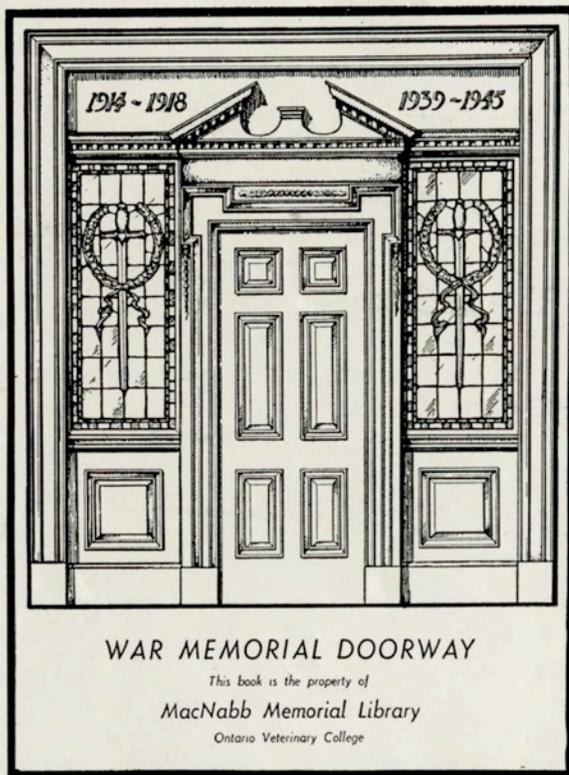


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LIGATION OF THE HEPATIC ARTERY,  
A BACTERIOLOGICAL, SEROLOGICAL AND SURGICAL STUDY

A Thesis

Presented to the Faculty of the Graduate School  
of the University of Toronto  
in Partial Fulfilment of the Requirements  
for the Degree of

Doctor of Veterinary Science

by

Leon M. Cobb

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## BIOGRAPHICAL SKETCH

The author was born in the County of Gloucestershire, England, in 1933. He obtained his secondary school education at Sodbury Grammar School, gaining the Oxford School Certificate in 1950, and the Higher School Certificate two years later. The following October he entered the second year of the veterinary course at the University of Bristol on a Governor's Scholarship. On July 3rd, 1956, the author graduated with the degree of Bachelor of Veterinary Science, and was subsequently admitted as a member of the Royal College of Veterinary Surgeons. In the same year he enrolled at the School of Graduate Studies of the University of Toronto and commenced study at the Ontario Veterinary College as a Graduate student. The author accepted a lectureship in the Department of Medicine and Surgery in June 1957. In May of the following year he was granted the degree of Master of Veterinary Science by the University of Toronto. The author re-enrolled in the School of Graduate Studies of the University of Toronto in September 1959.

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## INTRODUCTION

The reason for the dual blood supply to the liver has intrigued experimenters for many years. One of the ways to study this system was to ligate either the arterial or the portal blood supply and note the effects. The early experimenters soon found that when the portal vein was ligated in the dog it rarely survived long enough to be removed from the operating table (54) (75). When they came to study ligation of the hepatic artery it must have been with great surprise that they noted the animals dying, after a day or so, from an acute clostridial hepatitis. Could it be then, that the arterial blood had a bacteriostatic activity which when curtailed by ligation of the hepatic artery allowed clostridia to proliferate freely in the liver and cause the death of the host? Little light was thrown on this subject until 1949, when Markowitz, Rappaport and Scott (56) demonstrated that the lethal effect of ligation of the hepatic artery could be prevented by the administration of penicillin. Their interpretation of these findings was that the function of the hepatic artery is to prevent anaerobes normally contained in the liver from multiplying; once the hepatic artery is ligated the anaerobes will multiply unless controlled by penicillin.

People studying the liver of healthy dogs found that it harboured a clostridium (42) (108), which was later identified with the anaerobe causing death from ligation of the hepatic artery. This organism was usually described as "welchii-like" and on occasions as

Clostridium welchii (98). The identification of the organism was taken up in 1959 by Cobb, McKay and Archibald (16), who isolated Clostridium chauvoei from the livers of three apparently normal dogs.

It became apparent that any investigation involving surgery to the hepatic artery must include a thorough survey of the bacteria of the liver. It was decided to survey dogs of all ages in case there was an age incidence for invasion of the liver by bacteria.

The assumption had often been made (15) (53) (98) that the clostridium of clostridial hepatitis (caused by ligation of the hepatic artery) and the clostridium normally harboured in the healthy dog's liver were one and the same. This point had never been proved. Therefore, once it became apparent which clostridium was normally present in the liver of the healthy dog (16), experiments were planned to investigate the part that this organism played in death, resulting from ligation of the hepatic artery. It was decided to prepare a vaccine from the organism under suspicion and to vaccinate a number of dogs. The survival of these dogs after ligation of the hepatic artery was to be taken as proof that the clostridium isolated from the liver of most healthy dogs was in fact responsible for death when the hepatic artery was ligated.

The fact that death from ligation of the hepatic artery could be prevented by large injections of penicillin was readily appreciated, when it was recalled that most clostridia are very sensitive to penicillin. That the dogs should continue to survive after the penicillin was withdrawn at seven days was extremely surprising and called for an

explanation. The possibility of the rapid development of a collateral blood supply saving the animals was readily tested by injection experiments. In these experiments animals that had been ligated were injected with vessel outlining plastics and the collateral blood supply traced. There remained another possible explanation and this was that the penicillin was unable to control completely the clostridial infection in the early stages after ligation. Such a subliminal infection might well stimulate antibody formation which could protect the animal after the withdrawal of penicillin. This hypothesis was backed by the finding of several workers (22) (26) (27) (98) that the dogs are very frequently ailing for the first week after ligation of the hepatic artery. In order to test this hypothesis ten dogs were subjected to ligation of the hepatic artery and penicillin treatment for one week. At the end of this time the serum of the animals was tested for the presence of specific antibodies to Cl. chauvoei. Serum was also taken preoperatively and at two and four weeks postoperatively and similarly tested. The finding of a significant increase in the ability of the serum to protect susceptible laboratory animals against clostridial infection was sought. Such evidence would support the hypothesis that the protective function of penicillin against the lethal effects of ligating the hepatic artery can be taken over, in whole or in part, by naturally developed antibodies.

## REVIEW OF THE LITERATURE

Jackson and Hawn (42) in 1909 working with aseptically prepared emulsions of dogs' liver, frequently found that they contained a bacillus resembling the hay bacillus. Wolbach and Saiki (108), were persuaded by Jackson to carry out a more intensive study of this problem. Jackson and Hawn's (42) findings were verified by Wolbach and Saiki (108) who came to the conclusion that the liver of healthy dogs commonly harbours a spore-bearing anaerobe. Since that time there have been many reports of such an organism in the liver of the dog and it has usually been described as Clostridium welchii (1) (2) (88) (102) or a "welchii-like" bacillus (7) (52) (54) (98). However, in 1959 Cobb, McKay and Archibald (16) obtained the spore-bearing anaerobe, Clostridium chauvoei\* in pure culture from the liver of three out of four normal adult dogs. They postulated that this might well be the "welchii-like" bacillus referred to by previous workers. Wolbach and Saiki (108) obtained cultures of a spore-bearing anaerobe from the livers of 21 out of 23 dogs. They felt that the same organism was present in all the livers and proved this by cultural tests and morphological studies. They ventured to name the organism "welchii-like"; however, the photomicrographs they produced, and the description they give, have a much greater resemblance to Clostridium chauvoei

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\* Clostridium chauvoei is referred to as Clostridium fesari in the earlier editions of Bergey's manual (8).

than to Clostridium welchii. Bergey's manual (8) describes Cl. chauvoei as; Gram-positive rods 1.0 by 3.0 to 8.0 microns, usually showing a dark chromatic point near each end; spores ovoid, eccentric to subterminal, swelling the cells. Wolbach and Saiki (108) described the bacillus they studied as Gram stain variable 1.0 by 8-9 microns with oval subterminal spores, slightly greater in diameter than the bacillus.

Ellis and Dragstedt (22) were among the first to demonstrate the toxic potentialities of the liver anaerobes. Pieces of liver were cut free from the main body of the liver and left in the peritoneal cavity of a number of dogs. The dogs died within 48 hours. Examination of the peritoneal fluid from the dead dogs revealed a heavy growth of a spore-bearing anaerobe.

The question was often raised whether in fact the lethal effect of leaving a piece of liver free in the peritoneal cavity of a dog was due to the absorption of toxins from a bacterial peritonitis or to the toxic activity of liver autolysis per se. (1) (9) (21) (22) (58) (62) (103). Mason, Davidson, Mathews and Rastello (62), considered that the peritonitis was typically chemical and gave the name "aseptic liver autolysis" to the condition. Ellis and Dragstedt (22) on the other hand found that when they autoclaved the liver before it was placed in the abdomen of the dog there were no ill effects and that therefore the peritonitis must be bacterial in origin. With the advent of penicillin, Lewis and Wangensteen (47) settled the issue by preventing death from "aseptic liver autolysis" with penicillin therapy, thereby proving that

bacteria, and not the products of liver autolysis, were the cause of death.

Mason and Hart (61) have reported finding "welchii-like" bacilli in five biopsy specimens from the liver of five different human patients. Germain and Pennanae'h (28) described a case of Clostridium novyi infection in the liver of a man. The infection proved fatal.

Haberer (35) in 1905 first pointed out the lethal effect of ligating the hepatic artery and its branches in the dog. Haberer (35), and Huggins and Post (41) stressed that the success of the procedure depended upon making sure that all the aberrant arterial branches to the liver were found and ligated. They suggested that those animals that survived ligation of the hepatic artery should be injected with a blood-vessel outlining agent in order to demonstrate aberrant arterial vessels which might have been missed during the operation.

Huggins and Post (41) double-ligated the hepatic artery and its branches in nine dogs all of which died. In those that died in less than 48 hours the abdomen was found to be filled with blood-stained fluid, the liver was swollen, friable, and often had areas of necrosis. From similar necrotic areas Tanturi, Swigart and Canepa (98) were able to isolate a "welchii-like" bacillus. The findings of Huggins and Post (41) and Tanturi et al. (98) were confirmed by numerous workers (23) (27) (49) (56) (57) (88), and the reason for the mortality occasionally being less than 100 per cent was variously explained as a failure in

ligation technique, or the existence of a well developed collateral blood supply. Tanturi et al. (98) suggested that there might also be a seasonal variation in resistance and bacteriocidal function of the liver, for their experience was that the rate of survival varied according to the season in which the animals were operated upon.

Chau, Goldbloom and Gurd (15) ligated the hepatic artery and its branches in thirteen dogs, twelve died within 72 hours. They cultured the blood of the dogs prior to the ligation and again after ligation and noted that the peripheral blood stream was normally free of clostridia, but within three to four hours of the hepatic artery being ligated pure cultures of a "welchii-like" bacillus could be obtained.

In man, death from ligation of the hepatic artery has been recorded many times (32) (84). Graham and Connell (32) reviewing the literature noted that in twenty-seven cases of accidental ligation of the hepatic artery death occurred in seventeen of them.

Mitigation of the lethal effect of ligation of the hepatic artery by adding arterial blood to the portal vein has often been studied (29) (57) (67) (89). Narath (67) in 1916, was the first person to report a successful technique. Schilling, McKee and Wilt (89) improved the technique using an end-to-side anastomosis of the hepatic artery to the portal vein. Four out of eleven dogs survived the operation. It was while Markowitz, Rappaport and Scott (57) were attempting to improve upon these techniques that they noticed that large intramuscular doses of penicillin would save the lives of dogs even though there was no art-

erial blood supply to the liver. Markowitz et al. (57) followed up these findings by ligating the hepatic artery and all its branches in several dogs and protecting them with penicillin (300,000 I.U. of procaine penicillin for seven days). Most of the dogs survived, although a number of them were sick for several days. These experiments have been repeated many times (11) (15) (18) (34). There seems to be little doubt that penicillin is able to inhibit the growth of anaerobes within the liver and so prevent death of the animal.

Fraser, Rappaport, Tuylsteke and Colwell (27), Tanturi et al. (98) and Eze (23) all found that although dogs given penicillin therapy after ligation of the hepatic artery did not die they were often seriously ill. Fraser (24) using Thymol turbidity, Bromsulphaphthalein and other liver function tests was able to demonstrate obvious liver dysfunction from the first to the ninth postoperative day. Fraser (26) ligated the hepatic artery in twenty dogs and thirteen eventually survived; four died from gangrene of the gall bladder and one from what was described as ischaemic necrosis of the liver.

Attempts have been made by a number of people (26) (98) to replace penicillin therapy by a specific clostridial antiserum. Fraser (26) used Clostridium welchii, Clostridium oedematosus and Clostridium septicum antisera but was unable to obtain significant protection. Tanturi et al. (98) found that active immunization of dogs by Cl. welchii alpha toxin increased the average survival time from 37 hours to 98 hours. There is no record of anyone having used Clostridium chauvoei antiserum

to protect dogs.

The withdrawal of penicillin therapy seven to ten days after ligation of the hepatic artery is not usually reported as producing any ill effects. The reason for this rather surprising fact has never been completely understood. Many workers (15) (30) (70) (71) have postulated that a collateral arterial supply develops during the time that penicillin is protecting the hypoxic liver from clostridial proliferation. This hypothesis has been questioned by Tanturi *et al.* (98) and others (23) (27) (41) (69). Of the earlier authors studying ligation of the hepatic artery, Haberer (35) was the first to express a belief in the importance of the collateral arterial blood supply. More recently Popper, Jefferson and Necheles (70) (72) stated that if the hepatic artery and its branches were ligated, the phrenic collateral arterial system excised, the portal vein and bile duct stripped and all other collateral blood supply to the liver destroyed, death could not be prevented by penicillin therapy. On the other hand neither Fraser (27) nor Tanturi *et al.* (98) thought the slight increase in collateral arterial blood supply that did occur during the first week after ligation of the hepatic artery was of any significance. Eze (23) pointed out that if, as a number of workers had stated (15) (65), a single therapeutic level injection of penicillin was sufficient to protect a dog after ligation of the hepatic artery, there would hardly be time for a collateral blood supply to develop. Payer, Cerny, Kapeller and Minor (69) injected radiopaque material into the collateral arterial blood supply of the liver of dogs

from five to thirty-seven days after ligation of the hepatic artery and five days penicillin therapy. Their conclusion was that although a collateral blood supply was starting to develop by the ninth postoperative day it took approximately thirty-seven days before it had taken over fully from the hepatic artery. Rappaport, Lotto and Lougheed (78) using a series of three operations were able to stimulate the development of the collateral arterial blood supply to such an extent that it would completely support normal liver function. The collateral supply they noted was, from the left and right branches of the phrenico-abdominal arteries, small branches arising from the left gastric artery and vessels coursing along the length of the bile duct.

In 1959 Cobb, McKay and Archibald (16) reported the presence of the spore-bearing anaerobe Clostridium chauvoei in the liver of dogs. This organism seemed to fit very closely the characteristics of the spore-bearing anaerobe originally described by Wolbach and Saiki (108), and was very similar in morphological appearance to the "welchii-like" bacillus shown in the photomicrographs of Tanturi et al. (98), and Ellis and Dragstedt (22).

Bollinger (10) first made reference to Clostridium chauvoei in 1875 having cultured it from a case of symptomatic anthrax in the ox. The following year Fesar (24) commenced to classify the organism, a task that was completed by Arloing, Cornevin and Thomas (3) in 1887.

Clostridium chauvoei myositis in cattle and sheep is a relatively common disease (12) (17) (31) (43) (91) (93) (99), it has also been

reported in the horse (83), deer (4), pigs (20) (96), fresh water fish (14), and probably in whales (68) (83). The report on Cl. chauvoei infection in whales was made by Nielson (68) who described the method employed by the natives for catching whales in the Norwegian Fjords. Harpoons from the most heavily infected areas of the previous years "kill" were plunged deep into the back of the whale; it was then followed, sometimes for several days, until it finally succumbed to the clostridial infection. Folklore has it that the very first arrow was dipped into the carcass of an ox dead from a gaseous infection. Although Nielson (68) strongly suspected an infection by Cl. chauvoei he could not grow the organism. As will be shown later Cl. chauvoei unlike Clostridium welchii is an extremely fastidious bacterium.

Sterne, Thorold and Scheuter (97) and others (31) (60) (86) (90) (99) have reported the effective control of Clostridium chauvoei infection of cattle and sheep, by using formalized vaccines. Both penicillin and Clostridium chauvoei antiserum have been reported effective in controlling infections in cattle and sheep when given early in the disease (13).

Minett and Dhanda (66) reported that Clostridium chauvoei will live for a long period of time in the soil, but that it would not normally multiply there.

There are numerous reports of the recovery of clostridia from healthy tissues of normal animals (5) (16) (25) (55) (80) (95). Smith and Jasain (95) while investigating an outbreak of bacillary haemoglobin-

inuria in a number of herds, were able to culture Clostridium haemolyticum from the livers of apparently normal cattle. Reith (80) cultured clostridia from the muscle of normal pigs and Mitchell (55) has reported finding Clostridium welchii in the liver of a horse. Clostridium oedematiens, the cause of necrotic hepatitis in sheep has been recovered by Jamieson (43) from the livers of normal sheep. Aub, Brues, Ketyss and Nathanson (5) cultured an unidentified anaerobe from the exudate of ischaemic skeletal dog muscle, but until the work of Cobb, McKay and Archibald (16) in 1959 Clostridium chauvoei had not been identified in tissues of the normal or diseased dog.

## PART I

### BACTERIOLOGICAL SURVEY OF THE NORMAL CANINE LIVER

After the finding by Jackson and Hawn (42) of a spore-bearing organism in the canine liver, little was done to classify the organism although it was referred to whenever surgery involving the hepatic artery was discussed. Nor was there any attempt made to assess the degree of involvement of the liver or the age at which invasion by the organism began. From the high percentage of animals that died after ligation of the hepatic artery it was obvious that the "infection" was widespread throughout the dog population. A number of workers had said that they considered the organism was Clostridium welchii (1) (98), however, there are no reports of serum neutralization tests being undertaken to prove this point. In 1959 an organism identified as Clostridium chauvoei was recovered by the author (16) from three normal dogs in a preliminary investigation of the bacteriology of the canine liver. Chau, Goldblatt and Gurd pointed out that at least some of the bacteria found in the liver were probably held within the parenchyma. They had fed succinyl sulphathiazol orally on five consecutive days prior to ligation of the hepatic artery in order to preclude invasion of the portal vein by clostridia from the intestine, and found that ligation of the hepatic artery still caused death. This seemed to indicate that the clostridia had probably entered the liver prior to chemotherapy and were held there at least for five days.

The planning of the present survey was so arranged that the following questions could be answered.

a) What were the various species of bacterium present in the liver of the normal adult dog and what was the degree of infection with each species?

b) Was there any apparent preference of any particular species of bacterium for any part of the liver?

c) What was the incidence of each species of bacterium in the dog population surveyed?

d) At what age were dogs found to begin harbouring bacteria in the liver?

The method by which biopsy specimens were to be taken from the liver required careful consideration. Trusler, Reeves and Martin (103) had pointed out the necessity of taking extensive precautions for the aseptic removal of liver biopsy specimens because of the possibility of contamination by bacteria in the skin and muscle. The use of biopsy needles such as the Silverman needle was decided against because it would not have been possible to know from which lobe of the liver specimens were taken. An electrosurgical unit seemed to provide the best solution to the problem. When the skin was carefully prepared and the electrosurgical unit used to incise the abdominal wall and to cut out biopsy specimens it was thought that the possibility of contamination would be virtually excluded. While it was necessary to obtain a sufficient number of biopsy specimens from each dog to get a reasonable est-

imate of the normal bacterial content of the liver, taking a very large number might increase the risk of contamination without gaining further information. Therefore it was decided to cut twenty-four biopsy specimens from each liver for culture and one for histological examination.

#### Materials and Methods

The studies were confined to dogs between the ages of four months and six years. Pure-bred dogs were not used nor animals that were either obese or emaciated. The dogs weighed between fifteen and fifty-five pounds. The diet of the animals was not known before they were started on the experiment. During the time that the animals were in the laboratory they were fed kibble and canned meat. Upon entering the laboratory all dogs were given a thorough clinical examination and a protective dose of distemper-hepatitis serum (Lyostan<sup>R</sup>) was injected. Guinea-pigs weighing between 250 and 500 grams were used for the isolation and identification of clostridia.

#### Culture Media

The media chosen were those in which it would be possible to obtain growth of all bacteria that might be found in the canine liver.

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<sup>R</sup> Stevenson, Turner and Boyce, Limited, Guelph, Canada.

Media for both aerobic and anaerobic culture were selected.

Trypticase Soy broth<sup>R</sup> with five per cent dextrose (T.S.d.) was used for the anaerobic culture of specimens of guinea-pig liver.

Tryptose Phosphate broth<sup>R1</sup> (T.P. broth) was used for the culture of aerobes. This medium would also support the growth of clostridia within the liver biopsy for a day or so. The medium was freshly prepared for each operation and dispensed in pyrex test tubes, stoppered with absorbent cotton.

Fluid thioglycollate medium<sup>R</sup> (thioglycollate) was chosen for its suitability as a culture medium for the growth of anaerobes. Screw-cap pyrex test-tubes (20 x 150 millimetres) were half filled with the freshly prepared medium.

Thiol medium<sup>R1</sup> containing 1.5 per cent agar (thiol plate) was used as a solid medium for anaerobic growth. This medium was particularly designed for the culture of clostridia.

Blood-agar plates, freshly made with ten per cent sheep blood added, were used for both aerobic and anaerobic cultures.

Fermentation media used were 0.5 per cent dextrose, lactose, salicin and sucrose in trypticase agar base<sup>R</sup>. Litmus milk<sup>R1</sup> and nitrate broth<sup>R1</sup> were also used for the differentiation of clostridial species.

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R Difco Laboratories Inc., Detroit 1, Michigan, U.S.A.

R1 Baltimore Biological Laboratory, Inc., Baltimore 18, Maryland, U.S.A.

### Preparation of the Animal

Eighteen to twenty-four hours before surgery a six-inch-wide band of skin in the midline of the animal was shaved from the midsternal region to the level of the pubic brim. This area was then washed with warm water and pHisoHex<sup>R</sup> and the animal returned to its cage. Food was withheld until after surgery. One hour before surgery the animal was deeply anaesthetized with Neabutal<sup>R1</sup> (sodium pentobarbital) administered via the cephalic vein. Blood samples were then taken, the skin areas having previously been clipped and treated with 90 per cent ethyl alcohol. Samples were taken with a 20 gauge needle from a superficial vein when only a small sample was required, and with an 18 gauge needle from the heart when a large quantity of blood was required.

The surgical field was then prepared. The hair was clipped close to the skin from the midsternal region to the level of the pubis in a ten-inch-wide band. The skin was thoroughly washed with warm water and pHisoHex and the animal returned to its cage. Immediately prior to surgery the shaved area was again washed with pHisoHex, thoroughly rinsed and then sprayed with 90 per cent ethyl alcohol. Ten minutes later two and a half per cent iodine was applied to the skin with sterile swabs and the animal taken to the operating room.

The operating room technique was always carried out with a view to preventing air-borne contamination of biopsy specimens. Operations

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<sup>R</sup> Winthrop Laboratories of Canada Limited, Windsor, Ontario.

<sup>R1</sup> Abbott Laboratories Limited, Montreal, Canada.

were spaced so that whenever possible the operating room had been unused the previous day. Everyone coming into contact with the animal, while it was in the operating room, was required to wear a cap and mask and the surgeon and his assistant wore sterile gowns and two pairs of sterile gloves; the outer pair to be discarded immediately before the taking of biopsy specimens. Electrosurgery was carried out with a Bovie electrosurgical unit (Model "AG"<sup>R</sup>). Heavy needles were used to incise the skin and linea alba, and fine needles bent at right angles half way along their length were used to biopsy the liver. Needles were placed in culture medium after use and incubated to check for contamination.

The surgeon and his assistant scrubbed up thoroughly for four minutes and donned sterile gowns and gloves. The clipped area of the skin was again rinsed with alcohol, and iodine was applied with a sterile swab. The animal was draped and the skin incision made in the midline from the level of the xiphisternum caudally for six inches. The cutting power of the electrosurgical unit was set at 40 units and the haemostatic control at one minimum. Towels were immediately applied to the edges of the incision so that only the linea alba was visible. A fresh electrosurgical needle was connected up and the incision continued through the linea alba to the falciform ligament. Haemorrhage from the vessels in the falciform ligament was controlled either with haemostatic forceps or with the coagulation point of the electrosurgical unit. Ab-

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<sup>R</sup> The Liebel-Flarsheim Company, Cincinnati, Ohio, U.S.A.

dominal retractors were inserted and the liver exposed.

In order to obtain an impression of the degree of liver "infection" twenty-five biopsy specimens of approximately equal size (200 milligrams) were taken from various parts of the liver. Twelve specimens were cultured under anaerobic conditions and another twelve under aerobic conditions. One biopsy specimen was kept for histological examination, if it showed pathologic changes the experiment was abandoned. The left lateral, left central and right and left divisions of the gallbladder lobe were biopsied. The biopsy specimens were taken in pairs, one for aerobic and the other for anaerobic culture. Six specimens were taken from each lobe, i.e. a pair from the diaphragmatic surface, a pair from the gastric surface and a pair from the caudal edge of the lobe. It was not found possible to take samples from the right lateral and caudate lobes without running the risk of contusion and contamination to these and the surrounding lobes of the liver.

The lobes to be biopsied were grasped one at a time with Allis forceps, gently pulled into the field of view, and the biopsy specimens cut out with the fine electrosurgical needle. It was important to have the current as low as possible to avoid damaging the specimen. A setting of 20 to 25 units on the cutting power control and one minimum on the haemostatic power control was found sufficient. A fresh pair of forceps was used to transfer each specimen from the liver into the culture medium and the mouth of the test tube was flamed before and after inserting the specimen. The needle was sterilized between biopsy spec-

imens by touching it on to the abdominal retractors and running the current through it. The operation was undertaken as rapidly as possible. As controls, the electrosurgical needles were placed in culture medium (one in tryptose-phosphate and the other in thioglycolate broth). Also one tube of tryptose-phosphate and one of thioglycollate was held open for ten seconds close to the abdominal incision in case there was any bacterial air contamination. Samples of blood (half a millilitre each) were taken from the portal vein and hepatic artery of six dogs (H38,68,82,92,94,98) after the biopsy specimen taking was completed.

The tubes for anaerobic culture were placed in a Brewer anaerobic jar which was then partially evacuated, filled with hydrogen and the remaining oxygen burnt by platinum catalyst. All cultures were put into the incubator within half an hour of the last specimen having been taken. The incubator was set at 37 degrees centigrade. The aerobic cultures (T.P. broth) were observed and growth recorded at twenty-four hours, but the anaerobic jars were not opened before forty-eight hours. The tubes of T.P. broth not showing growth were re-incubated for a further twenty-four to seventy-two hours and the specimens in thioglycollate were incubated for a total of fourteen days. Whenever growth was noted smears were stained and the growth plated onto blood-agar medium for aerobic and anaerobic culture. The blood-agar plates were examined at twenty-four hours and again at forty-eight hours. Culture was also attempted anaerobically on thiol plates. More often than not both growth and gas production would be quite obvious in broth and yet the

plate cultures would be negative. This was because although Clostridium chauvoei would grow well in most broths it would only grow on plates under special circumstances. If the presence of Cl. chauvoei was suspected it became necessary for identification purposes to resort to guinea-pig inoculation. One millilitre of broth culture was injected into the hind limb of a medium sized (250 to 300 gram) guinea-pig, together with half a millilitre of tissue debilitating agent (ten per cent calcium chloride). When the guinea-pig showed signs of impending death it was killed and a 200 milligram portion of liver aseptically removed and placed into 60 millilitres of T.S.d. Impression smears were made of the guinea-pig liver to rule out Clostridium septicum since this organism is most often confused with Clostridium chauvoei, but it can usually be identified by the characteristic long chains that are seen on a stained liver impression slide. The T.S.d. broth culture was incubated anaerobically for twenty-four hours and then smears of the growth stained by Gram's stain. If the culture appeared to be pure a quarter of a millilitre of broth was injected into tubes of sucrose, lactose, dextrose, salicin, litmus milk, and tryptone broth (for Indole test). These tubes were then incubated anaerobically for twenty-four hours and the sugars examined after this time. Reaction in the litmus milk and tryptone broth was not recorded until after forty-eight hours incubation.

In later experiments it became possible to obtain growth of Clostridium chauvoei on plates by waiting until the guinea-pig was at the point of death before smearing the blood-agar plates with the liver

and heart blood. These plates were incubated under anaerobic conditions for twenty-four hours. Colonies could then be "picked" and the biochemical test made from T.S.d. broth cultures.

Final identification of Clostridium chauvoei was made by challenging guinea-pigs protected with specific clostridial antisera<sup>R</sup>. A twenty-four hour culture of the organism in T.S.d. broth was used to obtain a minimum lethal dose (see page 49). Two guinea-pigs were inoculated with one millilitre of Cl. chauvoei antiserum and two with one millilitre of Clostridium septicum antiserum. Twenty-four hours later the four guinea-pigs and two control animals were challenged with approximately twice the minimum lethal dose of the culture. This test was specific and only the guinea-pigs inoculated with Cl. chauvoei antiserum survived.

Occasionally the strain of Clostridium chauvoei examined was not sufficiently virulent to kill guinea-pigs, even with the aid of a tissue debilitating agent. In these cases confirmation by serum neutralization test was not possible and the organism was identified as Cl. chauvoei if it had the following characteristics; Gram-positive rods, one micron by four to ten microns, occurring singly or in short chains, rarely in filaments; Gram's stain variable in old cultures; spores terminal or subterminal, oval and slightly wider than the parent rod; acid and gas production in dextrose, lactose, sucrose and salicin; litmus milk slowly clotted and indole not produced. The organism could not be

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<sup>R</sup> Burroughs Wellcome and Company, Beckenham, London, England.

subcultured on blood-agar or thiol plates under aerobic or anaerobic conditions.

### Results

The bacteriologic findings revealed that the liver of a high proportion of the dogs harboured Clostridium chauvoei. Of the twenty adult dogs surveyed, seventeen harboured clostridia; Cl. chauvoei was identified in sixteen and Clostridium welchii in one (Table I).

Biopsy specimens harbouring Clostridium chauvoei usually produced gas in T.P. broth within twenty-four hours of being removed from the animal. Specimens in thioglycollate tubes often did not show signs of gas production until after thirty-six to forty-eight hours of incubation. It was never found possible to obtain growth of Cl. chauvoei on blood-agar plates or thiol plates by subculture directly from broth cultures. This in itself was a useful diagnostic pointer for when gas and growth were noted in culture tubes, yet it was not possible to obtain growth by subculture onto plates, Cl. chauvoei was suspected. The one time that Clostridium welchii was identified from a liver biopsy specimen gas formation was much greater and appeared several hours before the earliest Cl. chauvoei growth was recorded. When Cl. chauvoei was suspected in a culture and the twenty-four to forty-eight hour old culture injected intramuscularly into a guinea-pig, death generally occurred

after twelve to forty-eight hours. The injected limb was swollen, tender, and discoloured. There was extensive subcutaneous oedema of the ventral abdominal wall. A necropsy revealed extensive subcutaneous haemorrhage and oedema with gas formation spreading forward from the affected limb. The muscles of the injected limb were darkened and friable. The viscera appeared to be normal, except that the liver was often pale. If the guinea-pig was chloroformed when it was very close to death, liver impression smears would often show Gram-positive rods occurring singly or in chains of two or three. Liver smears and heart blood from these animals would usually produce growth on blood-agar or thiol plates under anaerobic conditions. At twenty-four hours the colonies were one to two millimetres in diameter often resembling water droplets, transparent or semitransparent surrounded by a wide zone of complete haemolysis (Figure I). The colony outline was often irregular and the surface flattened, occasionally with a central peak. Spreading colonies were occasionally noted.

Clostridium chauvoei fermented lactose, sucrose, dextrose and salicin with production of acid and gas. Salicin fermentation is not widely accepted as a characteristic of Cl. chauvoei (8) (93), but its occurrence throughout these experiments was invariable. Litmus milk was fermented with clot formation after twenty-four hours. The Indole test was negative.

A biopsy specimen from the liver of a guinea-pig infected with half a millilitre of twenty-four hour T.S.d. broth culture (dog number

H18) was sent to M. Stearne of the Anaerobic Department of Burroughs Wellcome<sup>R</sup> for identification. Dr. Stearne confirmed the identification of the organism as Clostridium chauvoei.

Of 273 biopsy specimens producing bacterial growth from 27 dogs, 256 were positive for Clostridium chauvoei and one for Clostridium welchii (Table I). Four biopsy specimens from three dogs (H17,24,68), contained Streptococcus faecalis. Six specimens from three dogs (H23,26,71) produced Staphylococcus aureus, and six specimens from one dog (H17) produced Escherichia coli.

#### Interpretation of Results

It became obvious after the survey had been in progress some time that any interpretation of the results would require the animals to be divided into two groups. This was because dogs over one year old almost invariably harboured bacteria whilst this was not so for those younger than one year. If, therefore, all but one of the dogs surveyed had been less than one year old the percentage of dogs harbouring bacteria would have been considerably lower than if all the dogs sampled had been over one year old. For this reason the emphasis of the results is put on dogs over one year old (adult), and those under one year (pups) are considered only when the question of the age at which infection

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<sup>R</sup> Anaerobic Department, Burroughs Wellcome, Beckenham, Kent, England.

appears is studied.

The survey was planned in order to answer the following questions.

a) What were the various species of bacterium normally present in the liver of the adult dog and what was the degree of "infection" with each species?

Any survey of bacterial population made from the limited number of biopsy specimens taken could not be expected to give a completely accurate account of the bacterial flora of any one liver. However it was considered better to take a limited number of specimens from many livers rather than many specimens from a few livers. It was also realized that there was some slight possibility that the bacterial flora contained in the top centimetre of liver tissue might not be the same as that two centimetres below the surface. However, the difficulties involved in obtaining deep tissue samples without contamination from the superficial tissue were too great to make the procedure practical. Within these limitations it was thought that the hepatic bacteria of the dog population of the region might closely parallel that of the twenty adult dogs sampled (Table III). From these dogs Clostridium chauvoei was isolated 231 times, Staphylococcus aureus seven times, Escherichia coli six times, Streptococcus faecalis four times and Clostridium welchii once.

b) Was there any apparent preference of any particular species of bacterium for any part of the liver?

It was not possible to answer this question directly because there was no way of assessing the number of bacteria in any one biopsy specimen. It was only possible to say it did not appear as though there was any area of the liver that was less likely to harbour a particular species of bacterium than any other.

c) What was the incidence of each species of bacterium in the dog population surveyed?

Clostridium chauvoei was isolated from 16 of the 20 adult dogs surveyed. Staphylococcus aureus was isolated from 3 of the 20 adult dogs surveyed. Streptococcus faecalis was isolated from 3 of the 20 adult dogs surveyed. Escherichia coli was isolated from 1 of the 20 adult dogs surveyed. Clostridium welchii was isolated from 1 of the 20 adult dogs surveyed.

d) At what age were dogs first found to harbour bacteria in the liver?

Of the 27 dogs of all ages biopsied, seven were less than one year old (Table II). Of these seven, one was ten months old. Two were eight months old and four six months old. All the biopsy specimens from the six-months-old pups proved sterile. Of the two eight-months-old pups one had Clostridium chauvoei in every biopsy specimen and the other was completely negative. The ten-months-old pup provided a mixture of Cl. chauvoei, Streptococcus faecalis and Escherichia coli. From such a small number of dogs it is not possible to draw any firm conclusions. The impression gained was that it was not until sometime around the

middle of the dog's first year of life that the liver began to harbour Clostridium chauvoei and other bacteria.

e) Were the bacteria cultured, circulating in the blood stream at the time of biopsy or situated in the liver tissue?

There existed a slight possibility that bacteria cultured from a liver biopsy specimen might in fact, have been passing through the blood vessels of the liver tissue at the time that the biopsy specimen was taken. In order to rule out this possibility, blood samples approximately a half a millilitre each were withdrawn from the hepatic artery and portal vein in six dogs immediately after the 25 biopsy specimens had been taken from the liver. These samples were cultured aerobically and anaerobically in T.P. broth. In all these dogs the blood samples proved sterile and yet the liver samples were positive. The conclusion was drawn that it was extremely unlikely that the bacteria cultured from liver biopsies were from the circulating blood.

#### Conclusions

There can be little doubt that the liver of a large number of adult dogs harbours Clostridium chauvoei, an organism which is highly pathogenic in other species of animal when stimulated under suitable conditions.

## PART II

### EVALUATION OF THE PATHOGENICITY OF CLOSTRIDIUM CHAUVOEI IN THE FATAL HEPATITIS CAUSED BY LIGATION OF THE HEPATIC ARTERY

It is a well established fact that ligation of the hepatic artery and its branches in the adult dog causes a rapidly fatal illness in a high percentage (15) (23) (26) (55) of cases. A post mortem examination of the liver of such animals reveals extensive, gaseous, foul-smelling areas of necrosis and gangrene. Masses of Gram-positive bacteria are seen in stained smears from the liver and from the fluid in the peritoneal cavity. This bacterium is identical in appearance with that cultured from biopsy specimens taken from the liver of normal dogs. It was thought reasonable to assume that ligation of the hepatic artery and its branches in the normal dog, in some way, allowed the harboured anaerobes to multiply and cause the death of the animal. This, however, was only an assumption. Positive proof required firstly, the identification of the causal clostridium and secondly, protection of the animal by vaccination or specific antiserum prior to ligation of the hepatic artery.

The isolation of Clostridium chauvoei from the livers of a very high proportion of the dog population made it seem reasonable to postulate that this was the bacterium that normally caused fatal clostridial hepatitis. It was decided therefore to prepare a vaccine from a culture

of Clostridium chauvoei isolated from a liver biopsy specimen. This vaccine was to be administered to a series of dogs and, after allowing sufficient time for them to develop an immunity, the hepatic artery and its branches were to be ligated. Survival of a significant proportion of these dogs would be interpreted as meaning that Cl. chauvoei was normally the bacterium responsible for death from ligation of the hepatic artery.

#### Materials and Methods

The dogs for these experiments were required to be in good condition, weighing between 35 and 55 pounds and aged between two and six years. All dogs were inoculated with distemper-hepatitis serum (Lyosan) (one millilitre per five pounds body weight) several days before commencing on an experiment.

Guinea-pigs were used in the preparation and testing of the vaccine. They were mature guinea-pigs of both sexes, ranging in weight from 250 to 350 grams. In order to investigate the possibility that the liver of the guinea-pig might normally harbour bacteria, six guinea-pigs were taken from various litters and killed with chloroform. Two lobes of the liver were aseptically removed. Each lobe was placed into a 100 millilitre bottle of T.S.d. One bottle was incubated aerobically and the other anaerobically for fourteen days. All the cultures proved

to be sterile.

#### Preparation of the Vaccine

The problems involved in preparing a vaccine that was safe, yet potent and free from bacterial contamination were manifold. Advice from M. Sterne (Burroughs Wellcome Research Laboratories, London, England) who did much of the original work on making the commercial production of Clostridium chauvoei vaccine feasible was of great assistance.

First of all, it was necessary to isolate Clostridium chauvoei in pure culture. This was made difficult by the fastidiousness of Cl. chauvoei. At the time of preparation of the first vaccine it had resisted all attempts to encourage growth as a surface colony on solid media. This ruled out the possibility of being able to "pick" isolated colonies for cultural purposes. However, it was found that the guinea-pig could be relied upon to produce a pure culture of the organism within its liver when injected intramuscularly with a broth culture of Cl. chauvoei. Although the liver of the normal guinea-pig had always been found sterile a constant check was kept upon cultures for bacterial contamination. Another problem was to treat the organism in such a way that it would stimulate a high level of immunity without affecting the animal clinically. Formalin had been advocated by numerous workers (60) (90) (99) as a means of killing bacterial cultures without destroying their immunizing power. Therefore, in

early experiments with vaccines a formalized washed culture was used. The broth culture was centrifuged, the supernatant fluid discarded, and the bacilli and spores washed several times with distilled water. This method did not produce a sufficiently potent vaccine. The reason was most probably because a large proportion of the immunizing antigens were washed away during its preparation. Finally a formalized alum-precipitated, whole broth culture was used.

The strain of Clostridium chauvoei used for the preparation of the vaccine was obtained from the liver of a dog (H18). Samples of the liver from a guinea-pig killed by an intramuscular inoculation of strain H18\* had been sent to another laboratory<sup>R</sup> to confirm the presence of Cl. chauvoei. Parts of the same guinea-pig's liver, weighing approximately one gram each, were kept refrigerated at five degrees centigrade as a source of Cl. chauvoei for vaccine production. The strain H18 was relatively virulent and never failed to kill guinea-pigs on intramuscular injection, even without the aid of tissue debilitating agents.

A section of guinea-pig liver infected with strain H18 was removed from refrigeration and ground up aseptically with two millilitres of normal saline. One millilitre of the suspension was injected into a guinea-pig together with one tenth of a millilitre of a ten per cent sol-

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\* Strains of Clostridium chauvoei were given the number of the dog from which they were isolated.

<sup>R</sup> Burroughs Wellcome Limited, Anaerobic Department, Beckenham, England.

ution of calcium chloride in distilled water. When the guinea-pig was obviously ailing it was killed with chloroform and a section of the liver weighing approximately one gram was placed in a 100 millilitre bottle containing 60 millilitres of T.S.d. The broth culture was incubated anaerobically at 37 degrees centigrade for forty-eight hours. Stained smears were examined to check the purity of the culture and the whole broth culture made up to a strength of 0.6 per cent formalin using a forty per cent solution of formaldehyde. The formalized culture was then incubated aerobically at 37 degrees centigrade for forty-eight hours. At the end of this time two millilitres of vaccine were removed in order to check for sterility. One millilitre was inoculated into each of two tubes of meat broth. One tube was cultured aerobically, the other anaerobically, for one week. In the meantime a seventy-five per cent suspension of potash alum ( $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ )<sup>R</sup> was autoclaved and two millilitres added to the formalized culture to make a final strength of two and one half per cent. The pH was raised to 6.8 using normal sodium hydroxide. The increase in pH caused increased precipitation of the culture. The vaccine was kept at room temperature for forty-eight hours and then used.

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<sup>R</sup> Aluminum and Potassium Sulphate, Merck and Company, Inc., Rahway,  
New Jersey, U.S.A.

#### Administration of the Vaccine

The vaccine was inoculated subcutaneously into the neck of the dog to be protected. A dosage rate of one millilitre per ten pounds body weight gave satisfactory protection. Only one inoculation was required to stimulate a protective antibody titre within two weeks.

#### Operation for Ligation of the Hepatic Artery

The liver receives most of its arterial supply from the branches of the hepatic artery. The common hepatic artery arises from the coeliac axis and is usually joined by the pancreatico-duodenal branch of the cranial mesenteric artery and at least one branch of the right gastro-epiploic artery before it reaches the hilus of the liver. As Haberer (35) and others (55) (98) have pointed out, if one wishes to deprive the liver of its arterial blood supply it is necessary to ligate, not only the common hepatic artery but also the pancreatico-duodenal and right gastric branches. The occurrence of aberrant arterial branches (usually in the hepato-duodenal ligament) have also been reported (35) (78), any one of which if missed might cause the operation to be ineffective.

Most techniques for deprivation of arterial supply to the liver required the isolation of the hepatic artery and all its branches (right gastro-epiploic and pancreatico-duodenal) and careful ligation of each (climbing ligature technique) (23) (26) (57). Such techniques

involved extensive dissection, often resulting in excessive scar tissue formation in and around the hilus of the liver, the portal vein and the bile ducts. A simpler, quicker and less traumatizing technique was that suggested by Markowitz (51) in which the portal vein and bile ducts were isolated from the hepato-duodenal ligament. The whole of the hepato-duodenal ligament (which contained the hepatic artery and its various branches) was then ligated with heavy silk. This method proved to be very effective. All six of the control animals succumbed to ligation of the hepatic artery\* by this technique.

#### Preparation of the Animal

Food was withheld from animals for twenty-four hours prior to surgery. The animals were anaesthetized with Nembutal. An area of skin eight-inches-wide was closely clipped in the midline from the midsternum to the level of the pubic brim. The skin was thoroughly washed with pHisoHex, rinsed with water and sprayed with 90 per cent ethyl alcohol. The area was next painted with two and a half per cent iodine. The alcohol and iodine treatments were repeated when the animal was on the operating table, immediately prior to surgery.

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\* The ligation of the hepatic artery will be used to denote the operation in which the hepatic artery and all its branches are ligated before they enter the liver. This is the only operation that was performed on the hepatic artery.

### The Technique for Ligation of the Hepatic Artery

The climbing-ligature technique for ligation of the hepatic artery used by previous workers (23) (26) (57) had the disadvantage of often causing an excessive amount of scar tissue. The technique used in these experiments was designed to minimize the stimulation of the formation of scar tissue while still ensuring the interruption of all arterial blood flow through the porta of the liver.

A midline skin incision was made from the level of the xiphisternum to an inch or so beyond the umbilicus. Towels were clipped to the skin edges and the incision continued through the linea alba to the falciform ligament. An abdominal retractor was inserted and the falciform ligament split. The proximal duodenum was grasped and pulled towards the animal's left flank. This manoeuver exposed the loop of the common hepatic artery as it lay at the rim of the epiploic foramen. The common hepatic artery was ligated at this point with number three silk. No attempt was made to separate the autonomic nerve fibres from around the hepatic artery or to preserve lymphatic vessels in this region. The pylorus was next grasped and pulled caudally thus revealing the common bile duct as it lay superficially in the hepato-duodenal ligament. The bile duct was easily lifted out of the surrounding tissue and retracted with a loop of silk. By pulling the duodenum caudally and to the left the portal vein could be exposed as it lay at the edge of the hepato-duodenal ligament. The covering of areolar tissue, lymphatic vessels and nerve fibres was lifted off the portal vein from the region

of the gastro-duodenal vein to the branching of the portal vein at the hilus of the liver. The lesser omentum was torn close to the hepato-duodenal ligament. A silk ligature was then passed around all the structures in the hepato-duodenal ligament except the portal vein and bile duct. The ligature was tied approximately half an inch from the hepatic porta. If the dog weighed over 45 pounds it was usual to divide the hepato-duodenal ligament lengthwise and ligate it in two parts. The abdominal incision was closed using chromic catgut in the muscle and silk sutures in the skin. Throughout the operation great care was taken to avoid touching the liver for it had been noticed previously that the slightest digital pressure would cause a local congestion of the liver.

When all observations had been completed on six of these animals they were killed by a large intravenous dose of sodium pentobarbital. The femoral artery was cannulated and the animal injected with ten per cent formalin and then with Vinylite<sup>R</sup>. They were subsequently dissected to confirm the efficacy of the ligation technique and to look for any development of a collateral arterial blood supply.

#### Antiserum Protection Tests

A second series of six dogs was inoculated with Clostridium

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<sup>R</sup> Gordon-Lacey Chemical Products Co., Inc., Maspeth 78, New York, U.S.A.

chauvoei diagnostic serum<sup>R</sup> in order to give them protection against Cl. chauvoei infection. Subsequently the hepatic artery in these animals was ligated. Survival of a significantly high proportion of inoculated dogs would be taken as evidence that Cl. chauvoei was normally responsible for the death of dogs after ligation of the hepatic artery.

The animals were each inoculated with Clostridium chauvoei diagnostic serum at a rate of ten millilitres per forty pounds body weight given subcutaneously on the first day, and half this dose intravenously on the second day. Twenty-four hours later the hepatic artery was ligated.

#### Control Animals

Two groups of control animals were used.

The first group was used to test the efficacy of the method for ligation of the hepatic artery. Six non-vaccinated adult dogs underwent the routine operation for ligation of the hepatic artery and its branches.

In the second group four adult dogs were sham-ligated. Sham-ligation consisted of following the routine technique for ligation of the hepatic artery except that all ligatures were cut free immediately after being tied. In addition, the autonomic plexus that encircled the hepatic artery was severed. This control was thought necessary to con-

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<sup>R</sup> Burroughs Wellcome and Company, Bekenham, London, England.

firm that the lethal effect of ligation of the hepatic artery operation arose from stoppage of the arterial supply and not through trauma to other structures in the hepato-duodenal ligament.

### Results

The vaccination and antiserum protection experiments were carried out in order to test the thesis that Clostridium chauvoei was the pathogen causing death of dogs after ligation of the hepatic artery.

#### Vaccination Experiment

All of the ten dogs vaccinated with freshly prepared formalized alum-precipitated Clostridium chauvoei vaccine fourteen days prior to ligation of the hepatic artery survived until killed four to seven weeks later (Table IV). However, the postoperative recovery period was not always uneventful. All but two of the dogs showed some degree of reaction to the operation, usually a listlessness and disinclination to eat lasting for about a week. Vomiting was also a common postoperative symptom. Four of the dogs (H49,63,78,96) were jaundiced for ten days to two weeks postoperatively.

At necropsy one dog (H49) that had been jaundiced for two weeks had an atrophied gallbladder, the liver was fibrosed and there were about 20 millilitres of bile-stained fluid in the peritoneal cavity. Two of

the dogs (H63,97) had become progressively thinner after surgery; at necropsy the carcass of both were seen to be grossly emaciated and in one (H63) there was extensive scarring of the left central and left lateral lobes of the liver, while in the other (H97) the scarring was diffuse throughout the liver. The other seven dogs (H42,56,74,78,93,96,99) appeared to be quite normal at necropsy except for a slight reduction in size of the liver and fibrosis of the gallbladder. A section of liver approximately five grams in weight was taken aseptically from the dog H78 at the time of death. Anaerobic culture in T.S.d. revealed a pure culture of Cl. chauvoei. This dog had been off food for five days postoperatively and jaundiced for ten days, but was apparently healthy at the time it was killed.

The results of these experiments indicated that while vaccination against Clostridium chauvoei would protect an animal from the lethal effect of ligation of the hepatic artery there were still some unexplained ill effects from the operation.

#### Antiserum Protection Experiment

Of the six dogs inoculated with Clostridium chauvoei diagnostic serum for two days prior to ligation of the hepatic artery four (H44,46,53,73) survived until killed at five to seven weeks after surgery (Table V). One dog became progressively emaciated and died three weeks postoperatively. The sixth dog (H107) was found dead on the morning of the third postoperative day after having made an apparently uneventful

recovery from the surgery. The necropsy revealed the gangrenous liver typical of a clostridial infection. The infection involved two thirds of the liver. Stained smears of the liver and the peritoneal fluid showed an organism resembling Cl. chauvoei. It was not possible to isolate the clostridium as it could not be encouraged to cause death in guinea-pigs. Much reliance could not have been put on bacteriologic findings because the dog may have been dead some hours before it was examined.

#### Control Experiments

The hepatic artery was ligated in six normal adult dogs. All six died from acute clostridial hepatitis.

The dogs had recovered from the anaesthetic and were walking around in from three to twelve hours after ligation. Between 18 and 96 hours after surgery they started to become comatose and dyspnoeic. The heart rate and respirations were greatly increased. Vomiting was a common sign of impending death. The animals usually died within 12 to 24 hours after the onset of the first symptoms. Necropsy revealed a typical clostridial hepatitis in the liver of all six dogs. Large areas of the livers were infiltrated with gas; some showed areas of infarction. There was a characteristic sour odour about the liver. Smears made from the gangrenous areas invariably revealed a heavy count of rods which stained Gram-positive and closely resembled Cl. chauvoei.

The second group of four control animals was used to confirm that the proliferation of clostridia within the liver was due to in-

terruption of the arterial blood flow and not to trauma to the surrounding structures. A sham-ligation was performed on four dogs. All four animals made completely uneventful recoveries.

#### Injection Findings

Six vaccinated dogs were subsequently injected with Vinylite in order to outline the arterial system. Several weeks after the injection a careful dissection was made of the liver. In five of the six dogs (one injection failed) Vinylite obviously arising from branches of the phrenic arteries was noted in the liver parenchyma. The area of liver supplied by these vessels was however, very small, and would hardly have amounted to one hundredth of the volume of the liver. Vinylite was also noted in blood vessels in the liver around the portal vein and vena cava. These again were very small vessels and arose from the walls of the adjacent portal vein and vena cava. In none of the injected dogs was there any evidence that the ligation of the hepatic artery was unsuccessful.

#### Discussion

Adult dogs inoculated with vaccine prepared from Clostridium chauvoei cultures were able to resist the usually lethal operation of ligation of the hepatic artery. It would seem reasonable therefore, to

assume that Cl. chauvoei was the pathogenic agent in this disease. The results of the serum protection experiments substantiated these findings.

### PART III

#### LIGATION OF THE HEPATIC ARTERY, PROTECTION BY PENICILLIN AND CLOSTRIDIUM CHAUVOEI ANTIBODY FORMATION

It was well known that penicillin administered at a therapeutic level would control the clostridial hepatitis that usually occurred after ligation of the hepatic artery (57). Its effect was very similar to the protection given by Clostridium chauvoei vaccine. The animals would often be ailing for a few days postoperatively and then recover. However, penicillin, unlike vaccine-stimulated antibodies was foreign to the body and fairly rapidly excreted; the level therefore required to be maintained by daily injections.

The surprising fact was that after about the sixth postoperative day the penicillin therapy could be safely terminated. Two findings early in this study, made it possible to investigate this problem further. The first was the identification of Clostridium chauvoei as the potential pathogen in the dog's liver, and the second was that the dog could be stimulated into antibody production by a controlled inoculation of Cl. chauvoei (vaccination). This being so, it then seemed plausible to postulate that during the postoperative recovery period, when the dogs were often ailing, they were in fact, suffering from a sublethal clostridial infection. This infection, it was thought, might stimulate antibody production sufficiently to control the clostridial infection when the penicillin was withdrawn several days later.

In order to investigate this hypothesis the hepatic artery was ligated in ten adult dogs and they were treated with penicillin for seven days. To detect and measure any rise in antibody titre a blood sample was collected at the time of ligation, at one week (day after penicillin withdrawn), at two weeks, and again one month after the operation.

Animal protection tests were used to measure the protective ability of the serum as there was no accurate in vitro test for measuring immunity. While an in vitro test might show the presence of some antibodies they might not be those associated with, or necessary for, the protection of the animal. In many ways the dog would have been the most satisfactory test animal to use, however, the problems involved in calculating a lethal dose of Clostridium chauvoei for 50 per cent of animals (L.D.50) in such a varied population were insurmountable. Instead guinea pigs were used as they were available in sufficient numbers from a reasonably uniform community. Serum from vaccinated dogs was used to test the suitability of the technique to measure antibody protection.

A control of four dogs was used in which the hepatic artery and branches were sham-ligated and penicillin was given postoperatively, for six days. This control could rule out the unlikely possibility of trauma to the structures of the hepato-duodenal ligament and the repeated injections of penicillin stimulating antibody formation.

### Materials and Methods

Ten adult dogs were used for ligation of the hepatic artery and four for sham-ligation. The method of selection and feeding of these dogs was the same as for previous ligation experiments (see page 30).

The penicillin used for these experiments was a suspension of penicillin G procaine in water, which contained 300,000 international units (I.U.) per millilitre (Crysticillin)<sup>R</sup>. The suspension was such that high blood levels could be obtained in a very short space of time and a therapeutic level was maintained for approximately 24 hours after each injection.

A dosage rate of approximately 300,000 I.U. of penicillin per 50 pounds body weight was used. Daily injections were made for six days postoperatively.

#### Surgical Technique

The preoperative preparation, the anaesthesia, and the surgical technique for ligation of the hepatic artery were the same as those used in the vaccination experiments (see page 36). In addition, an injection of penicillin was made into the muscle of the hind limb of the dog within fifteen minutes of the completion of ligation.

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<sup>R</sup> E.R. Squibb & Sons of Canada, Limited, Montreal.

The four control dogs were similarly treated, except that the ligatures were only tied momentarily and the autonomic plexuses around the common hepatic artery were severed. The control dogs were given the appropriate dose of penicillin for the six days after sham-ligation.

All dogs were carefully watched after the operation and those that would not eat the routine kennel diet were tempted with milk, sugar and canned dog food.

#### Collection of Serum Samples

Fifty millilitres of blood were collected from each animal at the time of surgery, and one, two, and four weeks postoperatively. In order to facilitate the collection of the "one, two and four week" samples the animals were rapidly injected intravenously with Pentothal<sup>R</sup> (sodium pentothal). Sufficient Pentothal was given to keep the animals motionless for from five to fifteen minutes. The blood was then drawn from the heart with an eighteen gauge two-inch needle and immediately placed in the refrigerator. The serum was pipetted off after two hours and kept in the refrigerator until needed for the guinea-pig protection tests.

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<sup>R</sup> Abbott Laboratories Limited, Montreal, Canada.

#### Preparation of the Clostridial Culture

In order to measure the protective capacity of the serum antibodies it was necessary to prepare a pure culture of Clostridium chauvoei which had to be capable of consistently causing disease. The amount of culture needed to cause death had to be in manageable volume and had not to vary from one day to the next.

These requirements were met by using the strain of Clostridium chauvoei H18, which was known to be consistently infective to guinea-pigs. It had also been used to produce a satisfactory vaccine. A large volume of whole broth culture was prepared and pipetted into small sterile screw-top tubes. The tubes of culture were kept in the refrigerator at five degrees centigrade for three weeks before use.

In order to prepare the whole broth culture a section of guinea-pig liver (approximately one gram) infected with Clostridium chauvoei was taken from the refrigerator (see page 32) and placed aseptically in a 100 millilitre bottle containing 60 millilitres of trypticase soy and dextrose (T.S.d.). The broth culture was incubated anaerobically for 48 hours at 37 degrees centigrade. A stained smear was then made to test for the purity of the culture. The whole broth was next shaken well and five millilitres pipetted aseptically into each of 12, seven millilitre sterile screw-top tubes. The tubes were stored in the refrigerator at five degrees centigrade. When they were required they were placed on the bench until they reached room temperature.

#### The Bacterial Concentration of the Broth Culture

Using Prescott and Breed's (74) method for counting bacteria in a solution the broth was found to contain approximately 35 million bacteria per millilitre.

#### Establishment of a Minimum Lethal Dose 50

Having prepared the Clostridium chauvoei culture it was next necessary to calculate the lethal dose 50 (L.D.50); that is the amount of culture that would kill 50 per cent of injected guinea pigs.

A tube of the broth culture prepared three weeks previously was taken from the refrigerator and warmed to room temperature. In order to obtain a rough estimate of the amount of culture that would be required to kill, a group of three guinea pigs was injected in the deep muscles of the thigh with decimal dilutions. One guinea pig was injected with one millilitre of broth culture, one with one tenth of a millilitre and the other with one hundredth of a millilitre. The guinea pig that received one millilitre died within 24 hours. The guinea pig that received one tenth of a millilitre of broth culture died after 48 hours and the guinea pig that received one hundredth of a millilitre did not show any ill effects. A further series of injections was made in which two guinea pigs received two tenths of a millilitre of culture each, two received one tenth of a millilitre each, and two one twentieth of a millilitre each. Those guinea pigs receiving two tenths of a millilitre

of culture died within 24 hours, one of those receiving one tenth of a millilitre died and the other lived. Both the guinea pigs that received one twentieth of a millilitre of culture survived. From this it was suspected that the L.D.50 was in the region of one tenth of a millilitre of broth culture. A further four guinea pigs were injected with one tenth of a millilitre of culture each; two died and two survived. Subsequently, whenever a fresh tube of culture was removed from the refrigerator it was tested by giving four guinea pigs intramuscular injections of one tenth millilitre. Invariably an L.D.50 of one tenth of a millilitre was obtained, even with a culture six weeks old.

#### Method of Measuring Serum Protection

Two methods for measuring levels of serum protection were considered. In one method a large volume of the serum to be tested was injected subcutaneously into guinea pigs for two days prior to challenge by the broth cultures. The other method consisted of mixing a smaller volume of the serum with the challenge dose of broth culture and heating them together at 37 degrees centigrade in a water bath before injection into guinea pigs.

It was important to know which of these two methods was the most sensitive, for it was thought that the protective power of the serum might well be very small. In order to compare the sensitivity of these two techniques they were tested against a known positive serum;

the serum used was from H74, a dog that had been vaccinated four weeks previously.

#### Method A

Four guinea pigs were used. They were each injected subcutaneously with ten millilitres of the serum from H74 on one day and eight millilitres on the second day. On the third day three of the guinea pigs were challenged with the Clostridium chauvoei culture, the fourth was kept as a control. The challenging doses of the culture for the three guinea pigs were three L.D.50's, six L.D.50's and ten L.D.50's. All three test guinea pigs died within 72 hours. The control guinea pig survived.

#### Method B

Four guinea pigs were used. In this method the maximum amount of serum that could reasonably be injected intramuscularly into a guinea pig was used. This was five millilitres. Three tubes of serum culture mixture were kept at 37 degrees centigrade in a water bath for one and a half hours. A fourth tube of serum only was incubated as well and acted as a control. Mixed with the five millilitres of serum in each of the three tubes was three L.D.50's, six L.D.50's and ten L.D.50's of culture. After one and one half hours of incubation the contents of each of the four tubes was injected into four guinea pigs. For convenience of injection half the contents of each tube was injected

into each hind leg. The guinea pig that received 10 L.D.50 died within 24 hours. The three remaining guinea pigs survived.

The impression gained from a comparison of these two methods was that method B would probably be the most satisfactory method to use.

A fixed volume of each sample of serum (five millilitres) was tested in pairs of guinea pigs against increasing multiples of the L.D.50. The lowest multiple of the L.D.50 which would kill both guinea pigs in the presence of the serum under test was recorded; this number was taken as an index of the protective power of the serum (Table VI).

Two guinea pigs were used for each challenge. Firstly, two tubes each containing five millilitres of the serum to be tested plus one L.D.50 were incubated for one and a half hours at 37 degrees centigrade. The two guinea pigs were then injected with approximately two and a half millilitres of mixture into each hind leg. If both the guinea pigs died the test was terminated and the serum recorded as being ineffective against one L.D.50. If either or both of the guinea pigs lived the test was repeated with two more guinea pigs using two L.D.50's; if both guinea pigs died the test was terminated. The serum this time was recorded as being ineffective against two L.D.50's. If either or both guinea pigs survived the test was repeated against three L.D.50's and the results interpreted in the same way.

## Results

### Ligation of the Hepatic Artery and Penicillin Therapy

The hepatic artery and its branches were ligated, and seven daily injections of penicillin given to ten dogs (H58,59,61,62,66,75,79,83,84,85) (Table VI). Of these dogs only seven survived long enough for the final sample of blood to be collected (four weeks). All surviving dogs were killed after six weeks. Only two of the dogs (H58,79) that lived for six weeks had a completely uneventful recovery. Most of the dogs were off food for about a week, and two of them (H59,64) had a transitory jaundice for two to three weeks. Of the three dogs that did not survive for four weeks (H62,83,84) one was killed because it contracted tracheobronchitis; the other two dogs died from what appeared to be a bile peritonitis. At necropsy the abdomen of these two dogs contained from 50 to 500 millilitres of a bile-stained fluid, the gallbladder was shrunken and surrounded by fibrinous material and the liver was irregularly scarred. The lesions in the liver of these two dogs resembled closely those described by Fraser (26) and attributed to hepatic ischaemia from ligation of the hepatic artery.

### Results of Guinea-pig Protection Tests

Thirty-six samples of serum were tested for their ability to protect laboratory animals (guinea-pigs) against an infection of Clostridium chauvoei (Table VI).

The preoperative serum samples from the normal adult dogs apparently did not give any protection to guinea pigs and this situation was not improved by ligation of the hepatic artery and penicillin therapy. In fact none of the samples gave protection that could be regarded as being significant. The reading for the vaccinated dog (H74) was seven. That is, it was not until a level of seven times the L.D.50 of the culture was injected that the serum failed completely to protect guinea pigs. It was certain, therefore, that the dog could be stimulated to produce a measurable quantity of antibody against Clostridium chauvoei; ligation of the hepatic artery and penicillin therapy did not appear to produce this effect.

The reason for the survival of the "ligated" dog after the withdrawal of penicillin still remained uncertain.

The amount of collateral arterial supply that had developed after ligation of the hepatic artery had been studied by injection techniques (page 42) and found to be of little significance. There remained the possibility that the reason there was no need for protection of the animal with penicillin or antibodies after seven days was because the danger of infection had passed.

## PART IV

### A HYPOTHESIS TO ACCOUNT FOR THE ABSENCE OF ANY DETECTABLE QUANTITY OF ANTIBODIES TO CLOSTRIDIUM CHAUVOEI IN THE SERUM OF DOGS FOLLOWING LIGATION OF THE HEPATIC ARTERY

#### Introduction

During studies of the changes that occur in dogs after ligation of the hepatic artery the author had frequently noted infarcts in the livers from eight to eighteen hours after surgery. It was thought that these infarcts might be linked with the death of the animal. This was substantiated when it was observed subsequently that in dogs destroyed at the first sign of toxæmia these infarcts were occasionally found to contain masses of clostridia while the rest of the liver remained normal. If then the nidus of infection was restricted to these infarcts it seemed reasonable that penicillin would quickly control the clostridia in these few areas. This being so, there was a likelihood that before the end of a week the active infection in these areas would be destroyed and any further protection made unnecessary. In order to substantiate the part that infarction played in the setting up of a clostridial infection, the hepatic artery was ligated in six dogs and the livers re-examined when the animals showed the first signs of toxæmia.

### Materials and Methods

The selection of dogs and the technique of ligation of the hepatic artery and its branches was identical with that used in previous experiments. The skin incision was closed with a subcuticular suture line to facilitate preparation of the animal prior to re-examination of the liver.

The first signs of toxæmia were a disinclination to walk, a glassy appearance to the eyes and mild dyspnoea. A little later on the animal would lean against the sides of the cage when forced to stand up. The animal was anaesthetized with Nembutal at the first signs of distress and the skin carefully prepared as for surgery. The abdomen was re-entered and the liver removed aseptically. Impression smears were immediately taken from infarcted areas and from five areas of apparently healthy hepatic parenchyma. A part of each infarct was removed for histological examination. Five, one gram specimens of apparently normal hepatic tissue were aseptically removed for anaerobic culture in thio-glycollate broth and another five were fixed in formalin prior to sectioning for histological examination. The sections were stained with haemotoxylin and eosin.

### Results

In three of the six dogs examined (H108,114,116), clostridia were identified from a direct smear of an infarcted area and of the twenty-five infarcts examined histologically, eight revealed the presence of clostridial proliferation (Table VII). None of the thirty specimens of normal liver (non-infarcted tissue) showed clostridia on histological examination, although from thirty similar areas clostridia were cultured on twenty-three occasions.

One dog (H118) was autopsied after four days, not because it was ailing but because it was thought that by that time the animal should have shown some signs of sickness if it was going to die from clostridial hepatitis. The liver of this animal was not infarcted and in fact, apart from a uniform congestion it appeared quite normal. However, the hepatic tissue adjacent to the gallbladder was bile-stained and friable. The gallbladder was oedematous and surrounded by fibrinous material.

### Discussion

The experiments in this section were planned to evaluate the part that infarction of the liver played in setting up conditions for clostridial infection.

The results clearly indicated that at approximately twenty hours after ligation of the hepatic artery some degree of infarction was

present in most cases and that often clostridia, in an apparently active stage of growth, could be seen on histological examination of these infarcts.

While it is interesting to find that in the one dog that did not show signs of a toxæmia there should also be an absence of infarction, it would be unwise to overemphasize any finding in one animal.

The results point to the possible importance of infarction in the pathogenesis of clostridial hepatitis. If this were so, penicillin might only be required to control a very localized clostridial infection, an infection which might easily be controlled in a week. In which case the animal might not need any further protection against clostridia.

## DISCUSSION AND CONCLUSIONS

It was surprising to find such a high incidence of Clostridium chauvoei "infected" livers in the dog population surveyed, particularly when it was recalled that it was Clostridium welchii that had always been suspected of inhabiting the canine liver. The canine population surveyed was confined to one area (Ontario) and most probably the incidence, as of most infections, will vary from one district to another. However it is of some significance that the only drawings and photomicrographs previously published show a "welchii-like" organism closely resembling Cl. chauvoei. It is probably that the methods used would have isolated Clostridium welchii in a mixed Cl. chauvoei - Cl. welchii culture. Not only would Cl. welchii have dominated but it would have had no trouble growing on the blood-agar plates used. The fastidiousness of Cl. chauvoei makes it relatively easy to overlook in a mixed culture, and almost impossible to isolate without resorting to guinea-pig inoculation.

It is interesting to postulate how the clostridia are held in the liver. That they are not in a form susceptible to penicillin is easily demonstrated by giving a course of penicillin followed by ligation of the hepatic artery. An experiment which produces typical clostridial hepatitis. Probably then the clostridium is in the spore form within the liver. Animals inoculated with spore containing vaccine are still found to harbour Clostridium chauvoei, a finding which might point to the harboured spores being protected within a living cell; possibly

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of the reticulo-endothelial system. It is a great pity that when Rous and Beard (85) made their classical studies on the extraction of von Kuppfer cells from the liver of dogs they failed to make anaerobic cultures.

The other species of bacterium isolated from the liver of the dog are probably of little significance, and no attempt was made to differentiate possible contaminants from probable normal flora. It is interesting to note, however, that in one dog examined during preliminary experiments and not recorded here (H10) seven biopsy specimens were taken from the liver and they all produced a pure culture of Streptococcus faecalis. The same dog was re-examined three weeks later and eleven biopsy specimens taken; eight of these eleven biopsy specimens produced a pure culture of Streptococcus faecalis. Escherichia coli might equally be a contaminant or a harboured bacterium. Staphylococcus aureus is a common skin and air contaminant.

The technique for ligation of the hepatic artery was thought to be almost foolproof. The flow of blood was stopped through all vessels in the hepato-duodenal ligament except the portal vein which was carefully lifted away to avoid any restriction of its blood flow. The effect on the penicillin protected liver after ligation by this technique differed somewhat from that reported by Markowitz, Rappaport and Scott (56), in their original observations. The latter workers, using a climbing suture technique for ligation of the hepatic artery found that penicillin protected the dog's liver from all ill effects and after sev-

eral weeks the liver was in perfect condition. This could not be said of the dogs ligated by the technique described here, for there was often fibrosis of the liver, sometimes shrinking, and invariably the animal was ailing for a week or more after surgery. These findings are in line with those of Fraser, Rappaport, Vuylsteke and Colwell (27), who made liver function tests during the post-ligation period and found evidence of hepatic failure from two to nine days after surgery. They also observed in some cases an ischaemic necrosis of the liver several weeks after ligation of the hepatic artery and seven days penicillin therapy. Fraser et al. (27) used the climbing ligature technique for ligation of the hepatic artery.

The success of the vaccination experiments not only proved conclusively that Clostridium chauvoei was the causal agent in death from ligation of the hepatic artery but it also gave a satisfactory method of protecting dogs prior to experimental surgery involving the hepatic artery; an able substitute for penicillin therapy. The failure of commercial antiserum to protect one of the six dogs inoculated might suggest that in some cases a higher dosage rate would be necessary.

The apparent absence of antibodies for Clostridium chauvoei in the serum of dogs after ligation of the hepatic artery and penicillin therapy was surprising in the light of the original hypothesis; which it will be remembered postulated antibody protection of the ligated dog after cessation of penicillin therapy. The evidence from the experiments on infarcts, however, shed a new light on the subject. In retro-

spect it would seem surprising for a bacterium as fastidious as Clostridium chauvoei to grow when the portal vein was still bringing large volumes of oxygen to the area. Infarction however, would provide a much more anaerobic environment. There can be little doubt that it is in the infarcted areas that infection is initiated. As might be expected most of the infarcts were found at the periphery of the liver lobes; where the collateral circulation would be least. It was also in these areas that the stained impression smears were found to be positive for clostridia. It might be argued that the growth of clostridia in these areas produced the infarcts. However, if the liver of a dog is watched constantly after ligation it will be seen that the infarcts usually appear within an hour. It will also be recalled that there were numerous infarcts without clostridia. It might be reasonably assumed that penicillin could rapidly control the clostridial infection in the infarct and it would be unlikely if the infection were not eliminated within seven days. Barring the unlikely event of spontaneous infarction after one week it might be hypothesized that the animal would be out of danger and any protection such as Clostridium chauvoei antibodies would be redundant. This is only a hypothesis, but supported by the following findings.

Eze (23), Milnes (65), and Chau, Goldbloom and Gurd (15) report that penicillin therapy can be withdrawn one or two days after ligation of the hepatic artery.

Huggins and Post (41) noted that the hepatic artery can be ligated in stages without producing death of the animal (such a technique

might possibly obviate infarction).

One dog (H118) that did not die within four days of ligation of the hepatic artery was found at autopsy to have a liver free from infarction.

To draw a comparison with another species, 'Black disease' in sheep (necrotic hepatitis) is an acute infection, usually of adult sheep, caused by Clostridium novyi. One of the diagnostic pathologic lesions is the presence within the liver of one or more infarcts. While it is not always wise to draw conclusions between different species this might seem to indicate that infarction plays a more important part in the multiplication of clostridia than was previously supposed.

## SUMMARY

A survey of the bacteria of the liver of twenty-seven dogs of all ages was made (648 biopsy specimens were cultured from the 27 dogs). Briefly the findings were:-

The liver apparently begins to acquire a bacterial flora in approximately the middle of the first year of the dog's life.

From the 648 biopsy specimens cultured Clostridium chauvoei was isolated 231 times, Staphylococcus aureus seven times, Escherichia coli six times, Streptococcus faecalis four times and Clostridium welchii once.

A new technique for the ligation of the hepatic artery and its branches in the dog is described. Six out of six dogs, ligated by this technique developed typical clostridial hepatitis within eighteen to thirty-six hours of surgery.

A description is given of a method for the preparation of a formalized alum-precipitated vaccine using a freshly isolated strain of Clostridium chauvoei. Ten dogs were vaccinated and after allowing two weeks for the development of antibodies, the hepatic artery was ligated in each dog. None of the vaccinated dogs developed clostridial hepatitis. These dogs were subsequently injected with a blood-vessel outlining substance in order to assess the degree and importance of the development of a collateral arterial supply to the liver. The collateral arterial supply was found to be insignificant.

Six dogs were inoculated with commercial Cl. chauvoei antiserum. Only one of these animals died after ligation of the hepatic artery. This

animal developed typical clostridial hepatitis.

The hepatic artery was ligated in ten dogs and they were protected for seven days postoperatively with penicillin. A search was then made for Clostridium chauvoei antibody activity which it was thought might be the reason for the animals surviving after the penicillin therapy was discontinued. The findings were negative.

An hypothesis is put forward to account for the absence of measurable amounts of antibody production to Clostridium chauvoei in the dogs' sera tested. The hypothesis is based upon the localization of the clostridial infection within areas of infarction in the liver. The hepatic artery was ligated in six adult dogs and the livers of five of them examined within twenty-four hours. Infarcts were consistently found. In a number of the infarcts active clostridial proliferation was noted. No clostridial proliferation was ever found on histological examination of the tissue between infarcts, although Clostridium chauvoei could usually be cultured from these areas. It is proposed that clostridial infection in these areas would be readily controlled by penicillin and that this might make any further protection of the animals after seven days of penicillin therapy unnecessary.

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TABLE I  
BACTERIOLOGICAL SURVEY OF  
THE LIVER OF DOGS OVER ONE YEAR OF AGE.

Animal Number	Age (Yrs.)	Wgt. (lbs.)	NUMBER OF BIOPSY SPECIMENS POSITIVE FOR BACTERIA <sup>A</sup>				
			Left Lat.	Left Central	G-B(left)	G-B(right)	Total
H 13	3	38	-	1	-	2	3
H 15	2	41	-	-	-	3	3
H 18	1	30	6	6	6	6	24
H 23	3	55	1 (2 Staph) <sup>XX</sup>	(1 Staph)	-	1 (1 Staph)	6
H 25	3	34	-	-	-	-	0
H 26	2	27	-	(1 Staph)	(1 Staph)	(1 E.coli)	3
H 27	2	33	-	-	-	(1 Cl.welchii)	1
H 30	2	55	6	6	6	6	24
H 32	2	42	-	4	2	4	10
H 29	2	38	3	3	5	6	17
H 33	5	46	6	6	6	6	24
H 38	2	29	1	1	-	3	5
H 68	2	43	1	2 (1 Strep)	1	3	8
H 69	6	50	6	6	6	6	24
H 71	3	31	6	6	6	6 (1 Staph)	24
H 82	4	50	5	3	6	6	20
H 92	2	30	1	-	2	3	6

TABLE I (cont'd.)

Animal Number	Age (Yrs.)	Wgt. (lbs.)	NUMBER OF BIOPSY SPECIMENS POSITIVE FOR BACTERIA <sup>x</sup>				
			Left Lat.	Left Central	G-B(left)	G-B(right)	Total
H 94	4	45	3	1	4	3	13
H 98	5	50	6	6	6	6	24
H 95	5	35	-	-	-	-	0

G-B(left) -- Left part of Gallbladder lobe:  
G-B(right) -- Right part of Gallbladder lobe.

<sup>x</sup> Numbers not bracketed are for Clostridium chauvoei

<sup>xx</sup> Numbers bracketed are for bacteria other than Cl. chauvoei e.g.

Staph. - Staphylococcus aureus  
Strep. - Streptococcus faecalis  
E. coli - Escherichia coli  
Cl. welchii - Clostridium welchii

TABLE II  
BACTERIOLOGICAL SURVEY OF  
THE LIVER OF DOGS UNDER ONE YEAR OF AGE

Animal Number	Age (Mos.)	Wgt. (lbs.)	NUMBER OF BIOPSY SPECIMENS POSITIVE FOR BACTERIA*				Total
			Left Lat.	Left Central	G-B(left)	G-B(right)	
H 17	10	26	-	(1 Strep.) (2 E.coli)	1 (E.coli)	(1 Strep.) (E.coli)	9
H 22	6	15	-	-	-	-	0
H 36	8	30	6	6	6	6	24
H 41	6	15	-	-	-	-	0
H 52	6	17	-	-	-	-	0
H 64	6	19	-	-	-	-	0
H 87	8	20	-	-	-	-	0

\* Numbers not bracketed are for Clostridium chauvoei

TABLE III  
SUMMARY OF BACTERIAL ISOLATIONS FROM THE LIVERS OF DOGS

Age	Number of Dogs	Total No. of Specimens Taken	Total Number of Isolations			
			<u>Cl. chauvcei</u>	<u>Cl. welchii</u>	<u>Strep. faecalis</u>	<u>Staph. aureus</u> <u>E. coli</u>
Over 1 yr.	20	480	231	1	2	7
Under 1 yr.	7	168	25	-	2	6
Total	27	648	256	1	4	7

TABLE IV

RESULTS OF EXPERIMENTS IN WHICH DOGS WERE VACCINATED WITH CL. CHAUVOEI VACCINE

Animal Number	Age (Yrs.)	Killed at (Wks.)	Postoperative Progress	Necropsy Findings
H42	2	6	Off food first five days.	Nothing abnormal except fibrosis of gallbladder.
H49	3	5	Off food first eight days. Jaundiced 2nd to 4th week.	Liver extensively fibrosed and reduced in size. Bile-stained fluid in peritoneal cavity.
H56	3	7	No ill effects.	Liver reduced in size, otherwise normal. Gallbladder fibrosed.
H63	4	5	Off food for seven days, Jaundiced from approximately 10th to 20th postoperative day.	Extreme emaciation. Liver scarred on left lateral lobe. Gallbladder fibrosed.
H74	2	4	Off food for first two days.	Nothing abnormal found, except fibrosis of gallbladder.
H78	4	6	Off food first five days. Jaundiced from 7th to 17th postoperative day.	Liver a little smaller than usual but otherwise quite normal. Gallbladder fibrosed.
H93	3	5	No ill effects.	Nothing abnormal except shrunken gallbladder.
H96	4	5	Off food for seven days. Jaundiced from 5th to 7th postoperative day.	Liver apparently normal except for fibrosis of the gallbladder.
H97	4	4	Off food for ten days. Became extremely thin taking only enough food to keep alive.	Diffuse scarring of whole liver.
H99	5	5	Off food for four days.	Liver apparently normal except for fibrosis of the gallbladder.

TABLE V

## DOGS PROTECTED WITH CL. CHAUVCEI ANTISERUM AND THE HEPATIC ARTERY LIGATED

Animal number	Age	Animal died (D) or killed (K) after (weeks)	Postoperative progress	Necropsy findings
H44	2	7 (K)	No apparent ill effects.	Liver normal. Gallbladder fibrosed.
H46	3	5 (K)	No apparent ill effects.	Liver a little smaller than normal. Gallbladder fibrosed.
H53	3	5 (K)	Off food for first five days. Jaundiced from 4th to 8th postoperative day.	Liver scarred. Gallbladder fibrosed.
H65	4	3 (D)	Became progressively emaciated. Jaundiced from 4th postoperative day until death.	Liver reduced in size and extensively fibrosed.
H73	4	6 (K)	Off food for first ten days.	Liver normal. Gallbladder fibrosed.
H107	3	3 (D)	Died on the third day.	Typical of clostridial hepatitis.

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TABLE VI

## LIGATION OF THE HEPATIC ARTERY WITH PENICILLIN PROTECTION

Animal number	Animals killed (K) or died (D) after: (days)	Necropsy findings	Protective index *			
			Pre-op.	1 wk.	2 wks.	4 wks.
H58	40 (K)	Liver normal. Gallbladder fibrosed.	1	2	2	2
H59	40 (K)	Liver reduced in size. Gallbladder fibrosed.	2	2	3	2
H61	40 (K)	Left lateral lobe of liver fibrosed. Gallbladder and liver surrounding adhered to abdominal wall.	1	2	1	2
H62	18 (K)	Infected with tracheobronchitis and destroyed. Liver apparently normal. Gallbladder fibrosed.	2	2	2	-
H66	40 (K)	Liver apparently normal. Gallbladder fibrosed.	2	1	2	2
H75	40 (K)	Two large areas of fibrosis at the tip of the gallbladder lobe. Gallbladder fibrosed.	2	2	2	2
H79	40 (K)	Liver apparently normal. Gallbladder fibrosed.	1	2	2	2
H83	10 (D)	Liver necrosis with scar formation. Large volume of bile-stained fluid in abdomen.	2	1	-	-
H84	42 (D)	Carcass extremely emaciated, liver diffusely fibrosed. Approximately 500 ml. of bile-stained fluid in abdomen.	2	3	2	-
H85	42 (K)	Liver reduced in size, but normal in structure.	3	2	3	3

\* The lowest multiple of the LD50 against which the serum was completely ineffective.

TABLE VII  
EXAMINATION OF THE LIVER FOR INFARCTION AFTER LIGATION OF THE HEPATIC ARTERY

Animal number	Liver re-examined after (hours)	Number of infarcts	Number of infarcts containing clostridia	Number of areas of apparently normal hepatic tissue containing clostridia	On direct smear		Histological examination		Culture
					On direct smear	Histological examination	On direct smear	Histological examination	
H108	24	3	1	2	0	0	0	0	4
H114	18	5	1	2	0	0	0	0	3
H115	20	4	0	0	0	0	0	0	5
H116	20	6	1	2	0	0	0	0	5
H118	96	0	0	0	0	0	0	0	4
H119	18	7	0	2	0	0	0	0	2

Figure 1

CLOSTRIDIUM CHAUVOEI

Primary isolation by direct smear onto blood-agar from the liver of a guinea-pig. The guinea-pig had been inoculated twenty-four hours previously with a broth culture of Cl. chauvoei.

Figure 2

CLOSTRIDIUM CHAUVOEI

Thirty-six hour old colonies of Clostridium chauvoei. Primary isolation from guinea-pig heart blood. The guinea-pig had been inoculated twenty-four hours previously with a broth culture of Cl. chauvoei (x 2).

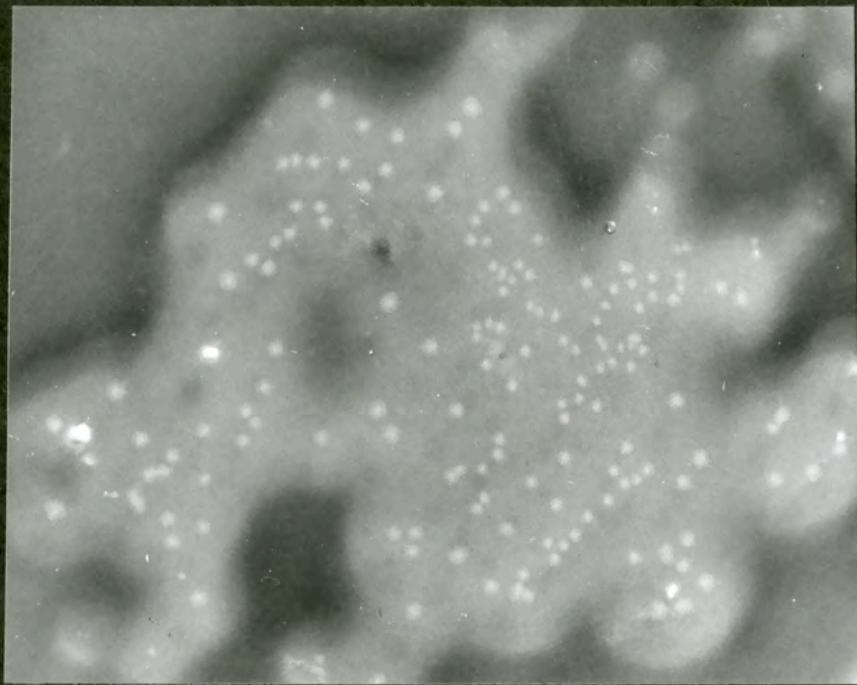


Figure 3

CLOSTRIDIUM CHAUVOEI

Forty-eight hour old colony of Clostridium chauvoei. (Methylene blue; x 80).

Figure 4

CLOSTRIDIUM CHAUVOEI

Periphery of a forty-eight hour old colony of Clostridium chauvoei. An enlargement of an area similar to the one outlined on Figure 3. (Methylene blue; x 1000).

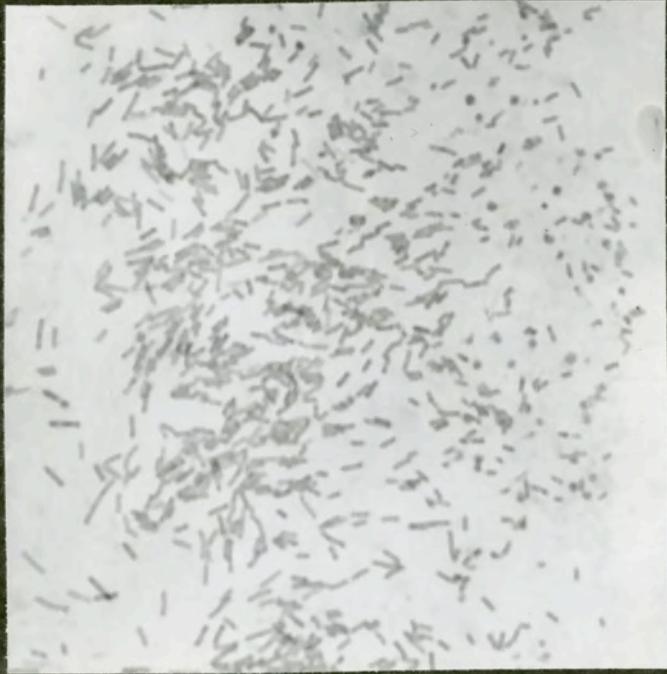
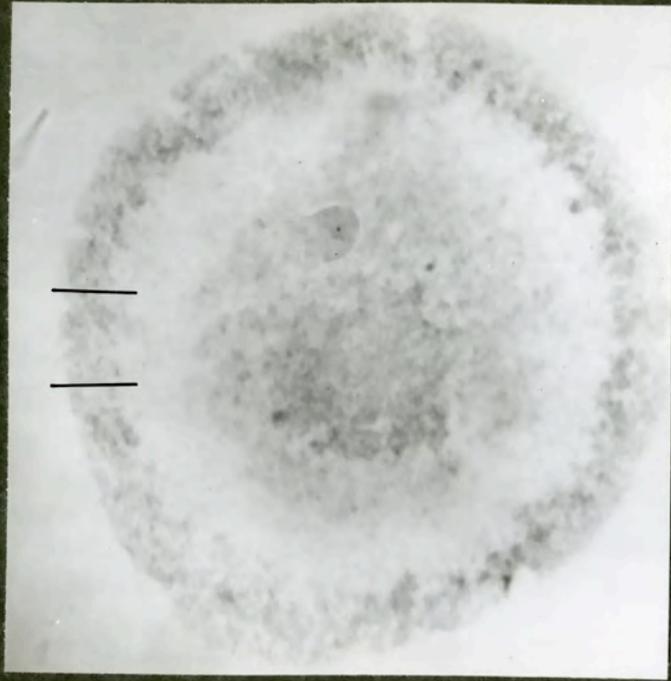




Figure 5

CLOSTRIDIAL HEPATITIS

Liver of an adult dog that died thirty-six hours after ligation of the hepatic artery. A gaseous necrosis affects most of the gallbladder lobe of the liver (x 1/2 actual size).

Figure 6

HEPATIC FIBROSIS

Liver of a 50 lb. adult dog two weeks after ligation of the hepatic artery and penicillin therapy. The scars (arrowed) are probably the result of infarction. The general shrinkage of the organ may be due to loss of centrilobular tissue (actual size).



Figure 7

ANAEMIC INFARCT

An anaemic infarct in the liver of a dog eighteen hours after ligation of the hepatic artery. Penicillin was not given. (Actual size).

Figure 8

HAEMORRHAGIC INFARCT

A haemorrhagic infarct (arrowed) in the left central lobe of the liver of a dog twenty hours after ligation of the hepatic artery. Penicillin was not given. (x 1/2 actual size).



Figure 9

CLOSTRIDIUM CHAUVOEI

Smear made from an infarcted area of liver. The dog was killed when it commenced to show signs of a toxæmia. The arterial supply to the liver had been interrupted eighteen hours previously. (Gram's stain; x 1800).

Figure 10

CLOSTRIDIUM CHAUVOEI

Photomicrograph of an infarcted area of liver. The dog was killed when it commenced to show signs of a toxæmia. The arterial supply to the liver had been interrupted eighteen hours previously. (Haemotoxylin and Eosin; x 1200).

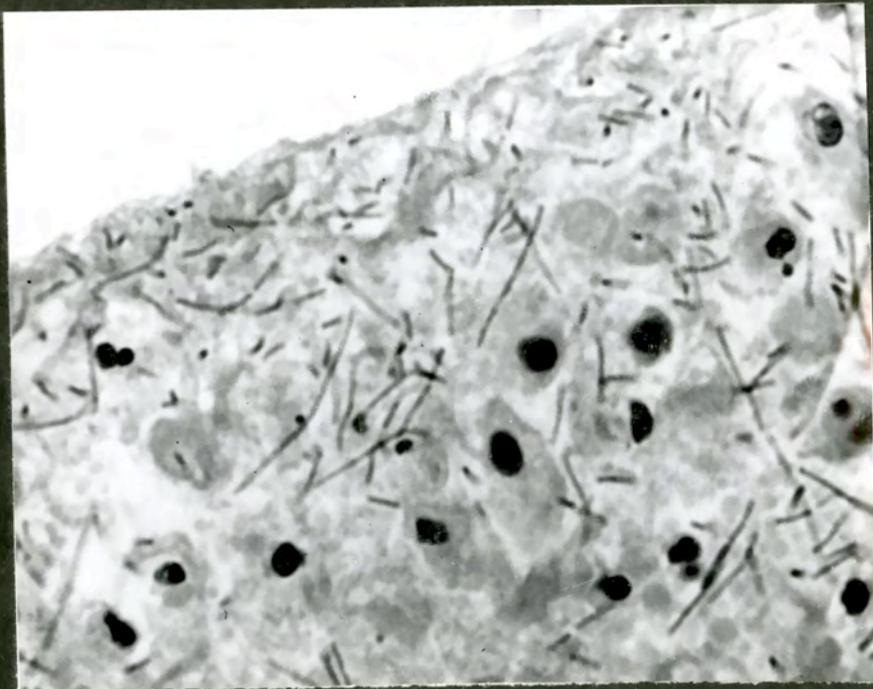
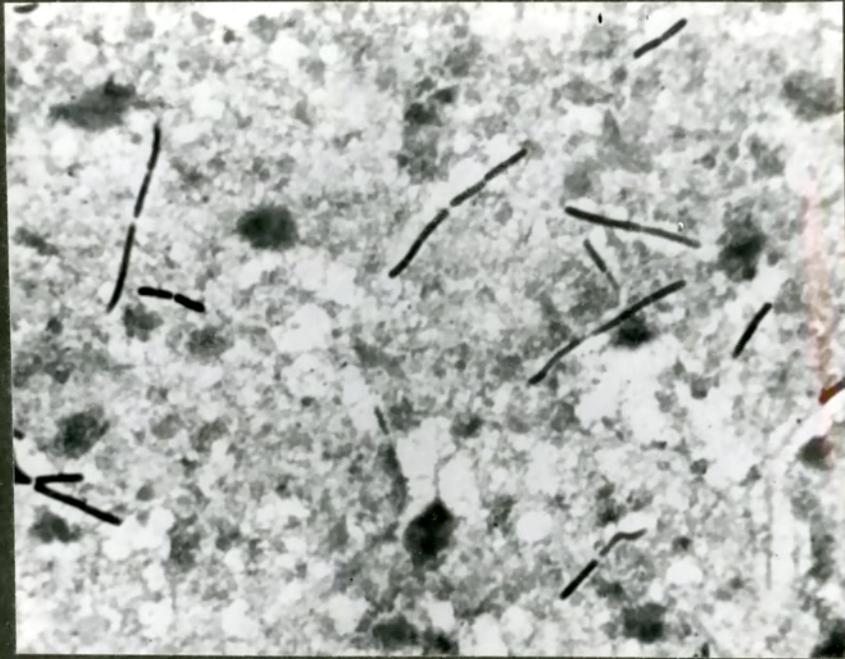


Figure 11

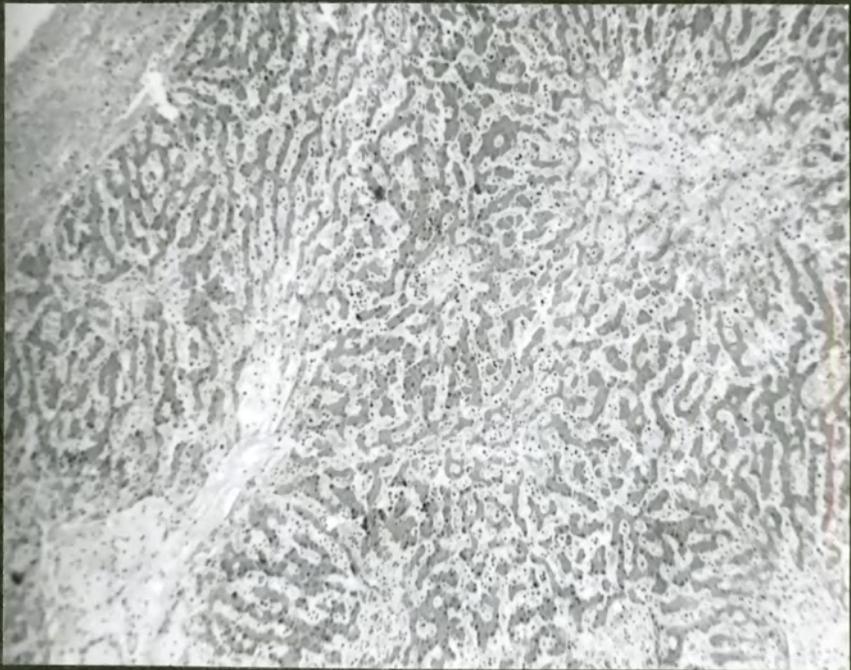
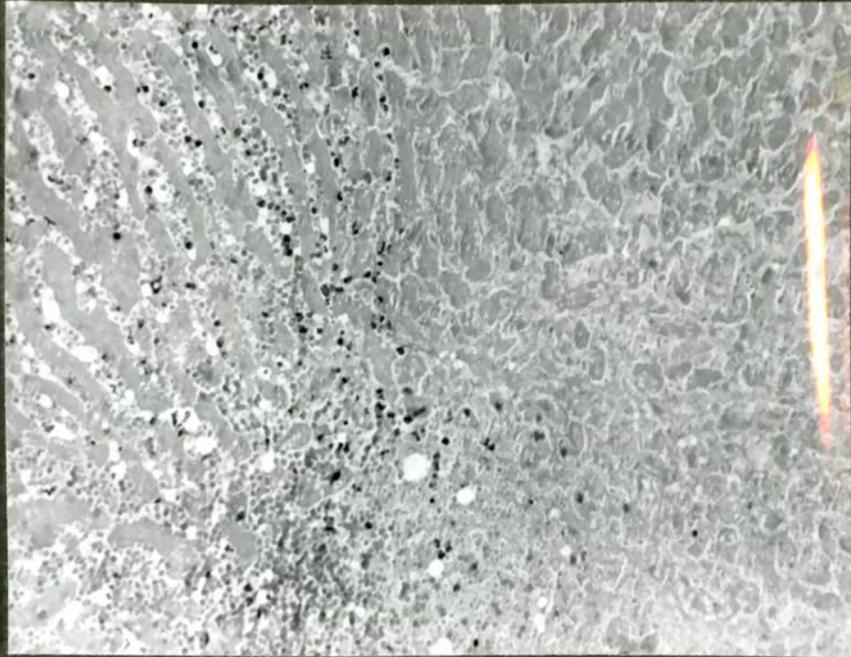
INFARCT

Photomicrograph of the periphery of an infarct. At a higher magnification the bacilli of Cl. chauvoei could be seen. (Haematoxylin and Eosin; x 270).

Figure 12

ISCHAEMIC NECROSIS

Photomicrograph of the liver of a dog two weeks after ligation of the hepatic artery and penicillin therapy. The lesions closely resemble those of an ischaemic necrosis. (Haematoxylin and Eosin; x 180).





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