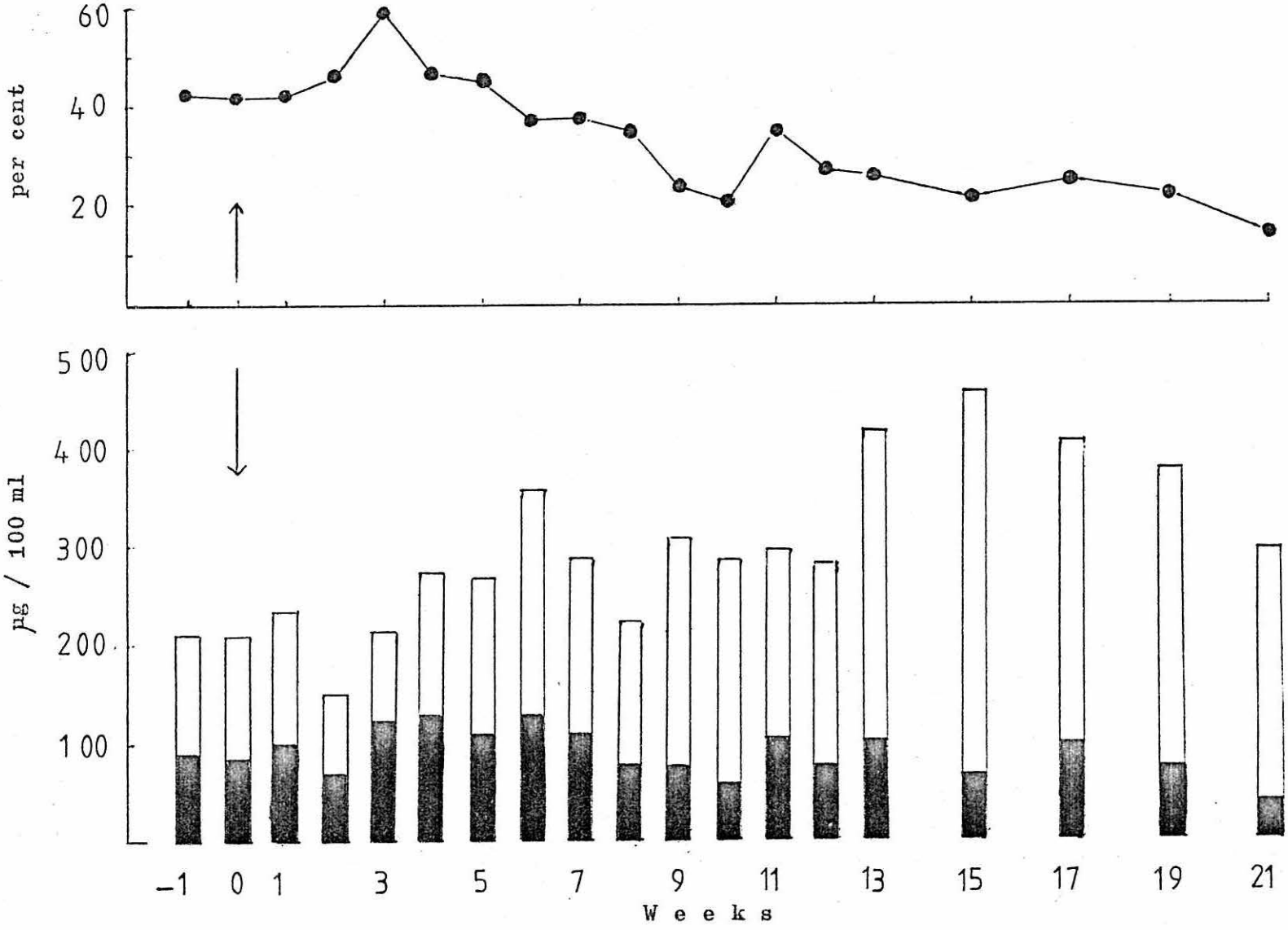


Fig. 26. Mean plasma iron, ▩ ; iron-binding capacity, □ ; and saturation concentrations o—o ; in 6 sheep each infected with 518 F. hepatica cysts. ↑ = infection.

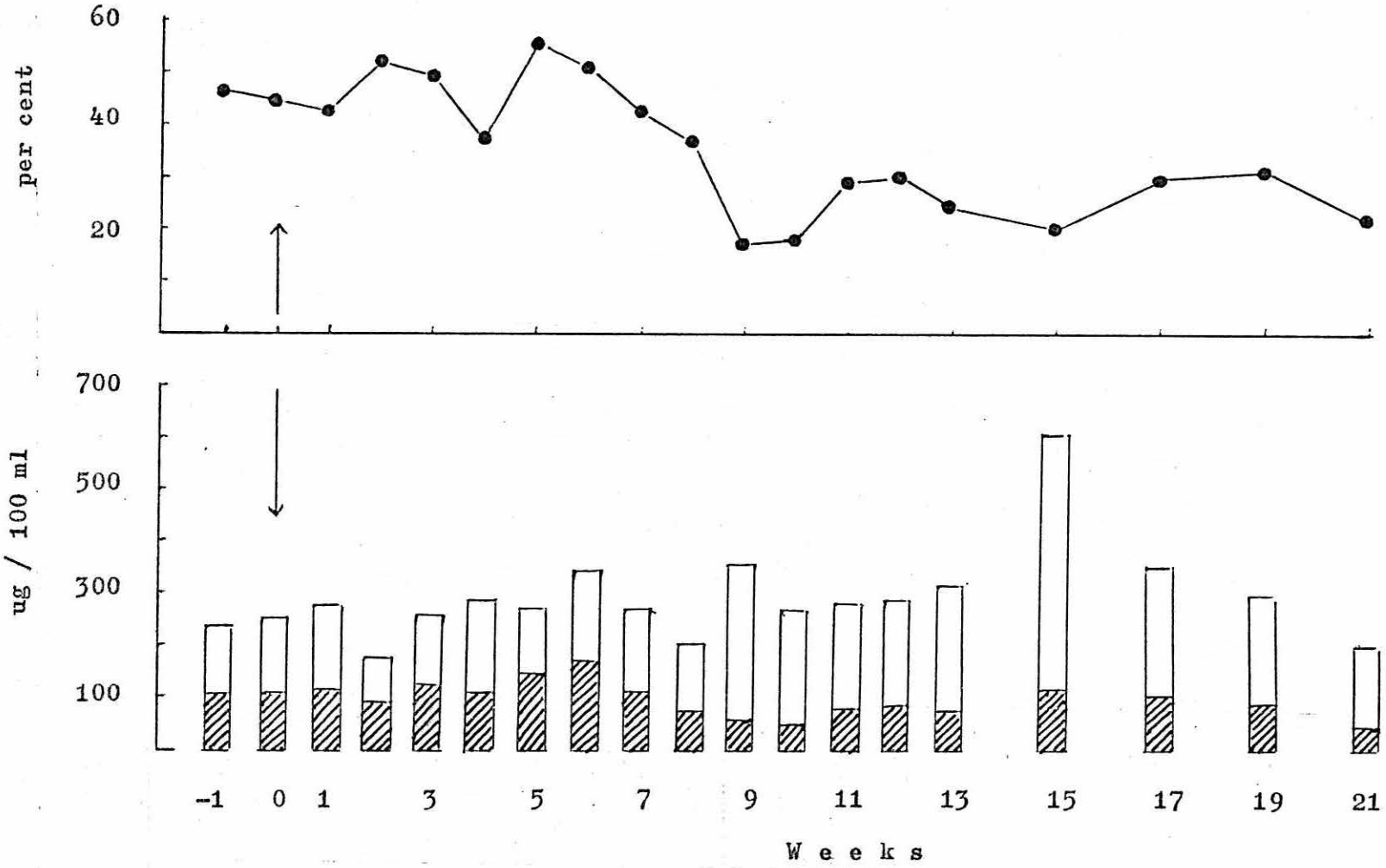


Fig. 27. Plasma iron ▨ , iron-binding capacity □ , and saturation concentrations o—o in sheep No. 1 infected with 518 F. hepatica cysts. ↑ = infection.

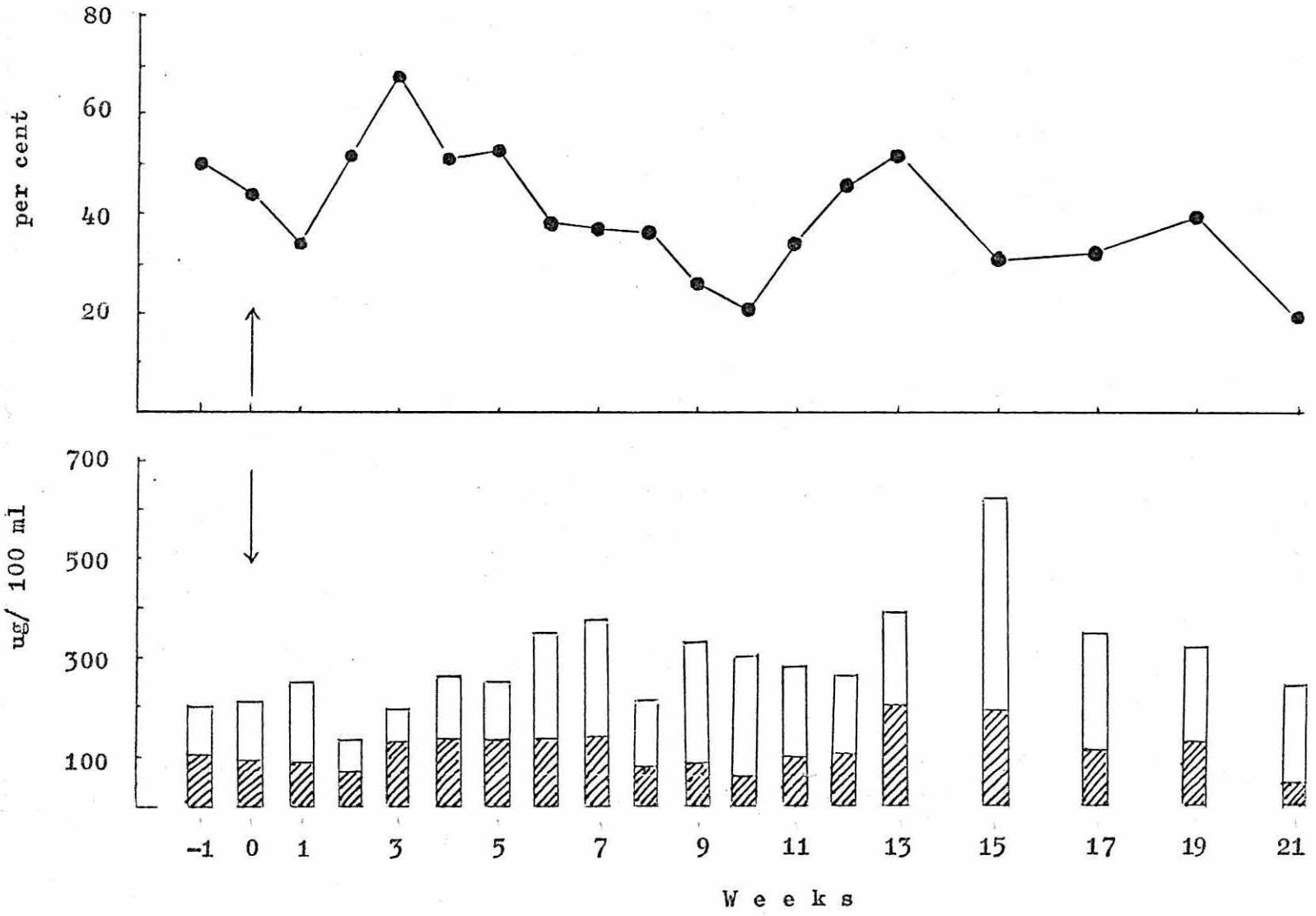


Fig. 28. Plasma iron ▨ , iron-binding capacity □ , and saturation concentrations o—o; in sheep No. 2 infected with 518 cysts of F. hepatica. ↑ = infection.

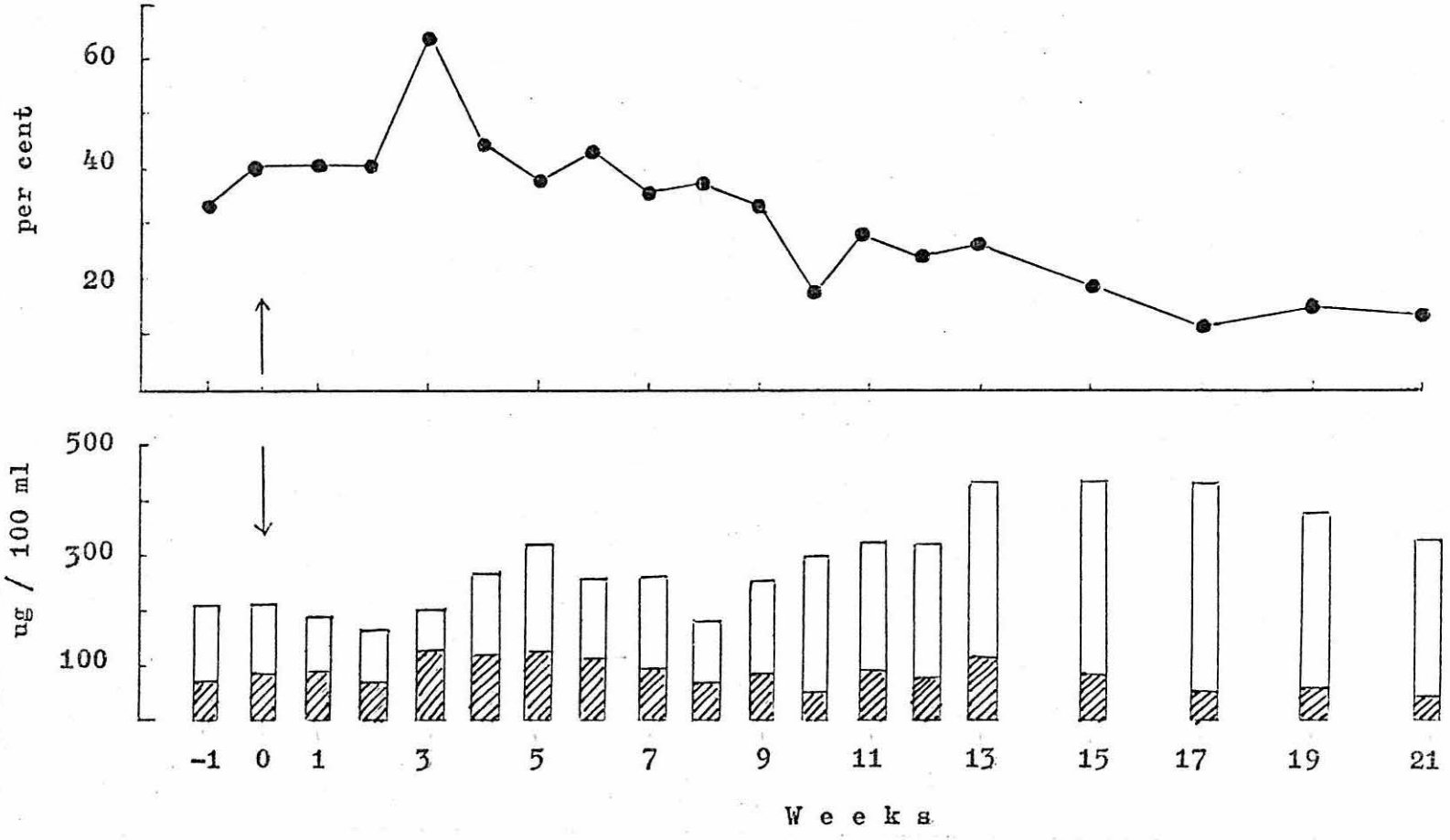


Fig. 29. Plasma iron ▨ , iron-binding capacity □ , and saturation concentrations o—o , in sheep No. 3 infected with 518 F. hepatica cysts. ↑ = infection.

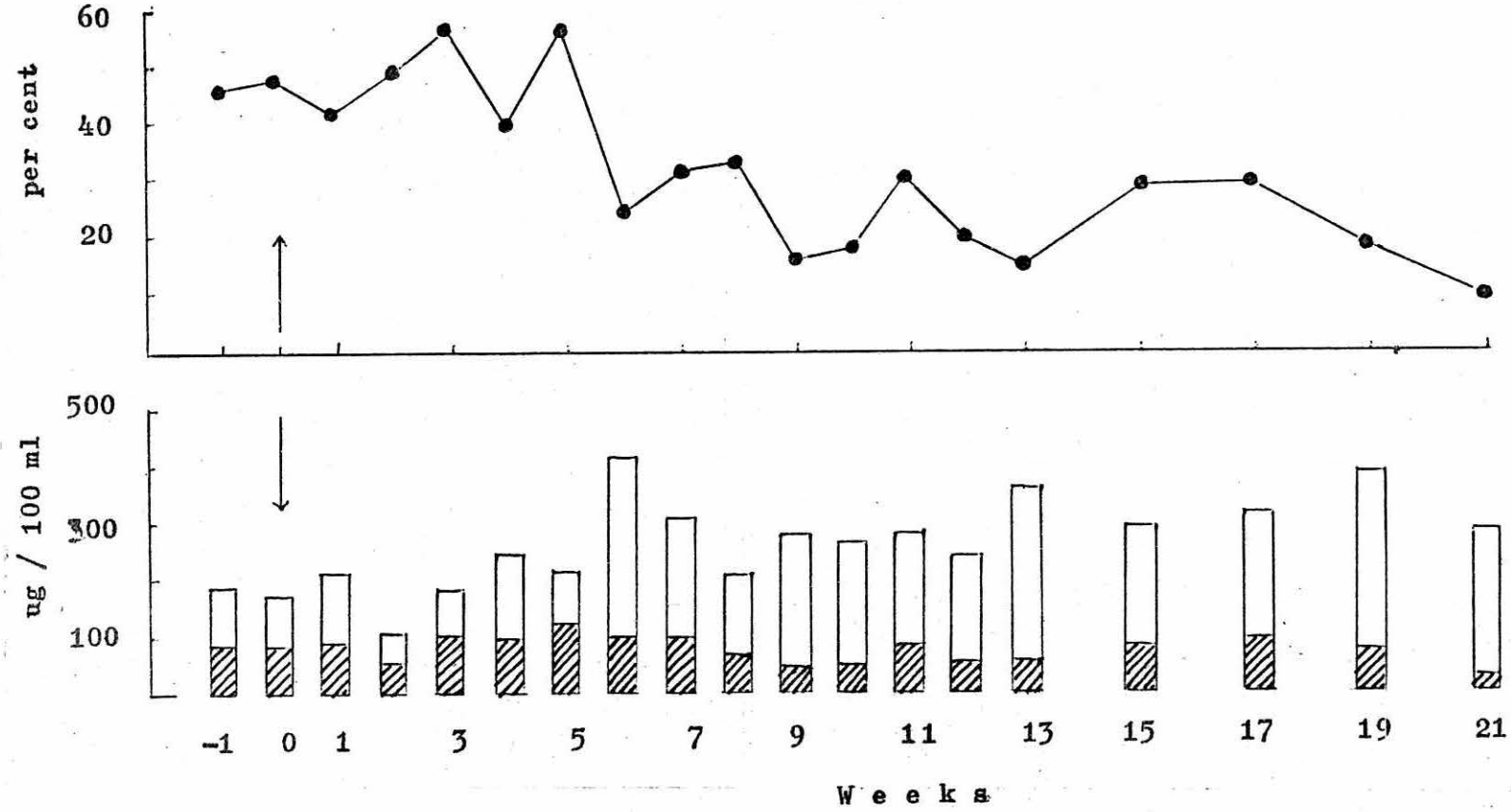


Fig. 30. Plasma iron ▨ , iron-binding capacity □ , and saturation concentrations o—o , in sheep No. 4., infected with 518 F. hepatica cysts. ↑ = infection.

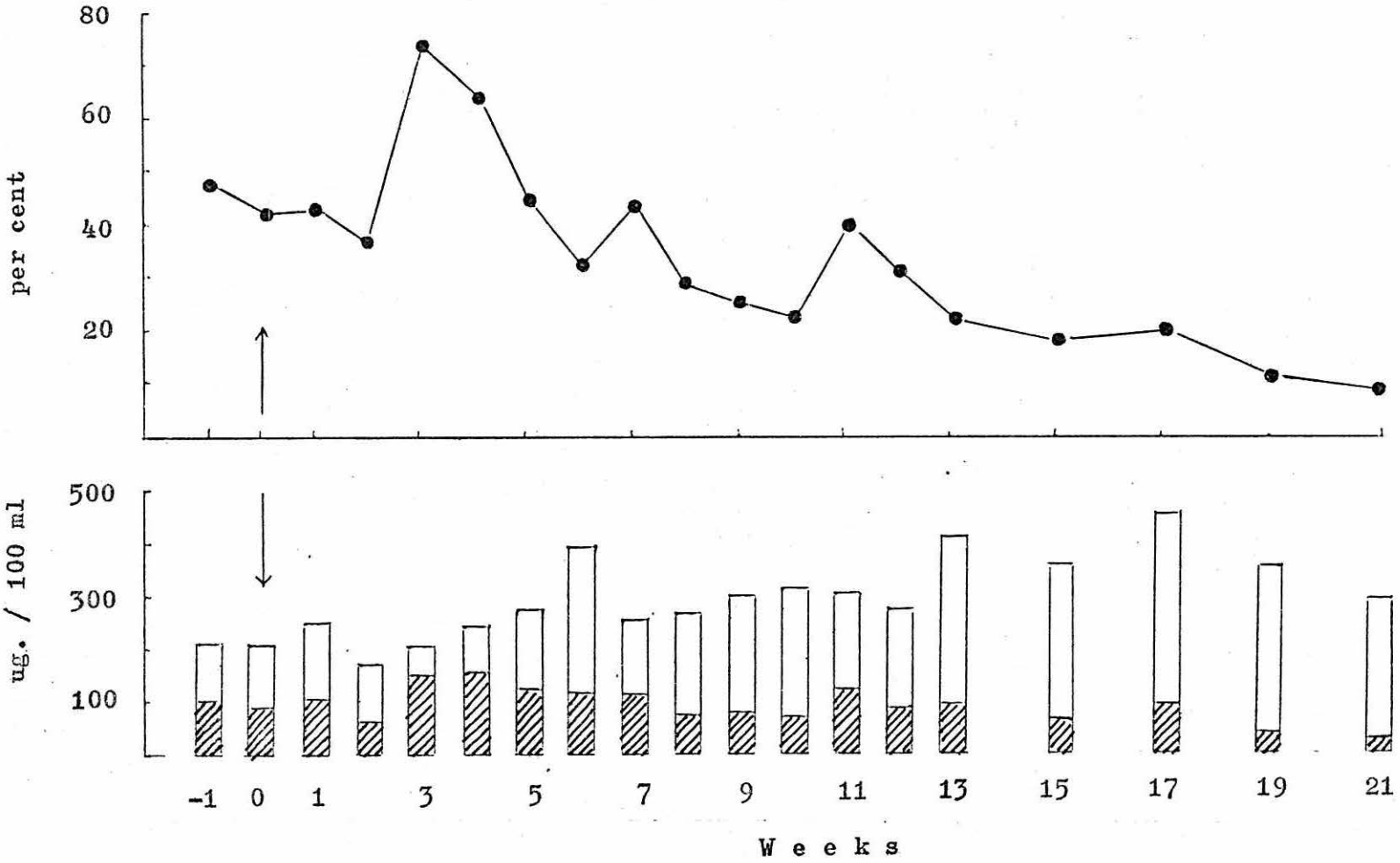


Fig. 31. Plasma iron ▨ ; iron-binding capacity □ , and saturation concentrations o—o ,in sheep No. 5., infected with 518 F. hepatica cysts. ↑ = infection.

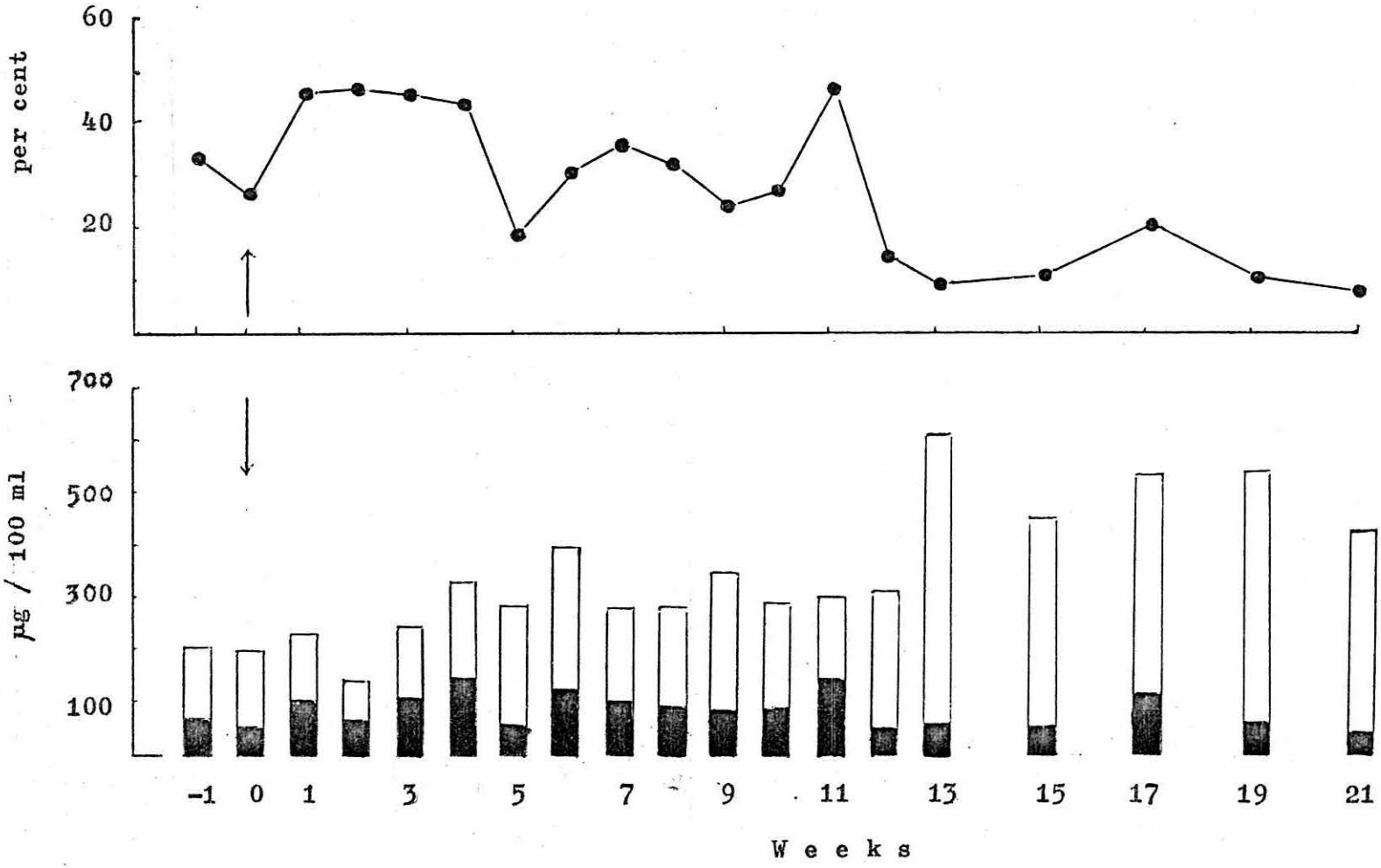


Fig. 32. Plasma iron ■ , iron-binding capacity □ , and saturation concentrations o--o, in sheep No. 6., infected with 518 F. hepatica cysts. ↑ = infection.

Table VIII. Mean ($\bar{x}$) and Standard Deviations (SD) of plasma iron values and plasma iron-binding capacity (in ug/100ml) and the saturation concentration (%) in infected sheep.

Weeks of infection	Plasma iron $\bar{x}$ ± SD		Iron-binding capacity $\bar{x}$ ± SD		Per cent saturation $\bar{x}$ ± SD	
-1	92.4	17.2	213.1	14.3	43.3	7.0
0	87.3	16.7	209.9	21.4	41.7	6.8
1	98.6	12.4	235.9	27.7	41.9	3.3
2	70.4	10.5	152.2	22.5	47.0	5.7
3	127.3	15.9	214.2	26.0	60.3	10.4
4	128.6	21.0	274.3	30.0	47.3	9.3
5	118.8	31.0	269.5	30.4	44.9	13.4
6	130.1	22.3	360.9	51.6	36.9	8.3
7	111.8	16.0	292.3	44.2	38.5	3.9
8	78.1	7.4	226.3	35.3	34.9	3.2
9	74.5	14.8	312.0	37.9	24.2	5.9
10	62.4	11.8	291.6	18.4	21.3	3.6
11	106.0	22.2	297.1	16.7	35.7	7.0
12	76.8	21.9	285.0	26.8	27.2	8.3
13	103.3	53.2	423.4	93.4	25.6	13.8
14	-	-	-	-	-	-
15	66.7	31.1	462.8	120.3	21.7	6.8
16	-	-	-	-	-	-
17	98.7	21.7	409.4	72.6	24.9	7.2
18	-	-	-	-	-	-
19	76.7	30.3	382.0	76.6	21.5	11.0
20	-	-	-	-	-	-
21	39.4	8.7	298.9	68.7	14.1	5.1

b) At the 8th week, preinfection, the levels were elevated and showed a slight tendency to increase till the 12th week. In sheep no. 2, however, the values increased by week 9 and then gradually decreased till the 12th week.

c) Higher values were mostly observed between the 13th and 19th week of exposure. Maximum values were reached at week 15 in sheep nos. 1 and 2 followed by a gradual decline till the end of the experiment. In the other animals a marked rise was noticed at week 13, particularly so in sheep no. 6. This was followed by a drop at week 15 and then an increase in week 17. The values were relatively lower during the terminal week.

iii) Saturation concentrations:

The percentage saturation varied with the changes in plasma iron concentrations and the iron-binding capacity and the changes were fairly similar in all the sheep. Generally the saturation concentrations were higher than preinfection levels during the first 5 weeks of infection. This was followed by a gradual decrease reaching a minimum by about the 9th or 10th week. By the 11th week, the values were elevated, then decreased and remained low in sheep no. 3 and 5 but further increased during the 15th - 19th week in the other animals.

COWS:

The plasma iron, iron binding capacity and percentage saturation are given in Table IX and Figs. 33-34.

Table IX Plasma iron and iron-binding capacity (ug/100ml) and percent saturation in infected cows.

Weeks of Infection	Cow No. R			Cow No. B		
	Plasma iron	Binding capacity	% Saturation	Plasma iron	Binding capacity	% Saturation
-1	220.5	450.0	49.0	185.1	374.9	49.4
0	219.3	397.4	55.2	197.4	377.1	52.3
1	182.7	349.7	52.2	117.0	349.7	33.4
2	183.7	349.9	52.5	117.0	350.0	33.4
3	-	-	-	-	-	-
4	162.0	471.7	34.3	160.4	336.9	47.6
5	180.9	401.3	45.1	194.8	375.8	51.8
6	177.4	559.4	31.7	169.8	401.0	42.3
7	133.3	310.2	43.0	144.9	426.1	34.0
8	168.8	467.9	36.1	167.1	427.0	39.1
9	139.2	455.0	30.6	132.6	372.9	35.5
10	134.9	44.20	30.5	126.0	396.8	31.7
11	-	-	-	-	-	-
12	159.1	365.9	43.5	140.9	293.2	48.0
13	263.0	732.0	35.9	188.0	565.4	33.2
14	-	-	-	-	-	-
15	207.1	467.3	44.3	154.0	450.0	34.2
16	-	-	-	-	-	-
17	235.2	470.3	50.0	-	-	-

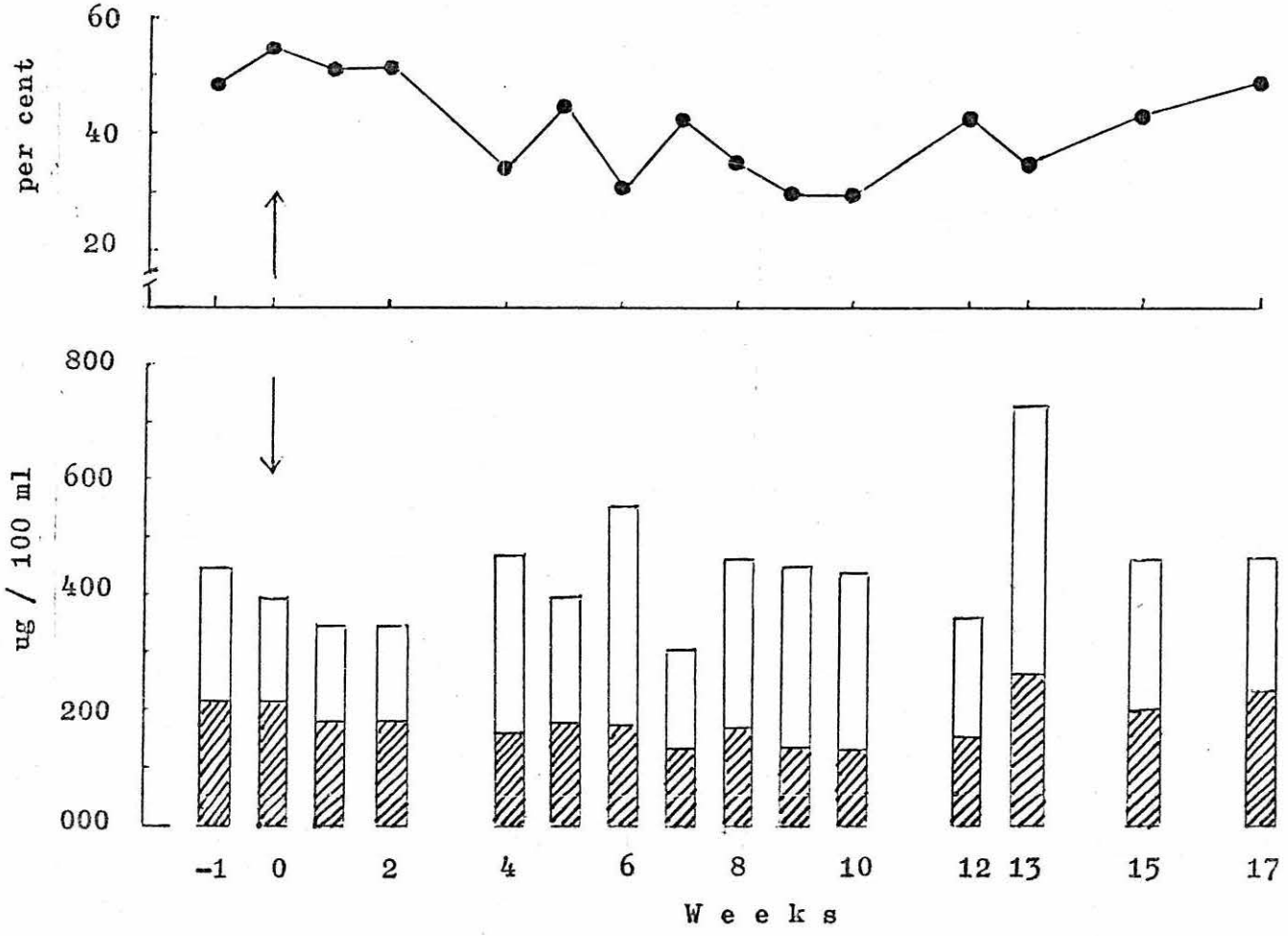


Fig. 33. Plasma iron ▨ , iron-binding capacity □ , and saturation concentrations o—o , in cow R., infected with 1880 F. hepatica cysts. ↑ = infection.

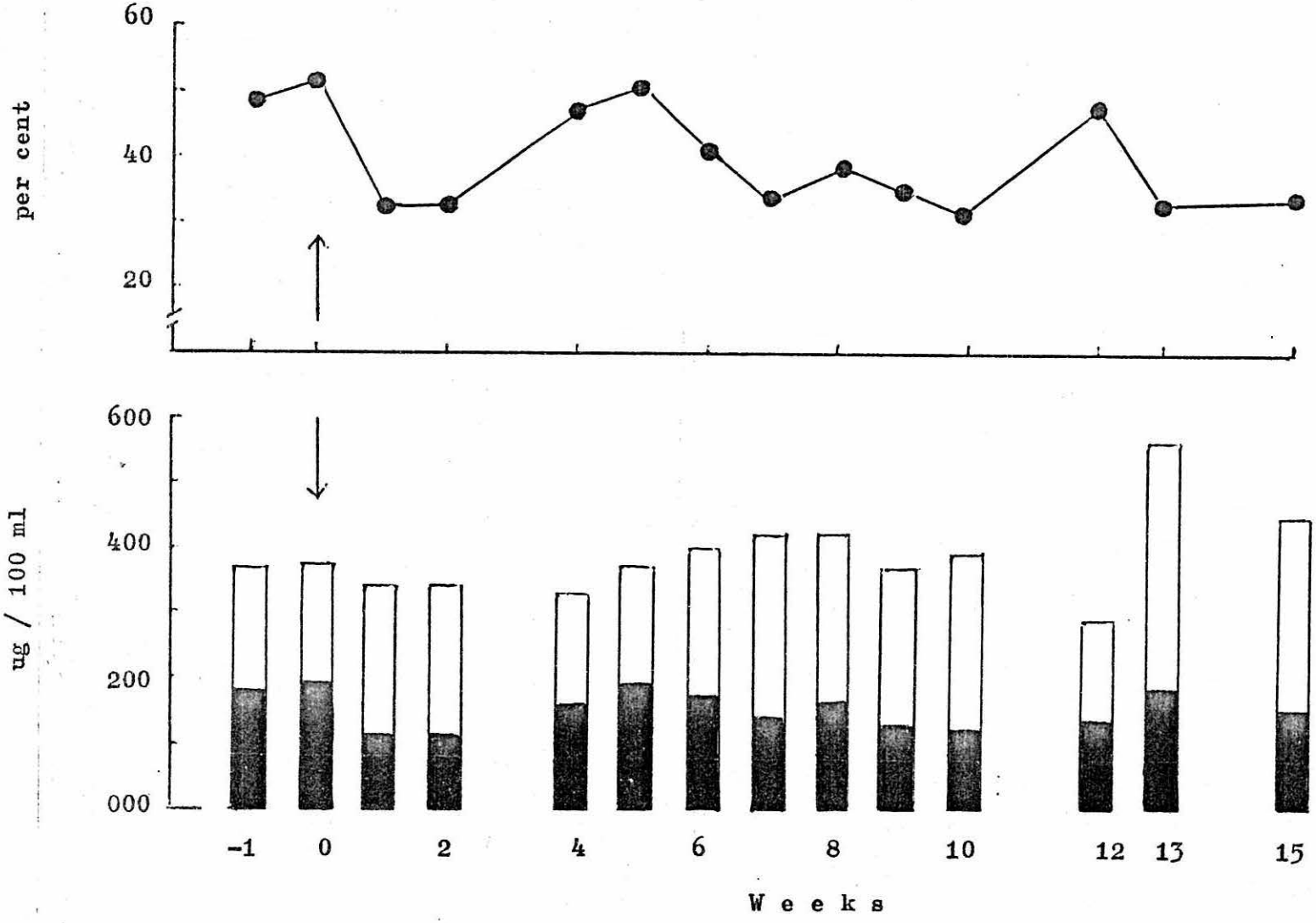


Fig. 34. Plasma iron ■ , iron-binding capacity □ , and saturation concentrations o—o , in cow B., infected with 1880 F. hepatica cysts. ↑ = infection.

i) Plasma Iron:

In cow no. R the plasma iron slightly decreased by the first week of infection and then fluctuated, with a tendency to decline till the 10th week. The concentrations again increased reaching a maximum by the 13th week and dropped slightly at the terminal two weeks.

In cow no. B, the plasma iron markedly decreased by the first and second weeks of exposure but increased to initial levels by the 5th week. Afterwards, the changes resembled those described for the previous cow.

ii) Plasma iron-binding capacity:

In animal no. B the iron binding capacity was slightly decreased at the first week of infection, remained almost similar till the 4th week and then increased during the subsequent 4 weeks. Then the levels decreased till the 12th week. Maximum values were recorded at the 13th week of infection.

The general trend of the plasma binding capacity in cow no. R was similar to that of cow B except that low values were observed at the 7th and 9th weeks.

iii) Saturation concentrations:

In cow B the saturation concentrations sharply decreased by the first week of infection. This was followed by a gradual increase till the 5th week and then a similar decrease till about the 10th week. The concentrations were temporarily elevated at week 12 but were low during the

terminal 2 weeks.

Cow R showed a general decrease till the 10th week of infection followed by a gradual increase thereafter.

Fluke recoveries:

The numbers of flukes recovered from infected sheep and cows are shown in Table IXa.

Table IXa Fluke recoveries from F. hepatica infected sheep and cows.

Animal spp.	Animal No.	No. of cysts* given	No. of adult flukes	Per cent recovery
Sheep	1	518	218	42.1
	2	"	208	40.1
	3	"	252	48.6
	4	"	203	39.2
	5	"	died	-
	6	"	225	43.4
Cows	B	1880	94	5.0
	R	"	157	8.4

*The cysts used for infecting sheep and cows are from the same batch of metacercariae.

EXPERIMENTAL III

Liver samples, collected from the three groups of sheep in the first experiment, were freeze-dried and prepared for atomic absorption spectrophotometry. Three samples were digested per animal and 5ml fractions from each of these three samples were pooled together for iron determination.

Formalin-fixed tissue samples (liver, spleen, kidney, lymph nodes, pancreas and lungs), collected from sheep and cows of the first 2 experiments were processed in paraffin and the sections stained for ferric and ferrous iron as described before. The stained sections were then photographed and the iron content estimated visually.

Results:

The liver iron concentrations in controls and infected sheep are given in Table X. Analysis of variance revealed that controls had significantly higher ($p < 0.01$) liver iron concentrations than either of the two infected group.

Table X Liver iron concentration (mg/100g dry weight) in controls and two groups of sheep infected with *F. hepatica*.

Group	A (Controls)				B (Single infection)				C (Reinfected)			
Sheep No.	1	2	3	8	4	5	10	12	6	7	9	11
Liver iron	70.0	49.0	77.0	77.0	36.4	13.9	36.0	8.4	46.6	37.8	51.1	27.4
Mean + SD	68.25 ± 11.5				23.67 ± 12.7				40.57 ± 9.0			

b) The histochemical staining for ferric and ferrous iron did not reveal any difference regarding the abundance of one form over the other. The estimated iron contents are given in Table XI.

Table XIa Estimate of tissue iron content after staining with prussian blue stain for ferric iron.
a) sheep of experiment no. 1 (killed 32 weeks P.I.)

Group	Liver	Spleen	Lymph node	Pancreas	Lungs*	Kidney*	Remarks
A	++	++++	+	-	-	-	Controls
B	±	+++	-	-	-	-	Infected once
C	±	NS**	-	-	-	-	Reinfected

Table XIb b) sheep and cows of experiment no. II (killed 24 weeks P.I.)

Animal spp.	Liver	Spleen	Lymph node	Pancreas	Lungs*	Kidney*	Remarks
Sheep	±	NS**	±	-	-	-	
Cows	±	+++++	-	-	-	NS**	

* A few sections seen
** Not stained
Scale: -, not detectable; ±, just detectable +++++, abundant.

It can be deduced from the above tables that infected animals had less iron in their livers than controls but had considerable amounts in the spleens.

An extra sheep and a goat were killed 7 weeks after infection to collect immature flukes. Liver sections, stained for ferric iron, were found to contain marked amounts of the metal (++±).

EXPERIMENTAL IV

In the light of the changes in plasma ascorbic acid content observed in the previous experiments, it was decided to investigate the possible alterations in tissues of infected animals, at different stages of infection.

Forty-two white rats (273-292g) were used; 27 were infected orally each with 15-20 F. hepatica metacercariae while the others remained as uninfected controls. The rats were kept in galvanised cages and fed on standard rat diet.

The animals were killed in groups at different intervals. Groups I, II, III and IV consisted of 9 rats each, 6 infected and 3 controls killed after 27 days, 50, 77 and 113 days of infection respectively. The last group (V) comprised 6 rats, 3 infected and 3 controls killed 197 days P.I.

Immediately after sacrificing the rats, the livers, spleens and kidneys were dissected out and weighed, whenever possible. Parts of the liver were frozen in liquid nitrogen and later on freeze-dried for iron and copper determination. Samples were also taken for glycogen and total protein estimation and for histology and histochemistry. The left kidney, spleen (or part of it at times) and a known weight of the liver were homogenised for Vitamin C determination.

Methods have been described previously.

Results:

i) Body weight:

Mean initial and final body weights are given in Table XII.

Table XII Mean body weight (g.) in rats

Group	Description	Initial weight	Final weight	Gain
I	Controls	-	-	-
	Infected	-	-	-
II	Controls	-	-	-
	Infected	-	-	-
III	Controls	273.7	349.3	75.6
	Infected	322.3	403.8	81.5
IV	Controls	291.3	366.3	75.0
	Infected	310.3	384.8	74.5
V	Controls	282.0	357.0	75.0
	Infected	316.3	378.3	62.0

The mean body weights were only recorded for groups III, IV and V and it shows that while the net weight gain was almost similar in controls, that of infected rats decreased with time.

ii) Organ weights:

Means of the organ weights can be seen in Table XIII and Fig. 35.

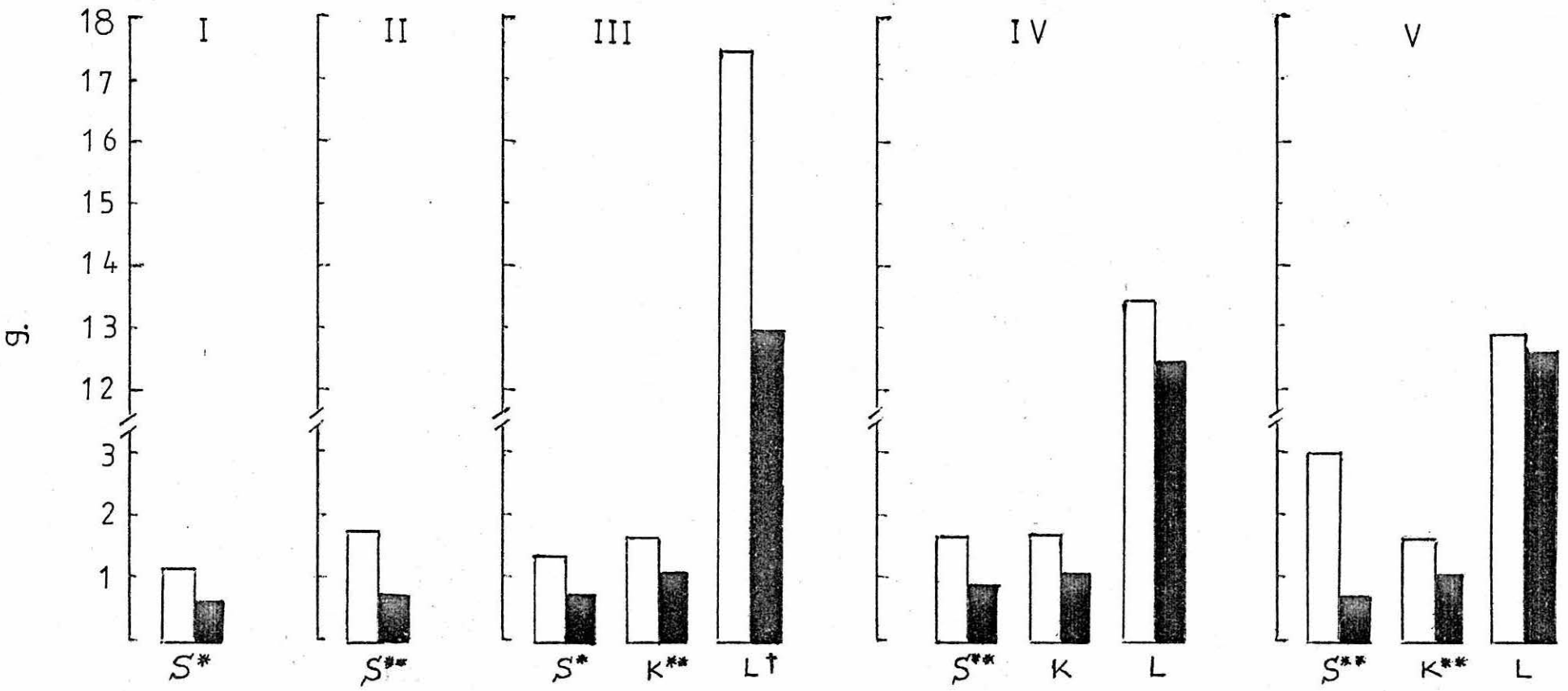


Fig. 35. Organ weights in rats: ■ controls; □ infected. Groups I, II, III, IV and V killed after 4, 7, 11, 16, and 28 weeks of infection with F. hepatica respectively. S = spleen; K = kidney; L = liver; * =significant at p = 0.05; ** = significant at p = 0.01; † = significant at p = 0.1.

Table XIII Means ($\bar{x}$) and standard deviations (SD) of organ weights (wt. in g.) in controls and infected rats in the different groups.

Group	Description	Liver		Spleen		Kidney	
		$\bar{x}$ ±	SD	$\bar{x}$ ±	SD	$\bar{x}$ ±	SD
I	Controls	-	-	0.62*	0.05	-	-
	Infected	-	-	1.13	0.34	-	-
II	Controls	-	-	0.64**	0.01	-	-
	Infected	-	-	1.77	0.24	-	-
III	Controls	13.0+	1.54	0.73*	0.02	1.09**	0.10
	Infected	17.47	3.37	1.31	0.38	1.57	0.11
IV	Controls	12.47	0.41	0.93	0.17	1.07	0.09
	Infected	13.47	3.45	1.90	0.328	1.70	0.65
V	Controls	12.70	0.20	0.77**	0.12	1.08**	0.01
	Infected	12.91	0.38	2.96	0.76	1.61	0.13

* significant ($p < 0.05$); ** highly significant ($p < 0.01$); +significant at $p < 0.1$.

It can be noticed that infected animals always had higher organ weights than control animals especially with regard to the spleen. The students 't' test revealed that the difference in spleen weight between infected and control rats were just significant ($p < 0.05$) in groups I and III but was highly significant ($p < 0.01$) in groups II, IV and V.

The differences in kidney weights were highly significant in groups III and V but not in group IV, although the difference is apparently greater. No statistical significance in liver weights was observed at $p < 0.05$ or

$p < 0.01$. However, in group III the difference was significant at $p < 0.1$.

iii) Tissue ascorbic acid:

The mean values for tissue ascorbic acid content are given in Table XIV and Fig. 36.

Table XIV Means ($\bar{x}$) and standard deviations (SD) of tissue ascorbic acid concentrations (mg/g wet weight) in control and infected rats.

Group		Liver		Spleen		Kidney	
		$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
I	Controls	0.282	0.017	0.771^{+}	0.029	-	-
	Infected	0.250	0.064	0.623	0.109	-	-
II	Controls	0.310	0.012	0.762**	0.090	-	-
	Infected	0.244	0.062	0.495	0.043	-	-
III	Controls	0.216	0.010	0.576*	0.011	0.135	0.004
	Infected	0.216	0.040	0.434	0.072	0.116	0.027
IV	Controls	0.249	0.026	0.514*	0.103	0.129*	0.030
	Infected	0.233	0.042	0.358	0.057	0.080	0.020
V	Controls	0.249	0.027	0.534**	0.029	0.131*	0.003
	Infected	0.239	0.054	0.263	0.031	0.107	0.011

* Significant $p < 0.05$; $^{+}$ significant at $p < 0.1$;
** Significant $p < 0.01$.

Again it could be noticed that uninfected rats had higher tissue ascorbic acid than infected ones. Student's 't'-test carried out between controls and infected animals for each group showed that the differences in liver ascorbic acid

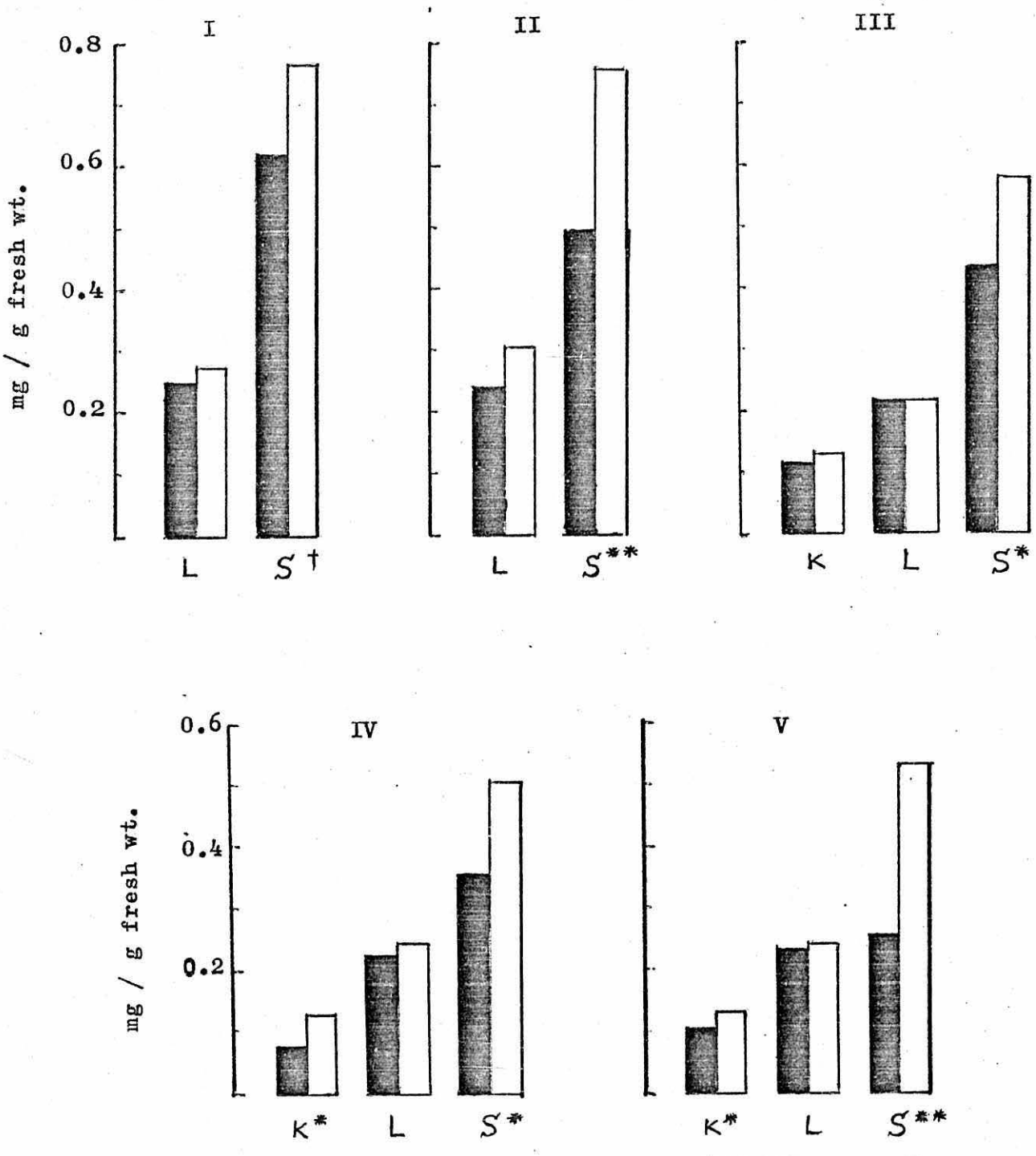


Fig. 36. Mean tissue ascorbic acid content in rats: □ controls; ■ infected with F. hepatica. Groups I, II, III, IV, and V killed after 4, 7, 11, 16 and 28 weeks respectively. † , *, or ** means significant at p = 0.1, 0.05, or 0.01 respectively.

were not statistically significant. Those for spleen ascorbic acid were significant in all groups except group no. I; in this group the difference was just below the level of significance at $p < 0.05$ but was significant at $p < 0.1$.

The differences in the kidney ascorbic acid were significant in groups IV and V.

iv) Liver total proteins and glycogen:

The mean values for liver total proteins and glycogen content are presented in Table XV and Fig. 37.

Table XV Means ($\bar{x}$) and standard deviations (SD) of liver glycogen and total protein content (g/100g) in control and infected rats.

Group	Description	Total proteins		Glycogen	
		$\bar{x}$	SD	$\bar{x}$	SD
I	Controls	19.00	2.16	0.569	0.080
	Infected	18.07	0.80	0.382	0.190
II	Controls	20.00+	1.70	1.028**	0.180
	Infected	15.90	2.50	0.160	0.060
III	Controls	18.00**	0.80	0.465*	0.040
	Infected	16.00	0.50	0.353	0.050
IV	Controls	19.70	1.90	0.842*	0.107
	Infected	19.50	0.70	0.562	0.164
V	Controls	19.22*	0.50	0.625**	0.160
	Infected	17.00	1.00	0.074	0.030

* Significant at $p < 0.05$

** Significant at $p < 0.01$

\+ Significant at $p < 0.1$.

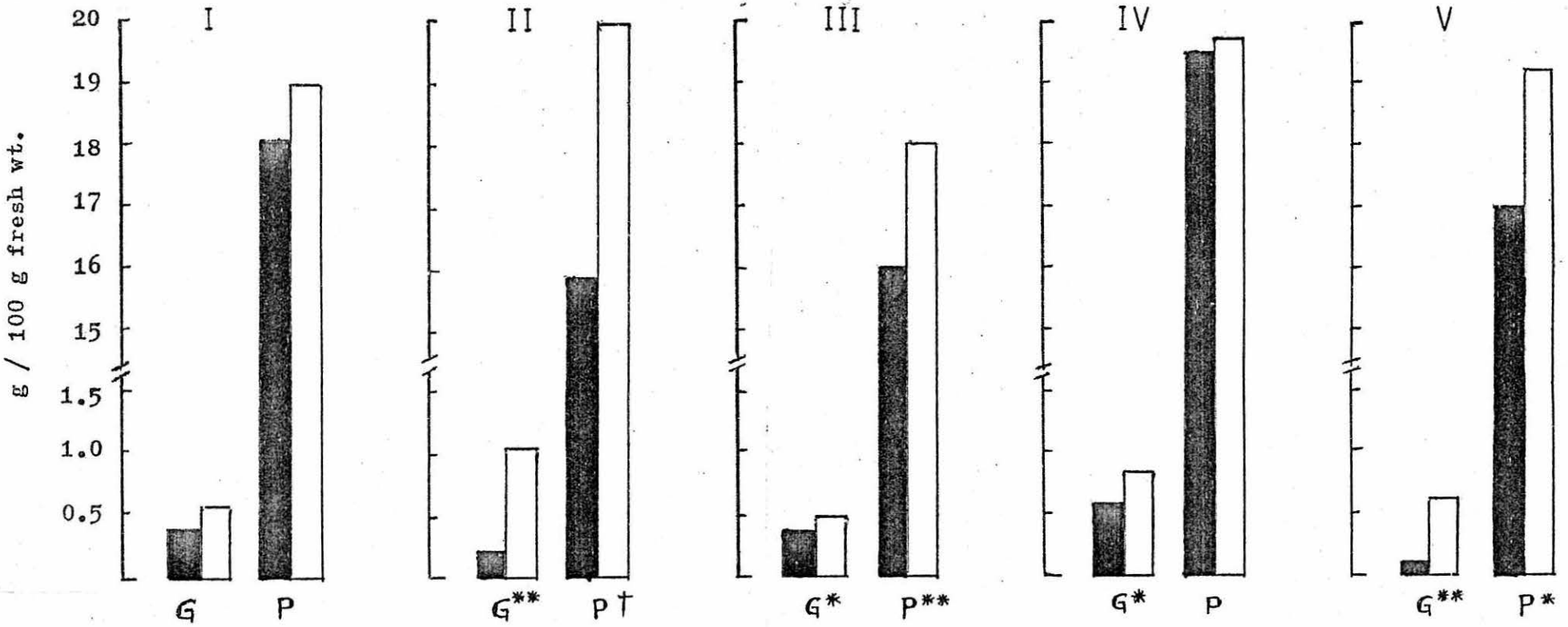


Fig. 37. Mean values for liver glycogen (G) and total proteins (P) in rats: ☐ controls; ▬ infected with F. hepatica. Groups I, II, III, IV and V were killed after 4, 7, 11, 16 and 28 weeks of infection respectively. †, *, or ** means significant at p = 0.1, 0.05, or 0.01 respectively.

The liver total proteins and glycogen were always higher in control animals than in infected ones especially with regard to glycogen content.

The differences in the total proteins are significant at either $p < 0.05$ or $p < 0.01$ in groups V and III respectively; those for group III were just below the level of significance at $p < 0.05$. The differences in glycogen contents were significant in all groups except the first one.

iv) Total liver iron and copper:

The mean values for liver iron and copper are given in Table XVI and Fig. 38.

Table XVI Mean ($\bar{x}$) and standard deviations (SD) for liver iron and copper (mg/100g dry wt) in control and infected rats.

Group	Description	Iron		Copper**	
		$\bar{x}$	SD	$\bar{x}$	SD
I	Infected	83.00	21.00	2.20	0.26
II	Infected	91.00	47.10	4.00	1.10
III	Infected	72.80	17.70	3.20	0.95
IV	Infected	59.97	25.75	3.00	1.06
V	Infected	58.97	21.60	2.90	0.74
	Controls	75.28	5.28	1.20	0.07

**Highly significant $p < 0.01$.

Analysis of variance did not reveal any significant differences between the mean liver iron concentrations in

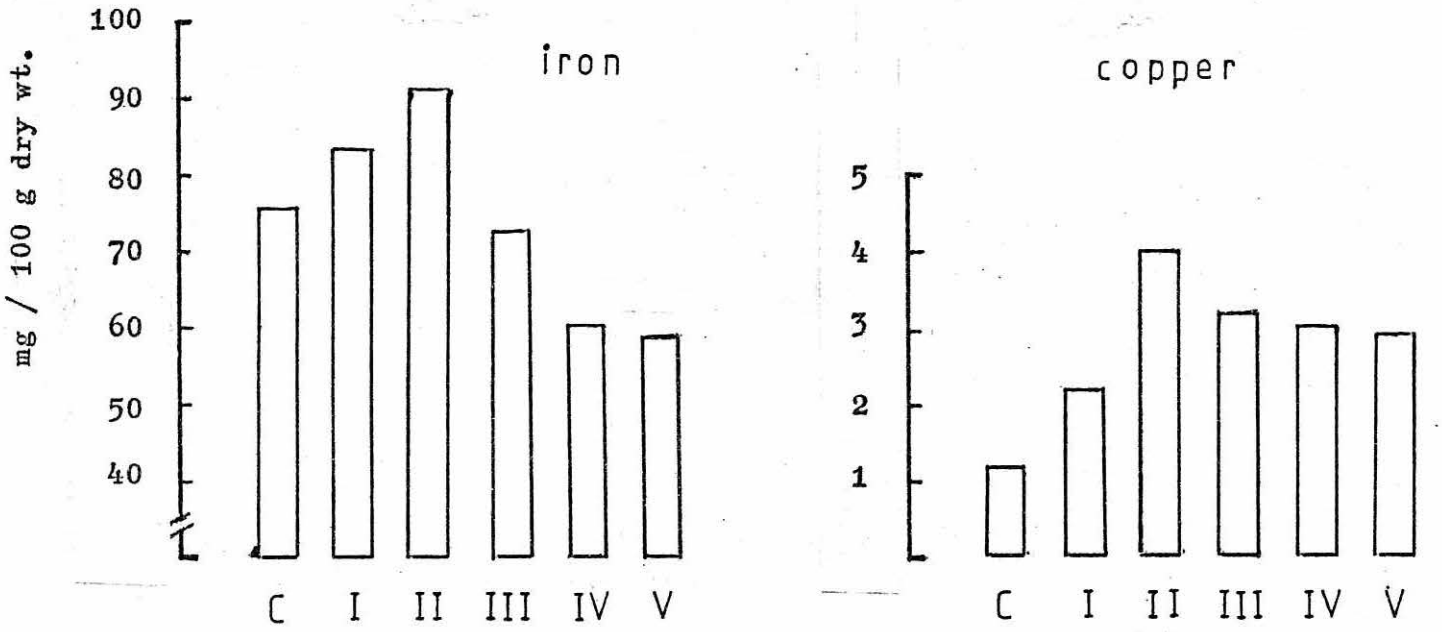


Fig. 38. Mean liver iron and copper concentrations in controls and in F. hepatica infected rats. C = controls; groups I, II, III, IV and V killed after 4, 7, 11, 16 and 28 weeks of infection respectively.

controls and each of the other infected groups. However, the data show that the liver iron concentrations were higher in infected groups killed at 4 and 7 weeks of infection than in controls, but were lower in animals killed at the 16th and 28th weeks post-infection. On the other hand, the copper values were significantly higher in all infected groups than in controls.

v) Histochemical staining of liver iron:

Estimated liver iron contents in infected rats are presented in Table XVII.

Table XVII Evaluation of iron concentration in the livers of infected rats killed at different intervals after infection; prussian blue stain for ferric iron.

Group	Liver iron content		Remarks
	Prussian blue stain	mg/100g dry wt* (mean)	
I	±	83.00	Killed 4 weeks P.I.
II	+++	91.00	Killed 7 weeks P.I.
III	±	72.80	Killed 11 weeks P.I.
IV	+	59.97	Killed 16 weeks P.I.

*These values were put down again for the sake of comparison.
± just detectable; +++ considerable.

The results show that rats killed at 7 weeks of infection had more stainable iron and those killed at 16 weeks had the least stainable iron. This seems to agree with the iron values obtained after digesting the tissues and reading in the atomic absorption spectrophotometer.

EXPERIMENTAL V

The previous experiments indicate that F. hepatica can affect the ascorbic acid balance in infected animals; plasma and tissue levels decreased suggesting that a state of ascorbic acid deficiency occurs during the course of the disease. In view of the important role played by Vitamin C in healing and regeneration processes, supplementation of ascorbic acid to fluke infected animals should, theoretically, lessen the severity of infection and enhance the healing of damaged liver tissue caused by young and adult parasites. To test this hypothesis the following experiment was designed.

Thirty hooded rats, 18 males (265-339g) and 12 females (142-186g) were divided into 2 groups (A and B) at random on body weight basis. Each group consisted of 15 rats, 9 males and 6 females and the animals were kept in galvanized cages and fed on standard rat diet.

Group A was supplemented daily with Vitamin C (L-Ascorbic acid), 1% in drinking water; this started a week before infection and continued for 14 weeks thereafter. It was calculated that each rat would receive about 0.22g ascorbic acid per day.

Each rat was infected with about 15-20 F. hepatica metacercariae. Body weight was taken weekly and after 14 weeks the rats were killed, organ weights recorded and tissue ascorbic acid, liver total proteins and glycogen determined as previously described.

Results:

i) Body weight:

Mean body weights are recorded in Table XVIII and Fig. 39.

Table XVIII Mean ($\bar{x}$) and standard deviations (SD) of the weekly body weights (g) and percentage gains in infected rats; group A supplemented with ascorbic acid in drinking water.

Weeks of infection	Group A						Group B					
	Males			Females			Males			Females		
	$\bar{x} \pm$	SD	Gain %	$\bar{x} \pm$	SD	Gain %	$\bar{x} \pm$	SD	Gain %	$\bar{x} \pm$	SD	Gain %
-1	276	29	-	164	7		287	28		165	14	
0	283	24	2.54	171	5	4.27	300	25	4.53	169	13	2.42
1	288	22	4.35	173	5	5.49	307	23	6.97	171	14	3.64
2	292	19	5.80	172	3	4.88	314	21	9.41	173	13	4.85
3	300	18	8.70	176	4	7.32	324	18	12.89	178	13	7.88
4	300	17	8.70	174	4	6.10	321	19	11.85	175	12	6.06
5	298	17	7.97	174	4	6.10	323	17	12.54	179	13	8.48
6	300	13	8.70	174	3	6.10	324	16	12.89	178	11	7.88
7	296	11	7.25	175	6	6.70	321	15	11.85	178	13	7.88
8	302	16	9.42	176	5	7.32	325	16	13.24	182	13	10.30
9	304	16	10.14	179	9	9.15	325	14	13.24	185	11	12.12
10	309	15	11.96	180	8	9.76	327	14	13.94	186	11	12.73
11	312	15	13.04	183	9	11.58	329	14	14.63	187	11	13.33
12	313	15	13.41	185	10	12.80	331	13	15.33	187	11	13.33
13	-	-	-	-	-	-	-	-	-	-	-	-
14	316	16	14.49	183	9	11.58	334	13	16.38	188	10	13.94

Male rats of group A showed a steady rise in body weight during the first 3 weeks of infection. The weights remained almost constant till the 8th week and again progressively increased till the end of the experiment. The

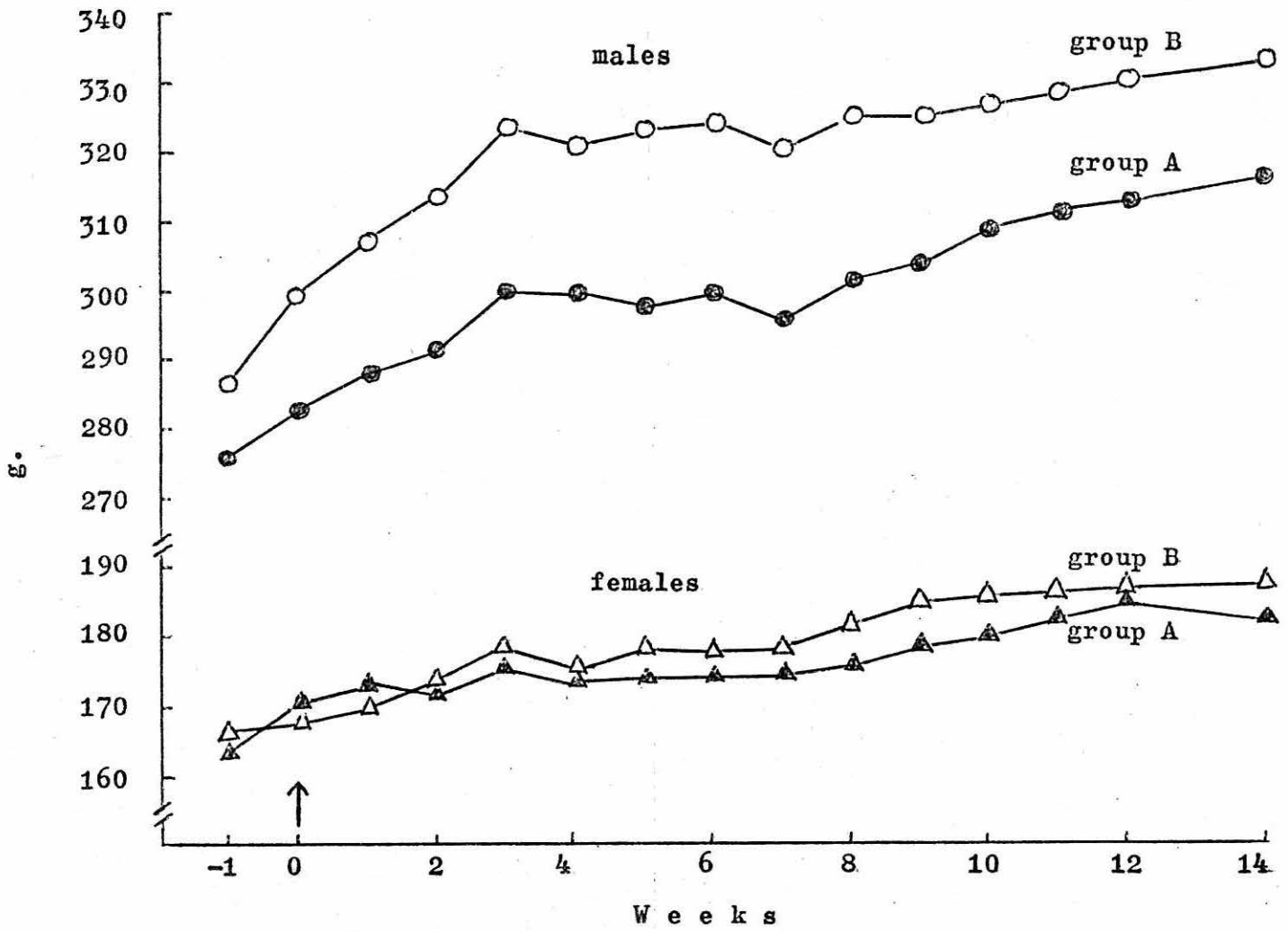


Fig. 39. Mean body weight in 2 groups of rats infected with F. hepatica.
Group A was supplemented with ascorbic acid in drinking water. ↑= infection

picture was similar in group B animals except that the initial rise was more marked while the second rise was less than in group A. Students' 't'-test revealed that the differences in the percentage weight gains between the two groups was significant at $p < 0.05$; the gains being higher in group B males.

Females of both groups showed similar changes to those of males except that the initial increase in body weight was not very steady in group A females and the terminal weight was low. However, the differences in the percentage weight gains in females was not significant ($p > 0.05$).

ii) Post mortem examination:

Group A:

The rats were in good shape. Most changes were observed in the liver. The organ was dark brown in colour, slightly enlarged and the hepatic lobes had slightly rounded edges. In some rats the hepatic lobules were very prominent. The liver lobes were adhering to each other and to other visceral organs. The common bile ducts were dilated and thickened. An average of 3 flukes were recovered from each liver and two rats had no fluke burden. The spleens were slightly bigger than normal.

Group B:

The livers were enlarged, surfaces were uneven and their consistency was fragile. The colour was pale brown and in many the cut surface was obviously greasy. Adhesions between the liver lobes and other internal organs were observed. The bile ducts were also thickened and enlarged and harboured adult flukes. Similarly, an average of 3 flukes were recovered from each liver and again two rats had no fluke burden.

The spleens were slightly enlarged and in a few rats there were lung abscesses, especially in the diaphragmatic lobes.

iii) Histopathological findings:

Group A (supplemented with ascorbic acid):

Liver: Generally the liver cells showed cytoplasmic granular degeneration. In most sections the hepatic lobule structure was preserved but the central veins were slightly stenosed and irregular in shape, elongated, triangular and star-shaped. Thin connective tissue strands were seen connecting the central veins with adjacent portal canals. In some sections, some central veins were dilated, congested and surrounding paranchyma degenerated and replaced by fibrous tissue. Portal canals were slightly fibrosed, oedematous and infiltrated with eosinophils. In others lobulation of the hepatic parenchyma was quite marked; thin and sometimes thicker connective tissue septae divided the liver parenchyma into lobules of various sizes. In these lobules

the normal radial structure is lost. Biliary ductule hyperplasia was seen in the interlobular tissue which was also oedematous and infiltrated with large lymphocytes, fibroblasts and a few eosinophils. Interlobular arteries were hypertrophied and a noticeable amount of greenish brown pigment was also observed. Regenerating cells, usually in small groups, were seen and these had well-defined cellular boundaries and were deeply stained. Binucleate cells were not uncommon.

Congestion of central and interlobular veins together with sinusoidal dilatation and congestion were common findings. Eosinophilia was not at all an important feature.

Spleen: The spleen trabeculae were thin but became more prominent towards the centre of the section. The lymphoid tissue was diffuse and the red pulp was flooded with red cells, indicating sinusoidal dilatation. The central arteries of the white pulp were hypertrophied. Again, eosinophilic infiltration was not a feature.

Lungs: Lung sections revealed the presence of abscesses. Alveolar septae were thickened and small bronchi hypertrophied. Associated with the changes in the bronchi there were diffuse and focal aggregates of lymphocytes. Congestion of lining tissue was also noticed.

Kidney: Congestion and haemorrhages in the renal cortex was seen.

Pancreas and Heart: No salient abnormality was observed in either of the two organs.

Group B (without ascorbic acid supplementation):

Liver: The changes observed are similar to those described for livers of Group A but with some differences:

a) Besides the cytoplasmic granular degeneration the hepatic cells had also distinct and round vacuoles in the cytoplasm, denoting fatty degeneration.
b) Central veins were very much stenosed.
c) Portal canals and interlobular septae were relatively more enlarged with connective tissue.
d) Sinusoidal dilatation and congestion was less marked.
e) Wide separation of hepatic cords was occasionally seen.
f) More eosinophils were seen in the portal tracts and interlobular tissue than in the previous group.

Spleen: Thicker trabeculae and some eosinophilic infiltrations were observed.

iv) Fluke recovery and measurements:

See Table XIX.

Table XIX Number of flukes recovered and means ± SD of their length (L) and width (W) in Group A rats, supplemented with ascorbic acid and Group B rats without ascorbic acid supplementation.

Group	No. of Flukes	L (mm)	W (mm)	L x W
A (15 rats)	41	11.878 ± 3.5	7.61 ± 1.6	94.27 ± 44.8
B (14 rats)	40	11.583 ± 2.92	7.167 ± 1.185	85.32 ± 33.0
t =		0.406 NS	1.396 NS	1.008 NS

NS = not significant at p = 0.05.

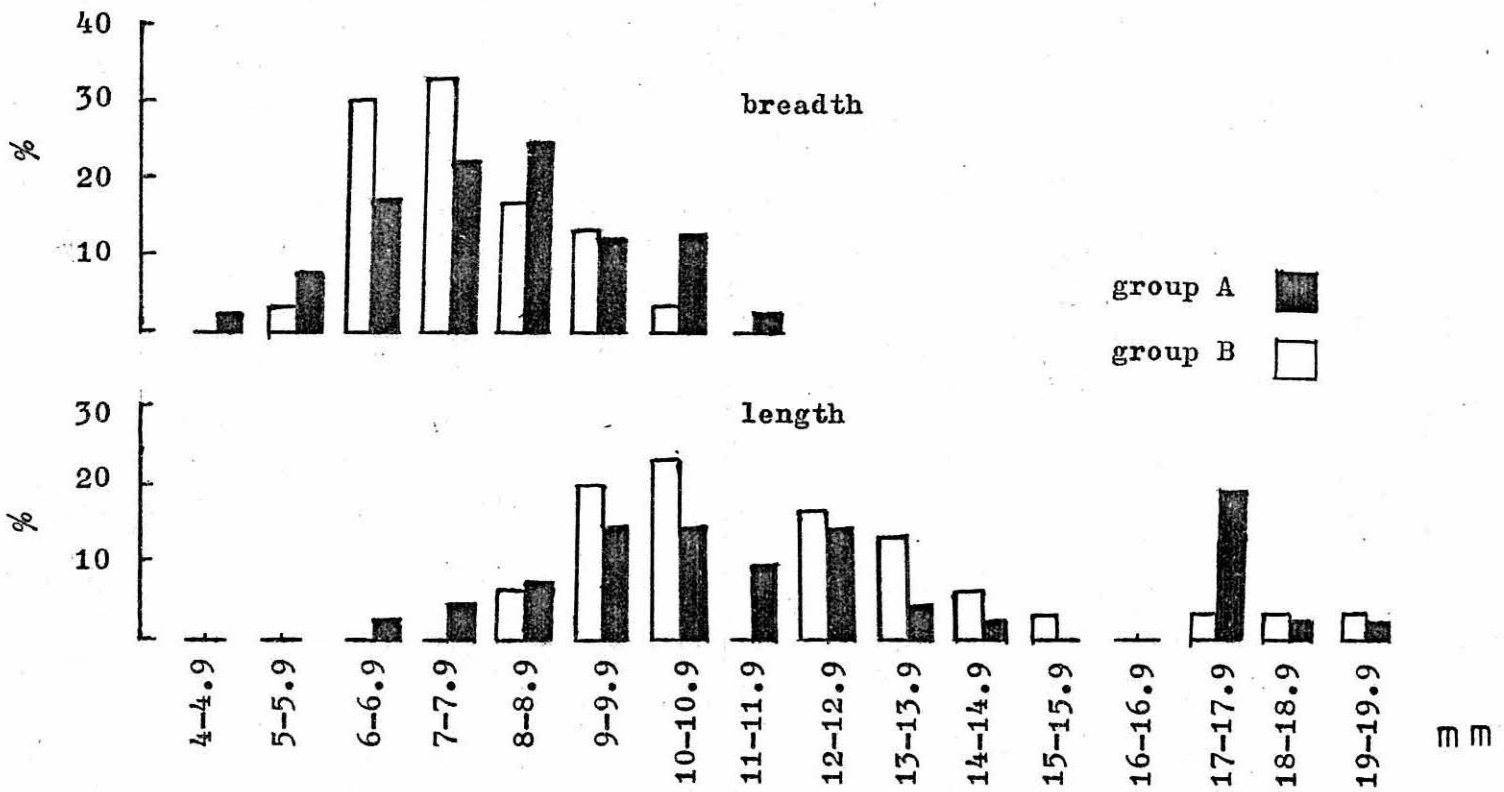


Fig. 40. Frequency distribution of the length and breadth of flukes recovered from 2 groups of rats infected with F. hepatica. Group A animals were supplemented with ascorbic acid in water.

The frequency distribution of the length and widths of the flukes recovered from each group of rats is shown in Fig. 40.

v) Organ weights:

The mean organ weights are given in Table XX.

Table XXa Mean ($\bar{x}$) and standard deviations (SD) of organ weights (wt in g) and the corrected organ weights (cor. wt. in mg/g = $^{\text{organ wt}}/_{\text{body wt}}$) in infected rats a) Group A, supplemented with ascorbic acid
b) Group B

a) Group A

Organ	Males				Females			
	wt. (g)		Cor. wt. (mg/g)		wt. (g)		Cor. wt. (mg/g)	
	$\bar{x}$ ±	SD	$\bar{x}$ ±	SD	$\bar{x}$ ±	SD	$\bar{x}$ ±	SD
Liver	12.8	1.9	40.28	4.18	8.1	0.4	44.13	0.83
Spleen	0.59	0.13	1.87	0.44	0.65	0.22	3.52	1.14
Kidney	1.15	0.07	3.65	0.21	0.83	0.05	4.51	0.29
Heart	1.50	0.25	4.77	0.80	1.22	0.07	6.57	0.24

b) Group B

Organ	Males				Females			
	wt. (g)		Cor. wt. (mg/g)		wt. (g)		Cor. wt. (mg/g)	
	$\bar{x}$ ±	SD	$\bar{x}$ ±	SD	$\bar{x}$ ±	SD	$\bar{x}$ ±	SD
Liver	13.4	1.6	40.05	3.31	7.2	0.5	38.13	1.01
Spleen	0.70	0.19	2.11	0.63	0.49	0.13	2.60	0.65
Kidney	1.14	0.08	3.42	0.21	0.75	0.07	3.96	0.24
Heart	1.63	0.11	4.90	0.36	1.14	0.04	6.07	0.27

The statistical analysis using the students 't'-test revealed the following:

a) Males of each group had significantly heavier liver, kidney and heart weights than females.

b) The differences in organ weights between males of Group A and B were not significant.

c) Females of Group A had significantly heavier livers than those of Group B.

With regard to the corrected weights, the analysis showed the following:

a) In Group A, females had significantly greater spleen, kidney and heart weights than males.

b) In Group B, females had significantly higher kidney and heart weights than males.

c) Males of Group A had significantly higher kidney weights than those of Group B.

d) Females of Group A had significantly heavier liver, kidney and heart weights than those of Group B.

iv) Tissue ascorbic acid content:

Mean ascorbic acid concentrations in tissues are given in Table XXI.

Table XXI Mean ($\bar{x}$) and standard deviations (SD) of tissue ascorbic acid (mg/g fresh wt) in infected rats.

Organ	Group A				Group B			
	Males		Females		Males		Females	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
Liver	0.189	0.018	0.169	0.021	0.203	0.025	0.171	0.012
Spleen	0.541	0.115	0.353	0.018	0.540	0.107	0.380	0.025
Kidney	0.171	0.016	0.118	0.016	0.165	0.029	0.104	0.005
Heart	0.085	0.014	0.084	0.021	0.082	0.007	0.083	0.008

It could be observed that males of each group had higher liver, spleen and kidney ascorbic acid content than

females of the same group, and the differences were statistically significant ('t'-test) in the case of livers in Group B ($p < 0.05$) and were highly significant ($p < 0.01$) for spleen and kidney in both groups.

The differences between either the males or the females of both groups were not significant.

vi) Liver total proteins and glycogen.

Total liver proteins and glycogen can be seen in Table XXII.

Table XXII. Mean ($\bar{x}$) and standard deviations (SD) of liver total proteins and glycogen (mg/100g) in infected rats.

Parameter	Group A				Group B			
	Males		Females		Males		Females	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
Total proteins	23.1	2.6	23.8	1.5	18.6	3.4	25.7	1.7
Glycogen	0.383	0.09	-	-	0.409	0.14	-	-

Students 't'-test revealed that:

a) Male rats of Group A had significantly higher ($p < 0.01$) total protein content than males of Group B.

b) Females of Group B had significantly higher liver total proteins than the males of the same group ($p < 0.01$).

The difference in liver glycogen content was insignificant.

Experimental VI

The Effect of Ascorbic Acid Supplementation in Guinea Pigs Infected with F. hepatica

In the previous experiment (Experimental V), the effect of vitamin C supplementation was investigated in rats infected with F. hepatica. Exogenous ascorbic acid seems to have little effect on rats, which are actually capable of synthesising the vitamin and maintaining adequate tissue levels. It was thought that ascorbic acid supplement could have a more obvious influence on animals that are incompetent of endogenous synthesis. Therefore guinea pigs were used in this experiment. The animals were fed on a scorbutogenic diet and a known amount of vitamin C was given orally every day. The purpose of the experiment is to compare the effects of two dose levels of ascorbic acid (a maintenance dose of 0.5 mg/animal/day and a high dose of 20 mg/animal/day) on the following:

a) Liveweight gains
b) Course of infection, fluke development and pathological lesions
c) Tissue ascorbic acid concentrations
d) Phagocytic and bactericidal ability of polymorphonuclear leukocytes (PMN). These parameters under (d) are given special attention because PMN leukocytes have an important defence role towards micro-organisms and that fluke infected animals are reported to be more susceptible to bacterial invasion. Further, the effect

of different dose levels of ascorbic acid on the activity of PMN leukocytes was investigated due to the essential part vitamin C plays in the function of these cells.

Experimental design:

24 white female guinea pigs, 111 days old and weighing 412-479 g were used. They were kept under the same conditions of management and fed on normal guinea pig diet (RGP pellets, The Christopher Hill Group Ltd., Poole). Nineteen days before infection the animals were divided into 2 groups, A and B, of 12 guinea pigs each and the normal diet was replaced by an ascorbic acid-free diet (QGP pellets, same company) which continued until the end of the experiment. Two days before infection a regime of ascorbic acid supplementation was started; a daily maintenance dose of 0.5 mg in 1 ml of 2% sucrose was given to each animal of group A and a high dose of 20 mg in 1 ml 2% sucrose was given to each member of group B. (The maintenance dose was raised to 5 mg 34 days later and the reasons will be given in due course). The vitamin was administered with the aid of a hard plastic tube attached as a mouth piece to a 5 ml syringe. The tube was introduced far at the back of the tongue and the right dose delivered. The animals were soon accustomed to this procedure without difficulty.

Two days after the commencement of vitamin C supplementation, 8 animals from each group were infected orally with about 33 F. hepatica metacercariae (cysts) and

the remaining animals served as uninfected controls. Body weight was recorded weekly.

Phagocytic and Bactericidal activity of PMN leukocytes:

Six weeks after infection, surviving infected animals and controls were anaesthetised with ether and 4 ml of blood were drawn aseptically by heart puncture in a syringe containing 1 ml of acid citrate dextrose anticoagulant (USPH USP formula; 8.0 g citric acid + 22.0 g sodium citrate + 24.5 g D-glucose per litre H_2O). Total leukocytes and PMN cells were counted in a Neubauer haemocytometer. Crystal (gentian) violet was used to stain the white cells (0.3 ml of 0.5% solution in glacial acetic acid + 9.7 ml H_2O). The differential counts were done immediately after total leukocyte counts on the fresh preparation in the haemocytometer under the high dry objective (Williams and Chase, 1976).

The phagocytic activity of the PMN leukocytes was assessed by their ability to reduce the Nitroblue tetrozolium (N.B.T.) dye to insoluble formazan which is precipitated as black granules in their cytoplasm. The method described by Park, Fikrig and Smithwick (1968) was utilized as follows: 0.1 ml of blood was transferred into siliconed concave slides and mixed with an equal volume of N.B.T. solution (a mixture of equal amounts of 0.2% N.B.T. in 0.85% saline and 0.15M phosphate buffered saline, pH 7.2). The slides were placed in petri dishes (which were kept moist by pellets of wet cotton wool), incubated for 15 minutes at 37°C and then

kept for a further 15 minutes at room temperature. The blood/N.B.T. mixture was again mixed by a platinum loop and smears made. After drying, the latter were counter-stained with Wrights stain. The films were examined under the microscope with oil immersion and 100-500 PMN leukocytes (heterophils and eosinophils) were counted and the number of N.B.T. positive cells recorded; absolute numbers were calculated from the total PMN leukocyte counts and the percentage of N.B.T. positive cells.

The bactericidal capacity of PMN leukocytes was assessed according to the method described by **Williams and** Chase (1976): One ml of the citrated blood containing a known number of PMN cells, was transferred to sterile stoppered siliconed glass tubes 150 x 15 mm and mixed with an equal volume of bacterial culture containing a known number of viable Salmonella dublin in the lag phase in $M/_{15}$ phosphate buffer, pH 7.1. The number of bacteria was 2-3 or more times the number of PMN leukocytes. The mixture was incubated at 37°C in a shaking water bath and the tubes were inverted 2-3 times every 5-10 minutes to ensure thorough mixing. After 45 minutes a 1 ml aliquot was dispensed into siliconed test tubes and diluted 1:5 with $M/_{15}$ phosphate buffer and centrifuged at 50 g for 10 minutes. The clear supernatant was drawn carefully with a Pasteur pipette and 0.1 ml samples of this fraction were serially diluted in phosphate buffer and 0.1 ml of the dilutions spread on the surface of Oxoid "Sensitest" agar plates for the determination of viable extracellular bacteria; this was

done by colony counting after 24 hours incubation at 37°C. The pellet was resuspended in 3 ml of the phosphate buffer and homogenised in a high speed MSE homogenizer to disrupt the cells. Samples of this homogenate were treated similarly to the supernatant for the determination of leukocyte-associated bacteria. The number of killed organisms and their ratio to the number of leukocytes was then calculated. It should be remembered that a total bacterial count was made on the original suspension at 0 hour of incubation and that the total supernatant and pellet viable counts were expressed as bacteria per ml of the original suspension.

Post-mortem examination, tissue ascorbic acid determination and fluke recovery:

Following death or sacrifice, the animals were dissected and examined for pathological abnormalities. The liver, kidney, spleen, heart and adrenals were weighed and samples from these organs were homogenised for ascorbic acid determination as described previously. Samples for histopathology were taken from all the above organs plus pancreas, intestines and lungs. Adult flukes in the bile ducts were recovered and the peritoneal cavity was inspected for flukes. The rest of the liver was sliced into 1 cm slices, put in a plastic container containing physiological saline and incubated at 37°C for 2 hours. Emergent flukes were collected, counted and measured while being stretched between two glass slides.

RESULTS

Body Weight:

Mean body weights for infected and control guinea pigs in the two groups A and B are recorded in Table XXIII and Fig. 41. It can be seen from the figure that changes in body weights before infection were very similar in the two groups; there was a steady increase during the first 7 weeks, when the animals were fed a normal diet, and then the weights remained almost constant till the 10th week, during the time of feeding the scorbutogenic diet. Following infection after the 10th week, infected animals of group A still maintained a constant weight till about week 13. Group B exposed animals, on the other hand, slightly increased in weight till week 12 and then remained constant till week 15.

Control animals increased in weight from the 11th to the 15th week but the increase was very slight in group A controls. This was followed by a temporary drop at week 16 and then by further increase thereafter.

Statistical analysis ('t'-test) for body weight data during the first 7 weeks of experimentation, the three weeks preceding infection, and the 4 weeks following infection, revealed the following:-

a) No significant differences between controls and infected animals in either of the two groups.

b) No significant differences between controls of groups A and B.

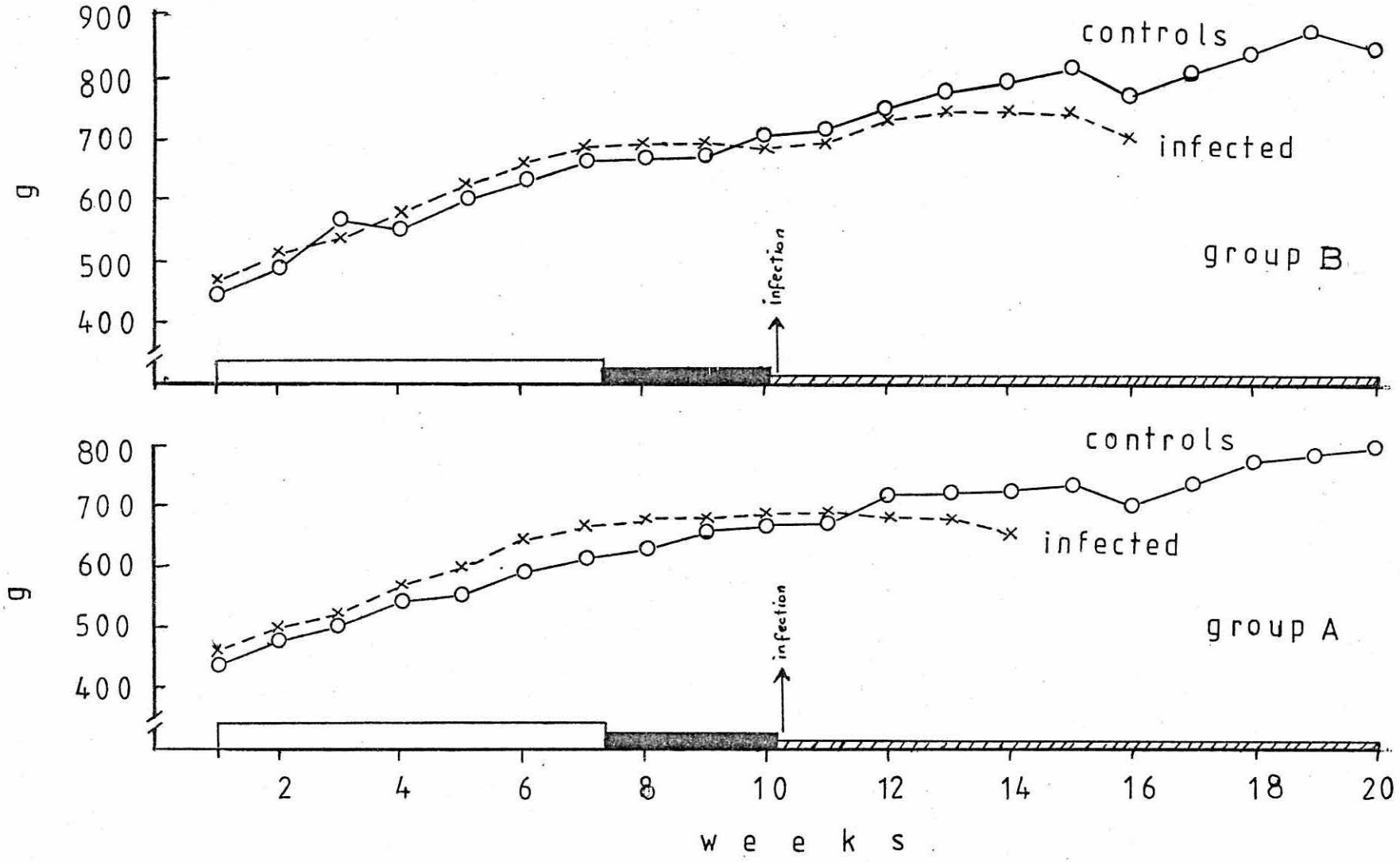


Fig. 41. Body weight in guinea pigs infected with F. hepatica. Group A : given 0.5 mg (later 5.0 mg) ascorbic acid / animal /day; group B: 20 mg / animal / day. ☐ Normal diet; ▬ scorbutogenic diet; ▨ scorbutogenic diet + ascorbic acid.

Table XXIII. Mean body weight in experimental guinea pigs: Group A, low vitamin C dose; Group B, high vitamin C dose.

Week	Group A (12 animals)				Group B (12 animals)			
	Infected (8)		Controls (4)		Infected (8)		Controls (4)	
	$\bar{x}$	± SD	$\bar{x}$	± SD	$\bar{x}$	± SD	$\bar{x}$	± SD
1	450.62	10.25	439.75	24.92	454.75	14.93	452.25	21.54
2	494.37	8.21	488.00	27.82	500.87	20.19	497.40	32.94
3	521.87	20.27	512.00	39.67	538.25	24.69	562.00	36.25
4	566.75	31.27	547.50	38.50	570.12	24.13	557.75	42.08
5	594.87	49.20	556.50	51.70	615.50	24.83	606.75	59.03
6	645.25	39.22	597.25	56.26	655.12	21.04	633.75	63.97
7	668.5	39.13	616.25	64.65	679.62	28.24	665.25	63.61
8	680.12	51.19	631.75	63.02	692.12	35.76	673.75	63.46
9	673.12	45.24	660.75	55.37	682.25	38.07	680.00	74.79
10	688.37	45.61	666.00	58.49	689.25	43.18	696.25	83.10
11	686.62	49.31	684.67	58.52	706.00	41.89	715.5	80.08
12	687.62	70.02	716.00	58.94	744.25	46.12	747.25	81.58
13	680.37	86.38	726.33	67.15	746.50	45.72	778.25	89.89
14	651.37	113.20	727.67	65.33	748.12	53.31	791.50	90.56
15	708.40*	31.86	733.67	75.92	740.62	50.50	810.50	106.09
16	684.40*	33.75	699.00	70.75	700.50	56.87	765.00	90.95
17	707.00*	64.84	739.67	70.71	689.83+	62.95	805.00	93.17
18	609.50**	39.5	767.33	78.26	711.00**	120.00	834.25	93.88
19			775.00	88.80			863.25	99.97
20			784.67	95.73			837.75	97.38

* average of 5 surviving animals

** average of 2 surviving animals

\+ average of 6 surviving animals

N.B. Weeks 1-10 preinfection.

Weeks 11-20 postinfection.

c) Infected animals of group B had significantly higher body weights than those of group A only during the post infection period ($P < 0.002$).

When control values were analysed during the 10th-20th weeks, it was found that group B controls (high vitamin C dose) had significantly heavier weights ($p < 0.02$) than those of group A (low vitamin C dose).

Course of infection:

Between the 4th and 6th weeks of infection, 3 guinea pigs from group A (low vitamin dose) died. Before death, the animals looked sluggish and dull with drooping heads and sunken eyes. Their fur became ruffled and was lost from parts of the head and shoulder. At this stage the vitamin C dose was raised from 0.5 mg/animal/day to 5 mg because death was thought to be enhanced by the vitamin deficiency and we wanted to keep the animals for other tests. After the 6th week similar symptoms appeared in two animals of group B which also died. The significant observation is that the above symptoms and deaths occurred two weeks earlier in the group supplemented with the low vitamin C dose.

Control guinea pigs of both groups looked perfectly healthy and developed no ill symptoms. One animal, however, showed signs of paraplesia but recovered fully in due course and the reason was thought to be mechanical damage.

Post-mortem findings and fluke recoveries:

The general necropsy findings in infected guinea pigs of both groups were as follows: Congestion of subcutaneous vessels, bloody-tinged hydrothorax (9.5 - 30 ml) and hydroperitoneum (18-95 ml) were constantly observed. The liver was apparently enlarged and oedematous. Large fibrin clots and small fibrin tags lining older fluke tracks were seen. The fibrin clots were well organised in places forming firm adhesions between the liver capsule and other visceral organs. Necrotic nodules were seen in surface and cut surface. The latter was greasy and the liver consistency was generally friable; ventral lobes were relatively harder. Gall bladders were distended and bile ducts were slightly thickened.

The spleens were enlarged and nodular. The kidneys were also slightly enlarged while the intestines were congested.

Control animals had no salient pathological abnormality.

The number of flukes recovered and their measurements can be seen in Table XXIV. Fifty-four flukes (mean length and width in mm, 5.92 ± 2.11 and 2.13 ± 0.75 respectively) were recovered from group A infected animals, and 60 flukes (mean length and width, 6.59 ± 1.96 and 2.61 ± 0.78 respectively) were recovered from animals of group B; flukes collected from the latter group were significantly longer ($p < 0.1$) and broader ($p < 0.001$).

Table XXIV Fluke recoveries and measurements in infected guinea pigs.

Group	Animal No.	No. of cysts	No. of flukes	% recovery	Measurements Length	Width
	1	28	5	17.86	4.20 ± 0.98	1.20 ± 4.00
	2	30	7	23.33	7.21 ± 1.46	3.14 ± 0.83
A	3	31	13	41.93	4.50 ± 1.94	1.85 ± 0.57
	4	32		died		
	5	34	12	35.29	6.79 ± 2.46	2.50 ± 0.61
	6	35	5	14.28	7.20 ± 1.83	2.10 ± 0.20
	7	35	12	34.28	6.00 ± 0.98	1.87 ± 0.30
	8	38		died		
	9	29		died		
	10	30	10	33.33	7.00 ± 1.55	2.55 ± 0.65
	11	30	7	23.33	7.86 ± 0.87	3.00 ± 0.80
B	12	31	7	22.58	5.00 ± 1.77	2.36 ± 0.51
	13	31	14	45.16	6.00 ± 1.08	2.11 ± 0.63
	14	33	7	21.21	6.50 ± 2.12	2.36 ± 0.69
	15	35		died		
	16	39	15	38.46	7.07 ± 2.48	2.77 ± 0.89

Means ($\bar{x}$) Standard Deviations (SD); NS = not significant

Group	Flukes recovered			Measurements	
	Total No.	No./animal	% recovery/ animal	Length (mm)	Width (mm)
A	54	9.0 ± 3.4	27.83 ± 9.99	5.92 ± 2.11	2.13 ± 0.75
B	60	10.0 ± 3.4	30.68 ± 9.00	6.59 ± 1.96	2.61 ± 0.78
t		0.465	0.474	1.7419	3.3115
P<		NS	NS	0.1	0.001

Frequency distribution histograms for the fluke lengths and widths can be seen in Fig. 42a and b. It can be observed that fluke length in the case of group A showed a normal and symmetrical distribution and that 81.4% of the flukes had a width ranging from 1-2.9 mm. On the other hand, 78.3% of the flukes recovered from group B had a width ranging from 2-3.9 mm.

Organ weights:

Mean organ weights are shown in Table XXV.

Table XXV Mean ± SD of organ weights in experimental guinea pigs a) organ weight in g; b) adjusted weight = organ weight/terminal body weight in mg/g.

Organ		Liver		Spleen		Kidney		Heart		Adrenal	
Group/ description		a	b	a	b	a	b	a	b	a	b
A	I	39.17 ± 8.53	63.92 ± 18.84	2.89 ± 1.03	4.54 ± 1.53	2.51 ± 0.41	4.18 ± 1.24	1.59 ± 0.29	2.5 ± 0.68	0.25 ± 0.04	0.40 ± 0.12
	C	36.67 ± 3.24	50.39 ± 4.24	0.99 ± 0.10	1.35 ± 0.10	2.27 ± 0.18	3.14 ± 0.39	1.77 ± 0.24	2.41 ± 0.10	0.24 ± 0.02	0.33 ± 0.04
B	I	48.03 ± 6.13	72.0 ± 8.18	2.97 ± 1.01	4.50 ± 1.75	2.79 ± 0.38	4.22 ± 0.76	1.7 ± 0.2	2.55 ± 0.31	0.31 ± 0.09	0.47 ± 0.14
	C	43.15 ± 12.46	51.52 ± 12.04	1.20 ± 0.12	1.47 ± 0.29	2.32 ± 0.35	2.79 ± 0.25	1.97 ± 0.26	2.39 ± 0.27	0.24 ± 0.08	0.29 ± 0.07

I = Infected; C = Controls.

Statistical analysis ('t'-test) of the above data revealed the following:

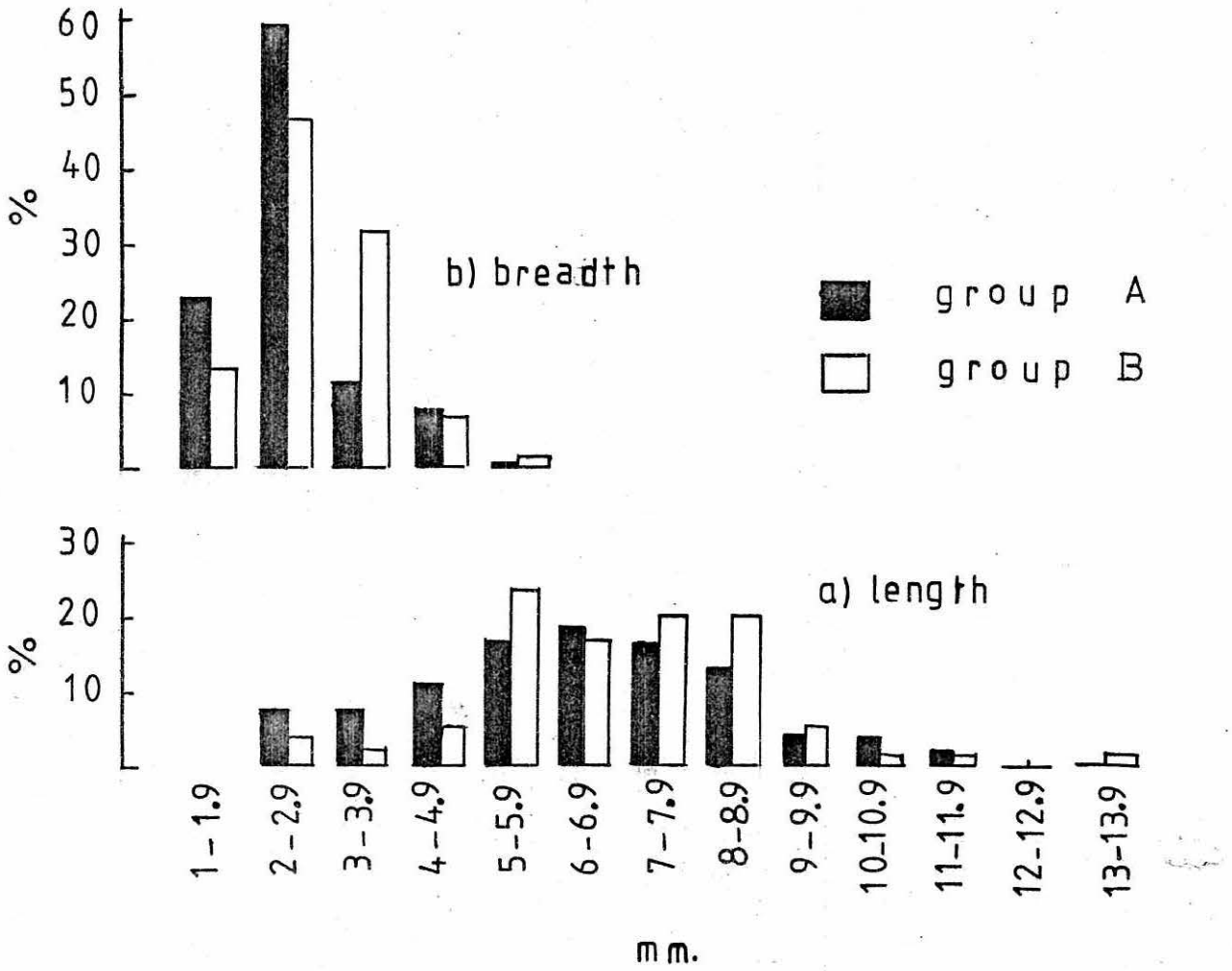


Fig. 42. Frequency distribution of the lenght and breadth of flukes recovered from fluke - infected guinea pigs. Group A given 0.5 (later 5) mg ascorbic acid / animal / day; group B given 20 mg ascorbic acid / animal / day.

a) Group A:

i. Infected animals had significantly heavier spleens than controls ($p < 0.01$).

b) Group B:

i. Infected animals had bigger spleens than their respective controls.

ii. The adjusted weights (organ weight/ terminal body weight) for liver, kidney and adrenals were significantly higher in infected guinea pigs ($p < 0.02$; 0.01 and 0.1 respectively).

The differences in adjusted weights between controls of group A and those of group B or between infected animals of the two groups were not statistically sound. In general, infected animals had heavier organs than controls.

Tissue ascorbic acid concentrations:

Results of the tissue concentrations of ascorbic acid are presented in Table XXVI. 't'-test of significance was applied to the obtained data and the following was revealed:

1. The differences in tissue ascorbic acid contents between controls and infected animals in either group was not statistically significant except for the heart values which were significantly higher in exposed animals ($p < 0.01$).
2. Ascorbic acid values obtained for infected guinea pigs of group A were not significantly different from those obtained for group B.

3. Although control animals of group B had consistently higher tissue ascorbic acid levels than those of group A, none of the differences attained significance.

In general, the tissue ascorbic acid concentrations were always higher in the infected animals of group A than in their controls but in group B this was true only for kidney and heart values; liver, spleen and adrenal concentrations were higher in controls.

Table XXVI Mean and SD of tissue ascorbic acid concentrations (mg/100g) in experimental guinea pigs.

Group Description	Liver	Spleen	Kidney	Heart	Adrenal
	A (Low vitamin C dose)				
Infected	7.96 ± 2.58	24.37 ± 15.09	5.49 ± 1.88	6.59 ± 1.06	24.05 ± 6.80
Controls	5.65 ± 0.86	17.80 ± 3.63	3.96 ± 0.25	3.23 ± 0.52	19.65 ± 3.49
	B (High vitamin C dose)				
Infected	6.84 ± 1.77	16.98 ± 3.37	5.02 ± 1.12	7.26 ± 1.21	21.10 ± 12.98
Controls	8.76 ± 3.37	23.42 ± 4.69	4.76 ± 1.43	3.67 ± 0.96	24.51 ± 9.79

Phagocytic and bactericidal activity of polymorphonuclear leukocytes:

The white blood cell picture and the number of N.B.T. positive PMN leukocytes are shown in Table XXVII.

Table XXVII Mean total white blood cell (WBC) counts; lymphocytes (L); neutrophils (N); Eosinophils (E); Monocytes (M); basophils (B) and the N.B.T. positive PMN cells in experimental guinea pigs. NS = not significant.

Group	Description	WBC	Differential count					N.B.T. positive cells	
			L	N	E	M	B	%	No.
A	Infected	10895 ± 4230	3408 ± 1512	4820 ± 1968	1311 ± 873	1172 ± 686	348 ± 222	1.6 ± 0.49	78.2 ± 53.97
	Controls	8725 ± 3090	5306 ± 1706	2929 ± 1151	116 ± 44	337 ± 225	36 ± 21	1.66 ± 0.47	69.2 ± 45.8
	t =	0.756	1.519	1.192	2.4	2.106	2.548	0.167	0.234
	p =	NS	NS	NS	0.05	0.1	0.05	NS	NS
B	Infected	11306 ± 5616	3459 ± 2325	5100 ± 2869	1541 ± 1053	1087 ± 616	166 ± 90	2.5 ± 0.86	141.04 ± 49.78
	Controls	7200 ± 1870	3858 ± 761	2864 ± 1104	129 ± 80	302 ± 142	46 ± 25	2.0 ± 1.41	62.42 ± 33.87
	t =	1.2017	0.282	0.807	2.317	2.15	2.226	0.526	2.26
	p =	NS	NS	NS	0.1	0.1	0.1	NS	0.1

In either of the two groups, infected animals had higher WBC, neutrophil, eosinophil, monocyte and basophil

counts but lower lymphocyte counts and the differences were significant for eosinophils, monocytes and basophils.

The incubation of N.B.T. dye with citrated blood resulted in the formation of formazan which was seen as small black granules in the cytoplasm. Care was taken to differentiate these from artifacts. These granules were seen in heterophils, eosinophils and occasionally monocytes. The figures recorded in Table XXVII are those of N.B.T. positive heterophils and eosinophils, as both cell types are phagocytic, and are denoted together as PMN leukocytes. The numbers of PMN positive cells were not very different in blood of infected and control animals of group A, but they were higher in infected animals of group B as compared to their respective controls; the differences were not statistically significant. The reduction of the N.B.T. dye was taken as an indicator of the phagocytic ability of PMN leukocytes.

Details of the test for the bactericidal ability of PMN leukocytes against Salmonella dublin are presented in Table XXVIII, and the results show the following:

1. The PMN leukocytes of fluke-infected guinea pigs had a greater bactericidal capacity than controls, particularly so in group A animals supplemented with the low vitamin C dose.
2. In group A, the mean numbers of S. dublin killed per phagocyte (total number of killed bacteria/number of PMN neutrophils + eosinophils) were 2.964 ± 0.817 and 1.597 ± 0.54 for infected and control animals respectively. Considering the blood neutrophils only

Table XXVIII Bactericidal capacity of polymorphonuclear leukocytes for Salmonella dublin

N + E = Neutrophils + Eosinophils; N = Neutrophils

Group		No.	Incubate: 1ml blood+1ml bacterial suspension			After 45 minutes incubation at 37°C					
						Viable counts x 10^7			Killed bacteria		
			PMNs/ml N+Ex10^6	PMNs/ml N x 10^6	Bacteria/ ml x 10^7	Super-natant	Cell-associated	Total	Total x10^7	No./ phago-cyte N+E	No./N
A Low vitamin C dose	Infected	2	6.045	3.681	3.300	1.300	0.2220	1.5220	1.7780	2.94	4.83
		3	7.313	5.861	3.900	1.396	0.2820	1.6780	2.2220	3.04	3.79
		5	7.781	7.205	5.000	1.968	0.2506	2.2186	2.7813	3.57	3.86
		6	1.667	1.593	0.572	0.246	0.0900	0.336	0.2360	1.42	1.48
		7	7.850	5.759	5.800	1.616	1.1580	2.774	3.026	3.85	5.25
	Control	17	2.837	2.722	0.856	0.1888	0.0750	0.2638	0.5922	2.09	2.17
		18	2.285	2.149	0.550	0.2484	0.0897	0.3381	0.2119	0.93	0.99
		19	5.032	4.865	1.266	0.5364	0.1260	0.6624	0.6036	1.20	1.24
		20	2.028	1.980	0.670	0.1888	0.0414	0.2302	0.4398	2.17	2.22
B High vitamin C dose	Infected	10	10.252	9.175	5.800	2.096	0.3060	2.4020	3.3980	3.30	3.70
		11	8.606	6.314	3.900	1.000	0.5040	1.5040	2.3960	2.78	3.79
		14	5.860	3.118	1.730	0.752	0.1947	0.9467	0.7833	1.34	2.50
		16	1.847	1.793	0.572	0.248	0.0600	0.3080	0.2640	1.43	1.47
	Control	21	3.125	2.873	1.266	0.5352	0.1173	0.6525	0.6135	1.46	2.13
		22	4.648	4.502	0.856	0.2420	0.0660	0.3080	0.5480	1.18	1.22
		23	2.737	2.688	0.550	0.2000	0.0831	0.2831	0.2669	0.97	0.99
		24	1.462	1.394	0.670	0.2020	0.0783	0.2800	0.3897	2.66	2.79

(i.e. dividing the number of killed bacteria by the total number of neutrophils) the mean number of killed bacteria per neutrophil were 3.842 ± 1.3069 and 1.655 ± 0.547 for infected guinea pigs and controls respectively. In both cases 't'-test revealed that the differences were statistically significant ($p < 0.05$).

In group B, given the high vitamin C dose, the corresponding figures were 2.212 ± 0.848 and 1.692 ± 0.669 bacteria/phagocyte (neutrophils + eosinophils) or 2.865 ± 0.9529 and 1.782 ± 0.7211 bacteria/neutrophil for infected and control animals respectively.

3. Control figures were not very different between the two groups.

It is of interest in this context to record the results of an exploratory experiment on the phagocytic activity of blood neutrophils of sheep experimentally infected with F. hepatica. Details of these sheep were given in Experiment I (p. 38). The N.B.T. reduction test was carried out, as described above, initially on blood from 2 infected sheep during the first 7 weeks of infection and then on blood from 8 infected and 4 control animals during weeks 11 and 12 post-infection. The results are summarised in the following two tables:

Table XXIX Absolute numbers and percentages of N.B.T. positive neutrophils in 2 infected sheep during the first 7 weeks of exposure.

Sheep No.	N.B.T. positive PMNs	Weeks of infection 0	1	2	3	4	5	6
10 infected with 268 cysts in a single dose	No.	49	94	63	255	181	353	267
	%	3.0	3.5	4.4	10.0	10.5	14.9	13.1
6 infected with 536 cysts given in 2 equal doses 14 days apart	No.	50	88	98	144	154	104	114
	%	10.1	12.9	25.0	22.9	19.1	12.9	14.3

Table XXX Mean ± SD of absolute numbers and percentages of N.B.T. positive cells in infected and control sheep during week 11 and 12 of infection. Each group comprised 4 sheep.

Group	Week 11 N.B.T. positive cells %	No.	Week 12 N.B.T. positive cells %	No.
C=Controls	7.25 ± 1.92	144.3 ±52.27	9.0 ± 4.3	171.0 ±113.0
A=Single infection 268 cysts	8.40 ± 5.58	115.1 ±65.33	10.1 ± 9.85	171.6 ±127.3
B=Re-infected 536 cysts	14.0 ± 4.12	264.0 ±114.84	15.6 ±7.75	263.7 ±104.1

The results obtained for the two sheep showed that in both animals the numbers and percentages of the N.B.T. positive cells were increased during the first 7 weeks of

infection. The percentages of N.B.T. positive cells observed for group A animals (single infection) at weeks 11 and 12 were not very much different from those of controls but the absolute numbers were rather low at week 11. On the other hand, both the absolute numbers and percentages of N.B.T. positive cells were much higher in reinfected animals (group B) than in controls.

DISCUSSION

Body Weight:

The liveweight gains have been recorded in sheep of the present experiment as a clinical indicator in the course of infection. The results show that liveweight gains in controls were not largely different from those of infected animals during the first 7 weeks of exposure. In fact, exposed sheep had weights equal to or slightly more than those of controls and this may be explained by the general observation that fluke-infected sheep may maintain a very good body condition before emaciation occurs.

Low weight gains were observed in both infected and control sheep during the first week after infection. A constant daily ration consisting of hay and nuts was given to each animal throughout the experimental period. For some reason nuts were not available for that week and this seemed to have affected the body weight.

From the 8th week onwards, controls showed consistently higher weight gains than infected animals but the differences, despite being appreciable, were not statistically significant.

In rats, the initial and final weights were recorded in three groups only and the results indicate that body weight decreased with time in infected animals; rats kept for the longest period had the lowest gains.

Fasciola hepatica infection in sheep is reported to

cause loss of body condition (Sinclair, 1962, 1970a, 1973; Reid et al., 1970; Presidente et al., 1973) and this is attributable to inappetence and to reduced digestibility and inefficient food utilization in infected animals (Duwel et al., 1974; Coop and Sykes, 1977; Hawkins and Morris, 1978). Such findings have also been reported in cattle (Canale et al., 1973; Cawdery et al., 1977). In this study inappetence was only noticed in two sheep (Nos. 9 and 12), otherwise all animals were clinically normal. Sheep No. 2 (control) was off food for 2 days because of a febrile condition and was treated with antibiotics.

Reduction in food conversion efficiency in sheep, and hence decrease in body weight, could be produced by a burden of 45 flukes (Hawkins and Morris, 1978) or even less, i.e. 8-17 flukes (Buchwalder, Daetz, Hiepe and Schmidt, 1977). In the present study a mean burden of about 87 flukes per sheep did not seem to cause drastic changes in liveweight gains. Of course, when assessing growth rates in fascioliasis, not only fluke burdens but also the plain of nutrition (Berry and Dargie, 1976, 1978) and probably age and sex of infected animals should be considered. However, body weight loss is repeatedly reported in fascioliasis but Anderson et al. (1978) are of the opinion that it is of little value in assessing the impact of the disease.

Ascorbic Acid:

The cattle, sheep and rats used in this study are all considered competent of synthesizing vitamin C (see p. 21). The plasma ascorbic acid was determined as it reflects the state of ascorbic acid metabolism in the body (Brown et al., 1971b). Leukocyte ascorbate was not measured due to the difficulty and time consumed in isolating sheep leukocytes.

The results of the first experiment indicate that F. hepatica infection in sheep adversely affects ascorbic acid metabolism. The progressive decrease in plasma ascorbic acid which started shortly after infection and continued till the 9th week corresponds to the migratory phase of the parasite which is characterised by extensive tissue damage (Dow et al., 1968; Rushton and Murray, 1974). Since ascorbic acid is manufactured in the liver, the damage produced by migrating flukes is liable to cause an impairment in the vitamin synthesis. Hepatic destruction is expected to become more pronounced as the parasites grow bigger, especially during the 6th to 8th weeks when they are in the localized migratory phase (Dow et al., 1968). This might explain the progressive decline in plasma ascorbate during the first 9 weeks of infection.

It is also possible that the ascorbic acid produced is preferentially used in regeneration and repair of damaged tissue. Moreover the vitamin may be utilized by the flukes themselves. Thus, Pantelouris and Hale (1962) stated that liver flukes feed on material rich in iron and for them to survive they should free themselves of the excess metal.

Their findings suggested that vitamin C plays an important role in maintaining the iron in a diffusable form to facilitate its conveyance to the sites of excretion. So, a great part of the vitamin could be used in this process. Furthermore, ascorbic acid is albumin transported (Turner and Hulme, 1971) and the hypoalbuminemia, commonly encountered in fascioliasis, could reduce the capacity of its transport and contribute to the low plasma levels observed. It seems that in the migratory phase of the flukes there is more utilization and hence increased demand for ascorbic acid which is not fulfilled by the damaged hepatic tissue.

After the 9th week of infection the plasma ascorbic acid content gradually increased in all sheep except No.7 of group B, but was still below preinfection levels. This corresponds to the patent period when the flukes are in the bile ducts, and it is reasonable to assume that at this stage the liver damage due to migration is reduced, regeneration is enhanced and consequently more ascorbic acid is being synthesized. This could be supported by the fact that biochemical dysfunctions detected by liver function tests during the prepatent period begin to normalise after the flukes enter the bile ducts, indicating the repair of the damaged hepatic tissue (Sewel, 1966).

The findings of the second experiment in sheep and cattle confirm those of the first one in that F. hepatica adversely affects ascorbic acid status in the body. The levels in the sheep greatly decreased after infection and were minimal by the 10th week. The cows also exhibited a

decline in plasma ascorbic acid which was less marked and this can be explained by the following:

1. Hepatic damage as judged by the macro- and microscopic appearance was relatively less in the two cows.
2. In both animals the fluke recovery was low indicating the occurrence of fewer burrows and hence less tissue damage.
3. Due to the size of their livers, the remaining uninjured functional tissue is greater in the bovines and hence more ascorbic acid could be synthesized.

It appears that the hepatic reaction induced in the two cows caused little obstruction to migrating flukes and the parasites seem to enter the bile ducts earlier than in sheep. This may explain why the ascorbic acid level remained low for only 6-7 weeks of exposure as compared to 10 weeks in the sheep. Afterwards the plasma ascorbate started to increase and this seems to be conditioned by the entry of flukes in the bile ducts as discussed previously. However, in this extended experiment, the rise was seen to be only temporary and lasted for about 4-5 weeks in sheep and about 2-3 weeks in cows, after which the levels declined again. The latter decrease could be related to the tissue reaction evoked by adult flukes in the bile ducts and the consequent irritation and necrosis of surrounding parenchyma as well as to the degeneration of hepatic tissue by toxic products possibly released by the flukes.

Control figures for plasma ascorbic acid showed considerable fluctuations especially in sheep of the first

experiment, but these were insignificant compared to the changes in infected animals. Wide variations in blood ascorbate have been previously reported in sheep by De Mille et al. (1972) who related it, in their opinion, to the rather loose control of the vitamin biosynthesis. Similar fluctuations were also observed in goats (Richmond et al., 1940), and probably occur in other animals.

The results of tissue ascorbic acid in rats are complementary to those of the plasma levels in sheep and cattle. Tissue ascorbic acid becomes reduced after infection particularly in the spleen. It is interesting to note that the liver ascorbic acid concentrations in infected animals, although a bit lower, were not largely different from those of controls. This could be due to the rapidity of liver regeneration in infected rats and the over-stimulation to synthesize more ascorbic acid to maintain normal levels. Assuming that adequate amounts of the vitamin are produced, then there are two explanations for the low spleen and kidney levels observed in exposed rats:

a) That normal amounts are supplied to these organs but the utilization is excessive or
b) The vitamin is not efficiently transported from the liver to other tissues, probably due to reduction of plasma albumin.

Glucose:

Ascorbic acid is synthesized from glucose (Chatterjee, 1970; Gupta et al., 1973) and availability of the glucose substrate could be an important limiting factor in the biosynthesis of the vitamin. When discussing the availability of glucose, certain considerations should be borne in mind with regard to carbohydrate metabolism in ruminants:

a) Glucose is not absorbed per se in the small intestine because ingested carbohydrates are fermented to volatile fatty acids by the rumen microflora; acetic, propionic and butyric acids being major components.
b) Glucose is provided through the process of gluconeogenesis mainly from propionate and this is considered essential in ruminants (Leng et al., 1967).
c) Glucose is of less importance as an energy source and acetate oxidation plays a more effective role in energy metabolism.

The available glucose may be utilized through any one of several pathways, i.e. free glucose (for maintenance of blood levels), glycogenesis, glycolysis, pentose cycle and glucuronate pathways. The latter is of particular interest as it leads to ascorbic acid synthesis through the following steps:

Glucose ⟶ Glucose-6-phosphate ⟷ glucose-1-phosphate ⟷ Uridine diphospho glucose ⟶ uridine diphospho glucuronate ⟷ glucuronate ⟶ L-gulonate ⟶ L-ascorbic acid (see Kaneko, 1970b).

The present findings revealed no significant differences in plasma glucose levels between controls and infected animals. This may indicate that the free glucose pathway is actively maintaining the blood levels but provides no clues to explain the low plasma ascorbic acid contents in infected animals. However, the following could be speculated:

i) Glucose is utilized for vitamin C synthesis but the demand for the latter is greater than the rate of supply.

ii) Glucose is available but damage to the liver cells could have unfavourably affected the efficiency of the enzymatic reactions involved in its conversion to ascorbic acid.

iii) The availability of glucose is limited and the sugar is used preferentially in other metabolic pathways (i.e. maintenance of blood levels, glycoprotein synthesis) rather than for vitamin C synthesis.

The first two points may be operating together and have been discussed before. The third point, on the other hand, would imply that vitamin C synthesis is not an important physiological priority (Brown et al., 1970) and would contradict reports about the many essential functions of ascorbic acid in the various systems (see Wilson, 1974; Chatterjee et al., 1975).

However, limited glucose availability may be partly responsible for the low plasma ascorbate level observed. Inappetence, reduced rumination and inefficient utilization of metabolizable energy have been reported in F. hepatica

infection in sheep (Sinclair, 1962, 1973; Coop and Sykes, 1977). Moreover, the reduced liveweight gains observed in this study, as well as those reported by many others, indicate that food utilization is impaired. It could, therefore, be assumed that in fascioliasis the decreased food intake and utilization would be associated with a reduction in volatile fatty acid production and consequently retard the rate of gluconeogenesis.

If glucose availability is limited and the rate of gluconeogenesis reduced then enhanced glycogenolysis would provide for the required glucose. The present results indicate that this might be true as significantly lower liver glycogen contents were observed in fluke-infected rats when compared to controls; infected animals had also lower tissue ascorbic acid levels. Depletion of liver glycogen has been reported in F. hepatica infection in rabbits (Furmaga, 1963), sheep (Tsvetaeva and Gumen'shchikova, 1965; Rubaj and Furmaga, 1969) and cattle (Kadziolka, 1962). In cattle, reduced liver vitamin C concentrations were also found.

In conclusion, the above discussion suggests that inability to utilize glucose for vitamin C synthesis, probably because of its inavailability, may have contributed to the low tissue and plasma ascorbic acid encountered.

Cholesterol and Low-density Lipoproteins:

As stated in the introduction, acute or chronic ascorbic acid deficiency in man and animals can be associated with changes in the plasma and tissue lipids. The state of ascorbic acid deficiency which seems to accompany fluke infection might also be connected with alterations in the plasma lipid constituents.

In the present study plasma cholesterol and low-density lipoproteins were determined and the results revealed that the cholesterol content decreased while the lipoproteins increased during the course of the disease. The decrease in plasma cholesterol commenced very early in infection when the plasma ascorbic acid was also declining. The decrease in both parameters seems to have a similar aetiology but whether the low plasma cholesterol was influenced by a deficiency of ascorbic acid is not certain. Low plasma, or serum, cholesterol was, however, observed in ascorbic acid deficient monkeys and humans; such findings contrast with many reports that associate hypercholesterolemia with vitamin C deficiency (see p. 8 for references).

Contrary to cholesterol, the plasma lipoproteins significantly increased in infected animals. The low-density lipoproteins correspond to the β-fraction in electrophoretic separation and consist mainly of cholesterol and its esters, phospholipids and some triglycerides (see Bartley, 1970). Since cholesterol levels were decreased, the rise in the

plasma lipoproteins would likely be due to an increase in either phospholipids or triglycerides, or both. Unfortunately, the latter two were not determined.

Plasma β-lipoproteins (low-density lipoproteins) were found to increase in scorbutic guinea pigs (Bobek and Ginter, 1962; Banerjee and Ghosh, 1961; Banerjee and Bandyopadhyay, 1963) and monkeys (Banerjee and Bandyopadhyay, 1965). This was associated with a rise in phospholipids, cholesterol and non-esterified fatty acids in the former animal but with increased plasma triglycerides and free fatty acids in the latter (Banerjee and Bandyopadhyay, 1963 and 1965 respectively). The rise in phospholipids in guinea pigs was explained by the fact that lipid synthesis is decreased in vitamin C deficiency and the available fatty acids are transported mainly as phospholipids, while the increase in triglycerides and free fatty acids was related to increased fat catabolism.

It can be speculated from the present study that F. hepatica infection in animals causes a deficiency in ascorbic acid which indirectly leads to changes in the plasma lipid profile. At this stage there is little experimental evidence to support the above statement and further investigations are necessary before solid conclusions can be drawn.

Iron:

Anaemia is a well recognised symptom of fascioliasis. It is caused by the loss of blood through haemorrhages in the liver during the migration of young flukes and, more important, through ingestion of blood by adult parasites and its loss *via* bile into faeces. About 0.5ml of blood could be lost per fluke per day (Sewel *et al.*, 1968; Holmes and Maclean, 1971; Sinclair, 1972; Berry and Dargie, 1978) and this would result in a simultaneous loss of iron and proteins. Eventually iron-deficiency ensues and clinical manifestations of anaemia develop. In the present study plasma iron, the iron-binding capacity and liver iron concentrations were utilized as tests for iron metabolism in *F. hepatica* infection and the results are later discussed in relation to the plasma ascorbic acid picture observed.

The infected sheep showed more or less similar changes in plasma iron and the iron-binding capacity which were more pronounced than in the two cows. The results show that plasma iron concentrations were either maintained or increased during the first 7 weeks of infection, and the binding capacity was apparently elevated. This denotes increased iron transport to erythropoietic tissues as erythropsiesis would be stimulated to compensate for the blood loss caused by migrating flukes. Iron deficiency is not expected to occur at this stage because the metal in damaged erythrocytes is mostly recycled and utilized for

the formation of new haemoglobin. Symons and Boray (1967) reported increased plasma iron levels in F. hepatica infected sheep during the acute stages of infection particularly at week 6, and Sinclair (1968) and Berry and Dargie (1978) observed normal serum iron levels during the first 8 and 7 weeks respectively in exposed sheep. In calves, Furmaga and Gundlach (1978) found that the serum iron was maintained at normal levels during the first 7-10 weeks of infection but decreased thereafter and the decline was associated with increased transferrin levels.

In the present investigation the plasma iron decreased after the 7th week of infection and reached minimum values by the 9th to 10th weeks; these results are in agreement with those of Berry and Dargie (1978). At this time flukes enter the bile ducts and because of their haematophagic activities, considerable amounts of haemoglobin iron could be lost and iron deficiency may occur. Since the requirements for iron are normally satisfied from the diet, then increased iron absorption would be one way to correct the deficiency (Berry and Dargie, 1978). However, in a previous report Holmes and Maclean (1969) found that iron intestinal absorption was not increased in infected sheep. Alternatively, storage iron in ferritin and haemosiderin is mobilized to replenish the iron pool (Moore, 1961; Kaneko, 1970) but this will soon be exhausted as the disease progresses due to the limited amount of stored metal (Charlton and Bothwell, 1970). It is relevant to mention here

that in this study sheep and rats in late infections had low liver iron concentrations. However, failure of storage iron to meet the iron demand could explain the low plasma iron levels in the more advanced stages of the disease but does not elucidate the low concentrations observed in the early patent periods as the storage compounds are unlikely to be totally depleted by this time. In this respect Berry and Dargie (1978) maintain that the stored iron could service the plasma pool up to the 12th week of infection.

So iron depots might have been adequate but there was a failure in transport of the metal to acceptor sites. Iron is transported *via* plasma complexed to transferrin. The level of transferrin is considered directly related to the iron-binding capacity (Moore, 1961) and a deficiency in this globulin would lead to reduced plasma iron concentrations. The present findings, as well as others (Sinclair, 1965, 1968; Furmaga and Gundlach, 1978) show that the iron-binding capacity increased shortly after infection indicating that transferrin levels were actually elevated. This will invalidate the argument of defective transportation.

Excluding the inadequacy of storage iron and defective transportation, a third factor might have contributed to the low plasma iron observed, namely a disturbance of iron release into the plasma. Histochemical findings in this study indicate that liver iron is depleted in infected animals but considerable amount are present in the spleen. The liver contains both reticuloendothelial

cells and hepatocytes and it is the iron in the latter which is most easily released into the plasma which could have accounted for the low levels in the liver (Lipschitz et al., 1971). Sinclair (1965) reported a decrease in bone marrow haemosiderin both in infected sheep and in another uninfected group that was simultaneously bled (to mimic the blood loss) but contrary to the findings in the latter group the haemosiderin did not totally disappear in the infected animals. His findings also indicate that the injected ^{59}Fe was not fully utilized by the erythropoietic tissues and the unutilized isotope was retained in the iron stores. The above account suggests that not all the available iron was mobilized and indirectly implies a disorder of its release. In a way, this could be supported by the findings of Berry and Dargie (1978); their results indicate that in poorly fed animals infected with flukes, iron delivery to the bone marrow is slow and this was not related to a transport deficiency.

Iron metabolism could be influenced by ascorbic acid and the interaction between the two has previously been reviewed (see page 11). Experimental evidence indicates that vitamin C deficiency may cause generalised defective reticulo-endothelial cell function and consequently a disorder of iron release into the plasma. Thus Lipschitz et al. (1971) found that the concentration of non-haeme iron was significantly lower in the livers of scorbutic guinea pigs than in those of controls while the spleen concentrations were much higher in the former group. When ascorbic acid was given to

scorbutic guinea pigs 24 hours prior to death, the spleen concentrations were as low as those of controls but the liver content remained unchanged. This denotes that the vitamin favourably affected iron release. They also found that when denatured erythrocytes containing ^{59}Fe-labelled haemoglobin were injected into normal and vitamin C deficient guinea pigs, most of the radio-iron was detected in the livers and spleens of both groups at 45 minutes and by 3 hours 50% of it was released into plasma in normal animals, whereas hardly any release occurred in the scorbutic pigs. Even after 24 hours 63% of the radioactivity was still retained in the latter group compared to 36% in the former.

It has been shown in the current investigation that F. hepatica infection in cattle, sheep and rats is associated with low plasma and tissue ascorbic acid concentrations and it was concluded that a state of ascorbic acid deficiency can supervene during infection. At the same time the present findings (as well as others) revealed that derangements in iron metabolism occur during the course of infection. Some correlation can be visualized between the changes in the plasma ascorbic acid levels and those in plasma iron and saturation concentrations particularly so during the 7th-10th weeks when both parameters were declining and after the 10th week when their levels increased. On the basis of the involvement of ascorbic acid in iron metabolism, the present information indicates that the plasma iron concentrations were influenced by the

ascorbate level. Thus the rise in plasma iron concentrations observed after the 10th week of infection could be due to the improved body ascorbic acid status as reflected by the increased plasma content.

It can be concluded that ascorbic acid deficiency may play an important role in the pathogenesis of the anaemia of fascioliasis by affecting iron metabolism, particularly its release and mobilization, in diseased animals.

Effect of Ascorbic Acid Supplementation in rats:

So far, the results indicate that ascorbic acid status is considerably upset in F. hepatica infection in cattle, sheep and rats and it seems possible that ascorbic acid deficiency plays a part in the development and severity of the lesions produced. The role of ascorbic acid in healing and repair processes has been emphasized before and it was anticipated that supplementation of vitamin C to infected animals would enhance tissue healing and renders infection less severe. This was therefore tested in rats.

The present findings show that infected rats given vitamin C had lower body weight than the unsupplemented ones. The latter group had higher initial body weights and gained more during the prepatent period. The supplemented group, on the other hand, performed somewhat better later during the patent phase and this suggests that the effect of the vitamin is not immediate but becomes apparent after the flukes enter the bile ducts, i.e. by enhancing repair and regeneration.

In both groups the macroscopic appearance of the liver and the low fluke recoveries indicate that not very much damage was inflicted by the migrating parasites. However, the friable and greasy consistency of the livers of the non-supplemented rats and the presence of microscopic fatty changes imply that hepatic degeneration was more expressed in this group.

The fluke recoveries and their measurements were not statistically different between the two groups but a

higher proportion of larger flukes was recovered from the vitamin-supplemented rats. This indicates an earlier entry of the parasites into the bile ducts. Sinclair (1970b) recovered bigger flukes from reinfected lambs than from infected controls receiving a similar total dose in one single infection and suggested that in resistant animals flukes migrate to the bile ducts faster and develop to larger size. Earlier arrival of flukes into the bile ducts has also been reported in challenge infection in mice (Lang, 1967).

Eosinophilia was not a prominent microscopic feature but the number of eosinophils seen in liver and spleen sections of the non-supplemented rats were relatively more than in those of the vitamin C-supplemented animals. Eosinophilia is a main feature of fascioliasis and its occurrence is probably associated with histamine or histamine liberators released by the flukes (Deschiens and Poirier, 1950; Mettrick and Telford, 1963) or with the development of a hypersensitivity reaction because of infection (Sinclair, 1974). The absence of a marked tissue eosinophilia in the presently investigated rats may be a reflection of the mild nature of infection, or it could be that the liberated histamine was detoxified by ascorbic acid (Nandi et al., 1974) especially in the group given the vitamin.

The differences observed in tissue ascorbate, liver glycogen and total proteins were not consistent in any one

group, and do not by themselves show any favourable effect of the added vitamin. With regard to tissue ascorbic acid, however, it is likely that the unsupplemented rats were adequately stimulated to synthesize enough vitamin C to cope with infection and maintain normal tissue levels. In the rats given ascorbic acid, on the other hand, the following might have happened:

a) The exogenous vitamin absorbed was enough to saturate the tissues and this exerted a suppressive effect on endogenous synthesis.
b) Only a small amount of the added vitamin was absorbed to provide for the extra needed, if any.
c) The vitamin was not absorbed at all or was absorbed but entirely excreted.

The present findings do not allow for definite conclusions to be drawn as to the beneficial effect of ascorbic acid supplementation in fluke infected rats. It could be that the species competent of synthesizing ascorbic acid (i.e. rats) are not suitable for evaluating the effect of exogenous vitamin C and it is suggested that guinea pigs would be ideal for such purposes.

The effect of vitamin C supplementation in guinea pigs:

The present findings show that guinea pigs steadily increased in weight when normal diet, containing vitamin C, was fed and that the body weight remained constant when an ascorbic acid-free diet was given. This indicates that ascorbic acid is important for maintaining normal growth particularly for animal species that depend solely on exogenous sources. This is further supported by the finding that control and infected animals supplemented with a daily dose of 20 mg ascorbic acid maintained higher body weight than their counterparts given a daily dose of 0.5 mg (later 5 mg) ascorbic acid. Evans and Hughes (1963) concluded that the poor growth in guinea pigs fed diets low in ascorbic acid is not due to reduced food intake but to a metabolic effect involving tissue formation. It is important that the low dose of 0.5 mg/day given to guinea pigs has been generally proved sufficient to maintain life and prevent non-specific secondary effects produced by acute vitamin C deficiency, i.e. inappetence, loss of body weight and loss of blood through haemorrhages etc. (Ginter, Bobek and Ovecka, 1968).

Infection with F. hepatica caused a decrease in body weight which was more pronounced in the group given the low vitamin C dose, indicating that the vitamin could be an important nutritional factor which helps in withstanding the impact of infection. This is substantiated by the observation that symptoms of illness and mortality occurred two weeks earlier in the group receiving the low vitamin C dose and could have been total had the dose not been increased.

Very little difference was observed in necropsy findings and in the numbers of flukes recovered between the two infected groups. However, more flukes of bigger sizes were recovered from guinea pigs that received the high vitamin supplement. Bigger-sized flukes were also recovered from vitamin C-supplemented rats, and from the discussion on this point in the previous experiment (V) this is unlikely to be due to decrease in body resistance. It has been mentioned earlier that flukes need ascorbic acid to excrete the excess iron from their tissues which would, otherwise, be incompatible with their existence in the host's body (Pantelouris and Hale, 1962). It is probable that in animals given the high ascorbic acid dose there was more vitamin to be utilized by the flukes to rid themselves of the toxic metal and consequently they survived better and grew to larger sizes.

The results of this study indicate that F. hepatica infection is accompanied by a general increase in organ weights, particularly the spleen and confirm those of the previous experiments. Enlargement of the spleen has been observed in F. hepatica infected sheep (Sinclair, 1968), mice (Kruyt and Van der Steen, 1971) and rats (Gameel, personal observation) and seems to be related to its role in the formation and destruction of blood cells and in the defensive mechanisms of the body, which are expected to be enhanced during infections. The liver is the main organ affected in fascioliasis and its enlargement is largely related to inflammatory oedema, fibrous tissue deposition, thickening of bile ducts and regenerative processes which take place in

the course of the disease. On the other hand, damage to the liver limits its capacity to detoxify toxic substances. These toxic substances may cause various inflammatory changes in the kidney while blood is being filtered through that organ. Moreover, damage to the liver is often associated with oedema and retention of water and salt in the extracellular tissues and this, together with the possible inflammatory effects of toxic substances, may be the reason for the increased kidney weights. The increased adrenal weights may be explained by the fact that in stress conditions, such as fluke infections, the secretion of adrenocorticotrophic hormone is promoted and the latter causes adrenal hyperplasia probably to increase the production of cortisol and other steroid hormones. As for the heart it is known that in conditions associated with anaemia, which include fascioliasis, the work load of the heart is increased and this might lead to cardiac hypertrophy.

With regard to tissue ascorbic acid concentrations, controls of the higher ascorbic acid dose had higher concentrations in their livers, hearts, kidneys, spleens and adrenals than those of the lower vitamin C dose, especially in the latter two organs. This implies that the degree of tissue saturation varies with the dose of vitamin C given in normal animals. On the other hand, infected animals on the low vitamin dose had more tissue ascorbic acid concentrations than their respective controls or their infected counterparts receiving the high vitamin dose (except for heart values). This was again noticeable in the case of spleen and adrenals. Infected animals on the high dose level had more ascorbic

acid in their kidneys and hearts than their controls. Such findings and variations between the infected groups are not readily explainable and were contrary to expectations. However, it is possible that various tissues undergo some adjustments to retain more ascorbic acid in case of infection especially when the supply is insufficient or just so. In this context Jablonowski (1976) investigated the levels of vitamins B_2 and C in some tissues of guinea pigs infected with Ascaris suum larvae and found that in hypovitaminosis C infection resulted in an increase in ascorbic acid concentrations in the blood and livers of exposed animals but not the adrenals; his findings seem to confirm other reports (Jablonowski, 1970; Tarczynski, Krzymowska, Ziecik and Jablonowski, 1972).

The association of F. hepatica infections and bacterial diseases has been reported in the literature. Bacteria may be transported from the intestines by migrating flukes and cause disease, or dormant infections may flare up due to lowering of the host's resistance as a result of fluke invasion (Haiba, Geinidy and Sokkar, 1968; Taylor and Kilpatrick, 1975).

Thus the relationship between fascioliasis and increased susceptibility to Clostridium oedematiens has been indicated by many authors (Turner, 1930; Cvjetanovic, Berberovic and Matanovic, 1958; Djukic, Matic and Katic, 1970). Damm (1975) reported significant quantitative and qualitative changes in the intestinal bacterial flora of cattle infected with F. hepatica; Escherichia coli and enterococci constituted the highest proportions. Further, mycobacteria species were isolated from flukes collected from

infected cattle (Onet, Constantinescu and Sandu, 1978).

The possible association of F. hepatica infection and salmonellosis has been suspected by Dijkstra (1973) based on the observation that a decreased incidence of Salmonella dublin is paralleled with a decrease in liver fluke infections and that control of the latter resulted in a reduction in the numbers of S. dublin positive cases. On the other hand, Rodriguez Rebollo (1966) concluded that a direct relationship between salmonellosis and fascioliasis is unlikely and Damm (1971) supported that by finding no evidence between fluke migration and transport of salmonella, although he speculated that lowering of resistance might occur. Moreover, Taylor and Kilpatrick (1975) are of the opinion that both infections occur independently but they may be favoured by similar external factors.

Very recently, Aitken, Jones, Hall, Hughes and Collis (1978a) and Aitken, Hughes, Jones, Hall and Collis (1978b) demonstrated that cattle experimentally infected with F. hepatica became more susceptible to intravenous injections of S. dublin.

From the foregoing account it appears that F. hepatica infection may adversely affect the defensive mechanisms of the host and render it more vulnerable to bacterial invasion. The increased susceptibility could be due to reduced phagocytic and/or microbicidal capabilities of phagocytes and this has been investigated in the current study. It should be remembered that normal or increased phagocytosis is not necessarily associated with a normal or enhanced

bactericidal activity, as has been shown in children with chronic granulomatous disease; PMN leukocytes from those patients showed phagocytic ability but failed to kill the phagocytized bacteria (Quie, White, Holmes and Good, 1967).

The in-vitro conversion of N.B.T. dye to insoluble formazan is a principal test for estimating the redox activity of PMN leukocytes (Baehner, Boxer and Davis, 1976). This test was utilized in the present study in a simple way without in-vitro challenge.

Both neutrophils and eosinophils are phagocytic cells and were commonly seen in this study reducing the N.B.T. dye. The percentages of N.B.T. positive cells were not very much different between controls and infected animals of each group, but the values were generally higher in group B animals which had the high vitamin C dose. The absolute numbers, on the other hand, were higher in infected animals, especially those of group B. The above results seem to confirm those observed in sheep, which showed that the absolute numbers and percentages of PMN positive cells increased in F. hepatica infected animals especially during the first 7 weeks of exposure. Both sheep and guinea pig findings suggest that the phagocytic activity of PMN leukocytes increase in fascioliasis.

The bactericidal ability of PMN cells for S. dublin was estimated after incubation with guinea pig's blood and the numbers of bacteria killed were related to either the total number of neutrophils and eosinophils or to neutrophils only. The reason why eosinophils were considered

is because they are also phagocytic cells and do increase markedly in fluke infection and the possibility that they play a part in bacterial killing cannot be excluded. Moreover, a role for eosinophils in the immunological processes of the body has been suggested (Speirs, 1963). In any case the present study reveals that the bactericidal capacity of PMN cells, expressed as the number of bacteria killed per phagocyte was higher in infected guinea pigs than in controls. This may denote that F. hepatica induces quantitative and qualitative changes in PMN leukocytes that make them more active against micro-organisms. The number of blood PMN cells was also elevated in exposed animals and both the quantity and adequate function of these cells are necessary in bacterial resistance (Quie et al., 1967). The above findings are not surprising because animals exposed to any sort of infection, whether bacterial, viral or parasitic, are expected to mobilize their defensive mechanisms against the invading agent; some of these mechanisms would be specific, i.e. antibody production and others would be non-specific, i.e. increased phagocyte function. Generally, blood and tissue neutrophils increase in many parasitic diseases including fascioliasis and these cells may participate in the host's defensive mechanisms, especially during early infection (see Furmaga et al., 1975). One form of this participation may be increased microbicidal activity.

However, the current findings appear to contrast those of Jones, Hall, Hughes and Aitken (cited by Aitken,

et al., 1978b) who found that blood obtained from fluke infected cattle is less efficient in removing S. dublin in-vitro than blood drawn from healthy cattle and they related that to impaired phagocytosis by PMN neutrophils. Similarly, the present results would be contrary to the general concept of increased susceptibility in fluke-infected animals to bacterial invasion, if this is expressed solely in terms of phagocyte-bacterial interaction.

The question which remains to be answered is whether the phagocytic and bactericidal activities of PMN cells were influenced by the amounts of ascorbic acid supplemented. The present results revealed little differences in the above mentioned leukocyte functions between controls given low or high vitamin C dose and indicate that these functions are not crucially affected by ascorbic acid concentrations. This conclusion seems to be in line with Stankova et al. (1975) who found unimpaired hydrogen peroxide production and bacterial killing by PMN cells obtained from scorbutic guinea pigs. They pointed out that small amounts of the residual ascorbate in scorbutic leukocytes could be adequate to maintain normal functions.

Unexpectedly, PMN leukocytes of infected animals given the low vitamin C dose had more bactericidal capacity than their counterparts on the high vitamin dose. It is likely that PMN cells of the former animals were somehow stimulated to conserve and utilize ascorbic acid in a more effective manner than those of the latter group and become more lethal to bacteria. Unfortunately, it was not possible to measure the leukocyte ascorbic acid concentrations in this experiment but the results of the tissue ascorbic acid

contents do suggest that animals given the low vitamin C dose were better able to retain the vitamin in their tissues than those supplemented with the high dose and this could be true for blood leukocytes.

Shilotri and Bhat (1977) demonstrated that a daily mega dose of 2g ascorbic acid given to human volunteers for 2 weeks reduced the intraleukocytic bacterial killing for Escherichia coli and this effect was reversed when the vitamin supplement was stopped. It could therefore be argued that the high vitamin C dose (20 mg/day) given to guinea pigs in the present study was above the maximum requirements of these animals and that toxic effects, which lead to impaired phagocytic function, might have been precipitated. Considering the present results it can be seen that the high dose given did not produce any adverse effects in control animals and on the contrary it seems to have a beneficial effect as compared to a daily dose of 5 mg, i.e. better growth performance, high tissue concentrations of ascorbic acid and relatively more active phagocytosis and bactericidal capacity. It is, however, concluded that the dose of 20 mg/day was quite safe to guinea pigs. In this respect measurement of urinary excretion of ascorbic acid may be useful for better interpretation of the results in future experiments.

Although some of the results in this section were rather unexpected, the study in general disclosed some worthwhile findings, especially with regard to the changes in phagocyte functions which indicate a similarity between the animal species tested above. Further experiments of a more detailed nature would seem to be justified.

PART B

PLASMA PROTEINS

The plasma proteins are globular proteins, most of which are conjugated with carbohydrates (glycoproteins), lipids (lipoproteins) or metals (metalloproteins). They perform wide physiological functions, some of which are listed in Table 1 below.

Table 1 Some plasma proteins and their physiological functions (summarized from Turner and Hulme, 1971. Most of the information is obtained from human proteins).

Protein	Functions
Albumin	1. Maintenance of colloid osmotic pressure. 2. Transport of bilirubin, porphyrins, uric acid, acetylcholine, fatty acids, copper, calcium, zinc, excess vitamin A and C, progesterone, aldosterone, histamine and various drugs i.e. barbiturates, penicillin and sulphonamides.
Orsomucoid (or α_1-acid glycoprotein)	Inactivation of progesterone.
α_1-Antitrypsin	It is the most prominent α-globulin. Inactivates trypsin, chymotrypsin, auto activated plasmin and probably other proteases.
α_1-Lipoprotein (High density lipoprotein)	Transport of lipids i.e. Triglycerides, cholesterol, phospholipids, carotein, vitamin A and E.
Ceruloplasmin	It is an α_2-globulin concerned with copper transport but also acts as an oxidase (Ferroxidase).
Haptoglobulins	α_2-globulins which bind haemoglobin.
α_2-Macroglobulins	The major proteins in the α_2-globulins. It binds insulin and may act as an inhibitor of trypsin, chymotrypsin and plasmin.

Table 1 (Contd.)

Protein	Functions
Pre-β-lipoproteins (very low density lipoproteins)	Transport of lipids, mainly triglycerides.
β-lipoproteins (low density lipoproteins)	Transport of lipids and lipid soluble vitamins and hormones.
Haemopexin	Is a β-globulin which binds haeme.
Transferrin	Is the principal β-globulin of plasma. It transports iron and also has an anti-bacterial and anti-viral activity.
Immunoglobulins (IgA; IgE; IgM; IgG)	These γ-globulins are a group of structurally-related proteins mainly concerned with antibody formation against infections. Some have tissue fixing activities (i.e. IgE) and others activate complement (i.e. IgG and IgM).
Prothrombin	Is the precursor of thrombin which is necessary for the formation of fibrin from fibrinogen in the blood-clotting mechanisms.
Plasminogen	The precursor of plasmin which possesses a fibrinocytic activity.
Fibrinogen	Precursor of fibrin necessary for blood coagulation.

Of the above-mentioned proteins, the orsomucoids, haemopexin, haptoglobulins, α_1-antitrypsin, the immunoglobulins, α_2-macroglobulins, ceruloplasmin, transferrin and prothrombin contain relatively large amounts of bound carbohydrate, particularly the first four proteins. The α_1-lipoproteins, the pre-β- and β-lipoproteins, on the other hand, have a very high lipid content (Shetler, 1961; Turner and Hulme, 1971).

Albumin, most of the α- and β-globulins, fibrinogen and other blood coagulation proteins are synthesized in the liver while the immunoglobulins are formed by cells of the

reticuloendothelial system, although substantial amounts of these γ -globulins can be of hepatic origin (Dimopoullos, 1970; Turner and Hulme, 1971). In the normal animal the concentrations of the various plasma proteins remain constant with a balance between biosynthesis and catabolism or external loss, and normal values are fairly established in domestic animals. In the case of disease, the concentration of one or more of the proteins may increase or decrease. Increases usually denote enhanced synthesis and/or decreased catabolism, while a decrease could be the result of impaired synthesis or increased catabolism.

Abnormal plasma protein patterns occur in many conditions of acute or chronic inflammations (of an infectious, chemical or physical nature), nutritional deficiencies, stress and hormonal imbalance, nephrotic syndromes, hepatic disorders and neoplasia. Quantitative changes in plasma proteins in such conditions can easily be detected by electrophoretic separation, a useful technique in laboratory analysis. Most of the changes observed in the various abnormalities involve a decrease in albumin and an increase in globulins especially the γ -globulins. The decrease in the former usually indicates a pathological liver but is also seen in situations of excessive protein loss and hypercatabolism or when haemodilution occurs. The accompanying rise in the γ -globulins could be due to alteration in the rate of synthesis, antigenic stimulation of the reticuloendothelial system and production of antibodies or a compensatory reaction to restore the plasma osmotic pressure that would be reduced by the hypo-

albuminaemia, and thus act as a plasma volume expander (Leland, 1961; Rees and Clarkson, 1967; Dimopoullos, 1970; Turner and Hulme, 1971). The α -globulins increase rather non-specifically in many infections and traumatic conditions; they may contribute to restore a reduced osmotic pressure or help cellular restoration (Shedlovsky and Scudder, 1942; Pierce, 1955; Leland, 1961; Dimopoullos, 1970). The β -globulin zone comprises many individual proteins i.e. glycoproteins, β -lipoproteins, haemopexin, transferrin, complement, immunoglobulins IgM and IgA, and increases, or decreases, in this fraction could be due to a change in the concentration of any one or more of the component proteins (Leland, 1961; Patterson and Sweasey, 1968; Schalm, Jain and Caroll, 1975). However, elevations in the β -globulins are often connected with hepatic disorders and mostly denote a disturbance in lipoprotein metabolism (Dimopoullos, 1970). The albumin-globulin (A/G) ratio varies with the changes in the two protein fractions and the total proteins may rise, decrease or, generally, remain unchanged.

Plasma protein changes in disease:

Alterations occurring in the plasma proteins and electrophoretic fractions in some pathological conditions are summarised in Table II.

Table II Plasma protein picture in some disease conditions.

TP = total proteins; Alb = albumin; F = fibrinogen; A/G = albumin-globulin ratio. $\downarrow,\uparrow_1$ or $\downarrow,\uparrow_2$ = α_{1-}, α_{2-}; β_{1-} or β_{2-}globulin.

Disease condition	Animal	TP	Alb	Globulins			F	A/G	References
				$\alpha-$	$\beta-$	$\gamma-$			
Tuberculosis	Cattle		$\downarrow$	$\uparrow_2$	$\uparrow$	$\uparrow$			Seibert and Nelson (1942); Seibert et al. (1947).
	"	$\downarrow$							Weimer and Moshin (1953)
	"	$\uparrow$							Hudgins and Patnode (1957).
			$\downarrow$	$\uparrow_2$		$\uparrow$			Wrights (1967).
	"			$\uparrow_2$					Moses et al. (1975).
Johnes disease	"	$\downarrow$	$\downarrow$			$\uparrow$			Nigmatullin (1961).
	"		$\downarrow$	$\uparrow$					Schotman (1962).
	"		$\downarrow$		$\downarrow$	$\downarrow$			Patterson and Sweasey (1968).
Brucellosis	"					$\uparrow$		$\downarrow$	Rose and Amerault (1964).
	"	$\uparrow$				$\uparrow$			Rice et al. (1966).
	Sheep					$\uparrow$			Peredereev (1967).

Table II (Contd.)

Disease condition	Animal	TP	Alb	Globulins			F	A/G	References
				α-	β-	γ-			
Corynebacterium pyogenes	Cattle	↑	↓	↑	↓	↑		↓	Petrov and Tashev (1976).
Staphylococcus pyogenes	"	↑	↓	↑	↓	↑		↓	
Corynbacterium pseudo tuberculosis									
i) natural	Sheep	↑				↑	↑		Gameel (1974a)
ii) experimental	"	↓	↓		↑	↓	↑		Gameel and Tartour (1974).
Erysipelothrix infection	Swine	↑				↑			Freeman (1964).
	"	↑	↓	$\downarrow_2$	$\downarrow_1\uparrow_2$	↑			Papp and Sikes (1964).
Nephritis & Mastitis	Cattle		↓	↑		↑			Schotman (1962).
	"								
Chronic mastitis	"			↑	↑	↑			Liberg (1977)
Subacute Chronic pneumonia				↑	↑	↑			
Foot and Mouth	Cattle		↓			↑			Perk and Lobl (1961)
Hog cholera	Swine		↓	↑					Weide and King (1962).
Swine fever	"		↓	↑	↑				Schang et al. (1964).
	"			$\uparrow_2$	$\uparrow_1\downarrow_2$	↑			Matthaeus and Korn (1966).
Canine distemper	Dog		↓	↑					Snow et al. (1966).
Infectious hepatitis	"		↓						Beckett et al. (1964).

Table II (Contd.)

Disease condition	Animal	TP	Alb	Globulins ∝-	Globulins β-	Globulins γ-	F	A/G	References
Newcastle disease	Chick		↓			↑			Clark and Foster (1968).
Marek's disease	"			↑1		↓	↓		Jurajda and Napravnik (1974).
	"				↓	↑			Sharma et al. (1976).
	"		↑			↑2			Mayer et al. (1977).
Mycoplasma synoviae	"	↑	↓	↑	↑	↑			Sells (1976).
Caprine pleuropneumonia	Goats	↓					↓		Gameel (1976)
Anaplasmosis i) early stage	Cattle	↑		↑	↑				Dimopoullos et al. (1960);
ii)late stage		↑				↑			Roger and Dimopoullos (1962).
Trypano-somiesis	Sheep		↓			↑			Rees and Clarkson (1967).
Tricho-strongylosis	Cattle	↓	↓		↓	↓			Leland et al. (1958, 1959a)
			↓	↑					Schotman (1962)
	Sheep	↓	↓			↑			Leland et al. (1959b, 1960).
Ostertagiasis	Cattle	↓	↓						Nielsen and Aalund (1961).
	"	↓				↑			Marht et al. (1964); Ross and Todd (1965).

Table II (Contd.)

Disease Condition	Animal	TP	Alb	Globulins α-	Globulins β-	Globulins γ-	F	A/G	References
Gastro-intestinal helminths*	Cattle	↓	↓			↑		↓	Viana and Campos (1973).
Dictyocaulus filaria	Sheep		↓			↑			Favati and Della Corce (1963).
Dictyocaulus viviparus	**Cattle**		↓		↑	↑			**Sharma (1967).**
Paramphisto-miasis	Cattle	↑	↓	↑		↑			Romaniuk (1975).
	Sheep	↓	↓		↓				Zajicek et al. (1977).
Sarcocystosis**	Cattle	↓	↓		↑				Fayer and Lunde (1977).
Haemonchosis	Sheep	↓	↓			↑			Kuttler and Marble (1960).
	"	↓	↓					↓	Raisinghani et al. (1971).
	"		↓						Presidente et al. (1973).
Oesophagosto-miasis	Sheep	↓	↓	↓	$\downarrow_1\uparrow_2$	↑			Dobson (1965, 1967a).
	"	↑	↓	↓	$\downarrow_1\uparrow_2$	↑			Dobson (1967b).
	"					↑	↑		Gameel (1974).

* Animals infected with 2 or more of Cooperia, Haemonchus, Oesophagostomum, Trichuris, Bonostomum, Trichostrongylus, Strongylus and Monesia.

** The increase in globulins was due to a rise in both IgM and IgG.

Table II (Contd.)

Disease Condition	Animal	TP	Alb	Globulins α-	Globulins β-	Globulins γ-	F	A/G	References
Schistosomiasis i) S. mattheei	Sheep		↓			↑			Dargie et al. (1973)
	"		↓			↑			Van Zyl (1974).
ii) S. bovis (natural)	"		↓	↑	↑	↓			Gameel (1974b)
Strongylus vulgaris	Horse				↑				Amborski et al. (1974).
Verminous aneurysms	"	↑	↓	↑	↑				Ooms et al. (1976).
Hyostrongylus rubidus	Swine	↓	↓						Titchener et al. (1974).
Leishmaniasis	Dog				↑				Vita et al. (1973).
Dirofilariasis	"	↑			↑				Barsanti et al. (1977).
Capillaria obsingnata	Chick Pigeon	↑ ↓	↓		↑	↑			Berghen (1966).
Plasmodium gallinaccum	Chick	↓	↓	↑		↑			Rao and Cohly (1953).
Coccdiosis	Chick	↓		↓					Kumar and Rawat (1975).
Traumatic reticulo-peritonitis	Cattle		↓		↑ 2				Miclaus et al. (1973).
	"			↑	↑	↑			Liberg (1977).
	"		↓		↑	↑			Samadieh et al. (1978).
Ketosis	"		↓	↑					Schotman (1962)
	"		↓	↑	↑	↑			Tenca et al. (1976).

Table II (Contd.)

Disease Condition	Animal	TP	Alb	Globulins α-	Globulins β-	Globulins γ-	F	A/G	References
Mg-deficiency	Sheep		↓	↑		↑			Kiesel and Alexander (1966).
Muscular dystrophy	"	↓	↓	↑	↑	↓			Kursa (1976).
Leucosis	"		↓			↓			Liberg (1977).
*	"		|		↑				Radionov et al. (1977).
Stem cell carcinoma	Chick			↑	↑				Loliger et al. (1966)
Transmissible myeloma	"		↓	↑	↑	↑			

*The γ -globulins increased due to an elevated IgG.

Plasma protein changes in fascioliasis:

The liver occupies a key position in the biosynthesis of the majority of the plasma proteins. It is also the main site where the pathological lesions of fascioliasis occur. Therefore, the damage inflicted by the flukes on this organ would result in an altered plasma protein picture, and this has been reported in many investigations; a summary of these changes in sheep and cattle can be seen in Table III.

The changes in the plasma proteins in fascioliasis seem to follow a certain pattern according to the stage of infection. During the prepatent period, when the flukes are migrating in the liver parenchyma, an increase in total globulins occurs while the albumin virtually remains

Table III Plasma protein changes in ovine and bovine fascioliasis: TP = total proteins; Alb = albumin; F = fibrinogen; A/G = albumin-globulin ratio.

Animal Spp.	TP	Alb	Globulins ∝ -	Globulins β -	Globulins γ -	F	A/G	References
Sheep	↓	↓			↑			Kona (1957).
		↓		↑				Noguchi et al. (1958)
	↑	↓	$\uparrow_1\uparrow_2$	$\uparrow_1$	↑			Sinclair (1962).
	↓	↓						Lee et al. (1966)
	↓	↓						Ross (1967a).
		↓	↓	↓	↑			Ross (1967b).
	↑	↓		↑	↑	↓		Furmaga and Gundlach (1967).
		↓		↑				Roberts (1968)
	↑	↓	↑	↑	↑			Sinclair (1968b, 1970a).
		↓			↑			Reid et al. (1970b).
		↓	↓	↓	↓			Reid et al. (1970c).
	↑				↑			Sewel (1970).
		↓						Presidente et al. (1973).
	↓	↓		↑				Sinclair (1974)
		↑						Banting et al. (1975).
	↑	↓		↑				Berry and Dargie (1976).
*		↓	↑		↑			Kadhim (1976).
		↓			↑			Buchwalder et al. (1977).
		↓		↑				Coops and Sykes (1977).

*Fasciola gigantica

Table III (Contd.)

Animal Spp.	TP	Alb	Globulins			F	A/G	References
			∝ -	β -	γ -			
Cattle		↓	↓		↑		↓	Komine et al. (1955).
*		↓	↓		↑		↓	Weinbran and Coyle (1960).
	↑		↑		↑			Tomicki and Malinowska (1961).
		↓	↑		↑			Schotman (1962).
	↑	↓	↑	↑±	↑			Malinowska and Tomicki (1962).
	↑	↓	↑		↑			Vasilev (1963).
	↑	↓			↑			Hankiewicz (1965).
		↓	↑		↑			Ross et al. (1966).
*		↓		↑				Sewel (1966); Ross and Todd (1968).
	↓	↑±	↓±	↑	↑			Konrad (1968).
		↓		↑				Doyle (1971).
	↑	↓		↑				Teresio et al. (1973).
*	↑	↓		↑	↑		↓	Blancou et al. (1974).
	↑	↑	↓		↓±			Romaniuk (1975).
*		↓			↑		↓	Haroun and Hussein (1975).
*		↓			↑		↓	Haroun and Hussein (1976).
		↓			↑		↓	Shimada (1976).
	↑	↓		↑			↓	Anderson et al. (1977).

± = slightly increased or decreased;

* = Fasciola gigantica.

unchanged ; this leads to a corresponding rise in total proteins. This pattern changes when the flukes enter the bile ducts; a drop in total proteins occurs due to the onset of hypoalbuminaemia and sometimes hypoglobulinaemia. The magnitude of these changes varies with the number and viability of metacercariae given, the species of the parasite used and also with the animal species, their age and nutritional status.

Thus, Sinclair (1962) studied the plasma protein changes in 10 month old sheep infected with 600 F. hepatica metacercariae either as a single dose or as 4 doses of 150 cysts administered at weekly intervals (fluke burden, 170-221 flukes/animal). His findings showed that the α_2-globulins increased as early as one week after infection while the α_1-, β - and γ -globulins increased between the 5th to the 11th week of exposure; this was accompanied by a concomitant decrease in albumin and a slight increase in total proteins. The globulins started to decrease after the 11th week and by week 19 were around preinfection levels. Generally, animals receiving the divided dose showed a greater response to infection.

In sheep infected with 800-1100 F. hepatica metacercariae (burden of 278-410 flukes/animal), Furmaga and Gundlach (1967) observed that the total proteins increased from the first week of infection, reached a peak at week 9, remained similar till week 13 and then gradually declined; the levels were below those of controls by the 17th week. The decrease in albumin started the first week of infection,

reached a minimum at week 11 and subsequently remained low. The γ -globulins increased to a peak at week 11, plateaued till the 13th week and then slightly dropped but remained higher than normal levels. The β -globulins increased after the second week, reached a maximum at week 7 and thereafter gradually dropped, but the level was still higher than control values until the 18th week of exposure. In contrast to the previous findings, no change was detected in the α -globulins.

Acute F. hepatica infection was investigated by Roberts (1968) in sheep infected with 5000 metacercariae in a single dose or as multiple doses of 1000 cysts each (Recoveries, 1000-3500 flukes/animal at 6-9 weeks). In sheep receiving single doses, albumin levels steadily decreased and this was accompanied by a sharp rise in γ -globulins till the 3rd week of infection; the rise in the γ -fraction continued, but slowly till the 7-8th week. The β -globulins showed a slight tendency to increase but no change was noticeable in the α -fraction. The changes were similar in the multiple infected animals but contrary to Sinclair's findings (1962) the response was somewhat slower in development. Following treatment the γ -globulins temporarily increased while the albumin showed a slow recovery resulting in an increase in total proteins. Sewel (1970) also observed a marked hyper-proteinaemia (10.61 g%) due to an increase in mainly γ -globulins in acutely infected sheep during the 6-12 weeks following infection.

Sinclair (1968) compared the plasma protein alterations in two groups of 4 month old lambs infected with

500 F. hepatica metacercariae each, and where members of one group received a daily intramuscular injection of a synthetic corticosteroid. In the group not treated with the corticosteroid the α_1-, α_2- and γ-globulins increased 2 weeks after infection while the β-globulins were elevated two weeks later. This was accompanied by a hypoalbuminaemia resulting in a moderate increase in total proteins especially between the 2nd-8th week of exposure. Following the 10th week the globulin concentrations, and hence the total proteins, dropped. In the corticosteroid-treated sheep, the total proteins increased during the 2nd-4th week of infection mainly due to a rise in albumin, but very little change was noticed in the globulin fractions. Another similar experiment was conducted by Sinclair (1970b) but in addition the effect of reinfection (500 cysts/animal) after eliminating a 12 week-old primary exposure (300 cysts/animal) was investigated. The plasma protein picture in the corticosteroid treated and untreated animals during the primary infection was similar to those reported above although the albumin levels staid high for 6 weeks longer in the former group. After reinfection, animals of both groups showed increased total proteins which was initially due to a rise in albumin and lately due to increase in γ-globulins; the albumin, however, declined 11 weeks after infection, i.e. 4 weeks earlier, in the corticosteroid treated and re-infected animals when compared to the other group.

The plasma protein picture was compared in

splenectomized and normal sheep infected with 3000 F. hepatica metacercariae (mean burden, 758 and 586 flukes/animal in the two groups respectively) by Sinclair (1970a). In both groups the total proteins increased from the second week of infection due to a rise in γ -globulins, but these changes seem to occur earlier in the splenectomized sheep. In this latter group the γ -globulins started to decline 8 weeks after exposure and reached half the initial value by week 14 resulting in lower total protein levels, as compared to the normal infected animals.

Kadhim (1976) investigated the plasma protein changes in 6 sheep infected with 200 metacercariae (mean burden, 54.5 flukes/animal) of F. gigantica and in a similar number of controls. A marked decrease in albumin accompanied by an equivalent rise in γ -globulins commenced 10 weeks after exposure. The α -globulins remained slightly higher in exposed animals following the 4th week of infection while the β -globulins showed a significant sudden drop at the 16th week followed by a sharp rise by the 18th week and then remained higher than those of controls. No significant variation occurred in total proteins.

Berry and Dargie (1976) investigated the effect of F. hepatica infection (1000 cysts/animal) on sheep kept on high protein (13%) or low protein (6%) diets. In both groups a hyperproteinaemia developed starting the second week of infection, reached a peak at weeks 9-10 and subsequently decreased to a hypoproteinaemia by the end of the experiments. Changes in globulins closely paralleled those

in total proteins while the albumin continued to decline from the 3rd week of infection and till the end of the observation. The above changes were observed to be more pronounced in sheep fed on the low protein diet. In another experiment Berry and Dargie (1976) infected 6 sheep, kept on high protein diet, with 600 metacercariae and 16 weeks later the animals were switched to low protein diet. Similar changes to those reported above occurred in the plasma proteins but a marked decrease in total proteins and albumin took place when the animals were switched to the low protein diet.

Coop and Sykes (1977) in investigating the effect of subclinical infection on food intake and utilization in sheep, noticed a dose-related hyperglobulinaemia and hypoalbuminaemia from the 10th week of infection onwards. It may be mentioned here that fluke burdens of as low as 8-17 flukes/sheep have been reported to produce changes in the plasma protein profile (Buchwalder, et al., 1977).

The changes encountered in plasma proteins in fluke-infected cattle are almost similar to those reported in sheep. In experimentally-infected cattle Vasilev (1963) observed a hypoalbuminaemia associated with a hypergammaglobulinaemia and a dose-related increase in α-globulins. Little variation occurred in the β-fraction.

Ross, Todd and Dow (1966) infected calves with 200, 300 or 1300 F. hepatica metacercariae each and found that animals inoculated with the higher dose developed a gradual hypoalbuminaemia from the 5th week of infection; the levels sharply dropped at week 13, reached a minimum at week 15 and

then slightly rose but remained below control levels. The globulins, particularly the α- and γ-fractions, showed variable increases starting the 3rd week of exposure and remained constantly higher than control values.

Doyle (1971) reinfected calves with 1650 F. hepatica cysts after 16 weeks of a primary infection of 750 cysts; in one group the first infection was terminated before re-infection. Serum albumin declined at the 11th week of the primary exposure and by the 16th week the levels were significantly lower than control values. After reinfection (between the 18th-30th week) the levels increased and were higher than those of controls during the last 4 weeks. The globulins started to rise from the second week of the first infection to a maximum at the 14th week which was sustained up to the 16th week. Following reinfection the levels slowly decreased till the 24th week and subsequently the decline was more marked.

Haroun and Hussein (1976) infected Zebu calves with 500-950 F. gigantica metacercariae. They observed a gradual decrease in albumin levels from the 3rd to the 5th week and minimum values were reached at week 10-12 of infection. The γ-globulins were slowly elevated from the 1st week onwards and were highest at the 9th and 15th weeks. No change was observed in the α- or β-fractions and the A/G ratio was greatly reduced.

Twenty calves were inoculated with 1000 F. hepatica cysts by Anderson, Berrett, Brush, Hebert, Parfitt and Patterson (1977). They reported that the albumin

concentrations decreased at the 6th week of infection while the globulins slightly increased at week 23 with a resultant decrease in the A/G ratio. The increase in total proteins was only slight.

Generally, changes in plasma proteins associated with fascioliasis seem to be a slight hyperproteinaemia associated with a decrease in albumin and increase in α- and, particularly, γ-globulins; changes in β-globulins are variable. Such a picture has repeatedly been reported in natural infections in sheep (Kona, 1957; Ross, 1967a,b; Reid et al., 1970a), cattle (Weinbran and Coyle, 1960; Malinowska and Tomicki, 1962; Hankiewicz, 1965; Sewel, 1966; Ross, 1968; Konrad, 1968; Blancou, Cheneau and Bouchet, 1974; Romaniuk, 1975; Haroun and Hussein, 1975; Shimada et al., 1976) and in experimental infection in rabbits (Secretan and Bickel, 1960; Vinciuniene, 1960) and rats (Thorpe, 1965).

The Plasma glycoproteins:

The glycoproteins are conjugated proteins which contain carbohydrate groups attached covalently to the polypeptide chain. The linear or branched chain of glycoproteins may contain from two to dozens of monosaccharide residues, usually of two or more kinds. Often the terminal monosaccharide is a negatively charged residue of N-acetylneuraminic acid, a sialic acid. Glycoproteins are widely distributed in the animal body and have a considerable biological significance. In vertebrates most of the glycoproteins are extracellular in occurrence and function and

these include the cell-coat glycoproteins, various digestive enzymes, mucoproteins of mucous secretions, glycoproteins of extracellular basement membranes, antibodies and the blood proteins. Plasma contains a large number of these carbohydrate conjugated proteins which play different roles as transport proteins, hormones, enzymes, inhibitors, complement and coagulation factors, blood group specific substances and antibodies (Gottschalk, 1972; Lehninger, 1975; Podhradska, Podhradsky and Andrasina, 1976).

Most of the information available regarding the metabolism of glycoproteins in health and disease comes from studies done on the human subject and comparatively little work has been reported in the various physiological and pathological conditions in domestic animals.

In man, over 30 distinct plasma glycoproteins are recognised whose levels are maintained constant in health but may change (mostly increase) in many physiological and pathological conditions (Winzler, 1965; Heide and Schwick, 1973). These changes can be assessed by different means: a) by electrophoresis of the glycoproteins, b) by estimation of the different protein-bound sugars and c) by measurement of individual glycoproteins. Electrohporesis separates the plasma glycoproteins into different fractions designated as albumin, α -, β - and γ -globulin glycoproteins. In the majority of situations the changes in the electrophoretic patterns are usually observed as elevations in the α -globulins and less so in the β -globulin glycoproteins (Heiskell, Carpenter, Weimer and Nakagawa, 1961; Patterson and Sweasey, 1968;

Podhradska et al., 1976). These changes are not specific because the different electrophoretic fractions contain many individual glycoproteins which cannot be identified by this technique. Estimation of plasma glycoproteins from the concentrations of its bound sugars is not specific either. The over 30 plasma proteins contribute different amounts of the different sugars and it is difficult to relate the increase of a particular sugar to a particular protein. In this context it may be mentioned that about half of the protein-bound hexoses of plasma is contained in seven of its proteins, i.e. orosomucoid, α_1-glycoprotein, haptoglobulin, α_2-macroglobulin, transferrin, fibrinogen and γ-globulins (Winzler, 1965). So for accurate estimations, the various glycoproteins should be individually quantitated. However electrophoresis and determination of protein-bound sugars are frequently used probably because they can be done cheaply and more easily.

The plasma glycoproteins in humans:

Countless reports are available on the plasma glycoprotein content of normal humans and on the changes occurring in patients with different pathological conditions. Significant increases in the glycoprotein of plasma have been associated with age, sex and pregnancy and, pathologically, with neoplastic diseases; tuberculosis; pneumonia; diabetes; rheumatic fever; rheumatoid arthritis; neoplastic, obstructive or infectious liver diseases; fractures; thermal injury; lupus erythematosus; myocardial infarction; gout; endocarditis; varices; ischemic heart disease; Bilbarziasis and many other

inflammatory conditions (Winzler, 1955; Heiskell et al., 1961; Shetlar, 1961; Macbeth and Bekesi, 1962; Dutt, Chatterjee, Khanade and Rajain, 1975; Hussein, Hafez, El Banna and El Hady, 1975; Mehta and Vankataraman, 1975; Snyder, Durham, Iskandrian, Coodley and Linhart, 1975; Jonsson and Wales, 1976). Details of the above conditions are beyond the scope of this study.

Plasma glycoproteins in animals:

The plasma glycoprotein contents, measured by electrophoresis or by estimation of one or more of the protein-bound monosaccharides, have been reported in normal cattle (Dimopoullos, Schrader and Fletcher, 1959; Pant, Rowat and Gupta, 1959; Dzulynska, Lutowicz and Kedzierska, 1962; Grant and Anastassiadis, 1962; Grant, Dale and Anastassiadis, 1966; Patterson and Sweasey, 1966; Gaunce and Anastassiadis, 1967; Dzulynska, Walkowiak and Skubiszewski, 1968; Channon and Anastassiadis, 1971; Zahrai, 1971), sheep (Dzulynska et al., 1962; Grant et al., 1966; Patterson and Sweasey, 1966; Walkowiak, Reklewska and Dzulynska, 1971), pigs (Dzulynska et al., 1962; Grant et al., 1966; Patterson and Sweasey, 1966; Gaunce and Anastassiadis, 1967; Channon and Anastassiadis, 1971), mare (Wojcik and Ewy, 1965), fowl (Grant and Anastassiadis, 1962; Gaunce and Anastassiadis, 1967; Dzylynska, Potemkowska, Walkowiak and Fabijnska, 1969; Channon and Anastassiadis, 1971), rabbits (Hudgins and Patnode, 1957; Prusiewicz-Witaszek and Dzialoszynski, 1967; Walkowiak, Brylinska, Krawczynska and Starzynski, 1972), guinea pigs (Weimer, Redlich-Moshin and Nelson, 1955; Hudgins

and Patnode, 1957; Walkowiek et al., 1972), rats (Hudgins and Patnode, 1957; Dail and Richmond, 1966; Grant et al., 1966), mice and hamster (Walkowiak et al., 1972). Differences in the plasma protein-bound carbohydrate contents between some animal species, between age groups within one species and during pregnancy have been pointed out by many of the above-mentioned authors.

Changes in the plasma glycoproteins in animals having different pathological conditions were reported and it seems that most of these reports were concerned with small laboratory animals and, to a lesser extent, bovines. Thus the effect of cancer, bacterial infections, nutritional muscular dystrophy and experimentally-induced inflammations on the plasma glycoprotein picture have been investigated in laboratory animals. Catchpole (1950) observed a marked increase in the total serum glycoproteins of mice bearing transplantable tumours and also in the water-soluble glycoproteins of the connective tissue bordering these tumours. The increase in the latter was suggested to be the source of the elevated circulating glycoproteins. Skipski, Katopodis, Prendergast and Stock (1975) found that the total lipid-bound sialic acids in serum were increased to more than two fold in rats with Morris hepatoma. Similarly, Bernacki and Kim (1977) observed elevated levels of both serum sialyltransferase activity and protein-bound sialic acid in rats affected with transplantable metastasizing mammary tumours and the increases were thought to originate from the tumour tissues.

Weimer et al. (1955) infected guinea pigs with Brucella suis and observed a significant rise in total serum

glycoproteins, seromucoid and γ-globulin polysaccharide content after infection. Hudgins and Patnode (1957) also noticed an increase in serum total glycoproteins and α_2-glycoproteins in guinea pigs, rabbits and rats infected with the tubercle bacillus; only rabbits had elevated γ-globulin glycoproteins.

Oppenheimer and Milhorat (1961) produced muscular dystrophy in rabbits fed Vitamin E-deficient diet and observed that the protein-bound hexoses, hexosamine and sialic acids increased during the development of the myopathy. On the other hand, mice with hereditary muscular dystrophy showed decreased, rather than increased, concentrations of the above-mentioned sugars as compared to their control littermates.

Experimentally-produced inflammation can also affect the plasma glycoprotein picture. Injury produced in rats by either the subcutaneous implantation of a sponge or by surgical removal of a small area of the skin was followed 1-2 days later by a marked increase in the total protein-bound hexosamine and this was due to elevation of the α- and β-globulin hexosamine contents (Neuhaus, Balegno and Chandler, 1960). Chandler and Neuhaus (1964) also produced injury in rats by a) subcutaneous implantation of a sponge, b) by laparotomy and partial hepatectomy and by c) carbon tetrachloride poisoning. They precipitated the seromucoid fraction after intraperitoneal injection of C^{14}-labelled algal hydrolysate and C^{14}-labelled glycine and found that in the first and third type of injury the percentage of the seromucoid protein and its radioactivity significantly increased;

in the second type of injury the radioactivity also increased but the protein concentration was near normal. They attributed this to synthesis of a new protein in response to injury rather than to the release of preformed proteins.

The effect of subcutaneous injection of sterile turpentine in rabbits was investigated by Podhradska, Podhradsky and Andrasina (1976); 48 hours after inducing the inflammation the α_2- and the β_{-2+3-}globulin glycoproteins (separated by electrophoresis) markedly increased and this was associated with increased concentrations of serum glucosamine, mannose, and fucose. Quantitative analysis revealed increased haptoglobulins and ceruloplasmin contents. Increased concentrations of haptoglobulins were previously observed in rabbits with a similarly induced inflammation (De Vonne et al., 1970).

The response of the serum/plasma glycoproteins to different pathological insults in bovines has also been reported. Grant et al. (1966) reported low values for serum proteins and protein bound hexosamine in cattle affected with chronic fluorosis. Patterson and Sweasey (1966) reported significantly higher protein-bound hexoses, hexosamines, sialic acid and seromucoid in cattle showing clinical Johnes disease than in normal animals. They also noticed marked elevations in the above protein-bound carbohydrates in two cases of chronic mastitis and in one case of actinomycosis. The same authors, Patterson and Sweasey (1968) separated the serum glycoproteins (by electrophoresis) of normal bovines and others with clinical Johnes disease and observed higher α - and β-globulin glycoproteins but lower albumin and

γ -globulin glycoproteins in sera of infected animals; all fractions were expressed as percentages of the total protein-bound hexoses.

Murti and Ray (1972) noticed significant elevation in the total hexoses, seromucoid and fucose of sera obtained from cattle affected with horn cancer.

In bovine trypanosomiasis Magaji (1975) observed that cattle infected with Trypanosoma brucei brucei had elevated serum protein-bound hexoses, hexosamines, sialic acid and seromucoid levels especially during the first 4 weeks of infection. Similar findings were noticed in T. vivax infection but the values remained high for 8 weeks after the challenge, and the elevations seemed to be related to the degree of parasitaemia. The same author also reported high protein-bound hexoses in cattle challenged with virulent Mycoplasma mycoides.

In dogs, Shetlar, Bryan, Foster, Shetlar and Everett (1949) reported an increase in serum polysaccharides following inflammation produced by any of the following: sterile turpentine abscess, bacterial abscess, talc granulomas or surgical operations (i.e. laparotomies); maximum elevations occurred 3-6 days after the initial injury.

Dogs vaccinated against distemper or animals showing the clinical disease and others with bacterial endocarditis had significantly higher serum mucoprotein-tyrosine levels than controls especially in the endocarditis group (Hammerstrom, Adams, Bussman and Lillehei, 1952).

Kloppel, Franz, Morre and Richardson (1978) found that serum concentrations of lipid soluble sialic acid was significantly higher in tumour-bearing dogs and the authors suggest the assay of the lipid-soluble sialic acid as a tool for diagnosing canine tumours.

Very few reports seem to exist on diseases of other animals. However, significant elevations in serum protein-bound hexoses and seromucoid levels were associated with experimentally- induced acute or chronic Eryseplothrix rhusiopathiae arthritis in pigs (Sheltar, Shetlar, Payne, Neher and Swenson, 1958). Strongylus vulgaris infections in ponies resulted in increased β-globulin glycoprotein (Amborski, Bello and Torbert, 1974).

In sheep, Goryachev (1968) observed a hyper α-glyco-globulinaemia and a decrease in protein-bound hexoses 13-33 days after experimental infection with F. hepatica, but normal values were restored after treatment. Romaniuk (1975), on the other hand, reported high serum sialic acid content in ovine fascioliasis or paramphistomiasis or both diseases when compared to normal sheep.

MATERIAL AND METHODS

Blood samples were collected from 12 male and 6 female Welsh mountain sheep and from 2 cows before and after being infected with F. hepatica metacercariae. Details of all animals and the experimental design can be seen in Part A, experimental I and II. The plasma was separated as described in the general material and methods of Part A.

Total proteins:

The plasma total proteins were determined by the biuret method of Weichselbaum (1946) adapted to a single channel autoanalyser. The analysis was carried out by the Pathology Laboratory, Caernarvon and Anglesey Hospital, Bangor, North Wales.

Electrophoretic separation of the plasma proteins:

This was achieved in a Shandon electrophoretic apparatus using cellulose acetate membrane (CAM) strips (120 x 25mm) and barbitone buffer, pH 8.6. The method used is that recommended by the Shandon manufacturers (Shandon Instrument Applications, No.11, 1965).

i) Plasma proteins:

Reagents:

a) Buffer:

7.36 g Barbitone

41.20 g Sodium barbitone

4.00 L H_2O.

20 ml 5% thymol in isopropyl alcohol were then added to prevent growth of micro-organisms.

b) Stain:

0.2 g ponceau S in 100ml 3% trichloroacetic acid.

c) Fixing solution:

5% trichloroacetic acid (TCA).

d) Washing solution:

5% acetic acid.

Procedure:

The CAM strips were wetted in the buffer first by being floated to soak from underneath and then completely immersed in the buffer. They were then removed, gently blotted and stretched across the bridge gap of the electrophoresis tank and placed on the two shoulder pads with a 1cm overlap at each end. About 1 µl quantities of the plasma samples were uniformly streaked on the CAM strips using a capillary tube and a constant electric current of 0.4 milliamps/cm width was applied for 1½ hours. At the end of the run the strips were fixed, stained for 10 minutes and then washed in 3 changes of 5% acetic acid. The strips were then blotted between two pads of filter paper and allowed to dry at room temperature; they were put between two sheets of filter paper and pressed under a heavy book to prevent curling. The strips were then scanned in a Carl Zeiss

scanner (M40111) at 540nm and the scanned areas evaluated by plannimetry.

ii) Glycoproteins:

Reagents:

a) Periodic acid:

2.5g periodic acid + 50ml 0.2M sodium acetate were made up to 500ml with H_2O.

b) 0.001 N HCl.

c) Potassium iodide solution:

10g potassium iodide in 100ml H_2O. Immediately before use 2.5ml of 0.1 N HCl were added.

d) Saturated ammonium thiosulphate solution.

e) Schiff's reagent:

2g Fuchsin base were dissolved in 400ml H_2O. 10ml of 2 N HCl and 4g of potassium meta-bisulphite were added. Some active charcoal was added and the mixture filtered.

f) 0.1 N (0.5%) Analar grade nitric acid (HNO_3).

Procedure:

2-3 µl quantities of the plasma samples were separated in CAM strips as described above. After electrophoresis the strips were dried at 80-100°C, placed in an alcohol bath for 10 minutes and then placed in the periodic acid solution for a further 10 minutes. The strips were rinsed in 0.001 N HCl and then transferred into the acidified potassium iodide

solution; the latter turned the strips and solution brown.

A few drops of saturated ammonium thiosulphate were added and the strips left till they became colourless. They were then rinsed in 0.001 N HCl and transferred to the Schiff's solution for 20-30 minutes until the glycoprotein bands were stained red-violet. The strips were then washed into three successive baths (15 minutes each) of 0.1 N HNO_3 and dried flat at room temperature between sheets of filter paper, scanning and evaluation of the electrophoretograms were as described before.

iii) Lipoproteins:

The plasma was prestained with Sudan black B before electrophoresis and the following method (McDonald and Ribeiro, 1959) was utilized:

A solution of Sudan black B was prepared by dissolving 1g of the dye into 100ml of ethylene glycol at 100-110°C. The mixture was thoroughly stirred for 5 minutes and filtered while still hot. It was allowed to cool at room temperature and then refiltered. 0.1mls of this dye solution were added slowly and with constant gentle shaking to 0.5ml of plasma and the mixture incubated for 20 minutes at 37°C. The samples were centrifuged at 4000g for 15 minutes to remove excess dye. About 0.03 µl of the prestained plasma was applied to CAM strips and electrophoresis performed as described before. The strips were scanned at 590nm and evaluated by plannimetry.

Determination of mucopolysaccharide components in plasma:

1. Total Hexoses:

Winzler's (1955) method modified by Nolan and Schmitt (1962) and applied to plasma.

Reagents:

a) 95% ethanol

b) 0.1N NaOH

c) 0.2 mg/ml Galactose-mannose standard: 0.1 mg/ml each of galactose and mannose.

d) Orcinol-H_2SO_4 reagent: 7.5 volumes of 60% H_2SO_4 mixed with 1 volume of 0.8g orcinol dissolved in 50ml 1 N H_2SO_4.

Procedure:

The proteins were precipitated by adding 5ml of 95% ethanol to 0.1ml plasma, mixed and centrifuged. The protein precipitate was again suspended in 5ml 95% ethanol, re-centrifuged and dissolved in 1ml of 0.1 N NaOH. The tubes were placed in an ice bath and 8.5ml of the orcinol reagent added and mixed. The tubes were capped with glass marbles and placed in a water bath at 80°C for 15 minutes. They were then cooled in ice and the colour allowed to develop in the dark. After 25 minutes the absorbance was read at 540 nm against a water blank; the blank (1ml H_2O) and standard (1ml galactose-mannose standard) were treated similarly.

2. Hexosamines:

The method described by Winzler (1955) and slightly modified by Niebes (1972) was utilized:

Reagents:

a) 95% ethanol

b) 3 N HCl

c) 6 N NaOH

d) Acetylacetone reagent: 1ml of acetylacetone in 50ml of 0.5 N $NaCO_3$, freshly prepared.

e) Ehrlich's reagent: 0.4g of p-dimethylamino-benzaldehyde dissolved in 15ml methanol and 15ml concentrated HCl, freshly prepared.

f) Glucosamine standard: 0.06mg glucosamine hydrochloride in 1ml H_2O (equivalent to 0.05mg free glucosamine).

Procedure:

The glycoprotein precipitate from 0.1ml plasma (procedure as for total hexoses) was suspended in 2ml 3 N HCl, the tubes sealed and placed in an oven at 100°C for 4 hours. After cooling at room temperature the tubes were opened, the contents neutralized with 1ml 6 N NaOH and then diluted to 4ml in graduated tubes. To 1ml hydrolysate (1ml H_2O blank and 1ml standard) 1 ml of the acetylacetone reagent was added, the tubes capped with glass marbles and placed in a boiling water bath for 15 minutes. After cooling 2.5ml 95% ethanol were added and mixed and then 1ml of Ehrlich reagent added. The

contents of the tubes were shaken and diluted to 6ml with 95% ethanol. After 30 minutes the absorbance was read at 530nm against the water blank.

3. Fucose:

Determined as described by Winzler (1955).

Reagents:

a) 95% ethanol

b) 0.1 N NaOH

c) H_2SO_4-H_2O mixture: 6 volumes of H_2SO_4 + 1 volume H_2O.

d) Cysteine reagent: 3g cysteine hydrochloride in 100ml H_2O.

e) Methylpentose standard: 20 ug of fucose or rhamnose/ml.

Procedure:

In duplicate test tubes the protein was precipitated from 0.1ml plasma samples as described for total hexoses and then dissolved in 1ml of 0.1 N NaOH. To the test tubes (and to 1ml H_2O for blank and 1ml of the methyl pentose standard) 4.5ml of ice cold H_2SO_4-H_2O mixture were added and mixed while the solutions were maintained cold in an ice bath. The tubes were then heated for 3 minutes in a boiling water bath and cooled in tap water. 0.1ml of the cysteine reagent were then added and immediately mixed. (This step was omitted from one of the serum samples to correct for non-specific colour development). After 90 minutes at room temperature in the dark the optical density was read at 396 and 430nm with

distilled water set at zero.

Calculation:

The fucose content of plasma was calculated from the difference in the readings obtained at 396 and 430nm, and subtracting the values without cysteine.

$$\frac{OD_{396}-OD_{430} \; - \; OD_{bl_{396}} - OD_{bl_{430}}}{OD_{st_{396}} - OD_{st_{430}}} \times 0.02 \times 1000 = \text{mg fucose/100ml}$$

bl = values without cysteine reagent added

st = standard

4. Sialic acids:

The method of Aminoff (1962) modified by Niebes (1972) was used.

Reagents:

a) 95% ethanol

b) 0.1 N H_2SO_4

c) 0.25 M periodic acid freshly dissolved in 0.1 N H_2SO_4.

d) 4% sodium arsenate in 0.5 N HCl

e) 0.1 N aqueous solution of 2-thiobarbituric acid, pH adjusted to 9 with 6 N NaOH.

f) N-butanol containing 5% (v/v) 12 N HCl.

Procedure:

The glycoprotein was precipitated from 0.1ml plasma as described for total hexoses. The precipitate was taken in 2ml 0.1 N H_2SO_4, the test tubes (capped with glass marbles) were heated in a water bath at 80°C for 30 minutes and then centrifuged for 3 minutes at 2000g. To 0.5ml hydrolysate (and to 0.5ml o.1 N H_2SO_4 for the blank) 0.25ml of 0.025M periodic acid was added and the tubes put in a 37° water bath for 30 minutes. The excess periodic acid was reduced by the addition of 0.25ml of 4% sodium arsenate. 2ml of 0.1 N thiobarbituric acid were added, the test tubes capped with glass marbles were heated in a boiling water bath for exactly 7.5 minutes. After cooling for 2 minutes in ice, 5ml of the N-butanol reagent were added. The pink butanolic phase was extracted, centrifuged at 1000g for 3 minutes and the absorbance read at 550nm against the blank.

Standard curve: 1-25 ug N-acetylneuraminic acid dissolved in 0.5ml 0.1 N H_2SO_4 and treated similarly to the test samples and blank.

5. Seromucoid:

The method used was that described by Winzler (1955) slightly modified by Patterson and Sweasey (1966).

Reagents:

a) 0.85% saline

b) 1.8 M perchloric acid

c) 5% phosphotungstic acid in 2 N HCl, freshly prepared.

d) 0.1 N NaOH

e) Reagents for the determination of total hexoses.

Procedure:

1ml plasma was diluted with 4ml saline and 2.5ml of 1.8 M perchloric acid added, dropwise and with shaking. After 10 minutes the test tubes were centrifuged and to 5ml of the supernatant 1ml of the phosphotungstic acid reagent was added and mixed. After 10 minutes the precipitated seromucoid was centrifuged down at 2000 r.p.m. for 10 minutes. The amount of seromucoid was determined from its hexose content; the seromucoid was suspended in 5ml 95% ethanol, centrifuged, dissolved in 1ml 0.1 N NaOH and the hexose content determined as described before (1).

RESULTS

A. The Plasma Proteins:

1. The plasma protein picture in 4 controls and 8 sheep infected with F. hepatica:

The mean weekly plasma protein changes for the 12 animals in the 3 groups A, B and C are shown in Table IV. Individual values are plotted in figs. 1-3.

Group B (infected with 268 cysts each in a single dose):

It can be seen that the plasma proteins of animals in this group showed more or less similar responses after infection, with some differences in timing and magnitude, see figs. 1a, b, c and d.

The total proteins generally increased from the second week of infection till the 10th week and then declined. By the terminal week of the observation, week 14, the total proteins were still higher than the initial levels. Two peaks could be recognised, one between weeks 2-6 and the other between week 7-11. Sheep No. 5 (fig. 1b), however, maintained almost a constant high level from the 4th to the 12th week of infection and declined slightly thereafter.

The albumin levels in all animals showed a downward trend starting the 4th week of infection and the lowest values were seen at week 13. In sheep No.10 (fig. 1a) minimum values were reached at week 9 and the levels showed an upward tendency till the final week of the experiment. The latter animals and No.12 (fig. 1d) showed more marked hypoalbuminaemia than the other two.

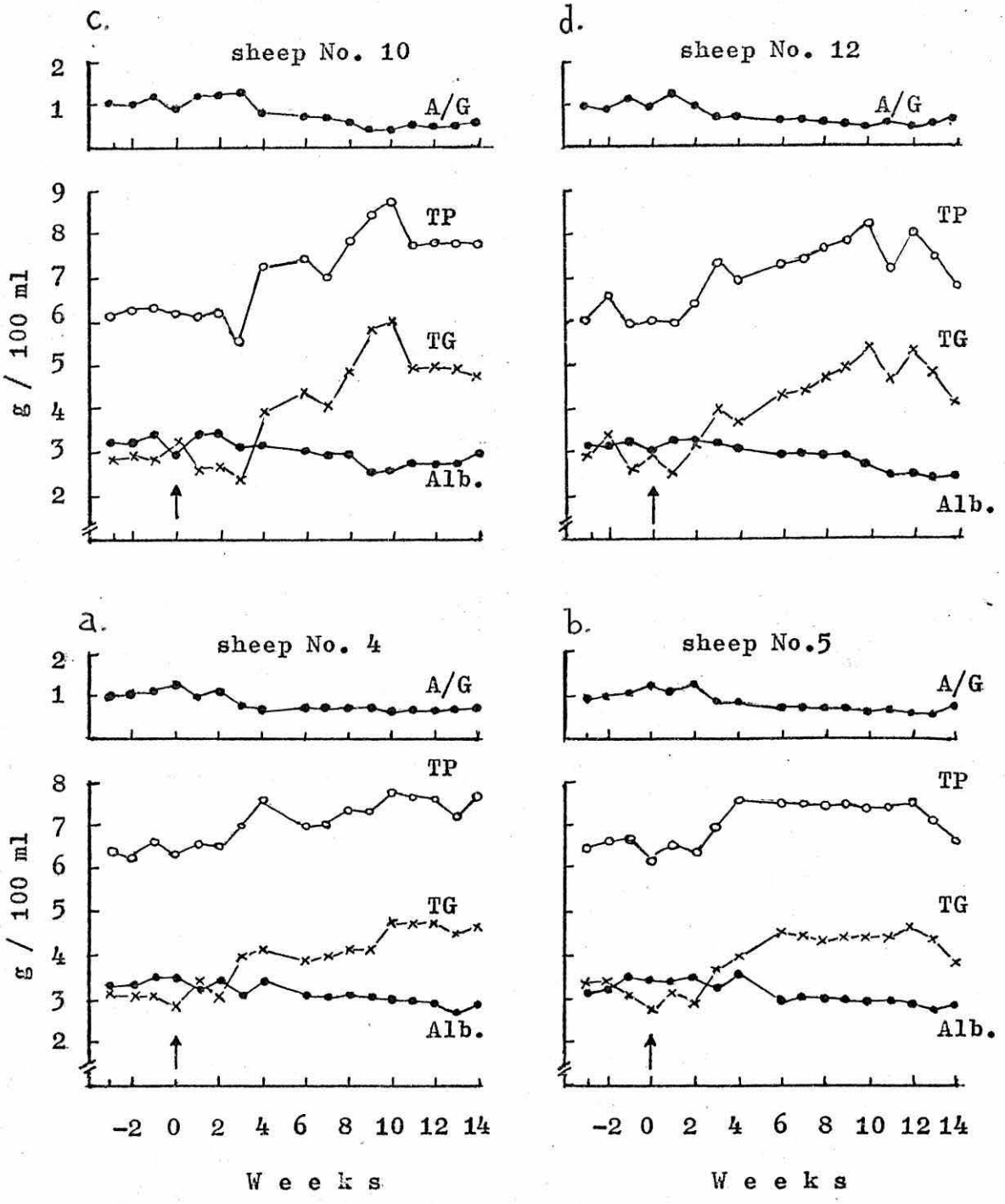


Fig. 1. Plasma protein changes in 4 sheep infected with 268 F. hepatica cysts given in a single dose. A/G, Albumin-globulin ratio; TP, total proteins; TG, total globulins; Alb., Albumin. ↑ = infection.

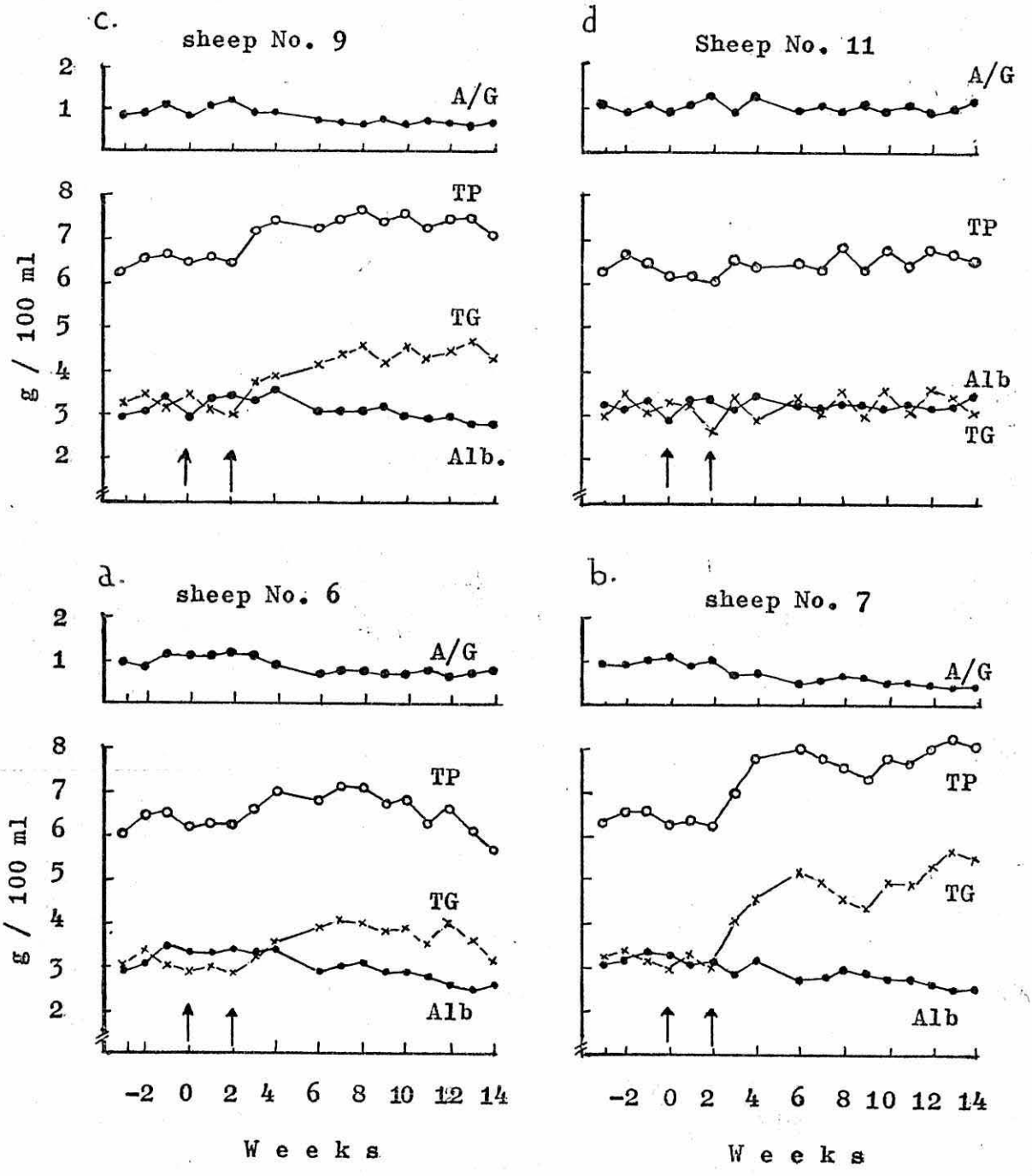


Fig. 2. Plasma protein changes in 4 sheep each infected with 536 F. hepatica cysts given in 2 equal doses 14 days apart. A/G, Albumin-globulin ratio; TP, total proteins; TG, total globulins; Alb., albumin. ↑ = infection.

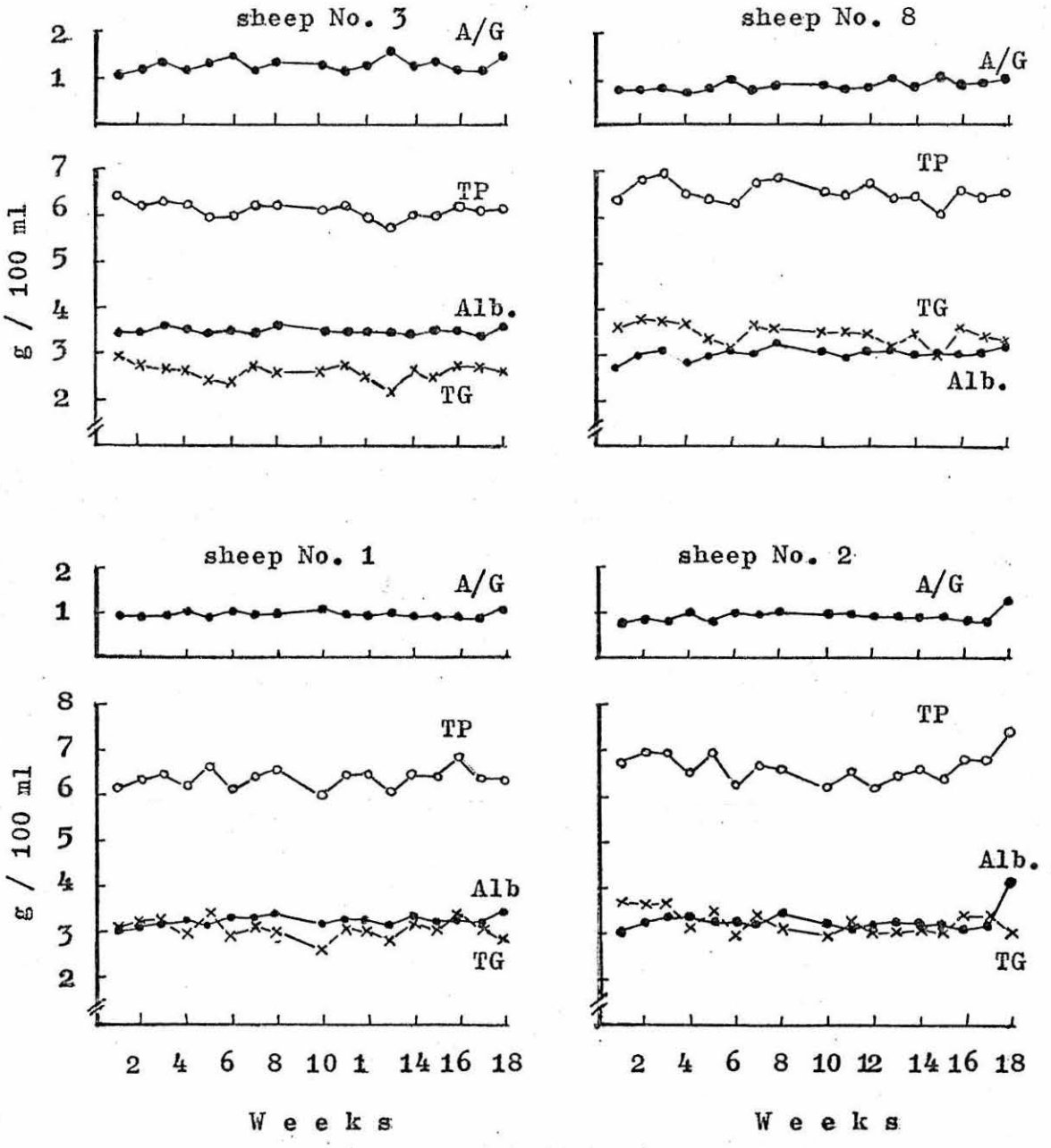


Fig. 3. Plasma protein picture in control sheep. A/G = Albumin-globulin ratio; TP = total proteins; Alb = albumin; TG = total globulins.

Table IV Means ($\bar{x}$) $\pm$ standard deviations of plasma proteins (g/100ml) in experimental sheep. T.P.=total proteins; Alb=albumin; T.Gls=total globulins. Group A, controls; group B, infected with 268 cysts in a single dose; group C, infected with 536 cysts given in two equal doses 2 weeks apart.

Wks	Group A			Group B			Group C		
	T.P.	Alb.	T.Gls.	T.P.	Alb.	T.Gls.	T.P.	Alb.	T.Gls.
-3	6.47	3.12	3.35	6.35	3.25	3.10	6.22	3.1	3.12
	0.22	0.25	0.3	0.15	0.05	0.16	0.13	0.12	0.13
-2	6.62	3.22	3.4	6.52	3.27	3.25	6.60	3.15	3.45
	0.29	0.19	0.39	0.18	0.04	0.21	0.07	0.05	0.05
-1	6.75	3.37	3.37	6.45	3.50	2.95	6.57	3.45	3.12
	0.30	0.20	0.43	0.29	0.12	0.16	0.08	0.05	0.08
0	6.42	3.27	3.15	6.27	3.27	3.00	6.30	3.12	3.17
	0.13	0.29	0.36	0.11	0.23	0.19	0.12	0.18	0.24
1	6.50	3.25	3.25	6.37	3.40	2.97	6.37	3.27	3.10
	0.34	0.18	0.44	0.29	0.07	0.33	0.15	0.11	0.16
2	6.22	3.37	2.85	6.45	3.45	3.00	6.27	3.37	2.90
	0.13	0.15	0.27	0.11	0.09	0.16	0.18	0.11	0.19
3	6.57	3.30	3.27	6.77	3.22	3.55	6.85	3.20	3.65
	0.19	0.14	0.33	0.69	0.08	0.68	0.26	0.19	0.32
4	6.62	3.50	3.12	7.40	3.40	4.00	7.17	3.42	3.75
	0.22	0.14	0.36	0.27	0.16	0.14	0.53	0.15	0.61
5	6.27	3.30	2.97	7.40	3.07	4.32	7.15	3.00	4.15
	0.22	0.14	0.33	0.19	0.08	0.26	0.57	0.16	0.69
6	6.45	3.25	3.20	7.32	3.05	4.27	7.17	3.02	4.15
	0.09	0.18	0.25	0.23	0.05	0.23	0.56	0.15	0.69
7	6.37	3.32	3.05	7.65	3.07	4.57	7.30	3.12	4.17
	0.26	0.13	0.36	0.21	0.08	0.29	0.37	0.11	0.46
8	6.17	3.32	2.85	7.82	2.92	4.90	6.92	3.07	3.85
	0.29	0.11	0.39	0.44	0.20	0.64	0.45	0.18	0.54
9	6.47	3.27	3.20	8.10	2.87	5.22	7.25	2.97	4.27
	0.23	0.16	0.30	0.49	0.13	0.62	0.45	0.15	0.55
10	6.25	3.30	2.95	7.57	2.85	4.72	6.92	2.97	3.95
	0.21	0.14	0.27	0.19	0.16	0.18	0.59	0.20	0.7

/Contd....

Table IV (Contd.)

11	6.67	3.32	3.35	7.80	2.82	4.97	7.22	2.87	4.35
	±	±	±	±	±	±	±	±	±
	0.23	0.15	0.32	0.21	0.08	0.29	0.56	0.24	0.63
12	6.47	3.25	3.22	7.45	2.75	4.70	7.15	2.80	4.35
	±	±	±	±	±	±	±	±	±
	0.25	0.09	0.27	0.23	0.09	0.25	0.83	0.31	0.92
13	6.65	3.72	2.92	7.25	2.87	4.37	6.87	2.87	4.00
	±	±	±	±	±	±	±	±	±
	0.52	0.41	0.33	0.50	0.16	0.40	0.87	0.37	0.99

The changes in total globulins followed closely the changes in total proteins; they increased from the 2nd week of infection and were highest by the 10th week.

Due to the increase in total globulins and the decrease in albumin the albumin-globulin (A/G) ratio decreased and this was again obvious from the second week of infection onwards.

Group C (infected with 536 cysts each given in two equal doses 14 days apart:

In this group animals Nos.6, 7 and 9 showed different responses in their plasma protein to fluke infection. Very little changes were observed in sheep No.11.

In sheep No.6 (fig. 2a) the plasma proteins increased from the 2nd post-infection to the 4th week and then showed little change till week 8; a gradual decrease was observed afterwards. In animal No.7 (Fig. 2b) the total proteins increased markedly from the 2nd week of infection to a maximum at the 6th week. This was followed by a temporary decrease till week 9 and then by an increase till the terminal week. Sheep No.9 (fig. 2c) on the other hand, showed similar changes till the 4th week of exposure and then the levels remained almost constant till the end of the observation.

The changes in plasma albumin were not very different in the 4 animals and were nearly similar to those described for the previous group.

Alterations in total globulins also followed those in total proteins.

Except in sheep No.11 the A/G ratio decreased after the 2nd week of infection.

Control animals, on the other hand, maintained constant levels for total proteins and the different protein fractions throughout the experiment; only slight reversible fluctuations were noticed, see fig. 3.

2. Plasma protein changes in 6 sheep infected each with 518 F. hepatica metacercariae:

The mean weekly plasma protein levels for the 6 sheep before and after infection with F. hepatica cysts are recorded in Table V and figs.4a and b. Individual values are presented in figs. 5-10.

The percentages and absolute values of the different plasma protein fractions showed similar changes in all animals and, except for albumin values, greatly matched each other. The description below will mainly apply to the absolute values.

The total proteins were slightly decreased till the 2nd week of infection and then increased till the 5th week. After a drop at week 6 the levels again increased to a maximum at week 12, maintained through week 13 and then steadily declined till the end of the experiment. The terminal level was about 20% less than preinfection values. Again two peaks were observed, one between the 2nd and 6th weeks and the other between week 6 and 11.

The plasma albumin decreased slightly after infection and till the 7th week and then more markedly till the 10th week. Between the 10th and 17th weeks a constant level was maintained followed by a steady decrease until the terminal week.

The α-globulins increased from the 3rd week post-infection till the 10th week. After a slight decrease at week 11

Table V Mean ($\bar{x}$) and standard deviations (SD) of plasma proteins in sheep infected each with 518 F. hepatica metacercariae.

Wks	Total proteins g/100ml	Albumin %	Albumin g/100 ml	Globulins ∝ - %	Globulins ∝ - g/100 ml	Globulins β - %	Globulins β - g/100 ml	Globulins γ - %	Globulins γ - g/100 ml	Globulins β + γ %	Globulins β + γ g/100 ml
-2	6.83	49.67	3.35	8.55	0.51					41.92	2.89
	0.18	1.15	1.38	2.2	0.15					3.08	0.17
-1	6.90	49.79	3.43	10.69	0.74					39.46	2.73
	0.24	2.18	0.14	1.75	0.14					2.09	0.15
0	6.60	51.01	3.37	12.04	0.80	14.97	0.97	21.89	1.43		
	0.24	1.06	0.14	1.5	0.1	1.94	0.12	0.54	0.07		
1	6.57	49.20	3.23	12.71	0.84	10.58	0.7	28.04	1.82		
	0.24	1.09	0.18	2.7	0.2	4.05	0.25	3.65	0.23		
2	6.42	49.12	3.15	10.60	0.68	11.52	0.73	28.95	1.86		
	0.13	2.75	0.15	2.4	0.16	4.78	0.29	4.64	0.29		
3	7.22	45.07	3.25	9.95	0.72	8.66	0.57	37.02	2.67		
	0.17	3.03	0.18	2.89	0.21	4.00	0.29	5.86	0.43		
4	7.58	40.93	3.10	13.22	1.00	9.89	0.75	35.94	2.72		
	0.25	3.18	0.21	3.24	0.28	4.82	0.37	5.22	0.38		
5	7.95	41.56	3.30	14.05	1.12	8.26	0.66	36.13	2.87		
	0.25	2.88	0.19	2.8	0.25	3.31	0.28	4.16	0.29		
6	7.33	41.81	3.07	17.21	1.16	8.29	0.58	32.34	2.39		
	0.11	1.89	0.15	0.81	0.29	2.07	0.15	1.9	0.11		
7	7.67	42.00	3.22	17.65	1.35	8.11	0.63	32.24	2.47		
	0.22	2.27	0.13	0.91	0.08	2.72	0.22	1.66	0.12		
8	8.17	36.56	2.98	20.52	1.70	10.38	0.87	31.13	2.61		
	0.25	1.89	0.13	3.53	0.25	2.88	0.24	1.29	0.19		
9	8.47	32.65	2.77	22.16	1.87	9.54	0.81	35.63	3.02		
	0.18	2.32	0.23	1.97	0.16	1.24	0.11	1.43	0.09		
10	8.40	30.04	2.52	23.78	1.99	8.33	0.69	37.51	3.12		
	0.41	2.87	0.19	1.51	0.15	1.62	0.16	1.58	0.26		
11	7.72	33.58	2.58	20.13	1.56	8.38	0.64	37.90	2.93		
	0.43	2.80	0.17	2.08	0.21	1.82	0.13	3.13	0.36		

/Contd....

Table V (Contd.)

12	7.28 ± 0.52	33.74 ± 3.20	2.45 ± 0.21	22.09 ± 1.37	1.61 ± 0.18	9.06 ± 1.82	0.66 ± 0.15	35.09 ± 2.66	2.56 ± 0.29
13	7.10 ± 0.61	35.66 ± 3.79	2.52 ± 0.21	23.09 ± 2.99	1.67 ± 0.25	9.55 ± 1.62	0.68 ± 0.13	31.68 ± 3.00	2.26 ± 0.40
15	6.27 ± 0.47	38.79 ± 4.83	2.42 ± 0.24	24.55 ± 3.12	1.53 ± 0.17	8.43 ± 1.44	0.53 ± 0.09	28.09 ± 5.69	1.78 ± 0.47
17	5.85 ± 0.26	41.88 ± 3.13	2.45 ± 0.22	20.24 ± 2.75	1.18 ± 0.15	9.58 ± 0.80	0.56 ± 0.06	28.30 ± 3.6	1.66 ± 0.24
19	4.85 ± 0.24	45.65 ± 3.01	2.22 ± 0.21	20.62 ± 2.13	0.99 ± 0.08	9.66 ± 1.91	0.47 ± 0.11	24.06 ± 3.61	1.16 ± 0.16
21	5.22 ± 0.46	39.16 ± 2.55	2.05 ± 0.27	22.30 ± 2.03	1.16 ± 0.1	10.18 ± 2.80	0.53 ± 0.14	28.28 ± 4.56	1.48 ± 0.26
23	4.72 ± 0.42	-	-	-	-	-	-	-	-

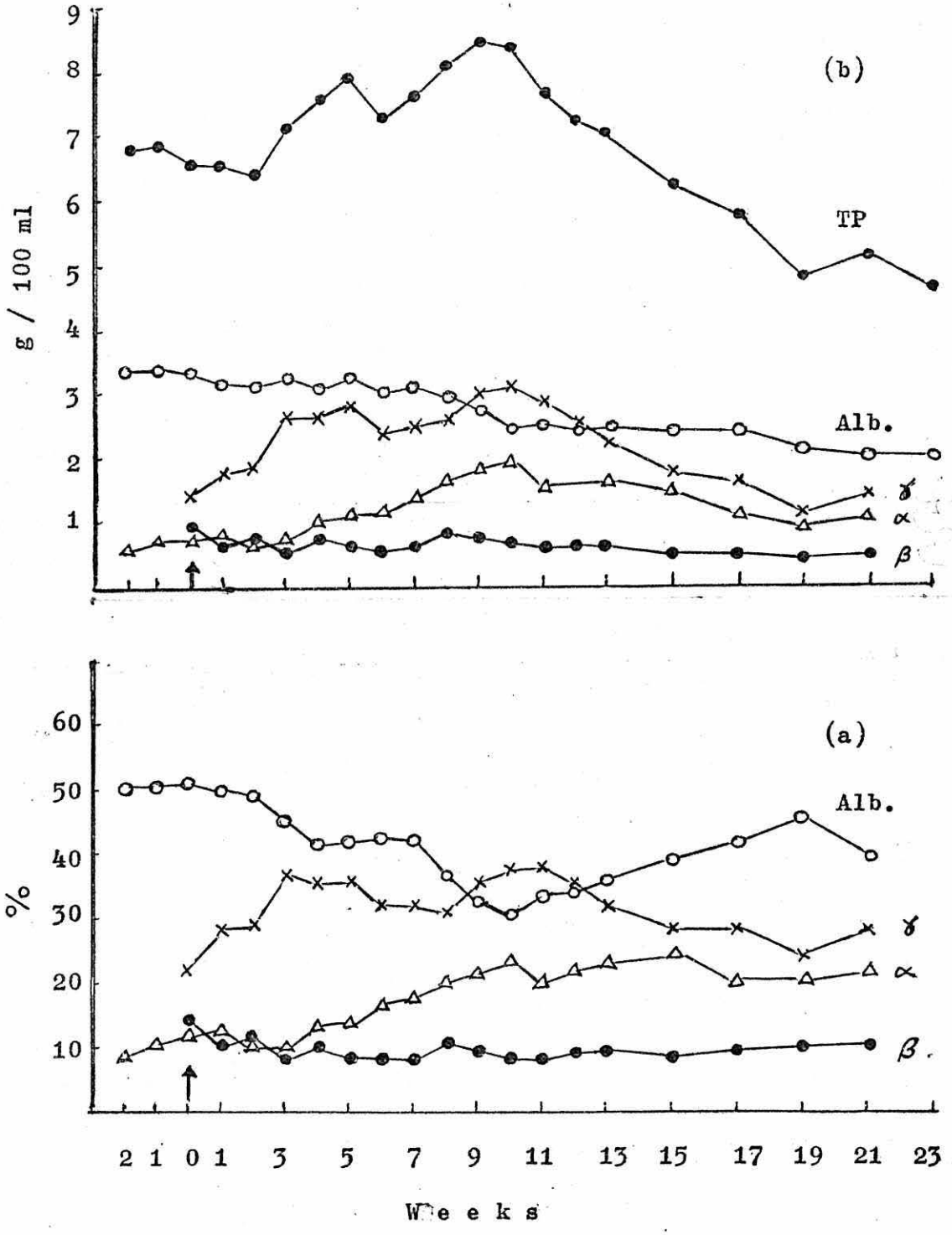
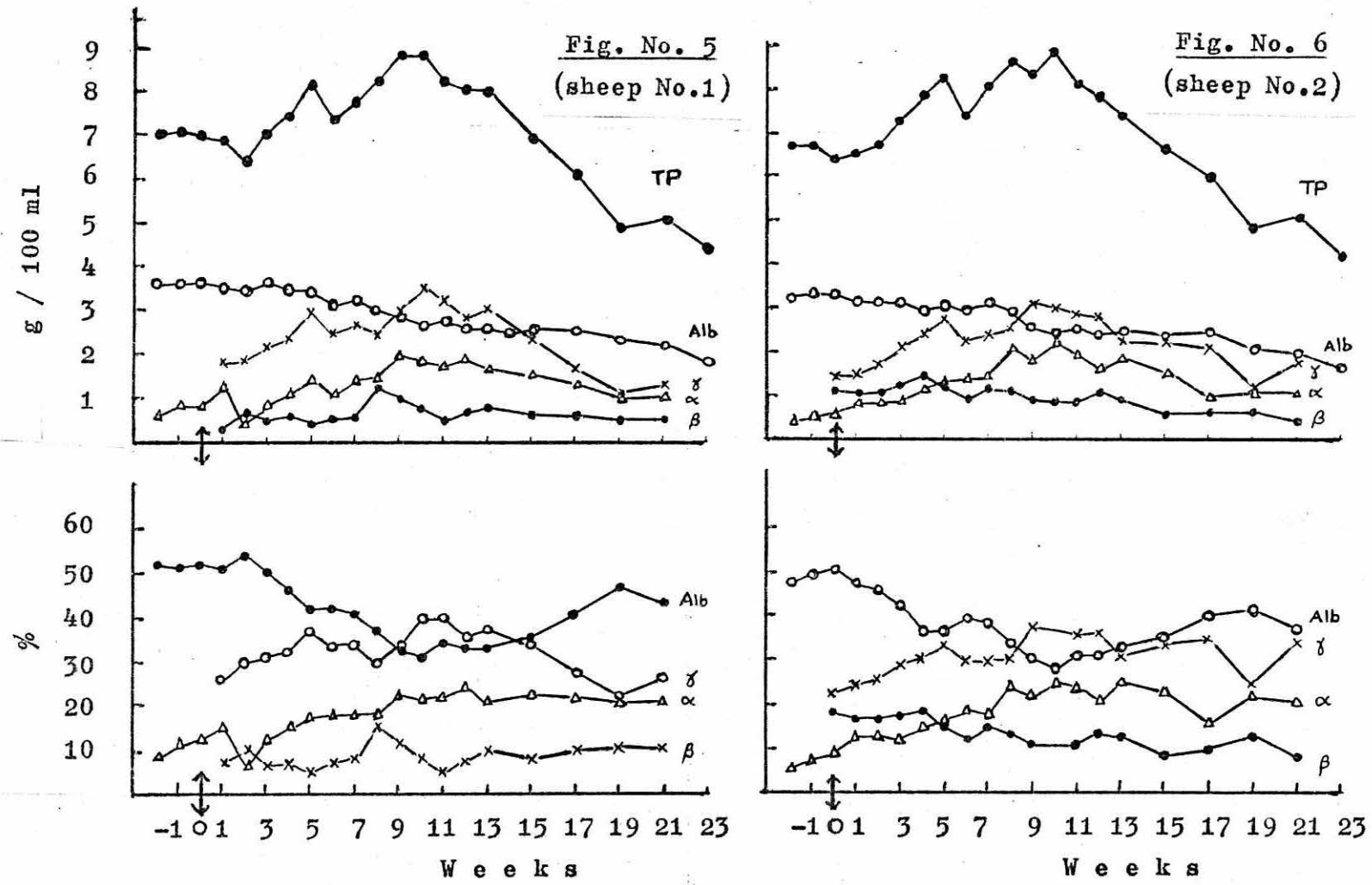
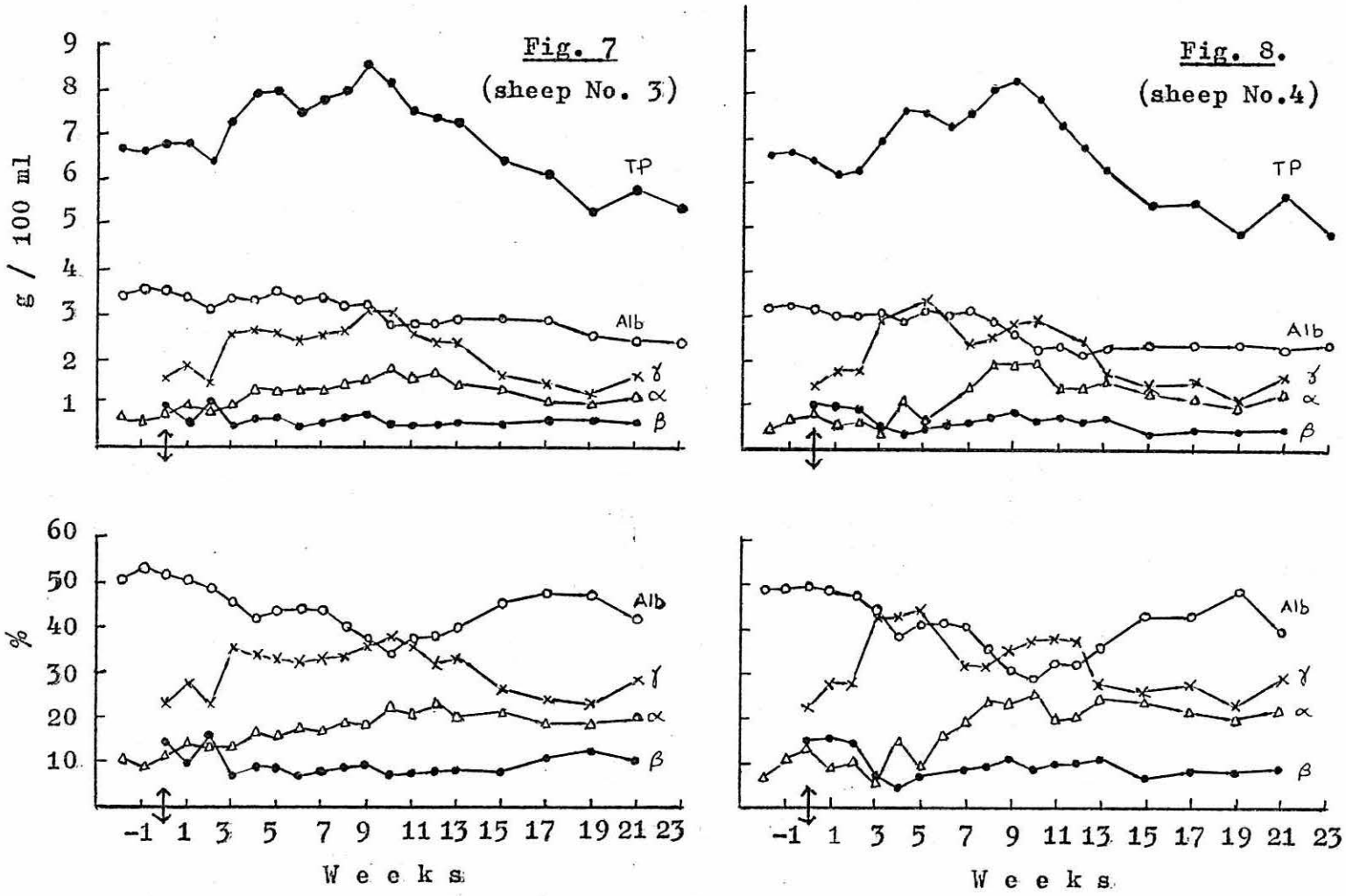


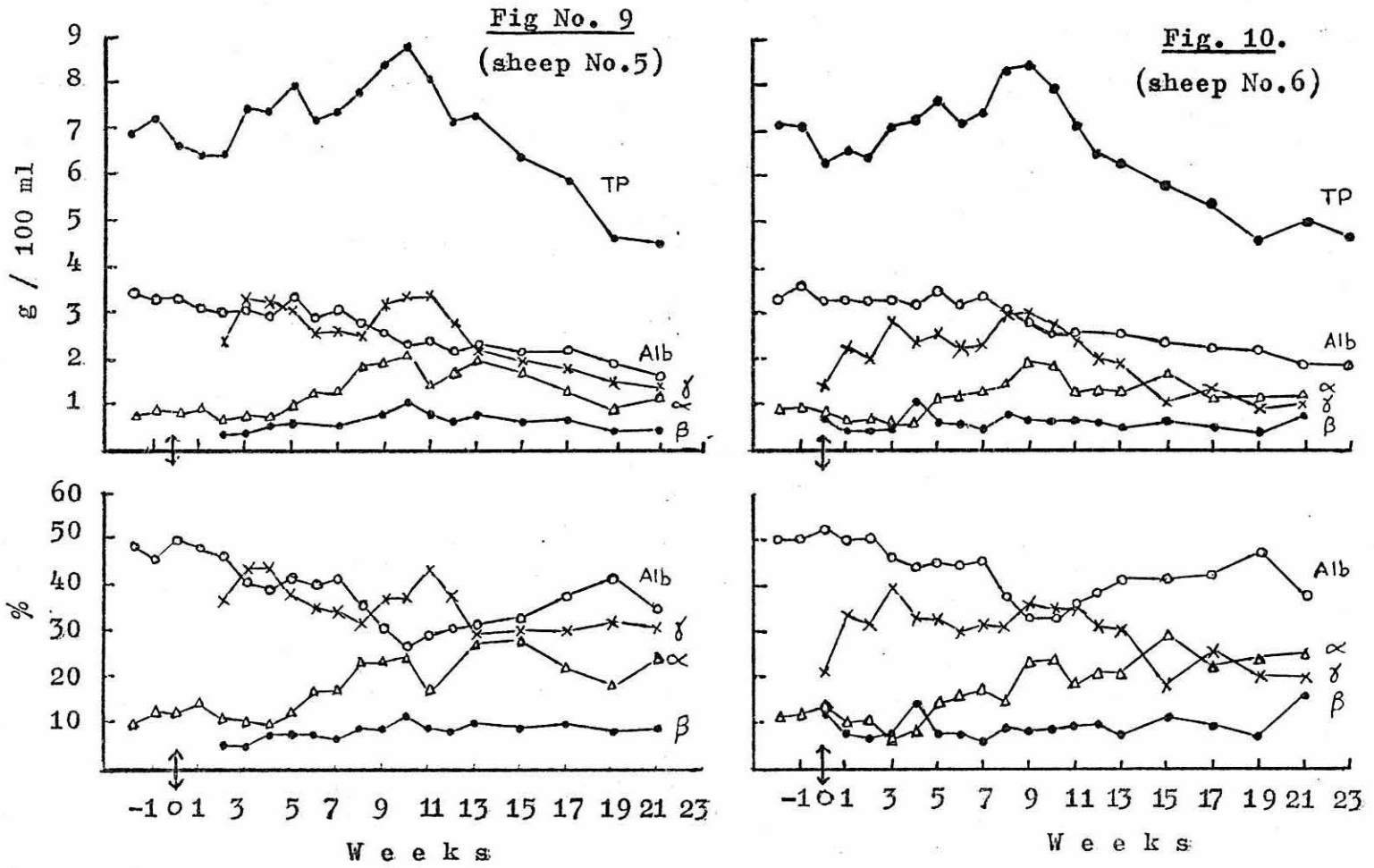
Fig. 4. Avarage plasma proteins in 6 sheep, each infected with 518 F. hepatica cysts. a) percentages and b) absolute values. TP = total proteins; Alb. = albumin; α, β, γ = globulins. ↑ = infection.



Fi s. 5 and 6. Plasma protein changes in sheep Nos. 1 & 2 each infected with 518 F. hepatica cysts. TP = total proteins; Alb = albumin; α, β, γ = globulins. ↑ = infection.



Figs. 7 and 8. Plasma protein changes in sheep Nos. 3 & 4, each infected with 518 F. hepatica cysts, respectively. TP = total proteins; Alb = albumin; α, β, γ = globulins. ↑ = infection.



Figs. 9 and 10. Plasma protein changes in sheep Nos. 5 & 6 respectively. Each animal was infected with 518 F. hepatica. TP = total proteins; Alb = albumin; α, β, γ = globulins. ↑ = infection

they remained nearly constant till the 15th week and then declined till the 19th week. The γ -globulins showed changes corresponding to those described for total proteins while the β -globulins changed very little with a slight tendency to decrease and in two animals (Nos. 1 and 5) showed a slight increase between the 7th and 11th week of infection.

3. The plasma proteins in 2 cows each infected with 1880 cysts of F. hepatica:

The percentage and absolute plasma protein values can be seen in Table VI and Figs.11 and 12.

Cow No. R:-

Figure 11 shows that the total proteins increased slightly after the first week of infection till the 4th week and then very slightly decreased till the 9th week. This was followed by a slight increase to a peak at week 11 and then the levels declined.

Plasma albumin showed a very slight increase till the 11th week and declined thereafter but the terminal values were still above pre-infection levels.

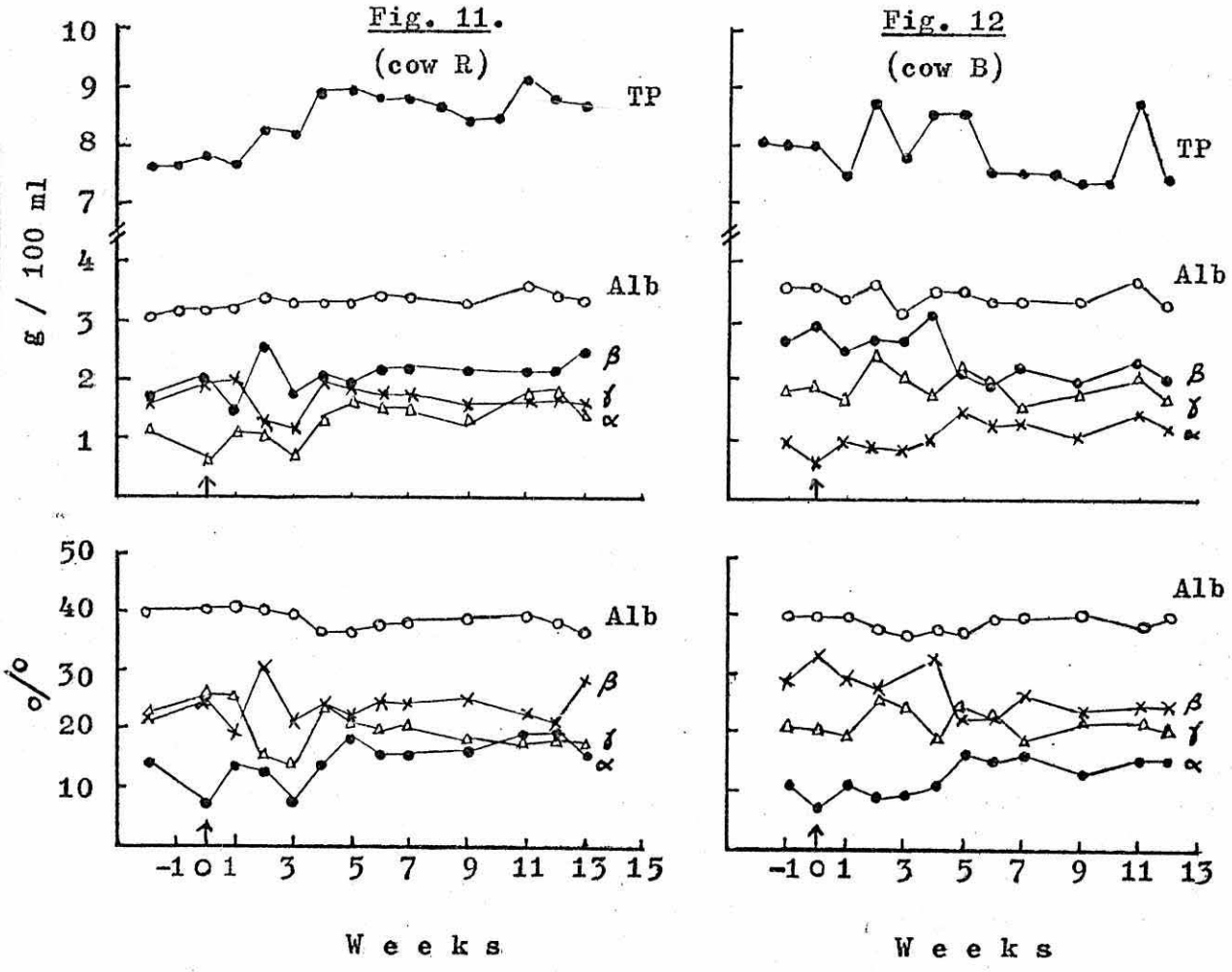
The α -globulins followed a similar pattern to total proteins, while the β -globulins showed a slight upward trend throughout. The γ -globulins decreased from the first week of infection till the 3rd week and then returned to pre-infection levels at week 4. Subsequently the levels were steadily, though slightly, decreased.

Cow No. B:-

It can be seen from Fig. 12 that the total proteins generally increased between the first and 6th week of infection

Table VI Plasma protein changes in cows infected with *F. hepatica*

Animal No.	Wks	Total proteins g/100ml	Albumin		Globulins					
					α-		β-		γ-	
			%	g/100 ml	%	g/100 ml	%	g/100 ml	%	g/100 ml
R	-2	7.6	40.48	3.08	14.37	1.18	22.58	1.72	22.58	1.72
	-1	7.6	-	-	-	-	-	-	-	-
	0	7.8	41.02	3.2	7.37	0.57	25.80	2.01	25.80	2.01
	1	7.7	41.56	3.2	13.70	1.05	18.94	1.46	25.79	1.98
	2	8.3	40.96	3.4	13.00	1.08	31.19	2.59	14.85	1.23
	3	8.2	40.24	3.3	7.56	0.62	21.56	1.77	13.99	1.15
	4	8.9	37.08	3.3	13.98	1.24	24.70	2.20	24.23	2.16
	5	8.9	37.08	3.3	18.37	1.63	22.27	1.98	22.27	1.98
	6	8.8	38.64	3.4	16.27	1.43	25.11	2.21	19.98	1.76
	7	8.8	38.64	3.4	16.20	1.42	25.06	2.20	20.09	1.77
	8	8.7	-	-	-	-	-	-	-	-
	9	8.4	39.28	3.3	16.37	1.37	25.60	2.15	18.75	1.57
	10	8.4	-	-	-	-	-	-	-	-
	11	9.1	39.56	3.6	19.20	1.75	22.78	2.07	18.45	1.68
	12	-	-	-	-	-	-	-	-	-
	13	8.8	38.64	3.4	20.70	1.82	21.83	1.92	18.82	1.66
	14	-	-	-	-	-	-	-	-	-
	15	8.7	36.78	3.2	16.41	1.43	28.44	2.47	18.37	1.60
B	-2	9.1	-	-	-	-	-	-	-	-
	-1	9.0	40.00	3.6	10.46	0.94	28.66	2.57	20.88	1.88
	0	9.0	40.00	3.6	6.56	0.59	32.81	2.95	20.62	1.85
	1	8.5	40.00	3.4	10.97	0.93	29.63	2.52	19.39	1.65
	2	9.8	37.75	3.7	8.89	0.87	27.57	2.70	25.79	2.53
	3	8.8	36.36	3.2	9.17	0.81	30.39	2.67	24.08	2.12
	4	9.6	37.50	3.6	10.61	1.02	32.95	3.16	18.94	1.82
	5	9.6	37.50	3.6	15.99	1.53	22.52	2.16	23.99	2.30
	6	8.6	39.53	3.4	14.88	1.28	22.64	1.95	22.95	1.97
	7	8.6	39.53	3.4	15.69	1.35	26.31	2.26	18.46	1.59
	8	8.6	-	-	-	-	-	-	-	-
	9	8.4	40.48	3.4	13.43	1.13	23.77	2.00	22.32	1.87
	10	8.4	-	-	-	-	-	-	-	-
	11	9.9	38.38	3.8	15.19	1.50	24.48	2.42	21.95	2.17
	12	-	-	-	-	-	-	-	-	-
	13	8.5	40.0	3.4	14.93	1.27	24.31	2.07	20.76	1.76



Figs. 11 and 12. Plasma protein changes in cow R and cow B, each infected with 1880 F.hepatica metacercariae. TP = total proteins; Alb = albumin; α, β, γ = globulins. ↑ = infection.

and then decreased till week 9. An alternate rise and drop in the levels occurred at weeks 11 and 12 respectively.

Very little change was observed in the albumin fraction but the α-globulins were elevated from the 3rd week post-infection till the 5th week and afterwards they were nearly maintained constant till the last week of the observation. The β-globulins became low at week 5 of infection and subsequently remained low. The γ-globulins were rather high between the 1st and 6th weeks of exposure.

B. The Plasma Gylcoproteins:

1. Sheep

a) Electrophoretic fractions:

The weekly mean percentages for the various plasma glycoprotein fractions in 6 sheep infected each with 518 metacercariae of F. hepatica can be seen in Table VII and Fig.13. Individual values are presented in Figs.14-19.

It can be seen that the albumin glycoproteins constituted the smallest fraction and their levels decreased from the 5th week onwards; a temporary rise was observed around week 13.

The α-globulins, on the other hand, had the highest percentages. They changed only slightly, showing an increasing tendency. The β-globulins generally decreased after infection till the 6th week, then increased till week 11-12 and subsequently the levels fell. The γ-globulins responded oppositely to the β-globulins.

b) Protein-bound mucopolysaccharide components:

Mean plasma total hexoses (T-H), hexosamine (Hine), sialic acid (S.A.), fucose (F) and seromucoid (S.M.) contents in 6 sheep infected with F. hepatica can be seen in Table VIII and Figs.20-24. Individual values are plotted in Figs.25-29. The ratios of these values to the corresponding mean plasma total proteins are presented in Table IX (see also Figs.20-24). The ratios

$$\frac{T.H}{S.A}, \frac{T.H}{F}, \frac{T.H}{Hine}, \frac{Hine}{S.A}, \frac{Hine}{F} \text{ and } \frac{T.H + Hine}{S.A + F}$$

are shown in Table X and Fig.30.

Table VII Mean ($\bar{x}$) ± standard deviations (SD) of the plasma glycoprotein fractions in sheep infected with F. hepatica.

Wks	Albumin %		Globulins %					
			α -		β -		γ -	
	$\bar{x}$	± SD	$\bar{x}$	± SD	$\bar{x}$	± SD	$\bar{x}$	± SD
-2	2.71	1.65	48.32	7.50	30.93	5.96	18.53	3.76
-1	2.50	2.00	47.21	4.38	30.95	5.09	19.12	3.70
0	-	-	-	-	-	-	-	-
1	5.84	0.95	51.73	5.97	19.34	6.51	23.76	3.99
2	4.84	3.54	51.58	10.16	22.03	9.15	21.48	6.31
3	1.58	1.14	60.44	4.15	16.10	1.60	25.62	4.17
4	3.74	2.34	50.28	6.80	17.88	8.86	27.85	3.64
5	2.18	1.65	57.28	5.19	18.99	1.32	20.88	6.49
6	1.73	0.85	58.45	2.38	26.65	3.72	13.04	3.61
7	1.76	0.79	59.06	3.67	(39.17 ± 3.33)			
8	1.25	0.55	59.63	4.73	27.56	2.25	12.14	1.62
9	0.96	0.66	57.42	6.28	30.12	9.23	10.95	3.39
10	-	-	-	-	-	-	-	-
11	1.60	0.45	53.61	9.27	33.25	8.26	9.90	1.70
12	0.82	0.6	64.87	4.77	24.93	2.40	8.14	3.03
13	3.29	2.19	53.75	6.38	29.37	8.81	13.90	0.87
14	-	-	-	-	-	-	-	-
15	0.78	0.68	61.73	3.22	12.33	2.82	23.72	6.75
16	-	-	-	-	-	-	-	-
17	0.91	0.79	60.39	4.25	13.90	3.49	24.28	5.65

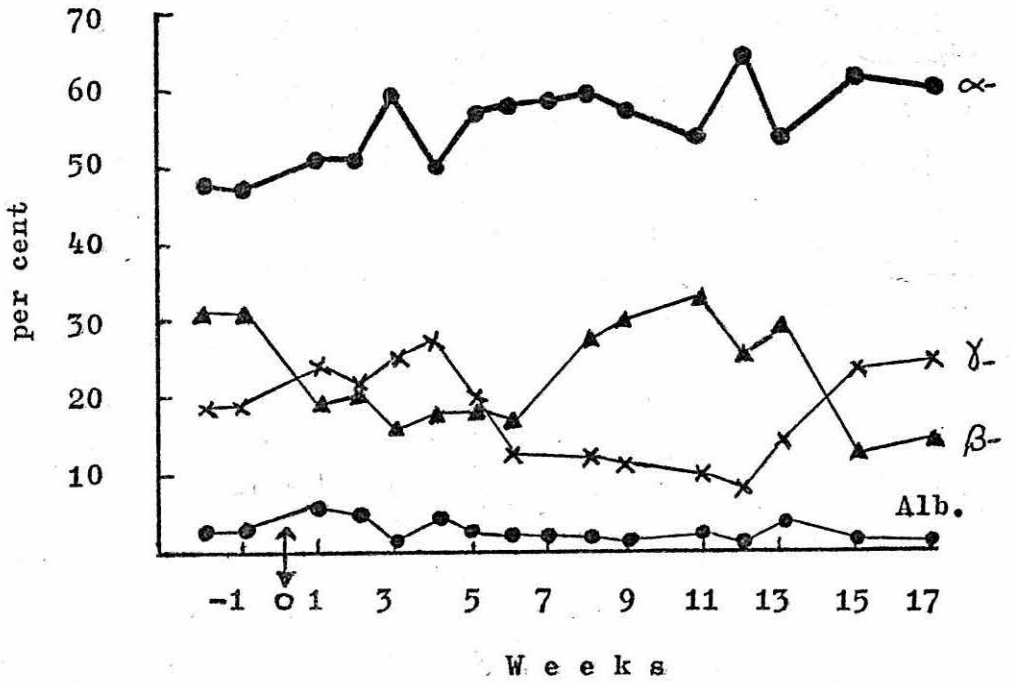
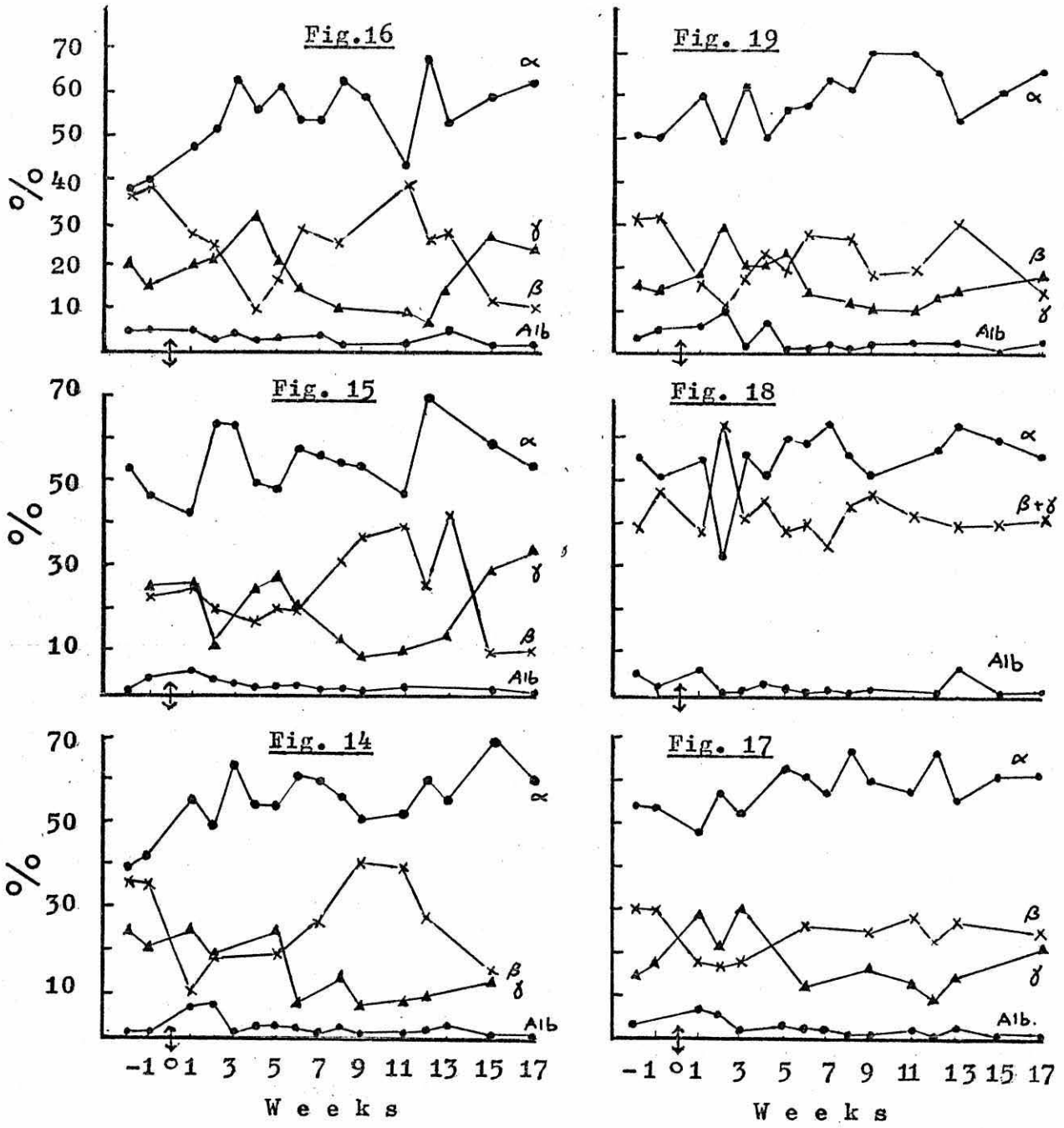


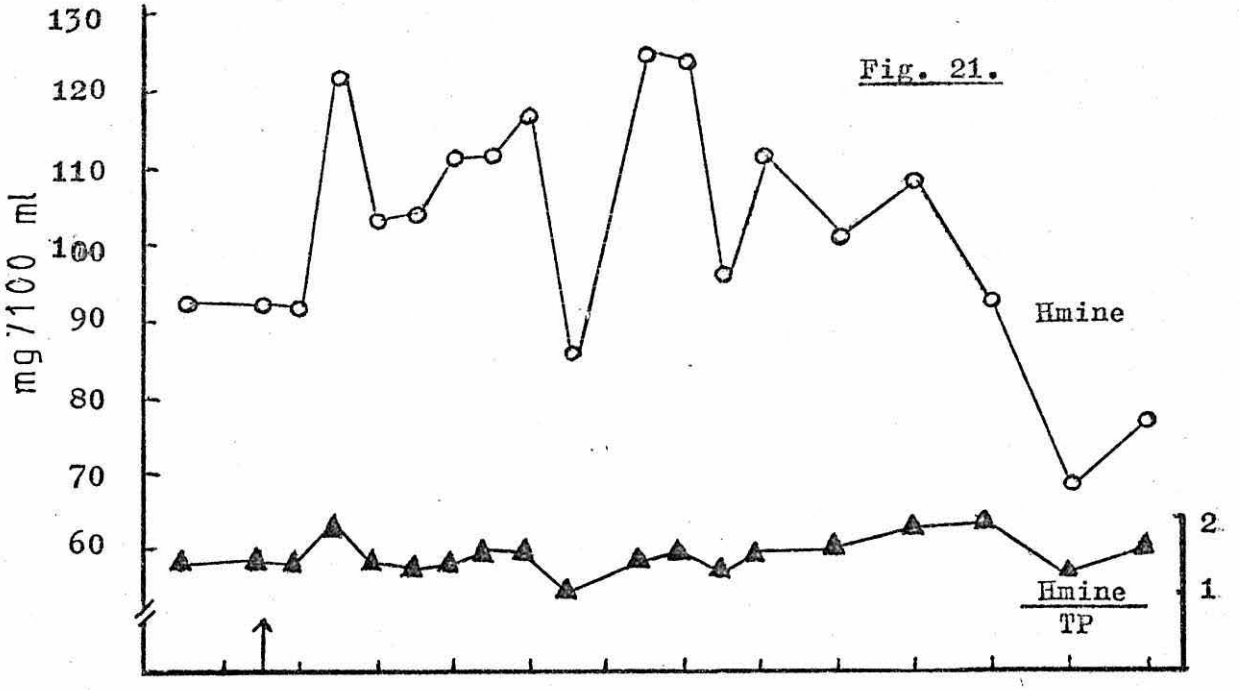
Fig.13. Mean plasma glycoprotein concentrations in 6 sheep (1 - 6), each infected with 518 F. hepatica cysts. Alb. = albumin; α, β, γ = globulins. ↑ = infection.

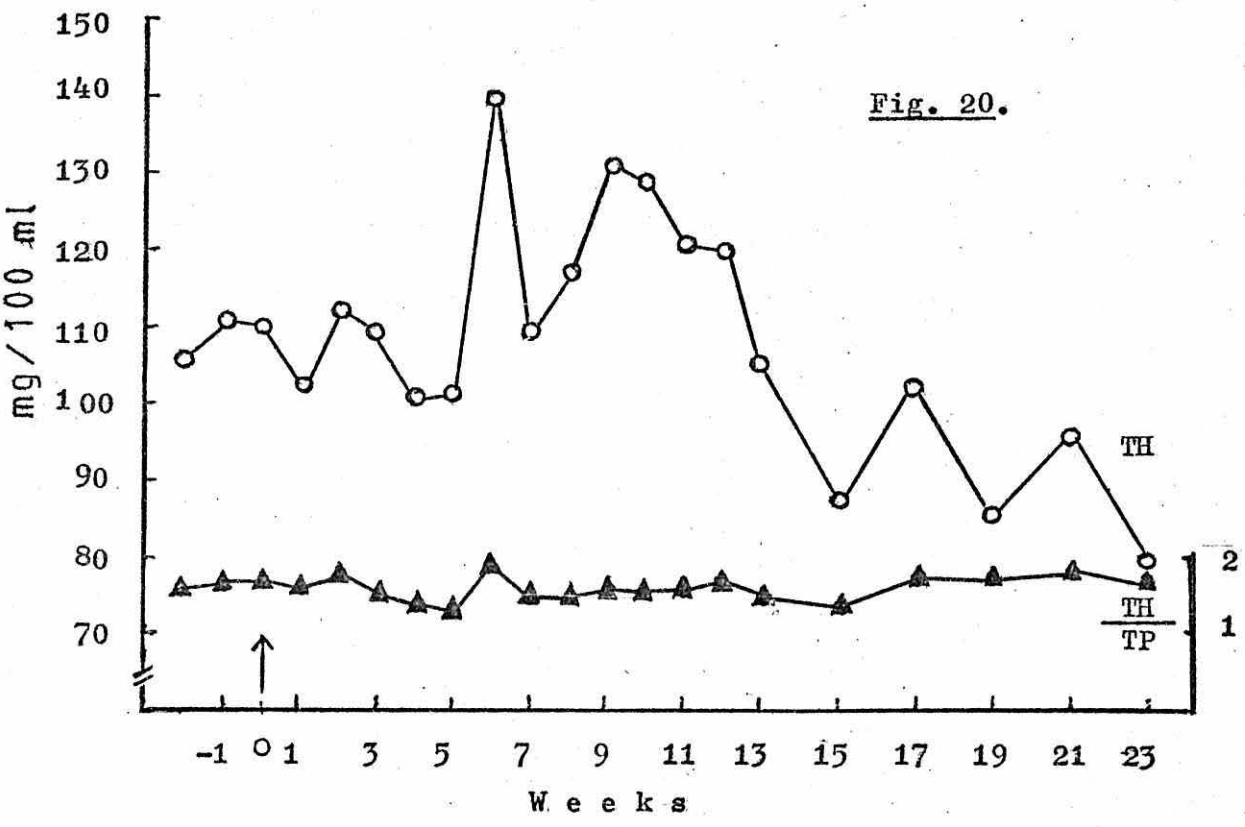


Figs. 14, 15, 16, 17, 18 and 19. Percentage distribution of the plasma glycoproteins in sheep Nos. 1,2,3,4,5 and 6, each infected with 518 F. hepatica cysts, respectively.
Alb = albumin; α, β, γ = globulins. ↑ = infection.

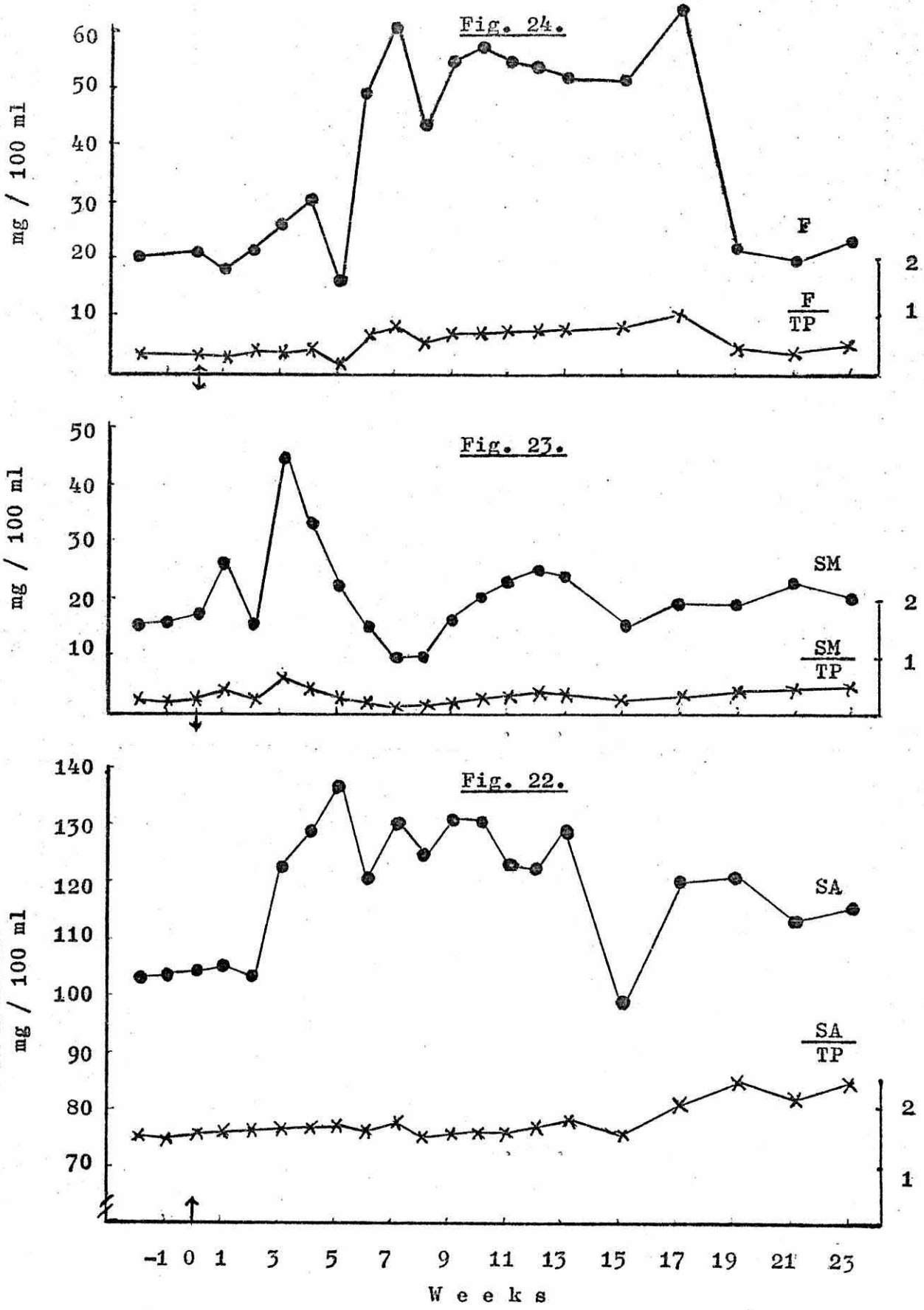
Table VIII Means ($\bar{x}$) $\pm$ standard deviations (SD) of the plasma glycoprotein components (mg/100ml) in 6 sheep experimentally infected with F. hepatica.

Wks.	Total hexoses		Hexosamine		Sialic acid		Fucose		Seromucoid	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
-2	106.64	5.20	91.89	14.02	98.48	11.49	20.23	2.66	15.61	1.67
-1	110.53	8.85	-	-	79.84	9.36	-	-	16.15	1.44
0	110.26	2.80	92.60	12.48	103.8	9.16	20.75	4.41	16.77	3.57
1	102.63	5.04	91.68	21.12	105.03	6.36	18.62	1.97	26.24	3.40
2	112.47	3.95	122.85	7.93	102.99	13.40	21.74	4.29	14.99	2.15
3	109.19	4.88	103.02	3.97	121.75	9.15	26.12	3.03	44.90	5.57
4	100.63	5.94	104.10	8.89	128.70	8.67	30.47	1.33	32.81	7.15
5	100.83	5.83	111.40	3.12	136.53	7.81	15.70	4.37	22.38	2.20
6	141.04	5.56	111.55	2.74	120.53	7.48	49.47	2.60	15.03	2.72
7	109.36	5.52	117.63	13.17	130.44	5.16	61.51	7.50	9.78	2.21
8	117.86	12.66	85.40	10.9	124.40	8.07	42.62	4.40	9.48	3.80
9	130.91	7.03			130.81	5.21	54.88	3.06	16.25	3.08
10	128.88	6.48	125.09	5.33	130.41	4.41	57.47	3.96	20.07	2.50
11	121.22	7.25	123.88	6.00	123.14	5.44	55.12	11.70	22.98	3.75
12	120.45	8.89	95.82	7.40	121.97	5.54	54.51	3.89	24.71	3.43
13	105.38	8.91	112.78	8.74	127.94	5.72	52.44	4.66	23.58	2.98
14	-	-	-	-	-	-	-	-	-	-
15	87.09	7.63	101.39	10.98	97.74	10.67	52.09	3.20	14.95	3.05
16	-	-	-	-	-	-	-	-	-	-
17	103.02	3.04	108.74	7.13	120.54	15.37	64.27	5.32	18.90	6.00
18	-	-	-	-	-	-	-	-	-	-
19	85.34	6.83	93.28	10.31	120.97	21.69	21.90	5.58	19.34	5.41
20	-	-	-	-	-	-	-	-	-	-
21	95.63	7.38	68.18	7.04	113.03	18.80	19.71	1.37	22.66	4.90
22	-	-	-	-	-	-	-	-	-	-
23	79.39	8.18	76.72	5.46	115.65	16.44	23.57	2.50	19.8	5.92





Figs. 20 and 21. Mean plasma total hexoses and hexosamine respectively, in 6 sheep each infected with 518 F. hepatica cysts. TH = total hexoses; TH/TP = ratio of total hexoses to total proteins; Hmine = hexosamine; Hmine / TP = ratio of hexosamine to total proteins. ↑ = infection.



Figs. 22, 23 and 24. Plasma sialic acid, seromucoid, and fucose respectively, in 6 sheep each infected with 518 F. hepatica cysts. SA = sialic acid; SM = seromucoid; F = fucose; TP = total proteins. ↑ = infection.

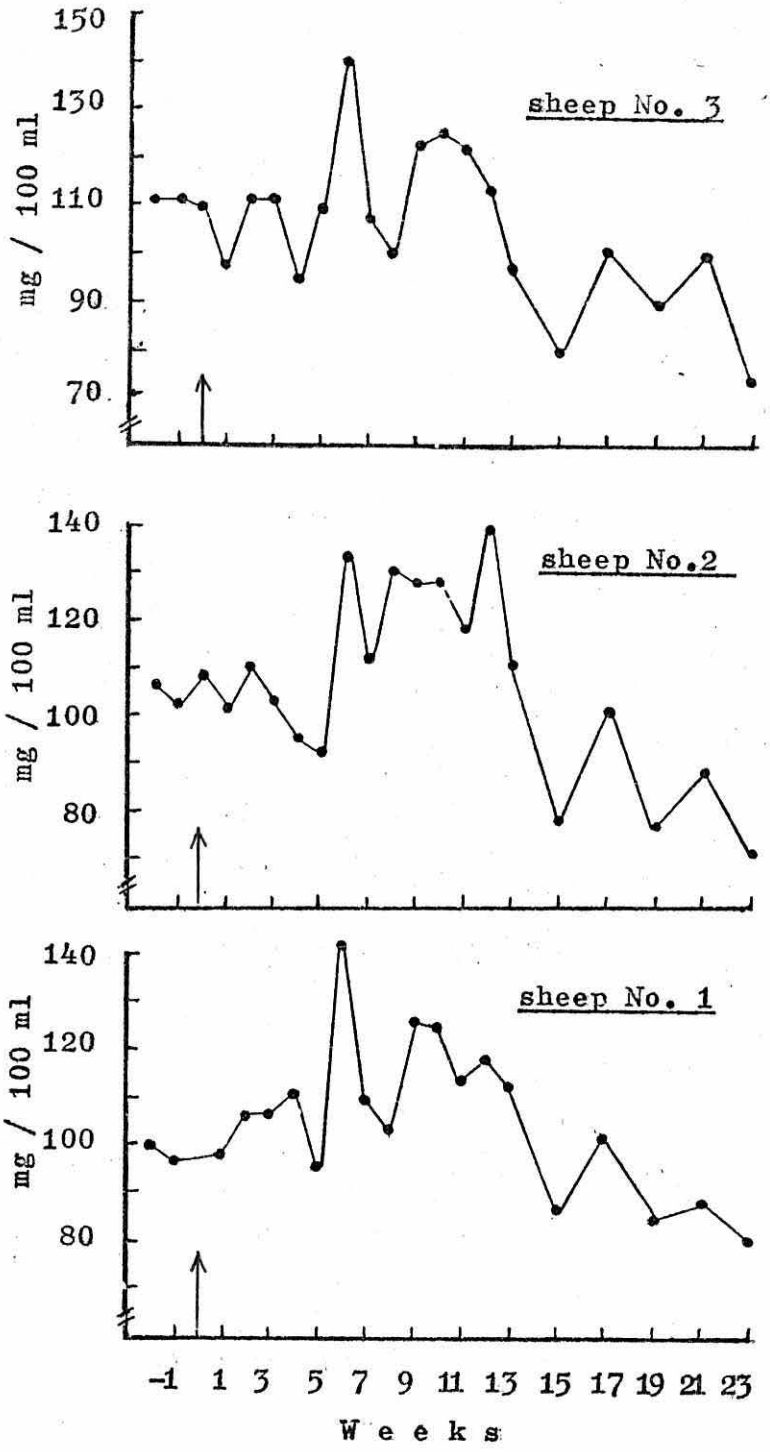


Fig. 25a. Plasma total hexoses in sheep Nos. 1,2,& 3, each infected with 518 F. hepatica cysts. ↑ = infection.

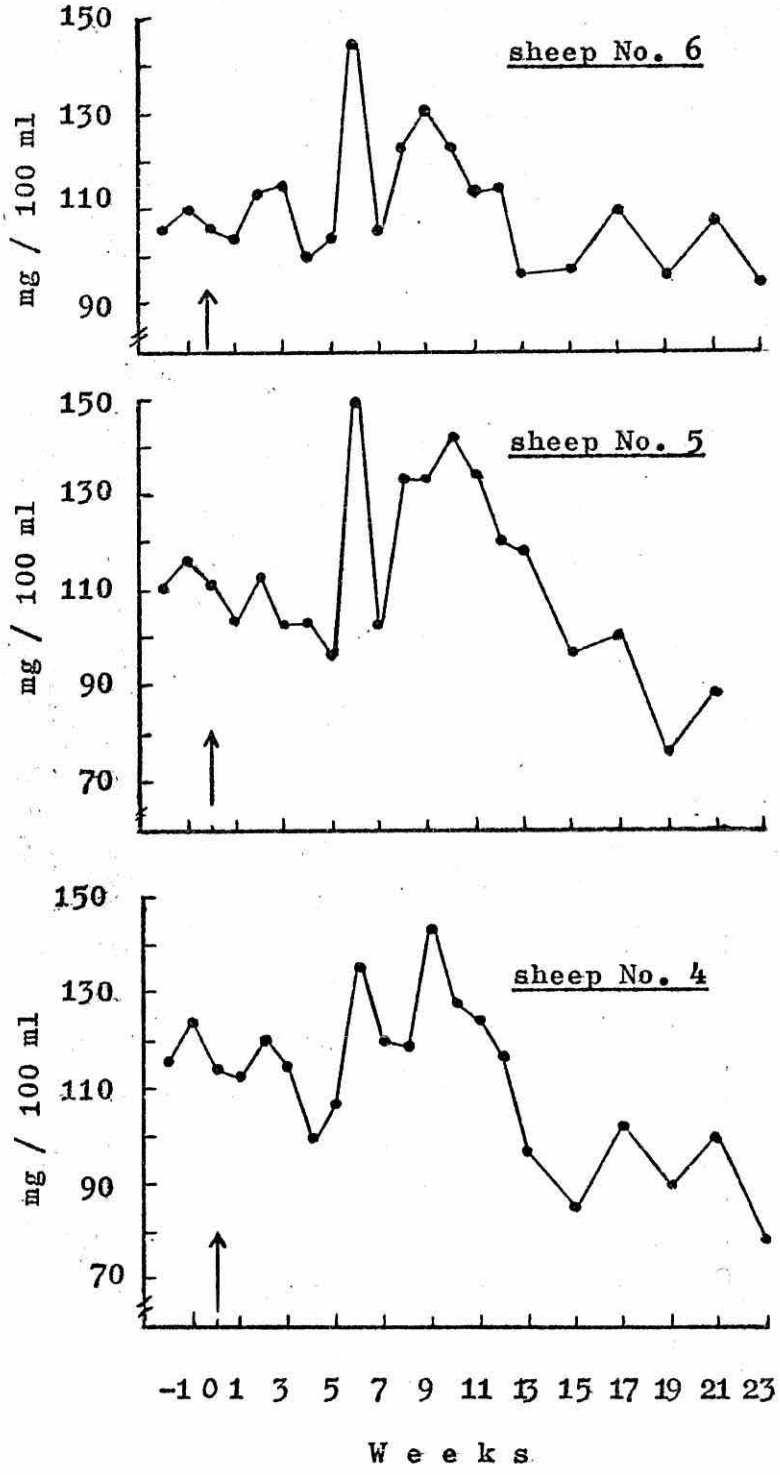


Fig. 25b. Plasma total hexoses in sheep Nos. 4,5,& 6, each infected with 518 F. hepatica cysts. ↑ = infection.

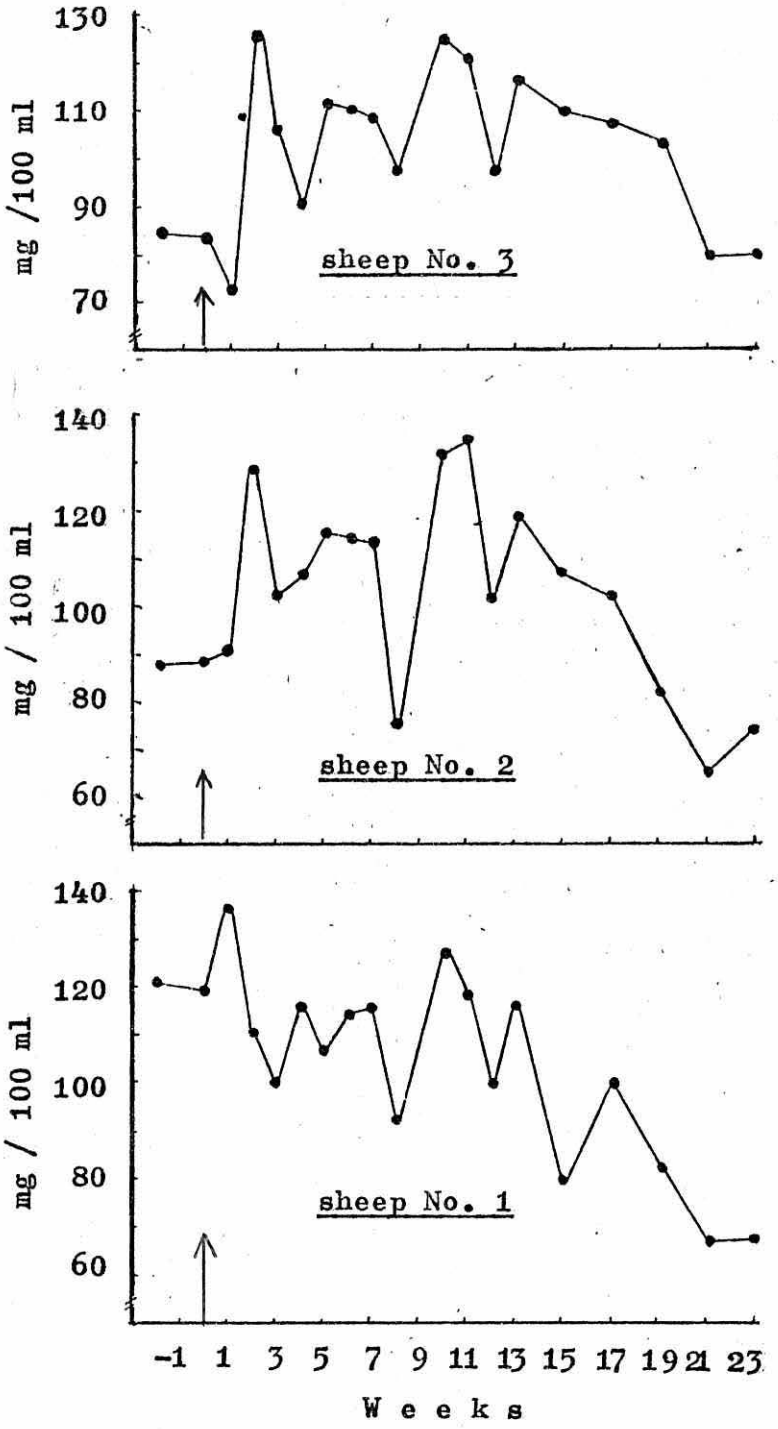


Fig. 26a. Plasma hexosamine in sheep NOs. 1, 2 and 3, each infected with 518 F. hepatica cysts. ↑ = infection.

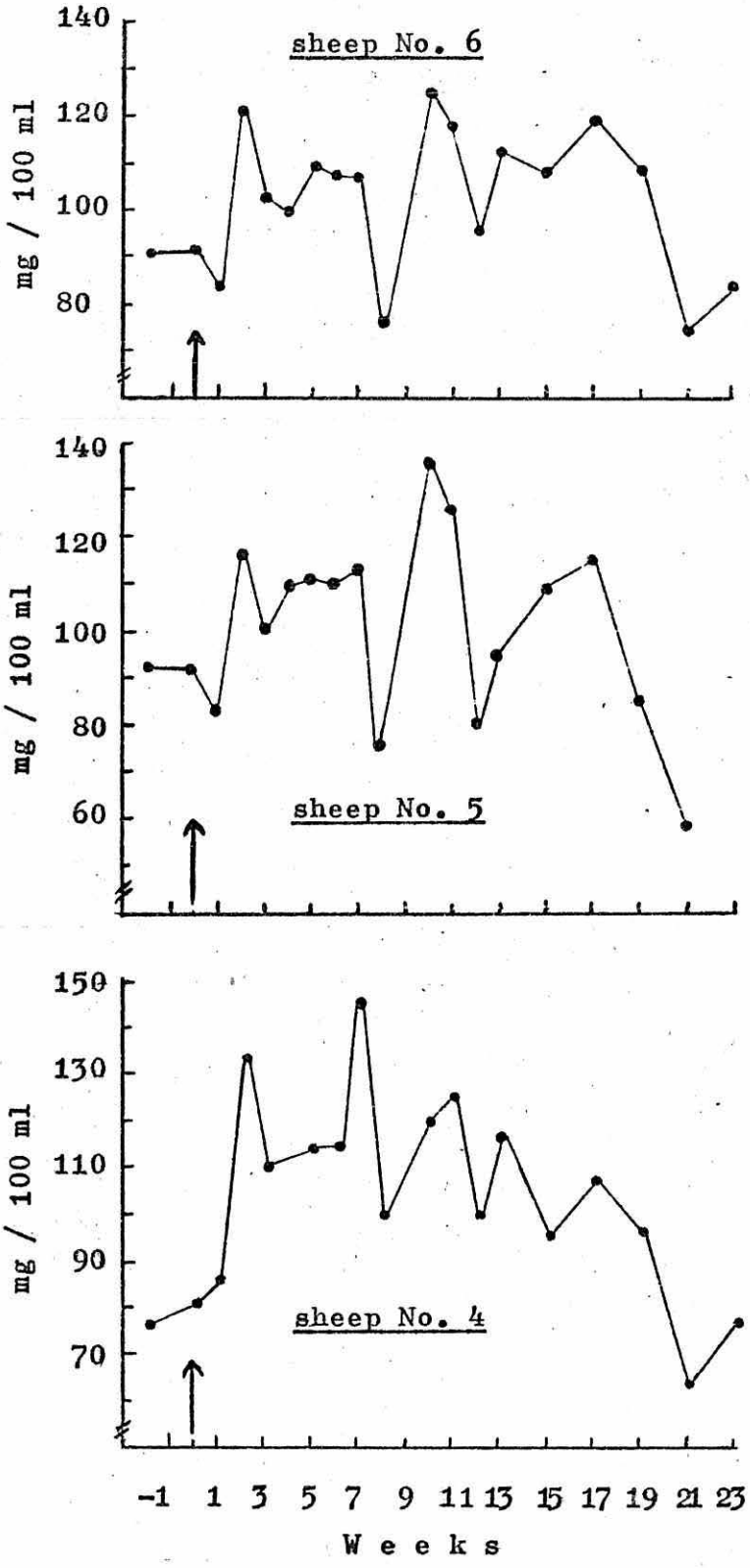


Fig 26b. Plasma hexosamine in sheep Nos. 4, 5,& 6, each infected with 518 F. hepatica cysts. ↑ = infection.

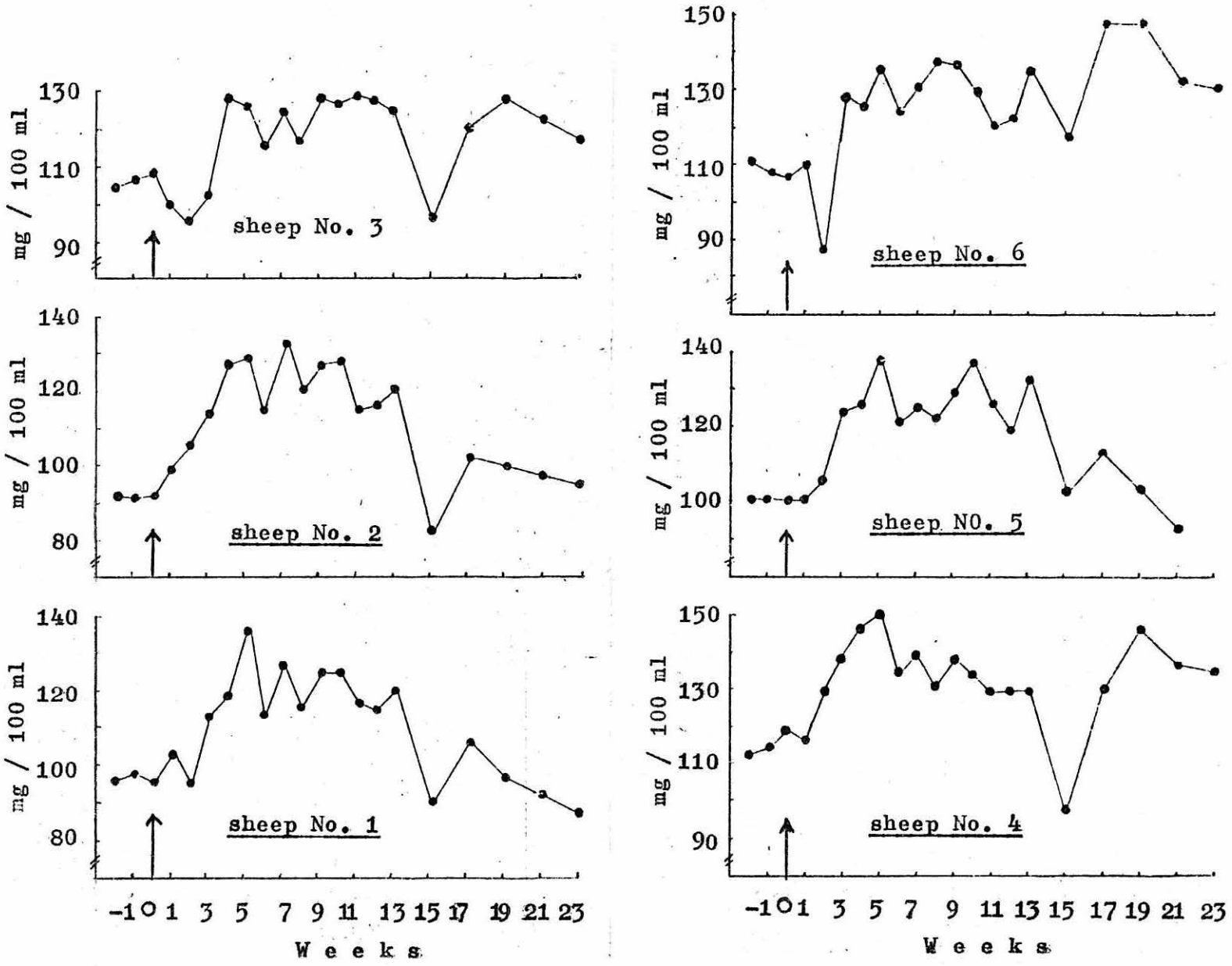


Fig. 27. Plasma sialic acid in 6 sheep, each infected with 518 F. hepatica cysts. ↑= infection.

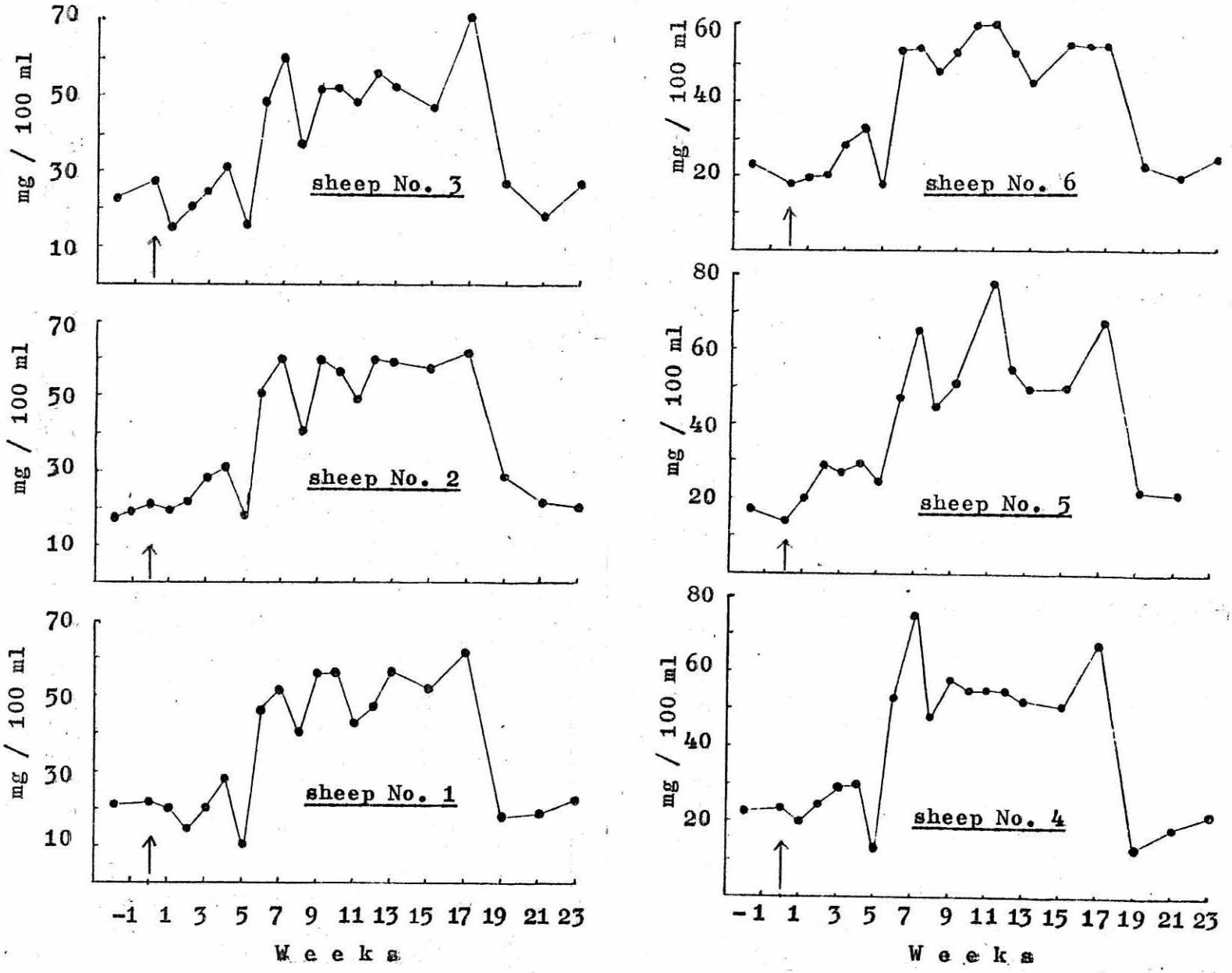


Fig. 28. Plasma fucose in 6 sheep, each infected with 518 F. hepatica cysts. ↑ = infection.

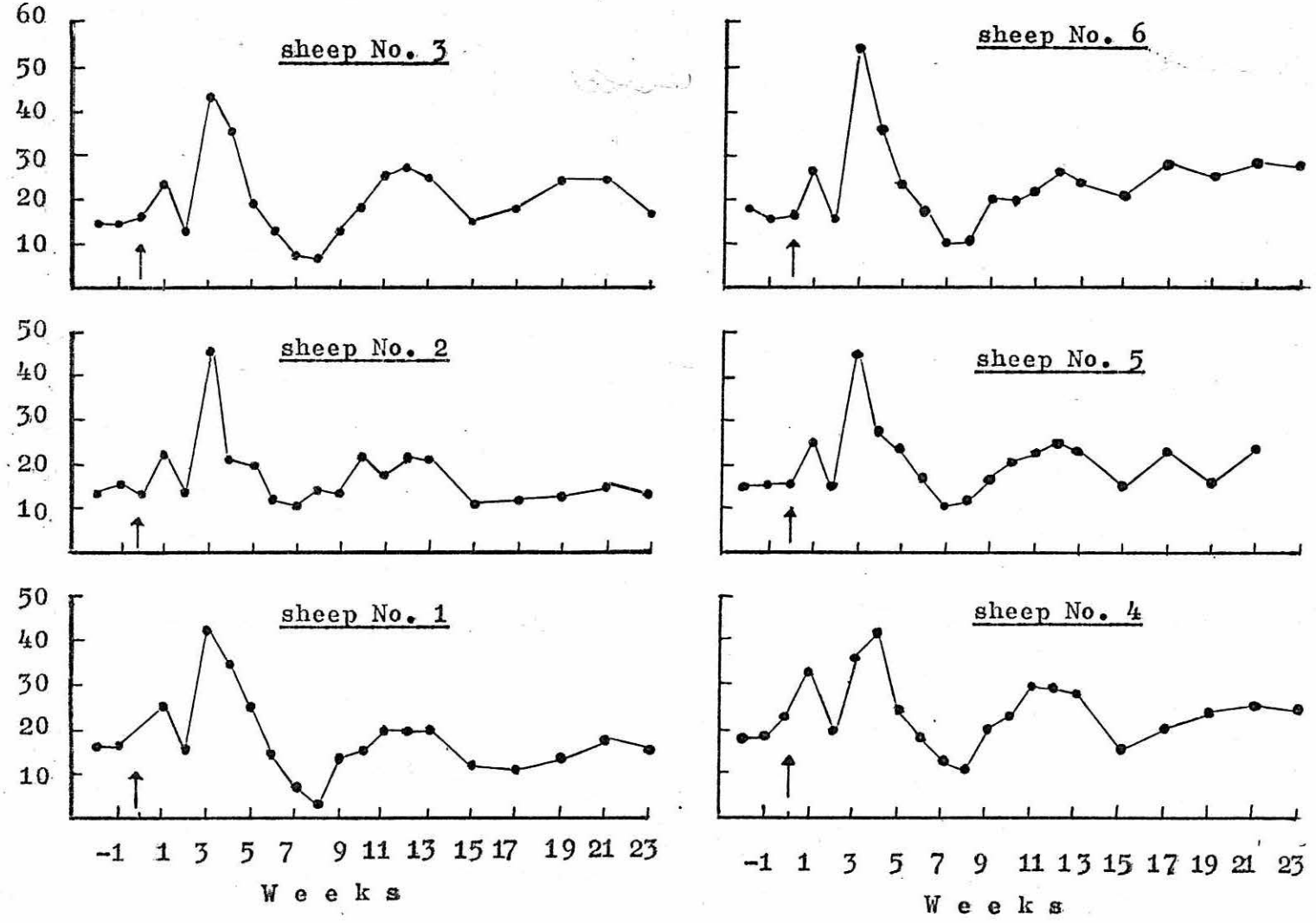


Fig 29. Plasma seromucoid (as hexoses) in sheep Nos. 1-6, each infected with 518 F. hepatica cysts. ↑ = infection.

Table IX Ratios of plasma glycoprotein components to plasma total protein in sheep infected with F. hepatica. T.H.=total hexoses; Hine=hexosamine; S.A.=sialic acid; F=fucose; S.M.=seromucoid. (Mean values).

Wks.	TH/P	Hine/P	S.A/P	F/P	S.M/P
-2	1.56	1.34	1.50	0.30	0.23
-1	1.60	-	1.50	-	0.23
0	1.67	1.40	1.57	0.31	0.25
1	1.56	1.39	1.60	0.28	0.40
2	1.75	1.91	1.60	0.34	0.23
3	1.51	1.43	1.69	0.36	0.62
4	1.33	1.37	1.70	0.40	0.43
5	1.27	1.40	1.72	0.20	0.28
6	1.92	1.52	1.64	0.67	0.20
7	1.42	1.53	1.70	0.80	0.13
8	1.44	1.04	1.52	0.52	0.12
9	1.54	-	1.54	0.65	0.19
10	1.53	1.49	1.55	0.68	0.24
11	1.57	1.60	1.59	0.71	0.30
12	1.65	1.32	1.67	0.75	0.34
13	1.48	1.59	1.80	0.74	0.33
14	-	-	-	-	-
15	1.39	1.62	1.56	0.83	0.24
16	-	-	-	-	-
17	1.76	1.86	2.06	1.10	0.32
18	-	-	-	-	-
19	1.76	1.92	2.49	0.45	0.40
20	-	-	-	-	-
21	1.83	1.31	2.16	0.38	0.43
22	-	-	-	-	-
23	1.68	1.62	2.45	0.50	0.42

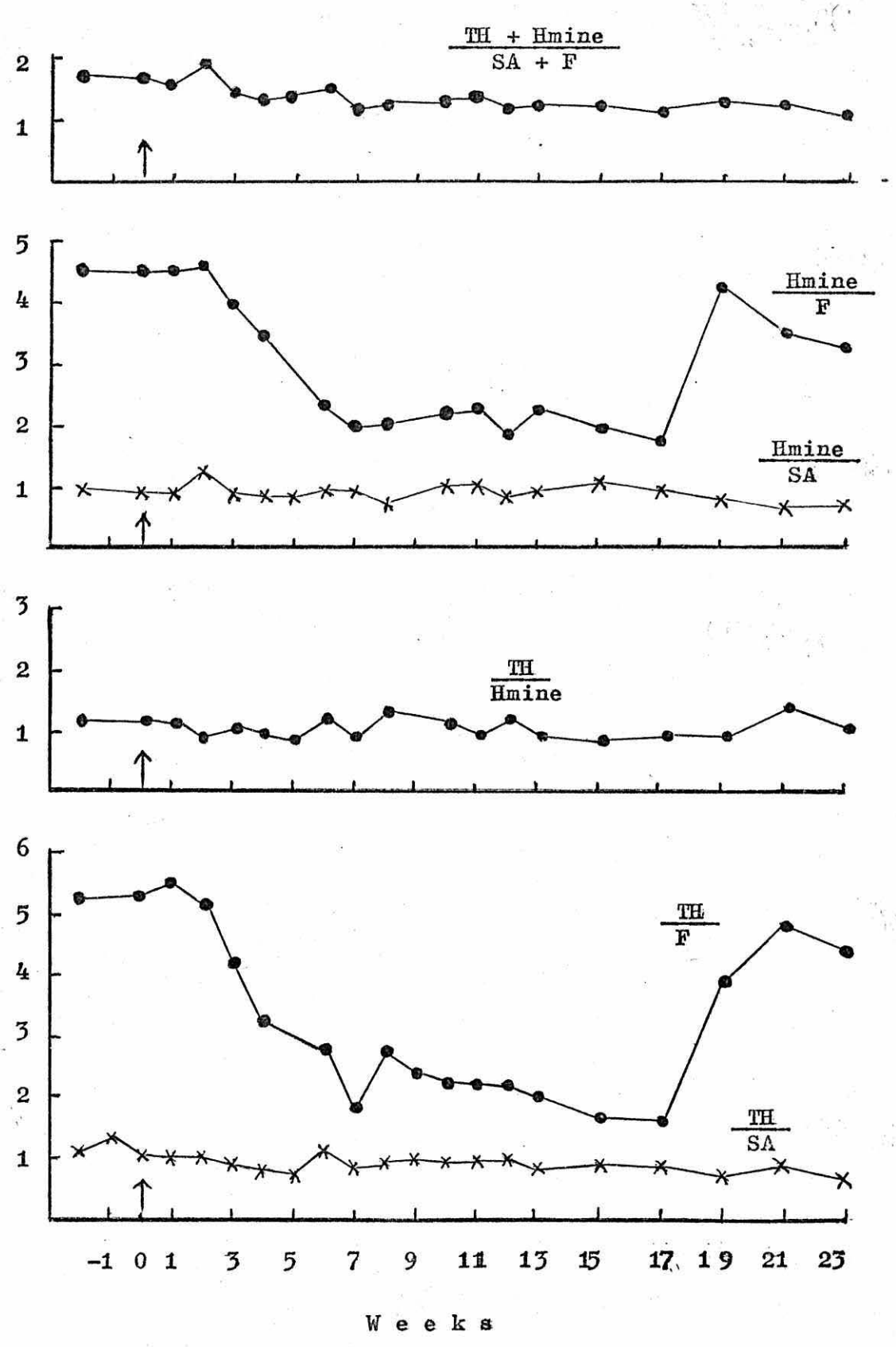


Fig. 30. Ratios of:plasma total hexoses (TH) or hexosamine (Hmine) to sialic acid (SA) or fucose (F); $\frac{TH}{Hmine}$; $\frac{TH + Hmine}{SA+F}$. ↑ =infection.

Table X Ratios of plasma total hexoses (T.H) to sialic acid (S.A), fucose (F) or hexosamine (Hine); Hexosamine to sialic acid or fucose; Total hexoses + Hexosamine to fucose + sialic acid (Mean values) in sheep infected with F. hepatica.

Weeks	T.H/S.A	T.H/F	T.H/Hine	Hine/S.A	Hine/F	$\frac{T.H+Hine}{S.A.+F}$
-2	1.08	5.27	1.16	0.93	4.54	1.67
-1	1.38	-	-	-	-	-
0	1.06	5.30	1.19	0.89	4.46	1.63
1	0.98	5.51	1.12	0.87	4.92	1.57
2	1.09	5.17	0.91	1.19	5.65	1.89
3	0.90	4.18	1.06	0.85	3.94	1.43
4	0.78	3.30	0.97	0.81	3.42	1.29
5	0.73	6.42	0.90	0.81	7.09	1.39
6	1.17	2.85	1.26	0.92	2.25	1.48
7	0.84	1.78	0.93	0.90	1.91	1.18
8	0.95	2.76	1.38	0.69	2.00	1.22
9	1.00	2.38	-	-	-	-
10	0.99	2.24	1.03	0.95	2.18	1.35
11	0.98	2.20	0.98	1.01	2.25	1.37
12	0.97	2.20	1.25	0.78	1.76	1.22
13	0.82	2.01	0.93	0.88	2.15	1.21
15	0.89	1.67	0.86	1.03	1.95	1.26
17	0.85	1.60	0.95	0.90	1.69	1.14
19	0.70	3.90	0.91	0.77	4.26	1.25
21	0.85	4.85	1.40	0.60	3.46	1.23
23	0.69	3.37	1.03	0.66	3.25	1.12

In general, the changes observed in the plasma glycoprotein components were not very different in all animals and the description given below will apply mainly to the mean values recorded.

Total hexoses:

Total hexoses showed minor changes during the preinfection period and the first 5 weeks post-infection. The values rose to a peak at week 6, fell at week 7 and were further elevated till the 9th week. Subsequently the total hexoses steadily decreased and after the 15th week they fluctuated widely and the downward trend was slight.

The hexose-protein ratio slightly declined between week 3 and 5 post-infection, increased at week 6 and then remained almost constant till the 15th week. Afterwards the levels increased again and were maintained constant till the end of the experiment.

Hexosamine:

There was little change in the plasma hexosamine content during the preinfection period. They increased to a sharp peak at week 2, declined at week 3 and again increased till the 7th week. A sharp drop was observed at week 8 followed by an increase. After the 11th week the levels slightly dropped and fluctuated widely till the 17th week and then further declined.

The hexosamine-protein ratio increased in the 19 weeks following infection and this was very slow during the first 13 weeks.

Sialic acid:

Again slight changes in plasma sialic acid content was observed in the preinfection period and the first 2 weeks of infection.

The levels increased sharply till the 5th week, then fell slightly and fluctuated around a constant level till the 13th week. A decrease to preinfection levels was observed at week 15 and this was followed by another sharp rise at week 17. After week 19 the sialic acid fell but only slightly.

The sialic acid/protein ratio increased very slowly till week 7, returned to initial levels a week later and again increased thereafter. The rise was more pronounced during the terminal 8 weeks, being about 60% of preinfection level at the end of the experiment.

Fucose:

The plasma fucose content increased slightly from the 1st week of infection to the 4th week, dropped at week 5 and once more increased. A high level (175% above initial levels) was maintained till week 17 and then the levels fell to pre-infection figures.

The fucose/protein ratio showed changes which corresponded with those described above.

Seromucoid:

The plasma seromucoid levels increased to a small peak at the first week of infection, declined by the 2nd week and sharply increased to about three-fold preinfection levels, by week 3. The levels then gradually decreased to below initial levels by about the 8th week and once more increased till the

12th week. The seromucoid content then declined to normal levels and then very slowly increased till the end of the experiment.

The seromucoid/protein ratio showed corresponding changes.

2. Cows:

a) Electrophoretic fractions:

The percentage glycoprotein values in cows Nos. R and B can be seen in Table XI and Fig.31.

Cow No. R:-

The percentage albumin glycoprotein values increased between weeks 7 and 11 with a peak at week 9. The α-globulins were elevated between the first and 4th week of infection, returned to preinfection values by week 5 and again slightly increased till week 7. This was followed by a decline till week 12.

The β-globulins behaved oppositely to the α-globulins. The γ-globulins, on the other hand did not appreciably change except for a slight rise during the 7th and the 11th weeks.

Cow No. B:-

The albumin glycoproteins also slightly increased from the 6th to the 9th week of infection then dropped and remained nearly constant till the end of the observation.

The α-globulin glycoproteins decreased at the first week of infection, returned to normal levels by the 2nd week and again declined by the 4th week. This was followed by a further

Table XI Electrophoretic distribution of plasma glycoprotein fractions in cows infected with F. hepatica.

Wks	Cow R				Cow B			
	Alb. %	Globulins % α-	β-	γ-	Alb. %	Globulins % α-	β-	γ-
-2	2.13	54.04	29.79	14.04	-	-	-	-
-1	0.63	42.01	36.99	20.38	2.44	47.86	29.57	20.12
0	2.36	48.95	32.20	16.49	2.81	48.07	27.02	22.10
1	-	-	-	-	-	-	-	-
2	2.70	45.94	39.77	11.58	1.91	37.26	28.98	31.85
3	0.75	60.38	24.90	13.96	2.17	41.54	32.12	24.15
4	1.94	61.16	20.39	16.50	3.46	48.70	26.51	21.32
5	1.59	47.45	35.99	14.97	0.48	37.08	42.82	19.62
6	1.23	50.10	35.32	13.35	2.75	41.21	36.26	19.78
7	2.91	52.32	30.23	14.53	3.06	42.51	34.56	19.88
8	3.54	53.69	26.55	16.22	4.38	45.17	34.21	16.23
9	-	-	-	-	-	-	-	-
10	5.19	49.61	20.78	24.41	4.81	45.19	30.77	19.23
11	2.05	43.95	35.38	18.62	6.54	40.65	32.52	20.32
12	-	-	-	-	-	-	-	-
13	2.17	51.01	32.25	14.57	3.42	36.75	42.73	17.09
14	-	-	-	-	-	-	-	-
15	3.54	-	-	-	-	-	-	-

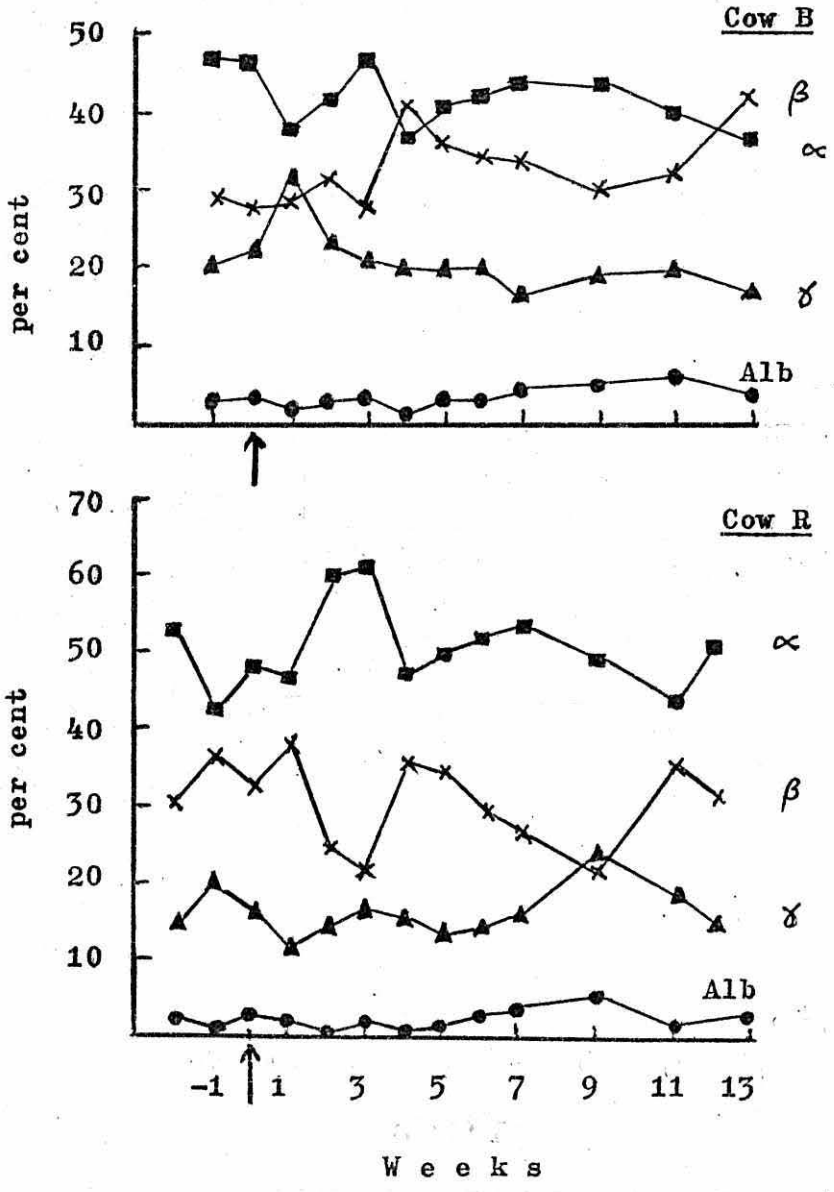


Fig. 31. Percentage values of plasma glycoproteins in cow No. R and cow No. B, each infected with 1880 F. hepatica cysts. ↑ = infection.

increase till week 7 and the values decreased after week 9. Again the β -globulins glycoproteins responded oppositely to the α -globulins. The γ -globulins glycoprotein increased at the first week, was normal by the 2nd week and remained almost constant will the end of the experiment. However, the levels tended to be low between week 6 and 9 post-infection.

b) Protein-bound carbohydrate components:

The plasma total hexoses, hexosamine, sialic acid, fucose and seromucoid in cows Nos. R and B infected with F. hepatica can be seen in Table XII and Figs. 32-36. The ratios of these protein-bound sugars to plasma total proteins are presented in Table XIII (also Figs. 32-36). The ratios of either total hexoses or hexosamine to fucose are shown in Table XIV and Fig. 37.

Total hexoses:

In both cows the total hexoses sharply dropped at week 2 and 3 of infection and then increased by week 4. After a decrease at week 5 the levels were elevated till week 11. This was more pronounced in cow No. R. After the 13th week the levels decreased slightly in cow No. R but sharply in cow No. B. The hexose/protein changes followed a similar pattern.

Hexosamine:

In cow No. R the plasma hexosamines decreased sharply at the 2nd week of infection, increased to slightly below pre-infection levels at weeks 3 and 4 and then declined but slightly. At week 8 the levels were normal again and at week 11

Table XII Plasma glycoprotein components (mg/100ml) in cows Nos. R and B infected with F. hepatica.

Wks.	Total hexoses		Hexosamine		Sialic acid		Fucose		Sero-mucoid	
	R	B	R	B	R	B	R	B	R	B
-2	118.7	126.3	-	-	96.0	97.0	18.00	31.30	11.62	12.00
-1	118.6	124.0	124.7	128.6	95.7	91.5	28.68	34.90	17.58	14.46
0	122.8	114.8	127.8	129.9	95.4	82.1	30.00	32.50	15.06	9.90
1	-	-	-	-	-	-	-	-	-	-
2	104.0	96.4	88.1	98.6	109.0	88.2	31.56	30.26	41.07	32.12
3	98.2	98.2	113.6	122.3	126.7	103.2	22.08	13.92	13.9	17.6
4	149.5	132.6	120.2	103.6	111.3	84.6	49.20	53.60	16.58	8.05
5	109.8	107.8	109.1	104.4	113.9	97.2	44.50	37.11	7.52	4.21
6	106.2	108.8	107.4	121.2	116.8	94.5	47.21	47.87	6.80	4.00
7	125.9	122.7	-	-	111.5	92.5	53.33	46.46	13.64	-
8	130.1	111.2	129.7	111.2	108.8	78.3	59.60	47.60	21.84	11.65
9	-	-	-	-	-	-	-	-	-	-
10	127.8	113.8	70.6	89.4	108.9	80.9	73.33	58.82	22.68	17.83
11	137.3	120.0	141.5	119.7	141.5	95.6	64.74	53.81	22.15	14.77
12	-	-	-	-	-	-	-	-	-	-
13	121.8	92.2	142.5	116.7	116.1	74.4	69.36	48.09	14.81	8.27
14	-	-	-	-	-	-	-	-	-	-
15	125.6	99.0	138.6	116.0	132.5	80.0	76.00	50.00	16.29	8.5

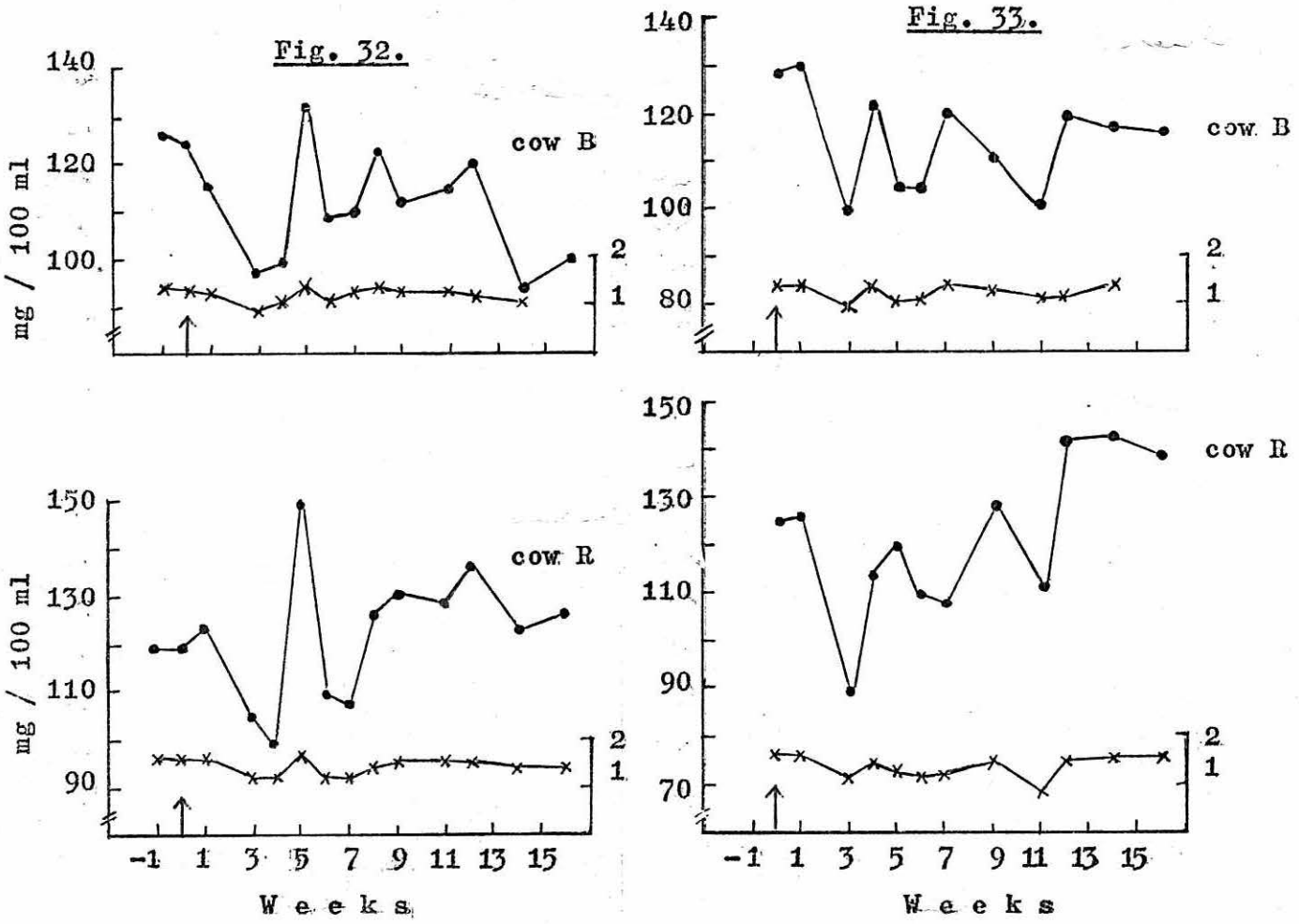


Fig. 32. Plasma total hexoses in cows R & B, each infected with 1880 F. hepatica cysts. ●—● = total hexoses; ×—× = ratio of total hexoses to total proteins.

Fig. 33. Plasma hexosamine in cows R & B, described above. ●—● =hexosamine; ×—× = ratio of hexosamine to total proteins. ↑ = infection.

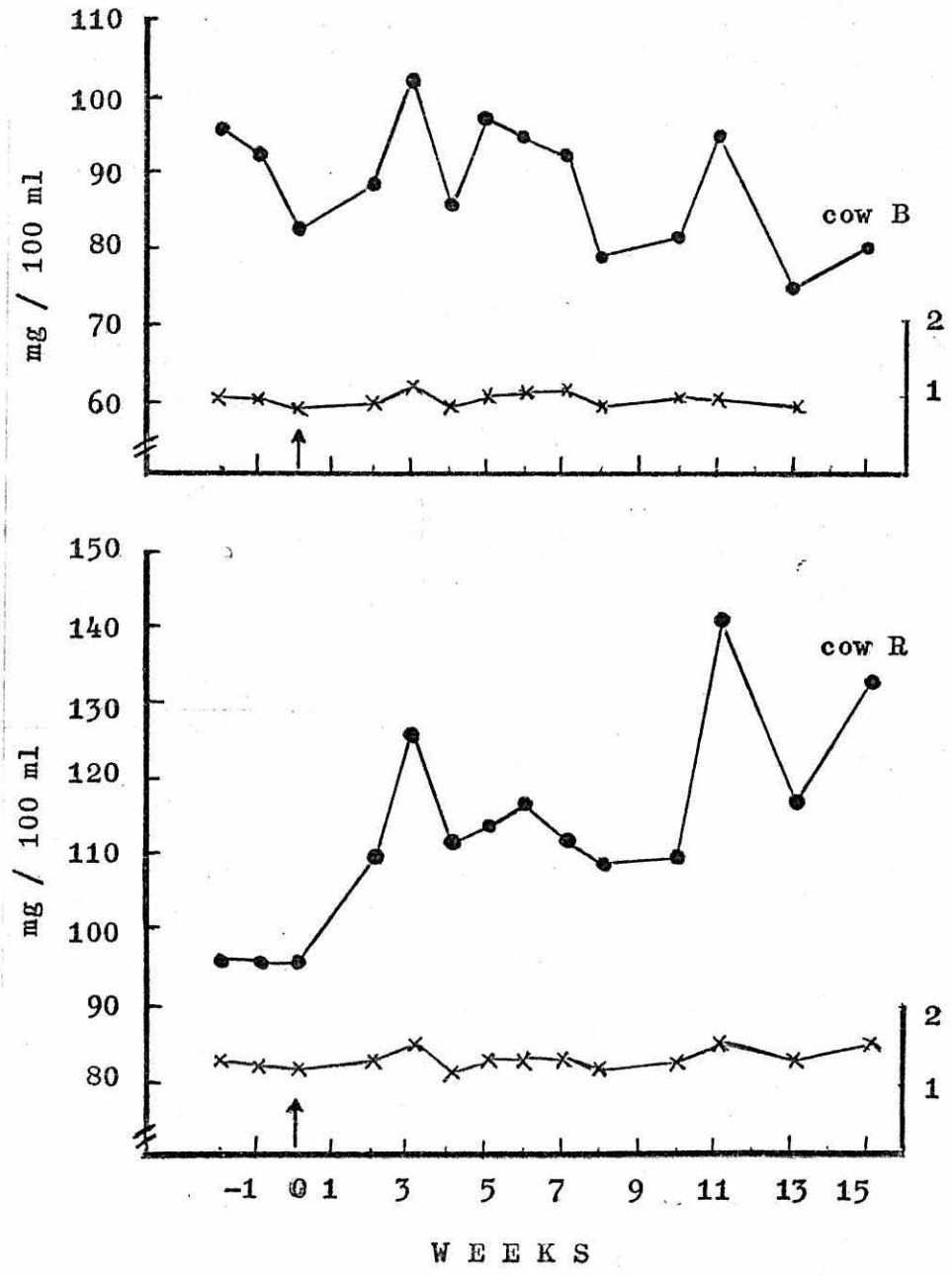


Fig. 34. Plasma sialic acid concentrations (●——●) in cows R & B, each infected with 1880 F. hepatica cysts. x——x = ratio of sialic acid to total proteins. ↑ = infection.

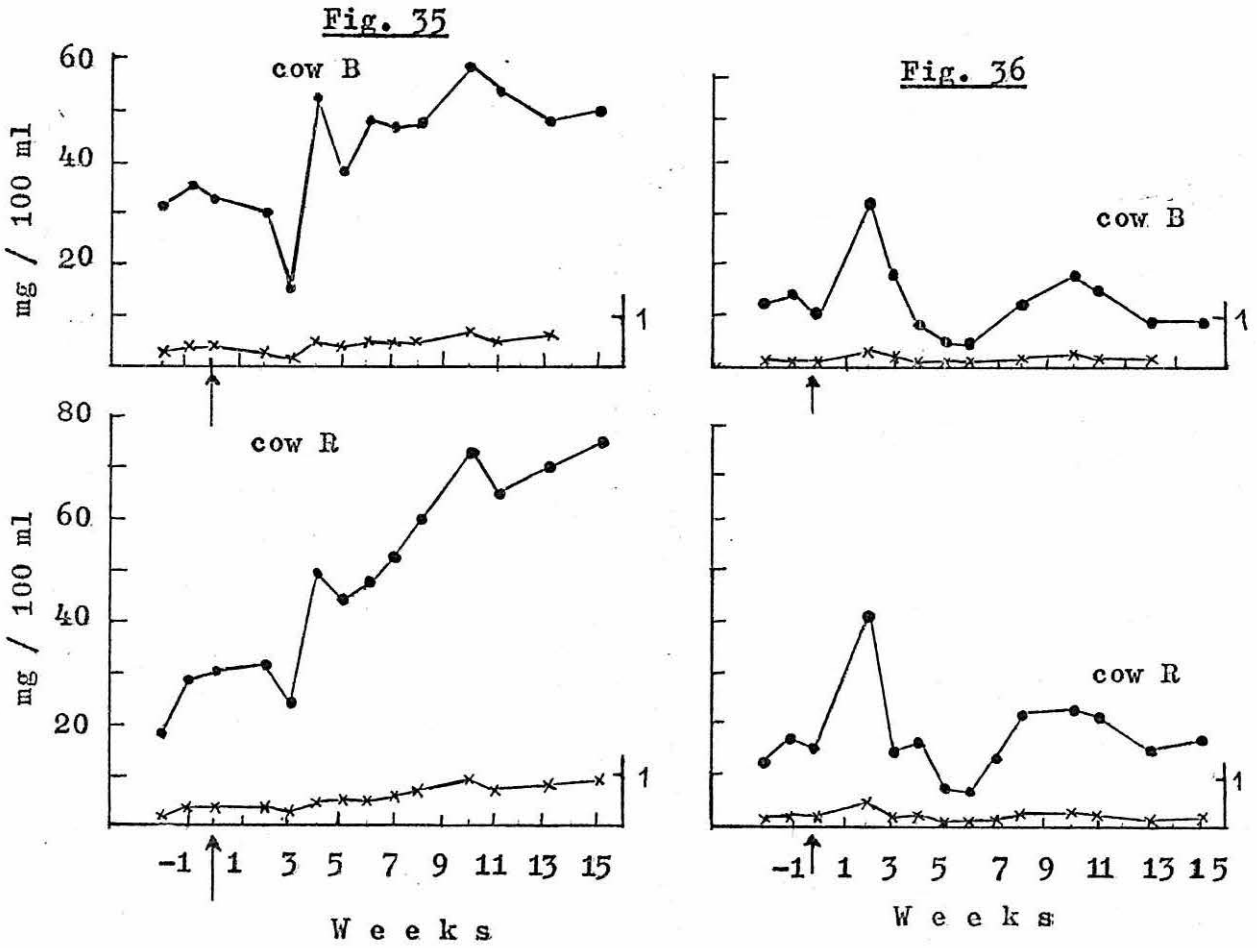


Fig. 35. The plasma fucose (o o) and fucose/total protein ratio (x x) in cows R & B, each infected with 1880 F. hepatica cysts. = infection.

Fig. 36. The plasma seromucoid (o o) and seromucoid/total protein ratio (x x) in cows R & B, each infected with 1880 F. hepatica cysts. ↑ = infection.

Table XIII. Ratios of plasma glycoprotein to protein in cows infected with F. hepatica. T.H. = total hexoses; Hamine = Hexosamine; S.A. = sialic acid; F = fucose; SM = seromucoid. P = total proteins. R and B = No. of the two cows.

Weeks	T.H./P		Hamine/P		S.A./P		F/P		SM/P	
	R	B	R	B	R	B	R	B	R	B
-2	1.6	1.4	-	-	1.3	1.1	0.2	0.3	0.15	0.13
-1	1.6	1.4	1.6	1.4	1.2	1.0	0.4	0.4	0.20	0.13
0	1.6	1.3	1.6	1.4	1.2	0.9	0.4	0.4	0.19	0.11
1	-	-	-	-	-	-	-	-	-	-
2	1.2	0.9	1.1	1.0	1.3	0.9	0.4	0.3	0.19	0.33
3	1.2	1.1	1.4	1.4	1.5	1.2	0.3	0.2	0.17	0.20
4	1.7	1.4	1.3	1.1	1.2	0.9	0.5	0.5	0.19	0.08
5	1.2	1.1	1.2	1.1	1.3	1.0	0.5	0.4	0.08	0.04
6	1.2	1.3	1.2	1.4	1.3	1.1	0.5	0.5	0.08	0.05
7	1.4	1.4	0.5	0.4	1.3	1.1	0.6	0.5	0.15	-
8	1.5	1.3	1.5	1.3	1.2	0.9	0.7	0.5	0.25	0.13
9	-	-	-	-	-	-	-	-	-	-
10	1.5	1.3	0.8	1.1	1.3	1.0	0.9	0.7	0.27	0.21
11	1.5	1.2	1.5	1.2	1.5	1.0	0.7	0.5	0.24	0.15
12	-	-	-	-	-	-	-	-	-	-
13	1.4	1.1	1.6	1.4	1.3	0.9	0.8	0.6	0.17	0.10
14	-	-	-	-	-	-	-	-	-	-
15	1.4	-	1.6	-	1.5	-	0.9	-	0.19	-

Table XIV Ratios of total hexoses (T.H) or hexosamine (Hine) to fucose (F) in cows Nos. R and B infected with F. hepatica.

Weeks	Cow No. R		Cow No. B	
	TH/F	Hine/F	TH/F	Hine/F
-2	6.59	-	4.03	-
-1	4.13	4.35	3.55	3.68
0	4.09	4.26	3.53	4.00
1	-	-	-	-
2	3.29	2.79	3.18	3.26
3	4.45	5.14	7.05	8.78
4	3.04	2.44	2.47	1.93
5	2.47	2.45	2.90	2.81
6	2.25	2.27	2.27	2.53
7	2.36	-	2.64	-
8	2.18	2.18	2.34	2.34
9	-	-	-	-
10	1.74	0.96	1.93	1.52
11	2.12	2.18	2.23	2.22
12	-	-	-	-
13	1.76	2.05	1.92	2.43
14	-	-	-	-
15	1.65	1.82	1.98	2.32

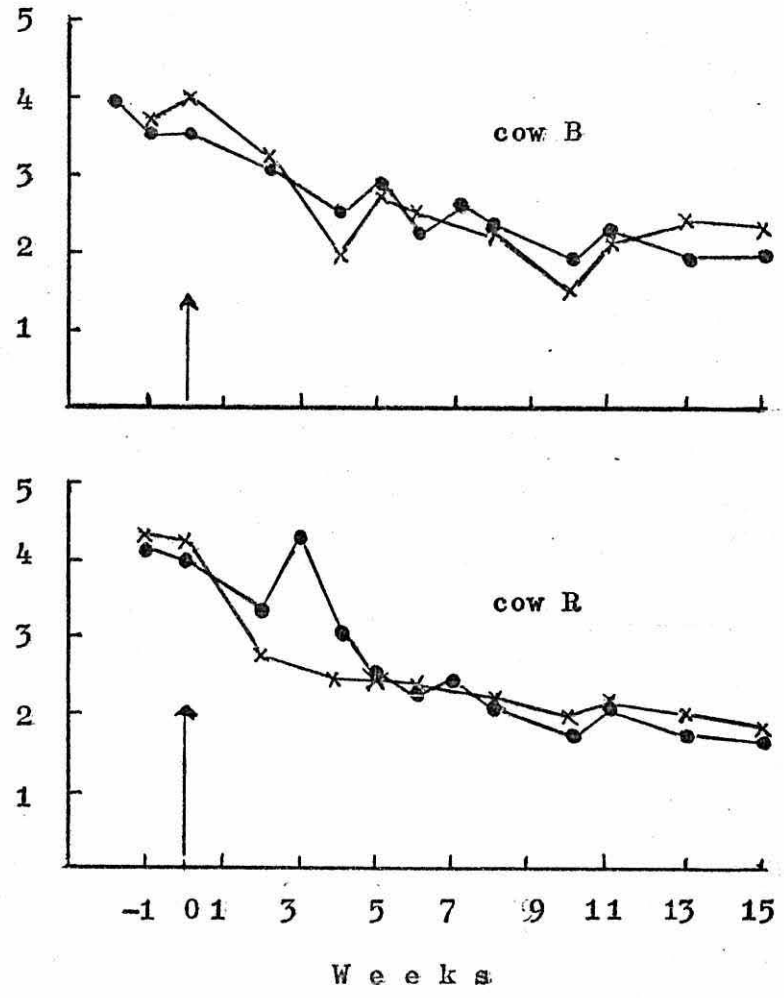


Fig. 37. Ratios of the plasma total hexoses (●—●) or hexosamine (x—x) concentrations to fucose in cows R & B, each infected with 1880 F. hepatica cysts. ↑ = infection

they were above preinfection values and remained constant till the end of the experiment.

In cow B the plasma hexosamine decreased a little by the 2nd week of infection and thereafter fluctuated widely till the end of the experiment. The hexosamines showed similar alterations.

Sialic acid:

The changes were more marked in cow No. R. The plasma sialic acid increased after infection to a first peak at week 3, declined at week 4 and then showed a slight downward trend till the 10th week. Maximum levels were observed at week 11. In cow B the plasma sialic acid content fluctuated widely but with a general downward trend. The sialic acid-protein ratios corresponded with changes in sialic acid in each cow.

Fucose:

Again cow R showed more obvious changes. The fucose levels increased steadily after an initial decrease at week 3 till the end of the experiment. Two peaks were observed, the first at week 4 and the other at week 10. In cow No. B the plasma fucose showed similar changes as in cow R but they were less marked especially after the 6th week of infection. The fucose/protein ratio followed a similar trend.

Seromucoid:

The changes were very similar in the two cows. The levels were sharply elevated at week 2 and then steadily decreased till week 6. Another increase was noticed afterwards to a maximum at the 10th week. Subsequently the levels decreased. A similar pattern was noticed for the seromucoid-protein ratio.

C. The Plasma Lipoproteins:

Sheep:

The weekly percentages of the different lipoprotein fractions of a plasma pool collected from 6 sheep infected with F. hepatica are presented in Table XV and Fig. 38.

The electrophoretic separation revealed 2 α-lipoprotein fractions and one β-fraction. The α-$_1$ lipoproteins increased slightly till the 3rd week of infection and then markedly decreased till the 8th week. This was followed by an increase till week 11 and then the levels decreased again. The α-$_2$ lipoproteins behaved similarly but they started to drop a week earlier and their levels approached pre-infection figures at weeks 12, 13 and 17. The β-lipoproteins, on the other hand, responded oppositely.

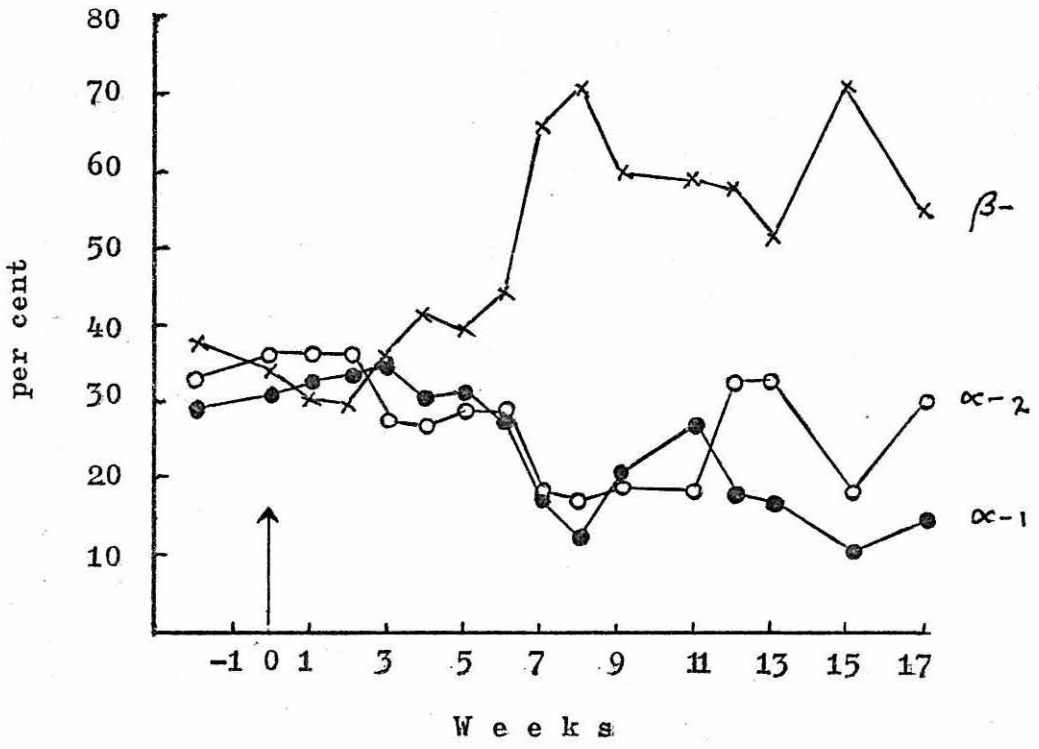


Fig. 38. Lipoprotein fractions in pooled samples of plasma obtained from 6 sheep, each infected with 518 F. hepatica cysts. ↑ = infection.

Table XV Plasma lipoproteins in sheep infected with *F. hepatica*

Weeks	Globulins %		
	α-$_1$	α-$_2$	β -
-2	28.56	33.11	38.33
-1	-	-	-
0	30.36	34.94	34.70
1	32.78	36.37	30.85
2	34.44	36.30	29.26
3	35.62	28.33	36.05
4	30.53	27.37	42.10
5	31.20	29.32	39.47
6	27.78	28.57	43.65
7	17.00	17.09	65.91
8	12.12	17.00	70.88
9	19.82	19.82	60.36
10	-	-	-
11	23.59	18.46	59.20
12	16.91	33.09	57.95
13	16.91	33.09	51.00
14	-	-	-
15	10.27	17.80	71.92
16	-	-	-
17	14.61	30.59	54.79

DISCUSSION

The Plasma Proteins:

The first experiment in sheep showed that a difference exists in the plasma protein profile between animals infected with a single dose of metacercariae and those receiving the divided dose. This could be very much related to differences in fluke burdens, as more flukes were recovered from the former animals, average of 345 flukes as compared to 258 in the latter group. This denotes that the sheep infected with a single dose might have experienced more liver damage and accordingly showed a greater response in plasma proteins. Animal No. 11 of the reinfected group had the least fluke burden and showed very little changes from normal.

The animals given the divided dose had double the number of metacercariae given to those infected with a single dose and the low fluke recovery in the former group suggests that a certain degree of resistance might have developed against the second inoculum. Reduced fluke recoveries have been reported in reinfected sheep (Boray, 1967; Sinclair, 1970b).

The second sheep experiment was continued for a longer period than the first one and it can be seen that the initial hyperproteinaemia, which was mainly due to elevated α- and γ- globulins, was soon reversed to a hypoproteinaemia after the 15th week of infection. The α- and γ-globulins returned to preinfection levels and the hypoproteinaemia was the result of a marked decrease in albumin associated with a slight decline in β-globulins. So, it is evident that the prepatent period is characterised by

elevated total proteins and globulins while in the patent phase these parameters decrease.

In general the present findings confirm those of many other reports and comparison will be restricted to a few of those. Sinclair (1962) detected similar changes in the serum proteins but the total proteins and globulins started to increase a little later in the prepatent period and decreased rather earlier in the patent period than in the present observation. Furmaga and Gundlach (1967) reported early changes in serum proteins but they did not notice any alteration in the α-globulin fraction. Similarly, Roberts (1968) observed no change in the α-globulin fraction. Kadhim (1976) infected sheep with F. gigantica and found that the serum albumin decreased rather late in infection, i.e. at the 10th week and this was associated with a concomitant increase in γ-globulins. The β-globulins increased after the 18th week while no change was observed in total proteins.

The present investigation showed that cows infected with F. hepatica exhibited different plasma protein pictures when compared to the above changes in sheep. In cow No. R the total proteins increased till the 4th week of infection and was maintained high till the end of the experiment. This was mainly due to increase in albumin, α - and β-globulins. The γ-globulins markedly decreased between the 1st and 4th week of exposure and remained slightly lower than pre-infection levels afterwards. In cow No. B the total proteins remained unchanged or slightly decreased during the course of

infection. The albumin and γ-globulins also showed very little changes. The α-globulins increased while the β-fraction decreased and remained low after the 5th week of infection. The above changes in cows seem to vary with the findings of Ross et al. (1966), Doyle (1971), Haroun and Hussein (1976) and Anderson et al. (1977) who reported a hypoalbuminaemia accompanied by an increase in γ-globulins in calves infected with F. hepatica. The different picture observed in cows of this experiment could be related to the very low fluke recoveries (94 flukes in cow B and 157 flukes in cow R) despite the evident pathological changes observed in the livers of these animals.

The hypoalbuminaemia observed in infected sheep of the current study can be explained on certain lines: a) increased albumin catabolism, b) decreased albumin synthesis, c) haemodilution. It has been shown by radio-isotope studies that the albumin turnover rates in rabbits infected with F. hepatica are changed only slightly during the migratory phases of the parasite but increase markedly when the flukes enter the bile ducts (Dargie et al., 1968; Dargie and Mulligan, 1971). Significant loss of proteins occur in rabbits, sheep and cattle due to the haematophagic activities of the flukes and to leakage of blood through the hyperplastic and inflamed bile ducts (Dargie and Mulligan, 1971; Nansen, 1970, 1971). Dargie (1973) reported that about 10gm of blood albumin are degraded daily in fluke infected sheep compared to about 4gm in controls, but this had little effect on the plasma albumin concentrations. He also stated that the concentration of extravascular albumin was reduced

in the course of infection indicating that the intravascular levels were constantly maintained from the extravascular pool.

To compensate for the lost albumin the liver will be stimulated to synthesise more of the proteins, and both intra- and extravascular levels could be maintained. Dargie (1973) concludes that the rate of albumin synthesis is actually increased in fluke infection. However, if the rate of albumin loss exceeds that of synthesis then the body will be in a negative balance. The rate of albumin synthesis is liable to decrease in fascioliasis and this will greatly depend upon the extent of liver damage and stage of the disease.

The plasma albumin levels may, however, be normal or insignificantly decreased but in the presence of haemodilution the albumin concentration per unit volume of the expanded blood would be low and give the impression of a marked hypoalbuminaemia; haemodilution has been observed in sheep infected with 1000 metacercariae of F. hepatica but not with 600 cysts (Berry and Dargie, 1978). A similar phenomenon might have occurred in sheep under the present investigation.

As has been mentioned the plasma albumin levels in the two cows were either not affected or slightly elevated. This could be attributed to the mild nature of infection and the light fluke burden observed in the two animals. In other words there was less blood loss and leakage through bile ducts, and adequate hepatic functional capacity, in terms of intact liver tissue, to synthesise albumin. However, increase in serum albumin has been observed in fluke-infected cattle (Romaniuk, 1975) and sheep (Banting et al., 1975). Sinclair

(1968, 1970) also noticed an initial increase in serum albumin in F. hepatica infected sheep treated with corticosteroids.

In sheep, the α- and γ-globulins were also shown to increase in this study during the prepatent period but returned to normal in the patent period. The β-globulins, on the other hand, were little affected. The α-globulins comprise many specific proteins (Turner and Hulme, 1971) and an increase in one or more of these components may lead to increase in the α-globulin fractions. However the α-globulins are said to increase unspecifically in many traumatic and disease conditions. The rise observed in the γ-globulins generally denotes antibody production against the invading parasite and, probably, against the toxic products they may secrete. Unlike albumin, the immuno-globulins are mainly synthesised by cells of the reticulo-endothelial system and their production is not crucially affected by liver damage. It should be mentioned that the immunoglobulins are also catabolised more than normal in fluke infection but their synthesis is greatly accelerated (Nansen, 1971). Contrary to the findings in sheep, no significant alterations were observed in the globulin fractions in the two infected cows investigated. The relatively low γ-globulin concentrations observed might indicate inadequate stimulation of the reticulo-endothelial system to produce antibodies.

The Plasma Glycoproteins:

Under normal conditions of health, the plasma glycoproteins are maintained constant but may be strikingly altered in disease conditions; some of these conditions, in both man and animals, have been previously reviewed (p.143).

As discussed above, the plasma proteins could undergo significant changes in fascioliasis. Since the plasma proteins are mostly glycoproteins a change in the carbohydrate moiety is very likely to occur, and this has been observed in the present study. In the sheep the electrophoretic separation of the plasma glycoproteins showed that the albumin fraction had the least intensity, average of less than 6%, and reflects a successful staining procedure. It was also observed that the percentage of albumin glycoproteins decreased throughout the experimental period in infected animals which seems to correspond with the decrease in plasma albumin concentrations in these animals. The α-globulin glycoprotein, on the other hand, constituted the highest proportion and contrary to albumin increased during the course of infection. This fraction contains most of the plasma conjugated carbohydrates (Channon and Anastassiadis, 1971; Jonsson and Wales, 1976) and comprises many individual glycoprotein components. These components, which include orosomucoid, α_1-antitrypsin, ceruloplasmin, haptoglobulins and α_2-macroglobulins, may increase during infection as part of the acute "phase reactants" (Snyder et al., 1975) and contribute to the high α-globulin glycoprotein levels. The acute "phase reactants" indicate an

abnormal state of excessive cellular proliferation and depolymerisation of connective tissue ground substance (Cameron, 1976); both processes may occur in the liver in fascioliasis. A hyper α-glycoglobulinaemia was reported by Goryacheo (1968) in F. hepatica infected sheep during the 2nd to the 5th week of infection and the levels returned to normal after treatment.

The β- and γ-globulin glycoproteins seem to react oppositely to each other. The γ-globulin glycoproteins seem to increase initially during the first 4 weeks of infection, declined to slightly below normal values till week 12 and again increased during the terminal 6 weeks. The initial increase would correspond to the rise observed in the γ-globulins which was explained before to be due to increased production of antibodies: Antibodies contain substantial quantities of protein-bound carbohydrates (Lehninger, 1975) and this may explain the increase in the γ-globulin glycoprotein observed. The decrease that followed afterwards was unexpected and is difficult to explain. However, the β- and γ-globulin glycoproteins migrated close to each other and on many occasions were very much overlapping and it is possible that this has led to some error in their evaluation. The changes observed in the electrophoretic glycoprotein fractions in the two cows were not very marked and also not consistent. Again this could be related to the light infection in these cows.

The present investigation also showed marked changes in the plasma protein-bound hexoses, hexosamine, sialic acid, fucose and seromucoid in all sheep and in one cow No. R; cow

No. B showed variable responses. Most of the discussion will be based on the sheep results. All sugars increased during the course of infection but the elevations were not in a parallel fashion. The total hexoses were elevated between the 5th and 13th week of infection, hexosamines between the 1st and 19th weeks, sialic acid increased almost throughout the experimental period starting the 2nd week, fucose from the 5th to the 19th week and seromucoid during the first 6 weeks of infection. In other words all the sugars increased during the prepatent and patent periods except the seromucoids which increased markedly in the prepatent phase. The ratios of hexoses, hexosamines and sialic acid to total proteins, on the other hand, were not changed when the sugar total concentrations were elevated but the ratios were increased when the concentrations were decreased. This suggests that hexoses, hexosamines and sialic acid increase in proportion to total plasma proteins during illness but in late infection their concentrations may exceed those of plasma proteins. This increase could be due to increased synthesis or, more likely, to release of glycoproteins from damaged liver tissue into the circulation. In case of fucose and seromucoid their concentrations changed proportionally to the levels of plasma proteins throughout the experimental period. Since all these sugars are protein-bound their ratios to total proteins may offer a more appropriate measure to glycoprotein changes in disease. Increased ratios may also denote the presence of unbound forms of these sugars in the circulation.

In most investigations the serum glycoproteins are

expressed as protein-bound total hexoses or hexosamines. Depending upon which one of the two monosaccharides was used different glycoprotein levels may be obtained. This is indicated by the finding that in advanced cancer an increase of 25% in serum glycoproteins was obtained when estimated as hexosamine but the increase was about 50% when the concentrations were expressed as total hexoses. Similarly in rheumatic fever the glycoprotein concentrations, estimated as hexosamine, increased very little but when estimated as total hexoses the concentrations increased by a 100% (see Mehta and Vankataraman, 1975). The present study indicates that the hexoses and hexosamine concentrations were comparable to a greater extent and the changes they exhibited were not very much different. This is indicated by the total hexoses/ hexosamine ratios which roughly fluctuated around 1. These results suggest that both monosaccharides can be used to estimate the glycoprotein changes in F. hepatica infection.

The sialic acid and fucose were also elevated particularly the latter which increased by over 150% of the preinfection levels. It is known that sialic acid occupies the terminal monosaccharide unit of the branched glycoprotein side chains (Lehninger, 1975), and that the terminal sialic acid may be replaced by fucose (Winzler, 1965; Channon and Anastassiadis, 1971). From the circumstances of this investigation it appears that the production of fucose was very much favoured to occupy the terminal units of glycoproteins. Sialic acid and fucose constitute the terminal units of glycoproteins and hexoses and hexosamine represent the interior units and the ratio of the two unit categories

gives an indication of the degree of carbohydrate branching (Channon and Anastissiadis, 1971). This ratio was calculated for the current data and results imply that more branching of the carbohydrate moiety occurred and this suggests that quantitative as well as qualitative changes occur in plasma glycoproteins in F. hepatica infection, both in sheep and cattle. The carbohydrate branching will be attributed more to the increase in fucose, as sialic acid seems to have increased in a comparable magnitude to both hexoses and hexosamines. The ratiosof either of the latter monosaccharides to sialic acid were little changed although they were rather low in the terminal 6 weeks. The ratio of total hexoses or hexosamine to fucose, on the other hand, dropped drastically during the course of infection but approached preinfection levels in the terminal 4 weeks of exposure; a very similar pattern was observed in both cows. This ratio (total hexoses or hexosamine/fucose) could be of clinical significance in fascioliasis if the present results in sheep and cattle are confirmed in future experiments.

Sialic acid is now considered to play an important role in the survival and viability of serum glycoproteins (Ashwell and Morell, 1974). Removal of even a small part of the terminal sialic acid would lead to the immediate recognition of the desialated glycoprotein and its removal by the liver. On the other hand, the presence of sialic acid residues on the liver receptors is essential for the binding of the desialated protein and its subsequent catabolism (Ashwell and Morell, 1974). The retention times of the plasma

glycoproteins in the circulation is thus directly related to the degree of sialiation (Morell, Gregoriadis, Scheinberg, Hickman and Ashwell, 1971; Barkai and Di Cesare, 1975). The increased plasma sialic acid observed in fluke-infected sheep and cows in the present investigation could therefore be very important to preserve and prolong the life time of the other plasma glycoproteins especially in chronic stages when the liver may be greatly damaged and glycoprotein synthesis impaired.

The current study shows that the plasma seromucoids behave differently from the other glycoproteins; they increased markedly during the first 6 weeks of infection and remained slightly above normal levels for most of the experimental period (weeks 10-13 and 17-23). This suggests that seromucoid can vary independently from other sugars, as has been observed in cases of parenchymatous liver diseases when the seromucoid levels decreased while that of total hexoses were considerably elevated. In other conditions, i.e. cancer and tuberculosis the increase in seromucoid was greater in extent than that in total hexoses (see Winzler, 1955). The seromucoid fraction is heterogeneous and contains a number of proteins mainly orosomucoid, 3.5S α_1-glycoprotein; haptoglobulin and albumin. The individual components contain differing amounts of hexoses, hexosamine, fucose and sialic acid (de Vaux St. Cyr, 1963; Bacchus, Kennedy and Blackwell, 1967) whose concentrations may be increased in pathological conditions and consequently elevate the seromucoid fraction.

Now the question arises as to the source of the increased plasma glycoproteins. More than one possibility exists:

a) Preformed glycoproteins from inflamed tissues or those locally formed in injury sites and those resulting from depolymerisation of connective tissue ground substances may be released into the circulation and elevate the plasma glycoprotein levels. Catchpole (1950) found high serum glycoproteins in tumour-bearing mice and related this to the increase in the soluble glycoproteins of connective tissues bordering the tumours. Further, Pirani and Catchpole (1951) demonstrated increased serum seromucoid levels in scorbutic guinea pigs which suggest that depolymerisation of connective tissue due to vitamin C deficiency might have contributed to the serum seromucoid.

Increased hyaluronidase activity in liver tissue and serum was observed in rats (Koizumi, Nakamura and Abev, 1967) and humans (Nakamura, Iwabori and Koizumi, 1970) having liver damage. Montfort and Perez-Tamayo (1978) found that in the reversible stages of liver cirrhosis in carbon tetrachloride-damaged rat livers, collagenase was always present in association with connective tissue. Both the increase in hyaluronidase and presence of collagenase suggest that connective tissue is constantly degraded in liver damage and this could ultimately increase the plasma glycoprotein components.

b) Glycoproteins may be increased in response to tissue proliferation rather than tissue destruction: This suggestion was first advanced by Shetlar, Foster, Kelly, Shetlar, Bryan and Everett (1949). Tissue proliferation

occurs in cancer and glycoproteins and lipoproteins increase in tumour tissues (Rapin and Burger, 1974). Increases in sialic-acid containing proteolipids and sphingolipids have been observed in sera of tumour-bearing rats and humans (Skipski, Barclay , Archibald & Stock, 1975; Skipski, Katopodis, Prendergast and Stock, 1975) and elevated serum lipid-soluble sialic acid has also been reported in tumour-bearing dogs (Kloppel, Franz, Morre and Richardson, 1978). Kloppel et al. (1978) maintained that the increase in the serum sialic acid could originate from the tumour cells whose rapid proliferation causes sloughing of cell membranes and their subsequent appearance in serum.

Acid mucopolysaccharides have been demonstrated in human leukocytes (Kerby, 1955) and rat thrombocytes (Odell and Anderson, 1957). Blood leukocytes often increase in many diseases and inflammatory conditions and could, therefore, contribute to the blood glycoproteins. In this respect Kloppel et al. (1978) state that the proliferation of white blood cells could have an effect similar to that of tumour cells in increasing serum glycoproteins.

c) The increased glycoproteins may be produced in the liver in response to tissue injury: There is sufficient evidence to believe that the liver is the main source of plasma glycoproteins. Werner (1949) observed increased serum glycoproteins in rabbits following removal of blood but this increase was not noticed when liver damage was induced. Spiro (1959) using labelled glucose demonstrated that most of the serum protein-bound glucosamine in rats was

synthesised in the liver. Shetlar (1961) observed that radioactive glucosamine injected intraperitoneally into rats was rapidly incorporated into protein-bound glucosamine, first in the liver and then into other serum fractions. Kukrel, Pancner, Louch and Winzler (1962) investigated the incorporation of S^{35} DL-methionine into seromucoid of dogs prior to and after hepatectomy and concluded that most of the seromucoid was synthesised in the liver and only a smaller part was of extrahepatic origin. Winzler (1965) found that after intravenous injection of labelled glucosamine in rats, the liver quickly concentrated about a third of the radioactive dose, and after being temporarily bound in the liver, appeared in the plasma proteins. He found that at least 80% of the radioactivity was present as glucosamine and the rest as sialic acid.

All the above reports point out the liver as the major souce of plasma glycoproteins.

To seek a reasonable explanation for the increase in plasma glycoproteins observed in sheep and cows of this study the foregoing three arguments should be considered. In Fascioliasis both tissue destruction and regeneration (proliferation) occur in the liver and either of these processes may contribute to the plasma glycoproteins and stimulate the intact liver cells to produce more glycoproteins. This may be maintained for a certain period but a stage might be reached when no more glycoproteins are released into the circulation, and the low glycoprotein concentrations seen in the terminal stages of the present experiment may support this view. Thus the decrease in total hexoses and hexosamine to below normal levels seen in this study may reflect failure of liver glyco-

protein synthesis and, probably, a decrease in the rate of tissue destruction in advanced stages. Recently, Hussein et al. (1975) in their study on human bilharziasis observed an increase in serum total hexoses, hexosamine, fucose and sialic acid in the early hepatomegalic-infiltrative phase of the disease but a decrease in the later fibrotic stages. Nakamura et al. (1970) showed that the serum and hepatic **hyaluronidase** levels increase in liver diseases but not in advanced liver fibrosis. Similarly Montfort and Perez-Tamayo (1978) found that the collagenase associated with connective tissue in cases of reversible liver cirrhosis is absent in the stages of irreversible hepatic cirrhosis. Both reports indicate that tissue depolymerisation, and hence release of glycoprotein constituents into the circulation, is decreased in advanced liver diseases, when synthesis is also expected to be reduced. These findings would lend some explanation to the results of Hussein et al. (1975) and also to the findings of the present study in fascioliasis.

Whether the increase in plasma glycoproteins observed in the current investigation is due to tissue proliferation or destruction or due to increased synthesis, the main source will be the liver, but it is possible that other tissues are involved. It has been demonstrated here that the γ-globulins increase tremendously in F. hepatica infection. These immunoglobulins are synthesised by cells of the reticulo-endothelial system which are mainly extrahepatic, and are known to contain considerable quantities of carbohydrates i.e. hexosamine, galactose, mannose, sialic acid, in the prosthetic groups. So it is reasonable to assume that the immunoglobulins contribute to the vascular glycoprotein pool. Moreover, an increase in the total white blood cell counts was commonly found in fluke-

infected animals and this leuckocytosis might have affected the plasma glycoprotein levels (Kloppel et al. 1978).

It might be asked, can the present findings be of diagnostic help in fascioliasis? It is premature to answer this question before further experiments are conducted. However, the pattern of change in the various plasma glycoproteins observed in infected animals currently investigated, especially with regard to seromucoid, sialic acid and fucose and the alterations in the total hexoses or hexosamine/fucose ratios could be clinically useful in assessing and following the progress of the disease. Measurement of specific glycoproteins of known functions and the chemical resolution of heterogeneous fractions, like seromucoid and sialic acid, would provide more information about glycoprotein changes in fascioliasis and allow for a better judgement as to which components could be utilized as diagnostic tools.

It should be mentioned that estimations of seromucoid (Keyser, 1964; Bacchus et al., 1967), fucose (Rosato, Seltzer, Mullen and Rosato, 1971) and sialic acid (Kloppel et al., 1978) have been advocated and utilized for the detection and differential diagnosis of various types of cancer in some animals and humans. Other uses of seromucoid determination were found in diagnosis of liver diseases (Keyser, 1964).

The plasma lipoproteins:

The present findings show that the electrophoretic separation of the plasma lipoproteins in sheep revealed 3 fractions, 2 having an α-mobility designated as α_1- and α_2-lipoproteins and the third moving in a β-position i.e.

β-lipoproteins.

Howell (1962) fractionated normal sheep blood and observed 3 electrophoretic fractions, the α-lipoproteins (41-45%), β-lipoproteins (28-33%) and chylomicrons (23-27%). Campbell (1963) recognised only two components, the α- and β-lipoproteins with the former constituting about 63% of the total area. Leat, Kubasek and Bullrin (1976) in their study of normal plasma lipoproteins found that the high density lipoproteins (HDL), low density lipoproteins (LDL) and very low density lipoproteins (VLDL) plus chylomicrons constituted about 76%, 20% and less than 5% respectively; on electrophoresis the former 3 fractions migrated in the α-, β, and pre β-position respectively while chylomicrons remained at the origin.

The present results obtained for the pre-infection period and the first 3 weeks of infection seem to agree with those of Campbell (1963) and Leat et al. (1976) in that the α- and β-lipoproteins constitute the major components with the former predominating, but are rather different from those of Howell (1962) who recognised chylomicrons as a third major component.

During the course of the current experiment the plasma lipoproteins started to change significantly after the 3rd week of infection. Both α-lipoprotein fractions decreased while the β-lipoproteins increased. This is in line with the findings of Howell (1962) in fluke-infected sheep but he also observed an increase in chylomicrons. Similarly these results agree with those previously reported in **part A of this thesis which** indicated that the LDLs (β-lipoproteins) were significantly elevated in fluke-infected sheep.

It has been repeatedly stated that fluke infection

mainly involves the liver and causes hepatitis, hyperplastic cholengitis and fibrosis. Flukes in the bile ducts, if present in large numbers, may obstruct bile flow and could lead to some degree of cholestasis; this might be aggravated by the presence of mucoid plugs which were often seen in bile ducts in association with the flukes. It is worth mentioning that distension of the gall bladders was a common finding in the sheep infected in this study, suggesting that obstruction to bile flow may occur. The hepatic changes caused by fluke infection is expected to cause disturbances in lipoprotein metabolism which is indicated by the present results.

Alterations in serum lipoproteins have been reported in liver diseases and biliary obstruction in man and other animals, and it may be relevant to quote some of these reports to explain the lipoprotein changes observed in fascioliasis. A decrease in the α-lipoprotein density was observed in rats with acute biliary obstruction, and humans with acute cholestasis (Seidel, Greten, Geisen, Wengeler and Wieland, 1972; McIntyre, Calandra and Pearson, 1974) and this was related to poor stainability of the lipid material or to altered mobility of the HDL (α-lipoproteins) in the β-position (Seidel et al., 1972). The above argument could lend an explanation to the decrease in the α-lipoproteins and increase in the β-lipoproteins observed in fluke-infected sheep.

In biliary obstruction in humans and rats the VLDL and LDL (β-lipoproteins) were found to increase due to a rise in their phospholipid and cholesterol contents, and the VLDL ran as β-lipoproteins on cellulose acetate electrophoresis (McIntyre et al., 1974; Agorastos, Boswell, Harry and McIntyre, 1976). Moreover Agorastos et al. (1976) state that in liver

diseases the excess triglycerides, which are normally transported in the VLDL or chylomicrons may abnormally be carried in the LDL (β -lipoprotein). Both altered mobility of VLDL with the β -lipoproteins and the abnormal transport of triglycerides by LDL may offer additional explanation to the increase in the β -lipoprotein percentages observed in F. hepatica infected sheep in the present study.

The present findings indicate that disturbance in lipid metabolism occurs in fascioliasis. The changes in plasma lipoproteins could be the outcome of a complex sequence of events and a detailed chemical investigation will be useful to understand more about these changes.

PART C

ENZYME SECRETION IN F. hepatica

General considerations:

Many helminth parasites pursue a complicated life cycle and may need to pass through more than one tissue barrier to complete a successful cycle. It is generally understood that parasites penetrate and migrate through the host(s) tissues mechanically, in many cases aided by special cuticular structures such as hooks and spines etc. A good deal of evidence is now accumulating that parasites may also secrete lytic secretions to break down tissues and hence both mechanical and enzymatic mechanisms may be involved.

Proteolytic activity has been suspected to be present in secretions of the hexcanth embryo of cyclophyllidian cystodes i.e. Taenia pisiformis, Taenia saginata, and the origin of these secretions was indicated to be the unicellular glands present in the hexcanth larvae (Sawada, 1961; Silverman and Maneely, 1955; Lethbridge, 1971). The contents of these unicellular glands disappear or become decreased after the larvae penetrate the integument of the intermediate host. Lewert and Lee (1955) also demonstrated collagenolytic activity in the early stages of the rapidly growing atrobilocercus but not in older forms or adult tape worms.

The histologic changes and tissue softening observed in the dermal and epidermal layers of the skin following the penetration of nematode parasites may indicate enzymatic secretions (Lewert and Lee, 1954). Collagenolytic and gelatinolytic activities have been demonstrated in living and extracts of Strongyloides ratti and Strongyloides simiae (Lewert and Lee, 1954). Similarly Devi, Murthy and Reddy (1969)

reported proteolytic activity in larvae and adults of Dracunculus medinensis in addition to a lipase and a "spreading factor". Matthews (1975) incubated larvae of Nippostrongylus americanus and Strongylus fulleborni with azocollagen at 37°C and found that they digest the azocollagen with subsequent release of the azo dye.

Relatively more work has been done to demonstrate enzymatic activity in trematodes and especially the schistosomes. As in other parasites, the secretion of enzymes was connected with the penetrative ability of the worm. Thus, Kloetzel (1968) showed that living eggs of Schistosoma mansoni possess a collagenase-like enzyme whose activity was diminished in the presence of immune serum. This was confirmed by Smith (1974) who demonstrated a true collagenase in S. mansoni eggs which degraded rat tail collagen and was inhibited by normal as well as immune sera from mice and monkies. The role of this enzyme was believed to assist the eggs to pass from the mesenteric venules, where they are layed, to the gut lumen to be excreted to the exterior.

The hatched schistosome miracidia were suspected to release enzymes when they penetrate the integument of the snail intermediate host and the source of the enzyme(s) was suggested to be the apical gland cells (Wajdi, 1966).

Mucopolysaccharid-like enzymes, such as hyaluronidase have been demonstrated in cercariae of S. mansoni (Kuntz, 1953; Lee and Lewert, 1957). Moreover, Lewert and Lee (1954) showed that cercarial secretions of S. mansoni digested azocollagen. They also found proteolytic activity in extracts of adult schistosomas but the lytic factor seems to be different in nature from cercarial enzymes and was not inhibited by the same substrates. Lytic activities in S. mansoni cercariae or

cercarial extracts have been demonstrated against casein (Stirewalt, 1963; Gazzinelli, Romalho-Pinto and Pellegrino, 1966; Dresden, 1972), haemoglobin (Lewert and Lee, 1956; Stirewalt, 1963; Stirewalt and Fregeau, 1966), elastin (Gazzinelli and Pellagrino, 1964; Gazzinelli et al., 1966; Dresden, 1972), azocollagen (Lewert and Lee, 1954, 1956; Dresden, 1972), hyaluronic acid (Levine, Garzoli, Kuntz and Killough, 1948) and gelatin (Lewert and Lee, 1954; Stirewalt, 1963; Dresden, 1972; Stirewalt, 1973). Lewert and Lee (1956) also reported lytic effect on hide powder and cartilage.

The source of these enzymes has been indicated to be the preacetabular glands (Stirewalt and Kruidenier, 1961) and Stirewalt (1973) confirmed histologically that the proteolytic activity was localized in those glands.

A globin-digesting enzyme was also detected in extracts of lyophilized adult S. mansoni (Grant and Senft, 1971).

The release of enzymatic substances by the different stages of parasites seem to be connected with the penetration or migration mechanisms and in this respect Matthews (1977) has presented a very good review about the passage of larval helminths through host tissues, in which the role of enzyme secretion by penetrating parasites has been indicated.

Enzymes secreted by F. hepatica:

Fasciola species pass through different stages of development before attaining maturity in the bile ducts, and to complete a successful life cycle they need to pass through more than one tissue barrier. These barriers are: the snail integument during miracidial penetration and cercarial

emergence, the intestinal wall of the definitive host after the metacercariae are ingested and excysted, the liver capsule and hepatic mass after the flukes pass the intestinal wall and then the walls of the bile ducts. Unusually, the young flukes may penetrate and migrate through the pancreas or lungs. In all cases the flukes may secrete enzymes to facilitate their passage through tissues.

Generally two groups of enzymes could be distinguished a) internal enzymes and b) external enzymes. The internal enzymes are concerned with intermediary metabolism of the parasites and include many enzymes of carbohydrate, protein and lipid metabolism; some of these enzymes are listed in Table I.

The external enzymes, on the other hand, include those secreted by the flukes to break down tissues during migration and to prepare food for internal utilization. Although adequate information seems to be available concerning internal enzymes relatively less is known about the secretion of external ones.

Faust (1955), cited by Dawes (1959), stated that the penetration of F. hepatica miracidia into the snail host is accomplished by digestive enzymes elaborated into the so-called "penetration glands" and discharged at the anterior end of the miracidia. Dawes (1959, 1960) investigated the penetration of F. hepatica and F. gigantica miracidia into the intermediate snail host and observed loosening, cytolysis and destruction of epithelial cells of the snail integument. He considered these changes to indicate the involvement of enzymatic activity. Polyakova and Sazanov (1965) did demonstrate the presence of hyaluronidase in F. hepatica miracidia. As in schistosomes, the source of these enzymes could be the miracidial glands which in F. hepatica consist of 3 types of cells; multinuclear

Table I Some enzymes of intermediary metabolism detected in F. hepatica.

Enzyme	Reference
Glycolytic pathways:	
Hexokinase	Prichard and Schofield (1968)
Glucose-6-phosphate dehydrogenase	"
Glucose-6-phosphatase	"
Phosphoglucomutase	"
Phosphomannoisomerase	"
Triose phosphate isomerase	"
Glycerophosphate dehydrogenase	"
Glyceraldehyde-3-phosphate dehydrogenase	"
Phosphoglycerate kinase	"
Phosphoglycerate mutase	"
Enolase	"
Lactic dehydrogenase	"
Phosphoglucoisomerase	Bryant and Williams (1962) and Prichard and Schofield (1968)
Phosphofructokinase and Aldolase	Bryant and Williams (1962), Mansour (1962) and Prichard and Schofield (1968).
Fructose diphosphatase	Mansour (1962).
Krebs cycle:	
Oxalosuccinic decarboxylase	Bryant and Williams (1962)
Ketoglutaric decarboxylase	"
Succinic dehydrogenase	"
Fumarase	"
Methylmalomyl-CoA mutase	Zoeten, Posthuma and Tipker (1969)
Propionyl-CoA carboxylase	"

/Contd....

Table I (Contd.)

Enzyme	Reference
Ornithine-Urea cycle	
Carbamyl phosphate synthetase	Janssens and Bryant (1968)
Arginino succinate lyase	"
Arginase and Oraithine trans-carbamylase	Campbell and Lee (1963) and Janssens and Bryant (1968).
Amino acid metabolism	
Glutamic-oxalacetic transaminase	Connelly and Downey (1968).
Glutamic-pyruvic transaminase	"
Glutamate dehydrogenase	Krvavica, Thommen, Prosenjak and Kucan (1967).
Glutamate synthetase	"
Aspartate transaminase	"
Alanine transaminase	"
Esterases and phosphatases	
Cholinesterase	Chance and Mansour (1953); Krvavica, Lui and Becejac (1967); Haites, Don and Masters (1972).
Alkaline and acid phosphatases	Saito (1961); Holton (1967); Probert and Lwin (1974).

For a detailed information about the biochemistry of the liver fluke, see the reviews of Pantelouois (1967) and Coles (1975).

ventral gland cells, apical gland cells and 2 lateral unicellular gland cells (Wilson, 1971). However, no direct evidence of cytolytic activity has been reported in these glands in Fasciola species.

Apparently little is known about the mechanism of emergence of the fluke cercariae from the intermediate snail host but Kendall and McCullough (1951) suggested that the emergence is a negative one.

After the metacercariae are ingested by the definitive host they excyst prior to penetration of the gut wall. The mechanism of excystment has been investigated by many authors (Susuki, 1931; Vogel, 1934; Schumacher, 1938; Hughes, 1959, 1963; - all cited by Dawes and Hughes, 1964; and Dawes, 1961, 1963) but no particular mention has been given to the role of enzymes secreted by the encysted cercariae in the process of excystment. Dawes (1963) concluded that during excystment the ventral sucker disrupts the cyst wall mechanically but Dixon (1966) suggested that the alteration in the structure and in the stainability of the cyst wall around the emergence hole and the separation of the lamellae of the ventral plug region could be due to action of enzyme(s) produced by the emerging flukes. The latter author also suggested that bile, which triggers the emergence phase, could activate an enzyme produced by the young flukes to induce muscular movement.

The juvenile flukes migrate through the intestinal wall on their way to the liver and Dawes (1963) showed that they make their way by ingesting a tract. In the liver the young parasites continuously and actively burrow through the liver mass and the degeneration of the hepatic cells surrounding the fluke tracts and the change in their staining reaction could indicate enzymatic involvement (Dawes, 1961b).

Enzymatic activity has been demonstrated in F. hepatica but little has been done to isolate and characterise specific enzymes. Thus Pennoit De Coomau and Van Grembergen (1942) and Halton (1963) demonstrated the presence of proteolytic activity in homogenates of adult F. hepatica and the only protease identified by the former authors was cathepsin C. Similarly Thorsell and Bjorkman (1965) found that intact F. hepatica secrete proteolytic substances against gelatin. Thorpe (1968) also demonstrated gelatinolytic action in liver flukes with open suckers and concluded that the lytic factor was derived from the gut, since flukes with ligated suckers were not able to effect gelatin lysis.

Howell (1966) demonstrated collagenolytic activity in living immature flukes as well as their sonicated extracts after incubation with azocollagen at 37°C. He found that the intensity of dye released was proportional to the amount of collagenase activity present.

Locatelli and Paoletti (1969) found that at pH 2.4 homogenates of adult F. hepatica had proteolytic action against bovine globin, serum albumin and globulins but the activity was relatively higher in the case of globin. They suggested that globin could be a suitable substrate to provide the flukes with some of their amino acid requirements.

Howell (1973) demonstrated proteolytic activity in adult F. hepatica extracts on casein and a gelatinolytic activity in fresh frozen fluke sections when mounted on blackened photographic plates. By comparing the digested areas on the gelatin plates with the fluke sections he concluded that the caecae were the main site of activity.

It can be concluded from the above account that there

are limited reports concerning the detection, characterisation and source of the enzymes which may be secreted by Fasciola species, especially so for F. gigantica. Moreover, definite information about the presence of enzymes such as elastase and hyaluronidase is apparently lacking and only one report described collagenolytic activity in immature flukes. In the present study the presence of elastinolytic and collagenolytic activities was investigated in metacercariae, immature and adult F. hepatica. The presence of hyaluronidase in adult flukes was also tested. Further, purification of some fluke enzymes, particularly elastase, was attempted.

The presence of hyaluronidase, elastase and collagenase in sheep plasma and the inhibitory effect of the latter against the former enzymes in-vitro were tested.

MATERIALS AND METHODS

Source of flukes, F. hepatica:

a) Immature flukes:

Obtained from a 6-weeks old infection in one sheep and one goat.

b) Mature flukes:

i) collected from the livers of 16 experimentally infected sheep

ii) collected from naturally infected ovine and bovine livers obtained from Bangor local abattoir.

After the flukes were collected they were washed in several changes of sterilized physiological saline (at least 4 washes till the flukes were clean of debris).

Part of the washed flukes were frozen in liquid nitrogen, freeze-dried, powdered and stored at -20°C till required.

Extracts of flukes were extracted as follows:
1.5g of freeze-dried flukes in 100ml of sterilized physiological saline, sonicated for 5 minutes and centrifuged at 15,000 r.p.m. in a cold centrifuge for 45 minutes. The supernatant was stored at 4°C and used as will be explained later.

Plasma:

Plasma was separated from blood of sheep and cows experimentally infected with F. hepatica.

Protease assay:

This was done according to the method of Charney and Tomarelli (1947).

Principle:

Digestion of a solution of azocasein substrate with proteolytic enzymes results in the formation of coloured components soluble in trichloroacetic acid (TCA). The colour in the TCA filtrate is directly related to the proteolytic activity.

Substrate solution:

2.5g azocasein (BDH) is dissolved in 50ml of 1% Na HCO_3 at 60°C with stirring. The pH is adjusted to 8.3 and the solution diluted to 100ml with distilled water (H_2O).

Enzyme source:

Live adult F. hepatica washed as described above and weighed.

Procedure:

2ml of the substrate solution were incubated with one or more flukes for 3 hrs. At the end of the incubation period the flukes were removed and the undigested azocasein was precipitated by the addition of 8ml of 5% TCA to each tube. The contents of each tube were then filtered through paper. To 5ml of the filtrate 5ml of 0.5N NaOH were added, and the colour developed read at 440nm against blank using a Unicam spectrophotometer. In the blank the 2ml of the substrate were substituted by an equal amount of carbonate buffer and incubated with similar numbers of flukes as in the test. Another blank consisted of only azocasein substrate which was incubated and treated similarly.

Hyaluronidase activity:

This was assayed by the turbidimetric method of Di Ferranti (1956) and confirmed by the colourimetric estimation of the N-acetylglucosamine-reacting material released from the substrate following the procedure of Reissig, Strominger and Leloir (1955).

a) Turbidimetric method:

Principle:

Turbidity develops when cetyltrimethylammonium bromide is added to a solution of acid mucopolysaccharide but not when added to chondroitinsulphate or hyaluroniate which has been depolymerised by hyaluronidase. The fall in turbidity is taken as a measure of the activity in depolymerizing the mucopolysaccharide and this is expressed as a percentage of that developed by the unaffected control substrate.

Reagents:

(1) Acetate buffer: 0.2M sodium acetate in acetic acid, pH 6, to which NaCl is added to give a concentration of 0.15M.

(2) Enzyme source: Live adult F. hepatica.

(3) Substrate solution: 50mg of sodium chondroitin sulphate dissolved in 100ml of the acetate buffer.

(4) Cetyltrimethylammonium bromide reagent: 2.5g dissolved in 100ml of 2% NaOH.

Procedure:

2ml of substrate were dispensed in each test tube and incubated with one or more live F. hepatica for different periods ranging from 1-4 hrs. at 37°C in a water bath. 2ml of acetate

buffer were used as blank and treated similarly. At the end of the incubation period 4ml of cetyltrimethylammonium bromide reagent were added and mixed by inversion. The turbidity was then measured at 400nm against the blank.

b) Colorimetric method:

Reagents:

(1) Potassium tetraborate: 0.8M solution and pH adjusted to 9.1 with KOH.

(2) p-Dimethylaminobenzaldehyde (DMAB) reagent: 10 gms of DMAB dissolved in 100ml of glacial acetic acid containing 12.5% (v/v) 10N HCl.

Procedure:

Similar to the turbidimetric method but at the end of the incubation period 0.5ml of the test samples and of blank were taken into each test tube and 0.1ml potassium tetraborate added. The tubes were heated for 3 minutes in a boiling water bath and then cooled in tap water. 3ml of DMAB reagent were then added, mixed and the tubes immediately placed in a water bath at 37°C. After 20 minutes the tubes were cooled in tap water and read without delay at 585nm. Standard solutions containing 0.05, 0.1, 0.16, 0.31, 0.63, 1.25, 2.5 and 5.0 mM N-acetylglucosamine were treated similarly to test samples and the readings obtained were plotted into a standard curve against the concentrations. The readings of the test samples were transformed to mM N-acetyl-glucosamine from this curve.

Collagenase activity:

This was determined by measuring the intensity of dye released from azocollagen substrate after incubation with an enzyme source.

Enzyme source:

Viable F. hepatica metacercariae

Immature F. hepatica

Adult F. hepatica or

Sonicated extracts of F. hepatica.

Substrate:

Azocollagen (Calbiochem) 1mg/ml of 0.1M phosphate buffer, pH 7.4.

Procedure:

5ml of the azocollagen substrate were incubated at 37°C with live adult flukes, live immature flukes, 30 viable cysts or 0.5ml of the extract, for varying periods of time ranging from 1-4 hours.

After incubation the flukes were removed and the tubes centrifuged at 2000 r.p.m. and the colour released was read at 530nm against a phosphate buffer blank. Two negative controls were treated similarly and consisted of a) 5ml of substrate suspension only, b) 5ml phosphate buffer incubated with flukes, cysts or extracts. Positive controls were also made by incubating 5ml of the substrate with 10, 20, 40, 80, 100, 120 and 160 µg of bacterial collagenase (Cl. histolyticum collagenase) each contained in 0.1ml of the phosphate buffer. The dye release per unit time was plotted against the enzyme concentrations and the enzymatic activity shown by the F. hepatica, metacercariae, immature or adult forms or their extracts were related to those obtained by the enzyme standards.

Elastase activity:

The method described by Naughton and Sanger (1961) was followed:

Enzyme source:

As for collagenase.

Substrate:

Dyed elastin prepared as follows:
2g powdered elastin (Sigma Ltd.) was suspended in 30ml of saturated aqueous solution of Congo red overnight, and the dyed protein filtered off. The elastin was well washed with water until the washings were clear of the dye and then dried by successive washings with acetone and ether.

The dyed elastin powder was used as a substrate to detect elastase activity and was suspended in 0.05M Na_2CO_3-HCl buffer (pH 8.8), 1mg elastin/ml buffer.

Procedure:

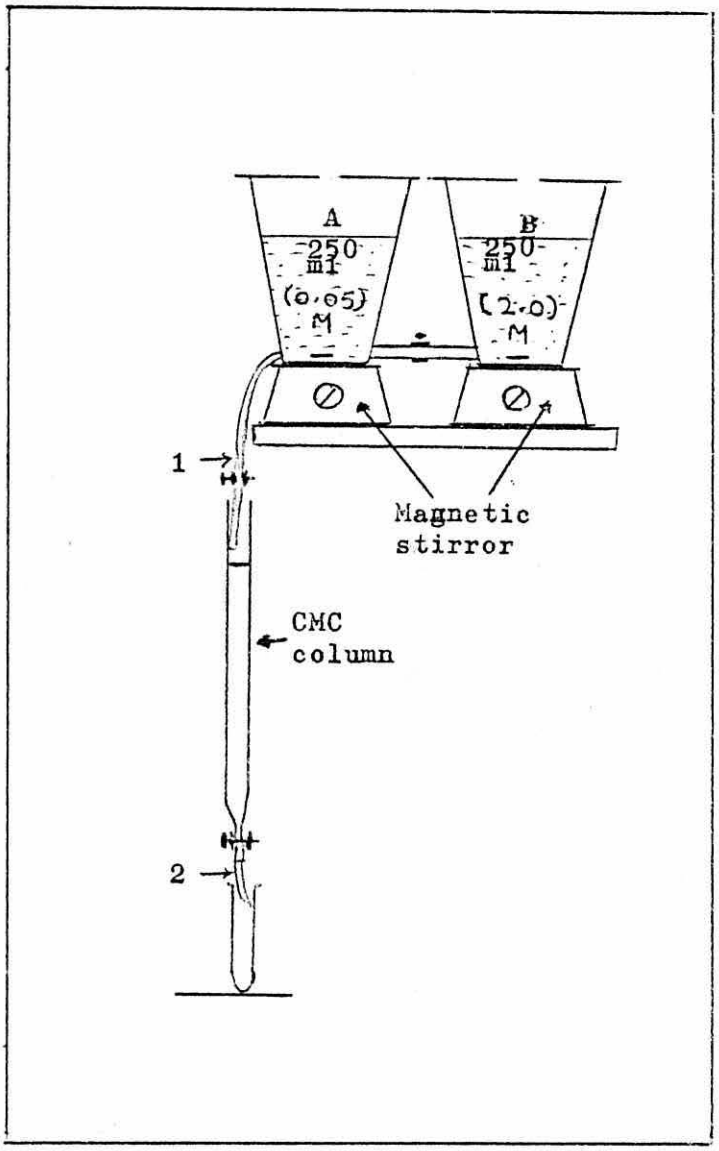
5ml of the substrate suspension was incubated with the enzyme source (cysts, immature and mature flukes and sonicated extracts) for varying time intervals, 1-8 hrs. in a 37° water bath. At the end of the incubation period the flukes were removed and the tubes were centrifuged at 2000 r.p.m. and the dye released in the supernatant measured at 495 nm against buffer blank. Negative controls containing either the buffer alone + the source of enzyme or the substrate suspension alone were treated similarly and read. Positive controls were also made by incubating 5ml fractions of the substrate suspension with 16.6, 25, 33.3 and 50 µg of pancreatic elastase (Sigma) each contained in 0.1ml of the carbonate buffer. The readings and enzyme concentrations were plotted in a standard curve and the activity released by the flukes or their extracts was related to the standard curve.

Elastase: purification procedure:

45g of freeze-dried fluke powder were stirred in 200ml 0.05M ammonium acetate buffer, pH 4.5, for 3 hrs at 5°C. It was then centrifuged at 2000 r.p.m. in a cold centrifuge (5°C) for 45 minutes. The supernatant was collected and the sediment re-extracted with 100ml of the buffer. The two supernatants were combined and brought to 45% saturation with ammonium sulphate (262g solid $(NH_4)_2SO_4$/l). The precipitated fraction was then dissolved in 100ml of 0.05M Na_2CO_3-HCl buffer, pH 8.8 and dialysed overnight in 10 litres of H_2O. The solution was then centrifuged at 2000 r.p.m., the precipitate washed in H_2O and freeze-dried.

40 mg of this final precipitate were dissolved in 4.0ml of 0.05M ammonium acetate buffer (pH 4.5), applied to a carboxy-methylcellulose column (10 x 1.5cm) and eluted by 0.05M ammonium acetate buffer whose molarity was continuously increased by addition of NaCl. The system used is described in Fig. 1. 10ml fractions of the elute were collected, their protein concentrations were estimated from the extinction coefficient at 280 nm. The molarity of the effluent was determined from their refractive index at a temperature of 21°C which were related to a standard curve made from the readings of different molar solutions (NaCl was added to 0.05M ammonium acetate buffer to give different molarities of 0.1, 0.5, 1.0, 1.5 and 2.0) against their refractive index at 21°C, see Fig. 2. Ideally a conductivity cell should have been used but that was not available. Elastolytic activity in the effluent fractions was assessed on 1ml fractions incubated with 5ml of the elastin substrate as described before.

Fig. 1 Diagramatic representation of the system used for CMC chromatography.



A and B are two identical beakers used as buffer reservoirs. Beaker A contained 250ml of 0.05M ammonium acetate buffer and beaker B contained 250ml of the same buffer whose molarity was brought to 2.0 by the addition of NaCl. As the buffer (0.05M) flowed from reservoir A into the CMC column, it was replaced by the 2M buffer from reservoir B, and the buffers in the 2 beakers were level. The molarity of the buffer in reservoir A was also gradually increased. The buffer flow from outlets 1 and 2 was adjusted to a rate of 1ml per minute.

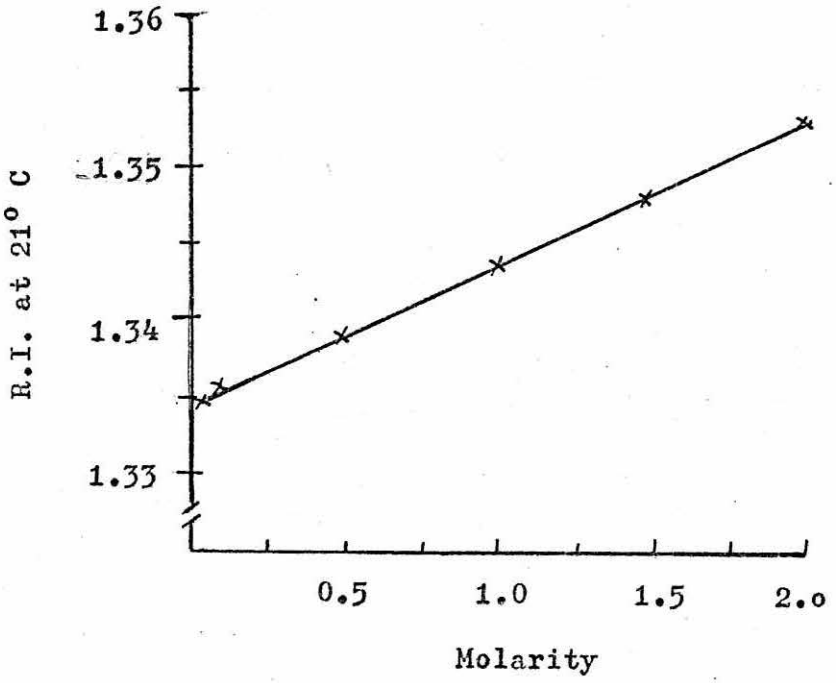


Fig. 2. Standard curve: Molarity of ammonium acetate buffer against refractive index (R.I).

Detection of enzymes in fluke secretions:

Flukes were incubated either in $M/_{15}$ phosphate buffer pH 7.2 or 0.05M Na_2CO_3-HCl buffer, pH 8.8 for 3 hours and then 1ml fractions of each buffer was incubated with 5ml azocollagen substrate or elastin suspension for detection of collagenolytic or elastinolytic activity.

Detection of collagenase, elastase and hyaluronidase in sheep plasma:

0.2ml of sheep plasma were incubated with 5ml of azocollagen and with elastin substrates for 3 hours in a 37^{o} water bath. The assay for proteolytic activity was carried out as described previously. As for hyaluronidase 0.2ml of plasma was incubated with 2ml hyaluronic acid (200 µg/ml) in sodium acetate-acetic acid buffer and incubated for 3 hours. The enzymatic activity was detected by measuring the release in N-acetylglucosamine reacting material in the incubation medium as described earlier.

Effect of sheep plasma on hyaluronidase and collagenase activities:

1ml of collagenase enzyme (20 µg/ml in $M/_{15}$ phosphate buffer, pH 7.2) or 1ml of hyaluronidase solution (1mg/ml in 0.85% saline) were pre-incubated each with 0.1ml of sheep plasma for 30 minutes at room temperature. Then 0.5ml of each of the pre-incubated enzymes was dispensed into test tubes containing the appropriate substrate; 5ml of azocollagen in phosphate buffer, pH 7.2 or 3ml of hyaluronic acid in phosphate buffer pH 6.4. The collagenase tubes were incubated at 37^{o} for 3 hrs and the hyaluronidase tubes for 16 hrs. Measurement of the azo dye and the N-acetylglucosamine reacting material released in the medium was done as described before.

N.B.

$M/_{15}$ phosphate buffers:

a) pH 7.2: 27ml of KH_2PO_4 (9.08g/lit) + 73ml of Na_2HPO_4 (9.465 g/lit).

b) pH 6.4: 71ml KH_2PO_4 + 29ml Na_2HPO_4.

RESULTS

Proteolytic activity:

The general proteolytic activity has been measured in whole adult flukes using azocasein as substrate. The results are shown in Table II. It can be seen that the azocasein was slightly digested during the first hour of incubation but the proteolytic activity increased during the second and third hours of incubation. It is also evident that the smaller flukes, in terms of fluke weight, had the highest proteolytic activity particularly during the first and second hrs. However, the activity was highest in larger flukes during the 3rd hr. of incubation.

Table II Average intensity of dye released by 1 adult *F. hepatica* from 5ml azocasein after 1, 2 and 3 hours incubation.

Weight range (g)	No. of flukes investigated	Readings at 440nm 1st hr.	2nd hr.	3rd hr.
0.00 - 0.09	18	0.019 ± 0.011	0.106 ± 0.090	0.201 ± 0.121
0.10 - 0.19	18	0.024 ± 0.014	0.218 ± 0.179	0.319 ± 0.229
0.20 - 0.29	32	0.005 ± 0.004	0.103 ± 0.082	0.341 ± 0.141
Average reading	(68)	0.0142 ± 0.0137	0.126 ± 0.119	0.287 ± 0.187

Hyaluronidase activity:

This has been demonstrated by measuring the decrease in turbidity of the mucopolysaccharide substrate, developed by the addition of cetyltrimethylammonium bromide, after being incubated with flukes. Table III shows that the turbidity decreased during incubation indicating the presence of hyaluronidase activity in *F. hepatica*. In some tubes the colourimetric method was used and

the presence of N-acetylglucosamine reacting material was demonstrated and confirming the breakdown of the chondroitin sulphate substrate. No change in turbidity and no colour developed by the colorimetric method was seen in control tubes without flukes.

Table III Turbidimetric assay of hyaluronidase activity after incubation of flukes with chondroitin sulphate for 3 hours. 35 adult F. hepatica were used.

Tubes	Average reading/fluke at 400nm			
	0 hr.	2 hrs.	3 hrs.	4 hrs.
Test	0.86 ± 0.048	0.724 ± 0.046	0.705 ± 0.039	0.652 ± 0.068
Controls	0.855	0.850	0.870	0.840
% fall in turbidity	100.8	85.18	81.03	77.60

Collagenolytic activity:

F. hepatica metacercariae incubated with azocollagen released the azo dye from the substrate but very slowly. The average colour intensity of the dye released by about 30 metacercariae at 530nm after 6 hrs. incubation with 5mg azocollagen was 0.188. This is equivalent to the dye intensity released by 40 µg Clostridium histolyticum collagenase from a similar substrate concentration in 1 hr.

Immature flukes also released the azo dye from azocollagen and this was very slow during the first 4 hrs. of incubation but increased by the 6th hr. The activity was directly related to the substrate concentrations, see Table IV and Fig. 3.

Table IV Collagenolytic activity of immature flukes (6 weeks old) 5ml substrate fractions, containing 5mg azo-collagen, were incubated each with 5 immature flukes. Total of 60 flukes were used.

Substrate concentration mg/ml	Average reading/5 flukes at 530nm		
	2 hrs.	4 hrs.	6 hrs.
1	0.027 ± 0.002	0.097 ± 0.001	0.385 ± 0.04**
0.5	0.025 ± 0.002	0.077 ± 0.002	0.157 ± 0.024*
0.2	0.025 ± 0.003	0.073 ± 0.002	0.123 ± 0.03*

* Equivalent to the dye released by 40 µg Cl. histolyticum collagenase, incubated similarly, in less than 1 hr.

** Equivalent to the dye released by 40 µg Cl. histolyticum collagenase in 2 hrs.

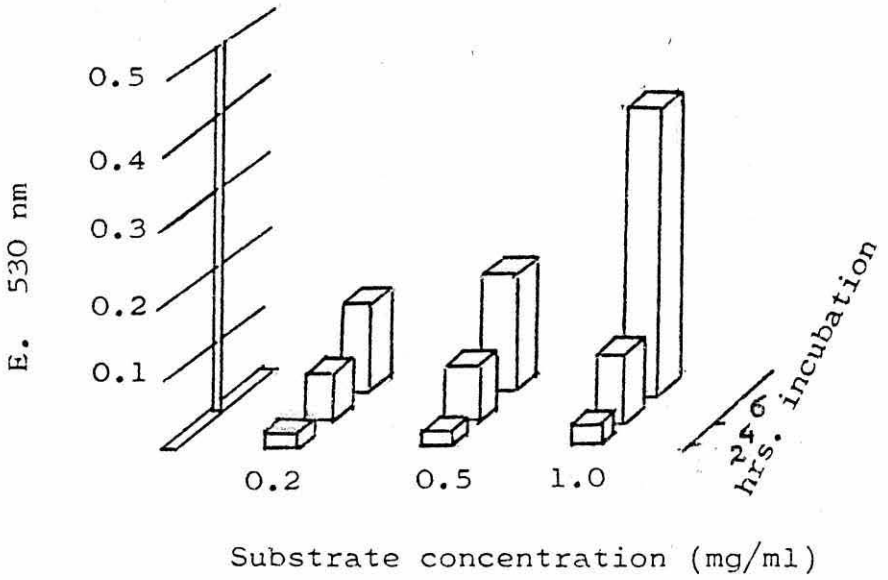


Fig. 3 Collagenolytic activity of immature F. hepatica. Average activity/5 flukes.

Mature F. hepatica had high collagenolytic activity as shown in Table V and Fig. 4 and the results can be summarised into the following points.

1. One mature fluke may have an activity equivalent to that of 40 µg Cl. histolyticum collagenase incubated with similar amounts of substrate (5 mg azocoll in 5ml buffer) for one hour.
2. One fluke no longer released azo dye from azocollagen after the 3rd hour of incubation.
3. The collagenolytic activity of flukes was directly related to the number of flukes and incubation time.
4. At least 3 flukes were required to completely digest 5mg azocollagen in 4 hrs. 6 flukes may digest 5mg in 2 hrs.

Collagenolytic activity was absent from the buffer medium when examined after pre-incubation with mature flukes for 3 hrs. This indicates that lytic substances were not secreted in the medium in the absence of substrate.

Table V Collagenolytic activity of adult F. hepatica. Mean activity per fluke after incubation with 5mg azocollagen.

No. of flukes	Reading at 530nm		
	1st hr.	3rd hr.	4th hr.
1	0.446 ± 0.287	1.00 ± 0.38	1.048 ± 0.775
2	0.537 ± 0.351	1.33 ± 0.55	2.05 ± 0.75
3	1.06 ± 0.66	1.42 ± 0.46	2.525 ± 0.32
4	1.27 ± 0.449	1.64 ± 0.520	2.325 ± 0.525
5	1.587 ± 0.41	1.90 ± 0.440	2.525 ± 0.325
6	1.50 ± 0.47	2.46 ± 0.75	2.50 ± 0.33

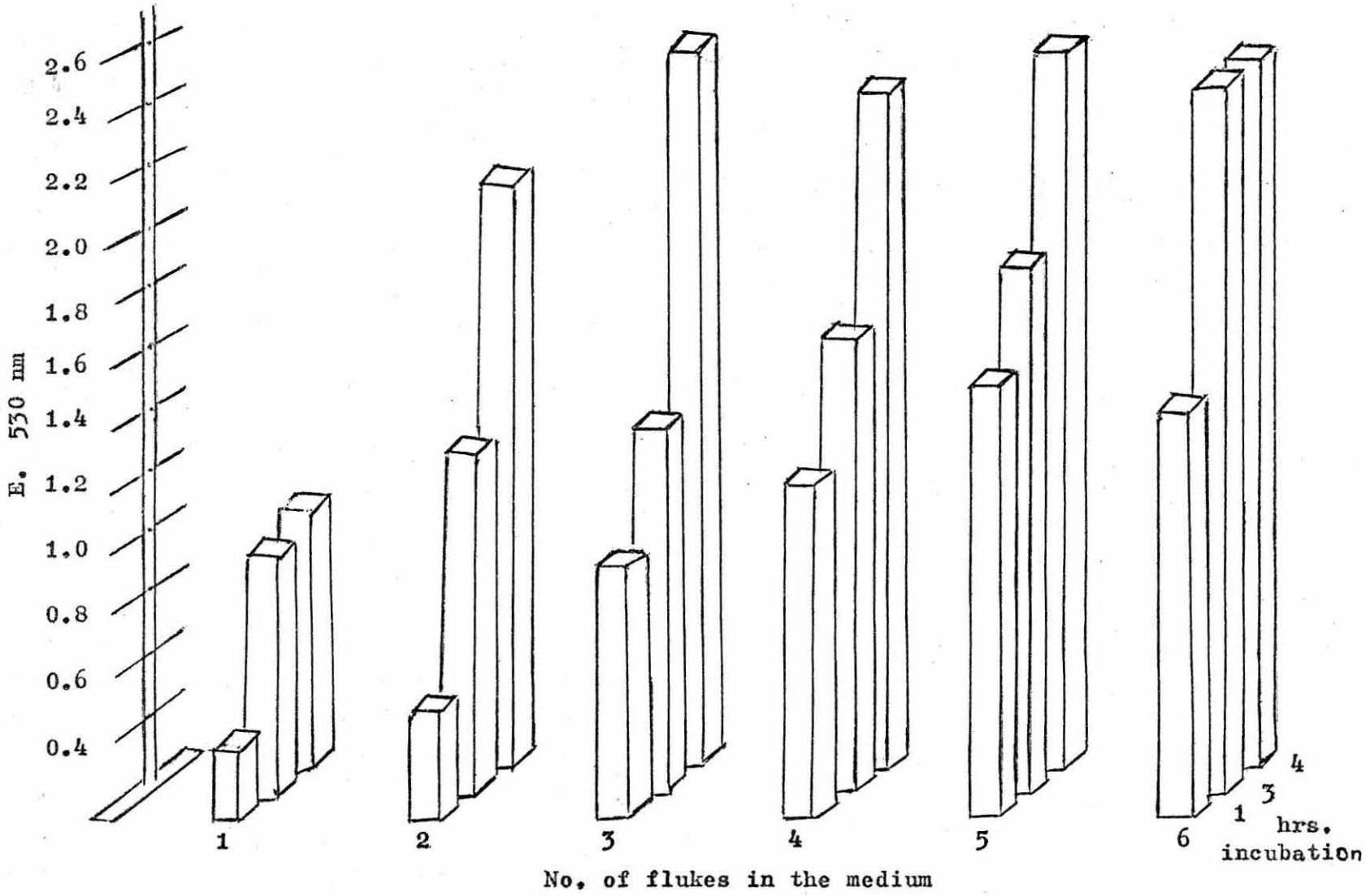


Fig. 4. Collagenolytic activity in mature F. hepatica.

Elastinolytic activity:

30 metacercariae of F. hepatica showed slight elastinolytic activity after 6 hrs. incubation with elastin.

Immature flukes, on the other hand, had a high level of activity which increased throughout the incubation period. The results are presented in Table VI and Fig. 5. Again the activity was directly related to the substrate concentration.

Table VI Elastinolytic activity of immature flukes. 5mg elastin suspension incubated with 5 immature flukes. 60 flukes were used.

Substrate concentration	Average reading/5 flukes at 495nm		
	2 hrs.	4 hrs.	6 hrs.
1.0	0.88 ± 0.039	1.06 ± 0.05	1.182 ± 0.25
0.5	0.49 ± 0.04	0.59 ± 0.045	0.665 ± 0.08
0.2	0.45 ± 0.032	0.512 ± 0.04	0.590 ± 0.05

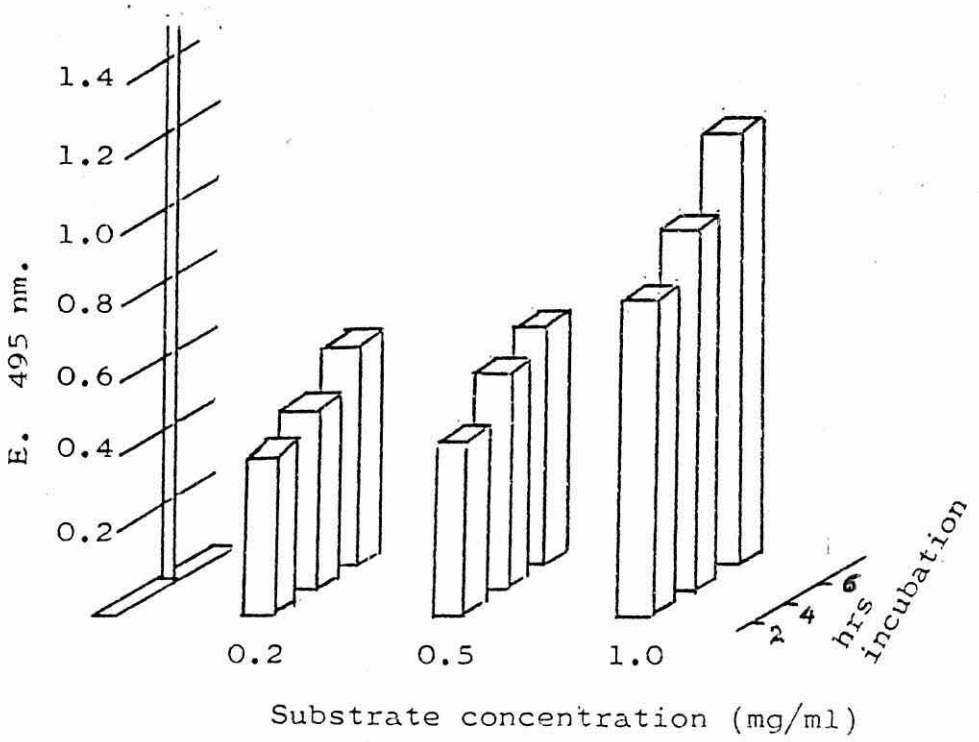


Fig. 5 Elastinolytic activity in immature F. hepatica.

Mature flukes:

Table VII and Figs. 6 and 7, show the average elastinolytic activity per adult fluke over a period of 8 hrs. incubation. The activity was directly related to time and the relationship was linear till the 7th week; no further breakdown of elastin occurred at week 8. It can also be seen from Table VII and Fig. 5 that medium sized flukes (0.1-0.19g in weight) had more activity than smaller or bigger-sized flukes.

Table VII Average elastinolytic activity per adult fluke incubated with 5mg elastin.

Weight range (g)	No. of flukes	Reading at 495nm			
		1st hr.	2nd hr.	3rd hr.	4th hr.
0.00-0.09	15	0.0335 ± 0.006	0.051 ± 0.019	0.075 ± 0.015	0.105 ± 0.005
0.10-0.19	21	0.0415 ± 0.027	0.159 ± 0.157	0.239 ± 0.206	0.306 ± 0.287
0.20-0.29	18	0.0277 ± 0.026	0.100 ± 0.076	0.147 ± 1.06	0.239 ± 0.158
Average activity	(54)	0.034 ± 0.065	0.104 ± 0.110	0.155 ± 0.150	0.238 ± 0.215
		5th hr.	6th hr	7th hr.	8th hr.
0.00-0.09	15	0.156 ± 0.056	0.226 ± 0.108	0.233 ± 0.105	0.223 ± 0.105
0.10-0.19	21	0.428 ± 0.381	0.369 ± 0.172	0.527 ± 0.195	0.545 ± 0.135
0.20-0.29	18	0.255 ± 0.179	0.279 ± 0.192		
Average activity	(54)	0.300 ± 0.288	0.311 ± 0.290	0.389 ± 0.160	0.377 ± 0.125

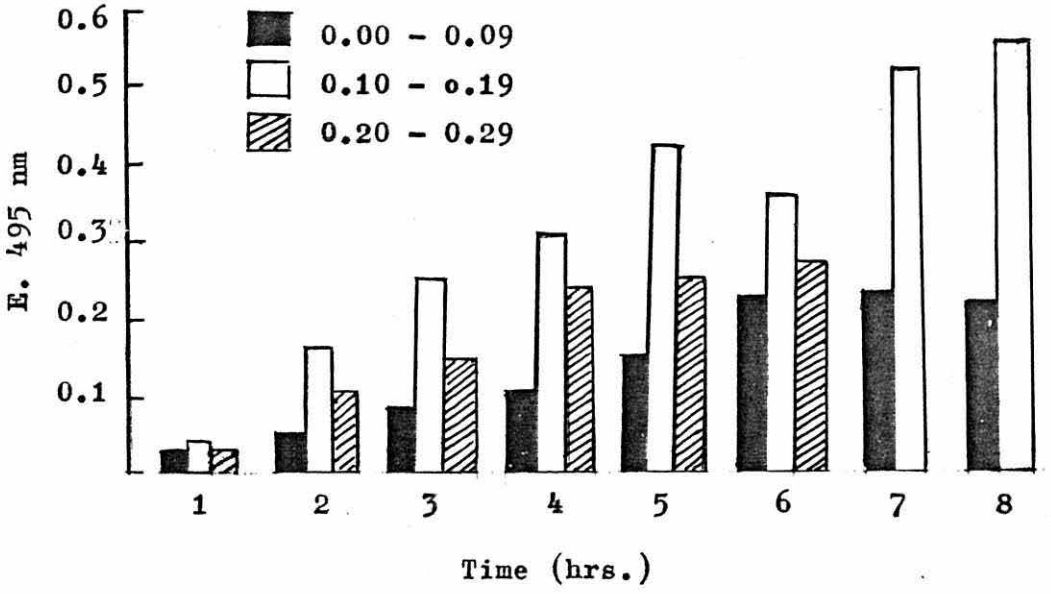


Fig. 7. Elasinolytic activity in adult F. hepatica of different weights.

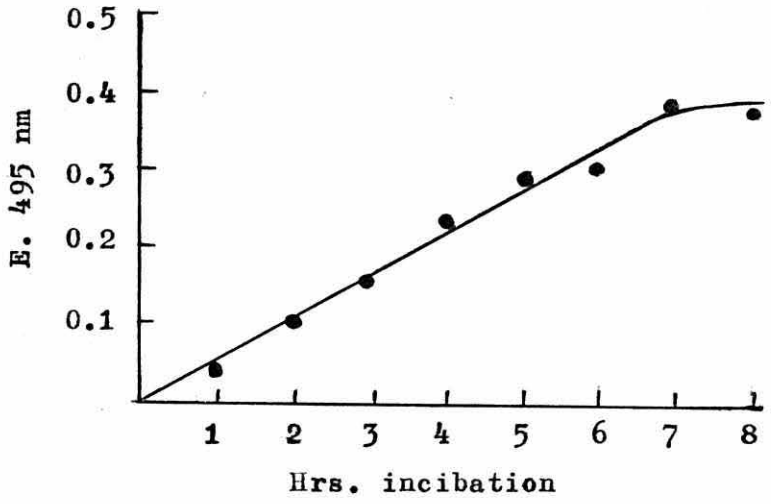


Fig. 6. Avarage elastinolytic activity per adult F. hepatica incubated with 5 mg Congo red dyed elastin in 5 ml carbonate buffer for different times.

Fluke extract also had a marked lytic action on elastin and this showed a linear relationship with time (see Fig. 8).

The activities of known amounts of pancreatic elastase incubated with 5mg elastin are presented in Fig. 9. From this figure it can be seen that a single immature or adult fluke incubated for 2 hrs. with 5mg substrate may have an activity equivalent to that of 22 µg or 13 µg elastase respectively.

Purification of fluke elastase:

The precipitation procedure of euglobulin from pancreatin was followed to precipitate a similar fraction from freeze-dried flukes. The results of the chromatography of this euglobulin-like precipitate on CMC, i.e. the elution curve, are shown in Fig. 10. It can be seen that the protein concentrations increased in the 3rd to the 13th fraction of the effluent especially so in fractions 8-11. The same fractions, 8-11, also showed the highest elastolytic activity and had molarities of 0.150, 0.225, 0.250 and 0.255 respectively.

Enzymatic activity in plasma:

i) Elastinolytic activity:

Sheep and cattle plasma were found to possess lytic activity against elastin. The changes in activity during the course of experimental fascioliasis was investigated in 6 sheep (each infected with 518 F. hepatica cysts) and 2 cows (each infected with 1880 cysts). The results are presented in Table VIII and Fig.11. In both the sheep and cows the elastino-

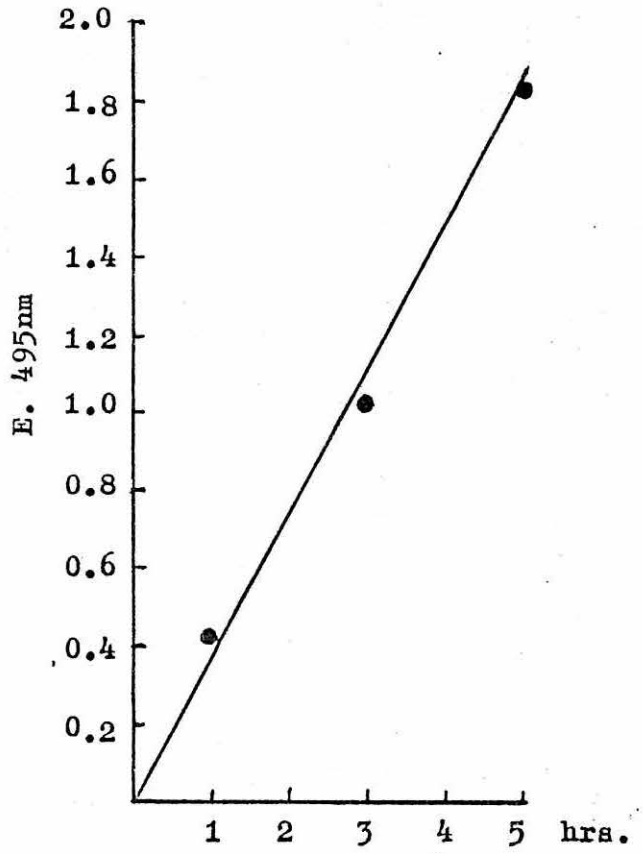


Fig. 8. Elastinolytic activity of 1 ml extracts of adult F. hepatica incubated with 5 mg Congo-red dyed elastin.

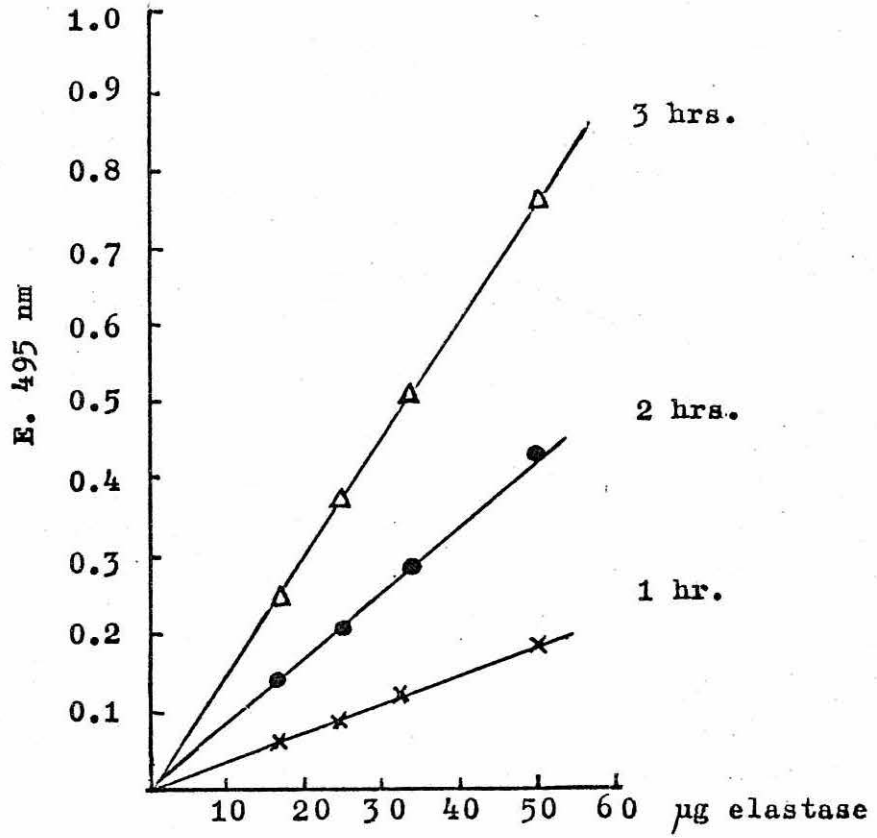


Fig. 9. Activity of different concentrations of pancreatic elastase icubated with 5 mg fractions of Congo red dyed elastin.

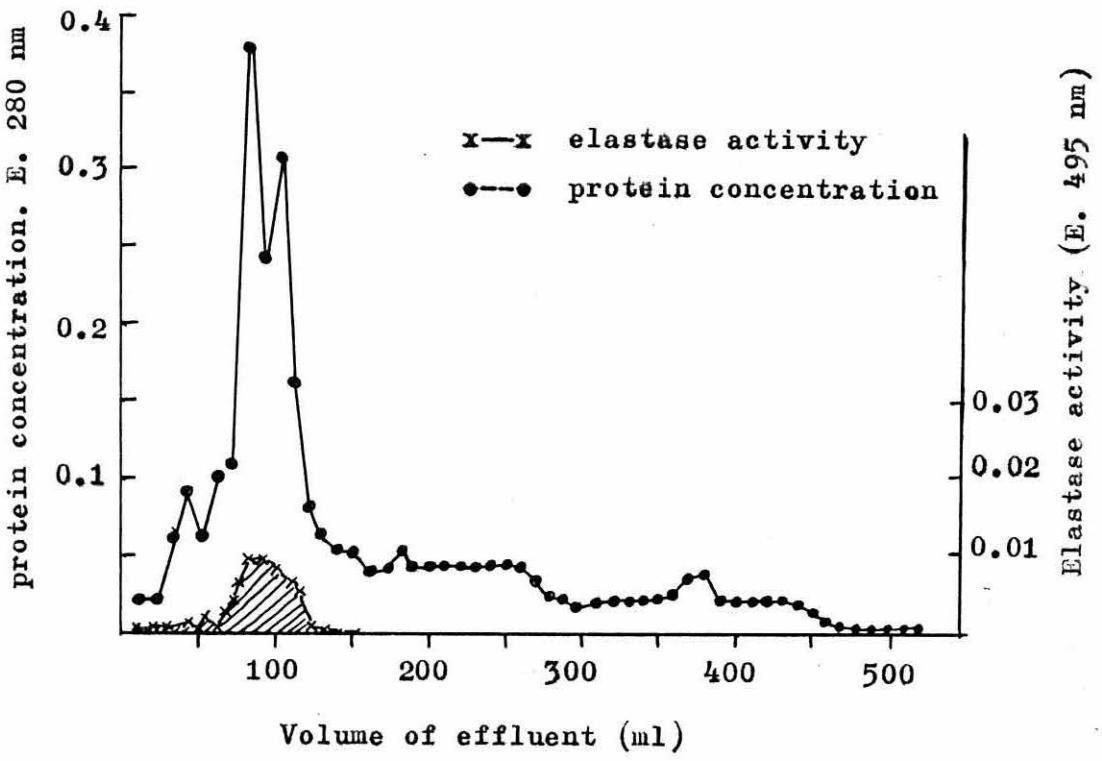


Fig. 10. Chromatography of "euglobin-like" precepitate obtained from freeze-dried adult F. hepatica on carboxymethylcellulose. See text for details.

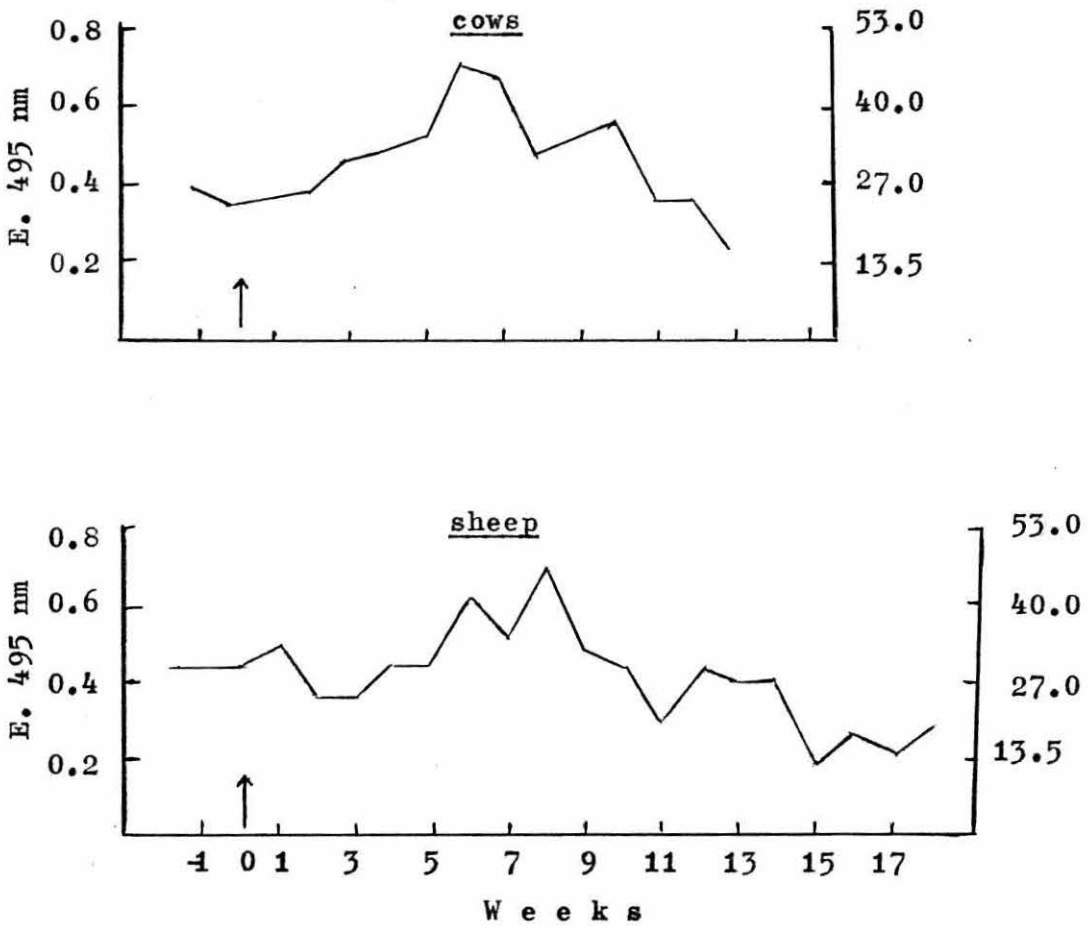


Fig. 11. Mean weekly elastinolytic activity in plasma of sheep and cows infected with F. hepatica.
0.2 ml plasma + 5 ml (5 mg) elastin substrate incubated for 3 hrs. ↑ = infection

lytic activity increased during infection and was highest at weeks 6, 7 and 8. Afterwards the level declined and this was more evident in sheep plasma which was tested for a longer period.

Table VIII Plasma elastinolytic activity in 6 sheep and 2 cows infected with F. hepatica. 5mg dyed elastin + 0.2ml plasma incubated at 37° for 3 hrs. Intensity of dye released is directly related to the enzymatic activity. (Mean values).

Weeks of infection	E. 495nm Sheep	Cows	Weeks of infection	E. 495nm Sheep	Cows
-2	0.430±0.040	-	9	0.457±0.066	-
-1	-	0.395±0.075	10	0.437±0.075	0.57 ±0
0	0.448±0.060	0.350±0.020	11	0.297±0.039	0.365±0.025
1	0.493±0.035	-	12	0.432±0.044	0.370±0.030
2	0.362±0.030	0.385±0.005	13	0.290±0.048	0.24 ±0.030
3	0.363±0.011	0.465±0.025	14	0.410±0.054	
4	0.438±0.055	0.495±0.075	15	0.182±0.011	
5	0.445±0.035	0.530±0.040	16	0.258±0.042	
6	0.618±0.071	0.715±0.115	17	0.222±0.033	
7	0.513±0.030	0.685±0.025	18	0.282±0.063	
8	0.708±0.076	0.485±0.025			

ii) Hyaluronidase and collagenase activity:

No activity against hyaluronic acid or azocollagen was detected in sheep or cows plasma.

The effect of sheep plasma on hyaluronidase and collagenase activity:

Known amounts of testicular hyaluronidase and Cl. histolyticum collagenase were pre-incubated with sheep plasma before being incubated with the appropriate substrate. Their activities were then assessed and expressed as percentages

of those of controls pre-incubated with buffer only and treated similarly. The results can be seen in Table IX and Fig. 12. It is evident that the addition of sheep plasma favoured the action of hyaluronidase during the preinfection period and the first 4 weeks of infection, but inhibited its action during weeks 5-11 post-infection. Afterwards the activity was only slightly inhibited. Figure 12 also shows that sheep plasma had an inhibitory effect on clostridial collagenase, which increased gradually during the first 7 weeks of infection and was more pronounced during the terminal 8 weeks.

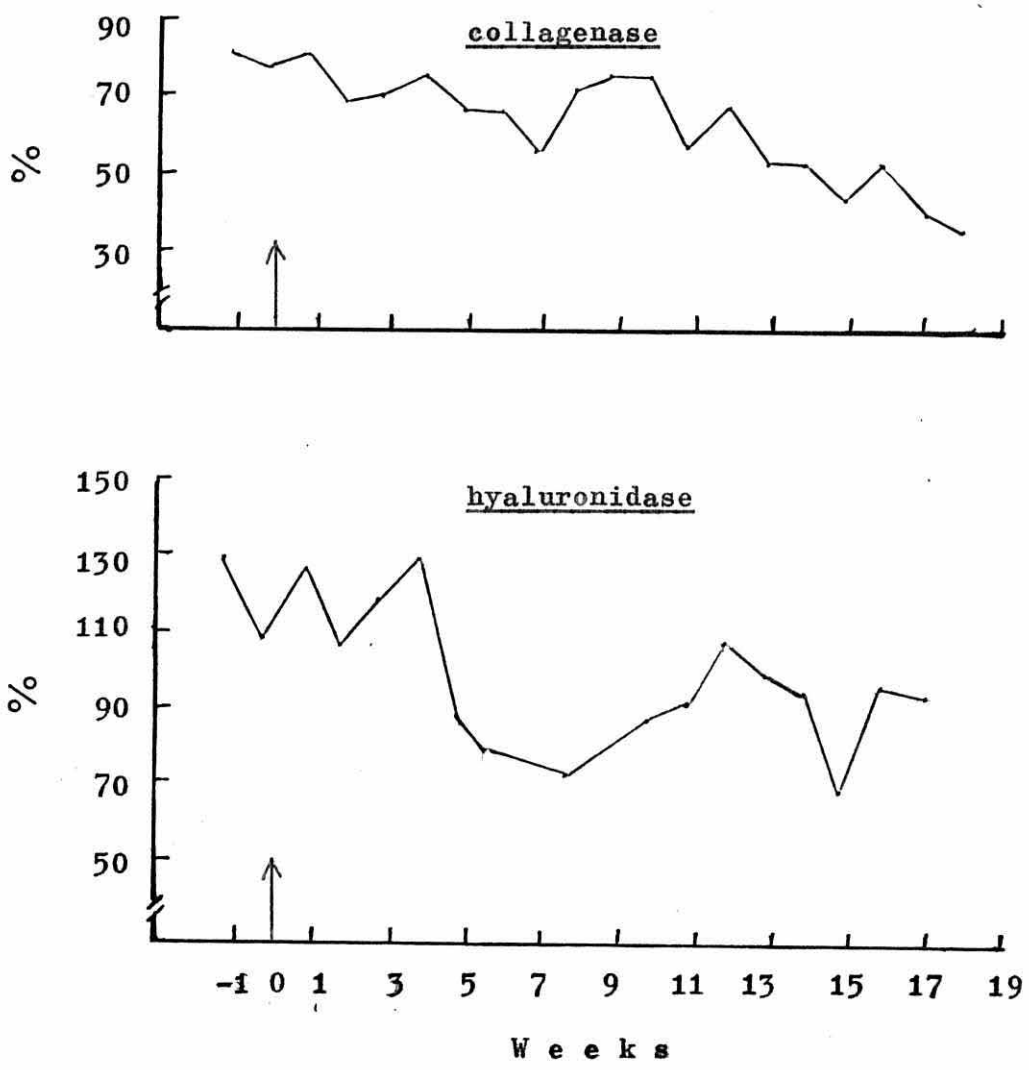


Fig. 12. Effect of plasma on hyaluronidase and collagenase activities
The enzymes were pre-incubated with plasma of sheep and cows experimentally infected with F. hepatica and then incubated with the substrate. The activities were expressed as percentages of those of controls treated similarly. ↑ = infection.

Table IX. The effect of sheep plasma on the activities of hyaluronidase and collagenase. Enzymes were preincubated with plasma and then with substrates (test). Their activities expressed as percentages of those of controls preincubated with buffer alone and then with substrates. Mean values.

Weeks of infection	Percentage activity : Test/Control x 100	
	Hyaluronidase	Collagenase
-2	-	-
-1	129.49 ± 11.8	81.25 ± 18.91
0	107.94 ± 6.35	77.45 ± 4.56
1	127.78 ± 7.85	80.00 ± 7.21
2	106.91 ± 8.89	67.97 ± 6.02
3	119.15 ± 5.50	70.14 ± 5.07
4	130.11 ± 4.81	76.19 ± 20.27
5	89.17 ± 4.06	66.67 ± 6.48
6	79.00 ± 3.67	66.00 ± 12.70
7	-	54.92 ± 7.03
8	74.4 ± 3.67	71.56 ± 5.02
9	-	76.53 ± 12.89
10	88.88 ± 4.23	75.31 ± 2.81
11	93.07 ± 12.55	55.86 ± 4.19
12	108.33 ± 5.24	67.60 ± 3.27
13	99.57 ± 8.80	52.46 ± 22.99
14	95.15 ± 5.21	52.88 ± 2.71
15	65.82 ± 3.82	44.24 ± 6.29
16	96.88 ± 4.93	53.06 ± 1.50
17	94.44 ± 4.66	40.50 ± 10.4
18		35.95 ± 1.72

DISCUSSION

Enzymatic activity in F. hepatica:

The present findings indicate that F. hepatica can secrete lytic substances against a wide range of protein and non-protein substrates. Collagenolytic and elastinolytic activities were demonstrated in metacercariae, immature flukes and adult parasites. Hyaluronidase-like activity was tested and demonstrated in mature flukes, but other stages, which were not examined here, might have such an activity. Many reports have previously described proteolytic activity in intact mature F. hepatica and their homogenates (Pennoit De Cooman and Van Grembergen, 1942; Halton, 1963; Thorsell and Bjorkman, 1965; Thorpe, 1968; Howell, 1973), and in immature flukes and their extracts (Howell, 1966) but little information seems to be available on the presence of enzymatic activity in miracidia or metacercariae. The present results suggest that all stages could possess enzymatic activity and contrary to the results of Howell (1966), who found very little collagenolytic activity in mature flukes, a powerful collagenase-like action was detected in adult flukes in this investigation. Apparently, the presence of hyaluronidase - or elastase-like enzymes in the liver fluke has not been reported. The presence of these latter enzymes in addition to collagenase or collagenase-like secretions in Fasciola species could be of importance for their penetration and migration in the host tissues as well as for their nourishment.

The ground substance of connective tissue is complex and contains abundant amounts of glycosamino-glycans. The main

constituents of the latter are hyaluronic acid and varieties of chondroitins and their sulphated esters which act as hyaluronidase substrates. Collagenous fibres are also present in all types of connective tissue i.e. in epithelial basement laminae, fibrous capsules, surrounding capillaries, sinusoids, muscle fibres and parenchymal cells, while elastic fibres are found where greater expansibility and tensility are needed, i.e. in arteries, ligaments, tendons, trachea, larger branches of the biliary tree etc. (see Bloom and Fawcett, 1968). Migrating flukes break through connective tissue in the intestinal wall, liver capsule and in the hepatic substance, and in so doing they may secrete penetrating enzymes, i.e. collagenases, elastase and hyaluronidase to depolymerise connective tissue. Moreover, liver flukes, whether they feed on blood or tissues, do need proteolytic enzymes to hydrolyse protein substances to provide them with their amino acid requirements and broadly to prepare food for internal digestion. In this respect, Thorsell and Bjorkman (1965) found that intact flukes liberated proteolytic material to the exterior and Howell (1973) concluded that the caecae epithelia synthesise enzymes which could be used for intracellular digestion or be subsequently released into the lumen for extracellular digestion; enzymes could be discharged outside the fluke body as a result of regurgitation.

The observation that the lytic action against azocasein and elastin was more in medium-sized flukes is difficult to explain and attempts to explain it might lead to many unrealistic speculations.

Whole flukes were used in preference to fluke extracts as an enzyme source and this was to exclude the involvement of tissue enzymes which may possibly be released during homogenization, or to avoid reduction in enzymatic activity due to inactivation by oxidation effects during tissue disruption (Howell, 1966).

Flukes incubated with buffer alone for 3 hours did not secrete proteolytic substances in the buffer medium; samples of the latter had no action on azocollagen or elastin. This indicates clearly that the presence of substrate acts as a direct stimulus for the flukes to secrete these proteolytic substances.

The question to be asked is that: does the enzymatic activity demonstrated in flukes against azocollagen, elastin and chondroitin denote the presence of true collagenase, elastase and hyaluronidase? The exact answer to this question will remain pending until these fluke enzymes are isolated and properly characterised, and because of uncertainty the terms collagenase-like, elastase-like and hyaluronidase-like enzymes or substances may better be used. Most of the doubts, however, regard azocollagen which may not be a specific substrate for collagenase. Milleman and Thonard (1959) maintained that azocollagen is a denatured protein and activity against this substrate is not a suitable test for true collagenase; the true collagenase hydrolyses undenatured collagen. However, many authors have used azocollagen to demonstrate collagenase activity (Oakley, Warrack and Van Heyningen, 1946; Lewert and Lee, 1956; Howell, 1966; Matthews, 1975; among others).

Chondroitin, and hyaluronic acid, are known specific

substrates for hyaluronidases and their solubilization, under optimum conditions set for known hyaluronidases, should be taken to denote the presence of a true enzyme. Similarly the digestion of elastin by flukes' secretions should indicate the action of true elastase; elastin is the ideal substrate for elastase and other proteinases have very little activity against it. The congo red-dyed elastin is a commonly employed substrate for the assay of elastases (Shotton, 1970).

Purification of fluke elastase or elastase-like substances was attempted first because it appears to be secreted in large amounts by both immature and mature flukes indicating that it has an important functional role as a broad action proteinase in the life of these parasites. Elastases are known to have a wide peptide bond specificity and can hydrolyse a wide variety of protein substrates besides elastin (Lewis, Williams and Brink, 1956). The purification procedure of euglobulin from pancreatin described by Naughton and Sanger (1961) was followed and an euglobulin-like precipitate was obtained and chromatographed on CMC. The results indicate that the elastase activity was eluted in fractions 8-11 of the effluent and that these fractions had the highest protein concentrations. Under the conditions of the system used these effluent fractions, 8-11, had molarities of 0.15, 0.225, 0.250 and 0.255 respectively. These findings seem to agree with those of Naughton and Sanger (1961) and De Cremoux et al. (1978) who found that elastase activity was eluted with 0.2M buffer, despite the different methods employed. The former authors were purifying elastase from pancreatin and the latter from alveolar macrophages.

It seems that this study is the first attempt to detect and purify elastase or elastase-like substances in

F. hepatica. A lot of work is to be done to confirm the presence of the true enzyme, purify it and study its characteristics. The purification procedure used here is not free from inaccuracies and may not be the right choice to purify elastase from parasites. More specific and accurate methods may be available and these should be sought in future studies. Similarly purification of other fluke enzymes, particularly those involved in tissue breakdown, i.e. collagenases and hyaluronidase, should be attempted after confirming their true existence. Advantages of such pure enzyme preparations, if successfully achieved, may be taken in producing specific antienzymes which could theoretically neutralize fluke enzymes and consequently retard or even prevent fluke penetration and migration. The idea of parasitic anti-enzyme immunity has been pointed out a long time ago (Chandler, 1935; Soulsby, 1957 cited by Howell, 1962) but it does not seem to have been seriously carried further. Antibodies are known to be produced by the host against flukes but their protective role is not firmly ascertained (Nielsen, 1976) and it might be advantageous to explore the possibility of utilizing anti-enzymes to induce resistance against flukes as an alternative or complementary means of protection.

Plasma elastinolytic activity:

Elastase is mainly found in the pancreas and pancreatic juice of many mammals and birds (Appel, 1974). Its presence has been demonstrated in human sera (Chao, Sciarra and Vosburg, 1962; Hall, 1966; Boumstark, 1967; Rinderknechi, Geokas, Silverman and Haverback, 1968), erythrocytes (Schonemann, 1967)

and granulocytes (Janoff, 1970, 1972) and is also reported to be secreted by macrophages obtained from man and animals (Janoff, Rosenberg and Galdston, 1971; Werb and Gordon, 1975; De Cremoux et al., 1978).

The present findings demonstrated the presence of elastinolytic activity in sheep and cattle plasma and indicated that the level of this activity changes during the course of experimental F. hepatica infection. The lytic action against elastin increased during the prepatent period and decreased in the patent phase. These findings can be explained by several assumptions: a) the flukes migrate through the liver mass and leave behind necrotic tracts which are replaced by connective tissue. This might specifically or non-specifically stimulate the host cells (in pancreas, granulocytes, tissue macrophages etc.) to produce elastase which by virtue of its wide proteinase activity may participate, together with other enzymes, in resorbing the newly deposited connective tissue. Maximum tissue damage and fibrosis is expected to occur during the localized migratory phase just before the flukes enter the bile ducts, and this corresponds with the period of maximum plasma elastinolytic activity. After the flukes enter the bile ducts the elastinolytic activity is expected to decrease because parenchymal damage is less in the patent period and the need for the enzyme will accordingly be reduced. b) It has been demonstrated in this study that flukes may secrete powerful elastinolytic substance(s) while migrating and breaking through tissues. These substances could diffuse through the sinusoids to reach the circulation, and contribute to the plasma elastinolytic activity. Flukes in the bile ducts might continue to secrete the lytic

substances but these are likely to be excreted to the exterior rather than diffuse in blood, and accordingly the plasma activity will not be increased.

c) Elastase inhibitors are known to occur in serum of humans and animals (Balo and Banga, 1949; Tolnay and Bagdy, 1959; Turino, senior, Garg, Keller, Levi and Mandl, 1969; De Cremoux et al., 1978). Roberts and Samuel (1957) showed that the serum inhibitor is found in the α-lipoprotein and Walford and Schneider (1959) reported the inhibitor to migrate electrophoretically at the junction of albumin and α_1-globulin. α_1-Antitrypsin (Heimburger and Haupt, 1966) and α_2-Macroglobulins (De Cremoux et al., 1978) have been reported as powerful inhibitors. It is difficult to visualize properly the role of these inhibitors in the present findings but they might have affected the plasma elastinolytic activity during the patent period of infection.

Inhibition of collagenase and hyaluronidase activities by plasma

The present results showed that Cl. histolyticum collagenase activity was decreased by plasma of sheep infected with F. hepatica. This indicates the presence in plasma of collagenase inhibitor(s) whose activity increased during the course of fluke infection. Of the plasma protein constituents the α_2-Macroglobulins (α_2-Mg) are known to inhibit the action of collagenases (Harpel, 1973). These α_2-Mg act as substrates for the enzyme and when they bind to each other a conformational change occurs in the macroglobulin in such a way that the enzyme molecules are entrapped and inhibited (see Werb, Burleigh, Barrett and Starkey, 1974). Another collagenase inhibitor, a β-serum protein, was also reported (Woolley, Roberts and

Evanson, 1976). The effect of these inhibitors may vary from one animal species to another. Thus, Woolley, Akroyd, Evanson, Soames and Davies (1978) found that the α_2-macroglobulins may account for about 60% of the total collagenase inhibitory capacity of dog's serum and the β-serum protein inhibitor for about 40%; the latter fraction was less effective in human serum accounting only for about 5%.

The inhibitory effect of plasma on collagenase as seen in the current findings could therefore be due to increase in mainly α_2-Mg. These macroglobulins are glycoproteins which may constitute about 80% of the α_2-electrophoretic fraction (Turner and Hulme, 1971). It has been shown in the previous section that the α-globulins and α-globulin glycoproteins of the same plasma samples used to detect collagenase inhibitors, were greatly increased during the course of infection; this increase could be due to elevated α_2-Mg levels. Another inhibitory effect on collagenase activity could be caused by changes in the plasma ionic concentrations. Analysis of the plasma samples (results not included in this thesis) revealed that calcium ion concentration decreased after the 10th week of exposure and remained low till the end of the experiment. This might have affected the collagenase activity as calcium ions are reported to promote collagenase activity (Seifter and Harper, 1970).

The current investigation showed that plasma obtained from fluke-infected sheep during the first 4 weeks of exposure had no adverse effect on the action of testicular hyaluronidase but the samples drawn during the 5th-11th weeks of infection were inhibitory to hyaluronidase. The period between weeks 5-11 of exposure corresponds to a phase of active

tissue destruction and the flukes may secrete hyaluronidase-like enzymes (as has been demonstrated) to depolymerise the hepatic connective tissue and facilitate easy burrowing. As a result, inhibitory factors against these fluke haluronidase-like enzymes may be released in the host's blood and tissues to antagonise this spreading factor(s).

A hyaluronidase inhibitor, known as Physiological Hyaluronidase Inhibitor (PHI), is known to be found in sera of man and animals (Mclean, 1942; Hobby, Dawson, Meyer and Chaffee, 1944) and its level increases in various pathological conditions involving tissue destruction and proliferation (Glick, 1950; Shack, Whitney and Freeman, 1950; Mathews and Dorfman, 1955; Shapiro, Bishop, Kuenzig, Tkaczeuski and Kamm, 1975). Fiszer-Fzafraz (1968) also found a hyaluronidase inhibitor in sera of cancer patients which seems to be different from PHI. So, the inhibition of hyaluronidase by fluke infected sheep plasma indicates that the level of PHI, or other hyaluronidase inhibitors, increases during the localized migratory phase of fascioliasis and this could represent a defence against the invading parasites.

Shacks et al. (1950) and Shapiro et al. (1975) reported increased PHI in scorbutic guinea pigs, presumably as a result of tissue depolymerisation which accompany vitamin C deficiency. It has been shown in the first part of this thesis that the plasma ascorbic acid concentrations in the same sheep, as well as in others, decreased after infection particularly during weeks 8-11. Tissue ascorbic acid content was also low in fluke-infected rats and it was concluded that deficiency of ascorbic acid may occur in fascioliasis. Whether the plasma PHI in fluke-

infected sheep was affected by a deficiency of ascorbic acid is a matter of speculation.

GENERAL DISCUSSION

GENERAL DISCUSSION

A good deal of information is now accumulating regarding the role of ascorbic acid in the functions of the various systems in the human and animal body and deficiency of this simple vitamin could lead to serious metabolic disturbances. The present study indicates that ascorbic acid deficiency may ensue in F. hepatica infections and this could have contributed to the development and severity of the clinical symptoms and lesions in infected animals.

Ascites and submandibular oedema were reported in fluke infected sheep (Sinclair, 1962, 1968, 1972). The latter was not observed in infected sheep of the present study but hydroperitoneum was observed. Moreover, the carcasses of the 6 animals infected with 518 F. hepatica cysts (experimental II part A) failed to set properly after slaughter even after 24 hrs. standing in the cold room; the tissues were moist, oedematous and slimy. The occurrence of oedema in fascioliasis can be explained by the many factors generally involved in the pathogenesis of oedema. Most important of these is the upset of the plasma colloidal osmotic pressure due, mainly, to decrease in plasma albumin with the subsequent movement of fluid from the circulation into tissues. Ascorbic acid deficiency may also, indirectly, contribute to the development of oedema. A relationship has been suggested between the vitamin and histamine detoxification (see p. 13). The latter increases capillary permeability which allows the loss of proteins and fluid from the blood. Histamine is present in many animal tissues and in mast cells, and there is a good correlation between the tissue histamine content and the number of mast

cells it contains (Bell et al., 1972). As in other inflammatory conditions the number of mast cells may increase in fascioliasis and these may contribute large amounts of histamine to tissues. The capacity to detoxify this amine, and consequently to prevent its vasodilatory action on capillaries, would be decreased in the case of ascorbic acid deficiency which is suggested to occur in fluke-infected animals. Accordingly, capillary permeability and escape of fluid from the circulation would be increased. Apparently the above argument has not been considered in the pathogenesis of oedema but has been taken to explain the occurrence of haemorrhages in scorbutic animals (Catterjee et al., 1975).

From the discussions on the changes observed in plasma iron and iron-binding capacity in fluke-infected animals, it was concluded that the decreased plasma iron levels were not primarily due to iron deficiency or to disturbance in its transportation but rather to defective release of the metal into the plasma. The defect in iron release could be caused by ascorbic acid deficiency which seems to occur in exposed animals. Besides affecting iron release, ascorbic acid deficiency may cause the formation of abnormal erythrocytes with decreased life span (Bloom and Fawcett, 1968). The role of ascorbic acid in iron metabolism in fascioliasis needs further investigations, which may be very helpful in understanding the pathogenesis of the anaemia in infected animals. In this respect the present findings revealed that liver copper concentrations increased in fluke-infected rats, especially in those killed at the 7th week of infection when the concentrations were more than three-fold those of controls.

This observation could be of some significance in the development of anaemia because copper may be released from the necrotic hepatic cells into plasma and cause intravascular haemolysis. Such a phenomenon is seen in humans suffering from primary biliary cirrhosis, chronic active liver diseases and Wilson disease, and the haemolysis is mainly due to the oxidative damage to erythrocytes by the released copper (Nutrition Review, 1977). It should, however, be mentioned that no visible haemolysis was observed in the plasma of fluke-infected animals used in this study.

The supplementation of vitamin C to F. hepatica infected rats and guinea pigs did not reveal firm conclusions as to the effect of the vitamin in ameliorating the symptoms and lesions in infected animals. However, ascorbic acid seems to have some beneficial effects in guinea pigs which are incapable of synthesising the vitamin. Infected animals on the low vitamin dose showed lower body weight gains, early appearance of illness symptoms and early mortality. These few observations are encouraging to further investigation of the effect of exogenous ascorbic acid on the pathology and clinical pathology of fascioliasis.

It was shown in this study that plasma cholesterol decreased while the low density lipoproteins increased during the course of experimental fascioliasis in sheep and a possible relationship between the above parameters and the changes in ascorbic acid levels was pointed out. However, the liver is assumed to be a major endogenous source for cholesterol synthesis (Bartley, 1970) and damage to this organ, as in fascioliasis, is likely to impair cholesterol synthesis.

Cholesterol in the liver is formed from acetate and if the argument about the probable decrease in volatile fatty acid production in fluke-infected sheep stands, then reduction in the acetate substrate would also lead to reduction in the rate of cholesterol formation. The increase in low density lipoproteins, on the other hand, could be due to increase in phospholipid or triglycerides, knowing that cholesterol levels were decreased. Inappetance and impaired food utilization, which accompany fluke infection, could lead to increased fat catabolism with the ultimate release of fatty acids and triglycerides in the circulation. Fatty acid transport is facilitated by albumin (Steinberg and Vaughan, 1965) and the hypoalbuminaemia, which occurs in fascioliasis,as shown here, may greatly limit their transportation. Alternatively, the fatty acids may be carried as phospholipids and a rise in this latter class would consequently increase the low-density lipoproteins. Le Bars and De L. Banting (1976) described changes in plasma triglycerides in fluke-infected sheep; the levels decreased during the 3rd to the 5th week and also at the 9th (66 days) of exposure. The present findings show that at similar periods after infection (between the 3rd-7th week and the 8th-10th week) the low-density lipoproteins were significantly increased in infected animals. Taking advantage of the findings of Le Bars and De L. Banting (1976) and knowing that plasma cholesterol levels were decreased in this study, then the rise in the low-density lipoproteins observed could mainly be due to a rise in phospholipids.

Disturbances in lipid metabolism have been observed in

fascioliasis by other workers. Fatty infiltration associated with increased total lipids and lipase activity (Zakhariv and Lemishko, 1975) and with elevated cholesterol content (Rubaj and Furmaga, 1969) were observed in livers of infected sheep and cattle. Qualitative and quantitative changes in fatty acid composition of serum and bile in infected cattle were also reported (Martincic, Fulgosi and Krvavica, 1973).

The changes observed in the plasma lipids in the current study, besides the possibility of being affected by the suggested ascorbic acid deficiency, could also be influenced by the tissue mineral levels. It seems that multiple interactions occur between minerals in the animal body and this may play a role in the regulation of serum lipid levels. Thus Klevay (1973) found that rats given water with high ratio of zinc/copper had elevated serum cholesterol and phospholipids. Sherman, Guthree and Wolinsky (1977) observed that restriction of dietary iron in maternal rats resulted in decreased zinc levels and increased copper concentrations in the livers and spleens of the iron deficient offspring. This was associated with an increase in serum triglycerides, non-esterified cholesterol and phospholipids, but it was not known whether the alterations in serum lipids were due to the iron deficiency or to the changes in zinc and copper levels.

The present study revealed significant changes in plasma iron and liver iron and copper concentrations in fluke-infected animals and these, together with the changes observed in plasma ascorbic acid and plasma proteins, might have influenced the plasma cholesterol and low-density lipoproteins observed. The above assumption, however, awaits experimental

confirmation.

From the search in the literature it appears that few studies have been done on the plasma lipoproteins and glycoproteins. Estimations of the different plasma protein fractions, from total protein values and electrophoretic concentrations, have been reported by many authors but these alone provide little information about changes occurring in their conjugated non-protein constituents. The latter should be determined simultaneously to give a more comprehensive picture of the overall changes occurring in the plasma proteins. Such an approach was attempted in the current study, but the data presented are far from complete.

The changes in plasma glycoproteins in many disease conditions have not been widely utilized for diagnostic and prognostic purposes. Recently, fucose and sialic acid estimations have been used in the diagnosis of neoplastic conditions (Rosato et al., 1971; Kloppel et al., 1978). This study also showed that the ratios of total hexoses or hexosamine/fucose values could be of diagnostic use in fascioliasis. Determination of specific glycoproteins of specific functions may be helpful in understanding more about the disease. Of particular interest are those glycoproteins which act as enzyme inhibitors (α_2-Macroglobulins, α_1-Antitrypsin); these may offer an explanation for the changes observed in the inhibitory effect of plasma of fluke-infected animals on the activity of certain enzymes, i.e. collagenase, elastase, hyaluronidase. The plasma glycoprotein inhibitors could play an important part in inhibiting the activity of endogenous enzymes in the blood or tissues of the host or

that of exogenous enzymes secreted by the invading flukes. Evidence has been provided here that young and adult flukes in the definitive host secrete enzymes which assist them in penetrating the host tissues. In this connection it should be remembered that the fluke enzymatic activity has been mostly related to the weight of the parasite and it may be more informative if the enzyme activity is related to the age of the flukes. Studies on the effect of storage on the enzyme activity of the encysted cercariae might be useful in explaining the changes in their virulence with storage and which could be of practical significance in experimental infection.

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