A CLINICO-PATHOLOGICAL STUDY

OF

BABESIA CANIS INFECTION

IN DOGS

by

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of the requirements for the degree of
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DEDICATION

TO MY WIFE

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1. INTRODUCTION

"The search for truth is in one way hard and in the other easy. For it is evident that no one can master it fully or miss it wholly. But each adds a little to our knowledge of Nature, and from all the facts assembled there arises a certain grandeur".

Aristotle

There is considerable evidence to suggest that the canine disease caused by Babesia canis has been recognized in South Africa for the greater part of two centuries. During the nineteenth century it was called malignant jaundice, malignant malarial fever and similar names but its causal organism was seen and described only in 1895 in Italy. Since then it has clearly become one of the most important infectious diseases of dogs over a large part of the world. Apart from the icy Arctica and Antarctica, Australia only among the continents, until very recently, was considered to be free of the disease. However, since it was diagnosed in Queensland in 1966 it is now to be found in all continents where infected vector ticks exist.

In South Africa where the climate varies for the greater part from tropical to temperate few dogs escape becoming infected fairly early in life, most of them probably during their first year. At the Onderstepoort small animal clinic, serving a limited area, some 500 dogs are treated annually for this disease.

During the early years of this century there was great interest in the transmitting ticks and in the clinical features of the disease. Later a great deal of work was done in connection with chemotherapy, attempts at cultivation of

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the parasites, attempts at transmission to other kinds of animals, the taxonomy of the organism and related aspects of its biology. There was early interest in the anaemia caused by the disease and the presence of bile pigments in the blood and urine, and numerous limited studies of particular aspects appeared in the literature. The first broader investigations of the clinical pathology of the disease were made by Maegraith, Gilles and their co-workers at the Liverpool School of Tropical Medicine. Their primary interest was in malaria, but owing to the very great degree of analogy between pathological processes in the two diseases and the ready availability of dogs as experimental animals they also paid attention to this disease. This was done by artifically infecting dogs by subinoculation of susceptible animals from infected ones.

As it has been observed at Onderstepoort that artificially infected and kennelled dogs in general get the disease in a milder form it was felt that a systematic study of actual field cases by modern clinical laboratory methods was due. This was motivated by the sometimes unsatisfactory results of specific chemotherapy and by sporadic observations of disturbed function of organs only secondarily due to the infection.

Quite apart from sentimental considerations (not by any means to be discounted) the economic value of dogs on farms, in homes, as trained tracker dogs in the Police, guide dogs for the blind and guard dogs in general was a factor not to be ignored. The great availability of material, moreover, and an opportunity of establishing a model in the laboratory

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concerned for similar characterization of other "tropical" infections of farm animals made babesiosis an attractive object of study. The term "babesiosis", it should here be mentioned, is used specifically in this work for Babesia canis infection of dogs, and not for other known or possible species of the genus.

Although the primary purpose of the investigation was the study of the clinical pathology of the disease it was deemed necessary to present a fairly comprehensive review of the other known facts which in many cases have some bearing on the results obtained. In this way this thesis attempts to present the whole disease as it affects the dog in all its vagaries.

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2. THE DISEASE CAUSED BY BABESIA CANIS: REVIEW

2.1 DEFINITION

Canine babesiosis is a peracute, acute, or chronic, febrile, non-contagious, infectious disease affecting chiefly dogs but also some other members of the Canidae. It is characterized typically by fever, anaemia, splenomegaly and a bounding pulse but has the potentiality of protean manifestations. It is caused by a protozoan parasite Babesia canis (Piana & Galli-Valerio, 1895).

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2.2 SYNONYMS

For the disease:-

Malignant jaundice (Hutcheon, 1893)

Malignant malarial fever (Hutcheon, 1899)

Bilious fever (Hutcheon, 1893)

Biliary fever (Jowett, 1909)

Piroplasmosis (Nocard, 1902)

Babesiosis (Fischer & Scheidemann, 1920)

Tick fever (various)

And translations such as fièvre bilieuse, jauness maligne des chiens, bosluiskoors, galkoors, etc.

For the causative organism:-

Pyrosoma bigeminum var. canis Piana & Galli-Valerio, 1895

Piroplasma canis Piana & Galli-Valerio, 1895

Piroplasma rossi Nuttall, 1910

Babesia rossi Nuttall, 1910

Rossiella rossi Nuttall, 1912

Although there is no cross-immunity, Babesia vogeli (Reichenow, 1937) also named B. major Reichenow, 1935, may in due course be included under B. canis.

2.3 HISTORY AND DISTRIBUTION

2.3.1 HISTORY

The early history of the disease caused by Babesia canis has been adequately reviewed by Henning (1956). As far as South Africa is concerned the disease had been recognized as a district entity for more than a century before Piana and Galli-Valerio saw and described the causative organism in 1895. Lounsbury, an entomologist, in 1902 collated much of this earlier information from writings, reports and conversations of various people who had known the disease during a considerable part of the nineteenth century. He also mentioned a letter of Lady Anne Barnard, a well-known personality and recorder of the local scene in the Cape Colony at that time. Dated November 29, 1779, this letter described a disease in dogs which was undoubtedly babesiosis.

Early authors in South Africa included Hutcheon (1893, 1896, 1899, 1907), Robertson (1901, 1906), Lounsbury (1901, 1902), Theiler (1904, 1905) and Jowett (1909). Koch (1897) identified the parasite in East African dogs. After this time there was a spate of interest in the disease which lasted for a number of years and has in fact not yet been dissipated. The nature, morphology, life-cycle and therapy were studied in great detail by Nuttall (1904), Nuttall & Graham-Smith (1905, 1906, 1907, 1909) and Nuttall & Hadwen (1909) in Cambridge, Christophers (1907) and Shortt (1936) in India, and by Kleine (1906), Deseler (1910), Toyoda (1913), Ziemann (1913, 1914) and Regendanz & Reichenow (1932) in Germany.

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These early days also saw great activity by French workers who included Marchoux (1900), Almy (1901), Nocard & Almy (1901), Nocard & Motas (1902), Brumpt (1919), Brumpt & Larousse (1922) and Brumpt (1938).

It is not claimed that this listing is complete but it does serve to demonstrate some of the interest that this disease and its causative parasite inspired.

2.3.2 DISTRIBUTION

Numerous authors have recorded their own observations in different countries and listed other countries where B. canis had been recognized. One of the early ones was Christophers, in 1907. He mentioned Italy, France, Senegal, Tanganyika, South Africa, India and Assam and concluded that the infection was present over Southern Europe, the whole of Africa and much of Asia.

Knuth & du Toit in their 1921 text added Hungary, Russia, Transcaucasia, Indochina, China, Tonkin and Puerto Rico.

Eaton in 1934 recorded the disease for the first time on the continental United States of America and according to Sanders (1937) it appeared to be fairly widespread in the southern States. The latter author also included the Philippines and England.

Cottier (1936) added North Africa and in the same year Markoff contributed places like Aleppo and Baghdad in the Middle East.

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Sutlić in 1942 reported that babesiosis was diagnosed for the first time in Croatia in 1939, and the most recent "first time" report is that of Hill & Bolton (1966) in Queensland on the eastern seaboard of Australia.

The disease is thus clearly of almost world wide distribution in tropical, subtropical and temperate zones. The degree of prevalence is conditioned mostly by the level of tick life i.e. of ticks capable of transmitting the disease. In South Africa it is holoendemic over a considerable part of the country. In the area surrounding Onderstepoort cases are encountered throughout the year with a relatively lower incidence during the colder winter months.

In the United States there is a fairly wide distribution. After Eaton's (1934) first report of its occurrence in Florida others followed: Sanders (1937) also in Florida, Merenda (1939) in a dog that had visited Texas, Grogan (1953) in Virginia, Seibold & Bailey (1957) in Florida, Rokey & Russell (1961) in Arizona, Malherbe (1962) in Pennsylvania, Delaware and New Jersey, Alperin & Bevins (1963) in California, and Buckner, Brock & Ewing (1965) in Oklahoma.

Uncomplicated babesiosis does not appear to cause severe morbidity in the United States and the present author was struck with the apparent degree of wellbeing of infected dogs as compared with the ordinary case seen in South Africa. There seems little doubt on meticulous blood smear examination the disease would be found to be considerably more widespread than is at present known. This could well be true also for other parts of the world where its presence is at present not suspected.

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2.4 SYMPTOMATOLOGY

2.4.1 Typical Symptomatology

The symptoms shown by the majority of cases of canine babesiosis have been described by a number of authors since the early days of its being known as a disease entity. Nocard & Motas in 1902 gave the first full account. They divided the forms observed into acute and chronic. In acute cases dogs were "profoundly" affected, with complete loss of appetite, a high fever (except in very young dogs), intense pallor of the mucous membranes due to blood destruction, frequently haemoglobinuria, and clinical jaundice in about half of them. Weakness was progressive until the animals showed apparent paresis and towards the end became comatose.

Chronic cases showed intense anaemia and only in rare instances haemoglobinuria or icterus. Fever, if present was noted only at the onset and lasted only a few days. They moreover showed emaciation, anorexia, weakness and a scurfy skin. Convalescence was found to take from six weeks to three months, with gradual return of appetite and improvement of mucosal colour.

The presence of haemoglobin in the urine never gave as dark a colour as that of blackwater fever in man and in many cases was evanescent or proceeded with aggravation till death supervened. They further made the point that haemoglobinuria was not necessarily present even in animals that died from the disease.

Theiler in 1904 described some more acute cases in which death supervened in 18 hours to a few days. He also mentioned digestive disturbances and vomition as not infrequent features of the disease.

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Since this time there have been frequent descriptions of the disease, all falling into more or less similar patterns.

The incubation period after natural infection by ticks has generally been given in the range of about 10 to 26 days (Lounsbury, 1901; Christophers, 1907; Nuttall, 1904). Shorter periods, often very few days, have been recorded with artificial infection, varying with the amount and virulence of infective material and the route of administration.

Using the usual course of the disease as the criterion it is convenient to divide cases of babesiosis into four classes:-

(a) Peracute forms. These are most often seen in very young dogs which the owners declare have been perfectly well up to about twelve or less hours previously. Many of these are presented in a state of medical shock with very low blood pressure, stagnation of blood flow, and circulatory failure. In these the limbs are flaccid and the animal virtually in a coma and not infrequently the temperature is well below normal. Severe haemoglobinuria is quite usually seen. The degree of pallor is dependent on the progress of erythrocytic breakdown. They respond poorly to treatment and tend to die in spite of specific therapy unless this is accompanied by energetic supportive measures.

(b) Acute forms. These represent the majority of cases seen in a country like South Africa where the disease is enzootic and the parasites of considerable virulence. Fever is fairly constantly present, with temperatures ranging from 102 - 105°F. Malaise, depression, anorexia, some dyspnoea, disinclination to move, rapidly developing pallor of the mucous membranes, a swaying gait and a full, bounding pulse are usually found. In most cases the spleen is readily palpable and may reach several times its normal size. Less striking but also present, may be a detectable enlargement of the liver.

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Haemoglobinuria may or may not be evident. Icterus supervenes in severe, protracted and neglected cases, and the urine becomes very heavily darkened with bile pigments. Debility is progressive and if the dog lives long enough emaciation becomes extreme.

(c) Chronic forms. These are seen less frequently in South Africa then the acute, perhaps most usually during the winter months when tick life is of comparatively low intensity. These animals show temperatures that may be irregularly elevated, development of severe or slight anaemia, a capricious appetite, and progressive loss of condition.

(d) Atypical forms. These need special mention as babesiosis may be regarded on occasion as an arch-simulator, resembling a wide variety of other diseases. They too are most often seen during the winter months and only occasionally during the height of tick activity. They have been discussed in detail elsewhere (Malherbe & Parkin, 1951; Malherbe, 1956). More recently another account has appeared (Jacquin, 1963) which covers a substantially similar range of clinical aberrations.

The criteria applied to diagnosis by Malherbe & Parkin in their 1951 paper may be reviewed as follows:-

The mere finding of parasites in the blood of a sick dog did not necessarily mean that all the symptoms shown were due to babesiosis. There might be a concurrent disease present, or since immunity in babesiosis is not normally sterile, any acute attack of organic or febrile disease might provoke an exacerbation of a labile B. canis infection, so that the two diseases might exist side by side. Subinoculation of blood into susceptible pups with positive

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results would provide no proof since the pups would usually become infected if the patient were in a state of premunition. In a country like South Africa, where the disease is enzootic, this would usually be the case.

For practical purposes the atypical cases had to be evaluated on the basis of their response to specific therapy for babesiosis. This response had to be reasonably rapid and complete, and the animal had to recover without any supportive treatment aimed at the particular atypical symptoms present. Diagnosis, or at least a presumptive diagnosis of B. canis infection was of course regarded as desirable in all these cases.

In rare instances where specific treatment is applied late, the confirmation by rapid response to specific treatment might fail on account of the development of secondary changes e.g. insufficiency of the liver or kidneys or both, or the presence of concurrent disease such as rickettsiosis (ehrlichiosis). For the purpose of the present study however such cases were not included as they could be regarded as equivocal.

2.4.2 Atypical Symptomatology

Some of the more striking forms recorded by Malherbe & Parkin (1951) and others are the following, given under headings of most obvious system involvements.

2.4.2.1 Respiratory symptoms. These frequently simulated pneumonia in the degree of dyspnoea present but no consolidation could be found on physical examination. Prompt

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response to specific therapy against B. canis was the rule. Anaemia was found in these cases and blood smear examination usually gave positive results. It was concluded that dyspnoea even in the absence of any degree of pulmonary oedema was due to poor oxygenation and myocardial weakness, also of anoxic origin.

Marked catarrhal involvement of the upper respiratory tract, resembling that of distemper, characterized some cases, which however gave either macroscopic or clinico-pathological support for a diagnosis of babesiosis. Response to specific therapy was quite striking. Though these catarrhal symptoms were undoubtedly occasioned by a lowering of body resistance it was clear that the treatment of these cases would be foredoomed to failure if the correct primary aetiology were not recognized.

2.4.2.2 Circulatory symptoms. These were represented by circulatory derangements and oedemas involving the subcutis, accumulations of transudate in the body cavities, and on occasion purpuric lesions. Ascites in young dogs less than one year old, sometimes in slightly older dogs, was in some cases associated with severe subcutaneous oedema of the ventral abdominal region, the skin in this area often giving the impression of transparency. Such animals usually ran normal or subnormal temperatures and parasites were often very rare or not to be found at all. Response to specific therapy was striking. The temperature rose to normal within 24 hours and the ascitic fluid was absorbed progressively with diuresis of urine with low specific gravity. Disappearance of the

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excess fluid was usually complete in seven to twelve days and the specific gravity of the urine returned to normal at the conclusion of the period. The daily drop of weight could be followed up to that point after which the process was reversed as the dog went on to full recovery.

Up to the time of the occurrence of ascites due to hepatic cirrhosis following poisoning with aflatoxin during the past few years, peritoneal transudation was almost invariably found to be due to babesiosis.

A more curious phenomenon noted on a number of occasions was the appearance of asymmetrical localized oedemas of the skin, involving the face, one or both ears, joints, a line along one mandibular ramus, the periorbital region, the lips or the whole head. Disappearance of such lesions within 24-48 hours after specific treatment was the rule.

2.4.2.3 Purpura haemorrhagica was described for the first time by Malherbe & Parkin (1951). Haemorrhages varying from pinpoint petechiae to ecchymotic patches up to three or four cm in diameter (with irregular outline) were described as involving the sclerae, the iris, the buccal or gingival mucous membranes and usually the skin of the abdominal wall on either side of the prepuce and the skin on the inside of the thighs. Some of these cases voided red, whole-blood containing, urine, and on occasion even red coagula. Similar clots have been observed in the faeces, indicating haemorrhage into the posterior bowel, and in the mouth from gingival bleeding.

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Temperature elevation and parasites in blood smears were irregularly observed, and the course was very variable.

Response to specific therapy was very satisfactory, extravasations disappearing progressively over a few days. This was accompanied by a return of appetite and wellbeing.

2.4.2.4 Nervous system. Symptoms referable to this system have been reported on several occasions since an early one by Parant in 1905. Other authors include Cuillé & Darraspen (1927), Purchase (1947), Frean (1950), Reusse (1954), Jacquin (1963) and recently Basson & Pienaar (1965).

These have taken the following forms: various paralyses, inco-ordination of the hind legs, paraesthesias, hysterias, goose-stepping, fits, tics of ears and other parts, peripheral nerve palsies and symptoms involving only single limbs. These cases in their varied nature were thus representative of aberrations of both the central nervous system and the peripheral nerves.

They too responded well to specific therapy in the great majority of cases, although very occasionally irreversible nerve degenerations did not allow of complete clinical recovery.

2.4.2.5 Digestive symptoms. Some degree of constipation is seen in many ordinary cases of babesiosis, probably due to fever and inactivity and can therefore not be regarded as atypical as has been done by some authors.

Parkin (1931) described a very acute case in which ulcerative stomatitis was prominent and in which all symptoms

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disappeared within two or three days of specific treatment. Cases of gastritis with vomiting were described by Belonje in 1944 and these responded equally well.

Another rather different category of cases with gastric symptoms, possibly with diarrhoea, due to secondary renal failure is also encountered and requires supportive treatment over and above specific chemotherapy. This is of course not always successful and depends basically on the number of functional nephrons left.

2.4.2.6 Ocular forms have also been recorded and can be added to similar lesions found in other protozoan diseases such as toxoplasmosis, trypanosomiasis and leishmaniosis of dogs. Lavier & Fombeure (1922) found in a systematic examination of a dog which had died of acute babesiosis that, though the peripheral blood contained few parasites, the capillaries of the retina and ciliary body were "positively bulging" with parasitized cells. Other cases of theirs showed transitory keratitis and iritis and in one there was a large opaque "body" in the aqueous humor of the left eye. In this latter instance the iris and cornea were not affected.

2.4.2.7 Pathogenesis of atypical babesiosis. It is a well-known pathological phenomenon that interference with the blood supply of an organ or any portion of an organ will result in modification or loss of function of that organ or portion. The degree of functional loss would be determined by the degree of disruption or interference and the extent of the area served by the particular blood vessel.

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Knisely and co-workers reported in 1947 on a series of studies of capillary circulation in normal and diseased patients the existence of what they termed the "sludging phenomenon" in diseases of widely varying aetiology. This intravascular agglutination or "sludging" of blood cells in capillaries has also been observed in infections with various protozoa. Fairley (1946) in a text book description of malaria, mentioned "a number of manifestations, classified as acute pernicious malaria, which are mainly dependent on internal sporulation and localized blockage of the capillaries by Plasmodium falciparum in different organs, such as the brain, heart, intestines and spleen by parasitized corpuscles which adhere to one another and to the capillary endothelium". He held this fact responsible for the diverse symptomatology of malignant tertian malaria.

It has recently been suggested (Zuckerman, 1964) that purpuric lesions could on occasion be associated with the phenomenon of auto-immunity. These lesions could result from thrombocytopenia and capillary damage, the platelets and endothelium possibly sharing an antigenic component.

Maegraith (1948) has discussed the situation in malaria in great detail. Neurological manifestations, most notably in P. falciparum malaria, he described as "protean". Signs listed by this author included apoplectic and epileptiform convulsions, frontal lobe syndromes, bulbar paralysis, cerebellar disturbances, meningeal symptoms, polyneuritis, tremors, and a variety of mental disturbances. In his discussion of the pathogenesis he considered it clear that general and local circulatory changes and endothelial damage

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were of fundamental importance in the general development of malaria, and that the local tissue damage was governed essentially by the development of anoxia arising from the generalized anoxaemia, the circulatory changes, alteration of endothelial permeability and possibly from histotoxic effects.

A very recent report on cerebral malaria (Plasmodium falciparum) in South Vietnam by Daroff, Deller, Kastl & Blocker (1967) stated that 19 cases of malaria out of 1200 handled over a ten month period at one hospital had been of the cerebral form. Their bibliography gave a sample of the many investigations of this syndrome during the period since the outbreak of World War II. They stated that almost every neurological sign and symptom had been described in the literature of malaria. Their own cases exemplified five groupings: (1) disturbance of consciousness, up to coma, (2) acute organic mental syndrome with signs of confusion, disorientation and intellectual deterioration (without depression of consciousness), (3) movement disorders such as extremity myoclonus, tremors and chorea, (4) focal neurological signs, and (5) acute personality changes like paranoid psychosis or delusions.

They commented on the still existing disagreement as to the basic pathological changes in cerebral malaria. "Some feel that thrombosis or plugging of intracerebral vessels by pigments, infected, clumped erythrocytes, and endothelial proliferation results in the cerebral dysfunction.

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Others deny actual vessel occlusion and blame the cerebral anoxic changes on stagnation, anaemia, and particularly on the decreased oxygen-carrying capacity of the infected erythrocytes. Ridgon & Fletcher emphasize cerebral oedema as the most constant and earliest change. Perivascular hemorrhages are frequently seen." These authors added that the pathophysiological process, whatever its exact nature, was completely reversible.

In babesiosis it is evident that the tissue changes are qualitatively very similar and are present to a variable extent in all cases, causing deviant symptomatology when more severe or extensive. Anoxic damage to capillary walls leading to increased permeability assists in the process of stagnation and sludging, no doubt aggravating the local and distal anoxia and creating the conditions favourable to oedema development and the occurrence of haemorrhages of various sizes. Generally speaking the changes are reversible as shown by good response to specific treatment but may be serious enough to prejudice recovery of the patient, as is in fact seen in many cases of the cerebral form.

A further and important reason for the development of gross oedemas and ascites in babesiosis is to be sought in the alteration of serum proteins which are quite severely affected in this disease. The parenchymatous damage to the liver, discussed elsewhere in this work, can be held to be responsible for a lowering of the plasma albumin as the result of the loss of synthesis by the liver cells. The plasma colloid osmotic pressure is decreased and the conditions for escape of fluid from the capillaries are thereby

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aggravated. Ascites, formerly regarded as requiring portal hypertension for its development, results readily from hypoalbuminaemia alone in both acute and chronic liver disease. Weir (1947) has proffered important influences in the genesis of ascites as "lowering of serum proteins, disproportionate decrease in the serum colloid osmotic pressure, dietary factors, disturbance of the ability of the liver to handle water, and altered permeability of the capillaries". The ready reversibility of ascites due to babesiosis suggests that these influences are in fact responsible for its development.

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2.5 CHEMOTHERAPY

2.5.1. Trypan blue

The first systematic search for an effective chemotherapeutic agent against B. canis infection was conducted by Nuttall & Hadwen (1909) and they found the dyes trypan blue and trypan red to be effective in curing most cases of the disease. Their previous attempts had shown quinine bihydrochloride, tartar emetic, sodium-methyl-arsenate, methylene blue and beta-naphthylamine to have no curative action, as was the case with arsacetin and soamin. Of the two dyes, trypan blue was found to be the more potent, causing rapid and positive degeneration and disappearance of parasites. They noted, however, the disadvantage of staining of tissues and mucous membranes and the tendency to abscess formation after subcutaneous injection. From their experiments they reported that some dogs relapsed in a benign fashion and went on to recover in spite of persistence of the infection. They believed further as had been noted by Robertson in 1906 that infection in babesiosis persisted in an inapparent form for a considerable time, in fact for years, rendering the blood still infective to susceptible animals. This is commented upon in section 2.7. The specific action of trypan blue was confirmed by Jowett in South Africa in 1909 and by Bumann in Germany in 1910.

Meyer (1912) was not much impressed with trypan red and found that it was toxic, particularly to the kidneys, which were already affected by the infection itself.

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Trypan blue held its own as the only specific therapeutic agent for the greater part of three decades in spite of its manifest disadvantages. These were examined by Carmichael in 1942 and consisted of a marked depressant action on the heart and nervous system involving a sometimes long and careful convalescence. Subcutaneous injection was unsatisfactory since there was a strong tendency for sterile abscesses to form, with necrosis of the skin, and sloughing. This necessitated use of the intravenous route which was found often to be very difficult in very young and collapsed patients. Relapses were of increasingly frequent occurrence and, as previously noted, the staining of tissues, and in addition soiling of the hands of the person treating the animal, made it unpleasant to use.

'Pirobleu', a combination of trypan blue and bile salts, was during this period produced by Sandoz and was initially preferred by Theiler and by workers in North Africa and India. The ultimate judgment however was that its sphere of usefulness corresponded to that of trypan blue itself. (Parkin, 1931).

2.5.2 The Acridines

Domagk & Kikuth reviewed the whole matter in 1930 and gave some new views. They stated that after trypan blue treatment there was no "sterilisatio magna" (in Ehrlich's sense) but that dogs would remain a source of infection after apparently successful treatment. They considered the natural healing processes of the body to have been aided without

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the animals having been freed of the parasites. They blamed this fact for the frequency of relapses which then responded only poorly to further injections of trypan blue, and ascribed this to rapid development of drugfastness.

These workers thereupon evaluated numerous drugs and of the available ones found only an acridine, 'Trypaflavin', to be useful at a dosage of 5 mg per kg. They recommended a single dosage of 15 mg per kg, or 10 mg per kg, administered intravenously on two occasions a week apart, in order to produce "sterilization" of the animals. The dogs were then however again fully susceptible after about 4-6 weeks. They had previously been quite unable to "sterilize" with trypan blue at any dosage. 'Trypaflavin' was better tolerated intramuscularly than trypan blue and was regarded as more effective and a preferable drug. After similar work by a number of authors and with analogous drugs: 'Gonacrine', 'Flavacrine', 'Flavacridine', (generically euflavine B. Vet. C), similar conclusions were arrived at. The final judgment was that it was very effective against babesias and related organisms not very susceptible to trypan blue, was on the whole better tolerated systemically, but had to be given strictly intravenously on account of its irritating and even necrotizing properties (Kikuth, 1935) outside the veins. This author though enthusiastic about its wider spectrum of activity gave as disadvantages the abovementioned tissue irritation, a yellow discoloration of tissues and its relatively higher price.

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2.5.3 Quinuronium sulphate

Kikuth then announced the introduction and successful use of 'Acaprin' (quinuronium sulphate B. Vet. C) which possessed very distinct advantages. Smaller doses could be used more successfully and either subcutaneous or peroral routes were effective. Moreover the problem of staining of tissues did not arise. The dosage in general use came to be 0.25 mg/kg as a 5% solution and in South Africa it became the usual form of treatment. This drug represented a great advance in simplifying treatment and making it more effective, but was not completely free from disadvantages. Injection was sometimes followed by moderately severe parasympathetic stimulation and hypotension manifested by restlessness, muscular spasms, salivation and defaecation.

After some years however relapses became increasingly common on intensive use in particular areas. Clearly the parasites were developing drug resistance which was transmitted to succeeding generations of the parasite.

2.5.4 The Aromatic Diamidines

In 1939 Lourie & Yorke published the first results of their trials with a series of new drugs, the aromatic diamidines, synthesized by May & Baker, Ltd. Ten such preparations were tried out on puppies infected with a virulent strain of B. canis. Almost all of them exerted some influence on the parasite but three showed definitely curative effects. The most active was found to be 4:4 diamidinostilbene (later named 'Stilbamidine') but the

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toxicity level was too near the therapeutic level. Of 16 clinical cases treated by Daubney & Hudson (1941) 13 recovered completely, two died (being too advanced) and one relapsed after receiving too small a dose. More than half of the treated animals showed transient face swelling and two became "asthmatic". Hyperaesthesia and restlessness were further features.

Carmichael & Fiennes (1941) carried out trials on 116 unselected cases with natural infection using 4:4 diamidino-diphenoxy-propane, the second of the three diamidines. Ten dogs relapsed and four died, but the curative effect in the remainder was perfectly satisfactory. They concluded that this drug (later named 'Propamidine') was a good one.

The activity of the third member of the group, 4-4'-diamidino-diphenyl-ether, was investigated by Carmichael (1942). He treated 25 unselected cases of babesiosis with consistently good results, with no toxicity and no relapses. The same author remarked in 1965 that subcutaneous injection of the 'Phenamidine' as it was later named, could prove to be irritating if the solution was too concentrated. (This applies to all the diamidines.) Transitory swelling of face and lips might occur occasionally. The drug is currently marketed as the di-2-hydroxy-ethane-sulphonate and given the trivial name of phenamidine isethionate.

The officially recommended dosage (Br. Vet. Codex, 1953) is 0.3 ml of the 5 per cent solution, w/v, per kg.

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At Onderstepoort, where clinical trials were also carried out at the same time as the East African workers, a dosage of 0.4 ml was decided upon, and has been used for more than 25 years, still, at the present time, with very good results. Apart from the higher dosage it is a regular practice at the Onderstepoort Outpatient Clinic to weigh all dogs accurately at the time of registration, before computing dosage.

In a number of areas in South Africa a great falling off of efficacy of the drug has been reported with relapses as a troublesome occurrence. This can almost without doubt be ascribed to erroneous guesses of weight and the use of the lower dosage rate. It has been shown by Fulton & Yorke (1941) that a single simple underdosage with 'Stilbamidine' produced drug resistance that persisted for 42 generations through dogs and puppies, over 28 months of passage. Not only had this strain become resistant to 'Stilbamidine' but it was also shown that the related drug 'Propamidine' had lost its effectiveness. What was even more striking was that this parasite had also acquired resistance to quinuronium sulphate, an unrelated drug. Quite conceivably this process could be repeated in any intensively treated holoendemic area to the point that the drug becomes much less useful as a result of the frequency of relapses.

The experience over many years at Onderstepoort has shown 'Phenamidine' not to be devoid of some small degree of

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toxicity. Occasional allergy-type reactions have occurred, but seldom of any serious import. They usually respond satisfactorily to antihistamine treatment. What happens more often is a hypotensive episode after treatment, with vomiting. As a rule this is not serious unless a dog is already in a medically shocked condition at the time of presentation. It becomes necessary in these cases to give supportive treatment such as hypertensive agents, blood transfusions and possibly resorting to the use of trypan blue which for a number of years has been kept as a reserve drug for such occasions.

2.5.5 'Berenil'

In 1955 Farbwerke Hoechst A.G. introduced a different formulation of a diamidine, viz. 4-4'-diamidino-diazoaminobenzene-diaceturate under the trade name of 'Berenil' for the treatment of babesiosis and trypanosomiasis. This has proved effective for B. canis infection at a dosage rate of 3.5 to 4 mg per kg (Botha, 1964 and Bauer, 1967) and has been used to good effect in areas where resistance to 'Phenamidine' has developed. The toxic effects are qualitatively similar to the drugs described earlier. Occasional vomiting after injection, necrosis and abscessation at the site of subcutaneous injection, and relapses were reported by Botha (1964). He experienced results better than with 'Phenamidine' in his practice area where considerable drug resistance to the latter drug was already evident. Several reports have however,

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been received from practitioners and encountered in the literature of encephalitis-like brain symptoms after 'Berenil' treatment, notably after repeated treatments, or after dosage of over 10 mg per kg (Enigk & Reusse, 1955; Bauer, 1967). The occurrence of drug resistence has not yet been reported and relapses are said to have responded well to increased dosage.

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2.6 PATHOLOGY

2.6.1 MACROSCOPIC PATHOLOGY

There is a considerable range of possibilities in connection with the gross appearance of the cadaver and the organs of a dog that has died from babesiosis. This will be readily evident from the diversity of symptomatology described in section 2.4. The changes depend largely on the degree of acuteness and duration of the disease. Dogs may die from the peracute form where there is no loss of condition, a variable degree of anaemia, splanchnic pooling of blood, and the presence or absence of severe haemoglobinuria. The cause of death is medical shock.

Acute and more chronic cases show a variable picture. Some or all of the following lesions may be observed: irregular loss of condition, varying degree of anaemia development, icterus, slight oedema of subcutaneous and intramuscular tissues, hydrothorax, hydropericard, ascites, oedema of the lungs, at times bronchopneumonia, subepi- and subendocardial petechiae, petechiae on the spleen, tumor hepatis with colour from pale yellow to mahogany brown, hyperaemia of kidneys affecting particularly the medulla and more or less bilirubinuria or haemoglobinuria. Enlargement of the spleen, sometimes grossly so, with tight smooth capsule and well rounded borders is a usual finding in the majority of cases.

In older dogs and in neglected, more chronic cases, the autopsy, apart from anaemia, icterus and congested internal

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organs may well show the typical picture of some organic failure. It may be one of cardiac, pulmonary, or renal failure. An animal may have died from uraemia after specific babesiacidal treatment - with gastritis, possibly enteritis, stomatitis and grossly pathological kidneys. The degree of icterus as will be seen from the chapter on liver function testing is mainly dependent on the degree of liver damage.

There are many accounts of the macroscopic morbid anatomy including those of Nocard & Motas (1902), Graham-Smith (1905), Christophers (1907), Knuth & du Toit (1921), Maegraith, Gilles & Devakul (1957) and Basson & Pienaar (1965). With small variations here and there, their observations fall into the same framework, and many authors remark on the wide range of variation.

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2.6.2 MICROSCOPIC PATHOLOGY

An early description of histological findings in babesiosis was that of Graham-Smith (1905). He noted that as a general rule capillaries tended to contain many more infected erythrocytes than did the larger vessels. He found this particularly noticeable in the lungs. The heart showed good myocardial striation, dilated capillaries and small haemorrhages with many parasites in the capillaries. In the liver the central vein and sinusoids were extremely dilated, the parenchymal cells near the central vein distorted or destroyed, and the portal vessels dilated. There were many leucocytes and red cells in the sinusoids. Splenic enlargement he found to be due to a massive amount of blood in the pulpa, and the trabecular vessels were dilated with many leucocytes. In the kidneys he found blood vessels generally dilated and the capillaries loaded with infected red cells. Similar changes were found in the adrenals, the pancreas, the brain and spinal cord, intestines and lymph glands. He found a varying number of infected red cells in the bone marrow.

Phagocytosis of erythrocytes has frequently been commented upon, inter alia by Graham-Smith, 1905; Neitz, 1938; and Maegraith, Gilles & Devakul, 1957). This feature is evident according to Maegraith et al. in the peripheral blood and in many organs, particularly the spleen and liver. They have found this to involve the large mononuclears and on occasion the neutrophils, and that they engulf either

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parasitized or unparasitized erythrocytes, or both, apparently indiscriminately. This very active process in the spleen is seen to take place mostly in the large mononuclear histiocytes of the pulpa, quite remarkably resembling the picture of self-limiting infections of malaria, such as that caused by P. brazilianum. In this infection the phagocytosis is an evident part of the successful or immune response of the infected animal. This in all likelihood applies equally to babesiosis.

In the liver phagocytosis occurs in the Kupffer cells which frequently contain parasitized or unparasitized red blood cells as well as free parasites. As in malaria, the Kupffer cells are often swollen and may bulge into the lumen of the sinusoids or lie free and fully laden in these vessels.

Maegraith et al. remark further that the ingestion of parasitized and uninfected red cells, as in malaria, bears the same irregular relation to the degree of erythrocyte infection.

They describe phagocytosis in babesiosis as "avid" but feel that the process has not been fully elucidated. Red cells do appear to become "sticky" to each other and to reticuloendothelial cells, whether as a result of changes of surface electrical charges, precipitation of "fibrin" crystals, opsonization or of other mechanisms. As in malaria this still requires clarification. It does appear to be associated with the presence of immune bodies.

These authors found that whether livers were normal in size or enlarged there were definite histological changes

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featuring congestion and dilatation of the sinusoids and central veins. There were varying degrees of fatty changes in parenchymal cells, even necrosis, which were essentially centrilobular in distribution. The sinusoids tended to be widely dilated and filled with erythrocytes regardless of the degree of anaemia. They found no thrombosis, although engorged macrophages, fixed and loose, were usually evident. Destruction of the hepatic lobule was sometimes gross, extending in particular lobules from the central vein outwards. In extreme cases only a thin stratum of relatively normal-looking peripheral cells remained. Haemosiderin granules were often to be seen both in Kupffer and hepatic cells.

In the kidneys, the medulla was often congested and the cortex somewhat blanched. Most constantly they found a great prominence of vessels in the cortico-medullary junction. Both free and intracellular parasites were present in all the small vessels. The glomeruli were not actively changed but they were occasionally shrunken and the Bowman's capsule contained debris and coagulated material. Tubular epithelia showed various degrees of degeneration often with granules of haemoglobin or its derivatives. Inside the tubules casts of homogeneous material, with debris and sometimes red cells, were to be found.

The spleen was grossly congested, with much evidence of phagocytosis of both parasitized and unparasitized red cells. Lymphoid tissue generally appeared to be reduced in amount and blood vessels were congested and packed with red cells however anaemic the animal happened to be. The whole picture

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closely resembled that produced by fatal malaria due to P. falciparum in man.

The heart was found much less affected than in human malaria. Striation was well preserved but capillaries between the muscle bundles were dilated and congested, often containing infected erythrocytes and free parasites. They saw no thrombosis and no haemorrhages and apparently no specific concentrations or plugs of parasites or parasitized red cells as found in other organs.

In the adrenals they found no very pronounced lesions apart from congestion, usually of the medullary vessels but on occasion also of the cortical vessels. They did not observe any plugging or haemorrhages.

The lung picture was particularly variable, depending on the clinical type of involvement. It was sometimes quite unaltered but for the rest the picture varied from slight hyperaemia to massive pulmonary oedema, the latter usually in cases of shock. Septal vessels were usually dilated.

Cerebral involvement in malaria already has a very large amount of literature devoted to it. Maegraith reviewed the subject in his monograph in 1948, listing many references up to that date, and Daroff, Deller, Kastl & Blocker (1967) have given a large list of some of the more recent ones, together with their own findings which are discussed in Section 2.4.2.7.

Cerebral babesiosis has been reviewed by Malherbe & Parkin (1951), Jacquin (1963) and by Basson & Pienaar (1965).

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Maegraith, Gilles & Devakul (1957) did not observe the cerebral form amongst their experimental cases but found parasites in considerable numbers in brain vessels and a "layering" of parasitized cells along the endothelium of blood vessels in ordinary cases of babesiosis.

Basson & Pienaar described great congestion of blood vessels in the brain and haemorrhages involving large parts of the cortex and in the region of the caudate and dentate nuclei. They gave the histological features in great detail and described inter alia numerous closely grouped perivascular haemorrhages from small vessels and capillaries, accumulations of eosinophilic substance in the Virchow-Robins spaces and generalized oedema of the entire brain and meninges. Most of the capillaries and small veins throughout the brain were "tightly packed" with parasitized red cells and free parasites. Some larger vessels showed "definite pavementing" of the endothelium with parasitized red cells.

Neurones in the haemorrhagic and lytic areas of the brain "exhibited what is frequently regarded as anoxic changes viz. shrinkage, pyknosis, cytoplasmic basophilia and hyperchromasia. Necrosis of the cytons and glial cells was also present".

The histopathology has been given in some detail as it sheds very considerable light on the spectrum of symptomatology and the reason for many of the biochemical changes found in this disease.

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2.7 IMMUNITY

Before the advent of specific chemotherapy by means of trypan blue (Nuttall & Hadwen, 1909) several attempts were made to immunize dogs against babesiosis. Nocard & Motas (1902) reported on experiments which suggested that immune substances were developed in the serum of recovered dogs. Robertson (1906), working with a probably more virulent strain in South Africa, did not confirm the French workers' results and found that "recovered" serum did not give any protection at all. He found in fact that the blood of recovered dogs was capable of conferring the usual virulent disease upon subinoculation. In a different approach, Nuttall & Graham-Smith (1909) attempted to confer immunity by injecting successive doses of blood containing dead parasites (either dried or treated with phenol). Their results were discouraging and further attempts were abandoned as unnecessary in view of the availability of specific treatment.

Recent knowledge of the situation has been summarized by Neitz (1956). It is clear that complete or absolute immunity does not occur in babesiosis. Recovery from the disease does however confer a fairly strong resistance or tolerance to a subsequent infection with the same immunological strain. Exposure to other strains may be followed by relapse of variable severity, indicating anything from some protection to none at all.

Immunity in babesiosis as in many other protozoal infections is maintained by premunition, labile infection, latent infection or <u>immunitas non sterilisans</u>. The animal

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retains resistance to the same strain at least as long as a residual infection remains which in most cases would be for years. In enzootic areas this labile infection becomes reinforced periodically by exposure to the vector ticks so that in South Africa the majority of dogs suffer from only one overt infection in their life-time. The premune state may however be broken down (with renewed overt disease) by other diseases caused by bacteria, rickettsiae, protozoa, viruses, massive helminth infestations, by severe traumatism or by organic disease such as nephritis or pneumonia (Malherbe, 1950). Haemobartonellosis could in all likelihood be added to this list. "Immunity" in babesiosis can moreover with great regularity be broken down by splenectomy, with reappearance of parasites in the peripheral circulation. Neitz (1956) explains premunition on the generally accepted view that the reticuloendothelial system participates in the battle of protection by phagocytosis, and thus that parasitic multiplication is limited.

It follows then that a process of immunization by injection of infected blood followed by treatment with a specific drug on the appearance of parasites would be quite feasible in an enzootic area.

Evidence has now been presented (Bauer, 1966) that after the termination of the period of premunition, which is known to occur in the absence of continued exposure to infection, a period of sterile humoral immunity follows. It appears that these periods are quite variable, depending on the duration and extent of active infection, probably strain differences,

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and most likely also on the degree of "natural" immunity and the general health of the individual. In a holoendemic area, such as large parts of South Africa, it is probable that most dogs after the initial infection remain in a stage of premunition for their entire lives as a result of repeated reinforcement of the latent infection. If they are removed to an area where they are not subjected to further infection they would continue their premunition for a variable period, followed by a variable period of humoral immunity after which they would again become fully susceptible to infection. Immunity in the general sense could be regarded as essentially humoral produced and maintained by the presence of parasites, but can survive the parasites for a variable period, after which it may disappear.

Premunition on the one hand provides in an enzootic or holoendemic area an excellent protection against new morbidity for the individual animal but on the other maintains an excellent source of infection to the tick vectors.

Bauer (1966) working with splenectomized dogs found that after infection and treatment their blood remained infective to other dogs for a period. Later, they were no longer infective and yet could not be infected. Ultimately they again became fully susceptible. Schindler, Wokatsch & Schröder reported on similar findings in the same year. They were able to demonstrate a stage after premunition when dogs were immune and without infection as they were unable to infect susceptible animals by subinoculation. After a while they

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again became fully susceptible.

In the case of malaria the situation seems to be very similar. Maegraith in his 1948 monograph examined all the evidence available till that time. He felt that an immunity based solely on premunition, though advanced by some authors, lacked experimental proof, and was much more inclined to believe that a genuine humoral immunity could survive overt and latent infection. Evidence further showed that an infection treated promptly led to very short immunity whereas untreated patients or patients not treated for a year developed a very solid immunity. Good immunity was also produced by repeated infections.

The suggested course of events was as follows: The immediate reaction of the body to malarial invasion is the non-specific destruction of parasites brought about mainly by phagocytosis, the degree and success of which depend on the natural resistance of the host. The cells chiefly concerned with phagocytosis are the ordinary reticuloendothelial macrophages of the spleen and liver and to some extent of the bone marrow. The phagocytes of other organs are relatively unimportant. Once the disease becomes established, natural resistance becomes reinforced by the development of acquired immunity which gives rise to a specific increase in phagocytosis, mainly in the spleen and to a lesser degree in the liver. These cells proliferate and show greatly enhanced avidity for red cells, parasitized red cells, parasites in all stages of development, pigment and cellular debris. It is further suggested that at the early stage of the

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infection the phagocytosis associated with the natural immunity of the host is non-specific. When acquired immunity appears the phagocytes although presumably still retaining their normal function as scavengers for other material, become specifically active against any red cells, parasitized red cells and parasites.

He quotes work (by Brown & Broom, 1935) to show that in the presence of immune bodies and associated changes in the plasma protein there was an equal reduction of electrical charge at the surface of both parasitized and unparasitized cells, with the effect that these become much more susceptible to phagocytosis. Knisely is quoted as observing the precipitation of "crystals" around and about erythrocytes in the circulation of monkeys with P. knowlesi infection, and reporting that this made erythrocytes 'sticky' towards phagocytes which readily ingested them. He also mentions the extensive work of Taliaferro and his group during the 1930's and early 1940's. They found that erythrocytes from an infected animal were phagocytosed slowly when injected into a normal animal, but were ingested extremely rapidly in an animal with acquired immunity. They postulated the existence in immune animals of some humoral agent equivalent to the opsonins of bacterial infections.

The whole question of immunity to protozoa has been exhaustively examined in a symposium of the British Society for Immunology (Garnham, Pierce & Roitt, 1963), notably in the contributions of Bruce-Chwatt, Cohen & McGregor, and Zuckerman. In the same symposium Riek discussed immunity

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to babesiosis on the basis of his work on bovine babesioses and concluded the basis of immunity in non-bovine hosts was likely to prove similar to that obtaining in cattle.

The criteria for complete absence of parasites in an immune animal were examined critically by Joyner & Davies in 1967, working with B. divergens in calves, some splenectomized. In all their experiments the intravenous inoculation of 100 ml of blood had rarely failed to give positive results when blood had been taken during the initial two years following infection. Since on one occasion only a negative test was followed by positive results (which continued for more than two years), they concluded that a single test was not completely reliable. For this reason three consecutive negative tests were adopted as their criterion of the loss of infectivity. Even by such strict testing they concluded that their experiments had appeared to support the existence of sterile immunity.

In summary, the evidence in respect of malaria and other protozoal diseases, and of Babesia in general and B. canis in particular, supports the concept now generally held that immune processes in protozoa and in bacteria are fundamentally similar, to extend a conclusion by McGregor (1964) to protozoan diseases other than malaria. The premunition of protozoa would serve effectively to reinforce the humoral immune processes.

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3. THE CAUSAL ORGANISM

3.1 CLASSIFICATION

A number of attempts have been made to classify the piroplasms parasitic in domestic animals. According to Neitz (1956) a scheme was submitted by França in 1910 but the first systematic study was presented by du Toit in 1918. Modifications were presented by Wenyon in 1926, Donatien & Lestoquard in 1930, Yakimoff in 1931, Neitz & Steyn in 1947, and the whole subject was again reviewed by Neitz in 1956 (who cited the above references).

Difficulty arose in connection with Babesia canis on account of varying pathogenicity, absence of cross-immunity between similar parasites from different countries and variations in vector specificity. Reichenow (1935) held the view that several different species occurred in nature. Enigk (1944) however, disagreed with subdivision on the basis of vector specificity, and the factors contributing to the inability of certain ticks to transmit B. canis are still unknown.

Neitz & Steyn (1947) and Neitz (1956) then proposed a classification which placed the genus Babesia Starcovici, 1893, in the suborder Piroplasmidea Wenyon, 1926, and the family Babesidae Poche, 1913. The name Babesia canis included the following older names: Pyrosoma bigeminum var. canis Piana & Galli-Valerio, 1895; Piroplasma canis (Piana & Galli-Valerio, 1895); Babesia rossi Nuttall, 1910; and Rossiella rossi Nuttall, 1912.

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It now appears possible (Neitz, 1965) that in future serological tests may prove valuable in the determination of the identity of Babesia species. Work with complement fixation tests may for instance prove to be useful, as antibody formation in the case of B. canis has already been demonstrated by Schindler & Dennig (1962).

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3.2 MORPHOLOGY AND DIAGNOSIS

3.2.1 MORPHOLOGY

The morphology of Babesia canis excited great interest from the time that Piana & Galli-Valerio (1895) named the organism Pyrosoma on account of the pyriform shape of many of these parasites. Nearly every author in the first decade of this century devoted much attention and much space to painstaking descriptions of the organism. A good deal has been made of its amoeboid character, which would explain its diversity of shape as seen in fixed and stained blood films. Authors giving such descriptions included Nuttall (1904), Nuttall & Graham-Smith (1905, 1906, 1907, 1909) and Christophers (1907). Later Regendanz & Reichenow (1932) and Shortt (1936) also made their contributions to morphology.

The organism is fairly large, measuring anything from about 1 to 5 microns, largely depending on how many there are in an erythrocyte. Most of them are found inside red cells, but in heavy infections some are found between the cells. In shape they are in their most typical or characteristic form seen as double pearshaped (two in a cell). The rounded ends are free and the pointed ends in contact with or in the vicinity of each other. With Giemsa or Wright's stain they are blue at least in outline and may show a dot of chromatin inside, representing a nucleus. The amount of internal detail depends much on how efficient the staining has been. In ordinary routine staining the outline may be well demarcated in blue with no detail in the interior. This does not

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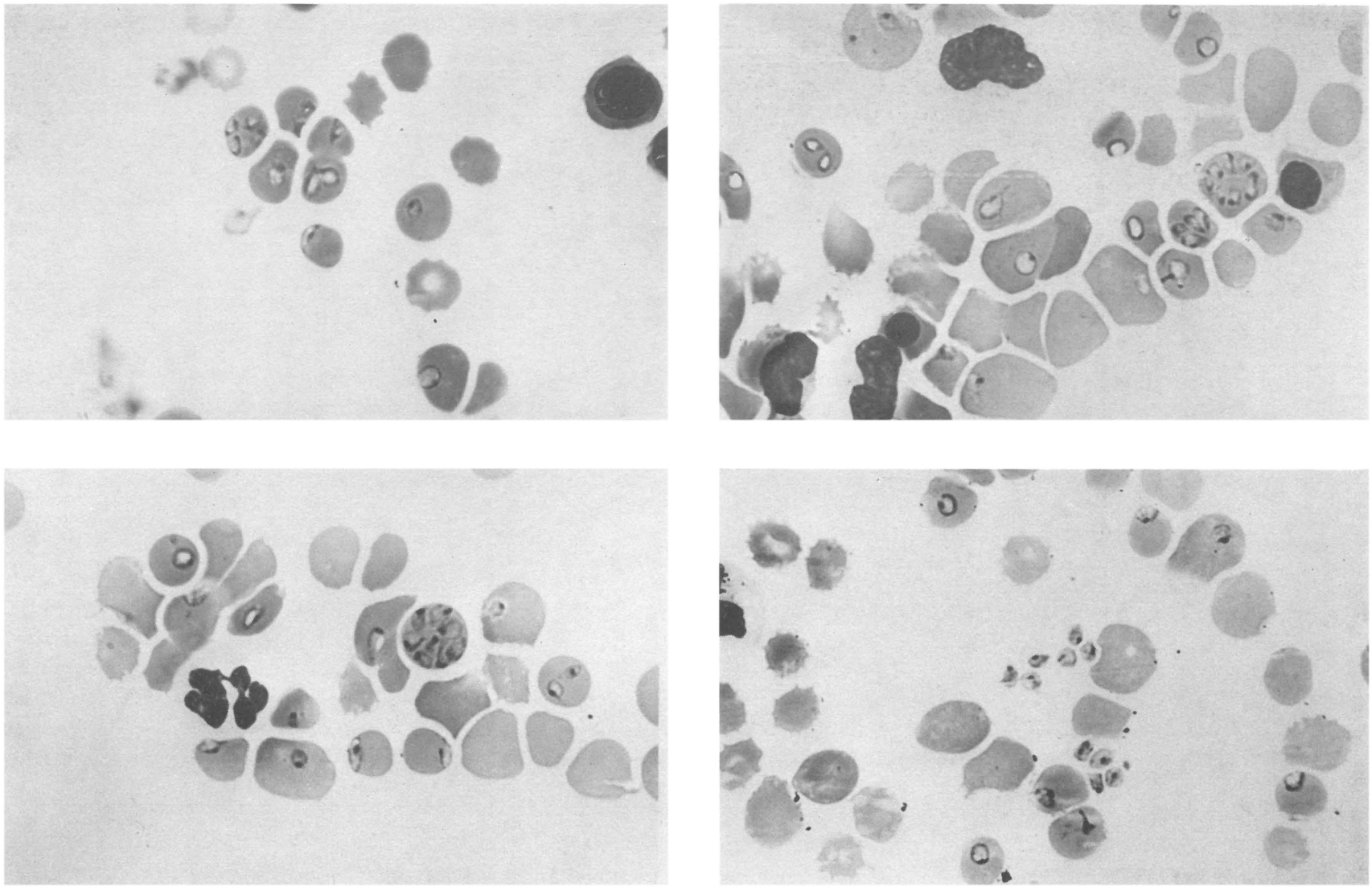
PLATE 3.2.1. PHOTOMICROGRAPHS FROM A SMEAR OF A YOUNG DOG WITH ACUTE BABESIOSIS. THE VARIATION IN SHAPES AND DISTRIBUTION OF PARASITES SHOULD BE NOTED AS WELL AS THE INCIDENCE OF ERYTHROBLASTS.



however, make them less easily recognizable.

There are many deviations from the pear-shape. Frequently they are well rounded or oval in outline or may appear in irregular amoeboid shape or as a narrow band stretching right across the cell. Mixed shapes are commonly seen. See Plate 3.2.1.

Multiplication is essentially by binary fission of these trophozoites in the red cells so that when there are more than one they occur most typically as exponential multiples of two, thus: 2, 4, 8, 16 and possibly 32. This aspect has been examined by Ewing (1965b) who concluded that in view of this fact budding or multiple fission could be ruled out. Where occasionally he found odd numbers there was usually one parasite obviously on the verge of binary fission. It would thus be wrong to regard the incidence of multiple parasites in a single cell to be the result of schizogony. The very occasional form that looks like budding is probably nothing more than a parasite fixed during amoeboid movement.

Occasionally phagocytosis of parasitized cells in blood macrophages may be seen, with parasites either well preserved or in a process of disintegration. Under these conditions unparasitized red cells may also be found in monocytes.

B. canis has been studied by electron microscopy by Bayer & Dennig (1961) and Simpson, Bild & Stoliker (1963). The original papers should be consulted for ultramicroscopical detail. Simpson et al. found that the parasites were confined by a definite cytoplasmic membrane and generally contained a

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single prominent nucleus bounded by a double membrane. The cytoplasm contained an extensively developed network of endoplasmic reticulum, small dense granules, organelles lined by a double membrane, and a rare food vacuole.

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3.2.2 DIAGNOSIS

Apart from the clinical features of the average or typical case of babesiosis, a definitive diagnosis is usually made from the examination of a thin smear made from capillary blood. This is obtained from the patient's ear in the following manner and observing the precautions as indicated.

(a) Use two slides that must be scrupulously clean, particularly of fatty material (finger prints).

(b) Clip the hair short on an edge of the ear and rub off any scurfiness. No moisture may be applied.

(c) With curved scissors roughly parallel with the ear margin (but with opposing curvatures) cut off a layer of epidermis. This is facilitated by doubling the ear at this point.

(d) Express one small drop of blood and touch it with a short edge of one slide (without any scraping).

(e) With the other slide on the table the first (with the blood) is placed on it with the top slide at an angle of about 45°, allowing the blood to spread sideways in the angle between the slides.

(f) Quite deliberately and without hurry the top slide is pushed away from its acute angle (which is maintained) allowing the blood to follow it until exhausted.

(g) This should result in a tongue shaped smear about $\frac{1}{2}$ in wide and $\frac{3}{4}$ - 1 in long. The whole operation should be done deliberately but unhurriedly,

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not allowing the blood to clot.

(h) The slide with the smear should be air dried as quickly as possible by waving, and can be fixed and stained immediately or at leisure.

In our experience this gives one the best chance of finding parasites when the level of parasitaemia is very low. The majority of parasitized cells seem to congregate along the edges of the "tongue" and in the serrated "tail" or tip of the tongue.

Ewing (1966) reported on a comparative study in which parasites were counted in the same animals using venous blood and capillary blood. He concluded that venous blood would reveal more B. canis trophozoites than would capillary blood but that if 100,000 or more erythrocytes were examined there was not any significant difference. His method for obtaining capillary blood was however by needle prick on the ventral surface of the ear. This was quite different from the method outlined above and it is submitted that the chances of collecting blood from a number of cut capillaries would be considerably better than by Ewing's needle prick method (which would sharply limit the number of capillaries drained). The latter technique would involve a greater "dilution" of capillary by venous blood to get sufficient material for a smear. In view of the virtually unanimous histological findings of parasite concentrations in capillaries it would be surprising if venous blood would contain as many or more.

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Ewing quotes several references to the use of bone-marrow smears but agrees that blood smear examination is preferable. He also mentions the use of thick films such as advocated by Mahoney & Saal (1961) in Australia for detection of B. bigemina and B. argentina. This procedure has not found favour at Onderstepoort. The writer has also not been impressed by thick smears for B. canis. While it conceded that a higher concentration of organisms can be examined, staining may be difficult and parasites distorted and liable to be confused with other cellular debris. They would also not be splayed out as in thin smears.

Winter (1967) re-examined fluorescence microscopy for the diagnosis of B. bigemina and B. argentina and concluded that it provided significant advantages over Giemsa, provided parasitaemia was not of too low an order. He certainly preferred it to the use of thick films. Ewing (1966) however felt that fluorescent-antibody techniques while giving good results could not be used "under ordinary, routine, diagnostic circumstances".

Complement fixation techniques have their usefulness largely limited to survey work and the laboratory. They are not likely ever to play any significant role in routine diagnosis. Mahoney (1964), investigating the value of complement fixation in B. bigemina and B. argentina concluded that it "should find a useful application in epidemiological and control work in the field" and could be accepted "as proof

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of current babesial infection even though the parasites in the blood were "too few for detection in thick films". He did not find that negative results could reliably be interpreted as proof of absence of infection.

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3.3 LIFE CYCLE

There have been a considerable number of studies of the life cycle of this organism as well as of the other babesias. Binary fission within the erythrocytes of the vertebrate hosts had been observed generally but the question of a sexual phase in the vector tick evoked great interest. Christophers (1907) described his observations of the development of B. canis in Rhipicephalus sanguineus and though using terminology indicating his belief in a sexual type of development ("gametogony") he concluded that he had not found proof of the absence of a sexual phase. Regendanz & Reichenow (1932) and Shortt (1936) in later studies of the parasite in Dermacentor reticulatus and R. sanguineus respectively, failed to find evidence of sexual forms and thus to confirm Christophers' observations. They found in the ticks that after many binary fissions in the intestinal epithelial cells they gave rise to a motile vermiform stage which migrated into the haemocoele and finally invaded the ovary and ova.

Controversy however continued, and other species of Babesia were studied by many workers. Neitz (1965) after considering all these studies felt that they had not produced convincing evidence as to whether or not a sexual phase occurred in the acarine vectors. The question thus remains an open one.

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3.4 TRANSMISSION

B. canis is transmitted naturally by various ticks in different countries. The association between severe illness and shortly previous exposure to massive tick infestation was appreciated well before the aetiology of the disease was clarified. Lounsbury (1901) gave the first report on experimental work in connection with the transmission of the parasite by *Haemaphysalis leachi* (Audouin) in South Africa. He showed transmission from the imago stage, when the infection was acquired, through the egg, larval and nymphal stages to the next imago stage. Brumpt (1938) much later showed that infection could be carried also from nymph to adult in the same generation.

The ubiquitous dog kennel tick, *Rhipicephalus sanguineus* (Latreille) is considered by Neitz (1956) to be the chief transmitter of the disease. Reports on the rôle of this vector have emanated from various places in Africa, North and South America, Asia and Europe. The most recent, albeit circumstantial, report has come from Australia, where Hill & Bolton (1966) have diagnosed the disease for the first time on that continent. This tick is very much more widespread over much of the world so that the potentiality of wider distribution of babesiosis does exist.

Brumpt (1919) in France and Neitz (1956) in South Africa demonstrated transovarian transmission in *R. sanguineus* from an imago of one generation to the imago of the daughter generation. The former author observed that infection could

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be retained through five generations of this species of tick maintained in the interim exclusively on hedgehogs, and that dogs could still be infected.

Christophers (1907) reported in India on both imago to nymph and imago to imago transmission through the egg stage while Shortt (1936) in the same country observed larva to nymph and nymph to adult transmission in the same generation. Reichenow in Germany (1935) recorded infections in dogs in all three stages from the imago of the previous generation.

Neitz (1965) summarized the proved vectors in a table which is reproduced in Table 3.4

Experimentally, the most usual mode of transmission is by subinoculation of blood from an infected or premune animal to a susceptible one by intravenous, subcutaneous or intraperitoneal routes.

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Table 3.4 BIOLOGICAL TRANSMISSION OF BABESIA CANIS BY MEANS OF TICKS (NEITZ, 1956)

Vectors	Country	Hosts	Larva	Nymph	Imago	Egg	Larva	Nymph	Imago	References
Dermacentor marginatus Sülzer = D. reticulatus Fabricius	Europe	3			X ———)				)	Brumpt (1919a)
					X ———)				)	Belitzer & Markoff (1930)
					X ———)			———)	———)	Regendanz & Reichenow (1932)
Dermacentor pictus Herm.	Russia France	3		X ———)						Enigk (1944)
Dermacentor venustus Banks = D. andersoni Stiles	France	3			X ———)				)	Brumpt & Larousse (1922)
Haemophysalis leachi (Andovin)	South Africa	3			X ———)				)	Lounsbury (1901)
				X ———)						Brumpt (1938)
Hyalomma marginatum Koch	Russia	3	X ———)						)	Enigk (1944)
Rhipicephalus sanguineus (Latreille)	India	3			X ———)			———)	———)	Christophers (1907)
			X ———)							Shortt (1936)
				X ———)						Reichenow (1935)
	Germany				X ———)		———)	———)	———)	Brumpt (1919b)
	France				X ———)				)	Neitz (1956)
	South Africa				X ———)				)	Steinhaus (1947)
	U.S.A.									Regendanz & Muniz (1936)
	Brazil									

X Stage at which infection is acquired.

) Stage at which infection is transmitted

3.5 CULTIVATION

There was great interest in the possibility of cultivation of B. canis and other piroplasms during the early years of this century. Deseler in 1910 reviewed the attempts made up to that time and included those of Ligniéres (1900), Dschunkowsky & Luhs (1904), Kleine (1906) and Nuttall & Graham-Smith (1908). Some of them believed that they had achieved some multiplication of parasites. Deseler, however repeated their work using media of similar composition and concluded from his attempts that he had not observed any increase of parasites in his cultures and that in fact there was a gradual decrease in number, with evidence of progressive degeneration.

Further attempts by Ziemann (1913), Knuth & Richters (1913), Toyoda (1913), Thompson & Fantham (1913) and Ziemann (1914) made it clear that the substrates were not suitable for survival and multiplication for any length of time and there was subsequently a loss of interest in cultivation.

In malaria there have been attempts over a much longer period since the discovery of the parasite in 1880 by Laveran (Polet, 1966). Investigations before 1943 were reviewed by Geiman, Anfinsen, McKee, Ormsbee & Ball (1946). Results obtained were similar to those for babesiosis. Significant progress was however made after this particularly since the World War II malaria programme, and new information became available about the growth requirements of plasmodia. Interest was stimulated anew in fundamental malarial research

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by drug resistance developing in certain strains of the organism and by the persistence of malaria transmission in socalled "problem areas" in spite of eradication procedures with drugs and residual insecticides. P. falciparum has shown itself as highly virulent in South East Asia and resistant to modern synthetic antimalarial drugs, resulting in reversion to quinine treatment. (Geiman, Siddiqui & Schnell, 1966). Success in cultivation of parasites by these workers, by Polet (1966), by Trager (1966), by Geiman et al. (1966) and others, has been only partial. The latter authors quote a 1948 statement by Geiman: "unlimited prolonged cultivation of mammalian plasmodia awaits better methods for the maintenance of the integrity of erythrocytes in vitro, the elaboration of specific biochemical and metabolic properties of plasmodia, the identification of unknown growth-promoting substances in plasma or the ability to provide an intracellular medium which will permit growth and multiplication free of the red cell". They feel that there have been stepwise advances and that chances are improving for a successful outcome. It is tempting to think that ultimately similar methods would also be effective for B. canis and that a suitable substrate will be found.

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3.6 PATHOGENICITY

3.6.1 PATHOGENICITY IN DOGS

An interesting phenomenon encountered in B. canis infection is that, unlike the Babesia infections in most other domestic animals, puppies up to the age of about three months are much more susceptible to a severe form of the disease and die much more readily from its effects than do older dogs. At the other end of their life span there is again a decreased tolerance when failing kidneys and other organs render the animal particularly prone to die from the secondary effects of the disease, even when specific treatment has eliminated the parasites. In an enzootic area this latter fact is not of great moment since practically all dogs acquire premunition much earlier in life.

A number of early authors have remarked on the comparatively high susceptibility of pups. These include Nocard & Motas (1902), Galli-Valerio (1904), Christophers (1907), Breinl & Annett (1909), and Donatien (1927). The only deviate opinion encountered was the text of Hagan & Bruner (1951), in which young pups were held to be relatively susceptible.

Neitz (1956) commenting on this phenomenon stated that a satisfactory explanation had not yet been given. He believed that the only anatomical and physiological difference was that in puppies involution of the thymus commenced within a few days after birth while in other species this only happened after several months. While this seemed likely it still lacked conclusive proof.

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Several workers have utilized passage through puppies in order to enhance the virulence of B. canis, notably Knuth & Richters (1913) and Maegraith, Gilles & Devakul (1957), who wished to use the more virulent disease for experimental work. Henning (1956) has suggested that one should regard the tick not merely as an intermediary but actually as the natural host of the protozoon, the canine host being merely a means of disseminating it. This is a nice point, but it could be argued that the more favourable milieu in the young puppy would tend to enhance the virulence of the organism.

A further consideration is the variation in virulence of B. canis found in different localities. In the perusal of literature of the first decade of this century one may observe the different results obtained by French workers as against the Cambridge group working with a locally maintained South African strain, in substantially similar experiments.

Babesiosis was encountered by Malherbe (1962) in the Eastern United States, notably in Pennsylvania, New Jersey and Delaware, in locally bred and kept dogs. In these cases their apparently good habitus was in striking contrast with the dejected habitus in South Africa, except when there was at the same time some other infection or some organic disease.

A statement sometimes made to the effect that imported and purebred dogs are more susceptible than indigenous dogs and crossbreeds, in a holoendemic area, does not in the experience of the writer appear to be true. "Imported" is

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here used in the sense of importation from an area where the disease does not occur. It is felt that in virtue of the fact that young pups are more susceptible than older ones to the virulent form of the disease, the generally older importations have some advantage, and are, in addition, generally better cared for. Purity of breed does similarly not produce any greater clinical severity.

It appears that the heaviness of tick infestation and the type of coat of the animal, together with the general state of health and quality of nutrition, are much more important factors than purity of breed, per se, and origin.

3.6.2 PATHOGENICITY IN OTHER ANIMALS

Until 1938 it was generally accepted in the absence of proof to the contrary that B. canis was specific for the dog. In that year however, Schoop & Dediè demonstrated the susceptibility of the silver fox to this infection. Previous attempts to infect two species of African jackals had, according to Neitz & Steyn (1947), failed, probably on account of their being in a state of premunition and consequently refractory to new infection.

In the early part of the century a number of workers attempted transmission from dogs to animals other than members of the Canidae. These authors include those listed in Table 3.6.2.1.

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TABLE 3.2.6.1

UNSUCCESSFUL ATTEMPTS AT TRANSMISSION OF B. CANIS

Authors	Animals subinoculated
Robertson (1909)	Horse, ox, sheep, cat, rabbit, guinea-pig, rat, mouse, fowl
Nocard & Motas (1902)	Ox, sheep, goat, cat, rabbit, guinea-pig, white rat, white mouse, fowl, pigeon
Galli-Valerio (1904)	Horse, ox, sheep, goat, cat, rabbit, white rat and mouse
Nuttall & Graham-Smith (1905)	Cat, ferret, hedgehog, guinea-pig, white rat
Bumann (1910)	Rabbit, guinea-pig, mouse

All these attempts failed. Thomas & Brown (1934) even tried infecting splenectomized cats without success.

After the successful transmission of B. canis to the silver fox by Schoop & Dediè there was renewed interest. Neitz & Steyn (1947) succeeded in infecting both entire and splenectomized black-backed jackals. In 1956 Neitz tabulated instances where it had been concluded that natural infection had taken place and that workers had in fact been dealing with B. canis, and also proved cases of artificial infection. This table is reproduced in modified form, with more recent additions, as Table 3.6.2.2.

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TABLE 3.6.2.2

DOMESTIC AND FERAL MEMBERS OF THE FAMILY CANIDAE

SUSCEPTIBLE TO BABESIA CANIS

Hosts	Countries	Mode of Infection	References
Dog	Africa, America, Asia, Europe, Australia	Natural and artificial	See Section 2.3
Side-striped jackal	East Africa	Natural	Nuttall (1910)
Wolf	Turkestan	Natural	Yakimoff & Schokhor (1917)
Red fox Silver fox	Germany	Artificial	Schoop & Dedié (1938)
Algerian jackal	Algeria	Artificial	Gayot (1946)
Black-backed jackal	South Africa	Natural and artificial	Neitz & Steyn (1947)
Cape Hunting Dog	South Africa	Natural	Neitz (1965)

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4. MATERIALS AND METHODS (GENERAL)

For the primary purpose of this study blood was collected from some hundreds of naturally infected clinical cases of babesiosis presented for diagnosis and treatment at the Onderstepoort Outpatient clinic. These represented the manifestations of the disease as encountered in actual practice and thus provided some advantage over experimentally produced cases which of necessity would have to be kept under more or less artificial conditions divorced from the stresses of everyday life. The majority of field cases thus studied provided only a single opportunity for collection of material as they were normally treated and discharged immediately. Severely affected dogs were where possible admitted to hospital for the necessary supportive treatment. Some of these were studied at intervals of a few days up to the stage of clinical health so as to follow the course of recovery. Finally, where desired, a smaller number of animals was experimentally infected for investigation of particular aspects.

Blood for haematology and for biochemical determinations was collected usually from a cephalic vein, or in small collapsed animals from the jugular, into suitable vials containing dried anticoagulants. Initially dried oxalates were used but later disodium ethylenediamine tetra-acetate (EDTA) was used exclusively for haematology and dried or concentrated heparin for chemical determinations. This material was processed under the conditions and within the time limits deemed desirable for accuracy.

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For convenience in recording and assessing the results of determinations within the framework of a progressive disease the affected animals were arbitrarily divided into three clinical categories reflecting roughly the progression of the disease. These could from their nature not be clear cut divisions as, inevitably, cases occurred which were borderline and had to be placed in one or other category where they best conformed in the judgment of the attending clinician to the chosen clinical criteria. The grouping was as follows:-

Category I. Early acute cases with detectable pallor of the mucous membranes and red cell counts of about three million or more per cubic millimetre.

Category II. Well developed cases with very pale to white mucous membranes and red cell counts usually ranging from less than one million to below three million per cu mm, but with no evidence of icterus.

Category III. Cases manifesting any degree of icterus, from faint to frank, regardless of red cell count which was often, though not necessarily, of the order of Category II.

The results of determinations were tabulated and subjected to statistical evaluation.

It was found that merely comparing means was not satisfactory on account of the scatter of values found within a category. Almost invariably and largely due to the steadily progressive nature of disease and the absence of a clearcut

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cut-off line, there was a greater or lesser degree of overlap between the values of the three categories. To determine whether there were real differences between the categories and to what degree these were statistically significant, the data were subjected to analysis of variance. This was computed by means of Snedecor's variance ratio "F" between the variance "between groups" (numerator) and the variance "within groups" (denominator). The values for this ratio were compared with those in statistical tables in the appropriate columns and rows for the degrees of freedom. Using tables for different levels of probability the degree of significance at which the null hypothesis could be rejected could be assessed. The method was as set out in the texts of Hoffmann (1963) and Lewis (1966).

The methods used for individual biochemical and other determinations were as given under their appropriate headings.

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5. THE CLINICAL PATHOLOGY OF THE LIVER IN BABESIOSIS

5.1 INTRODUCTION

Since the earliest days of the appreciation of this infection as a disease entity the symptom which has drawn the most attention (Hutcheon, 1899; Hutcheon, 1900; Almy, 1901; Robertson, 1901; Galli-Valerio, 1904) has been the frequent occurrence of icterus as evidenced by the yellow colour of the mucous membranes and skin. It was most evident in autopsies of dogs which had died from the disease and was seen particularly in the days before effective chemotherapy became known. It is today still seen in neglected or very severe cases.

The name "biliary fever" or "galkoors" by which the disease is most generally known in South Africa provides further evidence of the importance attached to this symptom in the lay mind. It has also been named in other countries and in other languages and times: malignant malarial fever, malignant protozoal jaundice, Gallenfieber, bösartige Gelbsucht, and fiévre bilieuse.

The participation of the liver in the clinical picture has its parallel in that of human malaria. As in the latter the picture in general is one of a centrifugally progressive degeneration, necrosis and disappearance of hepatic polygonal cells from the central vein of the lobule

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outwards leaving the periphery in the vicinity of the portal triad relatively unaffected until an advanced stage of the disease is reached (Andrews & Maegraith, 1948; Maegraith, 1948; Gilles, Maegraith & Andrews, 1953; Maegraith, Gilles & Devakul, 1957).

Very early in the present study it became clear that the liver became involved to some extent in the disease process long before clinical icterus was manifested. It was resolved therefore to examine this question by means of selected modern clinical laboratory procedures for assessing liver damage.

Svirbely, Monaco & Alford in 1946 reported on the comparative efficiency of various liver function tests in detecting hepatic damage produced in dogs by exposure to inhalation of xylidine, a toxic organic amine. Of these they considered the estimation of bromsulphalein retention (BSP test) to provide the most consistent results in that it detected very early liver damage. Rose-Bengal gave comparable results but presented difficulties in connection with turbidity of the plasma. Bilirubin retention gave erratic results and was not nearly as sensitive as the dye retention tests. Serum phosphatase was fairly useful but not conclusive when an early diagnosis was required. They found prothrombin time ratio to be technically more difficult than the BSP test and regarded the results as inconclusive. Urinary urobilinogen was less sensitive and less reliable and required daily analysis of 24 hour samples. These workers found the

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differential diazo test of van den Bergh to be of little value in early liver damage and obtained "direct" and "indirect" reactions only when the dogs were jaundiced and near death. The icterus index was of very limited use and the determination of fibrinogen and the albumin-globulin ratio did not give statistically significant results. Hanger's cephalin-cholesterol flocculation (1939) had been discarded at the outset for the reason that marked flocculation occurred in the serum of normal dogs, as indeed Hanger himself had found.

Drill & Ivy (1944) in an earlier study had obtained similar results when investigating the comparative value of BSP, serum phosphatase, prothrombin time, and intravenous galactose tolerance tests in dogs with livers damaged by twice weekly peroral doses of 0.5 ml of carbon tetrachloride per Kg. Their results indicated that though the tests mentioned might be expected to show a varying response according to the particular function or functions put out of action by the intoxicant they actually in practice showed merely a varying degree of sensitivity. In other words different tests under these circumstances of continued intoxication gave positive results after different stages of the development of liver damage.

In spite of the fact that these workers used a block comparator for the estimation of BSP retention as in the original method of Rosenthal & White (1925) they found this qualitatively the most sensitive in detecting the type of damage produced by carbon tetrachloride. Serum phosphatase (Bodansky, 1937) was considered from these

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few cases to be practically as sensitive as the dye retention. Next in order of sensitivity came prothrombin time (Ziffren, Owen, Warner & Petersen, 1942) and then the intravenous galactose tolerance test (Bassett, Althausen & Coltrin, 1941). Altogether only ten dogs were used but nevertheless the results were useful as an indication. In four of the animals, what they termed the "indirect van den Bergh" was performed with negative results after which this test was abandoned as being less sensitive than the others. These authors however concluded that bilirubin would have been detected if intoxication had been continued for a longer period. The method used was described as being "standard" procedure.

Most clinical studies of serum phosphatase appear to have been directed at using the test to differentiate between hepatocellular and obstructive icterus but even in this the overlap was so broad as to minimize its usefulness for differentiation. Experimentally workers found the sensitivity of serum phosphatase to be of the same order as dye retention tests in assessing liver damage produced by feeding dogs on a protein free diet, experimental hyperthyroidism, bile fistula dogs and in Drill and Ivy's carbon tetrachloride intoxication studies. It was considered technically a simple test and of value if bone disease could be ruled out. Svirbely et al. (1946) did not confirm this view in their study and regarded this test as inconclusive where early diagnosis was required while dye retention in their opinion met these requirements very adequately.

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It was thus decided for the purpose of the present study to run a battery of tests on ordinary "field" cases of B. canis infection presented for diagnosis and treatment at the Onderstepoort Outpatient clinic. These animals presented all variations and stages of development of the disease and gave a good cross-section of all types of the disease picture as met with in practice. From this it was anticipated that a clear picture could be obtained of the degrees of sensitivity of the various tests to the type of liver damage produced by this infection. For convenience the patients were divided into the three categories as set out in Section 4.

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5.2 BROMSULPHALEIN RETENTION

5.2.1 General Considerations

Rosenthal & White in 1925 introduced the use of a dye, sulphobromophthalein ('bromsulphalein', BSP) for the testing of liver function in humans. They showed that intravenously administered BSP was removed almost exclusively by the liver from the circulating blood. Numerous studies since that time have confirmed the general usefulness of this test, the uptake, storage, conjugation and excretion by the liver of this dye being a measure of both hepatic biochemical integrity and of hepatic blood flow. There are a number of steps limiting the rate of excretion into the bile, making the test a very sensitive indicator of hepatic dysfunction generally. Delay in the removal of dye from the blood stream may be due to hepatic necrosis or to fibrosis with a reduced (functional) parenchymal mass and subsequently depressed blood flow (Cornelius, 1963).

The comparative studies of Drill & Ivy (1944) and Svirbely, Monaco & Alford (1946) in connection with dogs subjected to liver damage by hepatotoxic substances were mentioned in Section 5.1. Later, in 1950, Hoerlein & Green published the results of investigations into the value of BSP retention in liver pathology arising from various infections, chemical irritants and dietetic deficiencies in dogs. These authors brought up the difficulty produced by the presence of bilirubin in appreciable amounts.

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This tended to invalidate attempts at exact quantitative assessment of liver damage. This issue was examined by Zieve, Hill, Hanson, Falcone & Watson (1951) who, starting from the view that the BSP test was not interpretable in the presence of moderate or severe icterus on account of regurgitation of the dye with bilirubin into the blood, suggested the use of a nomogram or a table of values to be used to assess the true levels of BSP retention. This could be considered as important or quantitatively significant if the percentage retention were in fact proportional to the amount of liver damage. It has, however, been pointed out (Cornelius 1963; Popper & Schaffner, 1957) that in evaluating BSP retention other factors need to be considered: the hepatic blood flow, the complex process of cellular BSP uptake, conjugation and excretion, and the patency of the biliary passage. Thus there are important additional factors affecting the blood flow wuch as shock, congestive heart failure, and reduced parenchymal mass as a result of fibrosis to be considered.

It is generally accepted that a retention of less than 5 per cent at 30 minutes after injection can be considered normal for the dog, using colorimetric methods of determination, and more or less arbitrarily taking a concentration of 10 mg per 100 ml as the 100 per cent retention standard at the customary dosage of 5 mg per Kg. Higher values given by the earlier workers quoted above were obtained with the use of commercial comparator blocks and can thus be regarded as only qualitatively comparable. Drill and Ivy

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for instance injected 5 mg per Kg and measured retention against 2 mg per Kg standards with a block comparator. They thus called 4 mg per 100 ml the 100 per cent standard and so indicated normal values as lying between two and 12 per cent at 30 minutes after administration.

Previous investigators of canine babesiosis have apparently not used BSP as a test of liver function. While clearly it would not give much information in icterus it was considered that a comparison of values between the three categories might well give a useful indication of the effect of the disease on excretion of the dye.

5.2.2 Materials and Methods

Some 52 dogs were used for this experiment, somewhat less than others since most of the first two categories could not be kept for the time required for the test.

For this study the usual 5 mg per Kg dosage was injected into one cephalic vein and a single blood sample collected from the contralateral vein exactly 30 minutes after the middle of the injection. The concentration of BSP in the plasma of the sample was determined colorimetrically and compared with a 10 mg per 100 ml standard representing 100 per cent retention.

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5.2.3 Results

The results in the three clinical categories are plotted as a scatter diagram to give an immediate visual comparison. See Fig. 5.2.3.

The trend of retention levels was quite evident. Although there was some overlap of values between the different categories the tendency was for BSP retention to rise as the diseased progressed. The overlap was merely a reflection of the continuous nature of the changes and the lack of clear delineation between the categories.

In the Category I cases, early acute ones, values remained substantially within normal limits and the mean was 4 per cent (s.d. = 2). When anaemia however became severe (Category II) the majority of tests indicated severe hepatic damage with a mean value of 24 per cent (s.d. = 20.6) retention, while in the third category, showing any degree of icterus, BSP retention figures were without exception substantially elevated above the normal range, the mean being 61.7 per cent (s.d. = 18.8).

In an analysis of variance the progressive rise in BSP retention during the course of the disease proved to be highly significant at a level of P less than 0.001, not only overall but equally so between successive categories. See Table 5.2.3.

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Fig. 5.2.3 SCATTER DIAGRAM INDICATING VALUES FOR BSP RETENTION IN THE THREE CATEGORIES OF BABESIOSIS

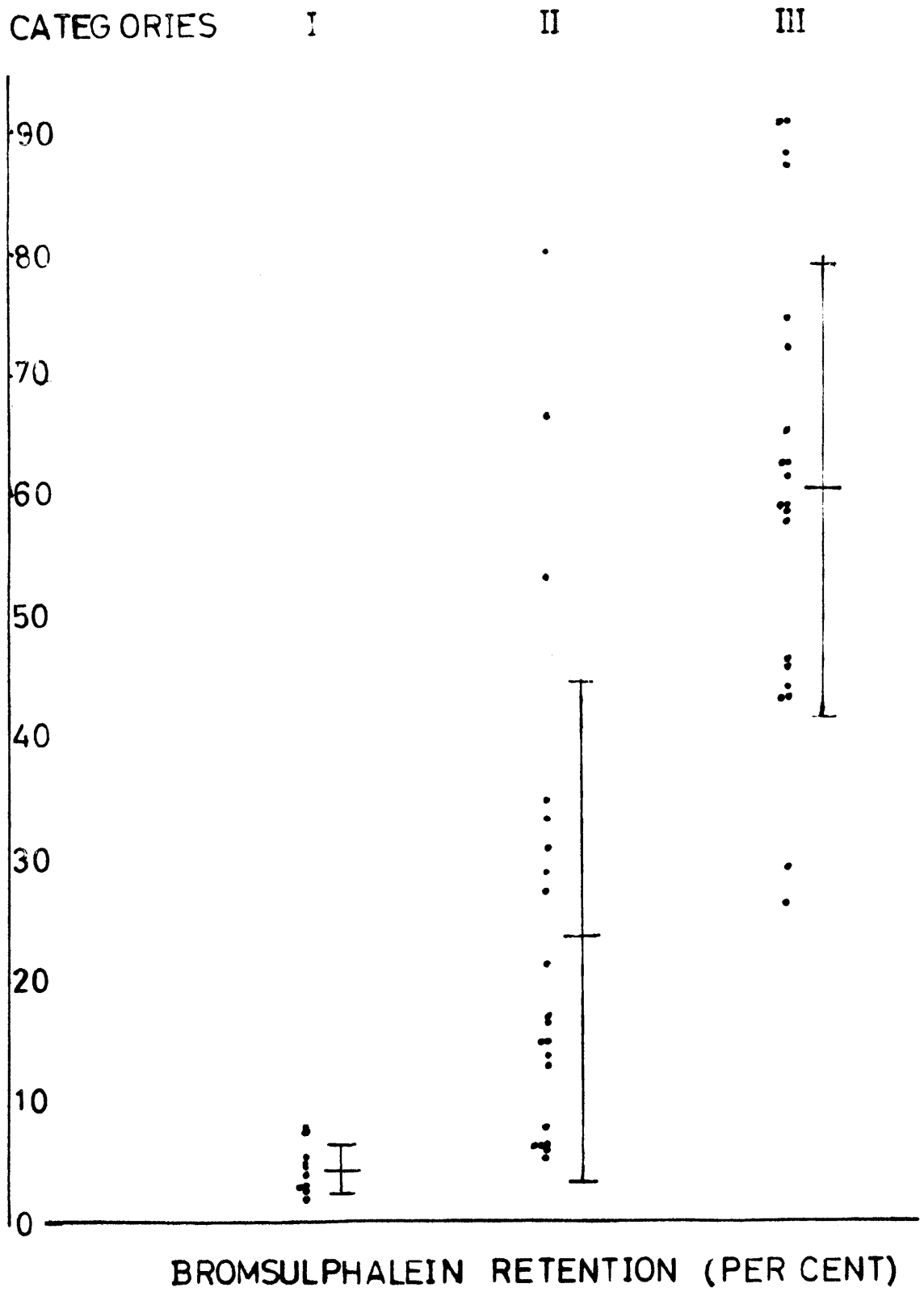

TABLE 5.2.3. STATISTICAL DATA

ANALYSIS OF VARIANCE: "F" RATIO

BROMSULPHALEIN RETENTION

Categories	"F"	Degrees of Freedom		Probability	Significance
I - II - III	40.3	DF_1 2	DF_2 49	$P < 0.001$	High
I - II	29.5	DF_1 1	DF_2 29	$P < 0.001$	High
II - III	36.5	DF_1 1	DF_2 40	$P < 0.001$	High

5.2.4 Discussion

Examination of the data given above makes it clear that the early acute cases of the first category showed virtually no rise of BSP retention above the usually accepted 5 per cent level. The few that were just above were deemed to be due to a degree of medical shock with consequently tardier flow of blood through the liver.

In Category II the development of liver damage was clearly associated with the development of severe anaemia and in this series of icterus cases (Category III) considerably retarded clearance of BSP was found in every instance.

The results tended to demonstrate sufficient development of liver damage to be clearly detectable by the BSP retention test from the point of severe anaemia onwards.

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An interesting observation relating to the second category was that animals giving the higher values, and being treated immediately with specific drugs, showed frank icterus on the following day (with further elevated BSP retention). This suggested the possibility that one might with some confidence predict the occurrence of a jaundiced state when severely anaemic patients show BSP retention of 80 or 90 per cent, regardless of whether they were treated or not.

A further point here to be noted is that although babesiosis is primarily a disease characterized by more or less intravascular haemolysis, sometimes in massive degree, icterus has never been observed in the absence of evidence of liver damage. Raised values for BSP already in the second, strictly nonicteric, category would support this contention, which has been found to hold true in many hundreds of liver function tests of different kinds. This renders a concept of "haemolytic icterus" an exceedingly doubtful one, as discussed elsewhere, certainly as far as dogs are concerned. (See Section 5.5).

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5.3 INFLUENCE ON PLASMA TRANSAMINASES

5.3.1 General Considerations

The foundations of the understanding of transaminating enzymatic mechanisms in the animal body were laid as long ago as 1937 by Braunstein & Kritsman. These consist of chemical reactions concerned with the transfer of the α-amino group of either aspartic acid or alanine (amino-acids) to α-ketoglutaric acid (a Krebs cycle metabolite) resulting in the synthesis of a new amino-acid glutamic acid and new α-keto-acids oxaloacetic or pyruvic acids in the systems currently utilized in diagnostic clinical chemistry. Credit for this application goes to the Wroblewski-Karmen-LaDue group at the Sloane-Kettering Institute in New York (LaDue, Wroblewski & Karmen, 1954; Wroblewski & LaDue, 1955; Karmen, Wroblewski & LaDue, 1955; Molander, Wroblewski & LaDue, 1955; Mason & Wroblewski, 1957) who were the pioneers in assessing the clinical value of determining the transaminase activity in human serum. This led to the establishment of a new and valuable addition to the battery of tests available to the clinical pathologist.

The initial work was done with serum glutamic oxaloacetic transaminase (SGO-T). This enzyme was found in the greatest concentration in certain body cells: in the case of man in heart muscle, skeletal muscle, brain, liver and kidney, in decreasing order. The clinical importance was found to arise from the fact that as a result of necrosis or degenerative processes in these cells the concentration of

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enzyme rose appreciably. Primary interest was accorded to its usefulness in the diagnosis of myocardial infarction where great elevation of activity was found, from 2 to 20 times normal during the first 24 hours after the onset, returning to the normal range within three to six days. This transaminase was unaffected by angina pectoris, coronary insufficiency, heart failure or cardiac arrythmias. It was therefore of great value where electrocardiographic results were equivocal. Enhanced plasma activity of the enzyme provided strong support for a diagnosis of infarction when Q waves were absent in the ECG, when the ECG pattern was obscured by previous infarction, and when there was a left bundle branch block. High values were recorded in various other conditions characterized by more or less extensive cellular damage, e.g. acute myocarditis, acute pancreatitis, haemolytic crises, extensive crush injuries and after surgery. Manso, Taranta & Nydick (1956) showed that enzyme activity was also enhanced by the administration of large doses of aspirin. Normal activity however obtained in pericarditis, pulmonary infarction, rheumatic fever, rheumatoid arthritis and acute cholecystitis.

Wroblewski & LaDue (1955) after their findings in myocardial infarction, investigated the concentration of SGO-T in liver disease such as caused by carbon tetrachloride poisoning, viral hepatitis, cirrhosis, obstructive jaundice and carcinomatosis. This investigation was coupled with determinations of serum bilirubin, cephalin flocculation, thymol turbidity, albumin-globulin ratio, alkaline phosphatase,

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cholesterol and cholesterol esters, bromsulphalein retention and prothrombin time. From these studies they concluded that SGO-T was impressively elevated as a result of acute liver cell injury due to tetrachloride poisoning and to viral hepatitis. Moreover this increased activity was found to be an index of liver cell injury not at all necessarily correlated with the usual tests of liver dysfunction. On account of the very considerable functional reserve of the liver considerable destruction is required to disturb these "functions". This evidence of liver cell injury or necrosis was certainly significant and useful and has led to considerable interest in various parts of the world. In Denmark in 1958 Madsen, Bang & Iversen reported on similar results in a large series of patients suffering from various liver diseases.

In a further series of publications Wroblewski & La Due (1956), Wroblewski, Jervis & La Due (1956) and Wroblewski & Cabaud (1957) showed the value of serum glutamic pyruvic transaminase (SGP-T) as an even more sensitive index of liver cell damage than SGO-T. In myocardial infarction SGP-T levels were found to be affected only at exceedingly high levels of SGO-T. In man then the picture emerged that if SGO-T was elevated, taking into consideration appropriate clinical and ECG findings, involvement of heart muscle was likely, whereas if both SGO-T and SGP-T were present in large amounts, particularly the latter, liver necrosis could be expected to be present. Confirmation of this work was provided by Pryse-Davies & Wilkinson (1958), particularly in viral hepatitis.

In the veterinary field fundamental work was carried

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out by Cornelius, Bishop, Switzer & Rhode (1959). They studied tissue transaminases by a modification of the procedure of Tonhazy, White & Umbreit (1950), and for the estimation of transaminase activity in the serum they used the methods of the Wroblewski group (Cabaud, Leeper & Wroblewski, 1956; Wroblewski & Cabaud, 1957) and of Reitman & Frankel (1957). Liver necrosis was induced in the various species by means of dosage with carbon tetrachloride in mineral oil.

SGO-T activity was increased following liver damage in horses, cattle, pigs and dogs, but in contrast SGP-T figures were affected only in dogs. This was a reflection of the fact that of these animals the liver of the dog only contained large amounts of GP-T. The work of Malherbe (1960) provided confirmation of these findings and in it he discussed the value of determination of transaminase activity in a variety of liver diseases such as occurring in the course of infectious canine hepatitis, babesiosis in horses, cattle and dogs, anaplasmosis in cattle, intra-hepatic cholestasis, hepatic carcinoma, purulent hepatitis, leptospirosis (L. icterohaemorrhagiae) in dogs, and in bacterial icterus of sheep. In this study a different method was used, that of King (1958) which gives figures roughly three times as high as those expressed as Sigma-Frankel units (Reitman & Frankel, 1957). Fundamentally the procedure consists of incubating serum or plasma with substrates consisting of α-ketoglutaric acid and aspartic

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acid or alanine to determine SGO-T or SGP-T respectively. Techniques of different authors, however, differ chiefly in respect of incubation temperature and duration, and the method by which the rate of reaction is determined. This rate is a measure of enzyme activity. For this, paper chromatography, spectrophotometric determination of the fall of extinction at fixed wavelengths, and chemical reactions of various kinds are used but there remains considerable doubt and confusion as to the comparative values of the units. Daly & Jordan (1959) have made a strong plea for the use of the King unit which is defined as that activity in 100 ml of serum which will convert one mol of amino-acid to keto-acid in one hour at 37°C. This activity is expressed in terms of the reactant formed and is measured colorimetrically. The units are of a convenient order as they avoid decimals or very large figures in the normal range.

In this laboratory the King method has been found to be eminently suitable and feasible with relatively unsophisticated equipment. Mohun & Cook (1957) devised a simple method, similar to King's, and gave comparative costs while also examining the resultant figures most critically. The differences in cost was found to be considerable and the results very satisfactory. In both these latter methods protein precipitation, and finally toluene extraction, are eliminated, thus reducing the number of steps leading to the desired end.

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Determination of transaminase activity in babesiosis, not previously done by investigators of this disease, seemed an attractive procedure for assessing the earliest beginnings of liver cell necrosis or degeneration as a result of the disease, and for following the progress of these changes during the course of the disease. Only passing mention had previously been made by the present author, (1960) of changes of transaminase activity in babesiosis.

5.3.2 Materials and Methods

In this study 89 dogs provided the source material and as mentioned above, the method of King (1958) was used for determination of the activity of the enzyme.

5.3.3 Results

These were subdivided into the three clinical categories and the determinations of SGP-T activity set out in a scatter diagram. See Fig. 5.3.3.

SGP-T was selected on account of its specificity for liver damage in the dog. As a routine, SGO-T was also determined simultaneously but the general pattern was that it was less sensitive in its response than SGP-T and that higher figures (for SGO-T) were almost without exception found only in specimens manifesting haemoglobinaemia.

Of the 35 dogs in the first category at least five gave values above the normal range, which has been found (Malherbe, 1960) to lie between about 30 and about 100 King units. This gave evidence that in a minority of cases of

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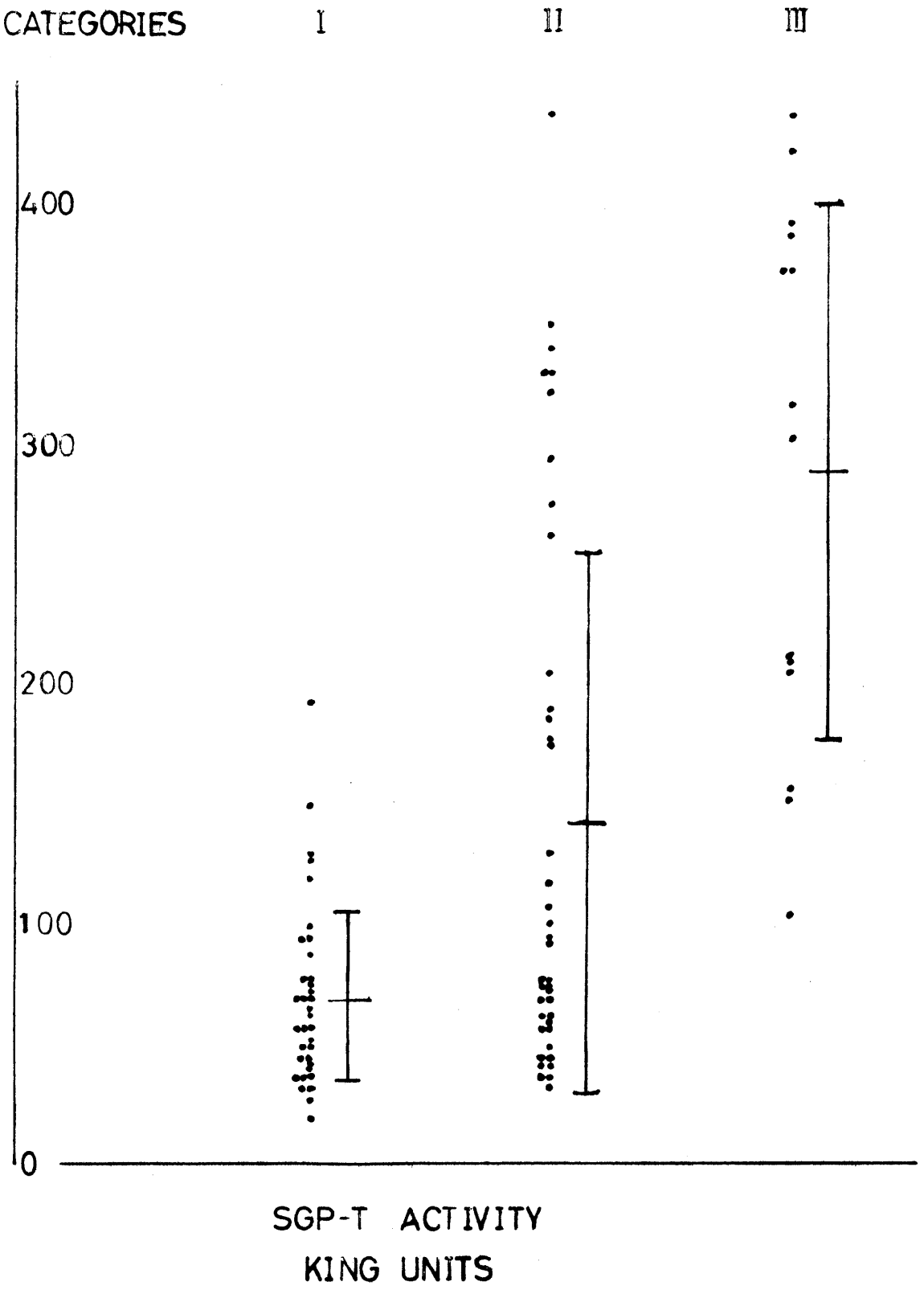
Fig. 5.3.3 SCATTER DIAGRAM INDICATING LEVELS OF GLUTAMIC PYRUVIC TRANSAMINASE ACTIVITY IN THE THREE CATEGORIES OF BABESIOSIS



early acute babesiosis there was already some slight degree of hepatocellular damage, sufficient to cause some elevation of enzyme activity in the plasma. The mean was 68 units (s.d. = 36).

The 40 dogs in Category II showed considerably more scatter, with more than half within the normal range, showing some lack of direct correlation between the degree of anaemia and the extent of liver cell involvement. Some very high values, indicating very extensive cell degeneration and necrosis, brought the mean to 139 (s.d. = 113), distinctly in excess of the normal range.

In the icteric dogs (Category III) all values were above the normal range and the mean was 287 units (s.d. 112).

Analysis of this series indicated a very high degree of significance at the level of P = 0.001 for the increase of transaminase activity during the progress of the disease. Similar evaluation between individual groups, I to II and II to III, showed significant increases at the same level of probability in both cases. See Table 5.3.3.

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TABLE 5.3.3. STATISTICAL DATA

ANALYSIS OF VARIANCE: "F" RATIO

PLASMA GLUTAMIC PYRUVIC TRANSAMINASE

Categories	"F"	Degrees of Freedom		Probability	Significance
I - II - III	40.3	DF_1 2	DF_2 49	$P < 0.001$	High
I - II	29.5	DF_1 1	DF_2 29	$P < 0.001$	High
II - III	36.5	DF_1 1	DF_2 40	$P < 0.001$	High

5.3.4 Discussion

The figures obtained in the first two categories illustrate the fact that there is no clear cut division between the two and that there is a considerable overlapping zone with no evidence of liver damage. Misleading histories (not necessarily deliberate) could have influenced the placing in one or other category to some extent. Generally however the red cell count was the guide to placing the cases but as there is in this disease quite often a precipitate haemolytic crisis the choice of category would in some instances be due to an accident of timing. Occasional cases of concurrent and unrelated hepatic pathology could also have affected a few of the figures.

The results obtained thus show that liver pathology commences in some cases at a fairly early stage of the disease and that it is progressive during the course of untreated infection.

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It is a frequent experience that dogs showing great pallor only on the day of treatment are found the next day to be markedly jaundiced, although parasites have disappeared from peripheral blood smears, indicating effective specific treatment. It has however, not been found possible to correlate the height of the SGP-T figure with the likelihood of such an occurrence.

A few case histories may illustrate this irregularity. Case 112/59 was a Category I animal clinically with a red cell count of 3.21 million per mm^3. Its plasma was reddish brown and total bilirubin was 2.70 mg per 100 ml, more of it unconjugated than conjugated. SGP-T was 190 King units. It was treated immediately but was icteric on the following day.

Case 32/59 (Category II) was admitted very pale with a history of 24 hours of inanition and malaise. BSP retention was 24 per cent, total bilirubin 0.65 mg per 100 ml with 0.45 unconjugated and SGP-T 260. Three days after admission and specific therapy it was still not eating but SGP-T had dropped to 176 units and the blood picture had deteriorated. No icterus developed. Blood urea nitrogen remained normal throughout indicating satisfactory kidney function. This animal made a slow but complete clinical recovery.

A Category I case, 114/59, on presentation had a red cell count of 3.01 million/mm^3, BSP retention of 38.4 per cent and SGP-T of only 54 units. The plasma showed evidence of marked haemolysis. Despite the low SGP-T the animal was severely icteric the next day with activity of this enzyme at 151 units and BSP at 64 per cent retention.

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Kidney function could possibly have been disturbed as shown by a blood urea nitrogen 27.6 mg per 100 ml on admission but the figures given rather suggest the possibility of a state of medical shock.

It would thus appear that SGP-T while a good indicator of the presence and extent of active degeneration and necrosis of liver cells, does not enable the clinician to predict the occurrence of subsequent icterus.

In summary, transaminase was found to be a useful indicator of the earliest stages of liver cell damage in babesiosis. Anoxia due to anaemia was obviously in the beginning not the major reason for such damage, and it could well be argued that anoxic damage resulted from defective sinusoidal blood flow as postulated by Maegraith (1948). This will be discussed more fully in Section 8.

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5.4 INFLUENCE ON PLASMA ALKALINE PHOSPHATASE

5.4.1 General Considerations

The measurement of serum alkaline phosphatase activity was brought into clinical use in the early 1930's when it had been found to be increased in certain types of bone disease (Kay, 1929, 1930). During that decade it received considerable attention (Roberts, 1933; Rothman, Meranze & Meranze, 1936; Cantarow & Nelson, 1937; Gutman, Olson, Gutman & Flood, 1940) in diseases of the liver and has subsequently become firmly established in the clinical chemical diagnostic evaluation of liver function (Cantarow & Trumper, 1962).

As far as dogs are concerned evaluations of the serum "phosphatase" activity were made by Drill & Ivy in 1944 and by Svirbely, Monaco & Alford in 1946, (see Section 5.1) and they found this a sensitive test of liver function.

Alkaline phosphatase is one of a group of phosphatases present in the blood and tissues and which hydrolyse organic phosphoric acid esters, primarily hexose monophosphate and glycerophosphate, liberating the phosphate ion. The different phosphatases are differentiated by the pH range in which each is most active. "Alkaline"

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phosphatase is most active at a pH of 9.3 but is also quite active at the pH of blood, around 7.4. It is present in blood, ossifying cartilage, intestinal mucosa, kidney, liver and other tissues (Gutman, 1959) and is believed (Bodansky, 1952) to be derived from osteoblasts for the greater part. The route of excretion from the body is almost entirely via hepatic cells into the bile in man and the dog. In cats, however, the kidney (Cornelius, 1963) also participates in excretion.

The amount of alkaline phosphatase activity has been expressed in a number of systems (Maclagan, 1959) of units per 100 ml depending on the substrates used and the conditions and duration of hydrolysis. Two of them extensively used today are Bodansky units (Bodansky, 1933) in the United States and King-Armstrong units (King & Armstrong, 1934) in Britain and in this country. Normal figures for man and dogs usually fall within the range of 1-4 Bodansky (Gutman et al., 1940) or 3-13 King-Armstrong units per 100 ml (Maclagan, 1959).

The King-Armstrong units used in the present study represent the number of mg of phenol which would be set free from a disodium phenyl phosphate substrate under the specified conditions of the method in 15 min. by 100 ml of plasma. The phenol is measured by its action on Folin and Ciocalteu's phenol reagent.

Figures in excess of the range given are obtained as a result of overproduction, as in young, growing animals,

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in animals suffering from rickets, osteogenic sarcomatosis and secondary hyperparathyroidism, or as the result of interference with the escape from the circulation via bile as seen in cholestatic conditions or in hepatitis. The question of this interference as the sole retention mechanism has been described as controversial, and Popper & Schaffner (1957) and Gutman (1959) have discussed the evidence concerning possibly increased formation in liver cells.

Much more recently Hill & Sammons (1967) have studied this aspect, using starch gel and paper electrophoresis for separating the isoenzymes of alkaline phosphatase. They showed that the enzymes from bone and from liver were not identical and postulated the following mechanism: "The normal serum level is the result of two factors, the rate of release of the enzyme from the tissues, principally liver and bone, and the rate of inactivation of the enzymes in the serum and body protein pool. In osteoblastic bone disease the elevated level is due to the rate of release of the enzyme exceeding the rate of inactivation. The raised level does not indicate an inability of the liver to excrete the enzyme via the biliary tract. In liver disease the increase in serum levels is a result of increased liberation of the enzyme from the sinusoidal surface of the liver cell and of regurgitation of the biliary enzyme back into the serum." The last word has no doubt not been said about this matter.

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Since not only the liver is involved in rises of plasma phosphatase activity it follows that in any evaluation the clinical circumstances should be given due weight, and that moreover this determination should be combined with others in the assessment of hepatic functional impairment.

Alkaline phosphatase activity follows more or less the following pattern in the differential diagnosis of jaundice. It is regarded as less sensitive than bromsulphalein retention for early disturbance of function. Transaminase activity is much more sensitive to liver cell damage than phosphatase, and between hepatocellular damage and cholestatic lesions, tend to react quantitatively in opposite ways. Transaminase activity rises moderately in obstruction and very much more in liver cell necrosis or degeneration while alkaline phosphatase shows considerably higher values in obstruction than it does in hepatitis and other forms of liver cell damage. Latner & Smith (1958) have in fact suggested the use of a transaminase/phosphatase ratio as being useful in the differential diagnosis of jaundice.

Working with dogs Freeman, Chen & Ivy (1938) found for instance values of the order of 100 K.A. units in experimental obstruction compared with 20 K.A. units in leptospirosis. Bloom (1957) has observed values of between 6 and 10 Bodansky units in dogs with hepatocellular damage while in obstruction they were usually much in excess of 10 Bodansky units. The peak value found by Malherbe (1959) in

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the course of a study of intrahepatic cholestasis (an obstructive lesion) was 499 K.A. units.

The difference however, is not always so clear cut since an obstructive element (presumably biochemical in nature) may supervene in severe hepatitis and the resulting levels may be as high as in obstruction (Gutman, 1959; Gornall & Bardawill, 1960).

5.4.2 Materials and Methods

For the purpose of this study heparinized blood was collected from 89 field cases of babesiosis and plasma alkaline phosphatase activity was estimated by the method of King & Armstrong (1934).

5.4.3 Results

Fig. 5.4.3 shows the scatter of plasma alkaline phosphatase values obtained in the three clinical categories.

Of the 28 dogs in Category I about one-third gave values above the accepted normal of 3-13 K.A. units. The mean however, was 12.4 (s.d. = 5.6) units.

In the second category, comprising 44 dogs, about half the figures were within normal range. The mean was 19.3 ($\pm$ 13.9) units.

In the case of the 17 icteric dogs the phosphatase values were almost invariably increased to levels commonly found in hepatitis and liver cell necrosis. In this group the mean value was 51 ($\pm$ 46.1) units and the two very high

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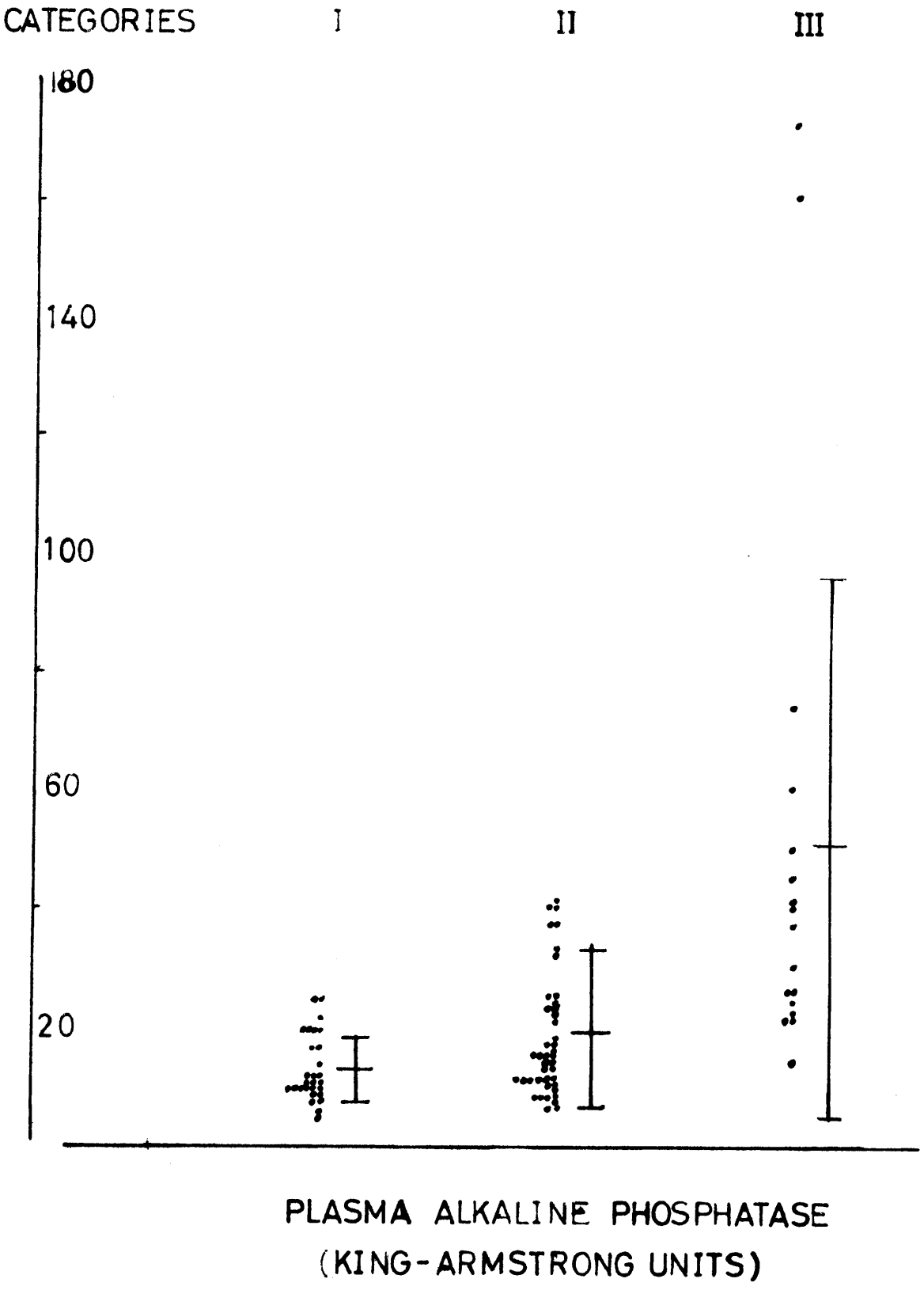
Fig. 5.4.3 SCATTER DIAGRAM INDICATING THE DISTRIBUTION OF ALKALINE PHOSPHATASE VALUES IN THE PLASMA OF THE THREE CATEGORIES OF BABESIOSIS



figures of 160 and 172 units recorded were considered to be of the order of those found in cholestatic conditions.

Statistical evaluation of the figures obtained showed that there were very significantly higher values as the disease progressed (at the level of P = 0.001). In a comparison however, between Category I and Category II values (on account of the many normal ones in Category II), the rise is significant only at P = 0.05, while between Category II and Category III it is highly significant at the P = 0.001 level. Hence the rise coinciding with the appearance of icterus makes the greater contribution to the overall increase. For statistical data see Table 5.4.3.

TABLE 5.4.3. STATISTICAL DATA

ANALYSIS OF VARIANCE: "F" RATIO

PLASMA ALKALINE PHOSPHATASE

Categories	"F"	Degrees of Freedom	Probability	Significance
I - II - III	46.45	DF_1 2 DF_2 86	$P < 0.001$	High
I - II	6.24	DF_1 1 DF_2 70	$0.05 > P > 0.01$	Poor
II - III	16.9	DF_1 1 DF_2 59	$P < 0.001$	High

5.4.4 Discussion

The exact significance of the higher than normal values in Category I must be assessed with some caution

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as babesiosis commonly affects dogs in the younger age group. Growing pups, up to the age of nine months, and a few animals showing signs of bone disease, were represented among the patients and these might reasonably be expected to give levels above normal limits on the basis of enhanced osteoblastic activity. This determination therefore does not provide clear evidence of hepatic functional impairment in the early stage of the disease.

In Category II the abnormal values tended to be considerably higher than the normal range, certainly higher than those assumed in Category I to be caused by osteoblastic activity. An appreciable incidence of liver pathology must then be responsible for these higher values.

The icterus group has been seen to have consistently increased values, some to very high levels, reflecting marked hepatocellular damage and even a degree of cholestasis. As these latter animals were proven cases of babesiosis (not mechanical obstruction) a case could be made for an assumption that anoxic damage was in these cases severe enough to inactivate the transfer system between the hepatic cells and the bile canaliculi, thus giving rise to phosphatase levels more usually found in obstructive lesions.

As far as human malaria is concerned serum alkaline phosphatase does not seem to have received anything more than very sporadic attention. Lippincott, Ellerbrook, Hesselbrock, Gordon, Gottlieb & Marble (1945) investigated liver function

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in cases of naturally contracted P. vivax malaria, including in their battery of tests alkaline phosphatase. These tests revealed little more than transient derangement of hepatic cellular function in some cases. The malarial infection had however been treated promptly with quinacrine. In a later report this group (Lippincott, Marole, Ellerbrook, Hesselbrock, Engstrom & Gordon, 1946) however described function tests in neurosyphilitic patients which had been treated therapeutically with P. vivax and in which the latter infection was allowed to continue for about 20 days of parasitaemia. The majority of these showed definite evidence of liver function impairment but alkaline phosphatase was not used for this series.

Maegraith (1948) in his monograph on pathological processes in malaria and blackwater fever, and Maegraith, Gilles & Devakul (1957) in a similar short study of canine babesiosis made no mention of serum alkaline phosphatase as an index of disturbed liver function.

In spite of its limitations this determination does show the trends of liver damage during the later progress of babesiosis.

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5.5 INFLUENCE ON BILIRUBIN METABOLISM

5.5.1 General Considerations

The most obvious change in the composition of blood plasma in conditions involving jaundice is the presence of bile pigments. This was remarked on in Section 5.1 in connection with babesiosis in the dog, where the popular names of the disease tend to stress the observation that jaundice is frequently seen in severe or neglected cases.

The most important bile pigment, bilirubin, is formed, briefly, as follows: At the end of the life-span of an erythrocyte it is engulfed by a reticulo-endothelial cell in which it disintegrates. The porphyrin ring of haemoglobin is opened and a bilirubin-iron-globin complex formed. Iron is released from the complex and combines with β-globulin for storage as ferritin or goes into the labile iron pool for re-use in the synthesis of haemoglobin. Excessive haemolysis and thus excessive release of iron gives rise to haemosiderosis which is an accumulation of insoluble particulate matter in the cells, appearing golden brown granules in routinely stained sections. Haemosiderin, which is a ferric hydroxide and mucopolysaccharide complex, is similar in structure to ferritin. The globin set free goes into the protein pool.

In milder degrees of intravascular haemolysis the haemoglobin set free is captured by haptoglobin, a colourless α-2 -mucoprotein globulin. This complex is removed from the blood stream by the reticulo-endothelial system. In

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the more massive haemolysis encountered in peracute and some acute cases of babesiosis these mechanisms are inadequate and the excess haemoglobin is spilled into the urine through the kidneys. The kidney threshold for haemoglobin is of the order of 130 mg per 100 ml of plasma in man. In such cases of babesiosis the plasma becomes a deep port wine red and a probably similar threshold is exceeded very considerably, resulting in haemoglobinuria. Haemosiderosis is a frequent histological finding in advanced cases of the disease.

The bilirubin resulting from normal breakdown of haemoglobin is taken up by the liver, conjugated, and excreted into the bile. Excessive formation of bilirubin however exceeds the capacity of the liver to cope with it and a build-up of unconjugated bilirubin in the plasma may be expected.

As long ago as 1883 Ehrlich found that if a mixture of sulphanilic acid, hydrochloric acid and sodium nitrite was added to serum containing bile pigments a violet colour was produced (With, 1954).

The foundations of the clinical application of this fact were however laid over thirty years later when van den Bergh & Muller (1916) demonstrated two different types of reaction in the serum of jaundiced humans, a "direct" one following immediately on addition of the diazo reagent (the mixture of Ehrlich) and an "indirect" one some time later. The "indirect" reaction could be elicited without delay if alcohol were added to the mixture, and was found

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primarily in cases characterized by haemolysis.

Chapman, Johnson & Karl in 1960 gave a modern review of the clinical application of the van den Bergh reaction in jaundice and assessed the present status of the test in the light of the newer knowledge of the identity of the "direct" and "indirect" reacting pigments, gained during the middle 1950's by chromatographic techniques (Cole & Lathe, 1953; Cole, Lathe & Billing, 1954; Billing & Lathe, 1956: Talafant, 1956; Schmid, 1956, 1957; Schmid, Hammaker & Axelrod, 1957; Arias & London, 1957; Schachter, 1957; Billing & Lathe, 1958).

The "direct" reacting fraction was shown by both the Cole-Lathe-Billing group in England and Schmid and his group in the United States to consist of two components which were named pigment I and pigment II, the formation of which was mediated by an enzyme, glucuronyl transferase, derived from the endoplasmic reticulum of the hepatic polygonal cells. This enzyme catalysed the transfer of the glucuronide radical from uridine diphosphate glucuronic acid to various receptors, in this case bilirubin. These glucuronides were shown to be readily soluble in water and the forms as secreted from the liver cells into the bile canaliculi. It was thus suggested that the basis of "direct" and "indirect" reactions was simply a matter of the solubilities of the ester glucuronides and of "free" or unconjugated bilirubin in water and alcohol respectively.

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While pigment II was certainly the diglucuronide the original pigment I of Lathe and his group was subsequently found not to be a monoglucuronide as had been reported but that pigment I after elution and rechromatographing yielded a mixture of bilirubin and bilirubin diglucuronide (Lathe, 1965). This problem has been the subject of a considerable amount of research, also by other groups, and it does not seem to have been finally settled. The alkali lability of the diglucuronide could provide the clue to the identity of pigment I (Lathe, 1965).

In human medicine the main interest in the van den Bergh diazo test has for the greater part of half a century been centred on its use to distinguish between "medical" or hepatocellular and "surgical" or obstructive forms of jaundice.

Watson (1956), working on the basis of the ratio between conjugated and total serum bilirubin, suggested that "low bilirubin ratios in cases of elevated total serum bilirubin are indicative of retention jaundice of various degrees. The lowest ratios (less than 20 per cent) are characteristic of haemolytic disease or constitutional hepatic dysfunction of the Gilbert type. While the majority of patients with jaundice due to parenchymal liver disease have bilirubin ratios in the same general range as found in cases of biliary obstruction (45 per cent to 80 per cent)

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there is a significant segment of cases with diffuse liver disease in which ratios below 40 per cent are encountered. In the range of 35 to 40 per cent there is a small likelihood of confusion with biliary obstruction, and below 35 per cent almost none." Other views have however, been expressed on this issue (Hoffbauer, Rames & Meinert, 1949; Klatskin & Drill, 1950; Zieve, Hill, Hanson, Falcone & Watson, 1951; Chapman, Johnson & Karl, 1960). These authors regard this determination as being of little value except in differentiating haemolytic jaundice from other types of icterus.

The truth seems to lie somewhere between the two viewpoints, certainly as far as typical or early cases are concerned. It is clear that the test has limitations. These arise from the fact that in unrelieved extrahepatic obstruction the hepatic cells and bile ducts inevitably and eventually become involved, so that shortly after the early phase a "mixed" or equivocal picture would be presented. On the other hand, in severe cases of hepatocellular damage, the excretory function of parenchymal cells may be interfered with to such an extent that the picture of cholestasis may well supervene. This lesion would be a biochemical one entirely, involving the transfer of bile from liver cells into the bile ducts.

For the purpose of the present study it was evident that the differentiation between bilirubin and its glucuronide conjugates could provide information of considerable interest, for the reason that the disease starts with haemolysis,

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frequently on a massive scale, progressing to anoxic liver damage. The picture presented would not be confused by any extrahepatic element. It could be anticipated that the test would reveal the extent and time of development of cellular damage and that the trends would be clarified by the figures obtained.

5.2.2 Materials and Methods

Specimens were collected from some 96 field cases of babesiosis and the plasma total and conjugated bilirubin estimated by the classical diazo procedure of Malloy & Evelyn (1937), slightly modified. Unconjugated bilirubin was calculated by difference between total and direct-reacting (one-minute) conjugated bilirubin. The results were allocated into three groups representing the three clinical categories and the findings plotted graphically as a scatter diagram. The percentages of total bilirubin represented in the individual cases by conjugated bilirubin were similarly plotted. For this latter purpose cases with 0.3 mg/100 ml or less of total bilirubin were left out of account since percentages were far too inaccurate at the portion of the colorimeter scale involved. The differences between categories were evaluated statistically by analysis of variance (variance ratio "F" method).

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5.5.3 Results

The scatter diagrams are presented in Fig. 5.5.3. The great majority of the 28 Category I cases had values of 1 mg/100 ml or less for total bilirubin. The exceptions, containing 1.6, 1.8, 2.7 and 2.95 mg/100 ml would represent borderline cases, with appreciable liver damage already present. The conjugated bilirubin percentages were with one exception less than 50. In Category II cases, 42 in number, almost half had levels of 1 mg/100 ml or less with a similar number between 1 and 5 mg/100 ml. Only two were higher than 5 mg/100 ml. Approximately 83 per cent of these revealed conjugate percentages of 50 or less. Category III showed very great scatter of total bilirubin values, ranging anywhere from 1.4 to 37.8 mg/100 ml. 25 of the 32 percentages conjugated were in excess of 50 representing only about 22 per cent of the cases in this category with some (albeit little) excess of the unconjugated over the conjugated moiety.

On statistical evaluation it was found that the mean values for total bilirubin (with S.D.) were respectively 0.70 ($\pm$ 0.08), 2.15 ($\pm$ 2.2) and 12.60 ($\pm$ 9.7) mg/100 ml. The overall rise was highly significant at P = 0.001 (See Table 5.5.3). The difference was less significant between Category I and II (only just missing P = 0.001) than between Category II and III where it was very highly significant at this level of probability.

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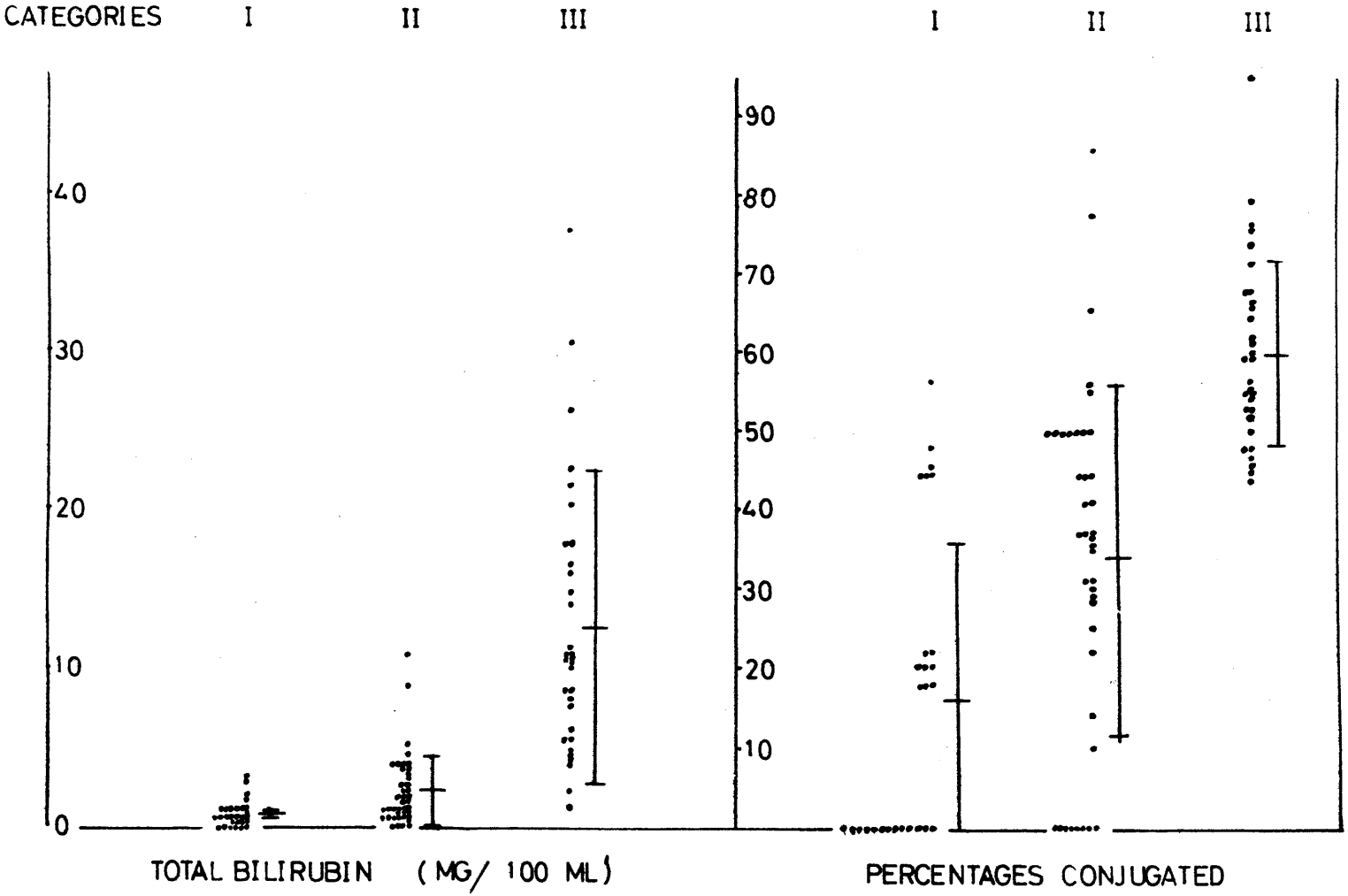
Fig. 5.5.3. SCATTER DIAGRAMS INDICATING THE SPREAD OF VALUES FOR TOTAL BILIRUBIN AND FOR THE PERCENTAGE OF BILIRUBIN IN CONJUGATED FORM IN THE THREE CATEGORIES OF BABESIOSIS



Similar examination of the conjugate percentages revealed means of 15.9 (± 20.2), 33.8 (± 22.3) and 60.2 (± 11.8). These differences were highly significant (overall) and between Category II and III at P = 0.001 and between Category I and II at P = 0.01. The statistical data are also presented in Table 5.5.3.

TABLE 5.5.3. STATISTICAL DATA

ANALYSIS OF VARIANCE "F" RATIO

TOTAL PLASMA BILIRUBIN

Categories Compared	"F"	Degrees of Freedom		Probability	Significance
I - II - III	42.6	DF_1 2	DF_2 99	$P < 0.001$	High
I - II	11.08	DF_1 1	DF_2 68	$0.001 < P < 0.01$	Moderate
II - III	45.70	DF_1 1	DF_2 72	$P < 0.001$	Very high

PRECENTAGE BILIRUBIN CONJUGATED

I - II - III	39.9	DF_1 2	DF_2 93	$P < 0.001$	High
I - II	10.16	DF_1 1	DF_2 62	$0.001 < P < 0.01$	Moderate
II - III	37.21	DF_1 1	DF_1 70	$P < 0.001$	High

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5.5.4 Discussion

The pattern of development of liver involvement in babesiosis is again shown up by these figures. In the first category cases most of the bilirubin present was unconjugated and resulted from haemolysis above physiological levels. A few cases which were regarded as borderline already showed some evidence of liver damage.

Category II presented a mixture of slight to moderately increased total bilirubin values with the unconjugated fraction still in a predominant position, suggesting haemolysis as the major source of the pigment. In the remainder the moderate increase in hepatocellular involvement was suggested by the more than 50 per cent levels for conjugated bilirubin.

There was a greater swing to the hepatitic range of values in the Category III cases with the great majority of conjugate percentages above 50. Total bilirubin was generally of a much higher order and some values so high as to suggest a biochemical cholestatic lesion. In these the conjugated fraction was very high.

From this series and from experience of this procedure over many years it has become clear that in B. canis infection clinical jaundice is never seen unless the liver cells are fairly heavily involved in the disease syndrome. No matter how peracute the disease no such thing as clinical jaundice from pure haemolysis has ever been found. In the more or less purely "haemolytic" stage of Category I the total bilirubin values are practically invariably of a low order

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as compared with those in which liver damage is evident.

In no case of this disease has "free" bilirubin ever been found to rise to anything like the levels found in cases of human neonatal jaundice with kernicterus, regardless of the peracuteness of the disease or the degree of haemolysis. In these severe cases there is often a severe "spilling" of haemoglobin into the urine, but never icterus unless the liver is already extensively involved.

Lettow (1962) in an extensive study of the diagnosis of liver disease in dogs, reviewed the literature in connection with a purely "haemolytic" icterus and quoted findings indicating that such an icterus, without involvement of liver cells, did not exist in dogs. He further found that in these animals hyperbilirubinaemia was not regularly present in liver disease but that if it was it could be regarded as very significant.

The question of bile pigments in plasma and urine in malaria has been discussed at length by Maegraith (1948). In this disease too he found that bilirubin determination supported the general conclusion that liver involvement occurred transiently or could be so severe as to dominate the clinical picture. Moreover, on the question of whether a pure haemolytic jaundice could exist in man without any involvement of the liver, he quoted conflicting views of older authors. Some held that jaundice in malaria, unaccompanied by bilirubinuria, was nearly always haemolytic in origin, whereas other workers stated categorically that there was a hepatic element in all forms of jaundice.

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The use of enzyme activity determinations since that time have helped greatly to clarify some of these points.

Gilles, Maegraith & Andrews (1953) and Maegraith, Gilles & Devakul (1957) reported on their findings in connection with bile pigments in babesiosis in a study of experimentally infected dogs. They estimated plasma bilirubin in 17 animals. In ten which were severely anaemic and clinically jaundiced bilirubin values were between 2.0 and 6.0 mg per 100 ml. Seven died, and three were killed in the early stages of recovery. Pathological changes were found histologically in the livers of all seven that died and of one of the killed group. The remaining seven animals which had bilirubin "within normal limits" were all anaemic but one, and none showed jaundice. Three of these died from the disease and the remainder were killed at various stages of recovery. Structural changes were seen in the livers of four.

In summary then it is evident that determination of bilirubin, while not capable of detecting very early liver damage, is nevertheless a useful means of throwing light on the progress from the initial haemolysis due to the activity of B. canis through to the stage where in some instances liver damage is so extensive as to present biochemical obstruction at the cellular level.

5.6 INFLUENCE ON SERUM PROTEINS

5.6.1 General Considerations

It has frequently been stated that changes in electrophoretic patterns cannot be regarded as characteristic of or specific for any particular disease. Marrack & Hoch (1949) for instance regarded any changes found in the same light as determination of erythrocyte sedimentation rate in that they were "a measure of the clinical state of the patient, rather than specific evidence of disease." They concluded that the greater precision of electrophoretic examination of pathological sera would provide no real advantage over the usual chemical methods.

Since that time however, there has been a progressive improvement in the available methods of electrophoresis. This has led to some qualification of the extreme view expressed above. In a 1952 study of electrophoresis by means of a modified Tiselius type of apparatus Polson & Malherbe found distinct differences between serum from normal dogs and ones suffering from babesiosis, rickettsiosis and distemper. These reflected more the response to forms of organic damage occurring in the particular disease than a constant picture representing the disease itself in all its phases.

Dimopoullos (1963) has given a comprehensive review of the whole situation, discussing, inter alia the origin,

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composition and significance of the various electrophoretic fractions. He remarked that changes in these fractions served only to indicate the status of the subject under study at the particular time and that any material changes in amount or proportion had to be related to specific conditions. He however, mentioned some exceptions where abnormal globulins appear, such as certain forms of carcinoma in man and plasma cell myelomatosis in horses. It has also been found (de Villiers, L.S., 1966, Medical School, University of Pretoria: personal communication) in a large general hospital that with cellulose acetate strip electrophoresis a number of clinically unsuspected cases of multiple myeloma in man have been revealed in routine specimens sent to the clinical laboratory.

Interpretation of electrophoretic findings is very considerably dependent on the particular technique being used, and even with the same method standards have to be established for the different animals in any particular laboratory.

There is a limited degree of unanimity on changes associated with liver pathology. Due to the overriding role of the liver in the biosynthesis of plasma albumin there is in pathological conditions a fall of the albumin concentration. This has been generally observed. Zöllner, Eymer & Scheid (1950), Groulade & Groulade (1953) and de Wael & Theunissen (1954) according to Dimopoullos (1963) represent authors who consider low albumin and elevated beta-

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globulins to be characteristic of liver disease. Nagel & Katz (1963) however found beta-globulins to be consistently low in severe hepatitis in man. Their further findings were that alpha-1-globulins did not vary significantly and that alpha-2-globulins were low in severe acute hepatitis. Gamma-globulins were elevated in both mild and severe cases.

Tella & Maegraith in 1965(b) reported on serum protein changes in monkeys with P. knowlesi malaria, mice with P. berghei malaria, and puppies with induced babesiosis. The canine patients were generally dead in five days and numbered about six. In this series the authors found a drop of serum proteins, notably in the albumin fraction, with little change in the globulin level. Electrophoretically however, alpha-1, alpha-2 and gamma were all increased while beta-globulin remained unchanged.

The present study was designed to investigate the effect of babesiosis on the total protein and the individual electrophoretic fractions during the course of the disease in a larger sample of field cases.

5.6.2 Materials and Methods

Sera were collected from 117 dogs for the purpose of this study. Of these, 37 were "normal", mostly young bitches, considered to be healthy and on the point of being oophorectomized. The remainder were diagnosed cases of babesiosis on presentation by the owners and were divided

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into the three not very clearly demarcated clinical categories (Category I: 34, Category II: 27, Category III: 19).

The total serum protein was estimated by means of Weichselbaum's (1946) biuret method. Fractionation was carried out on cellulose acetate strips in the Beckman Microzone Electrophoresis System. The samples of 0.25 µl of serum were subjected to a 250 volt current (3.5 - 5.8 m a) for 20 minutes in a pH 8.6 barbital buffer solution of 0.075 ionic strength and staining was done with Beckman Fixative Dye Solution based on Ponceau S. Scanning by means of a Spinco Model RB Analytrol with a Microzone scanning attachment completed a very satisfactory procedure giving highly reproducible results.

For statistical analysis of the results Snedecor's variance ratio "F" was employed. The probability levels were as indicated in the discussion of results.

5.6.3 Results

It was found at an early stage that a significant proportion of serum specimens was haemolysed due no doubt to the marked mechanical fragility of canine red cells. It was found that the haemoglobin complex migrated with the beta-globulin fraction during electrophoresis. For this reason it was decided to work only with absolute values (rather than with percentage composition) and to omit any consideration of beta-globulin.

Table 5.6.3.1 summarizes mean values found in "normal" animals and in the three clinical categories, and Fig. 5.6.3. graphically illustrates the distribution of values found. Statistical data are given in Table 5.6.3.2. The category of "normal" animals is designated for abbreviation as Cat. 0.

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TABLE 5.6.3.2 STATISTICAL DATA FOR SERUM PROTEINS
ANALYSIS OF VARIANCE "F" RATIO
TOTAL SERUM PROTEIN

Categories	"F"	Degrees of Freedom	Probability	Significance
0-I-II-III	1.02	DF_1 3 DF_2 113	P > 0.05	None
0 - III	1.92	DF_1 1 DF_2 54	P > 0.05	None
		SERUM ALBUMIN		
0-I-II-III	44.45	DF_1 3 DF_2 113	P < 0.001	High
0 - I	42.18	DF_1 1 DF_2 69	P < 0.001	High
I - II	7.35	DF_1 1 DF_2 59	0.001 < P < 0.01	Moderate
II - III	2.0	DF_1 1 DF_2 44	P > 0.05	None
		ALPHA-1-GLOBULIN		
0-I-II-III	2.0	DF_1 3 DF_2 113	P > 0.05	None
		ALPHA-2-GLOBULIN		
0-I-II-III	9.19	DF_1 3 DF_2 113	P < 0.001	High
0 - I	3.33	DF_1 1 DF_2 69	P > 0.05	None
I - II	7.43	DF_1 1 DF_2 59	0.001 < P < 0.01	Moderate
II - III	3.22	DF_1 1 DF_1 44	P > 0.05	None
		GAMMA-GLOBULIN		
0-I-II-III	17.42	DF_1 3 DF_2 113	P < 0.001	High
0 - I	23.48	DF_1 1 DF_2 69	P < 0.001	High
I - II	6.78	DF_1 1 DF_2 59	0.01 < P < 0.05	Poor
II - III	0.95	DF_1 1 DF_2 44	P > 0.05	None

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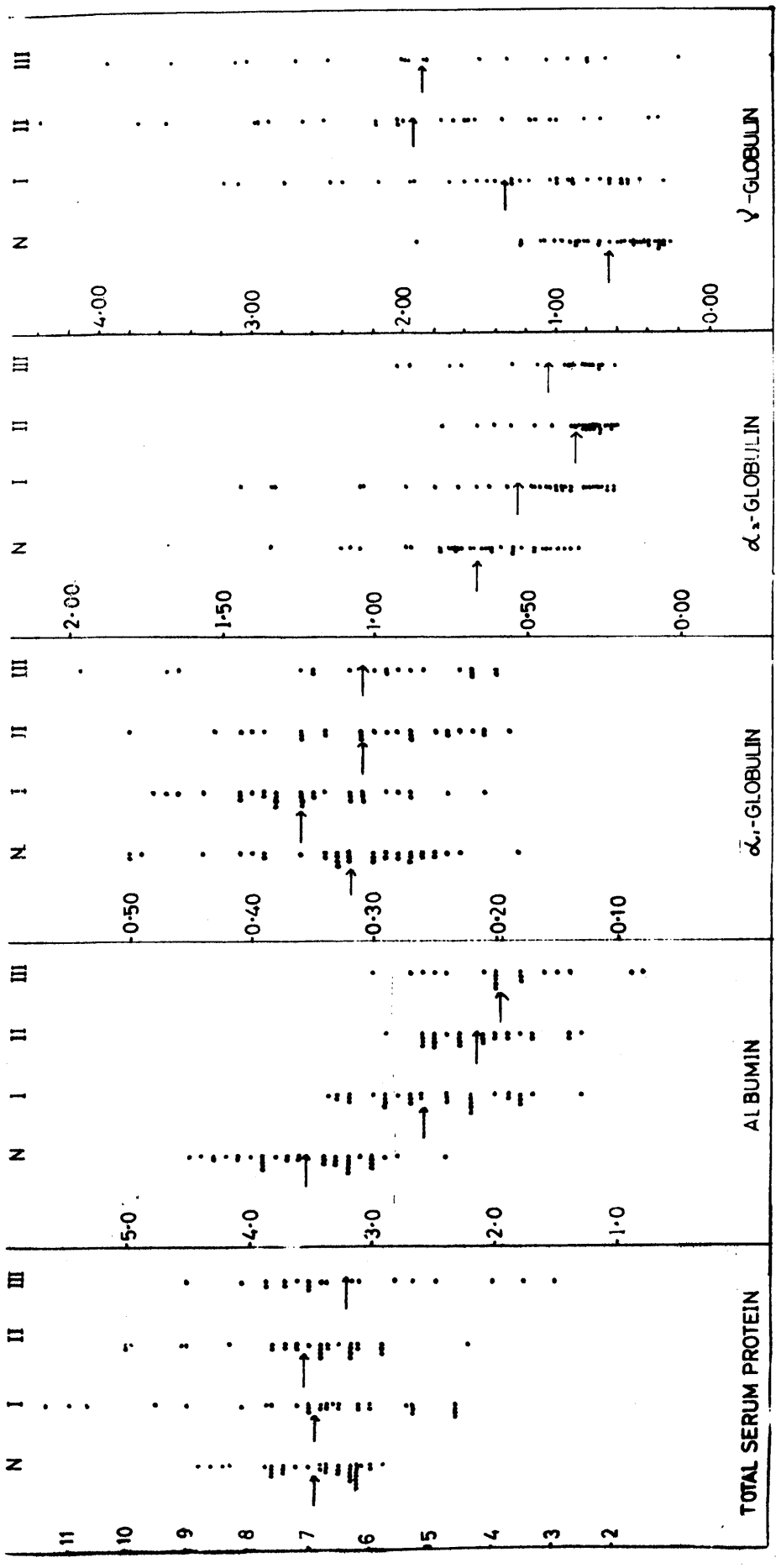
Fig. 5.6.3. ELECTROPHORETICALLY OBTAINED AND CALCULATED ABSOLUTE VALUES IN g/100 ml FOR TOTAL SERUM PROTEIN, ALBUMIN, ALPHA-1-, ALPHA-2- AND GAMMA GLOBULINS IN "NORMAL" DOGS AND IN THREE CATEGORIES OF BABESIOSIS



ELECTROPHORETIC SERUM PROTEIN FRACTIONATION

TABLE 5.6.3.1 MEAN (± S.D.)

SERUM PROTEIN FRACTIONS IN BABESIOSIS (gm/100 ml)

	TSP	Albumin	Alpha-1-globulin	Alpha-2-globulin	Gamma-globulin
"Normals"	6.90 ± 0.80	3.54 ± 0.50	0.32 ± 0.07	0.67 ± 0.23	0.66 ± 0.35
Cat. I	6.89 ± 1.77	2.58 ± 0.72	0.36 ± 0.07	0.54 ± 0.34	1.34 ± 0.77
Cat. II	7.01 ± 1.38	2.16 ± 0.43	0.31 ± 0.09	0.35 ± 0.14	1.93 ± 1.02
Cat. III	6.42 ± 1.59	1.95 ± 0.57	0.31 ± 0.09	0.44 ± 0.22	1.88 ± 1.04

Total serum protein. The scatter of high values represent mostly haemolysed sera so that all the means are probably somewhat on the high side. All categories are however similarly affected so that for the present purpose and with the use of only absolute fractional values this is not important.

The overall analysis of variance for total serum protein showed that there were no significant changes and that the null hypothesis could not be rejected. The lowest individual figures were found in Category III but they did not significantly affect the mean.

Serum albumin. There was a progressive fall of mean albumin values during the progress of the disease. This was highly significant at the level of P = 0.001. The fall from the "normal" group to Category I was significant at the same level, that from Category I to Category II at P = 0.01, while there was no significant difference between Category II and Category III. The decrease in the albumin fraction was thus clearly an early event in the course of the disease.

Alpha-1-globulin. There was no significant deviation from normal in this fraction.

Alpha-2-globulin. An overall decrease in this fraction was statistically significant at the probability level of 0.001. Between the "normals" and Category I there was as yet no significant difference, but between Category I and Category II a fall was statistically significant at the P = 0.01 level. From Category II to Category III there was a small but not significant rise.

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This change thus coincided with the development of severe anaemia.

Gamma-globulin. Statistically there was a highly significant overall rise of gamma-globulin at P = 0.001 and particularly between "normals" and Cat. I. Between Cat. I and Cat. II the difference was less significant (only at the P = 0.05 level) and between Cat. II and Cat. III not at all.

The rise in gamma-globulin was therefore an early event in babesiosis.

5.6.4 Discussion

The results obtained in this study in which extensive progressive involvement of the liver has been demonstrated, provide an interesting comparison with the findings of Nagel & Katz (1963) in their investigation of electrophoretic changes in benign as against severe cases of acute hepatitis in man. Their total serum proteins showed an immediate fall in both categories while in babesiosis the figure did not vary significantly. This was due clearly to a balancing-out between the albumin and gamma-globulin moieties in this disease, as may be seen from the figures in Table 5.6.3.1.

As was the case in Nagel & Katz's series, there was in babesiosis an early drop in albumin and an equally early rise in gamma-globulin, with this pattern well established from Cat. II cases onward. The appearance of icterus did not affect the issue.

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Maegraith, Gilles & Devakul reported on this point in 1957, when a small series of six dogs artificially infected with babesiosis they found a marked fall of total serum protein only in one instance.

From these findings it seems evident that the biosynthetic activity of the liver in the manufacture of albumin is prejudiced at an early stage of babesiosis and soon stabilizes at a lower level.

Elevation of gamma-globulin in hepatic disease is less easily explained. It is probably not due entirely to antibody formation. Popper & Schaffner (1957) state that gamma-globulin is not formed by hepatic cells but by mesenchymal elements such as plasma cells and reticulo-endothelial cells. The Kupffer cells in the sinusoids and mesenchymal cells in the portal tracts possibly synthesize it, as well as infiltrating histiocytes and plasma cells. Elevation of gamma-globulin in hepatic disorders can be related to stimulation and mobilization of these cell types. These authors also suggest that amino-acids, not being used by the damaged liver for albumin synthesis, become available for gamma-globulin formation. This may explain the reciprocal behaviour between albumin and gamma-globulin, as is clearly seen in the present study.

The Nagel & Katz series showed a rise of alpha-1-globulin in the benign cases of hepatitis, returning to within the normal range in the severe cases, while in babesiosis no significant change was noted. According to them the literature on this subject is controversial,

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with different authors giving high, normal or low figures.

Ceruloplasmin is known to rise in acute hepatitis but the quantitative contribution of this substance to the level of alpha-2-globulin is not important. Haptoglobin, one of its main components, has been found to be very low in the serum of hepatectomized animals (Drapanas, Kluge, Schenk, Schreiber & Stewarts, 1960) as Nagel & Katz also found in their severe cases of hepatitis. The latter authors even concluded that low values, below 0.30 gm/100 ml, bore "grave prognostic significance". In babesiosis there was a distinct overall fall in alpha-2-globulin, notably between Category I and Category II, which was stabilized between Category II and Category III. This was probably due to the counterbalancing effect of the increasing incidence of nephritis in the later stages of the disease. It has been found in the course of another investigation that there is a rise of alpha-2-globulin in uraemia in dogs. These two lesions evidently tend to compensate for each other in Category III.

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Malaria. It has been generally agreed for nearly four decades that in malaria there is, at least during some phases, a lowering of albumin concentration with coincident rise in globulins. Maegraith (1948) reviewed the considerable amount of literature on this subject up to that time. The most recent review found is that of Sadun, Williams & Martin (1966) in which the results of electrophoretic fractionation of serum proteins in human and animal malarias were examined. These showed some discrepancies and irregularities, particularly in respect of the alpha- and beta-globulins but albumin was regularly decreased and gamma-globulin increased.

A brief summary of their findings in two classes of humans (American Military personnel and civilians in Thailand), and those of the present study is given in Table 5.6.4.

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TABLE 5.6.4 SUMMARY OF SERUM PROTEIN CHANGES IN HUMAN MALARIA (P. FALCIPARUM) AND CANINE BABESIOSIS (B. CANIS)

Test	Human Malaria		Canine babesiosis
	U.S. Military Personnel	Thai civilians	Dogs
TSP	Decreased	Increased	Unchanged
Albumin	Decreased	Decreased	Decreased
Alpha-1	Unchanged	Increased	Unchanged
Alpha-2	Decreased	Unchanged	Decreased
Beta	Increased	Unchanged	-
Gamma	Increased	Increased	Increased

Note: Statistically not significant changes given as "unchanged"

Table 5.6.4 shows the substantial degree of agreement between the findings in these two similar diseases, reflecting in the main the response of the various serum fractions to involvement of the liver, common to both.

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5.7 INFLUENCE ON PROTHROMBIN TIME

5.7.1 General Considerations

Prothrombin is a protein constituent of blood plasma and is essential to the process of clotting. It is synthesized by the liver and requires the availability of the fat soluble vitamin K. It follows that its formation is dependent on both adequate liver function and the absorption from the ingesta of vitamin K and that a deficiency could result from hepatocellular damage or a lack of bile salts to emulsify the vitamin K containing fats in the intestinal contents.

There appears to be a fairly wide margin of safety in the prothrombin factor so that the coagulation time as measured by usual methods may remain within normal limits until more than 80 per cent of the blood prothrombin is lost.

For clinical purposes, since there is no direct method of assaying prothrombin quantitatively, it is convenient to determine the "prothrombin time" under rigidly controlled conditions. This gives very satisfactorily reproducible results, unlike determinations like "bleeding time" and "coagulation time" where too many factors can influence the result obtained.

Interest in estimation of prothrombin time in this study of babesiosis arose from two considerations:-

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(a) the frequently observed phenomenon of continued bleeding of Stomoxys calcitrans (stable fly) bites on the ears of dogs ill for some time, and

(b) as a liver function test bearing in mind the great functional reserve of this organ. Normal values are obtained in a considerable number of patients with some degree of hepatocellular damage.

5.7.2 Materials and Methods

Suitable oxalated specimens were collected from 71 dogs in the three clinical categories. The method of determination was the one stage prothrombin time test of Quick, Stanley-Brown & Bancroft (1935). In this procedure calcium is removed quantitatively with potassium oxalate, and the plasma added to a suspension of thrombokinase and calcium (Geigy). The whole process is carried out at exactly 37°C (waterbath) and the time taken from the addition of plasma till it coagulates measured by stop watch. The quantities used are standardized with the exception of the thromboplastin (thrombokinase) which is in excess.

The prothrombin times for the three categories were evaluated statistically as before.

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5.7.3 Results

The scatter of values obtained is presented graphically in Fig. 5.7.3. Mean values (with standard deviations) were respectively 16.9 ± 5.4, 20.05 ± 4.3, and 24.59 ± 6.7 seconds. There was an overall progressive increase in prothrombin time, significant at the P = 0.001 level.

TABLE 5.7.3 STATISTICAL DATA

ANALYSIS OF VARIANCE: "F" RATIO

PROTHROMBIN TIME

Categories	"F"	Degrees of Freedom	Probability	Significance
I - II - III	10.4	DF_1 2 DF_2 68	P < 0.001	High
I - II	5.1	DF_1 1 DF_2 46	0.01 < P < 0.05	Poor
II - III	9.24	DF_1 1 DF_2 52	0.001 < P < 0.01	Moderate

Category II values were higher than those of Category I at the level of P = 0.05 while Category III values were more significantly (P = 0.01) increased over Category II. The statistical data are given in Table 5.7.3.

With few exceptions the Category I values were within the normal range found by the method used.

In several icteric dogs followed serially after specific treatment at intervals of two or three days it was

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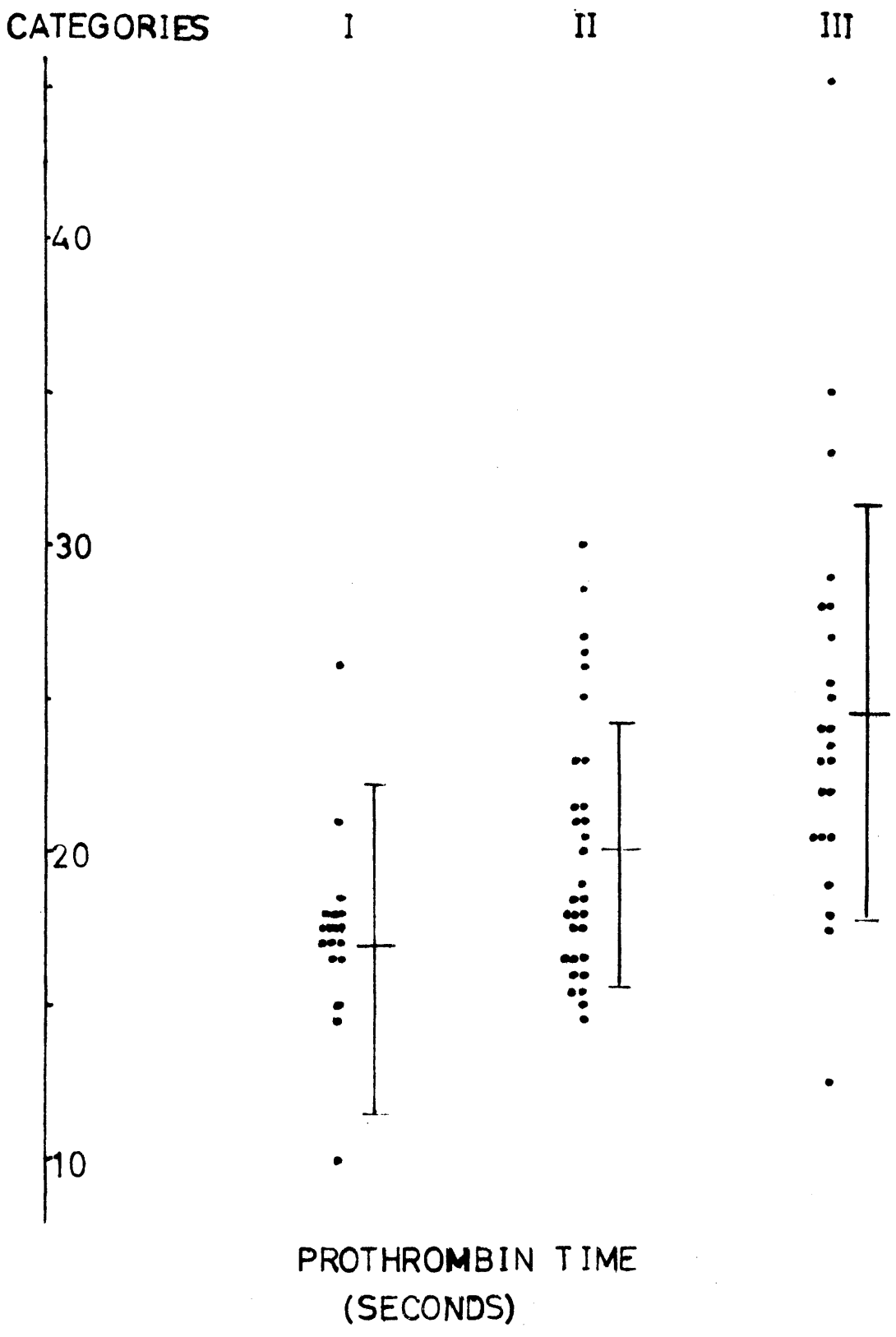
Fig. 5.7.3 THE SCATTER OF PROTHROMBIN TIME VALUES IN THE THREE CATEGORIES OF BABESIOSIS



found that initially high values dropped rapidly to normal even though icterus had not completely faded.

The highest prothrombin time was 45 seconds.

5.7.4 Discussion

The results obtained confirm that prothrombin time determination is not a very good liver function test in that it is relatively insensitive. Higher than normal values do however indicate liver pathology in this series since hepatocellular disease was the only likely reason for higher values.

This insensitivity appears to apply particularly to dogs. Drill & Ivy (1944) found it to be of a lower order of sensitivity, and thus less reliable, than alkaline phosphatase or bromsulphalein retention in detecting liver damage produced by carbon tetrachloride. They quoted work indicating that since the conversion rate of prothrombin was faster in dogs than in man this would compensate to some extent for a deficiency of prothrombin.

This and other studies of the effect of hepatocellular disease on prothrombin time have been reviewed briefly by Hoe (1960) and by Cornelius (1963) and have been assessed on the basis of poisoning by various hepatotoxic substances. In regard to babesiosis only passing reference has been made previously by Malherbe & Parkin (1951).

The fact that an appreciable incidence of delayed clotting has been demonstrated in the present investigation

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tends support to the idea that prolonged bleeding from insect bites on the ears of some affected dogs is due to a deficiency of "prothrombin".

In malaria prothrombin time has received little interest. Maegraith (1948) mentioned the findings of Fredricks & Hoffbauer (1945) and of Diggs (1945) that "no appreciable variations in prothrombin concentration have been recorded during malaria." Finally, the biochemical studies of Sadun, Williams & Martin (1966) did not include this as a measure of liver function.

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5.8 INFLUENCE ON CHOLESTEROL

5.8.1 General Considerations

Attempts have been made for over a century to make use of cholesterol levels in the blood in order to differentiate between the various types of icterus (Flint, 1862). The synthesis of cholesterol takes place for the greater part in the liver, other tissues such as the adrenal cortex, skin, intestine and the aorta also having some potentiality in this respect, though to a much smaller extent. The liver is however, concerned with the esterification of cholesterol (with fatty acids), and with oxidation and excretion.

In obstructive jaundice there is generally an increase of total cholesterol, without, as a rule, any change in the percentage of esters. Esterification is however depressed in hepatocellular disease, and in man Harper (1961) states, the total cholesterol may remain unchanged or even slightly reduced. Generally however, in dogs certainly, a degree of increase has been noted, possibly as a result of an element of obstruction at the cellular level.

Hoe & Harvey (1961) have given normal total cholesterol values as 186 $\pm$ 13.9 (range 110 - 286) for kennel dogs and 258 $\pm$ 36.3 (range 110 - 470) for domestic pets, but these figures seem to be rather high for entirely normal dogs. High blood cholesterol figures also occur in other diseases

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such as hypothyroidism, diabetes mellitus and advanced nephrosis which fact must be taken into consideration where such figures are found. Relative hypothyroidism is quite usual in obese domestic pets so that these could easily extend the range upwards.

The esterified cholesterol percentage is given by Hoe & Harvey (1961) as 65 ± 12 for kennel dogs and 62 ± 3 for household pets which conforms quite well with usually accepted figures.

As a liver function test this is subject to limitations. Hoe & Harvey have stated that it has a certain value in the diagnosis of liver dysfunction. Any dog with a normal total cholesterol and normal ester ratio would be unlikely to be suffering from any condition with liver involvement but, on the other hand, normal total cholesterol coupled with low ester ratio did not in their series necessarily indicate liver pathology. Apart from these there are also the metabolic and dietetic factors previously mentioned to be considered.

In the present study the effect of the infection on total plasma cholesterol in the three clinical categories was investigated. In a smaller series the percentage of esterification was also determined. As these cases were mostly young dogs the question of metabolic changes and biliary obstruction did not enter into consideration. Rises of total plasma cholesterol and the effect on esterification could thus be taken to reflect the progress of liver involvement in the disease in practically all cases.

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5.8.2 Materials and Methods

For the present study determination of total plasma cholesterol was carried out on 75 infected dogs while ester percentages were estimated in 46 of these. Plasma was treated with aldehyde-free glacial acetic acid and the cholesterol in it determined by means of a modified Liebermann-Burchard reaction. This comprises the production of a red colour when acetic acid solutions of certain sterols, including cholesterol, are treated with ferric chloride and sulphuric acid (Zlatkis, Zak & Boyle, 1953). The further procedure was that of MacIntyre and Ralston (1954) as set out by King & Wootton (1956). For fractionation of free and ester cholesterol all the cholesterol was extracted with a mixture of acetone and alcohol. Free cholesterol was quantitatively precipitated out of this extract with digitonin, the esters remaining in solution, and the digitonin precipitate was used to determine free cholesterol. The quantity of esters was derived by difference.

Statistical evaluation was by means of variance ratio "F".

5.8.3 Results

The scatter of results is shown graphically in Fig. 5.8.3. The means (with standard deviations) for the three categories were respectively 189.6 ± 37.8, 226.5 ± 56.7 and 260.9 ± 89.3 mg/100 ml for total cholesterol.

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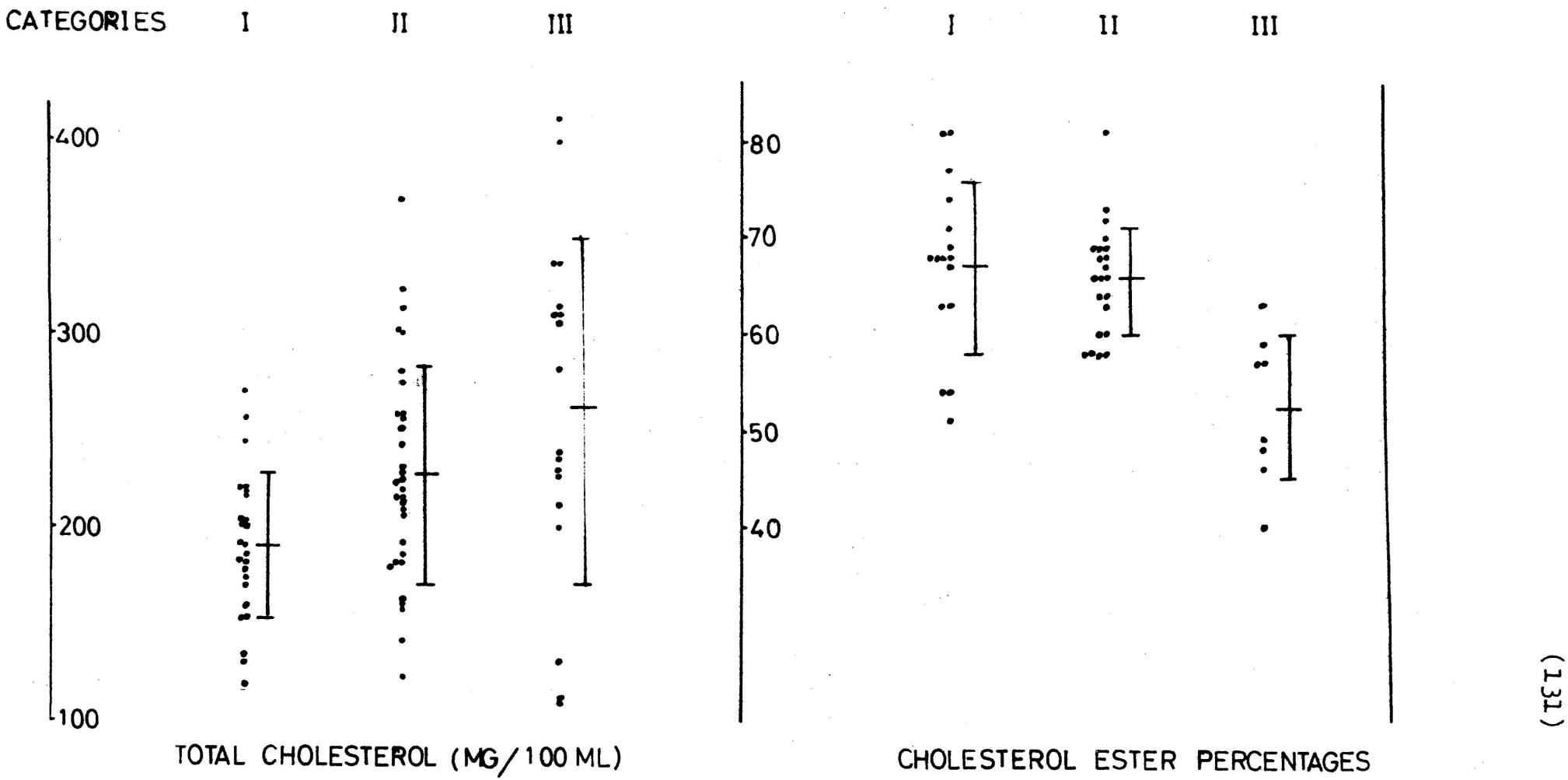
FIG. 5.8.3. SCATTER GRAPHS INDICATING THE DISTRIBUTION OF VALUES FOR TOTAL PLASMA CHOLESTEROL AND THE PERCENTAGE ESTERIFIED IN THE THREE CATEGORIES OF BABESIOSIS



The overall progressive upward trend was very nearly significant at the P = 0.001 level but highly so at P = 0.01. Analysis of variance between Category I and Category II showed a rise which was significant at P = 0.01. Between Category II and Category II however, although the mean rose, the difference was not statistically significant (at P = 0.05) no doubt on account of the very great scatter of values obtained. Statistical data are given in Table 5.8.3.

TABLE 5.8.3 STATISTICAL DATA

ANALYSIS OF VARIANCE: "F" RATIO

TOTAL PLASMA CHOLESTEROL

Categories	"F"	Degrees of Freedom	Probability	Significance
I - II - III	7.23	DF_1 2 DF_2 72	0.001 < P < 0.01	Moderate
I - II	7.85	DF_1 1 DF_2 55	0.001 < P < 0.01	Moderate
II - III	2.78	DF_1 1 DF_2 48	P > 0.05	NONE
CHOLESTEROL ESTER PERCENTAGE				
I - II - III	12.3	DF_1 2 DF_2 43	P < 0.001	High
I - II	0.42	DF_1 1 DF_2 36	P > 0.05	NONE
II - III	26.2	DF_1 1 DF_2 28	P < 0.001	High

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The ester percentages gave means (and standard deviation) of 67.3 ± 8.9, 65.8 ± 5.8 and 52.4 ± 7.8 respectively in the three categories. The overall decrease was found to be highly significant at P = 0.001 which was found to be almost entirely due to the drop between values for Category II and those for Category III (highly significant at P = 0.001).

5.8.4 Discussion

The results of this study show that while there is an early rise of total cholesterol (between Category I and Category II) this is not maintained on passing from the pale to the icteric dogs. The ester percentages, however, reveal a different picture in that in Category II they rise nearly proportionately with total cholesterol while the advent of icterus heralds a severe drop in percentage ester. It is clear then that in the icteric dogs there is well established liver damage evidenced by defective esterification of cholesterol in the liver.

No other studies on cholesterol and its esters in babesiosis have been found in the literature. Maegraith (1948) has however, reviewed the findings in malaria of a number of authors usually on only a few patients. From these it is clear that while there is no regularity about the total cholesterol level the fall in ester percentage is quite a usual finding in cases where the liver is involved.

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Looking at hepatocellular damage, <u>per se</u>, from any cause Cantarow & Trumper (1962) conclude that hypercholesterolaemia occasionally occurs in patients with mild acute hepatitis and cirrhosis but much less frequently than in obstructive jaundice. When this occurs, the increase is usually mainly in the free cholesterol while the ester ratio is diminished. In infectious hepatitis for instance, except in very severe cases, normal values for total cholesterol are the rule but the proportion of esters is frequently reduced.

In this study there is then general conformity of findings in connection with cholesterol metabolism with those of hepatitis in general and with those of malaria in man and animals.

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5.9 INFLUENCE ON PLASMA IRON

5.9.1 General Considerations

Iron is present in the body in small amounts but is vitally necessary for the formation of haemoglobin and of cytochrome and other components of respiratory enzyme systems. Very little is lost from the normal animal and the intake is closely related to the actual requirements of the body. Losses can be increased by any loss of blood or haemoglobin from the body. Whole blood contains about 40 - 60 mg/100 ml of iron while in the plasma the amount is of the order of 50 - 180 mcg/100 ml, bound to a beta-globulin as transferrin.

Low values for plasma iron may result from continuous loss from the body and depletion of the body stores. In man, values are high in pernicious anaemia where diminished haemoglobin formation is not due to iron deficiency, and also in acute hepatitis. The estimation of plasma iron has been used in the differential diagnosis of jaundice. The cause of this elevation in acute hepatitis is not clear but it is attributed to an increased liberation of ferritin from damaged hepatic cells. Intravascular haemolysis for any reason is associated with high plasma values because of the almost thousandfold higher content of iron in the haemoglobin spilt into the plasma.

It seemed likely that since both these latter conditions could be present at different times in babesiosis the

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determination of plasma iron would be of limited value as a liver function test. A small series of determinations was done as a random sampling of the type of result to expect.

5.9.2. Materials and Methods

Iron was estimated in the plasma of 36 clinical cases of babesiosis in the usual three categories.

The method used was that of King & Wootton (1956) in which iron is maintained in the ferrous state with ascorbic acid. A pink colour is produced when this reduced iron is treated with 2:2'-dipyridyl and this is compared colorimetrically with that produced by suitable iron standards.

5.9.3. Results

The variance in each of the three categories was so large that no statistically valid significance could be attached to the small but progressive rise in plasma iron mean values. (See Table 5.9.3). These were (with standard deviations): 251 ± 100, 291 ± 110 and 302 ± 124 mcg/100 ml. This is also clearly evident from the scatter diagram in Fig. 5.9.3.

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FIG. 5.9.3 SCATTER DIAGRAM INDICATING THE DISTRIBUTION OF PLASMA IRON VALUES IN THE THREE CATEGORIES OF BABESIOSIS.

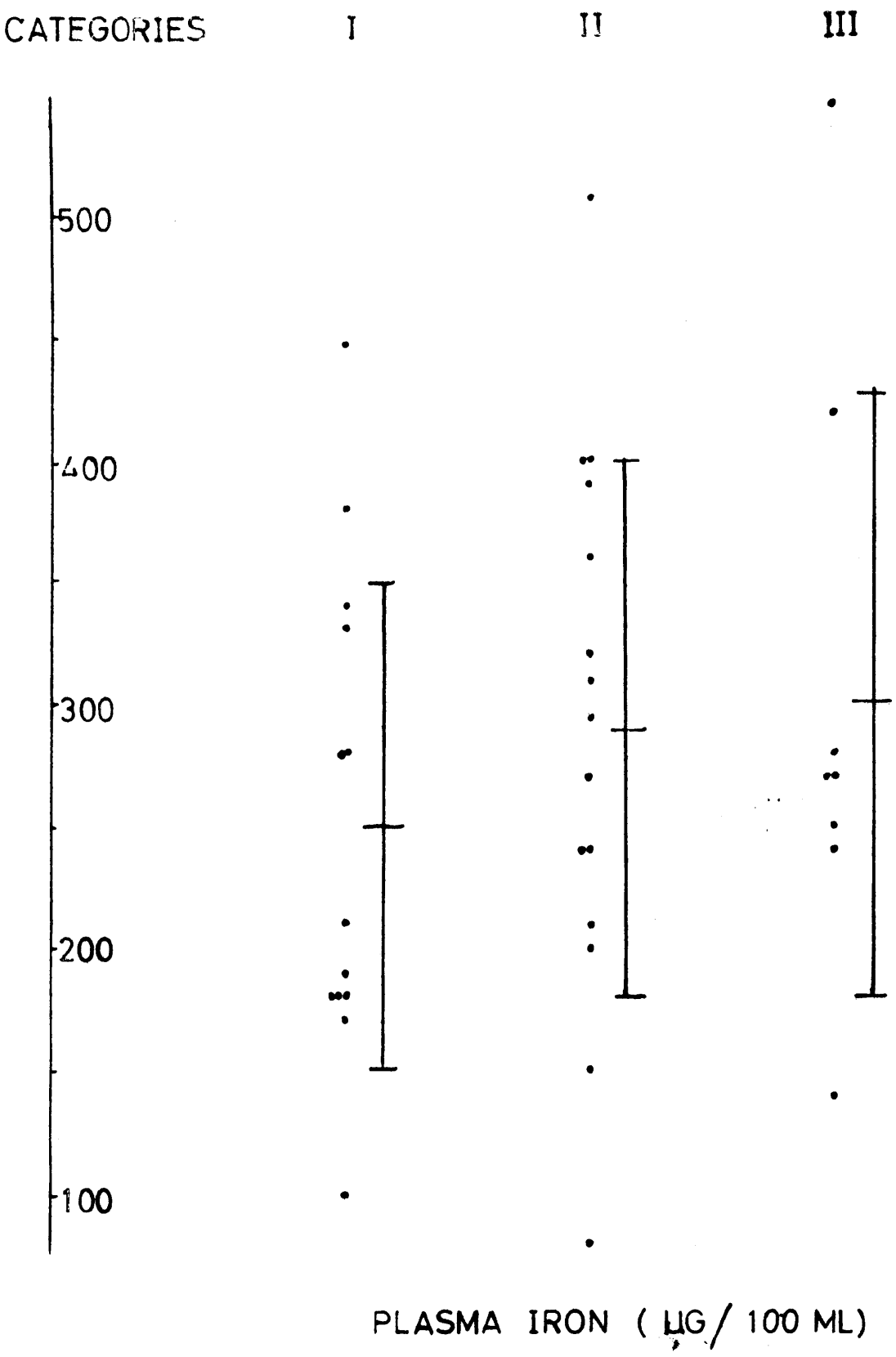

TABLE 5.9.3 STATISTICAL DATA

ANALYSIS OF VARIANCE: "F" RATIO

PLASMA IRON

Categories	"F"	Degrees of Freedom	Probability	Signifi-cance
I - II - III	0.68	DF_1 2 DF_2 33	P > 0.05	None

5.9.4 Discussion

As a liver function test the estimation of plasma iron was beset by the hazards of haemoglobinaemia associated with babesiosis in the more acute cases and or more or less "contamination" of plasma with haemoglobin resulting from the fragility of canine erythrocytes (which happens frequently in perfectly normal blood). There was thus a very wide range of values in each of the three categories and the test results could thus for this purpose be regarded as highly equivocal. While nearly all the Category III values were abnormally high and almost half of those of the Category I were within the normal range, the scatter in all categories was so large as to rob the procedure of any possibility of rejection of the null hypothesis.

Under these conditions the determination of plasma iron was almost predictably valueless as a test of liver function.

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5.10 INFLUENCE ON PLASMA URIC ACID

5.10.1 General Considerations

The determination of uric acid as a liver function test has enjoyed some vogue, although its limitations have always been appreciated as arising out of its lack of specificity. In the dog, hepatectomy is followed by an elevation in the blood and urine uric acid. (Cornelius, 1963). This is on account of the conversion of uric acid to allantoin by the liver in all dogs but the Dalmatian. Hoe & Harvey (1961) however, state that the active enzyme, uricase, is present in this breed in normal amount in the liver and kidneys. They suggest that the excretion of the comparatively small amount of allantoin in these animals may be ascribed to a lowered renal threshold for uric acid with decreased opportunity for oxidation.

There is some uncertainty about the site of formation of uric acid in man. Characteristically it is the end point of purine catabolism and is derived from nucleic acid or nucleoprotein breakdown. The probability is that most of it is formed in the bone marrow, possibly also in the muscles, and perhaps the liver in man and birds (Cantarow & Trumper, 1962). From the blood stream it is normally excreted mainly by the kidneys into the urine, to a lesser extent into digestive fluids, and in small amounts into sweat and urine.

It is considered on theoretical grounds that increases in the concentration in the blood stream are due to one or more of the following factors:

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(a) overproduction

(b) decreased destruction

(c) decreased renal excretion

Rises are found in haematological diseases such as some types of leukaemia, neoplastic disease of the bone marrow and polycythaemia, and also in conditions involving rapid breakdown of tissue. These are probably a reflection of the greatly increased production of uric acid resulting from the enormous increase in the number of short-lived cells. As far as the dog is concerned Cornelius (1963) suggests that the rise in liver disease is due mainly to the lack of conversion to allantoin.

Morgan (1959) however compared uric acid levels with bromsulphalein retention for liver function testing and found the latter much more sensitive and reliable. Hoe & Harvey (1961) concluded from the study of a large series that uric acid was of less value as an index of liver function than serum alkaline phosphatase.

Excretion through the kidneys may be prejudiced by any condition of severe renal functional shutdown and some very high values are found in the plasma in terminal stages of such conditions.

Since, as has been shown elsewhere in this work, both the liver and kidneys are very much involved in babesiosis it was decided to include determination of uric acid figures in a limited series of patients. Moreover there

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appeared to be no previous record of these in the literature of the subject.

5.10.2 Materials and Methods

Specimens for this purpose were collected from 71 dogs and the results evaluated as between the three clinical categories. Determinations were done on plasma by the method of Brown (1945), with modifications from time to time. In this method the phosphotungstic acid reagent was prepared by refluxing sodium tungstate with orthophosphoric acid and water. For colour development sodium cyanide or sodium cyanide plus urea solutions were used.

The three categories were evaluated statistically by means of variance ratio "F".

5.10.3 Results

Fig. 5.10.3. represents a scatter graph of values obtained in the three categories. The means (with standard deviations) were 0.70 mg/100 ml (± 0.26), 1.08 (± 0.59) and 1.55 (± 1.38) for plasma uric acid.

The overall increase of figures was moderately significant at the P = 0.01 level. Analysis of variance between Category I and Category II showed a moderately significant difference (P = 0.01) while, in spite of a few high figures in Category III the difference between Categories II and III were not significant at P = 0.05. The data are presented in Table 5.10.3.

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FIG. 5.10.3 SCATTER GRAPH INDICATING THE DISTRIBUTION OF VALUES FOR PLASMA URIC ACID IN THE THREE CATEGORIES OF BABESIOSIS

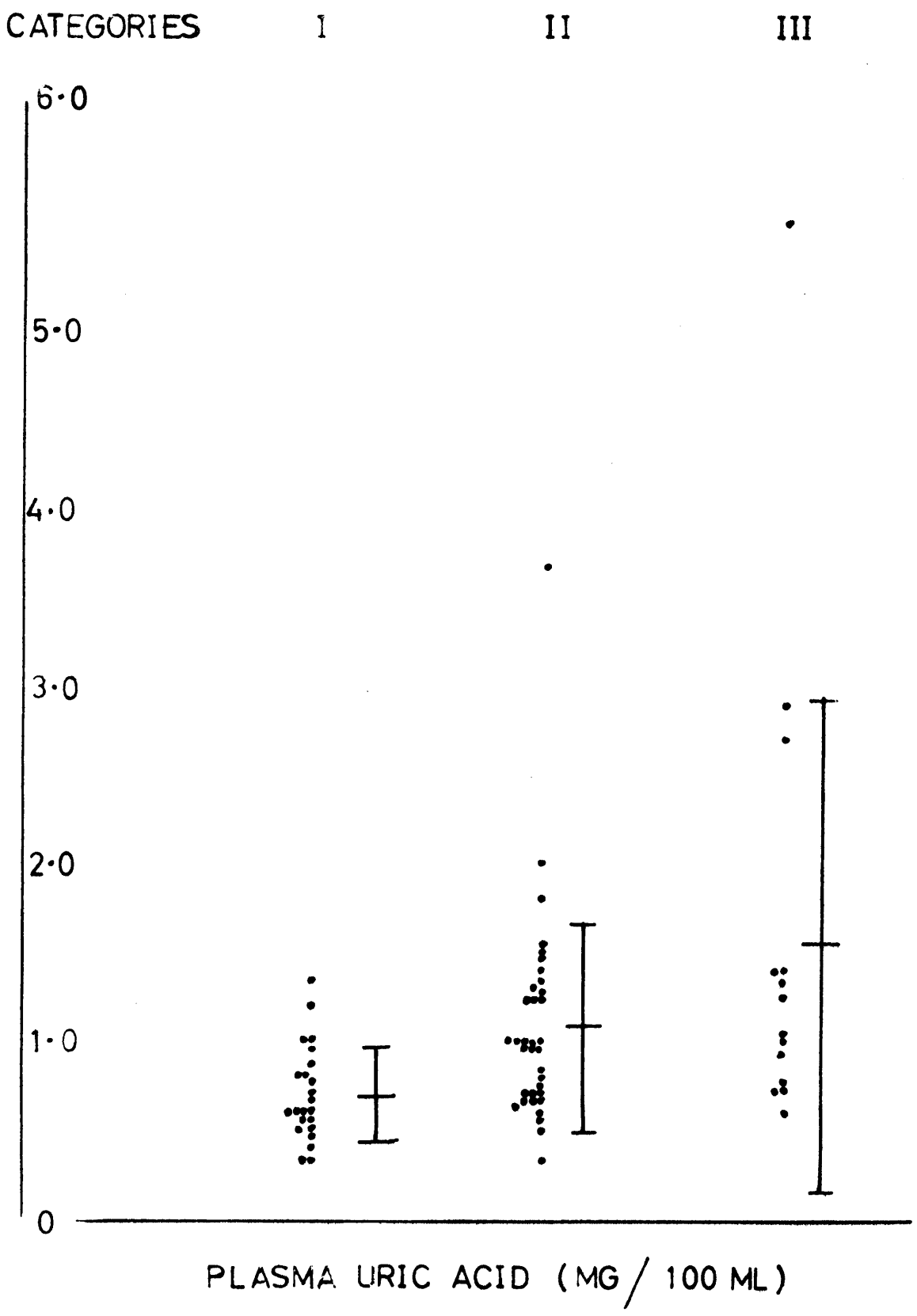

TABLE 5.10 3 STATISTICAL DATA

ANALYSIS OF VARIANCE: "F" RATIO

PLASMA URIC ACID

Categories	"F"	Degrees of Freedom	Probability	Significance
I - II - III	5.61	DF_1 2 DF_2 68	0.001 < P < 0.01	Moderate
I - II	8.00	DF_1 1 DF_2 55	0.001 < P < 0.01	Moderate
II - III	2.80	DF_1 1 DF_2 46	P > 0.05	None

5.10.4 Discussion

When compared with other parameters of liver function plasma uric acid does not give very definitive results. From the scatter graph it can be seen that the majority of values fell within normal limits which Bloom (1957) and Cornelius (1957) agree to place at between 0.1 and 1 mg/100 ml. These also conform with our own accepted figures. Hoe & Harvey (1961) have shown that severe liver damage may be present with no rise in uric acid. They stated that while in 100 per cent of their dogs with jaundice there was raised serum alkaline phosphatase, only 86 per cent showed a raised uric acid.

To illustrate the findings in the present study the abnormally high values are given with other determinations done at the same time in Table 5.10.4. From this it will be

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seen that in some of them the liver was gravely affected but high urea nitrogen values were regularly found. On the other hand the incidence of liver damage is very much greater than these findings would suggest (see the earlier sections). One must thus agree with the workers mentioned in the introduction that uric acid determination is not very useful or reliable as a liver function test. While shedding little or no light on organic function in babesiosis the findings are presented for the record.

TABLE 5.10.4
HIGH PLASMA URIC ACID VALUES WITH OTHER DETERMINATIONS IN INDIVIDUAL CASES

Patient No.	PUA	BUN	SGP-T	BSP	SAP (K-A)	Tot. Br.	Category
83/59	5.6	68.0	436	45.6	172	10.10	III
1748/63	3.66	79.1	-	-	31.7	-	II
1596/63	2.0	38.6	105	-	40.2	3.4	III
49/58	2.9	79.0	-	90.4	-	37.8	III
254/64	2.7	49.7	74	-	15.3	-	III
157/64	1.33	90.0	118	-	23.7	0.3	I

PUA: Plasma uric acid (mg/100 ml)
BUN: Blood urea nitrogen (mg/100 ml)
SGP-T: Serum glutamic pyruvic transaminase (King Units)
BSP: Bromsulphalein retention (per cent)
SAP: Serum alkaline phosphatase (King-Armstrong Units)
Tot. Br.: Total Bilirubin in plasma (mg/100 ml)

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6. THE CLINICAL PATHOLOGY OF THE KIDNEYS IN BABESIOSIS

6.1 Introduction

Involvement of the kidneys during the course of babesiosis was recognized by some of the early authors in this field from simple methods of urine examination. As long ago as 1902 Nocard & Motas noted the presence of albuminuria at all stages of the disease and described the kidneys as being "congested" at autopsy. Graham-Smith in a 1905 study of the morbid anatomy mentioned the finding of albumin and casts in most of his cases. Meyer in 1912 referred to kidneys "which are doubtless primarily affected by piroplasmosis." Damage of kidney epithelia with the presence of casts and protein was further discussed by Fischer & Scheidemann in 1920 and by Contis in 1926. One gains the impression that these findings were of less interest to most authors than the observed presence of bile pigments and of haemoglobin.

Maegraith, Gilles & Devakul in 1957 gave an account of their studies on pathological processes in B. canis infections. They used a highly virulent laboratory strain of the organism and enhanced the severity of infection by passage through at least one puppy before infection of their experimental animals which were of various ages and breeds. In this way some of their cases became oliguric or even anuric and they gave blood urea nitrogen (BUN) figures "commonly ranging from 60 to 90 mg per cent" and

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in one case reaching 290. They stated in connection with urine examination that protein was to be found within 24 hours of the first appearance of parasites in the blood and that it was detectable in the urine in progressively increasing quantities. This was accompanied in the later stages by evidence of tubular damage in the form of granular and hyaline casts. In their cases they observed low urinary volume in severe clinical forms. Oliguria and anuria were usually associated with uraemia and their development was independent of the appearance or otherwise of haemoglobin.

One case described by them showed proteinuria on the second day of overt infection accompanied by granular and hyaline casts on the seventh day. Oliguria (50 ml of urine in 24 hours) developed on the eighth day after inoculation and there was a cessation of urine flow on the ninth and tenth days accompanied by BUN figures of 284 and 214 mg per 100 ml respectively. On the following day urine flow returned to within normal limits (750 - 900 ml per day) and showed protein, some casts and a specific gravity (SG) of 1005. Five days after re-establishment of urine flow the BUN was 100 mg per 100 ml.

Some fulminating experimental cases in puppies showed an irregular degree of renal failure (although kidney involvement was to be seen on urine examination) presumably because the animals did not live long enough.

During the course of the present study urine specimens were collected where it was feasible under the circumstances of presentation of the sick animals, and at other

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times from hospitalized cases. BUN was determined more regularly as one of the battery of tests and the results assessed as between the arbitrary three clinical categories.

6.2 Urine Examination

Urine examination showed the variability that one could expect. Proteinuria was found to be usual and in fact present in all cases brought in as sick animals. The degree, however, was variable and did not show any particular pattern of severity in relation to nitrogen retention, the duration of the morbidity before sampling or to the degree of hepatic involvement. Many hundreds of urine specimens have been examined at all stages of the disease and serve to confirm this view. Table 6.2 gives a random sample of results of urine examinations in a few cases on presentation.

Early evidence of nephritic damage could generally be found in the first category usually in the combination of S.G. tending to be high, casts being rare and renal tubular epithelial cells (RTE) being present in the centrifuged deposit.

In the very anaemic (but not icteric) group the SG was variable and casts and RTE cells were more regularly demonstrated, while in the icteric group frank acute or subacute interstitial nephritis was shown to be present in all cases.

From analysis of urine thus it is clear that the kidneys are involved to an extent which generally tends to be

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TABLE 6.2 URINE FINDINGS IN A SAMPLING FROM THE THREE CLINICAL CATEGORIES

Lab. No.	Age	Sex	Breed	SG	pH	Prot.	Bilir.	RTE	Casts	BUN
CATEGORY I										
12/58	-	-	-	1043	5.5	++++	++++	++	++	-
157/64	12 m	M	Boxer	-	alk	++++	+	++	-	90.0
177/64	12 m	M	Dachs	-	-	++++	-	++	-	16.6
308/64	11 m	M	Spanl.	1040	7.5	+++	++	++	-	12.9
637/64	3 m	M	Alsat.	1038	5.5	+	++++	+		29.4
CATEGORY II										
67/58	-	-	Dob.	1031	6.2	+++++	++++	-	+++	53.4
1748/63	24 m	M	Boxer	-	acid	++++	++++	-	-	79.1
1734/63	12 m	M	Point.	-	acid	++++	+++	+	++	22.1
18/64	18 m	M	Dachs	1045	acid	+++	+++	+	++	22.1
21/64	10 m	M	Boxer	1013	5.0	++	++	-	+	42.3
254/64	7 m	M	Boxer	-	alk.	++++	++++	-	+	49.7
265/64	2 m	M	NDS	-	acid	+	++++	+	-	95.6
CATEGORY III										
6/58	8 y	-	-	1024	5.0	++++	+++	-	++++	60.8
9/58	-	-	NDS*	1013	5.5	+++	+++	-	++++	38.6
13/58	-	-	-	1009	5.5	++++	++++	-	+++	31.3
32/58	-	-	NDS	1025	acid	++++	++++	-	+++	88.3
49/58	-	-	Bull	1022		+++	++++	-	++++	79.0
54/58	-	-	NDS	1040	6.3	++++	+++	++	++++	51.5
299/64	18 m	M	Boxer	1020	4.5	++	++++	++	++++	75
254/65	18 m	M	Fox. T.	1028	5.5	+++++	+++++	+	+	50

*Nondescript breed.

more advanced as the disease progresses in time and severity.

6.3 Blood Urea Nitrogen

For the purpose of evaluation of kidney function as distinct from the foregoing evaluation of the clinical state it was not feasible to subject patients to lengthy clearance procedures. BUN determination was selected in spite of its limitations as a suitable parameter for indicating deteriorating kidney function during the process of and reflecting the intensity of effect of the Babesia infection.

Nitrogen retention in renal disease depends in general on the nature and extent of the renal lesion and upon extrarenal factors including prerenal deviation of water (oedema, vomiting, diarrhoea or fever) and excessive protein catabolism and/or deficient anabolism (Cantarow & Trumper, 1962). Oedema is only an occasional feature of the disease (see section 2.4.2). Vomiting and diarrhoea are not seen very often. Most dogs are off their feed typically, so that, with fever generally present, both excessive catabolism and defective anabolism would be operative. This would however apply to all cases and the effect is unlikely to be of the order of magnitude found in renal disease. The kidney lesion thus with due allowances for the above and for circulatory disturbances such as found in medical shock could be regarded as the agent mainly responsible for nitrogen retention.

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BUN was estimated by the titrimetric procedure of Hench & Aldrich (1926) based on the mercury combining power of blood. This procedure is derived from the principle that mercury combines with such products as urea, creatinine and uric acid when a solution of a mercuric salt is added to a solution containing these nitrogenous substances (in this case a protein free filtrate of blood) up to the point that the mercury combining power is satisfied. The excess mercury then appears in the solution and is demonstrated by the appearance within three seconds of a reddish brown, powdery precipitate when a drop of the solution to be tested is added to a drop of saturated sodium carbonate solution on a white spot plate.

While this method is not as accurate as the urease procedure it is sufficiently so to meet with clinical requirements and has therefore gained wide acceptance in clinical laboratories.

In the present study the results obtained were put into the three clinical categories and the values plotted on a scatter diagram. See Fig. 6.3.

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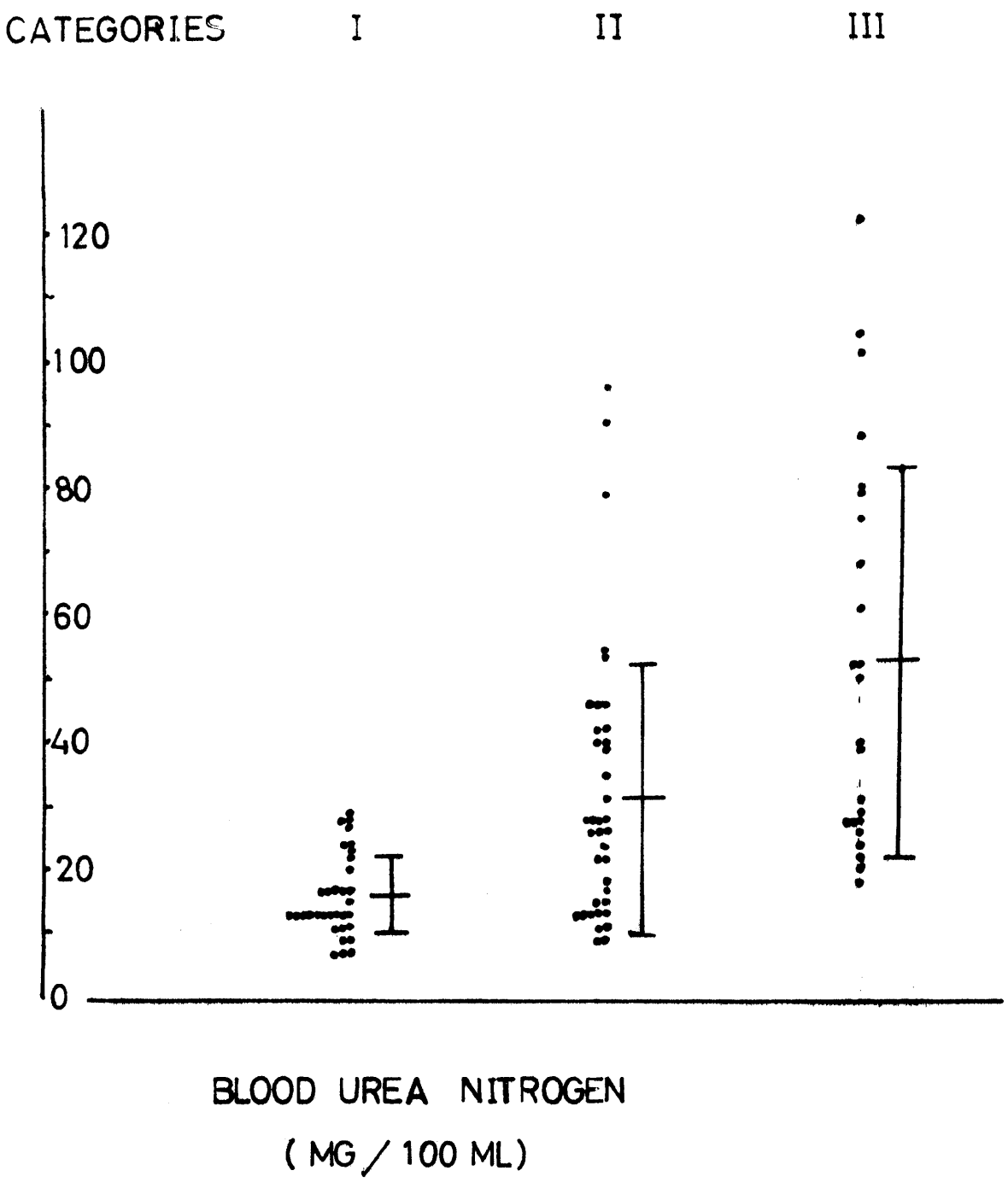
FIG. 6.3 SCATTER DIAGRAM INDICATING THE DISTRIBUTION OF BLOOD UREA NITROGEN VALUES IN THE THREE CATEGORIES OF BABESIOSIS



The majority of values found in the 34 early acute cases were within the normal range, usually taken to have an upper limit of about 20 mg/100 ml. Figures between 20 and 25 are not usually regarded as being particularly abnormal for dogs (Sion, 1966). There was one very high result (90 mg/100 ml) in a dog originally placed in Category I. As it was however not found to be typical of the group it was left out of consideration. The animal was a young one in an advanced state of medical shock with massive haemoglobinuria and severe parasitaemia. In spite of blood transfusion and specific treatment it died with a matter of hours.

The mean for Category I was 15.9 (SD 6.4), well with the normal range.

In the second category (39 cases) with simple severe anaemia, the tendency to higher values is clear with somewhat less than half of them falling within the normal range. The highest figure was 96 mg/100 ml. The mean at 31.3 (S.D. 21.2) was above the normal range.

Very nearly all the animals in the icteric (third) category showed nitrogen retention from mild to very severe degree. The mean BUN of the 24 cases was 52.7 (SD 30.4).

Analysis of variance (see Table 6.3) between all three groups showed a highly significant rise (at P = 0.001) as the disease progressed. Between Categories I and II the rise was highly significant at the same level of probability while the wide scatter of values in Category III left the rise highly significant at P = 0.01 but falling slightly short of the P = 0.001 level.

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TABLE 6.3 STATISTICAL DATA

ANALYSIS OF VARIANCE: "F" RATIO

BLOOD UREA NITROGEN

Categories	"F"	Degrees of Freedom	Probability	Significance
I - II - III	22.6	DF_1 2 DF_2 96	$P < 0.001$	High
I - II	16.6	DF_1 1 DF_2 71	$P < 0.001$	High
II - III	10.9	DF_1 1 DF_2 61	$0.001 < P < 0.01$	Moderate

6.4 Discussion

The above findings for BUN in South African field cases show a distinct qualitative trend. While it is conceded that in circumstances of lesser or greater virulence the quantitative picture is not completely regular it was here seen from urine examination that in early acute cases there was already evidence of kidney involvement without prejudice to kidney function. In severely anaemic animals the functional integrity of the kidneys in more than half was disturbed while in icteric animals nitrogen retention was a regular feature.

It has been estimated that the kidneys are able to function normally with 66 to 75 per cent of the original complement of nephrons out of action. This functional reserve thus allows the first category cases to reflect BUN values mostly within the normal range when urine analysis already shows evidence of renal involvement. As the disease progresses

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however the reserve becomes depleted and a progressive rise of BUN is evident.

Generally speaking, although the role of nephritis is considerable and is an important part of the disease picture, it is in the majority of cases reversible, requiring no particular treatment after specific babesiacidal treatment. In animals, however, that have suffered so severe a metabolic insult that they make a tardy recovery, kidney lesions may be exacerbated or may themselves contribute to the slow recovery. In older dogs, already approaching the border-line of kidney decompensation before contracting the infection the prognosis becomes very doubtful on account of loss of residual reserve capacity. They often die in spite of otherwise successful specific therapy. The proximate cause of death, then, unlike the great majority of cases of babesiosis, would be uraemia.

Supportive treatment aimed at this fact should certainly be instituted immediately when it becomes evident from BUN determination and urine examination that this is necessary. The development of metabolic acidosis as a result of renal failure also contributes to the necessity of such measures, and its recognition and correction would well be lifesaving in cases that fail to respond to specific therapy in the normal way.

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6.5 Comparison with Malaria

After a review of findings of many workers in the field of the different types of malaria Maegraith (1948) stated that "there is a considerable difference of opinion amongst workers on many features of acute renal involvement in malaria, but certain points emerge from the mass of apparently contrasting evidence, upon which there is general agreement. Most authors, for instance, have found that acute, as distinct from chronic, renal symptoms are commonest in Plasmodium falciparum infections." This is explained on the basis that this type of malaria tends to be acute and to act more vigorously and that circulatory and other changes of a general nature are most active in P. falciparum infections. Maegraith moreover quotes observations to the effect that renal failure accounts for half the deaths in blackwater fever, and states that apart from the manifest presence of haemoglobin and its derivatives in the latter form, the lesions of blackwater fever are identical in type and form, if not always in degree, with those of acute malaria. In fact a definite point is here made that tubular dysfunction may precede or follow haemoglobinuria and that this fact provides strong presumptive evidence of the nonspecific nature of its origin. It was concluded after exhaustive scrutiny of the evidence that large quantities of haemoglobin may pass through the kidney without inflicting serious damage to the renal tissue and without apparent interference with kidney function. He

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further discounted any great role of malarial parasites in the kidneys (generally scanty), of malarial "toxins" or parasitic metabolites, and of changes in the electrolyte concentration and body water balance, and concluded the most likely possibilities to be (i) that the cellular changes arise from a defect of function specific to the part of the nephron involved, or (ii) that localized tissue anoxia is developed and gives rise to nonspecific degeneration and necrosis. He suggested the development of a change in intrarenal blood flow and thus oliguria or anuria. General anoxaemia and tissue anoxia would follow on the loss of erythrocytes by parasitic action, loss of haemoglobin by degradation, and intravascular agglutination. Any incidence of shock would in itself interfere with the flow of blood through the glomeruli.

Holliday (1965) in a review of the causes, characteristics and management of acute renal failure in general, mentioned among common aetiologies haemolobinuria "such as seen in malaria (blackwater fever)" and shock resulting in severe reduction in renal blood flow and the consequent subjection of tubular cells to anoxic damage and acute tubular necrosis.

A group of workers in Thailand in 1967 (Sitprija, Indraprasit, Pochanugool, Benyajati & Piyarata) described acute renal failure in blackwater fever as well recognized and quoted Maegraith, Havard & Parsons (1945) as ascribing pathogenesis to renal anoxia. Maegraith & Findlay (1944) had suggested that the blood flow through the renal cortex

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might be bypassed into the medullary vessels and the subcapsular plexus, so aggravating the anoxia of tubular cells. Sitprija et al. were particularly interested in three malaria patients with no evidence of intravascular haemolysis, hypotension or shock. These had their renal function return to normal after antimalarial treatment. During their febrile period they had blood ureas ranging from 82 to 194 mg/100 ml and creatinin ranging from 2.08 to 3.65 mg/100 ml although biopsies revealed unremarkable histology, and urinary findings did not deviate notably for normality. They tended to the view that decreased renal blood flow during the period of heavy parasitaemia could explain renal failure at that time. In this respect they were in substantial agreement with Maegraith and co-workers cited above but considered that the physical presence of parasitized red cells and "sludging" merited further investigation. They conceded finally that if their patients had not been treated immediately acute tubular necrosis might have developed.

In a recent paper Berger, Birch & Conte (1967) stated that though renal tubular disease in falciparum malaria as manifested by haemoglobinuric nephrosis ("blackwater fever") was a well-established phenomenon, the occurrence of significant glomerular dysfunction was not well recognized. They did however, review such evidence as there was in the literature dating from 1898 on the subject of nephritis, and described their findings in three cases out of some 150 evaculated from Vietnam and

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who had received specific treatment in the war zone. They were studied clinically, biochemically and by renal biopsy when they relapsed in the United States. Characteristic laboratory findings included haematuria, marked proteinuria cylindruria and azotaemia (BUN up to 154 mg/100 ml) with marked impairment of creatinine clearance. The nephrotic syndrome was evidenced by protein excretion in urine in excess of 3.5 g/24 hr with hypoalbuminaemia.

Structural abnormalities of the glomeruli were present in the biopsy specimens obtained. These were non-specific in nature and resembled those seen in poststreptococcal glomerulonephritis. There was a complete lack of evidence of any antecedal streptococcal infection.

Although this series was small it tended to confirm the findings in a number of scattered reports, notably that of Spitz (1945) on the pathology of acute falciparum malaria. Gilles & Hendrickse (1963) and Edington (1967) have implicated P. malariae as the most common parasite producing the nephrotic syndrome in African children in West Africa, much more so than in the case of falciparum malaria.

As long ago as 1944 Maegraith & Findlay were stressing the non-specific nature of the lesions produced. They stated that they were similar to those seen in the crush syndrome, incompatible blood transfusions, concealed retroplacental haemorrhage, trauma of labour, excessive vomiting associated with pyloric stenosis, alkaline treatment and cholera.

The disease processes of malaria and babesiosis are so similar that in this case too one can conclude that they tend secondarily to produce similar lesions, essentially of non-specific nature.

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7. THE HAEMATOLOGY OF B. CANIS INFECTION

7.1 General Considerations

Babesia canis being a blood parasite, invading and multiplying in the red blood corpuscles and ultimately destroying them, exerts its most immediate effect in that medium. The earliest observers showed interest in the extent to which anaemia was produced in the disease. In 1902 Nocard & Motas presented by the standards of that period an exhaustive study on the haematology of canine babesiosis. Macroscopically, they noted that the blood became "pale, as if diluted with water"; coagulation was delayed; the clot was softer and paler than normal; the serum was pale to dark red as the disease progressed; and the impression was gained the erythrocytes had become much more fragile. In subacute cases serum exuding from the clot changed from pink to deep yellow with at times a greenish tint. Red blood corpuscles from citrated blood sedimented down to 1/5, 1/10 or even 1/15 of the total blood volume. From a normal red cell count of 6½ to 7 million the decrease was comparatively slow until the "haemoglobinuric crisis" when a precipitate drop brought the figure down to less than 2 million per cu mm. Haemoglobin values fell correspondingly from 12 to 13 per cent down to 6, 4 and 3½ per cent.

The white cell count was on the contrary increased to two, three or four times the normal figure, polymorphs being responsible for the greater part of the increase.

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This observation obtained in the more chronic forms of the disease. Anaemic changes were stated to include anisocytosis, polychromasia and nucleated erythrocytes, also more markedly in the protracted forms. Mononuclears were in these forms also found to be more frequent.

Galli-Valerio (1904) and Nuttall (1904) in later reviews of the disease provided no new information but quoted Nocard & Motas in extenso on their haematological data. Wright shortly afterwards (1905) published some haematological findings (based on a study of three dogs) and found them to be in substantial agreement. Christophers in 1907 identified up to 26 per cent of the leucocytes as monocytes.

Barratt & Yorke in a 1910 study investigated the question of whether haemoglobinuria was the result of haemoglobinaemia or not, and concluded that in fact it was. Haemoglobin appeared in the urine only when the plasma concentration exceeded 0.5 per cent (at which level there was some appreciable pinkness). As this threshold was exceeded in the plasma the urine haemoglobin appeared and rose sharply. They also determined what they called the haemoglobin: volume ratio i.e. the amount of haemoglobin in unit volume of erythrocytes. In the early part of the infection they found no change in the ratio, but there was an increase towards the end. A decrease was occasionally noted. Haemoglobin appeared in the plasma only upon extensive destruction of erythrocytes and not before. These standards of comparison have long since been superseded by more definitive ones, the Wintrobe indices.

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A decade later (1920) Fischer & Scheidemann described in acute cases investigated in China the usual anaemic and regenerative changes but in addition a hyperleucocytosis varying from slight to a leucaemic level. "Reiz" forms of lymphocytes were noted in varying frequency and also the presence of myelocytes.

Contis (1926) in describing cases encountered in Greece, also mentioned anaemic changes and leucocytosis. "Monoculears" were found to be increased in frequency up to about 12 per cent. These were mostly "large mononuclear lymphocytes". Small lymphocytes and neutrophils were not increased. He regarded the large mononuclears or large lymphocytes as transitional forms. In some cases he found eosinophiles to number up to 8 per cent. He also made mention of immature leucocytes and myelocytes.

Sanders in 1937 found a "varying degree" of leucocytosis and implicated chiefly polymorphonuclears in the increase.

In 1940 Landsberg & Eskridge offered the first study on the actual anaemia of babesiosis using modern methods of evaluation. They compared the physical and haematological changes in splenectomized and entire animals and found virtually no qualitative difference. Erythrocyte sedimentation rate was shown to be markedly accelerated. The corpuscular constants of Wintrobe (1934) did not show any particular trends during the course of the infection and they regarded the anaemia as being normocytic. This was in accord with the findings of Wintrobe (1961) in malaria. He regarded the

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haemolytic anaemia of this disease to be a case of "acute blood destruction", one of the accepted causes of normocytic anaemia.

Reticulocytes increased during the latter half of the infection as anaemia developed, reaching a figure of 23 per cent. Splenectomized dogs had a greater percentage of infected erythrocytes than those of similar age with spleens.

Landsberg & Eskridge also entered the lists with Eaton (1934), Simons (1939) and others on the question of special predilection of Babesia for reticulocytes. Eaton had postulated an "special predilection of reticulocytes" as he had found in cases of malaria he had studied, but Simons, who studied this question on three dogs found reticulocytes refractory to infection. He found this most noticeable as the blood in his cases showed a strong reticulocytosis and a considerable degree of infection, and he never saw a reticulocyte with a parasite.

Da Silva in 1944 described "target" cells, up to 17 - 22 per cent of all red cells. He demonstrated what he called a characteristic decrease of red cell fragility.

Maegraith, Gilles & Devakul (1957) could not confirm findings of Shirlaw (1938) and of Da Silva as to fragility and found saline fragility to be within normal limits at all stages of the disease. As far as mechanical fragility was concerned an appreciably increased effect was found only in haemoglobinaemic animals and during the last two or three days of fatal infection. They however, regarded the method

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of testing to be extremely artificial, the results probably bearing little relation to the situation in vivo.

In the same paper Maegraith et al. reported on leucocyte findings in a series of ten experimental dogs. Eight of these showed leucocytosis of up to 30,000 per mm^3 while in two leucopenia was found (resembling the leucopenia commonly found in malaria). They did not comment on the percentage composition of the cells.

In a broad review of diagnostic features of babesiosis, a French worker Jacquin in 1963 commented on the lack of unanimity as to the leucocyte picture as revealed in the literature up to that time. He did not come to any final conclusions on the subject.

As it appeared that natural field cases might well shed some further light on aspects of haematology that had been the subjects of disagreement a number of specimens were collected from dogs presented with babesiosis at various stages. A few dogs were also artificially infected in order to throw light on some controversial points.

7.2. Materials and Methods

Blood was collected, initially in "mixed" oxalates and later in EDTA (disodium ethylene diamine tetra-acetate) as anticoagulants and subjected to haematological examination. These specimens were all from diagnosed cases of babesiosis and the results were evaluated generally on the basis of the three clinical categories.

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The techniques used in the studies included the following:-

(a) Erythrocyte sedimentation rate. Freshly collected blood was placed in a standard Wintrobe haematocrit tube up to the 100 mm mark and this was set up exactly vertically and left for one hour. The amount of sedimentation could then be read off directly on the left hand scale. The results were recorded as mm/hr (Wintrobe) and no correction for anaemia was attempted.

(b) Haematocrit or Red Cell Volume. Immediately after ESR was complete the Wintrobe tubes were centrifuged at 3000 r.p.m. in MSE "Minor" clinical centrifuges for 30 minutes. It was known from experience that packing was complete for canine blood after this procedure. Results were expressed as a percentage.

(c) Red cell count. Counting was carried out as soon as possible using Hayem's solution as a diluent and Bürker-Turck or Spencer Bright-line counting chambers. Results were recorded as millions per cu mm.

(d) Haemoglobin concentration. In the early part of the work haemolysis was produced with 0.1 per cent sodium carbonate solution. Difficulty with standardization was experienced and later the much more acceptable cyanmethaemoglobin method was used (Drabkin's solution). Standardization with British Drug Houses cyanmethaemoglobin standard solution made determinations somewhat more accurate. Results were

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expressed as grams per 100 ml.

(e) Red cell fragility. A slight modification of the photometric method of Clark (Brown, 1963) was used. 0.5 per cent sodium chloride solution was found to be suitable for canine blood. Fragility in this solution is expressed as percentage haemolysis by comparison with complete haemolysis produced by the same volume of whole blood in distilled water. Volumes of 0.1 ml of heparinized blood were added to 10 ml of 0.5 per cent saline and to 10 ml of distilled water for the "test" and "standard" respectively. These were read in a colorimeter at 540 millimicrons against a distilled water blank after standing for ten minutes, and centrifugation.

(f) Reticulocyte count. The "wet" method was used, with slight modifications. One or two drops of blood were well mixed for about 15 seconds with an equal quantity of 0.1 per cent brilliant cresyl blue in 0.85 per cent sodium chloride solution on a watch glass, and kept covered for five minutes with a petri dish. From this a thin film was prepared in the ordinary way, air dried, fixed, and lightly counterstained with Giemsa solution. The percentage of reticulocytes was counted under an oil immersion lens in about 500 to 1000 cells with the aid of an Ehrlich ocular (which provides an adjustable square area for examination).

(g) Leucocyte or white cell count. The same haemocytometers (as for red cell count) were used after dilution with 2 per cent acetic acid solution to lyse the red cells,

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plus gentian violet, to facilitate visualization.

(h) Differential leucocyte count. Smears were prepared from fresh or newly collected anticoagulated (EDTA) blood by the method of Neser (1923). According to this procedure an elliptical smear is made by dropping a coverglass onto a small drop of blood on a slide, and sliding it very gently along the long axis of the slide. With practice sufficient skill and gentleness is developed to cause a minimum of distortion of white cells, and with the very much better distribution of white cells more accuracy is achieved than by customary methods. (It has for many years been the favoured method at the Onderstepoort Veterinary Research Institute). For staining the Romanovsky type Giemsa's stain was used after fixing with pure methanol or May-Grünwald solution.

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7.3 Results

7.3.1 Erythrocyte Sedimentation Rate (ESR)

It is evident from inspection of the scatter graph presented as Fig. 7.3.1 that there was a very great variation in the figures obtained within each of the three categories. Among the whole lot there were only very few normal values, usually taken to be up to about 5 or 6 mm/hr. A rise of ESR was thus a very early event in babesiosis. It was further evident that with the development of anaemia the ESR figures were generally higher. The means for the three categories (with calculated standard deviations which were notably large) were respectively 36.9 ± 27.6, 56.6 ± 26.7 and 66.8 ± 26.3 mm, thus a progressive rise. Analysis of variance overall showed significance at the P = 0.001 level, while similar analysis between categories showed a significant rise between Category I and Category II (P = 0.01) and no significant difference between Category II and Category III. See Table 7.3.1.

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FIG. 7.3.1 SCATTER DIAGRAM INDICATING THE DISTRIBUTION OF ERYTHROCYTE SEDIMENTATION RATE LEVELS IN THE THREE CATEGORIES OF BABESIOSIS

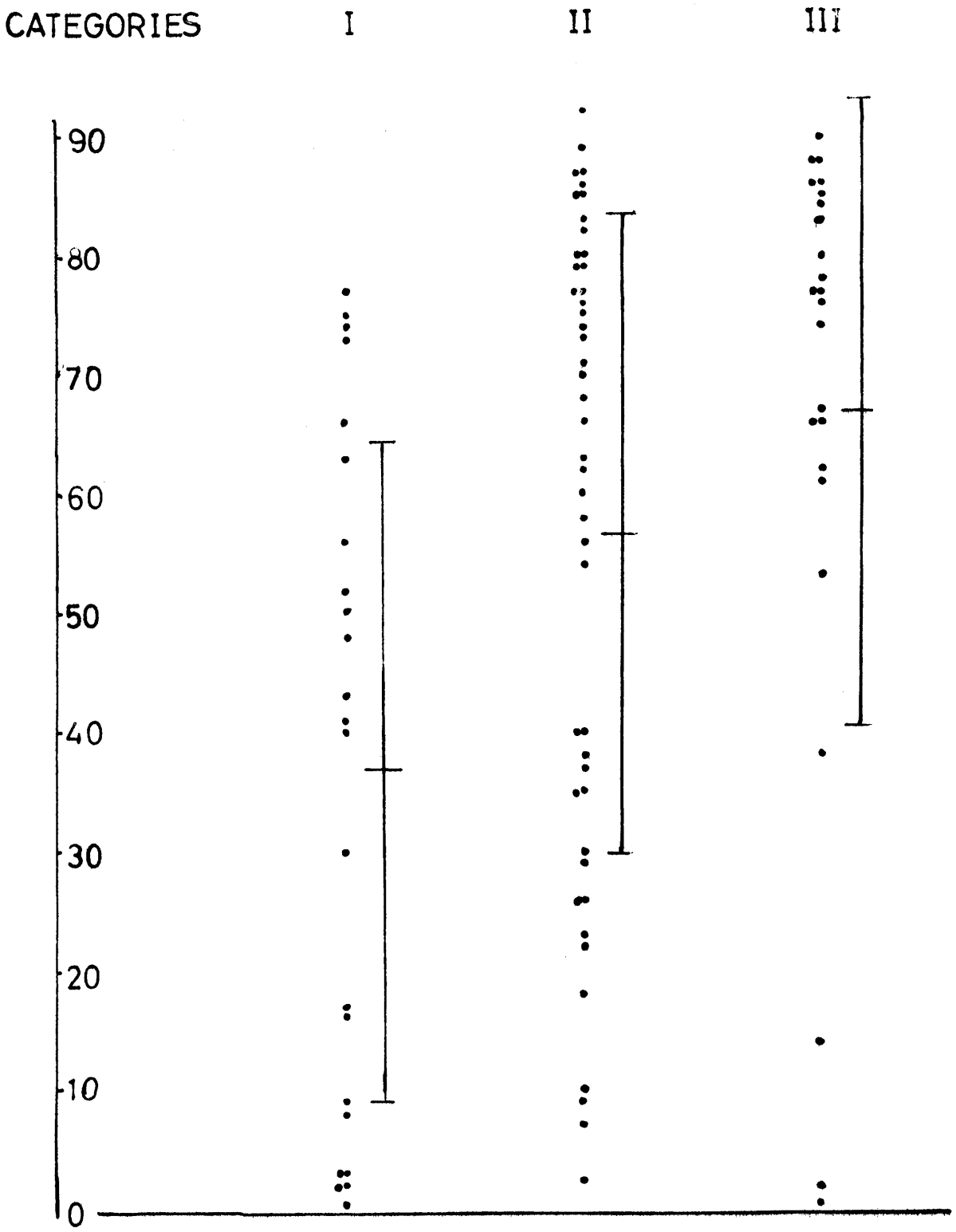

TABLE 7.3.1 STATISTICAL DATA

ANALYSIS OF VARIANCE: "F" RATIO

ERYTHROCYTE SEDIMENTATION RATE (WINTROBE)

Categories	"F"	Degrees of Freedom	Probability	Significance
I - II - III	7.71	DF_1 2 DF_2 91	$P < 0.001$	High
I - II	8.1	DF_1 1 DF_2 67	$0.001 < P < 0.01$	Moderate
II - III	2.3	DF_1 1 DF_2 69	$P > 0.05$	None

7.3.2. Haematocrit, Red Cell Count and Haemoglobin.

These were not graphed or compared since they constituted in a general way the frame of reference for subdivision of the categories, certainly for the first two, while Category III values, though generally reflecting severe anaemia, showed some variability. Icterus was observed on a number of occasions in dogs where the red cell count was in excess of 4 millions/ cu mm.

The figures obtained for about 99 field cases however, rendered examination of the type of anaemia by means of the Wintrobe indices possible.

The mean corpuscular volume for the three categories (with S.D.s) was 63.8 ± 14.0, 79.1 ± 16.4 and 72.3 ± 14.4 cu μ. Analysis of variance showed that an overall increase was significant at P = 0.001. This difference was greatest between Categories I and II ($P < 0.001$) and there was a small drop

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in the mean from Category II to Category III. When Categories I and III were compared it was found that there was stillan increase in mean corpuscular volume, but at a much lower level of significance viz. $P < 0.05$. See Table 7.3.2

TABLE 7.3.2. STATISTICAL DATA

ANALYSIS OF VARIANCE: "F" RATIO

MEAN CORPUSCULAR VOLUME

Categories	"F"	Degrees of Freedom	Probability	Significance
I - II - III	8.07	DF_1 2 DF_2 96	$P < 0.001$	High
I - II	15.22	DF_1 1 DF_2 70	$P < 0.001$	High
I - III	4.53	DF_1 1 DF_2 49	$0.05 > P > 0.01$	Poor
Overall according to Haematocrits	0.33	DF_1 1 DF_2 96	$P > 0.05$	None
MEAN CORPUSCULAR HAEMOGLOBIN				
I - II - III	7.4	DF_1 2 DF_2 94	$0.001 < P < 0.01$	Moderate
I - II	12.2	DF_1 1 DF_2 69	$P < 0.001$	High
I - III	3.8	DF_1 1 DF_2 48	$P > 0.05$	None

These results suggested that upon extensive destruction of existing red cells by the parasites there was a transient inflow of larger, less mature cells, followed by a tendency for the mean cell size to return nearly normal later in the disease.

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In order to examine the exact type of anaemia the results were rearranged into three groups representing the MCV figures in different degrees of anaemia where the haematocrits were 20 - 45 per cent, 12 - 19 per cent and 6 - 11 per cent. (It will be recalled that there was quite considerable variation in haematocrit in the icteric animals). It was now found that the means were 72.8 (±16.8), 72.5 (± 17.0) and 75.7 (± 15.5). Analysis of variance showed that the null hypothesis could not be rejected and that the anaemia was in fact normocytic.

These two arrangements are presented as scatter graphs in Fig. 7.3.2.1. The normocytic character of the anaemia is evident from these.

Mean corpuscular haemoglobin (MCH). An examination of these produced substantially similar results. Comparing the MCH values in the three categories there was a significant rise in Category II over I (at $P < 0.001$) while between II and III there was a small decrease. The means (with S.D.) were respectively 23.2 ± 5.6, 28.5 ± 6.0 and 25.9 ± 4.0 $\mu\mu$g. Rearranged according to the recorded haematocrits the means for the three degrees of anaemia were 26.3 ± 6.6, 25.5 ± 5.0 and 28.0 ± 5.4 $\mu\mu$g, with no significant differences. The two scatter arrangements are graphically illustrated in Fig. 7.3.2.2. which reflects a pattern similar to that for MCV. Statistical data appear in Table 7.3.2.

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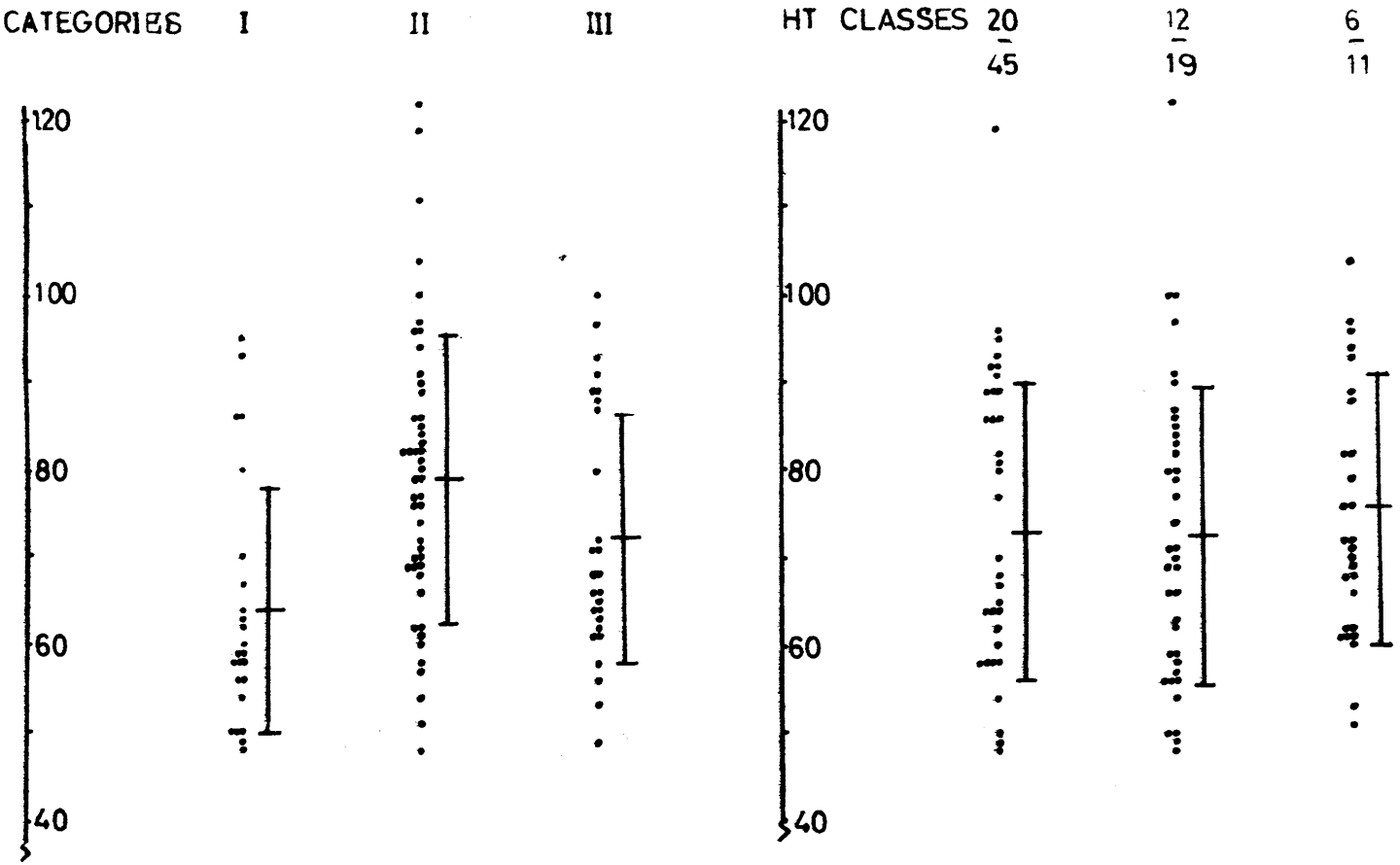
FIG. 7.3.2.1 SCATTER DIAGRAMS INDICATING MEAN CORPUSCULAR VOLUME VALUES ARRANGED ON THE BASIS OF THE CATEGORIES OF BABESIOSIS AND ON THAT OF THREE LEVELS OF HAEMATOCRIT VALUES

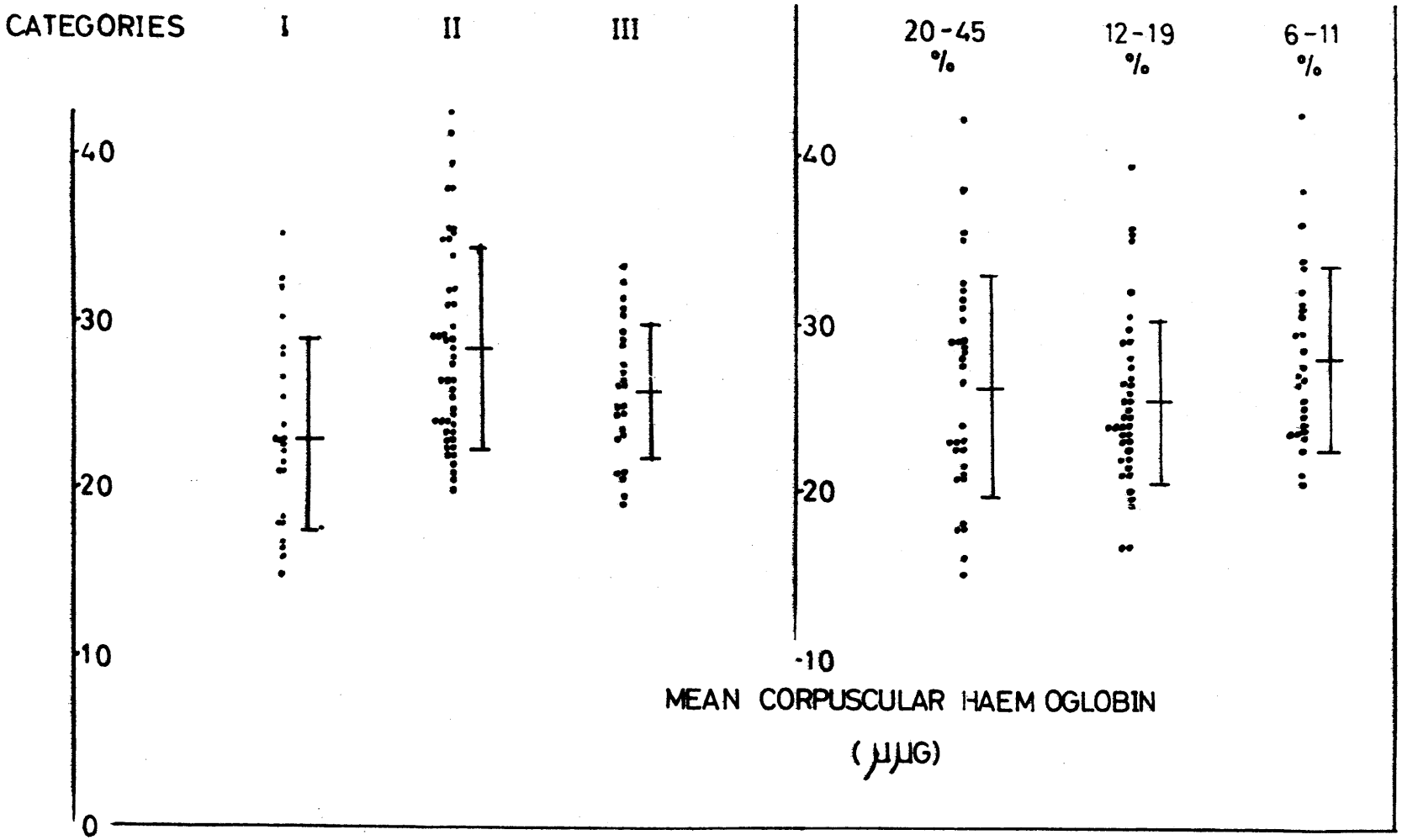


MEAN CORPUSCULAR VOLUME

COMPARED AS TO CATEGORIES

COMPARED AS TO HAEMATOCRITS

FIG. 7.3.2.2 SCATTER DIAGRAMS INDICATING MEAN CORPUSCULAR HAEMOGLOBIN VALUES ARRANGED ON THE BASIS OF THE THREE CATEGORIES OF BABESIOSIS AND ON THAT OF THREE LEVELS OF HAEMATOCRIT VALUES



Mean corpuscular haemoglobin concentration (MCHC). Here the means were so close together in the three categories, viz. 36.3, 36.7 and 36.3 per cent that further evaluation statistically and graphically was deemed unnecessary. Clearly the concentration of haemoglobin in the cells was not affected by the anaemia. The essentially normocytic and normochromic nature of the anaemia with only a transient macrocytosis was thus confirmed.

7.3.3. Osmotic fragility. The use of field cases for investigating fragility of red cells to hypotonic saline did not seem to be very promising since information as to the actual duration of illness was unlikely to be reliable. A few dogs were therefore artificially infected and saline fragility determined at two day intervals (three times weekly).

A dog which turned out to be immune gave a mean fragility percentage of 35.6 ± 12.5. A second had eight fragilities done before the first rise of temperature and they averaged 39.4 ± 7.7. At one determination after parasites appeared it was 25.2 per cent, a distinct drop in fragility, and after that it rose above normal to average 58.1 ± 7.9 during the further course of illness. Two other dogs gave a similar pattern and the figures obtained are given in Table 7.3.3.

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TABLE 7.3.3. PERCENTAGE OSMOTIC FRAGILITY BEFORE AND DURING INFECTION

Dog No.	Before clinical symptoms	First few days of infection	During established infection
3/68	25.6 ± 12.5 (9)*	-	-
4/68	39.4 ± 7.7 (8)	25.2 (1)	58.1 ± 7.9 (4)
13/68	41.5 (1)	28.2 (1)	69.1 ± 11.6 (4)
14/68	-	19.5 (3)	68.8 ± 11.6 (3)

*The bracketed figures indicate the number of determinations at two to three day intervals.

These few cases were insufficient for any definite conclusions but did seem to suggest that there was increased resistance to erythrocyte rupture for a few days immediately following the first rise of temperature and appearance of parasites in the blood, after which fragility was increased above the normal level for the remainder of the disease process.

Incidentally, case No. 13/68 died from babesiosis and case No. 14/68 became chronic with repeated exacerbations and remissions till it was treated after some five weeks.

7.3.4 Reticulocyte percentages. These were followed in a few cases artificially infected for the same reason as in section 7.3.3. Judging by these the pattern appeared to be just about what could be expected. In an acute case which died from babesiosis there was a moderate rise of reticulocyte percentage some days after clinical symptoms started, followed by a drop a day or two before the animal died, presumably from a degree of bone-marrow depression. In a more chronic case there was a marked increase during the period of severe anaemia and for a few days after treatment after which it subsided to normal levels with the return of the haematocrit to normal.

7.3.5. Leucocyte Count or White Cell Count (WCC). The figures obtained were arranged into classes constituting the three categories embracing 96 field cases of babesiosis. As in many other determinations of this study there was considerable variation in figures within the categories but the overall tendencies were clear. The means were 10.98 $\pm$ 3.7, 12.08 $\pm$ 5.9 and 22.80 $\pm$ 18.1 thousands per cu mm. The overall rise was statistically highly significant at P = 0.001. See Table 7.3.5. Between the first two categories however, with the variation as large as it was, the rise was not significant. The major rise was however, between Category II and III where the difference was highly significant at the P = 0.001 level. Clearly the marked leucocytosis developed comparatively late in the clinical development of babesiosis. Even here it will be apparent from the graph

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in Fig. 7.3.5.1 that in Category III about two thirds of the values were not unlike those of Category II and Category I in range. The overall difference is then brought about by the increasing incidence of greatly elevated WCC figures with the course of the disease.

TABLE 7.3.5. STATISTICAL DATA

ANALYSIS OF VARIANCE: "F" RATIO

LEUCOCYTE COUNT

Categories	"F"	Degrees of Freedom	Probability	Significance
I - II - III	10.87	DF_1 2 DF_2 93	$P < 0.001$	High
I - II	0.70	DF_1 1 DF_2 69	$P > 0.05$	None
II - III	13.81	DF_1 1 DF_2 70	$P < 0.001$	High

NEUTROPHILE COUNT

I - II - III	6.9	DF_1 2 DF_2 37	$0.001 < P < 0.01$	Moderate
I - II	0.01	DF_1 1 DF_2 27	$P > 0.05$	None
II - III	8.3	DF_1 1 DF_2 26	$0.001 < P < 0.01$	Moderate

It was evident that the rise was largely due to neutrophilic granulocytes. These were therefore calculated from the percentage differential counts as absolute values, and examined separately. The results obtained were qualitatively similar. The means were in order of categories 6592 ± 3807, 6733 ± 3217, 24,203 ± 24,950 per cu mm. The great scatter

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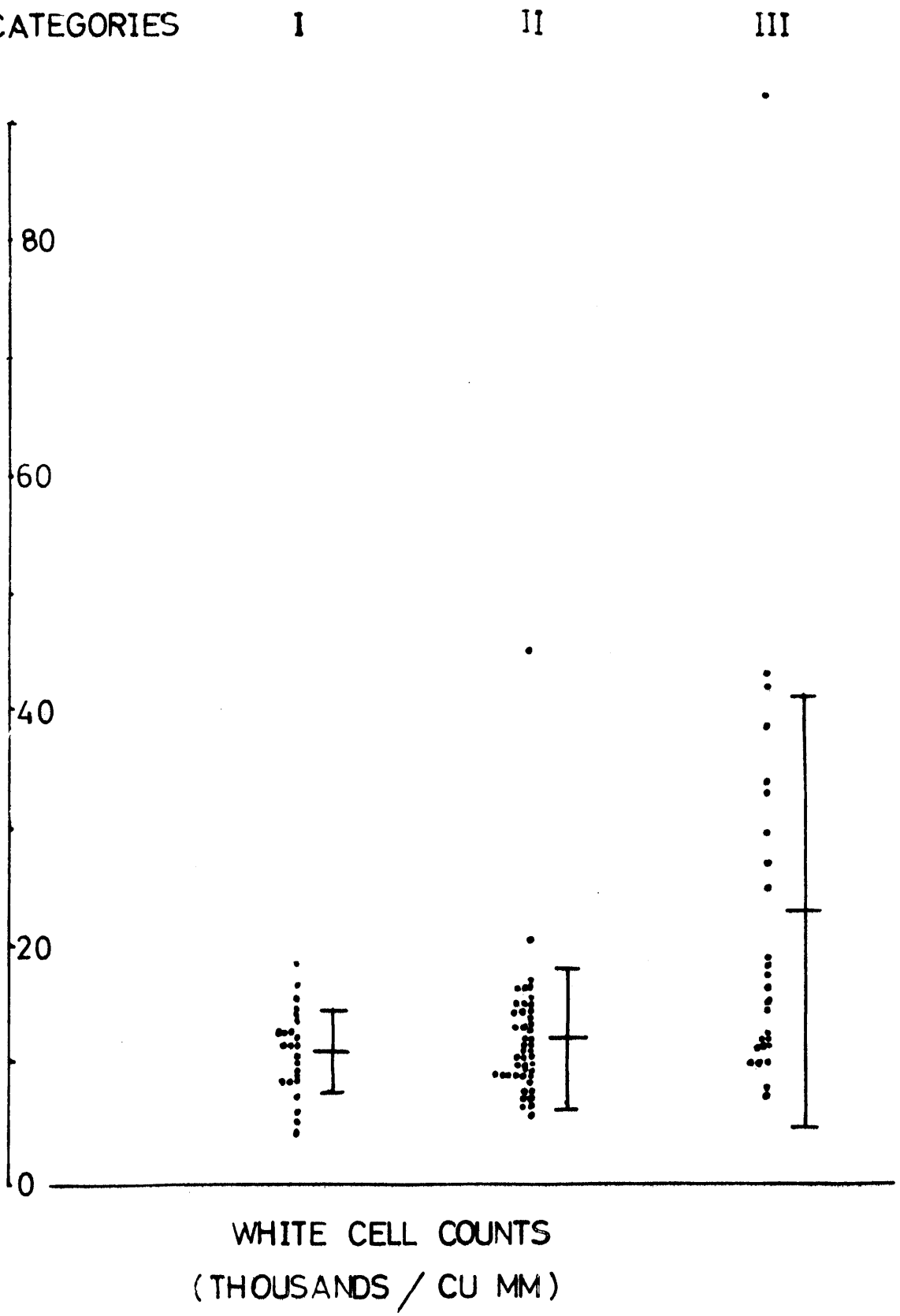
FIG. 7.3.5.1. SCATTER DIAGRAM

INDICATING THE DISTRIBUTION OF WHITE CELL COUNT

FIGURES IN THE THREE CATEGORIES OF BABESIOSIS



of values within categories militated against a high degree of significance, but, nonetheless the overall rise was significant at P = 0.01 with the rise between Category I and II not significant and that between Category II and III significant at P = 0.01. The scatter of absolute neutrophile figures is illustrated in Fig. 7.3.5.2.

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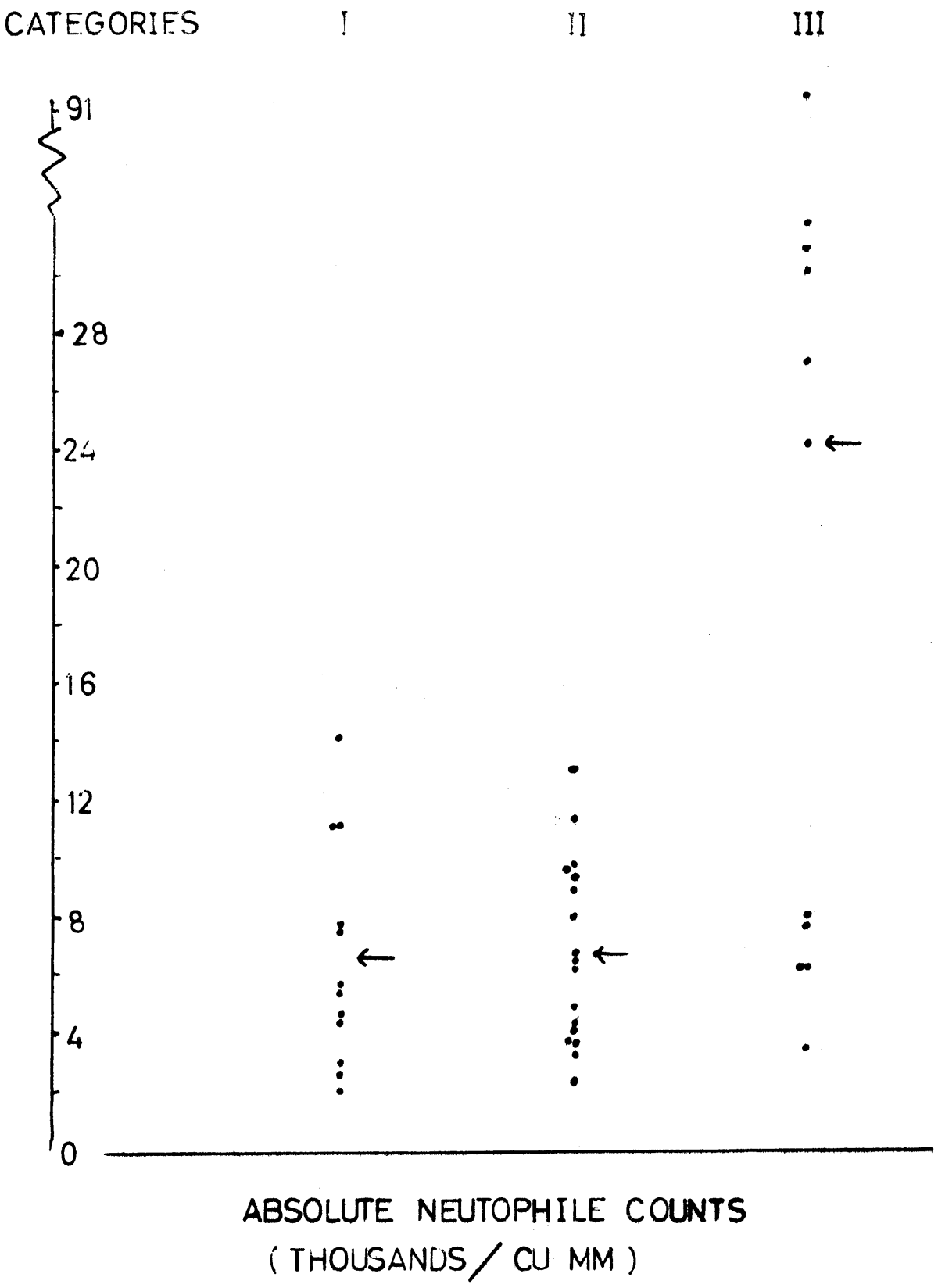
FIG. 7.3.5.2. SCATTER DIAGRAM INDICATING THE DISTRIBUTION OF CALCULATED NEUTROPHILE COUNTS IN THE THREE CATEGORIES OF BABESIOSIS



8. DISCUSSION AND CONCLUSIONS

In canine babesiosis, as Maegraith (1963) has pointed out in connection with malaria, infection gives rise to pathological consequences both locally at the point of host-parasite contact (i.e. in the infected erythrocyte) and remotely, in the other organic changes resulting to a greater or lesser degree from the primary insult. Clinically, the original parasite-host contact will lead to destruction of the invaded red cell and of nonparasitized red cells and the process may be terminated with a return to normality by specific babesiacidal chemotherapy.

In probably a majority of field cases treatment is delayed for various reasons and the disease progresses directly or indirectly to the remote pathological changes, which in sum represent babesiosis in its various potentialities and manifestations. The emphasis placed in the lay mind on icterus in the popular names given to the disease bears testimony to the frequency of such further pathology.

The anaemia derived from erythrocyte destruction does not in itself constitute an important pathogenetic factor until it becomes very pronounced. Until it reaches that stage there is probably little disturbance of the carriage of oxygen to the tissues. Local vascular disturbances may however, lead to anoxia within the tissues at a fairly early stage. The histopathology described in section 2.6.2 makes it clear that in virtually all organs there is dilatation and congestion of capillaries and other small vessels. Even in the presence of severe anaemia they are

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engorged and stagnated with parasitized and uninfected red cells. Maegraith (1963) has presented evidence of dynamic interference in intralobular blood flow in the liver (based on X-ray angiography) leading to a relative centrilobular haemostasis. He further quoted work providing evidence of severe reduction of the respiratory activity of liver cell mitochondria and stated that the biochemical lesion represented in fact a failure of oxygen acceptance and utilization leading to histotoxic anoxia, with severe local effects in the form of centrilobular necrosis.

Maegraith (loc. cit.) speculated on the nature of an active pharmacological factor circulating in the blood during malarial infection. Was it a "toxin" liberated by the parasite, or did the infection lead in some way to the release of non-specific substances with appropriate biochemical activity? He inclined to the latter belief, proof of which awaited further research.

In the present study field cases in three clinical categories of babesiosis were subjected to evaluation of liver and kidney function as well as of their haematology.

In early acute cases bromsulphalein retention was not affected unless there was an element of shock interfering with the flow of blood through the liver. There was however a highly significant rise of BSP retention with the development of severe anaemia and a still greater rise with the appearance of icterus. Serum glutamic pyruvic transaminase, a sensitive

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index of hepatic cellular damage or necrosis in the dog, showed a rise in some of the Category I cases but thereafter it was progressive all the way through at a high level of significance. Serum alkaline phosphatase did not provide clear evidence of hepatic functional impairment in the early stage of the disease. Some animals in the second category (severely anaemic, not icteric) showed marked rises while the icteric category showed consistently high values. Total bilirubin was little affected in Category I and was generally (if present) largely unconjugated as a result of the haemolytic process. There was some elevation of total bilirubin in Category II at a moderately significant level, with rises chiefly in the unconjugated moiety, and in Category III there was a highly significant rise, with the conjugated bilirubin generally well above half of the total. The picture presented showed the increasing contribution of liver damage as the disease progressed, with haemolysis providing a relatively decreasing percentage of total bilirubin.

An examination of serum proteins by electrophoresis showed the general situation that while total protein was in general not significantly increased or decreased albumin fell progressively during the course of the disease. Alpha-1-globulin remained unchanged whereas alpha-2 was decreased with the development of anaemia. Gamma-globulin was markedly increased at a very early stage of the disease, suggesting the possibility of a prompt manufacture of immune bodies, and, if currently investigated concepts of auto-immunity are substantiated, auto-immune bodies.

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The frequently observed incidence of prolonged bleeding, resulting from insect bites on the ears of dogs, stimulated investigation of prothrombin time in babesiosis. It proved to be a relatively insensitive as a liver function test, but an appreciable increase during the course of the disease, particularly in many individual cases, tended to support the idea that a lack of "prothrombin" was in fact responsible for delayed clotting seen in some dogs bitten on the ears by biting flies.

Cholesterol determination did not shed much light on the development of liver damage as far as the total content was concerned but ester percentages dropped very significantly with the development of icterus.

Plasma iron determination showed a very considerable scatter of values in all three categories and proved to be valueless for assessment of liver function. This applied also to plasma uric acid estimation which has enjoyed some vogue as a liver function test.

Ample evidence has thus been presented of liver damage, starting from a very early stage of the infection and progressing steadily in the untreated animal to the stage of overt icterus. Central degeneration and necrosis of hepatic cells, dilated and stagnant sinusoids, heavily phagocytic Kupffer cells, and a peripheral zone comprising cells varying from normal to highly degenerated ones according to the progress of the disease, have been well documented, notably by Maegraith, Gilles & Devakul (1957). This group have postulated some interference with or even cessation of

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intralobular flow with resultant starvation of liver cells of oxygen from the central vein outwards. They believe that the evidence collected points to a dynamic obstruction to blood flow arising from active constriction of the venous tree in the liver and that the circulation is not impeded mechanically by swollen liver cells or by macrophages.

The situation concerning involvement of the kidney was investigated by means of urine examination and determination of blood urea nitrogen. Urinalysis showed up evidence of some renal insult in the earliest stages of the disease but the very anaemic patients showed a rise of BUN in about half the cases and the incidence of icterus was regularly associated with nitrogen retention, sometimes at a very high level. No evidence was found that haemoglobin played any regular part in this process. It seems clear that an interference with the blood flow of the kidneys is more probably the operative mechanism. Local histotoxic anoxia as a result of this could very likely, as in the liver, lead to degeneration and necrosis of the tubular cells and the development of interstitial nephritis. Shock, leading to oliguria in peracute cases, could play a part in early death.

In the study of the haematology of the disease a number of interesting points emerged. The erythrocyte sedimentation rate was very variable but showed an increase right at the earliest stage of infection and a highly significant rise with the advent of icterus. Overall, the rise was progressive during the entire course of the disease, but it was clearly not due solely to the development of anaemia.

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Determination of the red cell count, haemoglobin and haematocrit permitted calculation of the Wintrobe indices. When the three categories were compared it was found that at a stage after massive destruction there appeared to be a temporary inflow of larger young cells from the bone-marrow. This is quite usual according to Wintrobe's 1956 text, not only in a disease like malaria but in any sudden blood loss. When all the mean corpuscular volumes were rearranged into three groups according to haematocrit the anaemia was found to be normocytic. The findings for mean corpuscular haemoglobin showed essentially the same pattern and analysis of the mean corpuscular haemoglobin showed that the concentration of haemoglobin remained between 36 and 37 per cent at all stages of the disease. Taken over nearly 100 patients thus the statement of Maegraith, Gilles & Devakul (1957) to the effect that the reduction in red cell count was usually associated with a roughly proportional fall in haemoglobin level in the blood could be confirmed. Ewing & Buckner recorded a similar finding in 1965. The anaemia of babesiosis is thus normocytic and normochromic, which is the usual effect of sudden blood loss or sudden blood destruction.

Saline osmotic fragility did not exactly follow the pattern suggested by Shirlaw (1938) or Maegraith et al. (1957) although a different method of assessment was used. Shirlaw reported increased fragility in B. canis infection whereas Maegraith and co-workers in their series did not find any change to outside the physiological range. Bohr (1946) in a study of erythrocyte fragility in acute infectious hepatitis

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in man found that it was consistently decreased in this disease. He found that the presence of bile pigment in the plasma had nothing to do with changes in fragility and that in fact the composition of plasma did not affect the matter. It thus seemed as if there was some change in the erythrocytes that was responsible for their increased resistance to haemolysis. Maegraith in his 1948 text went extensively into the question of the mechanism of haemolysis in view of the published papers up to that date and reached no final conclusion. This will be commented upon further on.

In the few cases where fragility was studied in babesiosis the results suggested a decrease in fragility during the first few days after the appearance of parasites followed by fragility increased above normal limits for the remainder of illness. The significance of this would have to await further study of possibly elaborated substances or changed properties of red cells which could lead to exacerbation of haemolysis (as discussed by Maegraith).

Red blood cells appear to be destroyed in two ways in babesiosis as in malaria, by haemolysis as a result of the presence of parasites and by greatly enhanced phagocytosis. The cumulative weight of evidence supports the present view that acquired haemolytic anaemia is an auto-immune disease. The suggested mechanism (Zuckerman, 1963) is that parasitized red cells may be sufficiently altered as to become auto-antigenic, with the result that auto-antibody is elaborated. Both parasitized and unparasitized red cells could become

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opsonized or coated with antibody and then become subject to removal from the circulation by macrophages of the blood filtering organs, most notably spleen and liver, this process thus leading to "excessive" anaemia.

This issue has however, been clouded by the accidental finding that the antiglobulin test becomes positive in any anaemia (even in the absence of any infective agent) and that it is associated with the presence of large numbers of reticulocytes.

This work is proceeding in connection with malaria and there is much reason to believe that findings will be found to apply equally for babesiosis. It was remarked by Zuckerman in 1964 that "it is recognized that reasoning is often by analogy and that analogy is not proof. However, the persistent recurrence of analogous observations may ultimately prove to have a significance of its own".

There has been a fair amount of unanimity in the literature of babesiosis that it is associated with leucocytosis. This has been shown to apply over a large number of cases in the present investigation and to constitute the most usual pattern. The occasional cases of leucopenia could well be the result of bone-marrow depression in very acute disease, although the possibly again of auto-immune sensitization of white cells has been suggested by Zuckerman (1964).

A matter which has not enjoyed unanimity is the identity of "large mononuclears" seen in babesiosis. Morphologically they can be followed back from neutrophiles as

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myelocytes and metamyelocytes, but on occasion the same can be said about the lymphocytic series. Consultation with an experienced cytologist (Gerneke, Onderstepoort, 1968: personal communication) revealed that to settle this matter would require an investigation of its own employing histochemical techniques. Purely on the basis of morphology the writer (and technicians concerned) were inclined to identify them as slightly to very immature cells of the neutrophilic series.

The actual cause of death in babesiosis has been briefly discussed by Maegraith and co-workers (1957). They recognized that it depended much on the duration of illness. In ordinary acute cases they ascribed it to respiratory failure with gasping and extensor spasms. In more protracted cases they implicated circulatory failure, usually associated with pulmonary oedema. They felt that these two modes of dying were fundamentally different as shown by the differing response to intravenous noradrenalin. In a shocked puppy with circulatory failure the response was quite spectacular whereas in the acute respiratory failure cases there was no effect whatever.

To these causes of death however must be added the role of the liver and the kidneys. This work has shown the great potentiality for damage of these organs by the disease. If animals live long enough, these in themselves, with associated metabolic and acid-base upsets (eg. severe metabolic acidosis in uraemia), become the proximate causes of death. This is seen frequently in the clinic when a dog is presented two or three days after specific treatment with the complaint that it has not recovered at all.

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Investigation in these cases of liver and kidney function usually reveals failure of one or other or both of these organs. Treatment then has to be directed towards the particular syndrome concerned. Anaemia itself is normally treated by blood transfusion, shock with nor-adrenalin, the liver with lipotropic agents and the kidneys with glucose-saline infusions (plus sodium lactate or bicarbonate as required for acidosis). Life-saving treatment thus becomes feasible only after the necessary clinico-pathological evaluation.

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9. SUMMARY

This thesis presents a broad survey of all important aspects of Babesia canis infection in dogs. In view of its diverse symptomatology the disease has been comprehensively investigated with particular emphasis on the effects of the infection on the liver, the kidneys and the haematology since these have been found to have a important influence on clinical events and on the type of supportive treatment to be instituted.

For experimental material major use was made of a large number field cases presented for diagnosis and treatment at the Outpatient Clinic of the Faculty of Veterinary Science at Onderstepoort. These were clinically assessed as belonging to one or other of three categories of severity and suitable specimens collected for laboratory evaluation. In a few instances dogs were artificially infected to clarify certain points but were on account of the artificiality of conditions not regarded as the best clinical material.

It was found that infected dogs could die from various causes: medical shock in peracute cases (particularly young dogs), liver failure, kidney failure, heart failure and occasionally pulmonary failure. Different combinations of these could be operative, and they could play an important part in morbidity and mortality even after elimination of parasites by appropriate specific chemotherapy.

The better understanding thus gained is designed to

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serve as a guide to what clinico-pathological examinations are required and thence to life-saving supportive treatment.

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