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BIOCHEMICAL STUDIES ON GEELDIKKOP AND ENZOOTIC ICTERUS

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DEDICATION

To my parents

CONTENTS

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Appendices:

SUMMARY

The haematology, chemical pathology, disturbed biochemistry, general enzymology and histopathology of geeldikkop, enzootic icterus and icterogenin intoxication in sheep are presented in full for the first time. The relationship of geeldikkop to enzootic icterus is demonstrated and both diseases are shown to be the result of sudden catastrophic failures of membrane permeability in various tissues of the body. The conditions are thought to be two different manifestations of a single disease entity, possibly low grade selenium intoxication. The acute manifestations are precipitated by various forms of non-specific stress the effects of which, on sheep from the areas where the diseases are enzootic, are described. One of the most important factors in this regard is thought to be a virus infection similar in nature to bluetongue. The chemical pathology and haematology of mild bluetongue infections in sheep are thus described in detail.

The chemical nature of the ovine bile pigments is described and methods are given for their isolation and identification, as well as for the isolation of bromsulphalein conjugates formed by the ovine liver.

Methods are given for the determination of copper in body fluids and tissues, the various copper fractions in blood, and iron and selenium in body tissues. The diagnosis of ovine liver, renal and adrenal dysfunction and myopathy by means of clinical laboratory tests is fully dealt with.

Intermediary metabolism in the ovine red cell is discussed and activity levels of numerous metabolic enzymes in these cells are presented for consideration.

Carbohydrate metabolism in the sheep as it is revealed by a study of the disturbances seen in geeldikkop forms an important theme of this thesis.

INTRODUCTION

Geeldikkop and enzootic icterus constitute together a serious annual threat to the sheep and wool industry in South Africa. The two conditions occur each year in the form of sporadic outbreaks or widespread epizootics throughout almost one-third of the country. Geeldikkop in its typical form is characterized by the sudden onset of a very severe photosensitization, an icterus of the intrahepatic cholestasis type, nephrosis, failures in carbohydrate metabolism, Addisonian electrolyte imbalances and numerous other interesting biochemical features, which will be described in this monograph. The symptomatology of the disease has been reviewed recently by Brown (1966). Enzootic icterus is typically an explosive haemolytic crisis coupled with a marked retention icterus, haemoglobinuria and acute nephritis. Both conditions are believed to represent different manifestations of a single disease entity, possibly a subclinical selenium intoxication (Brown, 1962, 1963, 1964; Brown & de Wet, 1962). The relationship of the two diseases is discussed at length in the present paper.

Both syndromes occur during the summer months throughout the semi-desert plains (geeldikkop) and mountain ranges (enzootic icterus) of the northwest Cape Province, southern Orange Free State, Gordonia, Namaqualand, Griqualand West and southern South West Africa. The epizootiology of both conditions has been fully described elsewhere (Brown, 1959b, Brown & de Boom, 1966). Within recent years the incidence of both has increased alarmingly, the reasons for which have also been set out elsewhere (Brown & de Boom, 1966).

When outbreaks occur in epizootic form, the two syndromes may be seen side by side in any particular flock or their appearance may alternate, while cases best described as suffering from a mixed, geeldikkop-enzootic icterus syndrome are by no means rare (Brown, 1966a; Brown, le Roux & Tustin, 1960). The acute episodes of both conditions are precipitated by a wide variety of severe non-specific stress conditions (Brown, 1959c, 1962, 1963, 1964, 1966a, 1966b; Brown, le Roux & Tustin, 1960; Brown & de Wet, 1962; Brown & de Boom, 1966). Although sheep on farms in the affected areas appear outwardly normal for the greater part of the year, profound biochemical differences have been shown to exist between them and sheep born and raised elsewhere (Brown, le Roux & Tustin, 1960; Brown & de Wet, 1962; Wagner, 1964; Wagner & Brown, 1966a, 1966b).

Annual losses from these diseases have not been computed with any degree os accuracy, but morbidity and mortality rates can be very high during severe epizooticf of either condition (Brown, 1959c, 1963, 1966a; Brown & de Boom, 1966). Sporadic losses from enzootic icterus are considerable, particularly following stock movements, while many chronic cases of this disease, which terminate fatally are not recognised as such (Brown & de Boom, 1966). The annual losses truly attributable to geeldikkop and enzootic icterus must also include large numbers of deaths occurring after the peak of the annual epizootics, due to chronic nephritis, Addisonian syndromes, septicaemic conditions and other complications, as well as considerable losses in production and breeding potential (Brown, 1966a). Added to this is the financial burden of supporting and nursing hundreds of lingering cases, many of which will ultimately terminate fatally. The vicious nature of the disease cycle and reasons for the increasing severity of each succeeding epizootic are discussed elsewhere (Brown & de Boom, 1966; Skinner, 1960).

The studies reported here formed part of a comprehensive investigation into he aetiology of geeldikkop and enzootic icterus, undertaken initially as a joint project of the Veterinary Research Institute, Onderstepoort, and the National Chemical Laboratories of the Council for Scientific and Industrial Research, and pursued subsequently by the author and his staff and co-workers at Onderstepoort and elsewhere. The historical aspects of this work are reviewed briefly in Chapter 1, and much of the earlier work of the author and his colleagues has already appeared in print (Brown, 1959a, 1959c, 1964, 1966a, 1966b; Brown & de Boom, 1966; Brown & de Kock, 1959; Brown & de Wet, 1962; Brown, *et al.*, 1960; de Kock & Enslin, 1958; Enslin & Wells, 1956; Gouws, 1965). The major part of this work has embraced extensive studies on the general chemical pathology and detailed biochemistry of the two conditions, the primary object of which was to attempt a definition of the probable nature of the aetiological agents concerned and to elucidate the pathogenesis of each syndrome. A few selected aspects of this work, of general interest, have been noted in earlier publications. This thesis represents an attempt to present as a coherent whole the results of all the work on the chemical pathology

and biochemistry of the two conditions, the greater part of which is now fully described for the first time. The relationship between the two syndromes is clearly defined; most of the pathogenesis of the symptom complex is elucidated and the various causes of death in the two syndromes are explained. The aetiology of both are shown to be complex and the probable nature of some of the factors concerned is indicated.

CHAPTER 1

Historical Notes on the History of Geeldikkop and Enzootic Icterus Research, Relevant to the General Theme of this Thesis

The work up until the time that the author and his colleagues commenced their studies on these two syndromes, has been adequately reviewed elsewhere (Brown, 1959a; Brown & de Boom, 1966).

A review of some particular aspects of the earlier studies is however essential background material for following much of the work presented here.

The greater part of the earlier studies on geeldikkop centred around three general themes, notably: Transmission experiments designed to prove an infectious cause of the condition; feeding and dosing experiments to establish the role of plants like *Tribulus terrestris*, L. (Zygophyllaceae) in its aetiology and studies designed to elucidate the pathogenesis of the photosensitivity, which is such a prominent feature in the syndrome.

The early attempts to prove the participation of an infectious agent in the aetiology of geeldikkop are detailed in Theiler's classic paper on geeldikkop (Theiler, 1918) and in a later review by the author (Brown, 1959a). This work proved fruitless and the thought was largely abandoned by the workers concerned. A considerable body of evidence has, however, geen gathered recently which indicates that an infectious agent might in fact be a most important factor in precipitating the acute attacks of the disease, whilst not being the direct cause as such (Brown, 1964, 1966b). This work is reported in detail in one of the latter chapters of this thesis, and reasons as to why the earlier transmission experiments failed are given.

Details of the work done to prove the role of *T. terristris* in the aetiology of the disease are to be found in the papers by Theiler (1918), Quin (1928, 1929, 1930), Sapiro (1950), Henrici (1952) and Brown (1959a). Although both Theiler and Quin were able to produce *some* cases of the disease by feeding the plant, they were not able to do so with any regularity, and all subsequent work has failed consistently to do so. Theiler's description of the histopathology of his cases raises some doubts as to whether he was in fact dealing with uncomplicated geeldikkop (Brown *et al.*, 1960). The fact that their positive results may have been produced with the assistance of an arthropod-borne infection cannot be ruled out—in fact, their descriptions of the general climatic conditions prevailing at the time they did this work, lend considerable weight to this idea. This thought will be discussed at greater length later in this paper.

The author and his co-workers commenced their own studies with a re-appraisal of the role of *T. terrestris* in the aetiology of the disease. Enslin & Wells (1956) were able to isolate crude saponins, the presence of which was reported earlier by Henrici (1952), in yields of 0·5 to 2·0 per cent of the dry weight of the plant. Subsequently De Kock & Enslin (1958) reported the isolation and characterization of four sapogenins from their crude material, namely diosgenin, ruscogenin, gitogenin and 25D-spirosta-3: 5-diene. Although the saponins and their aglycones were shown to be hepatotoxic, nephrotoxic and haemolytic (Brown, 1959a, 1959c, 1963) and capable of paralysing smooth muscle (Enslin & Wells, 1956), none of the characteristic features of geeldikkop have yet been produced by administering these compounds to experimental animals. The idea that *T terrestris* has anything to do with the disease, other than a secondary role as a stress-provoking agent, has now largely been discarded by the author and his colleagues (Brown, 1959c, 1962, 1963, 1964, 1966a, 1966b; Brown & de Boom, 1966; Brown & de Kock, 1959; Brown & de Wet, 1962). The work to be reported here lends further support to this particular contention.

A paper published by Quin (1931) was to initiate a long and fruitful series of research on photosensitization in sheep. Various fluorescent dyes were found to produce intense photosensitization, but without icterus, following intravenous administration in sheep (Quin, 1933a). This was followed up with Quin's classic work on the effects of surgical obstruction of the bile flow in sheep (Quin, 1933c) and the isolation from bile of a photodynamic substance, phylloerythrin, a pigment derived from chlorophyll by microbial digestion (Quin & Rimington, 1933). This pigment was subsequently shown to be the photodynamic agent responsible for the photosensitization in cases of geeldikkop. It was shown that the chief site of formation of this compound is in the rumen as a result of microbial activity and that it is absorbed mainly from the duodenum and small intestine and excreted in the bile of normal animals (Quin & Rimington, 1933). The mode of its excretion by the liver cell remained a mystery until Perrin (1958) showed that it was excreted in the bile adsorbed on to colloids such as bile acid complexes.

During the course of dosing experiments with various plants known to produce photosensitivity in sheep, Quin (1933b) found that the typical symptoms of geeldikkop could be reproduced by dosing to these animals the plants *Lippia rehmanni* (H. H. W. Pears) and *Lippia pretoriensis* (H. H. W. Pears), Verbenaceae. Phylloerythrin was once again demonstrated to be the photodynamic agent responsible for the photosensitivity in these cases, and it was found that the icterus developed by the animals was similar to that seen in natural cases of geeldikkop (Quin & Rimington, 1933). The conclusion was made that the plant contained a single factor which caused simultaneous insufficiency in the elimination of bile pigments and phylloerythrin (Quin, Rimington & Roets, 1935; Rimington, 1934; Rimington & Quin, 1934). The active principle of the plant was shown to cause a complete disappearance of bile pigment from the bile as well as causing a marked reduction in the flow of bile. These changes were shown to be transient, normal flow and pigment excretion being resumed about four to five days after the appearance of the disturbances (Quin, 1936).

The active agent was soon isolated from these plants; named icterogenin, its properties were studied and its structure partly elucidated (Rimington & Quin, 1935; Rimington, Quin & Roets, 1937; Roets, 1937). Shortly afterwards it was found that the plant *Lantana camara*, L. (Verbenaceae) induced the same symptoms and disturbances in biliary excretion as did the *Lippia* spp. (Steyn & van der Walt,

1941). The active principle was isolated, named lantadene A and its structure partly elucidated. It was shown to possess the same type of icterogenic activity as icterogenin. A similar, but physiologically inactive compound, named lantadene B was isolated at the same time (Louw, 1943; 1948; 1949).

Lantadene A was shown to be icterogenin by Barton and his co-workers, who also discovered with it in extracts from *L. rehmanni*, a closely related compound which they named rehmannic acid. Lantadene B was shown to be isomeric with rehmannic acid. Icterogenin, rehmannic acid and lantadene B were shown to be closely related pentacyclic triterpene acids and their structures were fully elucidated by the same school (Barton & De Mayo, 1954a; Barton & De Mayo, 1954b; Barton, De Mayo, Warnhoff, Jeger & Perold, 1954).

The icterogenic triterpene acids have proved to be valuable tools in the study of the interferences in biliary secretion in geeldikkop. A considerable amount of work has been devoted to the relationship between their chemical structure and their icterogenic activity, and a high degree of specificity has been demonstrated in this regard. Pure rehmannic acid and lantadene B have also been shown to be devoid of any icterogenic activity, but some new highly potent compounds have been discovered, which are even more effective icterogenic agents than icterogenin itself, e.g. 22β-angeloyloxy-3β-hydroxyolean-12-en-28-oic acid (Anderson, de Kock & Enslin, 1961; Brown & Rimington, 1964; Brown, Rimington & Sawyer, 1963; Brown, Anderson & de Kock, 1963; Heikel, Knight, Rimington, Ritchie & Williams, 1960). The use and relevance of these studies in relation to the geeldikkop problem are described later in this thesis. All efforts to isolate an icterogenic agent, similar in nature to the triterpene acids from the various plants incriminated in the aetiology of geeldikkop, have failed so far, and it seems unlikely from the work to be reported here that such an agent is responsible for the appearance of the disease.

Theiler (1918) must be credited with the first recorded thoughts on the possible biochemical mechanisms involved in the genesis of the photosensitivity and icterus seen in geeldikkop, in spite of the fact that he did not work in this direction at all. He recognised the peculiar nature of the icterus, now known to be an intrahepatic cholestasis (Brown, *et al.*, 1960), and his observations on the photosensitivity were remarkably close to what we know to be the case today. The first notes on the haematology of geeldikkop, albeit in the form of scattered tantalizing sentences, are also to be found in Theiler's classic paper. He noted the presence of an anaemia, particularly in the later stages of the disease, and although he did not attempt to explain its origin, he stated that it was not due to intravascular haemolysis. It is remarkable that his observations regarding the anaemia shown by many of his cases received scant attention from his immediate successors in the field of geeldikkop research. Had this been the case, the relationship of geeldikkop to enzootic icterus might have been recognised earlier.

Unlike geeldikkop, enzootic icterus received little attention as regards basic research until very recent times when the annual epizootics started to assume alarming proportions. The earlier work on the disease is reviewed elsewhere (Brown & de Boom, 1966; Pienaar & van der Merwe, 1966) and unpublished earlier studies on the chemical pathology of the condition are recorded in this paper.

Apart from the studies already mentioned, the chemical pathology and biochemistry of naturally occurring geeldikkop had received hardly any attention at all. Work on geeldikkop faltered somewhat after the untimely death of Quin, but in 1955 investigations were resumed on a large scale, this time as part of a joint research project between Veterinary Research, Onderstepoort, and the National Chemical

Laboratories of the Council for Scientific and Industrial Research. One of the approaches to the problem of the aetiology of the condition was to be a thorough study of the chemical pathology of the disease, and this became the particular responsibility of the author. At the time these investigations commenced there were simultaneous severe and widespread epizootics of both geeldikkop and enzootic icterus. Cases of both syndromes were freely available and research into the latter disease was incorporated into the major geeldikkop project. The decision to do this proved to be a fortunate one, for the collateral studies on both conditions soon showed their relation to one another and to some other rather ill-defined disease entities prevalent in the areas where both conditions are enzootic (Brown, 1963, 1964, 1966a; Brown & de Wet, 1962; Brown *et al.*, 1960). Both diseases have since been studied together on the assumption that they are in fact different manifestations of a single disease entity. The reasoning behind this assumption is fully set out later in this paper, which itself is a report of the bulk of the work done on the chemical pathology and biochemistry of the two conditions since its inception in 1955.

In conclusion it is fitting to record here our debt of gratitude to the Trustees of the Stock Diseases Research Fund, who in 1958 made available to this Institute a well-equipped mobile laboratory. The vehicle and its uses are described elsewhere (Brown, 1959b). Its acquisition made possible the greater part of the studies which are reported here.

CHAPTER 2

Experimental Facilities, Animals, Materials and Methods

Much of the work on the chemical pathology of the syndromes was done in the field under the conditions described below, and represents the results of comprehensive investigations at Vosburg (1958), Victoria West (1958, 1959, 1964), Somerset East (1958), Hofmeyr and Teviot (1959), Fraserburg, Sutherland and Loxton (1962), Beaufort West, Rietbron, Aberdeen, Murraysburg and Middelburg (1963).

The mobile laboratory mentioned in the previous chapter is equipped with a power generator, gas supply, refrigerators and a deep freeze unit, vacuum pumps and a highly efficient air conditioning system. It was the practice of the author and his staff to set up a temporary biochemical laboratory on the agricultural showgrounds of centres like Victoria West, Fraserburg and Somerset East during the investigations mentioned above. The mobile unit was used as a source of power, as a laboratory in which all the refined techniques used were carried out and in which all the sensitive electronic equipment or other valuable apparatus was housed. The various halls with their attached kitchens which are available on these showgrounds were converted into temporary chemical laboratories, in which most of the routine work was done. Once the temporary laboratory complex had been set up, the mobile laboratory unit ceased to be mobile for the duration of the investigation. Affected farms were visited and cases brought in for study in other independent motor vehicles. All the animals used in this work were collected from farms in the areas affected in this manner and were housed in the sheltered runs and pens of the showgrounds concerned. Use was made of roofed sheep pens to accommodate each animal separately.

This permitted a study of daily food and water consumption by each patient and prevented injury of critically ill animals by those in a better physical condition. Photosensitive patients were placed in completely shaded pens. Direct solar radiation of these cases was kept thus at minimum levels. The facilities created were thus ideal for performing detailed studies of the disease *in its natural setting*, in most of the areas where it occurs in the Karoo.

Cases representing all stages of geeldikkop were selected for detailed study from the hundreds of sick animals seen and examined during this work. Selection was made on grounds of the symptomatology observed and anamneses supplied by owners or their shepherds. The symptomatology seen in the various stages of the geeldikkop syndrome has been described in an earlier paper (Brown, 1966a). For the purposes of this discussion the cases used for study have been classified as follows:—

(i) *Prodromal cases:* These were sheep which showed at the time of examination no visible or outward signs of geeldikkop. In most instances a temperature of 104 to 106° was noted at the time of examination. The sheep concerned were in all instances selected initially in the early stages of this work for use as apparently normal controls, and were taken from flocks in which geeldikkop was present in the form of severe epizootics at the time. It was soon realized, almost after completion of the first few laboratory examinations, that these sheep were not as normal as they appeared to be on clinical examination and represented in fact prodromal cases of the disease. In most instances definite symptoms of geeldikkop made their appearance within two or three days of the initial examination. The fever observed in most of these animals at the moment of selection was dismissed at the time as being due to herding and struggling on capture. It was only later that the significance of this prodromal fever and its role in the general geeldikkop syndrome were realized (Brown, 1966a, 1966b). This aspect will be discussed more fully in one of the later chapters dealing with the role of stressors in the pathogenesis of geeldikkop. During the latter phases of this work many prodromal cases were selected by the presence of this fever reaction and were then allowed to develop geeldikkop up to a required stage of the disease before being subjected to further study. Such cases are amongst the animals in the following groups.

(ii) *Early cases:* These are cases of one to seven days standing. Many were collected on farms where the farmer during the course of daily inspections of his flock, noticed the animals presenting the first outward signs of the syndrome. A few were prodromal cases detected by the author and allowed to develop symptoms to the required stage of the disease. In many instances the duration of illness was estimated from the anamnesis supplied and the progress of the lesions seen at examination. The majority of cases of one to three days standing were highly photosensitive and the symptomatology included more or less hyperaesthesia, pruritis, pain, erythema and oedema of the exposed parts of the body, and mild to severe icterus. In cases of four to seven days standing photosensitivity had largely subsided but the lesions of photosensitization had progressed to severe oedema and incipient gangrene of the affected areas. Icterus was generally severe.

(iii) *Advanced cases—Group 1:* These are cases of 7 to 14 days standing and were selected as explained under (ii) above. In general these animals were no longer photosensitive, but the lesions of photosensitization were, in many, in an advanced stage and included severe oedema and necrosis of the affected areas. In more

fortunate cases a considerable amount of healing of such lesions had started to take place. Many of the animals in this group were *in extremis* when encountered, the reasons for which will become apparent in the course of later discussions in this paper.

(iv) *Advanced cases—Group 2:* Such cases were of 14 to 21 days standing and were either on the way to recovery with considerable healing of the lesions of photosensitization, or were either *in extremis* or in the throes of a rapid decline towards a fatal termination. Lesions in the latter cases included extensive necrosis and sloughing of the affected areas, with secondary infection of the gangrenous parts of the skin in some instances.

(v) *Recovered cases:* The duration of illness varied in these cases between 15 to 21 days. " Recovery ", for the purposes of this discussion, is defined as total subsidence of the acute phenomena of photosensitization, advanced healing of the effected areas (i.e. all sloughs replaced by healthy new skin growth), negligible icterus and recovery of gastro-intestinal motility, a reasonably normal habitus and appetite.

(vi) *Control animals:* It has been shown that marked differences exist between apparently normal Karoo sheep and those raised elsewhere in South Africa with respect to " normal " levels of many blood constitutents and " normal " levels of activity of certain enzymes present in ovine plasma (De Wet & Brown, 1966; Wagner, 1964; Wagner & Brown, 1966a, 1966b). This fact became apparent almost from the inception of this work. It was only within recent years when sufficient data became available that it was possible to offer statistical proof that this was indeed so. Acting, however, upon earlier assumptions, the practice was to collect a small number of clinically normal sheep, to use for purposes of comparison, from each area in which these investigations were carried out. Bearing in mind the existence of large numbers of prodromal cases of the disease during extensive outbreaks, these control animals were always obtained from farms on which no geeldikkop was present at the time. They were then housed, managed and treated in exactly the same way as all the patients used in this work.

In Appendix 1 brief descriptions of all the animals used in these studies are presented. They have been classified for ready reference as explained above, their place of origin is indicated and the most important symptoms shown are noted. A few further words of explanation are necessary with regard to the latter. The basic symptoms of the various stages of geeldikkop are the same in all cases of the disease (Brown, 1966a). The degree of icterus and photosensitization shown by each case is indicated in this appendix. " Lesions of photosensitization " means hyperaesthesia or pain, pruritis, erythema, oedema and secondary mechanical injury to affected areas of the body consequent to rubbing, scratching, biting and abolition of the blinking reflex, etc. Where the symptomatology of a particular case deviates from the general basic pattern, this is indicated, e.g. rhinitis, panophthalmia, dyspnoea, tachycardia, coronitis, etc. Many such symptoms belong properly to the precipitating agents in the syndrome and are discussed under this heading in a later chapter. Photosensitivity in early cases was established by shearing the wool off the back and head of the animals concerned, exposing them to solar radiation, and noting the appearance, within minutes of exposure, of hyperaesthesia, flinching, pruritis, pain, erythema and oedema. Unless otherwise stated, all cases presented more or less anorexia, gastro-intestinal atony and constipation. Furthermore, unless stated to the contrary, secondary bacterial infection of the lesions of photosensitization was minimal and kept to a minimum during the period of study by topical application of antibiotic or bactericidal preparations.

Table 1 represents a summary of the information contained in Appendix 1, with regard to the number of cases of each stage of the disease studied in this work.

TABLE 1.—*Number of cases of the different stages of the syndrome studied during this work*

(Details of these animals are to be found in Appendix 1)

Nature of cases	Number of cases
Prodromal cases	9
Early cases (1 to 7 days)	43
Advanced cases—Group 1 (7 to 14 days)	25
Advanced cases—Group 2 (14 to 21 days)	7
Recovered cases	8
Total number of affected animals studied	92
Clinically normal control animals	28
Total number of animals used in this work	120

All experimental and control animals were maintained on a diet of dry lucerne, ay and water, *ad libitum*, supplemented with a daily ration of 200 gm of crushed maize. In many cases a diet of mixed Karoo bushes collected each day from the surrounding veld was given *ad libitum* in addition to the above. This was done particularly where sheep were encountered which refused to eat either lucerne or maize (doubtless through unfamiliarity with such luxuries) and at the laboratory in Frazerburg where lucerne was in extremely short supply at the time. The Karoo bush mixture generally consisted of *Pentzia* spp., *Aridaria* spp., *Chenopodium album* L., *Atriplex semibaccata* R.Br., *Eriocephalus ericoides* Druce and *Salsola* spp., chopped coarsely and mixed.

All animals were examined three times daily and body temperatures were recorded at 8 a.m. and 10 p.m. Nursing care included specific treatment of eye and skin lesions and administration of alteratives and rumenatorics when necessary. All animals were sprayed on arrival with benzene hexachloride emulsions (" Dubbel-Benhex ", Klipfontein Organic Products) to prevent blowfly " strike " and to discourage attacks by bloodsucking flies, e.g. *Tabanus* spp. and *Stomoxys* spp.

Bleeding for analytical work was generally done early each morning before feeding and the various clinical and metabolic tests used were performed during the course of the day.

All the animals were generally sacrificed upon completion of the batch of blood studies and clinical or metabolic tests required from each. Material was then taken for biochemical and histopathological work and a detailed autopsy was carried out in each case. Slaughter for these purposes generally occurred two to seven days after acquisition of the animal concerned, depending on the studies being done. Animals brought in *in extremis* were generally destroyed after completion of a single batch of blood studies and clinical tests.

In the latter stages of this work the day-to-day progress of the disease in various cases was followed until either recovery or death supervened. (All animals whose numbers commence with V3- were used for these latter studies.) This enabled study of the sequence of biochemical events through each stage of the syndrome in individual animals.

The animals used for the studies on enzootic icterus, their management, feeding and care are described in Chapter 10.

All haematological determinations were carried out using standard techniques. The various methods employed in the analyses of blood, urine, bile and tissue constituents and in the assays of plasma or tissue enzyme activities are listed in Table 2. Similarly the methods used for organ function tests and other metabolic studies are given in this table unless otherwise stated in the text. The following methods, which are either original techniques or modifications of older procedures as adapted for field work in ovine pathology are to be found in Appendices 9–11: Plasma and tissue copper; tissue iron and the isolation of BSP conjugates from bile.

TABLE 2.—*Analytical methods used during the course of this work*

Method	Reference	Method	Reference
A. Whole blood, plasma or serum		*B. Red cells*	
Aldolase	Sibley & Lehninger, 1949	Aldolase	Sibley & Lehninger, 1949
Alkaline phosphatase	King & Wootton, 1956 (AAP method)	Fragility	Brown, 1963
		Glucose-6-phosphatase	Brown & Abrams, 1965; Swanson, 1950
Amino acids (total)	Hawk, Oser & Summerson, 1954	Glucose-6-phosphate dehydrogenase	Wagner & Brown, 1966a
Amylase	King & Wootton, 1956	Glutamic oxalacetic transaminase	King, 1958
Arginase	Cornelius & Freedland, 1962	Glutamic pyruvic transaminase	King, 1958
Ascorbic acid	King & Wootton, 1956 (DCPIP method)	Glutathione	Grunert & Phillips, 1951
Bicarbonate	Van Slyke, Stillman & Cullen, 1919	Glyceraldehyde phosphate dehydrogenase	Sigma Tech. Bull. No. 10, 1961
Bile acids	Irvin, Johnston & Kopala, 1944	Hexokinase	Crane & Sols, 1953, 1955
		Isocitric dehydrogenase	Taylor & Friedman, 1960
Bilirubin	Malloy & Evelyn, 1937	α-Ketobutyrate dehydrogenase	Elliot & Wilkinson, 1961
Calcium	Ferro & Ham, 1957	Lactic dehydrogenase	Wroblewski & La Due, 1955; Wagner, 1964
Catalase	Dobkin & Glantz, 1958; Wagner, 1964	Methaemoglobin reductase	Brewer, Tarlov & Alving, 1960
Ceruloplasmin	Houchin, 1958	Protoporphyrin	Grinstein & Watson, 1943
Chloride	Hawk, Oser & Summerson, 1954	Pyridine nucleotides (total)	Levitas, Robinson, Rosen, Huff & Perlzweig, 1947
Cholesterol (total)	King & Wootton, 1956	Phosphohexose isomerase	Bodansky, 1954
Cholinesterase	Augustinson, 1957		
Citric acid	Umbreit, Burris & Stauffer, 1964; Stern, 1957	*C. Tissue enzymes*	
Creatinine phosphokinase	Sigma Tech. Bull. No. 661, 1965	Bilirubin conjugation system	Grodsky & Carbone, 1957
Creatinine	Folin & Wu, 1919	Glucose-6-phosphatase	Brown & Abrams, 1965; Swanson, 1950
Fructose	Ashwell, 1957	Glutathione reductase	Racker, 1955
Glutamic oxalacetic transminase	King, 1958	Isocitric dehydrogenase	Ochoa, 1948
Glutamic pyruvic transasaminase	King, 1958	Succinic dehydrogenase	Sclater & Bonner, 1952
Haemoglobin	King & Wootton, 1956	Uridine diphosphoglucose	Strominger, Maxwell & Kalckar, 1957
Iron	King & Wootton, 1956	Uridine diphosphoglucose dehydrogenase	Pontis & Leloir, 1962
Isocitric dehydrogenase	Taylor & Friedman, 1960		
α-Ketoglutaric acid	Friedemann, 1957; Umbreit, Burris & Stauffer, 1964	*D. Liver function tests*	
Lactic acid	Barker & Summerson, 1941	Bromsulphalein clearance	Parker, 1948
Lactic dehydrogenase	Wróblewski & La Due, 1955; Wagner, 1964	Colloidal gold flocculation	Gray, 1940; Maclagan, 1946
Magnesium	Neill & Neely, 1956	Thymol flocculation	Maclagan, 1944, 1947
Oxalacetic acid	Neish, 1957	Thymol turbidity	Maclagan, 1944, 1947
Phosphate (total inorganic)	King & Wootton, 1956	Zinc sulphate turbidity	Kunkel, 1947
Phosphohexose isomerase	Bodansky, 1954		
Phylloerythrin	Perrin, 1958a	*E. Faeces analysis*	
Plasma Proteins:—		Bile pigments	Gray, 1953
(*a*) Total plasma proteins	Weichselbaum, 1946	Coproporphyrin	Rimington, 1958
(*b*) albumin and globulins	Kingsley, 1940	Protoporphyrin	Rimington, 1958
(*c*) filter paper electrophoresis	King & Wootton, 1956	Urobilinogen	Gray, 1953
(*d*) cellulose acetate electrophoresis	Van Zyl, 1966	*F. Urine analysis*	
(*e*) microzone electrophoresis	Van Zyl, 1967	Bile acids	Parker, 1948
Potassium	King & Wootton, 1956	Bile pigments	Gray, 1953
Pyruvic acid	Friedemann & Haugen, 1943	Coproporphyrin	Rimington, 1958
Sodium	King & Wootton, 1956	Urobilinogen	Gray, 1953
Sugar (glucose)	Lehman & Silk, 1952		
Urea nitrogen	Brown, 1957		
Uric acid	Caraway, 1955		

Anticoagulants for collection of blood samples and any special preservatives for labile blood constituents were in all instances as demanded by the methods cited. All determinations on blood or plasma were commenced immediately after collection of the samples and completed as soon as possible thereafter.

All enzyme assays done on liver, except where noted otherwise, were performed using 10 per cent homogenates in ice-cold 0·25M sucrose solution made in a manual all-glass Potter-type homogenizer. All homogenates were kept in ice baths at 0°C during use. Liver specimens for enzyme assays were collected immediately after slaughter of the sheep concerned and homogenized at once, the assays being completed as soon as possible thereafter. At the same time specimens of various organs were removed for histopathological studies and for mineral analyses (Cu, Fe and Se) and were placed in 10 per cent formalin solution (analytical reagent grade).

General urine examinations were performed on the urine voided by all the experimental animals whenever possible. Standard techniques of clinical urine analysis were employed. Collection of urine in the case of rams and wethers presented no obstacles, since the collection bottle and harness described in a previous communication were used (Brown, 1959b). These animals and the collection system mentioned were always employed when the daily (24 hour) excretion of any urinary constituent was studied. Collection of urine in the case of ewes was difficult under the temporary laboratory conditions. Whatever urine was voided by these animals during handling was collected in an enamelled mug. Bladder urine was collected from all animals at autopsy and subjected to the usual clinical laboratory tests.

Faeces were collected from all cases used in this work in standard faeces collection bags. Chemical estimations of various faecal constituents were performed by methods listed in Table 2. Samples from each animal were examined for the presence of helminth eggs and larvae by standard flotation techniques.

Studies on biliary constituents and the biliary excretion of phenolsulphonephthalein dyes were conducted on a number of animals representing various stages of the disease and provided with external biliary fistulae as described elsewhere (Brown, 1959b, 1963). The methods employed for analysis of biliary constituents were as described elsewhere (Brown *et al.*, 1963a; Brown, Anderson & de Kock, 1963b), or as listed in Table 2.

A Unicam S.P. 500 spectrophotometer was used for all enzyme studies; other photometric procedures were carried out on an Evans Electroselenium (E.E.L.) portable Model A photoelectric colorimeter. Fluorimetric assays were performed on an E.E.L. Model X136 fluorimeter. Paper electrophoresis of plasma proteins was performed using the E.E.L. paper electrophoresis apparatus and 34 × 5 cm Whatman No. 1 filter paper strips. Proteins were separated in a veronal: acetate buffer, pH 8·6, over 16 hours with a current setting of 2 milliamps per strip.

Chemicals used throughout were of " analytical reagent " grade. All solvents were redistilled or otherwise purified as described by Vogel (1959). The preparation of the reagents used in the determination of copper is described in Appendix 9. Compounds used for the preparation of substrates for enzyme assays or pure enzymes for the standardization of these were obtained from the Sigma Chem. Co. (St. Louis, Mo.) or from C. F. Boehringer (Mannheim, West Germany) and were of the highest purity offered by these manufacturers.

Not all the determinations and assays reported in the subsequent chapters were performed on each individual case. This would have been impossible under the circumstances concerned. During the initial phases of this work the largest number

of determinations and clinical laboratory tests which could be conveniently handled by the staff available were selected. This enabled obtaining a broad picture of the general chemical pathology of the disease, and allowed narrowing down the field of study in subsequent investigations to the most interesting aspects of this work. As the picture of the chemical pathology of geeldikkop and enzootic icterus gradually unfolded, many new determinations or clinical tests were introduced in order to follow up interesting data which came to light. The work entailed in its ultimate stages almost purely biochemical studies on tissues of affected animals. Certain batteries of tests were, however, always done on each case throughout this work in order to characterize the stage of the disease represented by the patient more accurately in terms of chemical pathology, e.g. tests of liver, kidney and adrenal function and certain aspects of the haematology rather than on symptomatology alone.

Since severe verminosis may introduce a chemical pathology of its own which may alter or obscure that germane to geeldikkop, particularly in the case of blood sucking or toxin producing worms, a careful search was always conducted at autopsy for helminth parasites. This was supported by examination of faeces for helminth eggs and larval forms. Most of the animals studied were free from worm parasites. About a quarter of the sheep presented old calcified lesions of oesophagostomiasis in the intestinal tract. In a few individuals very mild infestation with *Trichostrongylus* spp., *Nematodirus* spp. and *Ostertagia* spp. were found and in isolated cases fair infestations of *Stilesia hepatica*. Cestode infestations were limited to a few *Cysticercus tenuicollis* cysts in the abdominal cavities of some individuals, and a few adult tapeworms, probably *Thysaniezia* sp. in the intestines of two animals. It is unlikely that these very mild parasitic infestations in the few individuals concerned had any significant influence on the general chemical pathology and biochemistry in these cases of geeldikkop.

" Normal " values for Karoo sheep for the various blood and tissue constituents which were determined and for levels of enzyme activity in plasma and tissue are given in the appropriate places in the text. The statistical methods used in the compilation of such values are described elsewhere (De Wet & Brown, 1966; Sion, 1966; Wagner, 1964; Wagner & Brown, 1966a, 1966b).

CHAPTER 3

The Haematology of Geeldikkop

1. Leukocyte and Thrombocyte dyscrasias
2. Erythrocyte dyscrasias
3. The state of the bone marrow in geeldikkop
4. Iron metabolism in geeldikkop
5. Erythrocyte Sedimentation Rate
6. Concluding Remarks

1. *Leukocyte and Thrombocyte dyscrasias*

Total and differential leukocyte counts were performed on the blood of 51 cases representing all stages of the disease and of 18 of the control animals. Bloodsmears for differential counts were usually made in triplicate at the time of bleeding, fixed with methanol and stained with buffered Giemsa solution. Besides doing

differential counts on each smear, leukocyte morphology was studied and the number of thrombocytes present, relative to other cell types, was noted. Absolute thrombocyte counts were attempted initially but proved difficult and unreliable under the field conditions.

The experimental data relating to the leukocytes and thrombocytes of geeldikkop cases and control animals are reproduced in full in Appendix 2.

Absolute eosinophile counts were performed on the blood of all cases of geeldikkop, except those whose numbers are prefixed by V3-, and on the blood of all control animals. Since these counts were part of assays of adrenal function they will be presented in the appropriate place later in this thesis.

The data relative to the white cell counts are summarized in Table 3.

TABLE 3.—*White cell dyscrasias seen in geeldikkop cases*

1. Prodromal Cases.....	Severe leukopaenia in all cases. Mild to severe thrombocytopaenia in at least half the cases. Leukocytes very immature in all cases. Severe lymphocytopaenia throughout. Relative neutrophilia with marked "shift to the left". Mild bacteriaemia in two cases
2. Early Cases.....	Mild to severe leukopaenia in most cases. Leukocytes very immature in such cases. Generally a severe lymphocytopaenia and a severe relative or absolute neutrophilia with a marked "shift to the left". Thrombocytopaenia in two out of twenty cases. Mild bacteriaemia in one case
3. Advanced Cases (Group 1)..	Generally either a normal white cell count or a mild leukopaenia. Severe leukopaenia in a few instances. Mild to severe lymphocytopaenia and mild to severe relative or absolute neutrophilia in most cases. Marked "shift to the left" in a number of cases. No thrombocytopaenia. Mild bacteriaemia in one case
4. Advanced Cases (Group 2)..	Mild leukopaenia, lymphocytopaenia and neutrophilia in all cases. "Shift to the left" in one case. Thrombocytes normal
5. Recovered Cases.....	Leukopaenia, mild in most and severe in one case. Lymphocytopaenia and neutrophilia. "Shift to the left" and thrombocytopaenia in one case

As will be seen from this table very severe disturbances of the leukocytes were present in all prodromal cases, and took the form of an extremely severe leukopaenia and thrombocytopaenia. The leukopaenia was so severe that it was impossible to perform a total white cell count in each instance with any degree of accuracy. What leukocytes were present were so immature or undifferentiated as to make it impossible to carry out an accurate differential count in more than half the cases. The majority appeared to be juvenile forms of neutrophiles with a lesser number of large lymphocytes in some cases. A striking feature of these animals was the almost complete absence of thrombocytes from the bloodsmears of at least half the cases. The presence of a mild apparent bacteriaemia in two of these cases in the form of coccoid and bacilloid organisms is also noteworthy. A significant fact regarding these sheep, to which will be referred again shortly, is that they were collected from two farms in Vosburg at the height of one of the severest epizootics of geeldikkop

within recent times. The Vosburg district is incidentally one which is noted for severe outbreaks of the disease attended by high mortality. The severity of this particular outbreak surpassed anything of this nature within recent times for morbidity and mortality rates.

In the early cases of geeldikkop there was an apparent decrease in the severity of the leukopaenia in most cases, except in those taken from Vosburg (their numbers are prefixed with VB- in Appendix 2). In the latter instances great difficulty was still experienced in performing total and differential counts. Most of the leukocytes present on the smears appeared to be juvenile forms of neutrophiles and there appeared to be a distinct absence of cells which could be identified as lymphocytes with any certainty. Thrombocytopaenia was not a prominent finding in these cases. The remaining cases were collected in subsequent outbreaks around Victoria West and Beaufort West. Mild leukopaenia was present in about half of these animals; in the others the total count had apparently been restored to normal. Lymphocytopaenia and neutrophilia (which from the evidence available appeared to be mainly a relative neutrophilia) were striking features of these cases. In most cases there was a marked "shift to the left" with highly immature neutrophile forms predominating. Fair to mild thrombocytopaenia was seen in two cases only and bacteriaemia in one instance only.

Leukopaenia was not a general feature in the advanced stages of the disease, although it still persisted in a number of cases. Lymphocytopaenia and neutrophilia of a severe nature were, however, fairly general findings. The immaturity of the neutrophiles (i.e. shift to the left) was by no means as frequent a finding as in the early cases of the disease, but was none the less still present in quite a few animals. No disturbances of the thrombocytes could be detected in cases of this stage of the disease. No particular differences were apparent between the severe Vosburg cases (Sheep VB-L, VB-M and VB-N; Appendix 2) and those from elsewhere, in this stage of the disease. Mild bacteriaemia was in evidence in a single instance.

Cases of 14–21 days' standing (Advanced cases—Group 2) differed little from those of the previous stage of the disease with respect to their leukocyte pathology.

Leukopaenia was still persistent in recovered cases and might be severe in individuals as will be seen from the data presented. Lymphocytopaenia and neutrophilia remained prominent findings. A mild thrombocytopaenia was evident in one case.

The course of events as seen in the leukocytes of affected animals passing through all the stages of geeldikkop may be summed up as follows: The obvious clinical symptoms of the disease are preceded by a period of violent leukocyte upheaval, manifested as an extremely severe leukopaenia and thrombocytopaenia. The leukopaenia involves at this stage both granulocytes and agranulocytes, those present in the blood being highly immature and undifferentiated forms. These disturbances are associated with the febrile reaction described earlier and elsewhere (Brown, 1966a). As clinical symptoms appear and the disease progresses, so the severity of the leukopaenia and thrombocytopaenia decline, the latter passing over in most cases entirely. The nature of the cellular reaction changes; a clearly discernible lymphocytopaenia associated with what is most likely a relative neutrophilia are prominent features of early cases of the syndrome. Considerable immaturity of the circulating leukocytes, particularly the granulocytes is still very much in evidence. As the disease passes into its second week and the dramatic initial symptoms of photo-sensitization

subside, the leukocyte pathology becomes more stable and easier to define. Mild leukopaenia is still evident in many cases, but total counts within the accepted normal range are the rule. Lymphocytopaenia is a firmly established symptom and is associated with a neutrophilia which in many cases has started to assume an absolute character, particularly in animals where considerable skin necrosis and secondarily infected lesions are present. (The reader should compare the haematological data given in Appendix 2 with the clinical data presented in Appendix 1.) Although a neutrophilic " shift to the left " is very much in evidence in these cases, myelocytes have largely disappeared and the neutrophile population is composed largely of metamyelocytes (particularly stab cells), early segmenters, and fully segmented forms. Thrombocytopaenia is probably an event of the past in most of these cases. As the disease enters its final stages in which skin tissue necrosis and secondary infection dominate the symptom picture (sheep VB-O and VB-Y, see Appendix 2), leukopaenia may be moderate, lymphocytopaenia is still present but less severe than before, and neutrophilia is as can be expected most prominent. In animals in which considerable healing of the lesions of photosensitization is in evidence (sheep VB-Z1 and V1-16, see Appendix 2) the leukopaenia is correspondingly less severe and the neutrophilic shift " to the left " far less obvious. Differential counts are still largely abnormal in recovered animals in which neutrophilia is still a prominent sign, as can be expected from the presence of residual lesions in these cases. Leukopaenia may still be severe in individuals, while others (e.g. sheep VB-Z, see Appendix 2) may still show considerable evidence of the initial leukocyte disturbances.

The pattern of leukocyte aberrations in the latter stages of geeldikkop is not unexpected and is consistent with the symptomatology, i.e. massive skin necrosis, secondary infection, toxaemia and healing reactions. The disturbances seen in the prodromal and early cases are surprising, unexpected and important in explaining the pathogenesis of the disease. More will be said of these points later, but a few general words on leukopaenia, lymphocytopaenia and thrombocytopaenia are, however, most relevant to the discussion at this stage.

It is now an accepted fact that under conditions of stress the adrenal cortical secretion depresses the number of circulating eosinophils and lymphocytes and leads to an elevation in the number of circulating neutrophils (Schalm, 1961). The triad of neutrophilia, eosinopaenia and lymphocytopaenia following stressful stimuli is commonly seen in the stress of disease and may be anticipated in bacterial infections, traumatic injury, extensive burns, malignancy, severe haemorrhage or blood destruction, and at parturition (Schalm, 1961). A review of the literature on this subject is that of Gordon (1955). The use of circulating eosinophil counts in the evaluation of adrenal cortex function will be described later in this work.

The two cell types, eosinophils and lymphocytes, which are depressed by adrenocortical activity in stress, constitute some 63 per cent of the circulating leukocytes in the sheep and the neutrophiles which increase under the stimulus of stress represent about 30 per cent. Thus, initially, the fall in number of circulating eosinophils and lymphocytes may exceed the increase in number of neutrophils with the effect of an actual reduction in total leukocyte count as neutrophils move from the blood into the affected tissues (Schalm, 1961). Clark (1941) was one of the first workers in ovine pathology to demonstrate that atrophy of lymphoid tissue and lymphocytopaenia are a response to prolonged stress, e.g. in pregnancy toxaemia of ewes, or ketosis in wethers.

The marked immaturity of the leukocytes in the early stages of the disease is highly reminiscent of a " degenerative left shift " in which the total leukocyte count remains within the normal range or may be slightly elevated, while the occurrence of young granulocytes in the circulation is prominent. It reflects an inability of the bone marrow to rise to the occasion and put forth a large number of mature cells (Schalm, 1961).

Thrombocytopaenia is frequently the result of the toxic action of chemical, vegetable, animal or physical agents on the bone marrow, and is also observed during the course of blood disorders such as Gaucher's disease, haemolytic icterus, various other anaemias and infections (Bodansky & Bodansky, 1957). As will be seen later in this discussion, bone marrow hypoplasia is a common finding in geeldikkop. The thrombocytopaenia of geeldikkop is apparently of a very transient nature since natural cases of the disease never develop any bleeding tendencies, nor have we observed any disturbances of the clotting mechanism in our cases to date.

A search through veterinary literature for general information on leukopaenia is not very rewarding. There is general agreement that it is common to virus diseases and overwhelming infections (Schalm, 1961) while Coffin (1953) describes it only in connection with uncomplicated virus diseases, e.g. feline panleukopaenia and infectious canine hepatitis.

When all that is known about geeldikkop is considered, it is apparent that the following points from the above discussion must be borne in mind with regard to an interpretation of the leukocyte dyscrasias which have been observed: responses to severe stress, toxic bone marrow inhibition and uncomplicated virus infections. The mild bacteriaemia seen in a few cases is probably of no consequence since no evidence has ever been found that the disease is of bacterial origin. It is likely that the bacteria observed in the bloodsmears of these few cases were non-pathogenic invaders from the respiratory tract or gastro-intestinal canal which were able to proliferate during the temporary severe leukopaenia. The existence of this leukopaenia from before the onset of clinical symptoms of geeldikkop does, however, assist in explaining the failure of sheep suffering from the disease to resist the rapid spread of secondary infection of the lesions of photosensitization and thus the appearance of secondary symptoms like purulent rhinitis, keratitis and panophthalmia [which can appear even before the lesions of photosensitization are far advanced (Brown, 1966)].

2. *Red cell dyscrasias*

Full details of the work done on the haematology of the erythrocytes of geeldikkop cases and control animals are presented in Appendix 3, and the results are presented graphically in Figures 1, 2 and 3. The same stained bloodsmears used for the differential white cell counts were used for studies on red cell morphology. Examination of the erythrocytes of the prodromal cases from Vosburg was limited to an examination of stained bloodsmears since at that time the significance of these cases was not fully appreciated. Cases of a similar nature studied in later investigations elsewhere, and allowed to develop into different stages of the disease, showed essentially the same haematology as the early cases described in Appendix 3.

It is apparent from the descriptions of the bloodsmears given in this appendix that a very severe anaemia was present in all of the prodromal cases and many of the early cases of the disease. The changes seen represent an active outpouring by the bone marrow of immature erythrocytes and their precursors in what can only

have been an anaemia due to loss of red cells. The phenomena observed in the bloodsmears of these cases seemed to be of a transient nature since the bloodsmears of most cases of later stages of the disease showed little of note as regards red cell pathology.

When a study is made of the figures found for the red cell counts, packed cell volume and haemoglobin of the early and advanced cases, one is struck by the inconsistency of these data with what is observed in the bloodsmears of many of these animals. In the early cases particularly, the figures cited are generally higher than in the control animals and above the ranges given in the literature for these blood constituents. These trends are illustrated in Fig. 1 and 2.

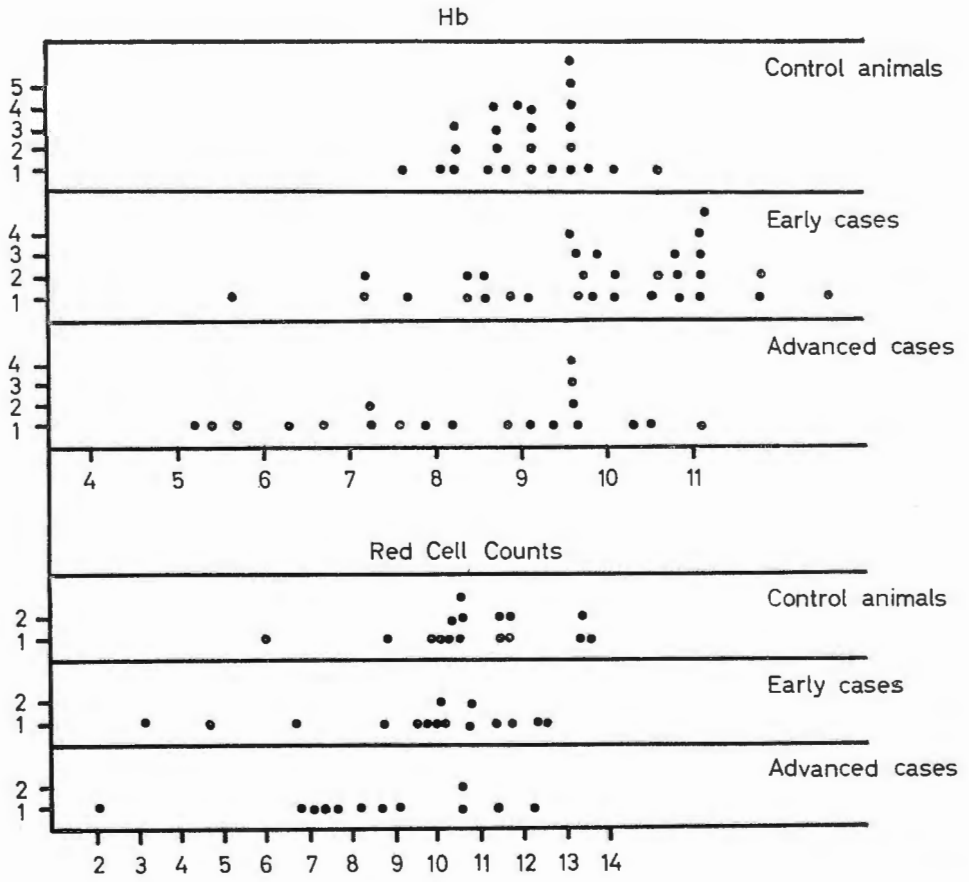

FIG. 1.—Haemoglobin and red cell counts in early and advanced cases of geeldikkop and in control animals. The figures on the abscissae represent gm% Hb and millions/cu mm red cells, respectively. Numbers of observations corresponding to these figures are plotted as ordinates

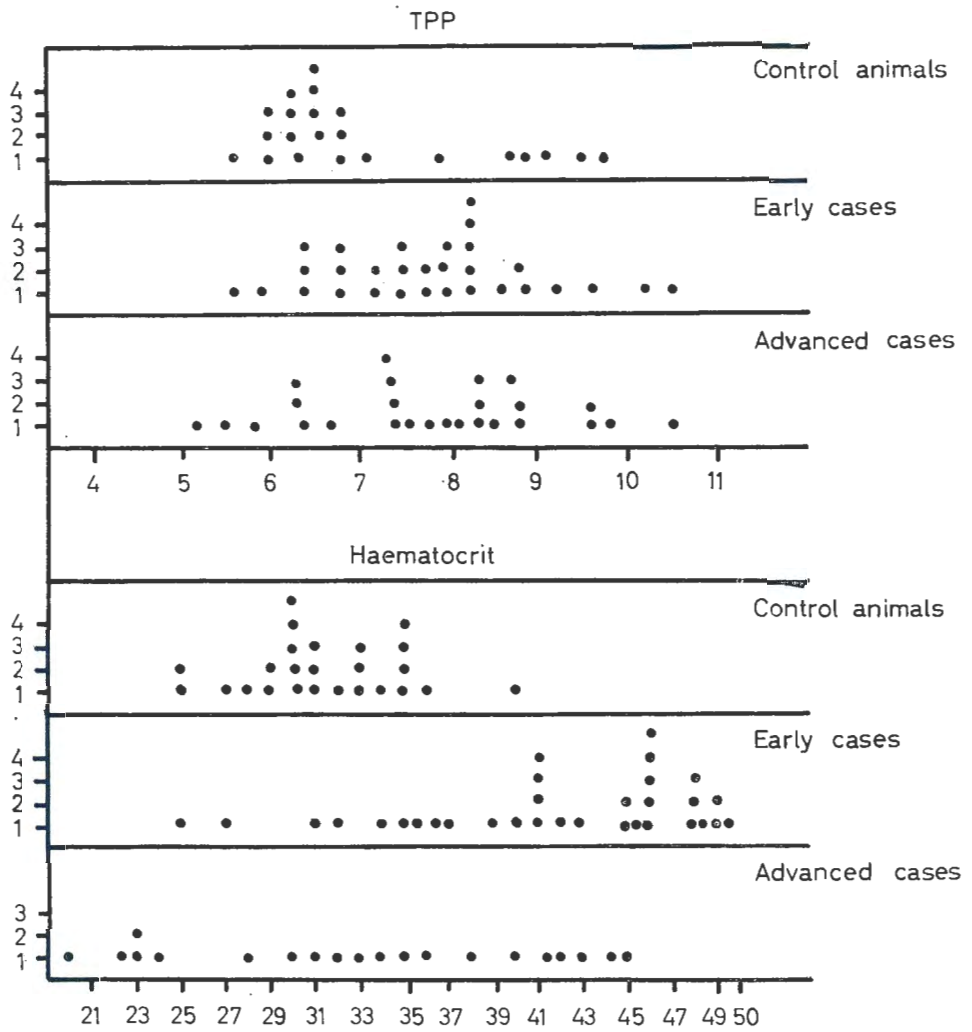

FIG. 2.—Total plasma proteins and haematocrit values in early and advanced cases of geeldikkop and in control animals. The figures on the abscissae represent gm% plasma proteins and % red cells respectively. The numbers of observations corresponding to these figures are plotted as ordinates

It is apparent from these figures that the most striking elevations of haematocrit and haemoglobin are to be found in the early cases of the disease and that in advanced cases a considerable degree of return to normal has taken place. The trends observed are similar to what has been described for the leukocytes. One is faced, however, in the interpretation of these data, with the question of reconciling the severe regenerative changes seen in the bloodsmears of prodromal and early cases with what is apparently a hypercythaemia in such animals. A considerable degree of macrocytosis is evident in many bloodsmears. This would tend to raise the haematocrit of the animals concerned but not the red cell count or the value for

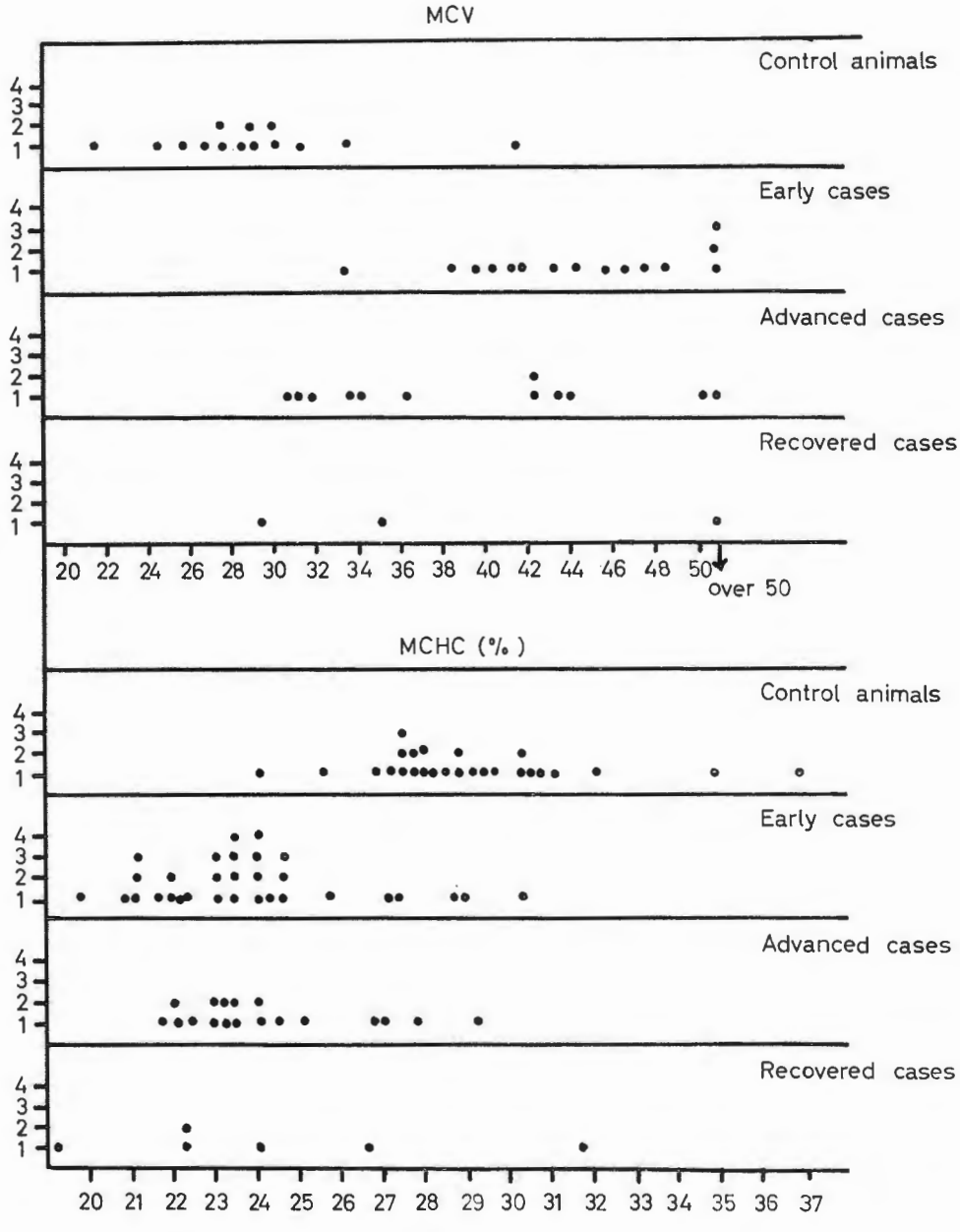

FIG. 3.—Haematological indices calculated for cases of early and advanced geeldikkop and control animals. The figures on the abscissae represent cubic microns for MCV and % for MCHC respectively. The numbers of observations corresponding to these figures are plotted as ordinates

haemoglobin. Since the values for haemoglobin and haematocrit show the same general shifts above those of the control animals (Fig. 1 and 2) something has obviously happened in the early stages of the disease leading to haemoconcentration and thus to spuriously high and relative rather than absolute figures for these tests. That this is the case, is demonstrated by the figures for total plasma protein values presented in Appendix 4 and Fig. 2, where the data are portrayed graphically. It will be seen from these data that the plasma proteins show the same rises above normal in the early cases and the same downward trend in the advanced cases as the haematocrit, red cell count and haemoglobin values. Since in any *acute* disease which is uncomplicated by hypoproteinaemic states, the plasma protein concentration is a reliable index of the fluid volume of the plasma, it is apparent that a considerable degree of haemoconcentration is present during the early stages of the geeldikkop. This is borne out by the autopsy examinations of such animals where dehydration was a common and prominent finding. The reasons for it will become apparent later.

In order to characterize the anaemia seen in the early cases in terms of conventional pathology, the absolute haematological indices have been calculated for each case in which sufficient data were available. The results for mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) are presented in Appendix 3 and portrayed in the case of the first two indices, which are sufficient for the purposes of this discussion, in Fig. 3.

A comparison of the indices calculated for geeldikkop cases with those calculated for the normal control animals, shows that the anaemia of geeldikkop is characteristically hypochromic and macrocytic in the early stages of the disease, becoming later hypochromic and normocytic in many advanced cases. Too few recovered cases have been studied to characterize the course of the erythrocyte disturbances in this stage of the syndrome.

When all the data pertaining to the haematology of geeldikkop are considered, it is clear that the disease embraces as an integral part of its pathology a *hypocythaemic hypochromic macrocytic anaemia* of more or less severity depending on the stage of the disease.

Such anaemias are encountered typically during the course of haemolytic diseases (Parker, 1948; Coffin, 1953; Schalm, 1965) and are transient in nature (Schalm, 1965).

3. *The state of the red bone marrow in geeldikkop*

The macrocytosis with hypochromia suggests that an unusual demand for red cells causes many immature cells to be discharged into the circulation during the early stages of the disease.

The next logical step in the study of the haematology of geeldikkop would thus be a study of the organ producing these large numbers of immature cells, namely the red bone marrow. With an active bone marrow one would expect to find in bloodsmears from cases of haemolytic anaemia precisely what has been described under the prodromal and early cases, particularly during the stages of remission of such an anaemia. Leukocytes are apt to be increased and immature forms may be numerous, while the thrombocytes are often more numerous than in normal blood. With extreme grades of marrow response such as may occur in the severe haemolytic anaemias, enormous numbers of nucleated red cells and immature leukocytes

may be present in bloodsmears (Parker, 1948). When the bone marrow is inactive or exhausted, no immature red cells are seen, reticulocytes are reduced, platelets few and in addition to reduction in total number of leukocytes, there is apt to be a scarcity of " stab " forms.

The presence of severe bone marrow hypoplasia in cases of geeldikkop has been noted in earlier publications (Brown, 1963; Brown & de Wet, 1962; Brown, *et al.*, 1960). It is seen most particularly in advanced cases of geeldikkop, cases of the " mixed geeldikkop-enzootic icterus syndrome " and in enzootic icterus itself (Brown, *et al.*, 1960). A detailed study of the bone marrow pathology of geeldikkop was largely beyond the scope of the investigations reported in this paper. The results of a brief study of a few Giemsa-stained bone marrow impression smears are noted in the appropriate place in Appendix 3. These smears were made from bone marrows which appeared hypoplastic on autopsy examination. Cases in which the haematological examination revealed moderate to severe anaemia (notwithstanding any haemoconcentration as mentioned earlier) showed a fair to marked decrease of red cell-forming elements and precursors, and in general of the myeloid series as well (sheep V1-3, V1-6 and V1-16, see Appendix 3). On the other hand animals in which the haematology revealed little evidence of anaemia showed evidence of active haemopoeiesis in their bone marrow smears (sheep V1-20 and V1-24, see Appendix 3).

The following would appear to be the sequence of events regarding the bone marrow as far as can be judged from the evidence presented in this chapter. The prodromal stage of the disease is marked by two singular events: A severe leukopaenia which is essentially a lymphocytopaenia and a mild to severe haemolytic episode. The net effect is a powerful stimulus to the red marrow to pour out large numbers of immature cells of both the erythrocytic and granulocytic series and to exhaust itself apparently of formed thrombocytes. This state of affairs continues for a number of days until bone marrow haemopoeisis has speeded up sufficiently to cope with the initial embarrassment. In a number of animals, however, possibly animals in which there are a few successive haemolytic crises, the bone marrow becomes exhausted and cannot rise to the occasion any longer; anaemia, granulocytopaenia and thrombocytopaenia are persistent findings and hypoplasia of the marrow is a striking autopsy finding.

4. *Iron metabolism in geeldikkop*

Haemochromatosis and cytosiderosis were prominent findings in a number of advanced cases of geeldikkop and in cases of a mixed geeldikkop-enzootic icterus syndrome (Brown, *et al.*, 1960). Iron-containing pigment was found deposited in the splenic tissue, lymph nodes and tubule cells of the kidney. Siderosis of the latter cells was particularly prominent in cases of the mixed syndrome in which the kidney cortices were seen at autopsy to be dark " gunmetal " grey in colour (Brown, *et al.*, 1960).

In order to characterize the anaemia of geeldikkop still further, a study was made of the plasma and tissue iron levels in a number of geeldikkop cases representing different stages of the disease and in some of the control animals. The results are presented in Tables 4, 5, 6 and 7.

TABLE 4.—*Plasma iron levels in control animals*

Sheep No.	mg%	Sheep No.	mg%
V1–7054	0·22	V1–7066	0·22
V1–7055	0·20	V1–7067	0·21
V1–7056	0·22	V1–7068	0·16
V1–7057	0·20	V1–7069	0·20
V1–7059	0·18	V1–7070	0·16
V1–7060	0·18	V1–7071	0·22
V1–7061	0·21	F–K2	0·13
V1–7062	0·16	F–12221	0·12
V1–7064	0·21	F–12222	0·12
V1–7065	0·22		

Range: 0·12–0·22 mg%
Average Value: 0·19 mg%

TABLE 5.—*Plasma iron levels in geeldikkop cases*

Sheep No.	mg%	Sheep No.	mg%
A. *Early Cases*		B. *Advanced Cases*	
V1–1	0·16	*V1–4	0·26
V1–2	0·52	*V1–5	0·16
*V1–3	0·24	*V1–6	0·44
*V1–11	0·22	*V1–7	0·32
*V1–13	0·28	V1–8	0·28
*V1–15	0·38	*V1–9	0·16
V1–17	0·20	V1–10	0·20
V1–18	0·30	*V1–12	0·32
V1–22	0·18	*V1–14	0·30
V1–23	0·16	V1–20	0·22
V1–24	0·20	V1–21	0·20
V1–25	0·18		
V1–26	0·10	C. *Recovered Cases*	
F–1	0·12	V1–19	0·20
*F–2	0·31	F–3	0·20
*F–5	0·20	*F–4	0·14

Ranges: 0·10–0·52 (early cases); 0·16–0·44 (advanced cases) (mg%)
Average Values: 0·23 (early cases); 0·26 (advanced cases) (mg%)

* Haematological examinations showed fair to marked anaemia.

As can be seen from Table 4 the plasma levels of iron in control animals varied little within the limits of 0·12 to 0·22 mg per cent with a mean value of 0·19 mg per cent. Table 5 shows marked elevations of the element in the plasma of many geeldikkop cases, reaching in some instances the plasma saturation level for iron (by analogy with other species, this should lie between about 0·360 to 0·500 mg per cent for the sheep). In such cases " iron-overload " may be present and cytosiderosis can be expected. The mean values for the plasma levels of iron were significantly raised in both early and advanced cases of the disease.

Liver and kidney levels of the element were also remarkably constant in the control animals, varying little between the limits of 9·0 to 14·8 mg per cent and 6·0 to 12·2 mg per cent respectively (Table 6).

TABLE 6.—*Liver and kidney iron levels in control animals*

(All values are expressed as mg per 100 gm of liver on a wet basis)

Sheep No.	Liver mg%	Kidneys mg%	Sheep No.	Liver mg%	Kidneys mg%
V1–7054.....	14·8	9·0	V1–7062.....	12·8	11·2
V1–7055.....	9·2	9·0	V1–7064.....	14·8	10·0
V1–7056.....	12·0	8·2	V1–7065.....	11·6	11·8
V1–7057.....	11·0	6·0	V1–7066.....	9·0	9·0
V1–7059.....	9·0	7·2	V1–7067.....	14·8	12·2
V1–7060.....	14·0	12·0	V1–7068.....	14·8	11·2
V1–7061.....	12·8	11·2			

Range: (*a*) Liver: 9·0–14·8 mg% (*b*) Kidney: 6·0–12·2 mg%
Average: (*a*) Liver: 12·4 mg% (*b*) Kidney: 9·9 mg%

TABLE 7.—*Liver and kidney iron levels in geeldikkop cases*

(All values are expressed as mg per 100 gm of liver on a wet basis)

Sheep No.	Liver mg%	Kidneys mg%	Sheep No.	Liver mg%	Kidneys mg%
V1–3.....	8·2	4·2	V1–14.....	7·4	3·8
V1–24.....	19·0	13·6	V1–20.....	18·8	29·6
V1–6.....	10·6	9·0	V1–14A.....	8·2	12·8
V1–7.....	10·6	7·4	V1–16.....	12·4	34·4
V1–9.....	8·8	6·0			

Range: (*a*) Liver: 7·4–18·8 mg% (*b*) Kidney: 4·2–34·4 mg%
Average: (*a*) Liver: 11·6 mg% (*b*) Kidney: 14·1 mg%

As can be seen from Table 7 increased iron values in liver tissue were found in only two out of nine cases of geeldikkop. In the case of kidney tissue, four cases out of the nine showed markedly raised values for the element. These findings are consistent with the general histopathology of the disease. Hepatic siderosis is seen very seldom indeed and deposition of iron in the kidneys seems to be found only in the types of cases mentioned earlier (Brown, *et al.*, 1960). As has been pointed out earlier cytosiderosis is to be expected only in these instances in which the plasma saturation level for the element is exceeded. This does not appear to be very frequent judging from the data presented in Table 5. It seems from the data available that the binding of most of the iron liberated in the haemolytic process, as the normal

storage form ferritin, is unimpaired. By the same token its uptake and release to storage sites by plasma transferrin is not disturbed, except in instances where " overload " has occurred.

It would seem from our studies thus far that the frequency of occurrence and duration of the hyperferraemia seen in geeldikkop is dependent largely on the severity and duration of the initial haemolytic episode. It is still present in at least half of the advanced cases studied and in none of the few recovered cases. Too few of the latter have, however, been studied in this respect to state definitely when the symptom disappears. More will be said regarding iron metabolism in the next chapter.

5. *Erythrocyte sedimentation rate*

The data pertaining to this determination are presented in Appendix 3. No deviations from the normal value accepted for the sheep have been found in any of the cases of geeldikkop. Accelerated sedimentation rates are found in such conditions as acute general bacterial infections, malignancy, arthritis, local suppurative processes and anaemia (Parker, 1948; Coffin, 1953). Elevated erythrocyte sedimentation rates are also to be anticipated in traumatic injury and considerable tissue damage and dermatitis (Schalm, 1965). Sedimentation tests are seldom applied to ovine blood because of the almost insignificant settling of the red cells even in severe disease states. Geeldikkop, in which there is tremendous tissue damage and generally considerable secondary bacterial infection of the lesions of photosensitization, is apparently no exception to this general finding.

6. *Concluding remarks*

The Vosburg cases (prefixed VB-) were some of the severest cases of geeldikkop ever seen by the author amongst many hundreds of animals suffering from the disease. They probably represent therefore the limits to which the pathology of geeldikkop can advance whilst still retaining its identity as a distinct syndrome. The disturbances in the leukocytes and erythrocytes of these animals were consistently uniform in their severity; the same will be seen to apply in various aspects of the chemical pathology discussed later.

Cases of a " mixed geeldikkop-enzootic icterus " syndrome are typically severe and invariably terminate fatally after an illness of about seven days. Such cases show signs characteristic of both syndromes and will be discussed in greater detail later. They represent, however, cases in which geeldikkop has lost its character of a distinct entity.

In Appendices 2 and 3 are presented " normal values " for the various haematological determinations done, which have been taken from various literature sources. When compared with similar values obtained from the control animals, some discrepancies are evident. The present data as regards the leukocytes are in general agreement with those from other sources but differ from these in being somewhat lower in all aspects pertaining to the erythrocytes. Since the control animals were shown to be free of significant helminth infestations and were in excellent condition, the differences must be attributed to the set of nutritional and environmental factors peculiar to the Karoo. These facts demonstrate some of the pitfalls inherent in using biological data of this nature for comparative purposes which have been obtained from animals maintained under very different conditions elsewhere.

CHAPTER 4

The General Chemical Pathology and Biochemistry of Geeldikkop

A. Liver Function in Geeldikkop

1. Introductory remarks
2. Bile pigment excretion and bile acid secretion
3. Blood, urine and faecal porphyrin levels
4. Copper metabolism
5. Iron excretion
6. Plasma protein levels
7. Total plasma cholesterol levels
8. Liver function tests
9. General discussion

1. *Introductory remarks*

Geeldikkop is amongst other things basically a disease involving disturbances in biliary secretion or excretion (Quin, 1933b, 1936; Quin & Rimington, 1933; Quin *et, al.*, 1935). A large part of the work reported here therefore involved studies on the definition of the nature of these disturbances and attempts to eludicate their pathogenesis. An attempt will be made to demonstrate during the course of this chapter that the disturbances of liver function in geeldikkop involve a sudden and almost complete regurgitation of many of the components of bile into the systemic blood circulation.

2. *Bile pigment excretion and bile acid secretion*

The typical icterus of geeldikkop was recognized by the earlier workers on the disease as being similar to that seen in mechanical obstruction of the bile duct (Quin, 1936; Quin & Rimington, 1933), and to that produced by icterogenin and plants containing this factor (Quin, 1933b, 1936; Quin & Rimington, 1933). The statements regarding the nature of the icterus were based upon then contemporary studies of the pathology of the disease and upon results of the qualitative Van den Bergh test for bile pigments in blood plasma. No details of any bile pigment determinations on the plasma of geeldikkop cases were ever published, nor does any detailed study of bile pigment metabolism in the disease appear to have been made prior to the present investigations.

The nature of the bilirubin conjugates present in sheep bile, their mode of formation and manner of excretion form the subject of one of the later chapters of this thesis. The present discussion will deal only with plasma levels of unconjugated bilirubin (hereafter referred to as *bilirubin*) and of conjugates of the pigment (hereafter referred to as *bilirubin glucuronides*) and the occurrence of the latter in urine from geeldikkop cases.

Bilirubin levels in the plasma of normal sheep are negligible, and have been determined by various authors to lie within the range 0 to 0·5 mg per cent (Cornelius & Kaneko, 1963). The traces of pigment that are present are protein bound and generally not detectable by the method used in these studies. Bilirubin determinations were performed regularly on plasma from all the control animals used in this work. In no instance was a value higher than 0 mg per cent recorded.

Levels of the pigment and its conjugates have been determined on plasma from all the geeldikkop cases used in these studies. For the purposes of this discussion, the cases of geeldikkop under consideration have been classified as before, but the various groups have been further subdivided as follows:—

Early cases: Group 1: Highly photosensitive animals showing negligible or mild clinical icterus

Group 2: Animals which were no longer photosensitive but in which the lesions of photosensitization were generally severe and clinical icterus was mild to severe

Advanced cases: Group 1: Cases with mild lesions of photosensitization in general, and mild icterus. An uneventful recovery could have been expected in each case

Group 2: Cases showing lesions of photosensitization and icterus varying from mild to extremely severe. Many cases were either *in extremis* when first seen or showed severe general symptoms unrelated to the photosensitization syndrome (Brown, 1966a).

In addition to the above classification all the cases studied have been tabulated within each subgroup according to the duration of illness and are listed in order of increasing bilirubin concentration. This manner of listing the cases studied is used in various tables throughout most of this thesis, since the plasma total bilirubin level provides a useful general means of assessing the severity of the biochemical disturbances in each patient, and hence the severity of the disease as a whole.

In the discussion following immediately, the severe Vosburg cases (sheep prefixed VB-) have been separated from the more characteristic generally encountered type of case (all animals prefixed V1-, V3- or F-).

Plasma bile pigment concentrations in typical early cases of the disease are presented in Table 8.

It is apparent from this table that hyperbilirubinaemia is present from the onset of symptoms. Icterus is preceded by photosensitivity but this symptom has largely passed over by the time the icterus is clinically obvious. Maximum total bilirubin levels are found between the second and third day of illness. A subsequent decrease in bile pigment concentration is seen in animals which will presumably recover (see, for instance, the following table where this trend is continued).

A noteworthy finding in these cases of geeldikkop is the presence of almost equal amounts of bilirubin and bilirubin glucuronides in the plasma of nearly all of the affected animals. In some instances most of the total pigment present is bilirubin with lesser amounts of its conjugates being in evidence (see e.g. the values for sheep V1-2, V3-14, V3-15, V3-20, V3-16 and V1-18).

Data relating to the plasma bile pigments of advanced and recovered cases of the disease are presented in Table 9.

TABLE 8.—*Plasma bilirubin levels in early cases of geeldikkop*

(All values are expressed as mg per 100 ml blood)

Salient Clinical Features	Duration of Illness	Sheep No.	Total Bilirubin	Bilirubin Glucuronides	Bilirubin
Group 1: Highly photosensitive. Mildly icteric	1–2 days	V1–25	1·0	0·6	0·4
		V1–3	2·0	1·2	0·8
		V3–22	3·5	1·8	1·7
		V3–23	4·4	2·4	2·0
		V3–21	4·5	2·0	2·5
		V3–24	6·9	3·8	3·1
		V1–2	6·9	3·0	3·9
	2–3 days	V1–23	0·1	0	0·1
		V1–22	0·4	0·2	0·2
		V1–24	1·6	1·2	0·4
		V1–17	2·6	1·4	1·2
Group 2: Severe lesions of photosensitization. Mild to severe icterus	1–2 days	V1–1	7·8	4·4	3·4
		V3–26	12·3	6·5	5·6
	2–3 days	V3–14	0·22	0	0·22
		V3–13	0·7	0·4	0·2
		V1–15	1·0	1·0	0
		F–5	2·8	1·4	1·4
		V3–15	3·90	1·04	2·86
		V1–13	4·6	2·6	2·0
		V3–27	4·8	3·0	1·8
		V3–20	6·0	2·7	3·3
		F–2	13·1	7·1	6·0
		V3–25	14·7	8·4	6·3
		F–1	15·0	10·0	5·0
	3–5 days	V3–16	0·36	0	0·36
		V3–10	0·62	0	0·62
		V1–26	1·6	1·0	0·6
		V3–11	2·4	1·22	1·20
		V3–6	4·0	2·4	1·6
		V3–7	5·0	3·2	1·8
	5–7 days	V1–11	0·1	0	0·1
		V1–18	8·2	3·8	4·4

TABLE 9.—*Plasma bilirubin levels in advanced and recovered cases of geeldikkop*

(All values are expresssed as mg per 100 ml blood)

Salient Clinical Features	Duration of Illness	Sheep No.	Total Bilirubin	Bilirubin glucuronides	Bilirubin
Advanced Cases					
Group 1: Mild lesions of photosensitization and mild icterus	7–8 days	V1–21	0·2	0·2	0
		V3–8	0·26	0	0·26
		V3–12	0·28	0	0·28
		V3–17	0·3	0	0·3
		V3–9	0·46	0	0·46
		V3–2	1·4	0·4	1·0
	8–10 days	V1–9	0·1	0	0·1
		V1–10	0·6	0	0·6
	10–14 days	V1–12	0·8	0·4	0·4
Group 2: Mild or severe lesions of photosensitization and mild or severe icterus. Many animals *in extremis* or showing severe general symptoms	7–8 days	V1–5	3·8	2·4	1·4
		V1–4	7·6	4·4	3·2
		V1–14	10·0	5·7	4·3
		V1–7	14·3	8·8	5·5
		V1–6	16·4	11·2	5·2
		V3–18	21·2	10·6	10·6
	8–10 days	V1–8	0·8	0·2	0·6
		V3–1	5·2	2·6	2·6
	10–14 days	V1–20	1·6	1·0	0·6
	14–21 days	V1–16	12·2	11·2	1·0
Recovered Cases.....	(±21 days)	V3–19	0	0	0
	(±17 days)	V1–19	0·1	0·1	0
	(±21 days)	V3–4	0·4	0·2	0·2
	(±21 days)	V3–3	1·6	0·6	1·0
	(±16 days)	F–4	4·9	2·9	2·0
	(±16 days)	F–3	5·5	3·6	1·9

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The generally low figures for total bilirubin in the plasma of the animals in Group 1, indicate a considerable degree of recovery, continuing the trend noted in the previous table (see also the symptomatology of these animals in Appendix 1). Most of the pigment in the plasma of these animals consists of bilirubin, rather than its glucuronides.

A steady increase in total plasma bile pigment concentration from the onset of clinical symptoms is apparent in the majority of advanced cases in the second group. Values for total bilirubin concentration of 10 to 20 mg per cent were seen quite frequently. Although considerable amounts of bilirubin contribute towards these high values, the conjugated pigment is generally present in slightly greater amounts. It accounts for almost all the total bilirubin in exceptional cases only, e.g. Sheep V1-16. Animals falling in this second group were generally critically ill and in most the syndrome of icterus and photosensitization had reached its acme. In some the severe general symptomatology was unrelated to the disturbances of pigment metabolism (Brown, 1966a).

Recovered animals may still show quite high total bilirubin levels depending on the interval between the onset of symptoms and examination of the particular case (compare Sheep F-3 and F-4 with the rest of the cases in this category which are of about five days longer standing).

Plasma bilirubin levels in the severe Vosburg cases were as shown in Table 10.

TABLE 10.—*Plasma bilirubin levels in the Vosburg cases*
(All values are expressed as mg per 100 ml blood)

Salient clinical features and duration of illness		Sheep No.	Total Bilirubin	Bilirubin glucuronides	Bilirubin
A. PRODROMAL CASES (Sheep No. VB-P, VB-Q, VB-R, VB-S, VB-T, VB-U, VB-V, VB-W and VB-X)		All cases	0	0	0
B. EARLY CASES (1–7 DAYS)	Duration of Illness				
Group 1: Highly photosensitive; mildly icteric	1–2 days	VB–H	11·26	6·25	5·01
		VB–I	12·50	6·90	5·60
Group 2: Severe lesions of photosensitization, mild to severe icterus	3 days	VB–A	18·75	10·63	8·12
		VB–E	20·63	12·50	8·13
		VB–F	25·00	15·63	9·37
		VB–C	31·25	20·00	11·25
		VB–B	37·51	24·38	13·13
	4 days	VB–G	37·51	21·88	15·63
	4–5 days	VB–J	20·63	13·75	6·88
	7 days	VB–K	40·62	25·63	14·99
C. ADVANCED CASES (7–21 DAYS) Severe lesions of photosensitization. Mild to severe icterus	7–8 days	VB–D	7·51	6·25	1·26
		VB–L	21·88	11·88	10·00
		VB–M	29·38	17·50	11·88
		VB–N	37·51	23·13	14·38
	14–21 days	VB–Z1	22·50	13·75	8·87
		VB–O	30·00	17·50	12·50
		VB–Y	37·51	24·38	13·13
D. RECOVERED CASES (21+ DAYS).	21+ days	VB–Z	1·88	1·25	0·63

It is apparent from this set of figures that the prodromal stage of the disease is not associated with hyperbilirubinaemia. This symptom appears simultaneously with the first clinical signs of the disease and its development is rapid. The data given in this table are indicative of the extreme severity which the icterus in geeldikkop may assume. Values for bilirubin are very close to those for its conjugated derivatives in the early stages of the disease, but become considerably lower in most cases as the disease progresses. The very high levels of bilirubin glucuronides found in the plasma of these animals are indicative of a complete block in the hepatic excretion of conjugated bilirubin. In many instances the gallbladder was found at autopsy to contain only small amounts of colourless or faintly yellow-tinged bile.

The total bile acid levels found in the plasma of seven of our control animals are presented in Table 11. These results are representative of plasma bile acid levels in apparently normal Karoo sheep. Although the " normal range " for these plasma constituents has not been statistically established for Karoo sheep, it can be considered as being 0 to 3 mg per cent for the purposes of this discussion. The values for bile acids in the control animals are compared in Table 11 with the levels of bilirubin glucuronides in the plasma of the same animals. Plasma bile acid levels in all stages of geeldikkop are represented by the figures presented in Table 12. The corresponding values for bilirubin glucuronides are again presented for comparison.

It is apparent from these figures that regurgitation of bile acids into the systemic blood circulation occurs from the onset of the clinical symptoms of geeldikkop, and maximum blood levels are reached during the first three days of illness in typical cases. Reconstitution of bile acid secretion by the liver, or lowering of the renal threshold for these substances seems to occur rapidly after the third day of illness in these cases, and coincides with a general decline in plasma bile pigment levels. Hyperbileacidaemia may still be a prominent finding in some severe advanced cases, e.g. Sheep V3-1A and V3-18 (Table 12), and is associated with severe hyperbilirubinaemia. In such cases there is obviously still severe interference with the primary hepatic or secondary renal pathways of excretion.

The results of the analysis of urine samples of a number of geeldikkop cases for bile pigments and bile acid salts, are presented in Table 13. The plasma levels of bilirubin glucuronides and bile acids of the cases concerned have been included for comparison. Data relating to he occurrence of bile pigments and bile acid salts in the urine of the control animals are reproduced in Table 14.

It is apparent from the data in Table 13 that slightly more than half the animals showed no or negligible bilirubinuria in spite of the high levels of conjugated pigment in their blood. In others, the bilirubinuria which was present was quite mild in comparison with the tremendous load of bilirubin glucuronide carried by the plasma.

As can be seen from Tables 13 and 14, bile acid salts were detectable in the urine of all the control animals, but were absent from the urine of nearly all the geeldikkop cases, even in animals where the plasma bile salt level was extremely high (Sheep V3-25 and V3-26). Any reconstitution of bile acid secretion which may occur as mentioned earlier, must obviously involve the primary biliary route and not urinary excretion.

Urobilinogen is generally always detectable in the urine of normal sheep. The controls used in this work were no exceptions (Table 14). This pigment was found to be absent from all the urine specimens obtained from the geeldikkop cases, even in animals which appeared to be recovering. In the latter, gastro-intestinal motility was still generally impaired and absorption from the gut therefore at a low level.

TABLE 11.—*Plasma bile acid and bilirubin glucuronide levels in control animals*

(All values are expressed as mg per 100 ml of blood)

Sheep No.	Bilirubin glucuronides	Bile Acids
V3–33	0	2·43
V3–31	0	2·19
V3–28	0	1·81
V3–29	0	1·48
V3–32	0	0·81
V3–5	0	0·52
V3–30	0	0·19

TABLE 12.—*Plasma bile acid levels in cases of geeldikkop, compared with plasma levels for bilirubin glucuronides*

(All values are expressed as mg per 100 ml of blood)

Sheep No.	Stage of Disease	Bilirubin glucuronides	Bile acids
V3–24	Early case: Group 1 (1–2 days)	3·8	32·32
V3–23	„ „ „ „ (1–2 days)	2·4	42·13
V3–21	„ „ „ „ (1–2 days)	2·0	9·23
V3–22	„ „ „ „ (1–2 days)	1·8	27·23
V3–26	„ „ Group 2 (1–2 days)	6·5	70·59
V3–25	„ „ „ „ (2–3 days)	8·4	46·03
V3–27	„ „ „ „ (2–3 days)	3·0	16·04
V3–20	„ „ „ „ (2–3 days)	2·7	1·90
V3–15	„ „ „ „ (2–3 days)	1·04	14·09
V3–13	„ „ „ „ (2–3 days)	0·4	0
V3–14	„ „ „ „ (2–3 days)	0	2·09
V3–7	„ „ „ „ (3–5 days)	3·2	4·38
V3–6	„ „ „ „ (3–5 days)	2·4	0·62
V3–11	„ „ „ „ (3–5 days)	1·22	1·48
V3–10	„ „ „ „ (3–5 days)	0	0·99
V3–16	„ „ „ „ (3–5 days)	0	0·76
V3–2	Advanced case: Group 1 (7–8 days)	0·4	0·62
V3–8	„ „ „ „ (7–8 days)	0	1·38
V3–9	„ „ „ „ (7–8 days)	0	2·95
V3–12	„ „ „ „ (7–8 days)	0	1·20
V3–17	„ „ „ „ (7–8 days)	0	1·62
V3–1A	„ „ Group 2 (7–8 days)	—	18·4
V3–18	„ „ „ „ (7–8 days)	10·6	80·73
V3–1	„ „ „ „ (8–10 days)	2·6	3·43
V3–3	Recovered case (±21 days)	0·6	1·57
V3–4	„ „ (±21 days)	0·2	0·81
V3–19	„ „ (±21 days)	0	1·19

TABLE 13.—*Qualitative examination of the urine of geeldikkop cases for bile pigments and bile salts—comparison with plasma levels of these compounds*

Sheep No.	Bilirubin Glucuronide in Plasma (mg%)	Plasma Bile Acids (mg%)	Bilirubin Glucuronides in Urine	Urinary Uro-bilinogen	Urinary Bile Acids
VB–K	25·6	—	3+	0	+
VB–G	21·9	—	2+	0	0
F–1	10·0	—	0	0	0
V3–25	8·4	46·0	2+	0	traces
F–2	7·1	—	traces	0	0
VB–I	6·9	—	+	0	traces
V3–26	6·5	70·59	0	0	0
V1–13	2·6	—	traces	0	0
F–5	1·4	—	0	±	0
V1–24	1·2	—	0	0	0
VB–N	23·1	—	+	0	0
VB–0	17·5	—	3+	0	+
V1–7	14·3	—	+	0	0
V1–16	11·2	—	0	0	0
V1–4	4·4	—	+	0	0
F–3	3·6	—	0	0	0
F–4	2·9	—	0	0	0
V3–1	2·6	3·43	traces	0	+
V1·5	2·4	—	traces	0	0
V1–20	1·0	—	0	0	0

TABLE 14.—*Qualitative examination of the urine of control animals for bile pigments and bile salts*

Sheep No.	Bilirubin glucuronides	Urobilinogen	Bile acids
F–K2	0	+	+
F–12221	0	+	+
F–12222	0	+	2+
V1–7054	0	+	+
V1–7055	0	+	+
V1–7056	0	+	3+
VI–7057	0	+	+
V1–7059	0	+	+
V1–7060	0	+	2+
V1–7061	0	+	+
V1–7062	0	+	3+
V1–7064	0	+	+
V1–7065	0	+	+
V1–7066	0	+	2+
V1–7067	0	+	+
V1–7068	0	+	+
V1–7069	0	+	3+
V1–7070	0	+	+
V1–7071	0	+	+

The faeces in all stages of geeldikkop are characteristically lighter in colour, acholic and severely desiccated [the latter as a result of the severe gastro-intestinal stasis which is present in the disease (Brown, 1966a)]. Qualitative tests for bilirubin and urobilinoids on faecal specimens from early cases yielded very much weaker positive reactions than those obtained with faeces pellets from the control animals. Negative reactions were obtained on the faeces of many advanced cases. Considerable amounts of both pigments appeared again in the faeces during the recovery phase, particularly when gastro-intestinal motility had been restored.

3. *Blood, urine and faecal porphyrin levels*

Earlier studies on the role of phylloerythrin as the photodynamic agent in geeldikkop have already been mentioned. These studies did not include any quantitative studies on the levels of this porphyrin in the blood or urine of geeldikkop cases.

As can be seen from the case histories given in Appendix 1, and the grouping of sheep in the previous tables, the stage of actual photosensitivity in typical uncomplicated geeldikkop occupies only the first one to three days of the duration of the syndrome, which as a whole may last for 14 to 21 days (Brown, 1966a). Since the porphyrin is usually excreted in the bile, it is of interest to determine whether the interference with its excretion is similar to that in the case of bile pigments and bile acids.

Coproporphyrin is also normally excreted in the bile of the sheep (Clare, 1944; Brown, 1967a) and it is thus also of interest to see whether its excretion is similarly affected.

The existence of a haemolytic anaemia in geeldikkop was postulated in the previous chapter. In a subsequent section evidence will be produced that some of the features of geeldikkop are a marked increase in red cell fragility and severe disturbances of erythrocyte carbohydrate metabolism. The levels of free protoporphyrin in the red cells of geeldikkop patients are thus also of immediate interest.

Values for plasma phylloerythrin and erythrocyte protoporphyrin for the control animals are presented in Table 15, while the corresponding data found in cases of all stages of geeldikkop are presented in Table 16. Values for bilirubin glucuronides in the plasma of the sheep concerned have been included in the latter table for comparison.

TABLE 15.—*Plasma phylloerythrin and erythrocyte protoporphyrin levels in control animals*

(*Note.*—Phyllo. = phylloerythrin; proto. = protoporphyrin)

Sheep No.	Plasma phyllo. mcg%	Red cell proto. mg %
V3–5	0·88	0·095
V3–28	5·70	0·060
V3–29	8·10	0·110
V3–30	13·80	0·074
V3–31	5·80	0·054
V3–32	2·99	0·052
V3–33	2·94	0·115

TABLE 16.—*Plasma phylloerythrin, erythrocyte protoporphyrin and bilirubin glucuronide levels in cases of geeldikkop*

(*Note.*—phyllo. = phylloerythrin; proto. = protoporphyrin)

Sheep No.	Stage of disease	Bilirubin glucuronide mg%	Plasma phyllo. mcg%	Red cell proto. mg%
V3–24	Early case: Group 1 (1–2 days).....	3·8	49·11	0·100
V3–23	,, ,, ,, ,, (1–2 days).....	2·4	46·98	0·045
V3–21	,, ,, ,, ,, (1–2 days).....	2·0	21·70	0·075
V3–22	,, ,, ,, ,, (1–2 days).....	1·8	46·31	0·039
V3–26	,, ,, Group 2 (1–2 days).....	6·5	84·95	0·215
V3–25	,, ,, ,, ,, (2–3 days).....	8·4	119·34	0·138
V3–27	,, ,, ,, ,, (2–3 days).....	3·0	38·19	0·096
V3–20	,, ,, ,, ,, (2–3 days).....	2·7	10·44	0·075
V3–15	,, ,, ,, ,, (2–3 days).....	1·04	18·40	0·045
V3–13	,, ,, ,, ,, (2–3 days).....	0·4	27·50	0·045
V3–14	,, ,, ,, ,, (2–3 days).....	0	3·45	0·03
V3–7	,, ,, ,, ,, (3–5 days).....	3·2	10·35	0·019
V3–6	,, ,, ,, ,, (3–5 days).....	2·4	22·95	0·015
V3–11	,, ,, ,, ,, (3–5 days).....	1·22	11·90	0·077
V3–10	,, ,, ,, ,, (3–5 days).....	0	9·10	0·050
V3–16	,, ,, ,, ,, (3–5 days).....	0	2·60	0·044
V3–2	Advanced case: Group 1 (7–8 days)...	0·4	8·50	0·070
V3–8	,, ,, ,, ,, (7–8 days)...	0	2·85	0·060
V3–9	,, ,, ,, ,, (7–8 days)...	0	2·20	0·077
V3–12	,, ,, ,, ,, (7–8 days)...	0	5·80	0·050
V3–17	,, ,, ,, ,, (7–8 days)...	0	2·86	0·126
V3–1A	,, ,, Group 2 (7–8 days)...	—	6·80	0·116
V3–18	,, ,, ,, ,, (7–8 days)...	10·6	136·01	0·150
V3–1	,, ,, ,, ,, (8–10 days)..	2·6	9·64	0·164
V3–3	Recovered case: (±21 days).....	0·6	12·60	0·090
V3–4	,, ,, (±21 days).....	0·2	4·50	0·102
V3–19	,, ,, (±21 days).....	0	0·98	0·075

Marked elevations of phylloerythrin occur in the plasma of affected animals from the onset of symptoms, and there is in general a good correlation between the plasma bilirubin glucuronide, phylloerythrin and bile acid levels. The presence of significant amounts of the porphyrin in the blood of affected animals is somewhat more persistent than in the case of bilirubin conjugates and bile acids, " normal " levels being reached again by the fifth day of illness in most cases. Highest values for plasma phylloerythrin are generally found around the second to third day of illness. All the animals listed under Group 1 in Table 18 were highly photosensitive, while those in Group 2 were no longer photosensitive. Some very high plasma levels of phylloerythrin are however still present amongst the animals in this group. Sheep V3-18 which falls into Group 2 of the advanced cases gave the highest figure yet found for plasma phylloerythrin in the present investigations. This sheep was also no longer photosensitive. The reader should notice that this particular animal also had the highest values in the two groups of advanced cases for plasma bile acids (Table 12) and bilirubin glucuronides.

The relevant clinical data and the figures presented here suggest a poor correlation between the presence of photosensitivity and plasma phylloerythrin levels. More will be said on this point in the discussion at the end of this section.

Allen (1956) has given the normal range of free protoporphyrin in ovine erythrocytes as 0·03 to 0·09 mg per cent. The figures obtained from the erythrocytes of the control animals are in the same order (range: 0·052 to 0·115 mg per cent, mean value: 0·08 mg per cent). The range for human erythrocytes is given as 0·024 to 0·052 mg per cent (Goldberg & Rimington, 1962). The erythrocyte of the sheep contains thus slightly more of this porphyrin than the human red cell.

It is apparent from the data presented in Tables 15 and 16 that values for red cell protoporphyrin higher than the upper limit of the normal range were found in two early cases (Sheep V3-26: 0·215 mg per cent and Sheep V3-25: 0·138 mg per cent) and in three advanced cases (Sheep V3-17: 0·126 mg per cent; Sheep V3-18: 0·15 mg per cent and Sheep V3-1: 0·164 mg per cent). Values for recovered cases fell within the " normal " range. Mild elevations of free protoporphyrin were thus seen in the erythrocytes of 20·8 per cent of the cases studied.

Such elevations of free protoporphyrin in circulating erythrocytes are found in erythropoietic protoporphyria, erythropoietic porphyria of man and cattle, anaemias of pyogenic infections, lead poisoning and in iron deficiency and haemolytic anaemias (Cornelius & Kaneko, 1963; Goldberg & Rimington, 1962). In the latter two instances a real or relative lack of iron could account for the accumulation of free protoporphyrin in the erythrocyte. Very little is known of erythrocyte protoporphyrin levels in ovine disease states. The elevations encountered in the cases of geeldikkop mentioned may be a manifestation of the haemolytic state which is present.

Plasma coproporphyrin levels were unfortunately not studied during the present field investigations owing to the large number of other determinations which were being done on the sheep used at the time. The urinary excretion of this porphyrin was, however, studied in the rams and wethers amongst the experimental animals. The animals used to control this part of the work were 16 clinically normal sheep purchased from various farms in the geeldikkop areas at a time when no outbreaks of the diseases were prevalent anywhere. These sheep were used for studies on the chemical pathology of mild bluetongue infections which will be discussed later in this thesis. The data obtained from these animals are presented in Table 17.

The first two collection dates in each group of animals (i.e. sheep 1–8 and 9–16) represent urine collections before injection with a mild strain of bluetongue virus. These figures may be considered as being " normal ". Temperature spikes occurred in approximately half the sheep about five days after injection (the date of the second urine collection in the table) and lasted for not longer than two days. Most of the reactors were amongst the first group. In a few cases, e.g. Sheep 1, 3 and 8, these fever reactions were associated with mild and transient elevations in the daily urinary output of coproporphyrin.

The data in this table are useful in indicating the small elevations which may occur in the daily urinary excretion of this porphyrin in mild diseases not associated with frank photosensitivity. Although the animals listed in the table were subject to a pyrexia of virus origin during the latter stages of the experiment cited, the figures make a useful comparison with those obtained from geeldikkop cases.

TABLE 17.—*Urinary coproporphyrin levels in normal sheep emanating from the Karoo infected with a mild strain of bluetongue virus*

(Values are expressed as mcg coproporhyrin excreted per 24 hr)

Sheep No.....	1	2	3	4	5	6	7	8
Date								
31–1–65.....	5·24	2·53	—	—	—	—	0·96	2·92
1–2–65.....	—	—	4·34	10·76	2·90	6·39	—	—
2–2–65.....	17·62	6·90	—	—	—	—	3·68	3·77
3–2–65.....	—	—	2·95	15·92	9·45	15·55	—	—
7–2–65.....	33·19	5·29	—	—	—	—	8·99	7·83
8–2–65.....	—	—	5·45	6·36	7·30	15·8	—	—
9–2–65.....	16·43	—	—	—	—	—	0·64	10·01
10–2–65.....	—	—	5·63	4·62	0·75	12·66	—	—
14–2–65.....	40·36	4·85	—	—	—	—	0	21·61
15–2–65.....	—	—	4·29	5·55	0·88	15·70	—	—
16–2–65.....	19·77	—	—	—	—	—	0·16	0
17–2–65.....	—	—	30·43	0	0	4·76	—	—
21–2–65.....	25·69	—	—	—	—	—	11·96	10·99
22–2–65.....	—	—	11·62	—	9·98	—	3·56	—
23–2–65.....	—	—	2·97	—	8·18	—	10·58	—
24–2–65.....	—	—	6·55	—	3·41	—	19·74	—

Sheep No.....	9	10	11	12	13	14	15	16
Date								
22–3–66.....	16·82	2·57	2·12	0·17	—	—	—	—
25–3–66.....	3·82	0·05	—	—	—	—	—	—
28–3–66.....	17·93	2·61	0	2·57	13·69	—	—	—
30–3–66.....	4·30	7·31	0·94	1·41	—	3·85	—	—
4–4–66.....	6·43	3·02	—	—	—	—	8·01	2·46
7–4–66.....	—	—	3·35	1·67	11·45	5·22	—	—
12–4–66.....	16·96	2·79	2·77	6·22	6·54	3·6	4·36	9·62

Range..... Sheep 1–8: 0–40·36 mcg per 24 hr
Mean Value..... Sheep 1–8: 9·07 mcg per 24 hr (58 determinations)
Range..... Sheep 9–16: 0–17·93 mcg per 24 hr
Mean Value..... Sheep 9–16: 5·45 mcg per 24 hr (32 determinations)

Very little is known of coproporphyrin excretion in normal sheep. In his earlier work Clare (1944) indicated that coproporphyrin is either absent from the urine of normal sheep or else present in extremely small amounts. It has been possible to confirm this earlier work and to quantitate the small amounts which are actually present by using the highly improved spectrophotometric techniques developed recently by Rimington's school (Rimington, 1958; Rimington & Sveinsson, 1950). The overall range found from 90 determinations on the urine of these 16 control animals for the 24 hourly excretion of this porphyrin is 0 to 40 mcg with a mean value of 7·26 mcg.

The data obtained from fifteen of the geeldikkop cases are presented in Table 18.

TABLE 18.—*Urinary coproporphyrin levels in geeldikkop cases*

Sheep No.	Stage of disease	Copro. (mcg/Litre)	Copro. (mcg/24 hr)
V3–24	Early case: Group 1 (1–2 days).....	366·7	454·6
V3–23	„ „ „ „ (1–2 days).....	226·9	115·7
V3–22	„ „ „ „ (1–2 days).....	172·8	240·2
V3–21	„ „ „ „ (1–2 days).....	96·7	22·1
V3–26	„ „ Group 2 (1–2 days).....	257·1	382·6
V3–25	„ „ „ „ (2–3 days).....	297·6	330·3
V3–27	„ „ „ „ (2–3 days).....	7759·0	4089·0
V3–20	„ „ „ „ (2–3 days).....	136·1	305·3
V3–15	„ „ „ „ (2–3 days).....	493·5	182·6
V3–13	„ „ „ „ (2–3 days).....	209·9	91·76
V3–6	„ „ „ „ (3–5 days).....	21·95	26·34
V3–2	Advanced case: Group 1 (7–8 days).....	42·06	10·52
V3–9	„ „ „ „ (7–8 days).....	54·64	10·93
V3–3	Recovered case: (±21 days).....	29·46	51·86
V3–4	„ „ (±21 days).....	4·27	5·72
V3–32	Control animal.....	66·46	31·52

Extremely high values for urinary coproporphyrin were found during the first three days of illness in both photosensitive and non-photosensitive animals. No attempt was made at this stage of the work to determine which isomer of the porphyrin was mainly present in the urine of these cases.

The faeces of sheep contain porphyrins of both endogenous and exogenous origin. The former enter the gut via the bile and are represented mainly by protoporphyrin and coproporphyrin. These same two porphyrins may be formed as exogenous products by gut micro-organisms acting either on haem-containing materials in the food or synthesizing them from simple substances (Goldberg & Rimington, 1962). The exogenous porphyrins present in sheep faeces include in addition those formed by bacterial degradation of chlorophylls, e.g. phylloerythrin, chlorins, etc. Mesoporphyrin and deuteroporphyrin formed by bacterial transformation of protoporphyrin are probably also present.

Faecal coproporphyrin and protoporphyrin levels were determined on 24 hour samples from a limited number of geeldikkop cases. The results are presented in Table 19. Seven clinically normal sheep purchased from farms in various parts of the Karoo and used for nutritional experiments at Onderstepoort served as controls for this work. The results of the porphyrin analyses done on 24 hour faeces samples from these sheep are presented in Table 20.

TABLE 19.—*Faecal coproporphyrin and protoporphyrin levels in geeldikkop cases*

(All values are expressed as mcg porphyrin per gm of dried faeces. Faeces were dried to a constant weight at 100° C and then powdered in a laboratory mill before analysis)

Sheep No.	Stage of disease	Copro.	Proto.
V3–24	Early case: Group 1 (1–2 days).....	0·037	26·92
V3–23	„ „ „ „ (1–2 days).....	0·022	46·02
V3–22	„ „ „ „ (1–2 days).....	0·032	31·36
V3–13	„ „ Group 2 (2–3 days).....	0·101	21·59
V3–2	Advanced case: Group 1 (7–8 days).....	0·320	93·19
V3–9	„ „ „ „ (7–8 days).....	0·312	30·66
V3–3	Recovered case: (±21 days).....	0·350	25·32
V3–4	„ „ (±21 days).....	0·370	22·55

Coproporphyrin: Range..... (*a*) Early cases: 0·022–0·101 mcg/gm
(*b*) Advanced cases: 0·312–0·320 mcg/gm
Mean value..... (*a*) Early cases: 0·048 mcg/gm
Protoporphyrin: Range..... (*a*) Early cases: 21·59–46·02 mcg/gm
(*b*) Advanced cases: 30·66–93·19 mcg/gm
Mean value..... (*a*) Early cases: 31·47 mcg/gm

TABLE 20.—*Faecal coproporphyrin and protoporphyrin levels in normal sheep emanating from the Karoo*

(All values are expressed as mcg per gm of dried faeces. Faeces were dried to a constant weight at 100° C and were then powdered in a laboratory mill and thoroughly mixed before analysis. C = coproporphyrin; P = protoporphyrin)

	Date	Sheep 9601	Sheep 9603	Sheep 9619	Sheep 12099	Sheep 12102	Sheep 12221	Sheep 12513
C	30/7/64	0	0	0	0·010	0	0	0
	31/7/64	0	0	0	0·021	0	0	0
	1/8/64	0	0	0	0	0	0	0
P	30/7/64	24·92	25·00	23·82	26·77	25·50	13·24	10·88
	31/7/64	21·71	22·71	21·79	22·23	11·30	31·96	17·75
	1/8/64	26·82	28·00	24·82	29·65	6·00	29·34	46·87
		Sheep 15582	Sheep 16624	Sheep 16626	Sheep 17206	Sheep 17211	Sheep 17220	Sheep 17234
C	30/7/64	0·042	0	0	0·382	0·052	0·051	0
	31/7/64	0·037	0	0	0·217	0·031	0·030	0
	1/8/64	0·062	0	0	0·082	0·164	0·064	0
P	30/7/64	23·68	19·62	23·38	19·36	25·57	24·66	24·87
	31/7/64	33·01	5·96	2·85	15·81	10·84	14·59	4·53
	1/8/64	30·86	4·04	1·08	14·47	8·83	14·92	1·49

Coproporphyrin: Range..... 0–0·382 mcg per gm
Mean Value.... 0·030 mcg per gm
Protoporphyrin: Range..... 1·08–46·87 mcg per gm
Mean Value..... 19·42 mcg per gm

Detectable amounts of coproporphyrin were present in the faeces of only 37 per cent of the control animals. Clare (1944) was only able to detect it in the faeces of 15 per cent of his experimental animals. The levels of coproporphyrin found in the faeces of the control sheep are far below those accepted for the faeces of healthy humans, i.e. 0 to 0·382 mcg per gm for sheep (our figures) as opposed to 0 to 36 mcg per gm for humans (Goldberg & Rimington, 1962; Eales, 1960). This may be a simple dilution effect due to the large amount of undigested plant material present in herbivore faeces.

Coproporphyrin was present in the faeces of all the geeldikkop cases noted in Table 19. In this respect, little difference is apparent between these sheep and the control animals.

Protoporphyrin is normally absent from the urine, but is present in bile and faeces (Dean, 1963; Goldberg & Rimington, 1962). The range found for this porphyrin in 42 determinations on the faeces of 14 normal control sheep is shown at the foot of Table 20. These ranges are very similar to those established for human faeces, e.g. 0 to 30 mcg per gm (Rimington, 1958) and 1 to 113 mcg per gm (Eales, 1960).

Faecal protoporphyrin was found to be within the " normal range " in four early and two recovered cases of geeldikkop, and markedly elevated in one of the two advanced cases noted in Table 19. No particular significance can be attached to this single elevated value at this stage.

Before leaving this discussion on porphyrin excretion it is essential that some of the findings in this regard which conflict with known facts be discussed. In the first instance Clare (1944) has stated that if phylloerythrin can be detected in the blood of animals, it is there in sufficient amounts to cause photosensititivy. At that time no reliable method was available to estimate the porphyrin quantitatively in plasma or body fluids. Clare was able to demonstrate the presence of phylloerythrin in the plasma of 93 per cent of authentic facial eczema cases in a photosensitive state. Only 4 per cent of his normal sheep had detectable amounts of the porphyrin in their plasma. This latter statement is contradictory to the generalization cited above. The amount of phylloerythrin which could be extracted from the plasma of his photosensitive cases *appeared* to be in the order of less than 0·01 mg to 1·6 mg per 100 ml blood, but was generally in the region of 0·1 mg per 100 ml. In the same paper he stated that the minimum amount of the porphyrin required to produce photosensitization following intravenous injections lay between 0·4 mg to 0·6 mg per kg body weight. (Using a dosage level of 0·5 mg per kg in sheep weighing 55 kg with a mean plasma volume of 1600 ml, this would give an immediate plasma concentration of 0·78 mg per cent or 780 mcg per cent—far above the levels found in our photosensitive sheep.) The porphyrin was found to disappear rapidly from the blood, although his animals were still photosensitive for a number of hours after phylloerythrin was no longer detectable. The following quotation from his paper is important in the present context: " The injection experiments demonstrate that phylloerythrin is a powerful photosensitizing agent, and that a single injection of 0·6 mg per kg is sufficient to cause marked photosensitivity. Furthermore this amount produces a detectable concentration in the blood for less than thirty minutes; *whereas in even the less severely affected cases of facial eczema traces of phylloerythrin were found over a period of several days* ". (The italics are mine).

As will be seen from Table 15 the phylloerythrin content of the plasma of the control animals was found to range from 0·88 to 13·80 mcg per cent, and as pointed out, considerably higher levels were found in non-photosensitive geeldikkop cases (Table 16). The following questions arise as a result of these findings:—

(*a*) How reliable is the method used by us?

(*b*) Is there a critical level of phylloerythrin in the plasma above which an animal will become photosensitive, and if so is there a variation in the level of tolerance of sheep to small but significant amounts of phylloerythrin in their plasma?

(*c*) Is the nature of the actinic dermatitis in advanced cases of geeldikkop such that there is so much impairment to dermal blood circulation, that further activation of the photodynamic agent cannot occur in the affected areas?

(*d*) Is it possible that another porphyrin which is simultaneously retained in the bloodstream, e.g. coproporphyrin, may act as an additional and perhaps more important photodynamic agent?

The method used to determine plasma phylloerythrin levels is that of Perrin (1958a). He has stressed the limitations of his method and has stated that it is applicable over the range of 50 to 3500 mcg per cent. The figures given here for our geeldikkop cases have been obtained by using the correction factors and reliability checks proposed by him. Our highest figures fall within the range of sensitivity of his method, while those from our photosensitive animals are in the order of the lower limit of linearity of this method. Such figures are therefore quite acceptable in terms of the method. The accuracy of the lower figures obtained from sheep in the later stages of the disease and from the control animals is open to question. Accuracy apart, the fact remains that low levels of the porphyrin are detectable in the blood of these animals.

If this fact is accepted then it seems probable that there is a critical concentration of the porphyrin in plasma above which sheep may become photosensitive, and it is not unlikely that there can be considerable individual variations in this respect. In a later publication, Clare (1955) cites earlier unpublished work by Perrin (1950) indicating that the " level of phylloerythrin in the blood necessary to cause photosensitization in sheep is quite low and may be less than the detectable limit of 0·005 mg per 100 ml ". Most of the control animals gave plasma phylloerythrin values of this order, but the critical level may be higher in individuals (e.g. control Sheep V3-30).

The answer to question (*c*) above lies in the facts that Sheep V3-25 and V3-13 showed extremely mild and healing skin lesions at the time the values given in Table 16 were obtained, while signs of photosensitization did not develop on newly shorn and shaved areas on the backs of other early cases of Group 2 or advanced cases when those animals were exposed to the sun.

There seems, from the figures given in Tables 17 and 18, to be a better correlation between the levels of urinary coproporphyrin and the incidence of photosensitivity, than with plasma phylloerythrin levels and the latter. Clare (1944) discounted coproporphyrin as being responsible for the photosensitivity in facial eczema. His findings in this regard are nevertheless most interesting. Significant amounts of the porphyrin were found in the plasma of 35 per cent of his facial eczema cases and in only 5 per cent of his normal animals, and also in the urine of 81 per cent of his photosensitive animals as against the urine of only 6 per cent of normal sheep. A point of particular interest was that the incidence of phylloerythrin in plasma from

affected sheep was found by Clare to be much higher than that of coproporphyrin (93 per cent compared with 35 per cent), whereas in urine the order was found to be reversed (coproporphyrin, 81 per cent and phylloerythrin, 65 per cent). When Clare injected coproporphyrin he found that symptoms of photosensitivity were produced by " an amount of the porphyrin sixty times the amount found in the blood of facial eczema cases ". On the other hand he stated: " In these experiments however, there appears to have been a rapid loss of coproporphyrin from the blood, *whereas in clinical cases a low but constant concentration of the pigment is maintained. Under such circumstances which could not be reproduced experimentally, the coproporphyrin may accumulate in the tissues and show some photosensitizing activity* ". (The italics are mine).

There are two major difficulties inherent in attempts to incriminate coproporphyrin as a photodynamic agent in geeldikkop from the data here presented. In the first instance coproporphyrin appears to be more easily excreted by the kidneys than phylloerythrin (as seen e.g. by the persistence of fairly high levels of the latter during later stages of the disease when urinary coproporphyrin excretion has fallen off considerably). It is known that over 95 per cent and possibly all of the urinary coproporphyrin is actually excreted by the kidney in the form of coproporphyrinogen which oxidizes spontaneously to coproporphyrin. Liver function is an important factor in determining the route of coproporphyrin excretion (Goldberg & Rimington, 1962). There is a good correlation between the urinary: faecal coproporphyrin ratio and the extent of injury of the hepatic parenchymatous cells in various conditions. Since it would appear that only coproporphyrinogen passes through the kidney and that renal tissue is apparently incapable of reducing coproporphyrin brought to it in the bloodstream to porphyrinogen, it must be concluded that the route of coproporphyrin elimination is governed by the ratio between porphyrin and porphyrinogen established in the liver. If freshly prepared coproporphyrinogen III is injected intravenously into normal rabbits a considerable portion of the dose is eliminated in the urine, whereas if the corresponding porphyrin is injected it is eliminated only in the faeces (Goldberg & Rimington, 1962). It is not known whether these findings pertain to phylloerythrin in the sheep. All the work done on it so far indicates that it is absorbed from the gut and is present in blood and bile as the porphyrin and not the porphyrinogen, in which case it may not be easily excreted by the normal sheep kidney.

The other difficulty is that the period of true photosensitivity in geeldikkop is very short (one to two days). The figures presented in this thesis have been obtained from 24 hourly samples made up of urine voided into a collecting bottle at very infrequent and irregular intervals. There is an oliguria in the early stages of geeldikkop and most of the 24 hour sample was often passed in the early morning when the animals were being handled. Under these conditions of collection it is virtually impossible to establish a relationship between the peak period of coproporphyrin excretion and maximum photosensitivity.

4. *Copper metabolism*

This aspect of the chemical pathology of geeldikkop is of particular interest, in view of the attempt which will be made to demonstrate that geeldikkop and enzootic icterus are two different manifestations of a single disease entity. It has been known for many years that conditions very similar to enzootic icterus may result from a high dietary copper intake (see e.g. Stamp & Stewart, 1953; Bull, Dick, Keast & Edgar, 1956; Bracewell, 1958). This line of work was actively pursued

by earlier workers on enzootic icterus at Onderstepoort and will be discussed in the appropriate section in this thesis. Nothing was known of copper metabolism in geeldikkop until the writer commenced the investigations reported here.

Normal values for the various fractions of copper present in the blood of Merino sheep at Onderstepoort have been compiled by Brown, Brink & Wagner (1967). The methods used for estimation of these fractions are given at the end of this thesis; the normal ranges found are reproduced for study in Table 25. Total plasma copper levels found in the control animals are given in Table 21. With the exception

TABLE 21.—*Total plasma copper levels in control animals*

(Values are expressed as mcg copper per 100 ml blood)

Sheep No.	Plasma Cu	Sheep No.	Plasma Cu
F–12221	75	V1–7062	80
F–12222	75	V1–7064	130
F–K2	133	V1–7065	130
V1–7054	125	V1–7066	70
V1–7055	100	V1–7067	130
V1–7056	100	V1–7068	100
V1–7057	100	V1–7069	75
V1–7059	150	V1–7070	100
V1–7060	100	V1–7071	100
V1–7061	100		

Range: 70–150 mcg % Mean value: 104 mcg %

of the five values ranging from 70 to 80 mcg per cent, all the other figures are well within the normal 80 per cent limits established by Brown, *et al.* The five low values mentioned are lower than the low outer 9 per cent limit established for Onderstepoort sheep, and the majority of values cited in Table 21 are lower than the median established for the same sheep (Brown, *et al.*, 1967).

The values found for total plasma copper levels in the prodromal cases of geeldikkop are presented in Table 22. These values are, with the exception of the figure obtained from Sheep VB-Q, also well within the established 80 per cent range. The exceptional value falls in the upper 9 per cent limit of the normal range.

TABLE 22.—*Total plasma copper levels in prodromal cases of geeldikkop*

(Values are expressed as mcg copper per 100 ml blood)

Sheep No.	Plasma Cu	Sheep No.	Plasma Cu
VB–P	140	VB–T	120
VB–Q	200	VB–U	140
VB–R	140	VB–V	160
VB–S	140	VB–W	120

Range: 120–200 mcg % Mean value: 145 mcg %

Total plasma copper levels in early, advanced and recovered cases of geeldikkop are presented in Tables 23 and 24. The ranges and mean values given at the foot of each table are compiled from the data arranged in chronological order irrespective of whether the sheep concerned were from Groups 1 or 2 of the early and advanced

cases. The top limit of the normal 80 per cent range for Onderstepoort sheep is 160 mcg per cent and that of the upper 9 per cent limit is 223 mcg per cent (see Table 25). It is apparent from the data presented in Table 23 that total plasma copper levels are above normal within two days of the appearance of clinical symptoms, while frank hypercupraemia is present within three days of the onset of illness.

The data in Table 24 show that there is a return to normal levels in the second week of illness in the Group 1 animals in which an uneventful recovery was likely. Extremely high total plasma copper levels are found, however, in the gravely ill animals of Group 2, maximum values being apparent on the seventh and eighth days of illness. Such very high values may persist in some cases up to three weeks after the appearance of the initial symptoms of the condition.

Normal total plasma copper levels are found in recovered cases.

TABLE 23.—*Total plasma copper levels in early cases of geeldikkop*
(Values are expressed as mcg copper per 100 ml blood)

Sheep No.	Stage of disease	Plasma Cu
VB–H	Early case: Group 1 (1–2 days)	280
VB–I	„ „ „ „ (1–2 days)	240
V1–25	„ „ „ „ (1–2 days)	267
V1–3	„ „ „ „ (1–2 days)	333
V1–2	„ „ „ „ (1–2 days)	269
V1–23	„ „ „ „ (2–3 days)	267
V1–22	„ „ „ „ (2–3 days)	167
V1–24	„ „ „ „ (2–3 days)	133
V1–17	„ „ „ „ (2–3 days)	233
V1–1	„ „ Group 2 (1–2 days)	268
VB–A	„ „ „ „ (2–3 days)	300
VB–E	„ „ „ „ (2–3 days)	320
VB–F	„ „ „ „ (2–3 days)	360
VB–C	„ „ „ „ (2–3 days)	380
VB–B	„ „ „ „ (2–3 days)	320
V1–15	„ „ „ „ (2–3 days)	300
F–5	„ „ „ „ (2–3 days)	158
V1–13	„ „ „ „ (2–3 days)	300
F–2	„ „ „ „ (2–3 days)	170
F–1	„ „ „ „ (2–3 days)	190
VB–G	„ „ „ „ (3–5 days)	280
VB–J	„ „ „ „ (3–5 days)	320
V1–26	„ „ „ „ (3–5 days)	253
VB–K	„ „ „ „ (5–7 days)	260
V1–11	„ „ „ „ (5–7 days)	133
V1–18	„ „ „ „ (5–7 days)	300

Range (1–2 days): 240–333 mcg%. Mean value (1–2 days): 276 mcg%
Range (2–3 days): 133–380 mcg%. Mean value (2–3 days): 280 mcg%
Range (3–7 days): 133–320 mcg%. Mean value (3–7 days): 258 mcg%
Overall range (1–7 days): 133–380 mcg%. Overall mean value (1–7 days): 271 mcg%

TABLE 24.—*Total plasma copper levels in advanced and recovered cases of geeldikkop*

(Values are expressed as mcg copper per 100 ml blood)

Sheep No.	Stage of disease	Plasma Cu
V1–21	Advanced case: Group 1 (7–8 days)	200
V1–9	,, ,, ,, ,, (8–10 days)	167
V1–10	,, ,, ,, ,, (8–10 days)	267
V1–12	,, ,, ,, ,, (10–14 days)	200
VB–D	,, ,, Group 2 (7–8 days)	200
VB–L	,, ,, ,, ,, (7–8 days)	400
VB–M	,, ,, ,, ,, (7–8 days)	360
VB–N	,, ,, ,, ,, (7–8 days)	320
V1–5	,, ,, ,, ,, (7–8 days)	434
V1–4	,, ,, ,, ,, (7–8 days)	533
V1–6	,, ,, ,, ,, (7–8 days)	434
V1–7	,, ,, ,, ,, (7–8 days)	434
V1–14	,, ,, ,, ,, (7–8 days)	233
V1–8	,, ,, ,, ,, (8–10 days)	233
V1–20	,, ,, ,, ,, (10–14 days)	300
VB–Z1	,, ,, ,, ,, (14–21 days)	240
VB–O	,, ,, ,, ,, (14–21 days)	480
VB–Y	,, ,, ,, ,, (14–21 days)	320
V1–16	,, ,, ,, ,, (14–21 days)	233
VB–Z	Recovered case: (±21 days)	120
V1–19	,, ,, (±17 days)	100
F–4	,, ,, (±16 days)	170
F–3	,, ,, (±16 days)	170

Range (7–8 days): 200–533 mcg%. Mean value (7–8 days): 355 mcg%
Range (8–10 days): 167–267 mcg%. Mean value (8–10 days): 222 mcg%
Range (10–14 days): 200–300 mcg%. Mean value (10–14 days): 250 mcg%
Range (14–21 days): 233–480 mcg%. Mean value (14–21 days): 318 mcg%
Overall range (7–21 days): 167–533 mcg%. Overall mean value (7–21 days): 382 mcg%
Range (recovered cases): 100–170 mcg%. Mean value (recovered cases): 140 mcg%

At the time when most of these studies were being made, the programme of work involved so many determinations on each case that it was impossible to include determination of the different blood copper fractions as well. During the late summer months in 1967 a further extensive outbreak of geeldikkop occurred in the north-west Cape and the writer was fortunate in being able to obtain six further early cases for study. These animals, numbered 22860 to 22865, are not listed in Appendix 1 since they were only used for study of a few selected aspects of the biochemistry of the disease. They were all cases of one to three days standing when removed from their home surroundings near Middelburg in the Cape Midlands. They were placed on a fast passenger train and sent to the laboratory. Studies thus commenced four to five days after appearance of the initial symptoms in these animals. They all showed icterus of varying degrees but were no longer photosensitive. All had varying degrees of kidney damage confirmed later at autopsy. These sheep are listed in Table 25 where an indication is given of the severity of the syndrome in each case.

31610—5

TABLE 25.—*Blood copper fractions in geeldikkop compared with the normal ranges established for these*

(All results, except those for ceruloplasmin are expressed as mcg per cent. Ceruloplasmin values are expressed as mg per cent)

Sheep No.	Total plasma Cu	Direct reacting Cu	Indirect reacting Cu	Cerulo-plasmin	Red cell Cu	Remarks
22860.....	213·0	4·1	208·9	37·8	107·6	Severely icteric, with severe nephrosis
22861.....	183·3	0	183·3	43·3	126·8	Severely icteric, with severe nephrosis
22862.....	157·1	0	157·1	33·4	83·0	Mild icterus and kidney lesions
22863.....	222·6	0	222·6	38·5	96·0	Mild icterus and kidney lesions
22864.....	175·0	0	175·0	33·7	114·2	Mild icterus and kidney lesions
22865.....	135·7	0	135·7	28·9	137·6	Mild icterus and kidney lesions
Normal 80% range.....	97–160	0–6·7	92–163	4·5–10·1	17·5–118	
Range including outer 9% limits.....	90–223	0–20·0	90–220	2·0–13·0	2·5–190	

Total plasma copper levels were within the upper 9 per cent limit of the normal range for Onderstepoort sheep, at the time of examination, but in most cases were higher than the range found for the control sheep emanating from a similar area. The indirect-reacting (firmly globulin bound) copper fraction was in general higher than the normal 80 per cent range. This was due to a marked elevation in plasma ceruloplasmin levels. Red cell copper levels were in general within the normal 80 per cent limits, and all within the upper 9 per cent range.

Liver and kidney copper levels were determined in nine control animals and nine cases of geeldikkop. The results are presented in Tables 26 and 27.

TABLE 26.—*Liver and kidney copper levels in control animals*

(Values are expressed as mg copper/100 gm wet liver)

Sheep No.	Liver Cu	Kidney Cu
V1–7056	34·99	0·42
V1–7057	20·00	0·42
V1–7059	18·33	1·67
V1–7061	33·34	0·42
V1–7064	20·84	1·67
V1–7066	20·84	5·00
V1–7068	20·84	9·17
V1–7070	32·50	9·18
V1–7071	24·40	3·33

Liver—Range: 18·33–34·99 mg per 100 gm. Mean: 25·12 mg per 100 gm.
Kidney—Range: 0·42–9·18 mg per 100 gm. Mean: 3·48 mg per 100 gm.

TABLE 27.—*Liver and kidney copper levels in cases of geeldikkop*

(Values are expressed as mg copper/100 gm wet liver)

Sheep No.	Stage of disease	Liver Cu	Kidney Cu
V1–3	Early case: Group 1 (1–2 days).....	43·33	5·83
V1–24	„ „ „ „ (2–3 days).....	13·3	5·0
V1–9	Advanced case: Group 1 (8–10 days).....	41·67	5·25
V1–14A	„ „ „ „ (10–14 days).....	43·33	3·33
V1–6	„ „ Group 2 (7–8 days).....	25·11	10·00
V1–7	„ „ „ „ (7–8 days).....	70·82	25·11
V1–14	„ „ „ „ (7–8 days).....	56·66	5·0
V1–20	„ „ „ „ (10–14 days).....	158·32	20·84
V1–16	„ „ „ „ (14–21 days).....	41·67	1·83

Liver—
Range (Advanced cases): 25·11–158·32 mg per 100 gm
Mean value (Advanced cases): 62·51 mg per 100 gm

Kidney—
Range (Advanced cases): 1·83–25·11 mg per 100 gm
Mean value (Advanced cases): 10·19 mg per 100 gm

Liver copper levels were higher in seven out of the nine geeldikkop cases than those in the control animals. No correlation between liver and kidney copper levels was seen in either group and in only two of the advanced geeldikkop cases were the levels of copper in the kidneys significantly higher than in the control animals.

Values for liver copper in the sheep are to be found expressed in various ways in the relevant literature, e.g. in parts per million of wet or dry tissue, mg per 100 gm wet or dry matter or mcg per gm wet or dry matter. These values can be readily compared with those presented here if the moisture content of sheep liver is known. This is given by Eden (1943) as 67 per cent and by McDougal (1947) as 80 per cent. Using a mean value of 75 per cent the range of liver copper in the sheep taken from various literature sources (Beck, 1956, 1963; Eden, 1943; Todd, Gracey & Thompson, 1962) can be calculated to be 0·05 to 17 mg per 100 gm liver on a wet basis. This range includes animals of all ages and both sexes on dry grazing or green pastures. As can be seen from Table 26 the liver copper levels of apparently normal sheep on the dry summer grazing of the Karoo are somewhat higher than those found in sheep running under various conditions elsewhere in the world. The values found in advanced geeldikkop cases are even higher. Kidney copper levels in sheep elsewhere in the world are given as 0·48 to 1·0 mg per 100 gm kidney tissue on a wet basis (Eden, 1943; Todd, *et al.*, 1962). In general sheep on the summer Karoo grazing have higher kidney copper levels than animals studied elsewhere. By comparison, extremely high values are to be found in some advanced cases of geeldikkop.

5. *Iron excretion*

Iron metabolism in geeldikkop has been discussed in the preceding chapter. The reader is reminded that hyperferraemia is a prominent feature in early and advanced cases of the disease. Plasma iron levels range from 0·10 to 0·52 mg per cent in early cases and 0·16 to 0·44 mg per cent in advanced cases. The range was

found to be 0·12 to 0·22 mg per cent in the control animals. The few recovered cases studied were found to have " normal " plasma iron levels. Liver iron values were found to be raised in two out of nine geeldikkop cases studied. Increased kidney iron levels were found in four of these animals.

6. *Plasma protein levels*

These have been determined in the majority of cases studied during this work. During the first three field investigations the author and his staff were only able to determine the levels of total plasma proteins, albumins and globulins without further fractionation of the latter group of proteins. The results are presented in Appendix 4 and are summarised in Table 28. The method used for the determination of total plasma proteins (Table 2) is one which gives precise and highly reproducible results and was used throughout this work. Separation of albumins and globulins was carried out during these three earlier investigations by a salting-out procedure (Table 2). Such methods are not as precise nor as reproducible as the subsequently used electrophoretic techniques. The separations are particularly effected by extremes of temperature such as are found under field conditions in the Karoo. Comparisons may be made of the results for albumin and globulin analyses between the various groups of sheep listed in Appendix 4 and Table 28, but not between these results and those obtained subsequently.

TABLE 28.—*Summary of results pertaining to plasma protein levels in animals studied during the first three field investigations*

Nature of cases	Total Plasma proteins gm%	Albumins gm%	Globulins gm%	A : G Ratio
Control sheep.....	5·64–6·80	1·94–3·80	2·80–4·35	0·52–1·35
Prodromal cases.....	4·69–5·33	3·20–3·63	1·04–1·81	1·76–3·42
Early cases: Group 1 (1–2 days).....	5·33–7·95	3·31–3·80	1·82–4·25	0·87–1·63
(2–3 days).....	5·60–8·40	3·30–3·50	2·30–4·90	0·71–1·43
Early cases: Group 2 (2–3 days).....	5·55–8·30	1·84–3·86	2·24–5·30	0·56–1·62
(3–5 days).....	5·97–6·90	2·65–4·28	1·69–3·42	0·81–2·53
(5–7 days).....	5·87–8·40	2·80–3·80	2·56–5·60	0·50–1·29
Advanced cases: Group 1 (8–14 days).	7·60–9·60	2·80–3·70	4·40–6·80	0·41–0·84
Advanced cases: Group 2 (7–8 days)...	5·55–9·60	2·10–4·48	2·14–6·50	0·33–1·59
(10–21 days).	5·87–7·75	3·0–3·41	1·92–4·75	0·35–1·77
Recovered cases.....	5·44–7·50	2·25–3·30	2·67–4·39	0·64–1·03

Total plasma protein levels in all the prodromal cases were lower than those found in the control animals. Such decreased levels could be absolute or relative, the latter being more likely and commonly due to water retention with subsequent haemodilution. Red cell counts, haemoglobin estimations and packed cell volume

determinations were unfortunately not done on these cases. It is therefore impossible to express any firm opinion about these figures. The reader is reminded of the severe disturbances seen in the haematology of these animals (Appendix 3). It is quite conceivable that these low figures could result from water retention following the stress during this stage of the disease.

Total plasma protein levels were found to be elevated in most of the early and advanced cases of the disease. It was demonstrated in the previous chapter that haemoconcentration (following dehydration) was a prominent feature in many of the early and advanced cases studied (see Appendix 3 and Fig. 2). This could account for the elevations noted in the plasma proteins, but it is apparently not the only factor concerned. Similar results were found in nearly all the recovered animals. If the data relating to the plasma globulins and A: G ratio presented in Table 28 and Appendix 4 are studied, it will be seen that the rise in total plasma protein levels is associated in many instances with an increase in the globulin fraction. This becomes more pronounced as the disease progresses.

It was possible during the last two extensive field investigations to perform electrophoretic separations of the plasma proteins of all the cases studied. The results are presented in Tables 29 (control animals) and 30 (geeldikkop cases). The method used involved separation on filter paper strips (Brown, 1964). Resolution of the globulin fractions of sheep plasma proteins is generally poor by this method (Horak & Clark, 1963; Brown, 1964). Sharper definition of these fractions was noted however in the case of many normal Karoo sheep (Table 29) and in cases of geeldikkop, particularly in the latter as the disease progressed (Brown, 1964). The main difficulty generally lies in resolution of the α and β-globulins. These have been recorded in Tables 29 and 30 as a combined fraction and where good resolution was obtained the individual figures have been given.

TABLE 29.—*Electrophoretic analysis of plasma proteins in control animals*

(Values for the various fractions are expressed as gm per 100 ml of plasma. TPP = total plasma proteins; Alb = albumins; Glob = globulins; Und = undifferentiated fractions; A: G = albumin : globulin ratio)

Sheep No.	TPP	Alb	Total Glob	α—Glob	β—Glob	$\alpha + \beta$ Glob (undifferentiated or total)	γ—Glob	Und	A: G
V3–5	7·00	2·94	3·40	—	—	1·47	1·93	0·64	0·86
V3–28	8·80	2·69	4·51	0·84	2·0	2·84	1·68	1·57	0·60
V3–29	9·84	2·57	6·25	1·50	2·53	4·03	2·23	1·01	0·41
V3–30	7·90	2·89	3·55	1·12	0·70	1·82	1·72	1·44	0·82
V3–31	8·60	4·11	3·83	0·89	0·79	1·68	2·14	0·63	1·08
V3–32	7·99	3·04	4·11	1·16	0·76	1·92	2·20	0·82	0·74
V3–33	9·28	3·82	3·24	0·77	1·35	2·12	1·13	2·18	1·18

TABLE 30.—*Electrophoretic analysis of plasma proteins in geeldikkop cases*

(Values for the various fractions are expressed as gm per 100 ml of plasma. TPP = total plasma proteins; Alb = albumin; Glob = globulins; Und = undifferentiated fractions; A: G = albumin: globulin ratio)

Sheep No.	Stage of disease	TPP	Alb	Total Glob.	α-Glob.	β-Glob.	$\alpha + \beta$ (undiff. or total)	γ-Glob.	Und.	A: G
V3-22	Early case: Group 1 (1–2 days).....	7·29	2·56	4·06	1·41	0·66	2·07	2·0	0·66	0·63
V3-23	,, ,, ,, (1–2 days).....	7·56	2·64	3·39	—	—	1·51	1·88	1·51	0·78
V3-21	,, ,, ,, (1–2 days).....	9·65	2·39	6·12	—	—	1·94	4·17	1·11	0·23
V3-24	,, ,, ,, (1–2 days).....	8·08	2·82	4·75	—	—	2·01	2·74	0·50	0·59
V3-26	,, ,, Group 2 (1–2 days).....	8·80	1·70	6·03	—	—	2·31	3·71	1·07	0·28
V3-14	,, ,, ,, (2–3 days).....	8·17	2·53	5·31	2·50	1·36	3·86	1·45	0·32	0·48
V3-13	,, ,, ,, (2–3 days).....	6·82	2·28	3·64	—	—	2·03	1·62	0·88	0·63
F-5	,, ,, ,, (2–3 days).....	6·40	2·44	3·84	—	—	1·91	1·93	0·71	0·48
V3-15	,, ,, ,, (2–3 days).....	7·90	1·83	5·17	1·81	1·27	3·08	2·09	0·87	0·55
V3-27	,, ,, ,, (2–3 days).....	7·56	2·12	4·86	1·88	1·47	3·35	1·52	0·55	0·48
V3-20	,, ,, ,, (2–3 days).....	8·60	2·36	5·73	—	—	1·83	3·89	0·49	0·61
F-2	,, ,, ,, (2–3 days).....	6·50	3·86	2·63	—	—	1·37	1·27	—	1·46
V3-25	,, ,, ,, (2–3 days).....	8·80	2·37	5·85	1·63	1·97	3·60	2·26	0·56	0·40
V3-16	,, ,, ,, (3–5 days).....	9·28	1·54	6·96	2·06	1·80	3·86	3·10	0·74	0·22
V3-10	,, ,, ,, (3–5 days).....	8·08	1·90	5·50	—	—	1·59	3·90	0·69	0·35
V3-11	,, ,, ,, (3–5 days).....	10·60	3·04	6·75	—	—	2·75	4·01	0·45	0·78
V3-6	,, ,, ,, (3–5 days).....	8·42	2·57	5·43	—	—	2·38	3·48	0·40	0·47
V3-7	,, ,, ,, (3–5 days).....	10·19	2·72	7·45	—	—	1·36	6·09	0	0·37
V3-8	Advanced case: Group 1 (7–8 days).....	8·42	2·59	5·81	2·59	1·40	3·99	1·83	0	0·44
V3-12	,, ,, ,, (7–8 days).....	8·60	2·12	5·99	2·38	1·35	3·73	2·26	0·47	0·35
V3-17	,, ,, ,, (7–8 days).....	5·46	1·46	3·80	—	—	1·70	2·10	0·19	0·39
V3-9	,, ,, ,, (7–8 days).....	7·38	2·32	5·05	—	—	2·35	2·70	0	0·50
V3-2	,, ,, ,, (7–8 days).....	8·60	1·93	6·03	1·78	0·91	2·69	3·33	0·62	0·32
V3-18	,, ,, Group 2 (7–8 days).....	11·10	2·12	8·40	3·46	2·28	5·74	2·66	0·55	0·25
V3-1A	,, ,, ,, (7–8 days).....	8·80	2·39	6·40	—	—	2·88	2·94	0·58	0·37
V3-1	,, ,, ,, (8–10 days).....	9·84	2·23	7·16	—	—	2·90	4·26	0·44	0·31
V3-19	Recovered case: (± 21 days).....	5·30	2·00	3·30	—	—	1·30	2·00	0	0·61
V3-4	,, ,, (± 21 days).....	7·56	2·54	5·01	—	—	2·10	2·49	0·42	0·51
V3-3	,, ,, (± 21 days).....	8·26	2·89	4·45	—	—	1·39	3·06	0·91	0·65
F-4	,, ,, (± 16 days).....	7·26	2·26	4·39	—	—	1·63	2·07	1·29	0·64
F-3	,, ,, (± 16 days).....	7·50	2·82	4·22	0·42	1·13	1·55	2·66	0·45	0·67

High figures for total plasma proteins were found in some of the control animals (Table 29). The figures presented in this table were obtained from blood samples drawn immediately after arrival of the animals at the temporary laboratory in Victoria West. This also applies to all the geeldikkop cases prefixed V3, some of which were *in extremis* on arrival. The plasma protein values found in all these animals reflect thus not only disturbances due to the disease itself, but disturbances like dehydration, which may result from transporting the animals over long distances from their home surroundings to the laboratory. As mentioned in an earlier report (Brown, 1964) normal total plasma protein figures and A: G ratios in the order of 0·76 were found in all the control animals after they had been allowed to settle down for a few days.

If the figures given in Table 30 are examined it will be seen that there is considerable variation in the total plasma protein levels of all the cases under consideration. The normal range for this determination has been established as 6·85 to 8·23 gm per cent in the Merino sheep (Van Zyl, 1967). At least half the cases of one to three days standing gave values within this normal range. Slightly lower or somewhat higher values were found in the rest. Of the thirteen cases of three to ten days standing noted in this table, ten showed fair to markedly increased total plasma protein levels. Normal values were the rule in the recovered animals.

Albumin: Globulin ratios range from 0·41 to 1·18 in the control animals, with most of the values above 0·74. Extremely low A: G ratios are found in early cases of geeldikkop, even in those of one to two days standing. In cases of one to three days standing the low A: G ratio is mainly due to increases in the γ-globulin fraction. In some cases the increased golbulin values are due to a rise in the $\alpha + \beta$-globulin fraction. Increased γ-globulin levels are very prominent in cases of three to five days standing, and are almost general in the advanced cases. In the latter group of animals increases in the $\alpha + \beta$-globulin fraction are also frequent. Recovery is marked by a return to normal with respect to both globulin fractions, in most cases.

The α-globulin fraction ranged from 0·84 to 1·50 gm per cent in the control animals. The range for Merino sheep has been statistically established as 0·68 to 1·38 gm per cent (Van Zyl, 1967). When elevations in the $\alpha + \beta$-globulin fraction were found in geeldikkop cases, such increased values were invariably due to an increment in the α-globulins (Table 30).

The changes in plasma protein levels which have been described above are illustrated in Fig. 4 and 5, where plasma protein electrophoretograms from typical cases of various stages of the disease are reproduced. The changes in γ-globulin concentrations are most noticeable.

As mentioned in one of the previous sections the author obtained six early cases of four to five days standing for further studies, early in 1967 (see Table 25 for details of these cases). At the time of their arrival the author and his colleagues were using the highly sophisticated technique of microzone electrophoresis on cellulose acetate membranes for separation of sheep plasma proteins. Analyses of this nature were performed on the plasma of these animals immediately after their arrival at

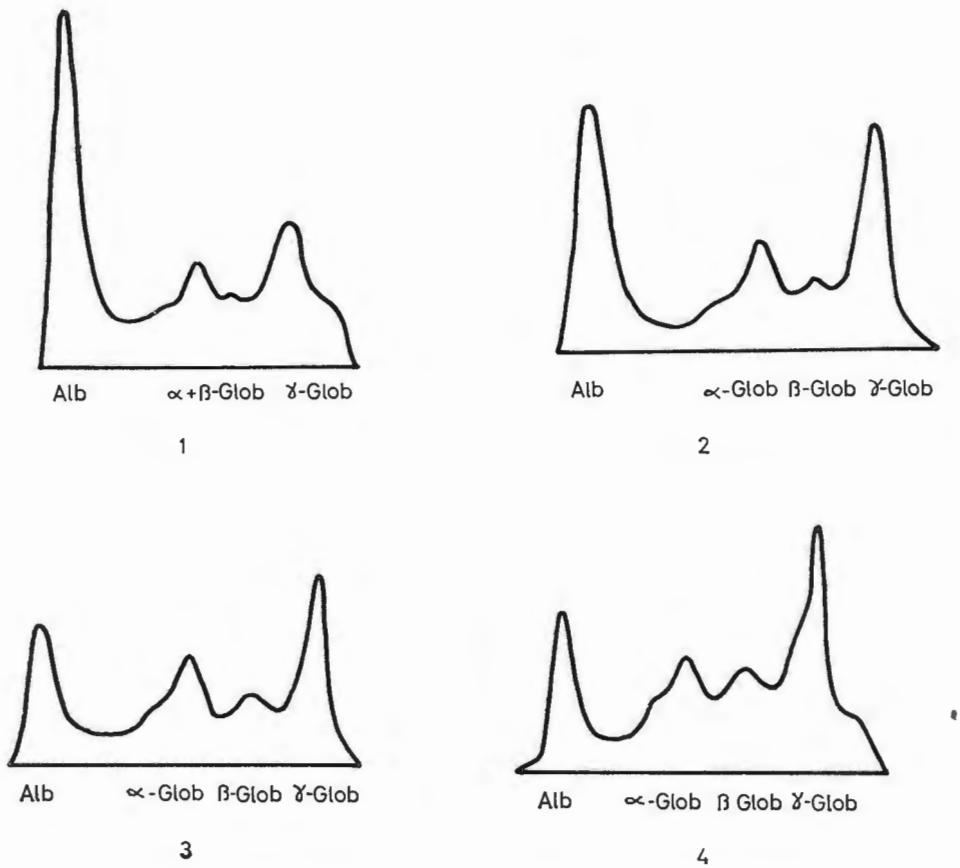

FIG. 4.—Plasma protein electrophoretograms found for control animals and cases of geeldikkop:
1 = control animal V3–5;
2 = early case, Group 1, V3–22 (1–2 days);
3 = early case, Group 2, V3–15 (2–3 days);
4 = early case, Group 2, V3–16 (3–5 days).

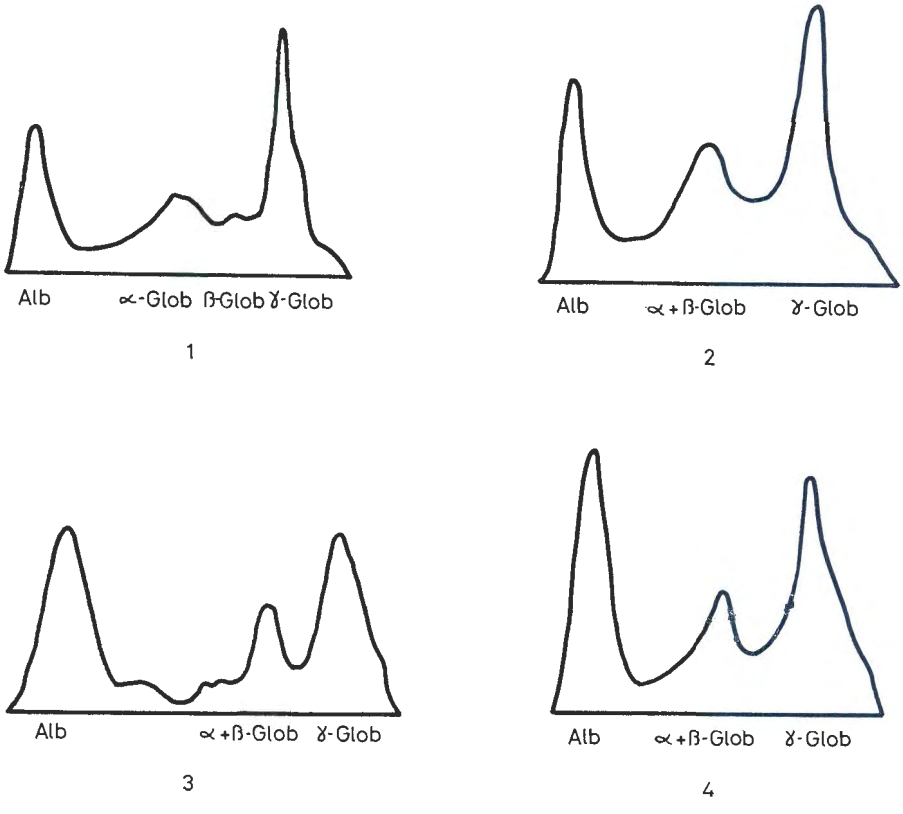

FIG. 5.—Plasma protein electrophoretograms found for cases of geeldikkop:—
1 = advanced case, Group 1, V3–2 (7–8 days);
2 = advanced case, Group 2, V3–1 (8–10 days);
3 = recovered case, F3 (16 days);
4 = recovered case, V3–3 (21 days).

Onderstepoort. The results are presented in Table 31. Total plasma protein levels were extremely high in this group of animals. This was due partly to dehydration following the train journey to Onderstepoort, and mainly to an absolute increase in the globulins in the plasma of these animals. The technique used permits

TABLE 31.—*Plasma protein levels in six early cases of geeldikkop as determined by microzone electrophoresis on cellulose acetate strips*

Sheep No.	Total Plasma Proteins gm%	Albumins gm%	Total Globulins gm%	$\alpha 1$ gm%	$\alpha 2$ gm%	β gm%	γ gm%	$\gamma - T$ gm%	A:G Ratio
22860.....	10·80	3·95	6·85	0·71	1·61	2·62	1·41	0·50	0·58
22861.....	11·04	4·20	6·84	0·92	1·58	2·50	1·31	0·53	0·61
22862.....	11·52	4·73	6·79	0·73	1·58	2·79	1·33	0·36	0·697
22863.....	8·76	3·14	5·62	0·67	1·35	2·25	1·01	0·34	0·56
22864.....	9·00	3·36	5·64	0·68	1·13	2·25	1·13	0·45	0·596
22865.....	9·36	3·95	5·41	0·62	1·25	1·87	1·25	0·42	0·73

Note: $\alpha 1$, $\alpha 2$, β, γ and $\gamma - T$ refer to the different globulin fractions (see Van Zyl, 1967). The normal (80%) ranges for the various fractions in sheep plasma have been established by Van Zyl (1967) to be: Total plasma proteins, 6·85–8·23 gm%; Albumins, 3·66–4·91 gm%; $\alpha 1$-globulins, 0·16–0·51 gm%; $\alpha 2$-globulins, 0·52–0·87 gm%; β-globulins, 0·27–0·61 gm%; γ-globulins, 1·02–1·96 gm%; γ-trailing fraction ($\gamma - T$), 0·05–0·56 gm% and A:G ratio, 0·82–1·76.

complete resolution of the globulins as indicated in Table 31. The increased globulin levels are shown in this table to be due to marked increases in the α 2-globulin levels and very marked increases in the β-globulin levels. γ-Globulin levels were within normal limits in these cases. The reader is reminded of the marked elevations of ceruloplasmin (a copper containing α 2-globulin) found in the plasma of these animals.

7. Total plasma cholesterol levels

Total plasma cholesterol was determined on blood samples from a few cases only. The results are presented in Table 32. Although the normal range for

TABLE 32.—*Total plasma cholesterol in geeldikkop cases and control animals*
(Values are expressed as mg cholesterol per 100 ml plasma)

Sheep No.	Stage of disease	Total cholesterol
F–5	Early case: Group 2 (2–3 days).....	295·5
F–2	,, ,, ,, ,, (2–3 days).....	295·5
F–1	,, ,, ,, ,, (2–3 days).....	290·1
F–4	Recovered case (±16 days).....	286·4
F–3	,, ,, (±16 days).....	295·5
F–K2	Control animal.....	236·4
F–12221	,, ,, ...	254·0
F–12222	,, ,, ...	218·0

this plasma constituent has not been established statistically for Merino sheep in South Africa, it can be taken as being in the order of 150 to 250 mg per cent for the purposes of this discussion. Moderate elevations of plasma cholesterol were found in the three early cases and two recovered cases examined in this regard.

8. *Liver function tests*

Besides the classical tests of liver function based on bile pigment, porphyrin and cholesterol metabolism, the results of which have been described above in this section, use has been made of the following batteries of tests to characterize the nature of the liver lesions in geeldikkop more exactly:—

(i) tests based on plasma protein synthesis or turnover, namely: the thymol turbidity and flocculation tests, the colloidal gold flocculation test and the zinc sulphate turbidity test;

(ii) tests based on the excretory functions of the liver, namely: bromsulphalein excretion and plasma alkaline phosphatase and amylase activity levels; and

(iii) tests based on the levels of activity of certain metabolic enzymes which are present in the body in highest amounts in metabolically active tissues like liver and muscle, namely: assays of glutamic oxalacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), isocitric dehydrogenase (ICD), lactic dehydrogenase (LD), phosphohexose isomerase (PHI), aldolase (Ald) and cholinesterase (Ch) in plasma. The use of the tests mentioned in (i), (ii) and (iii) in ovine icteric states has been noted in previous publications (Brown, 1962, 1963, 1964, 1966a, 1967a, 1967b). Normal values for activity levels of GOT, GPT, ICD, LD, PHI and Ald have been established statistically for Merino sheep in South Africa by Wagner & Brown (1966a). The normal (80 per cent) ranges found are reproduced in Table 33, for the purpose of this discussion. Similar values for the turbidity and flocculation tests and the plasma levels of activity of alkaline phosphatase, amylase and cholinesterase have not yet been established statistically for the Merino sheep running under South African conditions. The values given in Table 33 may be considered as "normal" for the purposes of this discussion.

TABLE 33.—*Normal ranges for the various liver function tests used in this work*

(*N.B.*—Units for each test are as defined in the original procedure)

Test	Normal range (units)
Thymol turbidity	0–5
Thymol flocculation	0–±
Colloidal gold flocculation	0–±
Zinc sulphate turbidity	0–7
Alkaline phosphatase	5–25
Amylase	less than 600?
Glutamic oxalacetic transaminase	41–212
Glutamic pyruvic transaminase	7–87·5
Iso-citric dehydrogenase	65–950
Lactic dehydrogenase	393–896
Phosphohexose isomerase	47–117
Aldolase	0–14·7
Cholinesterase	less than 1·0?

TABLE 34.—*Summary of results of flocculation or turbidity tests in geeldikkop cases and control animals*

(TT = thymol turbidity; TF = thymol flocculation; CG = colloidal gold; ZT = zinc sulphate turbidity

The units for each test are as described in the original procedure)

Nature of case	TT (Range)	TF (Range)	CG (Range)	ZT (Range)
Control animals	0·3–0·8	0	0	0·7–2·58
Prodromal cases	0 –1·2	0	0	0 –1·0
Early cases	0 –4·0	0	0–±	0 –8·5
Advanced cases (Group 1)	0 –2·6	0	0–±	0 –11·4
Advanced cases (Group 2)	1·6–5·0	0	0	0 –13·0
Recovered cases	0·7–1·4	0	0	1·0–3·5

TABLE 35.—*Bromsulphalein (BSP) clearance in cases of geeldikkop*

(The figures given represent the percentage of intravenously injected bromsulphalein cleared from the systemic circulation at the end of a thirty minute period after injection)

Sheep No.	Stage of disease	% BSP cleared from the blood
VB–1	Early case: Group 1 (1–2 days)	100
V1–25	„ „ „ „ (1–2 days)	65·4
V1–3	„ „ „ „ (1–2 days)	69·5
*V3–21	„ „ „ „ (1–2 days)	98·7
V1–1	„ „ Group 2 (1–2 days)	47·4
*F–5	„ „ „ „ (2–3 days)	100
V1–13	„ „ „ „ (2–3 days)	89·5
*F–2	„ „ „ „ (2–3 days)	100
F–1	„ „ „ „ (2–3 days)	87·4
VB–G	„ „ „ „ (3–5 days)	100
V1–26	„ „ „ „ (3–5 days)	100
V1–11	„ „ „ „ (5–7 days)	94·4
VB–L	Advanced case: Group 2 (7–8 days)	95·0
VB–N	„ „ „ „ (7–8 days)	95·0
V1–8	„ „ „ „ (8–10 days)	90·9
F–4	Recovered case (±16 days)	100
F–3	„ „ (±16 days)	100
† All the control animals		100

* These sheep were provided with external biliary fistulae for studies of excretion of the dye into the bile (see text)

† BSP tests were done as a routine on all the control animals mentioned in this report. All showed 100% clearance of BSP from the blood plasma within 30 minutes after injection.

(*a*) *Tests based on plasma protein synthesis or turnover:* The results of these are set out in Appendix 5 and summarized in Table 34. In general results for the turbidity tests were well within the normal range. Only three isolated instances of positive zinc sulphate turbidity tests were found amongst the 78 animals studied (early case, V1-15 and advanced cases, V1-12 and V1-16, listed in Appendix 5). No positive flocculation test results were found in any of the cases studied.

(*b*) *Bromsulphalein* (*BSP*) *clearance:* Disturbances in the clearance of injected BSP from the systemic circulation and in its excretion by the liver in early and advanced cases of geeldikkop were reported in previous communications (Brown, 1962, 1963). The results presented in Table 35 are representative of what can be expected thirty minutes after injection of a test dose of 0·2 mg/kg body weight. Up to 50 per cent of the injected dose can be retained in the bloodstream of early cases of one to two days standing. In early cases of two to seven days standing, 100 per cent clearance from the bloodstream is the general finding, but individuals have been encountered in which from 5 to 13 per cent of the injected dose remains uncleared at the end of thirty minutes (Table 35). In advanced cases 100 per cent clearance is the rule. Individuals are found in which 5 to 10 per cent retention of the dye is still present at the end of the test period (Table 35).

Three BSP conjugates have been observed in the bile of sheep, as well as small amounts of free BSP, appearing in this medium within 30 minutes after intravenous injection of the dye (Brown, 1963). It was decided during the course of the two most recent field investigations to examine the conjugates of bilirubin and BSP which appeared in freshly secreted bile of selected geeldikkop cases. Biliary cannulae were introduced into the common bile ducts of three typical icteric early cases of geeldikkop (V3-21, F-2 and F-5) under local anaesthesia. A test dose of 150 mg of BSP was given intravenously a few hours later and samples of bile were collected hourly for 12 hours after the injection. Urine was collected over the whole of this period, using the collecting bottles described elsewhere (Brown, 1959b).

The technique for the isolation and purification of BSP conjugates is described in Appendix 11. In the case of Sheep F-2 and F-5 blood levels of 38 and 45 mg per cent respectively were found one minute after injection and in both cases 0 mg per cent thirty minutes later. In none of the meagre bile samples or urine collected during the 12 hour test period for both sheep, could any trace of BSP be detected. The test was repeated on Sheep F-5 on the following day using an intravenous dose of 450 mg. Once more, no BSP was evident in the bile or urine samples collected during this time, and complete clearance from the blood was evident by thirty minutes after injection.

Sheep V3-21 also received 150 mg of BSP intravenously. A blood level of 35 mg per cent of BSP was found one minute after injection and at the end of thirty minutes this had fallen to 1·3 mg per cent. The animal passed 100 ml of urine during the two hours immediately following the injection. This urine contained 8·3 mg of BSP. During the next four hours 500 ml of urine containing a further 21·7 mg of BSP were voided. Thereafter no more BSP could be detected in the urine which was collected. At no time during the 12 hour test period could any BSP be detected in the small volume of bile which was passed. Thus this animal successfully eliminated 30 per cent of the administered dose of BSP via the urine, but since the blood levels of the dye were negligible during the final 11½ hours of the test period, it must have retained 70 per cent of the dose in its liver.

It is apparent from the data cited here that clearance of BSP from the blood circulation is apparently retarded during the very early stages of the disease, i.e. one to two days after symptoms are first noticed. That this impairment of clearance

from the blood is a transient one is demonstrated by the data given in Table 35 for cases of three to ten days standing. Although BSP is cleared rapidly from the blood by the liver parenchyme in this latter group of animals, it is obvious from the experiments cited that it is not being excreted into the bile, and since very little appears in the urine it must be retained in the liver cells. Wheeler, Meltzer, Epstein & Bradley (1958), in their studies of BSP excretion by the dog, conclude that hepatic removal of this dye involves two processes, viz. (1) secretion into the bile at a rate

TABLE 36.—*Plasma levels of alkaline phosphatase (A.P.), glutamic-oxalacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) in geeldikkop cases and control animals*

Sheep No.	Stage of disease	AP	GOT	GPT
V3–22	Early case: Group 1 (1–2 days)	21·4	293	35
V3–23	,, ,, ,, ,, (1–2 days)	60·5	475	38
V3–21	,, ,, ,, ,, (1–2 days)	16·8	438	25
V3–24	,, ,, ,, ,, (1–2 days)	48·8	427	35
V3–26	,, ,, Group 2 (1–2 days)	32·5	488	45
V3–14	,, ,, ,, ,, (2–3 days)	45·0	348	0
V3–13	,, ,, ,, ,, (2–3 days)	40·7	313	40
F–5	,, ,, ,, ,, (2–3 days)	17·1	261	48
V3–15	,, ,, ,, ,, (2–3 days)	39·6	598	63
V3–27	,, ,, ,, ,, (2–3 days)	12·9	475	29
V3–20	,, ,, ,, ,, (2–3 days)	14·2	468	11
F–2	,, ,, ,, ,, (2–3 days)	12·56	296	54
V3–25	,, ,, ,, ,, (2–3 days)	21·4	383	18
V3–16	,, ,, ,, ,, (3–5 days)	18·4	295	23
V3–10	,, ,, ,, ,, (3–5 days)	15·3	183	18
V3–11	,, ,, ,, ,, (3–5 days)	81·4	438	13
V3–6	,, ,, ,, ,, (3–5 days)	—	370	13
V3–7	,, ,, ,, ,, (3–5 days)	—	355	22
V3–8	Advanced case: Group 1 (7–8 days)	13·4	175	38
V3–12	,, ,, ,, ,, (7–8 days)	38·1	245	10
V3–17	,, ,, ,, ,, (7–8 days)	16·3	308	40
V3–9	,, ,, ,, ,, (7–8 days)	22·4	190	38
V3–2	,, ,, ,, ,, (7–8 days)	—	375	38
V3–18	,, ,, Group 2 (7–8 days)	—	555	63
V3–1A	,, ,, ,, ,, (7–8 days)	—	375	93
V3–1	,, ,, ,, ,, (8–10 days)	—	437	38
V3–19	Recovered case: (±21 days)	10·0	208	18
V3–4	,, ,, (±21 days)	22·4	203	18
V3–3	,, ,, (±21 days)	35·5	535	37
F–4	,, ,, (±16 days)	15·3	223	30
F–3	,, ,, (±16 days)	9·41	289	48
V3–5	Control animal	—	163	35
V3–28	,, ,,	5·33	95	43
V3–29	,, ,,	10·64	50	30
V3–30	,, ,,	8·19	93	31
V3–31	,, ,,	4·28	113	34
V3–32	,, ,,	6·67	135	38
V3–33	,, ,,	7·11	150	18
F–K2	,, ,,	8·2	311	117
F–12221	,, ,,	11·8	112	33
F–12222	,, ,,	10·5	99	60

limited by a transfer maximum, and (2) hepatocellular storage in an amount proportional to plasma concentration. In pathological conditions, normal storage may co-exist with virtually complete absence of biliary transport, suggesting that separate mechanisms are involved in the storage and transport of this dye. It is known from the work of various schools that BSP is actively secreted by the liver, although the precise site and mechanism of secretion are still subjects of considerable debate (Cook, Lawler, Calvin & Green, 1952; Andrews, 1958; Bollman, 1958; Hanzon, Knisely & Brauer, 1958).

(*c*) *Plasma enzyme assays used to establish the existence of liver pathology:* The levels of activity of alkaline phosphatase (AP), glutamic oxalacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) found in typical cases of geeldikkop and the control animals are presented in Table 36. Fair to marked elevations of AP were encountered in six out of the sixteen early cases of one to five days standing. Normal values were thus the rule. Fair to marked elevations of plasma GOT activity are seen in nearly all the early cases, such elevations being present from the onset of symptoms. Highest levels are generally encountered during the second or third day of illness, declining thereafter to reach normal levels in many advanced cases destined to recover. Extremely high values are however, still a feature of the severely ill Group 2 advanced cases, and moderate to high levels of the enzyme were even present in some of the apparently recovered cases.

With the exception of one advanced case (V3-1A), all the animals studied showed normal levels of plasma GPT activity.

The data for the assays of amylase (Am), cholinesterase (Ch), isocritic dehydrogenase (ICD), lactic dehydrogenase (LD), phospho-hexose isomerase (PHI) and aldolase (Ald) on the plasma of geeldikkop cases and control animals are presented in Table 37.

No significant deviations from normal were seen as regards amylase and cholinesterase, although in the case of the former enzyme more high values (within the normal range) were found in cases of one or two days standing than in cases representing later stages of the disease. ICD values were similarly elevated, within the normal range during the first two days of illness and in individuals thereafter. Lower values well within the normal limits are found in the advanced and recovery stages of the disease.

Extremely high levels of plasma LD activity are found during the first two to three days of the illness. Values within the normal range are frequent in cases of five to eight days standing, although fair to markedly elevated figures are still the rule, and may even be seen in recovered animals (e.g.Sheep V3-3) twenty-one days after the onset of symptoms.

PHI levels are also moderately or markedly raised during the first two or three days of illness, but appear to return fairly rapidly to normal levels thereafter. Ald activity is markedly increased in the plasma of early cases of the disease, highest values being encountered during the first two or three days of illness. Thereafter, the levels of the enzyme tend to fall again rapidly, particularly in animals destined to recover, e.g. the Group 1 advanced cases. As in the case of GOT and LDH extremely high values are still found in the severely affected advanced cases, and even in apparently recovered individuals (Sheep V3-3).

TABLE 37.—*Plasma levels of amylase (AM), cholinesterase (Ch), iso-citric dehydrogenase (ICD), lactic dehydrogenase (LD), phosphohexose isomerase (PHI) and aldolase (Ald) in geeldikkop cases and control animals*

Sheep No.	Stage of disease	Am	Ch	ICD	LD	PHI	Ald
V3–22	Early case: Group 1 (1–2 days).	467	0·002	535	1000	157	113
V3–23	,, ,, ,, ,, (1–2 days)	512	0·23	603	1120	171	115
V3–21	,, ,, ,, ,, (1–2 days).	109	0·42	433	1820	149	39
V3–24	,, ,, ,, ,, (1–2 days).	588	0	585	1020	140	96
V3–26	,, ,, Group 2 (1–2 days).	429	0	552	2080	318	131
V3–14	,, ,, ,, ,, (2–3 days).	139	0	68	1260	106	52
V3–13	,, ,, ,, ,, (2–3 days).	122	0·34	90	2000	98	52
V3–15	,, ,, ,, ,, (2–3 days).	157	0	148	2330	160	160
V3–27	,, ,, ,, ,, (2–3 days).	202	0	275	1290	165	88
V3–20	,, ,, ,, ,, (2–3 days).	89	0	598	2140	159	48
V3–25	,, ,, ,, ,, (2–3 days).	424	0·25	555	1490	177	87
V3–16	,, ,, ,, ,, (3–5 days).	244	2·74	80	1590	153	43
V3–10	,, ,, ,, ,, (3–5 days).	96	0	98	700	117	23
V3–11	,, ,, ,, ,, (3–5 days).	338	0	555	2800	155	51
V3–6	,, ,, ,, ,, (3–5 days).	—	—	388	1600	—	—
V3–7	,, ,, ,, ,, (3–5 days).	—	—	492	1720	—	—
V3–8	Advanced case: Group 1 (7–8 days).	323	0	400	620	128	13
V3–12	,, ,, ,, ,, (7–8 days).	338	0·08	260	1160	88	25
V3–17	,, ,, ,, ,, (7–8 days).	255	0·98	288	1770	24	19
V3–9	,, ,, ,, ,, (7–8 days).	264	0	97	530	128	20
V3–2	,, ,, ,, ,, (7–8 days).	—	—	308	500	—	48
V3–18	,, ,, Group 2 (7–8 days).	—	—	263	2820	170	211
V3–1A	,, ,, ,, ,, (7–8 days).	—	—	425	1220	—	74
V3–1	,, ,, ,, ,, (8–10 days)	—	—	328	1100	—	91
V3–19	Recovered case: (±21 days)	142	1·14	68	780	108	8
V3–4	,, ,, (±21 days)	742	0	467	500	—	38
V3–3	,, ,, (±21 days)	144	0	585	1830	—	208
V3–5	Control animal	—	—	205	410	—	—
V3–28	,, ,, ...	5·09	0·255	78	430	84	27
V3–29	,, ,, ...	72·3	0·165	50	410	50	9
V3–30	,, ,, ...	417	0	80	470	50	10
V3–31	,, ,, ...	10·9	0·23	118	465	59	27
V3–32	,, ,, ...	131	0	38	450	68	25
V3–33	,, ,, ...	102	0·1	250	590	80	34

9. *General discussion and summary of findings*

The histopathology of geeldikkop will be correlated later with the full picture of the chemical pathology in Chapter 9. It is pertinent to review briefly at this stage the salient points of the liver pathology (Brown, *et al.*, 1960). The most constant change noticed in the livers of the Vosburg early cases was an extensive bile pigmentation of the parenchymal cells. The pigment was scattered diffusely through the cells and it did not seem to have accumulated in any particular region of the lobules. Bile pigment was also present in the Kupffer cells. The parenchymal cells appeared swollen and hyaline degeneration could be demonstrated in some of them. A moderate degree of intra-cystoplasmic fat infiltration was also evident.

The *bile ducts were found to be empty and the bile canaliculi were prominent but with the exception of one case, no bile pigment could be demonstrated in them.* There was no evidence of any large scale necrosis of liver cells as described earlier by Theiler (1918). A few isolated liver cells showed necrobiotic changes, with pycnotic nuclei and eosinophilic cytoplasm. The presence of interstitial connective tissue was noticed in a few cases but to such a moderate degree that it did not seem to bear any relation to the disease as such. The same changes were apparent in the livers of the advanced Vosburg cases, but bile pigmentation of the parenchymal and Kupffer cells was much more severe.

The livers of the typical general cases of geeldikkop, designated V1- in the foregoing text and tables, differed from the Vosburg cases only in that bile pigmentation of the hepatic cells was less marked, the degree of fatty changes in isolated cells was milder and little evidence was seen of swelling of the liver cells. *The complete absence of bile pigment in the canaliculi and larger bile ducts was again a most prominent finding.*

The histopathology of the most recently studied cases designated V3- and F- has not yet been published. The most important findings as regards the liver are briefly the following: Very mild parenchymatous degeneration and fatty infiltration and pigmentation were observed in the livers of most of the early and advanced cases. Very mild portal cirrhosis with mild round cell infiltration into the portal tract was observed in about half the early cases. Slight bile duct proliferation was observed in two out of nineteen early cases studied, in two out of seven advanced cases and in two out of four recovered cases. Mild necrobiotic changes were seen in occasional cells in the livers of only two early and one advanced case. These changes consisted of a more eosinophilic cytoplasm and nuclear pycnosis. Haemosidersosis was rare. *Bile canaliculi were empty in most of the early and some of the advanced cases.*

The hepatic pathology of geeldikkop is thus distinguished by a singular lack of significant and generalized morphological changes in the cells of the liver or its architecture. There is no evidence of hepatitis, widespread necrosis or intrahepatic biliary thrombosis, cholangiolitis or widespread peri-cholangiolar cell infiltrations. In short, there is nothing in the histopathology to explain the severe disturbances which have been described in this chapter.

No extra-hepatic obstruction to bile flow is present and bile secretion apparently never ceases entirely. The gall-bladders of most of our cases were found at autopsy to contain from 10 to 400 ml or more of bile. The higher figures were often found where severe gastro-intestinal stasis was present and had been so for a number of days. In these cases the bile was highly concentrated and dark greenish-black and tarry in appearance. In the early and less severe cases the gall-bladders were generally found to contain small amounts of very pale greenish-yellow and often almost colourless bile. In other instances only about 10 to 20 ml of very viscous tarry fluid was present. In the early cases thus there is a severe diminution in the volume of bile secreted and the amount of pigment excreted. Fluid secretion carries on at this lowered and impaired level as is evident from the larger volumes of bile present in the gall-bladders of the advanced cases.

It is clear from the chemical pathology described that hyperbilirubinaemia is present from the moment clinical symptoms are first observed and rapidly increases in severity. Maximum levels appear to be reached between the second and third days of illness in animals destined to recover, the amount of bile pigment in the plasma

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declining steadily as the condition of the animal improves. In the severer type of case total bilirubin levels increase steadily until the case terminates fatally. In most of the early cases the plasma bile pigment consists of a mixture of almost equal amounts of bilirubin and bilirubin glucuronide. In the rapidly worsening advanced cases bilirubin glucuronide accumulates in the plasma in large amounts far in excess of the bilirubin.

The presence of large amounts of bilirubin in the blood indicates the existence of a haemolytic state, frank hepato-cellular injury or some other form of impairment of bilirubin conjugation. The presence of the very large amounts of bilirubin glucuronide in the plasma, particularly in that of the advanced cases, indicates impairment of excretion of this pigment by the liver cell, in the absence of any obvious intra- or extrahepatic obstructions to bile flow. The existence of a haemolytic syndrome in geeldikkop has been indicated in the previous chapter, the question of hepato-cellular injury will be discussed shortly and the integrity of the conjugation mechanisms will form part of the following chapter.

The marked decrease in faecal bile pigment levels and the absence of urinary urobilinogen in early and advanced cases of the disease also indicate a marked diminution in the hepatic excretion of conjugated bile pigment.

The flocculation and turbidity tests of liver function give in general negative results for hepato-cellular injury. This is not entirely unexpected in cases of up to 10 days standing. Positive reactions in these tests depend upon qualitative as well as quantitative changes in the plasma protein composition (Popper & Schaffner, 1957; Sherlock, 1958). Margen & Tarver (1956) have calculated by isotopic work on humans, the half-life of albumin in the body to be about 26 days. Assuming that the rate of protein catabolism does not change, it will take 16 days before a 50 per cent reduction in the rate of albumin synthesis leads to a decrease of 20 per cent in the plasma albumin concentration. Even a complete cessation of albumin production will result only in a 20 per cent decrease in plasma albumin after about 8 days (their figures). There is no reason to assume that this situation is peculiar to man only. Horak & Clark (1963) have deduced a similar turnover rate in sheep. It has been pointed out that marked quantitative changes involving the globulins are present from the onset of clinical symptoms in geeldikkop. From the data presented it is apparent that there is no decrease in plasma albumin concentration but an actual increase in the globulins as the disease progresses. This state in the sheep apparently does not give positive results in the flocculation or turbidity tests used. In the light of this discussion it is inconceivable that the marked alterations in the A: G ratio described earlier can be related to liver cell injury. These tests have been found to give negative results in common bile duct obstruction in the sheep (Brown, 1967a, 1967b).

Elevations in the serum concentration of certain enzymes as measured by their biochemical activity occur as a result of the following processes involving the liver:—

(1) escape from disrupted parenchymal cells with necrosis or altered membrane permeability (e.g. GPT, GOT, ICD and arginase); and

(2) lack of biliary excretion in obstructive icterus (e.g. alkaline phosphatase) (Cornelius & Kaneko, 1963). The first group of enzymes must be subdivided into two groups, namely (*a*) enzymes which are " liver specific " in that high concentrations are only present in hepatic tissue (e.g. GPT in dogs, cats and primates and arginase in all ureotelic animals); and (*b*) enzymes which are in high concentration in other tissues in addition to liver and are therefore not liver specific (e.g. GOT, ICD, PHI and Ald) (Cornelius & Kaneko, 1963).

Plasma alkaline phosphatase levels are useful in diagnosing obstructive jaundice and intrahepatic cholestasis in the human (Popper & Schaffner, 1957). The test is of little value in the diagnosis of such states in ovine medicine (Brown, 1967b, 1967c). This is also the experience of several authoris quoted by Cornelius & Kaneko (1963). Elevations of plasma cholinesterase have been noted in toxic hepatitis in mammals, but the diagnostic value of this test in animal liver disease has been questioned (Cornelius & Kaneko, 1963). Increased serum amylase values have been found in common bile duct obstruction in humans (Parker, 1948). The plasma activities of these enzymes and of GPT are of little assistance in resolving the problem of the nature of the hepatic injury in geeldikkop. The plasma levels of GPT do not rise in simple or complicated common bile duct obstruction in the sheep (Brown, 1967b, 1967c) and only very small elevations in plasma levels could be expected in hepatic parenchymal injury in the sheep, since the livers of mature sheep do not contain significant amounts of the enzyme (Cornelius & Kaneko, 1963).

The various disease conditions in which elevations of plasma GOT, LD, Ald, ICD and PHI activity may be encountered are listed in Table 38. GOT occurs in all metabolically active tissues of man and domestic animals. Low or variable values

TABLE 38.—*Conditions in which the various enzymes discussed may be markedly elevated*

Enzyme	Conditions	References
Aldolase.....	Early acute hepatitis, muscular dystrophy and myositis, neoplastic conditions, pulmonary infarction, extensive peripheral gangrene, acute haemorrhagic pancreatitis, acute myocardial infarction, acute infections, surgical interventions	Sibley, 1958; Dreyfuss *et al.*, 1958; Hauss & Leppelmann, 1958; Brown, 1967b
Lactic dehydrogenase.....	Muscular dystrophy, myocardial infarction, infectious viral hepatitis, neoplasia, acute pancreatitis, increased erythrocyte breakdown, acute infections, surgical intervention	Berger & Broida, 1962; Dreyfuss *et al.*, 1958; Hauss & Leppelmann, 1958; Hess, 1958; Hill, 1958; Wroblewski, 1958; White, 1958
Glutamic oxalacetic transaminase	Acute viral hepatitis, hepato-cellular necrosis, myocardial infarction, acute infections, skeletal muscle injuries and muscular dystrophy, surgical intervention	Hauss & Leppelmann, 1958; O'Brien *et al.*, 1960; Sherlock, 1958
Isocitric dehydrogenase...	Infectious hepatitis, neoplasia....	Bowers *et al.*, 1960; Sterkel *et al.*, 1958; Wolfson *et al.*, 1958; White, 1958
Phosphohexose isomerase.	Neoplasia, muscular dystrophy...	Dreyfuss *et al.*, 1958; White, 1958

are found in common bile duct obstruction in humans and in sheep (Sherlock, 1958; Brown, 1967b). It has been found elevated in the plasma of cases of experimental hepatic necrosis in horses, cattle, pigs, dogs and cats; in starvation in horses and

cattle and in paralytic equine myoglobinuria (Cornelius & Kaneko, 1963). LD occurs in the largest amounts in skeletal muscle with lesser amounts in heart muscle and liver (White, 1958; Wróblewski, 1958). ICD occurs in equal amounts in heart muscle and liver with lesser amounts in skeletal muscle (Wolfson, Spencer, Sterkel & Williams-Ashman, 1958). Normal values are found in cases of extra-hepatic bile duct obstruction in humans and sheep, but elevated values are encountered in acute liver injury (Bowers, Potter & Norris, 1960; Brown, 1967b; Sterkel, Spencer, Wolfson & Williams-Ashman, 1958; Wolfson *et al.*, 1958). Elevated levesls of the enzyme in plasma are regarded as being specific for acute liver injury in humans (Bowers *et al.*, 1960; Sterkel *et al.*, 1958). It is not known whether this applies to sheep (Cornelius & Kaneko, 1963). Slightly elevated or normal levels of Ald are encountered in human and ovine obstructive jaundice (Brown, 1967b; Sibley, 1958; White, 1958).

It is obvious from the above discussion that elevations of GOT, Ald, LD and PHI activity in the plasma may result from the sudden injury of a large number of cells of tissues rich in these enzymes. All the conditions listed in Table 38 or mentioned above, responsible for such elevations may be dismissed as being responsible for the high values found in cases of geeldikkop,with the exception of acute liver injury or myopathy. In view of what is described in the literature concerning plasma enzyme levels in the former condition and in view of the liver histo-pathology of geeldikkop described earlier, it is rather unlikely that the huge increments of some of these enzymes in the plasma of early and severe advanced cases of the disease are due to liver cell injury. If increased ICD levels are specific for liver damage in the sheep, then the normal levels of this enzyme throughout the course of the illness are further confirmation of this thought. Elevations of plasma arginase activity are a specific indication of acute liver injury in all ureotelic animals (Cornelius & Kaneko, 1963). Normal values are given for sheep plasma as 0 to 2·7. Assays of arginase activity were carried out on plasma from all six of the early cases of geeldikkop designated sheep 22860 to 22865. Values found ranged between 0·34 and 3·1 units. This is taken as being additional evidence in favour of an extra-hepatic origin of the increased plasma enzyme levels in geeldikkop. In one of the subsequent chapters, evidence will be produced that myopathy is actually responsible.

From what has been said so far, geeldikkop appears to be essentially a peculiar type of cholestasis in which the transport of conjugated bile pigment across the hepatic cell membrane is impaired and in which there is a marked decrease in the secretion of biliary fluid. The condition is aggravated by the apparent co-existence of a haemolytic syndrome. If the first statement is true, then it is of importance to see if the other components of bile are affected similarly.

Regurgitation of bile acid salts is present from the moment the symptoms first appear. Maximum blood levels are found during the first three days of illness in typical cases, thereafter bile acid secretion appears to become· econstituted. There is a good correlation between plasma bilirubin glucuronide, bile acid salts and phylloerythrin levels. Maximum blood levels of phylloerythrin are found during the first two to three days of illness. Urinary coproporphyrin excretion is markedly increased during the first three days of symptoms. Total plasma cholesterol is markedly increased in early cases of geeldikkop. Plasma copper levels are normal in the prodromal cases, rising rapidly after symptoms appear, to reach frankly hypercupraemic levels within three days of illness. In animals which recover plasma copper levels return to normal as biliary secretion or excretion is reconstituted. Extremely high plasma levels are found in the severe advanced cases, maximum

values being reached round about the seventh or eighth day of illness. Hypercupraemia may persist in such individuals for up to three weeks after onset. Marked hyperferraemia is similarly a prominent feature of the early and advanced cases of the disease. The position regarding BSP has already been discussed. It appears then that most if not all of the solid components of bile appear to be returned to the systemic blood circulation from the moment of onset of the first symptoms.

Further support for this line of reasoning is to be found in contemporary work by the author on biliary secretion and common bile duct occlusion in the sheep (Brown, 1967a, 1967b, 1967c). The 24 hourly output in bile of the compounds mentioned in the foregoing paragraph, with the exception of that of cholesterol, is given in Table 39. The data in this table were obtained from a group of six adult

TABLE 39.—*Mean and ranges of the* 24 *hourly excretion of various bile components in a group of six Merino wethers* (*after Brown*, 1967a)

Determination	Mean value found	Range found	Remarks
Bile acids (gm/24 hr).....	7·76	3·38–11·59	These values probably represent " fasting " levels of bile acid secretion
Copper (mcg/24 hr).....	133·0	12·9–1119·6	—
Iron (mcg/24 hr).....	155·1	4·1–408·0	—
Bilirubin (mg/24 hr).....	47·3	10–80	—
Coproporphyrin (mcg/24 hr)	79·8	3·7–400·6	—
Phylloerythrin (mcg/24 hr)	668·6	35·3–3545·5	The figures include " fasting " and feeding levels of excretion on a diet of green lucerne, crushed maize and water, *ad libitum*

Merino wethers weighing between 51 to 67 lb, maintained on a diet of green lucerne, crushed maize and water *ad libitum*. The mean weight of these animals was 55 lb (shorn), which was approximately the weight of most of the geeldikkop cases studied. The plasma volumes of these six sheep ranged between 1429 to 2000 ml with a mean value of 1676 ml (blood volumes ranged from 2067 to 2619 ml with a mean value of 2335 ml). Taking as a hypothetical case a sheep weighing 55 lb with a plasma volume of 1676 ml and secreting the biliary components indicated in Table 39 at the mean level given, let us consider what would happen if this animal developed a sudden total block to the excretion of the components of bile and had the entire 24 hourly output regurgitated into its systemic blood. The plasma concentrations of the bile components of interest which would theoretically be obtained at the end of 24 hours are indicated in Table 40. Coproporphyrin is indicated as the 24 hour urinary output. The figures for copper and iron are respectively 7·94 and 9·25 mcg per cent. The figure given in the table for copper is this value added to median plasma value found by statistical analysis, namely 129 mcg per cent (Brown, Brink & Wagner (1968), while that for iron is the value just cited added to 110 mcg per cent, the value generally taken by us as being the mean of the normal range for sheep.

TABLE 40.—*Plasma concentrations or urinary output of some components of bile which could theoretically be attained within 24 hours of blocking biliary secretion or excretion compared with those actually found in early cases of geeldikkop and common bile duct occlusion (both of one to two days standing)*

Bile component	Expected plasma concentration	Plasma concentration found in early geeldikkop cases (mean values)	Plasma concentration found in early cases of common bile duct occlusion (mean values)
Bile acid salts, mg%.....	463·0	36·3	—
Bilirubin glucuronide, mg%	2·82	2·11	2·86
Phylloerythrin, mcg%.....	39·9	49·8	5·0
Copper, mcg%.....	136	278	179
Iron, mcg%.....	119	313	123
	Expected urinary output	Urinary output found in early geeldikkop cases	Urinary output found in early cases of common bile duct occlusion
Coproporphyrin, mcg/24 hr	79·8	243	124

The expected plasma concentrations of the bile components of interest are compared in this table with the mean figures actually found in cases of geeldikkop of one to two days standing and in cases of common bile duct obstruction, one to two days after surgical occlusion of the duct (Brown, 1967b).

The " expected " figure for bile acid salts is just more than ten times higher than was actually found in early geeldikkop cases. The range of 24 hourly excretion for this bile component is a very wide one (3·38 to 11·59 gm/24hr), while the secretion is dependent on gastro-intestinal motility and an intact enterohepatic circulation (Brown, 1967a). It is more than likely that the low mean figure found in early geeldikkop cases (range = 9·23 to 70·59 mg per cent) is due to the severe gastro-intestinal stasis known to precede the onset of clinical symptoms (Brown, 1966a). The " expected " figure for bilirubin glucuronides is in good agreement with those actually found in early geeldikkop and common bile duct occlusion and that for phylloerythrin is in the same order as is found in geeldikkop. The figures for iron and copper agree well with those found in common bile duct occlusion but are lower than those found in geeldikkop. The higher figures found in this disease could be due to the intravascular haemolysis. This had been suggested in the previous chapter to be the case with iron. It was reported earlier and elsewhere (Brown, 1963, 1967c) that the superimposition of kidney lesions on an existing block to biliary excretion, leads to the rapid development of a frank hypercupraemia. Plasma levels of copper such as those found in geeldikkop are rapidly attained when sheep suffering from common bile occlusion are dosed with nephrotoxic agents.

The mean plasma phylloerythrin concentration, indicated in Table 40, for cases of common bile duct occlusion, is one obtained from patients without disturbances of renal function. It has been mentioned earlier in this chapter that phylloerythrin

may not be as easily excreted as coproporphyrinogen by the normal kidney. The amount excreted in the urine over 24 hours in an animal with uncomplicated bile duct obstruction, fed on green food *ad libitum*, is certainly far less than that of coproporphyrin, although its rate of production (and excretion by the normal liver) is greater (Brown, 1967b). The presence of kidney lesions would further reduce its renal excretion (Brown, 1967c) and hence elevate its plasma levels.

Bearing in mind the possible explanations given for the discrepancies mentioned, it seems from the above argument that most, if not all, of the constituents of bile are returned to the systemic blood circulation in geeldikkop. Since hyperbilirubinaemia is not present in the prodromal cases, the onset of the block to excretion of biliary constitutents is sudden, and the block is initially a complete one. In typical uncomplicated cases the interference with the excretion of biliary solids in general seems to last about three to four days, after which a rapid return to normal can be expected. The rate of reconstitution of secretion or excretion varies with the different compounds normally present in bile. It seems to be rapid in the case of the bile acid salts and biliary porphyrins, and slower in the case of bilirubin glucuronides, BSP and copper. In the severe cases, particularly those which will terminate fatally, impairment of biliary secretion and excretion is characteristically severe and of long duration for most of the constituents of bile.

Bile acid salts, bilirubin glucuronides, coproporphyrin and phylloerythrin are actively secreted into bile by the liver cells of the sheep while copper and iron are secreted by an active mechanism involving restricted filtration (Brown, 1967a). Since disturbances of the architecture of the liver are minimal in geeldikkop, and since there is a remarkable absence of serious morphological changes in the majority of the parenchymal cells of this organ, the block to the excretion of biliary components must be a biochemical one involving either the selective permeability of the liver cell membranes or the systems supplying energy for active transport of the various biliary components mentioned. These topics will be discussed in the chapters which follow.

One of the most interesting aspects of the chemical pathology of the bile pigments in geeldikkop is the extremely high levels to which conjugated bilirubin can rise in the plasma. This immediately poses the question as to why this pigment is not rapidly cleared by the kidneys since it is freely soluble in water over a wide pH range. The renal threshold for bilirubin in the sheep lies in the range of 0·9 to 3·5 mg per cent, being probably closer to less than 1 mg per cent in most animals (Brown, 1967b). Common bile duct occlusion in the sheep typically leads to bilirubinuria when plasma levels of bilirubin glucuronides reach 0·9 to 3·5 mg per cent and its increasing severity parallels that of the hyperbilirubinaemia (Brown, 1967b). Urobilinogen generally disappears from the urine two to four days after bile flow into the intestine has ceased. Bileaciduria and porphyrinuria develop rapidly within two days and are severe and sustained.

More than half the geeldikkop cases studied showed no bilirubinuria in spite of their high plasma bilirubin glucuronide levels. In others bilirubinuria was extraordinarily mild. Urobilinogen was, as could be expected, absent from the urine of early and advanced cases, but bile acid salts were also absent from the urine of all the early cases studied, in spite of the high plasma levels of these compounds.

The urine of geeldikkop cases is initially porphyrin free, but within two to three days of the onset of symptoms, coproporphyrin is eliminated in this medium in very large amounts. Phylloerythrin does not appear to be excreted in the urine of these animals in spite of its high plasma levels.

It seems thus that a block to the secondary renal excretion of many of the regurgitated biliary constituents may co-exist in geeldikkop with the hepatic block already discussed. This aspect of geeldikkop forms the subject of one of the following chapters.

Clinical icterus is preceded in geeldikkop by photosensitivity. Once the icterus has become obvious the photosensitivity has largely passed over. These statements are perfectly reconcilable with the chemical pathology as set out above. In uncomplicated bile duct obstruction clinical icterus is first discernible two to three days after bile duct occlusion at plasma levels of 2·5 to 3 mg per cent of bilirubin. This is also the case in the typical geeldikkop case. In the atypical severe cases such as those seen at Vosburg clinical icterus may be severe from the onset of symptoms. This is due to the severe intravascular haemolysis in such cases.

In previous publications (Brown, 1964, 1966a, 1966b) the statement has been made that the actual increase in plasma γ-globulin levels in geeldikkop is due to the influence of an infectious agent. This agent has been described as producing a mild febrile reaction which passes largely unnoticed by the flock owner or his shepherds some while before the symptoms of geeldikkop appear. Although clinically mild, the febrile condition produces profound biochemical disturbances which culminate in the appearance of the typical symptoms of geeldikkop. On the grounds of the symptomatology and pathology of the disease, this mild infection which precipitates the geeldikkop syndrome is thought to be of viral origin (Brown, 1966a). Although this important aspect of the disease will form the subject of one of the concluding chapters of this thesis, when a considerable body of evidence will be produced in support of the idea, the reader is reminded at this stage of the severe haematological upheavals found in the prodromal cases and of the fact that high plasma γ-globulin levels are present in geeldikkop from the moment of onset of the symptoms. The high levels of GOT, LD and Ald will be shown to be the result of myopathy due to the infection mentioned.

The plasma α and β-globulins may be increased in the early and particularly in the advanced cases of geeldikkop. The α2- and β-globulins contain lipoproteins. In obstructive jaundice, especially if chronic, the serum lipids are increased and are atypically combined with peptides in the form of at least three abnormal lipoproteins which migrate on human plasma electrophoretograms as β-globulins (Sherlock, 1958). The plasma lipoproteins have not been studied in geeldikkop and it is not possible to say to what extent they contribute towards the increments in the levels of these plasma proteins. Much of the increased α2-globulin is ceruloplasmin, as has been indicated. This increase follows on the disturbances in copper metabolism and is also a rapidly developing feature of common bile duct obstruction in the sheep, and a pronounced one where such obstruction is complicated by coexisting kidney pathology (Brown, 1967b, 1967c). Some of the increase in β-globulins may be due to an increase in transferrin (a β_1-globulin) which would have to occur to bind the iron being released by haemolysis and being regurgitated back into the systemic blood following obstruction of its biliary escape. The increase in transferrin, if it does occur, would take place once all the immediately available apoferritin in iron-storage organs has been saturated with this element. It is noteworthy that the increase in plasma β-globulins is generally seen from about five days after the onset of symptoms in geeldikkop. Increased transferrin levels are known to occur in some haemolytic syndromes, e.g. in phenylhydrazine induced anaemia (Moore & Dubach, 1962).

A marked increase in liver copper levels was found in most of the geeldikkop cases examined for this purpose. The following has been suggested to occur after regurgitation of biliary copper following common bile duct obstruction in the sheep (Brown, 1967b): Free biliary copper re-attaches to the apo-cuproproteins in the liver and as these become saturated, passes out into the bloodstream as ceruloplasmin; considerable amounts of copper appear to pass into the red cells as well as into the plasma α2-globulins; terminal rises in plasma copper are probably associated with binding of copper by other globulins, as is known to occur in human subjects (Cumings, Goodwin & Earl, 1955; Thompson & Watson, 1949). In most of the sheep with bile duct obstruction high red cell copper levels were associated with increased red cell fragility (Brown, 1967b). It is likely that the translocations of copper in geeldikkop proceed along similar lines. Increased red cell copper has the effect of increasing the fragility of already very fragile cells (see one of the following chapters) and aggravating the haemolytic state present. The high liver levels of the element result from rebinding of regurgitated copper and from copper accumulating as a result of haemolysis. The sheep is known to have a pattern of copper metabolism different from that of other animals. The concentration of liver copper is normally higher than in most other species. This derives from a diminished ability to restrict the storage of copper in the liver and not from an increased intake or greater absorption. Sheep liver has the ability to complex quite large amounts of copper in a relatively stable form and to lose the excess at a very slow rate of elimination (Beck, 1956; 1963; Adelstein & Vallee, 1962).

CHAPTER 5

The General Chemical Pathology and Bio-chemistry of Geeldikkop

B. The Enzymology of the Liver with particular reference to Bilirubin Conjugation and Energy Metabolism

1. Introductory remarks
2. Bilirubin conjugation
3. Carbohydrate metabolism
4. The tricarboxylic acid cycle
5. Glutathione reductase, ascorbic acid and catalase
6. General discussion

1. *Introductory remarks*

It was shown in the previous chapter that most, if not all, of the solid constituents of bile are regurgitated into the general blood circulation from the moment the symptoms of geeldikkop appear. It was postulated, on the grounds of the histopathological and chemical pathological studies cited and on the grounds of what is known regarding the secretion of the components of bile, that the block to biliary excretion in the disease is of a biochemical nature. The selective permeability of the liver cell membranes or the systems which supply energy for the active transport

of the various biliary components, appear to be involved. It seems unlikely, from the large amounts of bilirubin glucuronides and bile acid salts which appear in the plasma at the height of the geeldikkop syndrome, that the major conjugation mechanisms in the liver cells are involved. This chapter is devoted to a study of these mechanisms and some of the systems which supply energy to the liver cell as a whole.

2. *Bilirubin conjugation*

Proof that the main conjugates of bilirubin excreted in the bile of sheep are glucuronides, is offered in one of the later chapters. The discussion which follows is based on this postulate. The reactions for the synthesis of bilirubin diglucuronide in the liver parenchymal cell are:—

(*a*) UDP + ATP + nucleoside diphosphate kinase → UTP + ADP

(*b*) UTP + glucose-1-phosphate + UDPG-pyrophosphorylase → UDP-glucose + PP

(*c*) UDP-glucose + 2DPN + UDP-glucose dehydrogenase → UDP-glucuronic acid + 2DPNH

(*d*) 2UDP-glucuronic acid + bilirubin + UDP-glucuronyl transferase → 2UDP + bilirubin diglucuronide [after Granick & Mauzerall, 1961. UDP, ADP, UTP and ATP are uridine and adenosine di- and triphosphates respectively; UDPG is uridine diphosphoglucose; pp is pyrophosphate; DPN and DPNH are diphosphopyridine nucleotide (nicotine adenine dinucleotide) and its reduced form respectively].

The UDP-glucuronyl transferase (also known as UDP-trans-glucuronylase) is present in liver microsomes and is capable of transferring glucuronic acid to carboxyl, amine, alcoholic and phenolic groups. Other tissues are low in this enzyme activity (Granick & Mauzerall, 1961). Assay of the system as a whole provides a means of assessing not only the conjugation of bilirubin but also glucuronide conjugations in general. The method for studying the synthesis of bilirubin glucuronides by tissue homogenates proposed by Grodsky & Carbone (1957) is a useful tool in this respect and was used by the author. It depends upon the synthesis of bilirubin glucuronide by liver homogenates from added bilirubin. The results are expressed as a percentage of the added bilirubin which is conjugated during a test period of 30 minutes. The authors of the original procedure (Grodsky & Carbone, 1957) found a 15 to 30 per cent conversion of bilirubin by rat liver homogenates during this time. The results found after using liver homogenates from cases of geeldikkop and control animals are presented in Table 41. The values found for the control animals are in the same order as those found by Grodsky & Carbone for rat livers. Low values were found in about half the early and five out of seven advanced cases. All the early cases listed as Group 1 in this table were of one to two days standing while those of Group 2 reading from Sheep V3-14 down to V3-25 were cases of the same duration. The rest of the early cases were of three to five days standing. The lowest values were thus to be found within the first two days of illness. The advanced cases were all of seven to ten days standing. Low values were found in both groups of advanced cases.

UDPG and UDPG-dehydrogenase were assayed on a number of the liver homogenates from the cases mentioned in Table 41. The results are presented in Tables 42 and 43. Synthesis of UDPG appears to be unimpaired and maintained

TABLE 41.—*Bilirubin conjugating activity of the livers from cases of various stages of geeldikkop and control animals*

(The results are expressed in terms of the percentage of bilirubin added which was recovered as glucuronide)

Early cases	% Conjugation	Advanced cases	% Conjugation
V3–22 (Group 1).....	7·1	V3–8 (Group 1).....	16·6
V3–23 „	5·4	V3–12 „	14·5
V3–21 „	2·9	V3–17 „	4·7
V3–24 „	8·8	V3–9 „	8·3
		V3–2 „	3·7
V3–26 (Group 2).....	16·2		
V3–14 „	15·2	V3–18 (Group 2).....	8·8
V3–13 „	4·5	V3–1 „	6·6
V3–15 „	1·8		
V3–27 „	9·8	Recovered cases	% Conjugation
V3–20 „	12·7		
V3–25 „	2·5	V3–19.....	12·2
V3–16 „	20·5	V3–4.....	12·5
V3–10 „	20·6	V3–3.....	5·0
V3–11 „	22·1		
V3–6 „	15·8		
V3–7 „	10·3		
Control Animals	% Conjugation	Control Animals	% Conjugation
V3–33.....	21·0	V3–32.....	30·0
V3–31.....	25·5	V3–5.....	22·0
V3–28.....	59·5	V3–30.....	16·5
V3–29.....	20·5		

TABLE 42.—*Assays of UDPG on homogenates made from livers of geeldikkop and control animals*

Animal No.	Nature of case	μ-moles UDPG/ 100 mg liver
V3–21	Early case.....	4
V3–14	„	1
V3–13	„	11
V3–15	„	2
V3–27	„	1
V3–20	„	2
V3–16	„	4
V3–10	„	5
V3–8	Advanced case.....	2
V3–12	„ „	13
V3–9	„ „	8
V3–1	„ „	4
V3–4	Recovered case.....	3
V3–3	„ „	4
V3–33	Control.....	2
V3–28	„	4
V3–29	„	3

TABLE 43.—*Assays of UDPG dehydrogenase on homogenates made from livers of geeldikkop and control animals*

Animal No.	Nature of case	UDPG dehydrogenase units/ 100 mg liver
V3–22	Early case Group 1	150
V3–23	,, ,, ,,	200
V3–21	,, ,, ,,	195
V3–24	,, ,, ,,	225
V3–26	Early case Group 2	100
V3–14	,, ,, ,,	100
V3–13	,, ,, ,,	80
V3–15	,, ,, ,,	70
V3–27	,, ,, ,,	55
V3–20	,, ,, ,,	55
V3–25	,, ,, ,,	75
V3–16	,, ,, ,,	160
V3–10	,, ,, ,,	150
V3–11	,, ,, ,,	125
V3–8	Advanced case Group 1	85
V3–12	,, ,, ,,	80
V3–17	,, ,, ,,	85
V3–9	,, ,, ,,	90
V3–18	Advanced case Group 2	325
V3–1	,, ,, ,,	20
V3–4	Recovered case	45
V3–3	,, ,,	95
V3–33	Control	75
V3–31	,,	25
V3–28	,,	145
V3–29	,,	25
V3–32	,,	50
V3–30	,,	75

at a steady rate throughout the disease. It appears, however, from the results in Table 43 that there is a marked increase in the levels of activity of UDPG-dehydrogenase during the first five days of illness, elevated levels being present from the onset of symptoms. The milder advanced cases (of seven to eight days standing) destined to recover and the recovered cases show liver levels of activity within the range of the control group. Very high values may be found in the severely affected Group 2 advanced cases, e.g. V3-18.

3. *Carbohydrate metabolism*

Blood sugar levels of the control animals numbered V1-7054 to V1-7071, and animals F-K2, F-12221 and F-12222 ranged from 41·0 to 68·5 mg per cent with a mean value of 51·9 mg per cent. Blood sugar values found in the prodromal and the severe Vosburg cases are presented in Table 44, while those found in the more typical cases are presented in Table 45. Blood sugar levels ranged from 25·0 to 59·0 mg per cent, in the recovered animals with a mean value of 43·4 mg per cent.

TABLE 44.—*Blood sugar levels in the prodromal cases and severe Vosburg cases*

Case No.	Nature of case	mg%	Case No.	Nature of case	mg%
VB–P	Prodromal case.....	26·0	VB–F	Early case 3 days.....	15·7
VB–Q	,, ,, ...	47·0	VB–C	,, ,, ...	56·0
VB–R	,, ,, ...	11·5	VB–B	,, ,, ,, ...	47·0
VB–S	,, ,, ...	16·5	VB–G	,, 4 days.....	63·0
VB–T	,, ,, ...	19·5	VB–J	,, 5 days.....	52·5
VB–U	,, ,, ...	19·5	VB–K	,, 7 days.....	59·5
VB–V	,, ,, ...	56·0			
VB–W	,, ,, ...	18·0	VB–D	Advanced case 7–8 days	59·5
VB–X	,, ,, ...	31·5	VB–L	,, ,, ,,	24·0
			VB–M	,, ,, ,,	14·0
VB–H	Early case 1–2 days...	18·0	VB–N	,, ,, ,,	126·0
VB–I	,, ,, ...	12·5	VB–Z1	,, ,, ,,	19·0
VB–A	,, 3 days.....	22·0	VB–O	,, ,, ,,	25·5
VB–E	,, ,, ...	21·0	VB–Y	,, ,, ,,	17·0

TABLE 45.—*Blood sugar levels in the more typical geeldikkop cases*

Case No.	Nature of case	mg%	Case No.	Nature of case	mg%
V1–25	Early case 1–2 days...	61·0	V1–11	Early case 5–7 days...	82·5
V1–3	,, ,, ...	74·5	V1–18	,, ,, ...	51·5
V1–1	,, ,, ...	74·0	V1–21	Advanced case 7–8 days	65·0
V1–23	,, 2–3 days...	67·0	V1–5	,, ,, ,,	76·5
V1–22	,, ,, ...	61·0	V1–4	,, ,, ,,	52·5
V1–24	,, ,, ...	73·5	V1–14	,, ,, ,,	40·0
V1–17	,, ,, ...	48·5	V1–7	,, ,, ,,	191·0
V1–2	,, ,, ...	134·0	V1–6	,, ,, ,,	95·5
V1–15	,, ,, ...	54·5	V1–8	,, ,, 8–10 days	38·5
F–5	,, ,, ...	49·5	V1–9	,, ,, ,,	47·5
V1–13	,, ,, ...	90·0	V1–10	,, ,, ,,	44·0
F–2	,, ,, ...	49·5	V1–12	,, ,, 10–14 days	47·5
F–1	,, ,, ...	54·5	V1–20	,, ,, ,,	63·0
V1–26	,, 3–5 day....	62·0	V1–16	,, ,, ,,	50·5

The prodromal stage of the disease is characterized by an extremely severe hypoglycaemic state which accompanies the severe haematological upheavals and hypergammaglobulinaemia noted earlier. This hypoglycaemic state was persistent in many of the severe early and advanced Vosburg cases. In the latter group very low values were encountered mainly in cases *in extremis*. Values of over 100 mg per cent were generally found in animals *in extremis*, immediately before death. Such hyperglycaemias were usually accompanied by a glycosuria. These are essentially terminal changes found in many ovine diseases.

Hypoglycaemia is not a feature of the more typical cases of geeldikkop. The blood sugar levels are in many instances high for sheep and often well above the upper limit of the range in the control and recovered animals. This *hyperglycaemic tendency* is seen particularly in the early cases of the disease. Glucose tolerance tests were done on most of these animals. Typical results are presented in composite

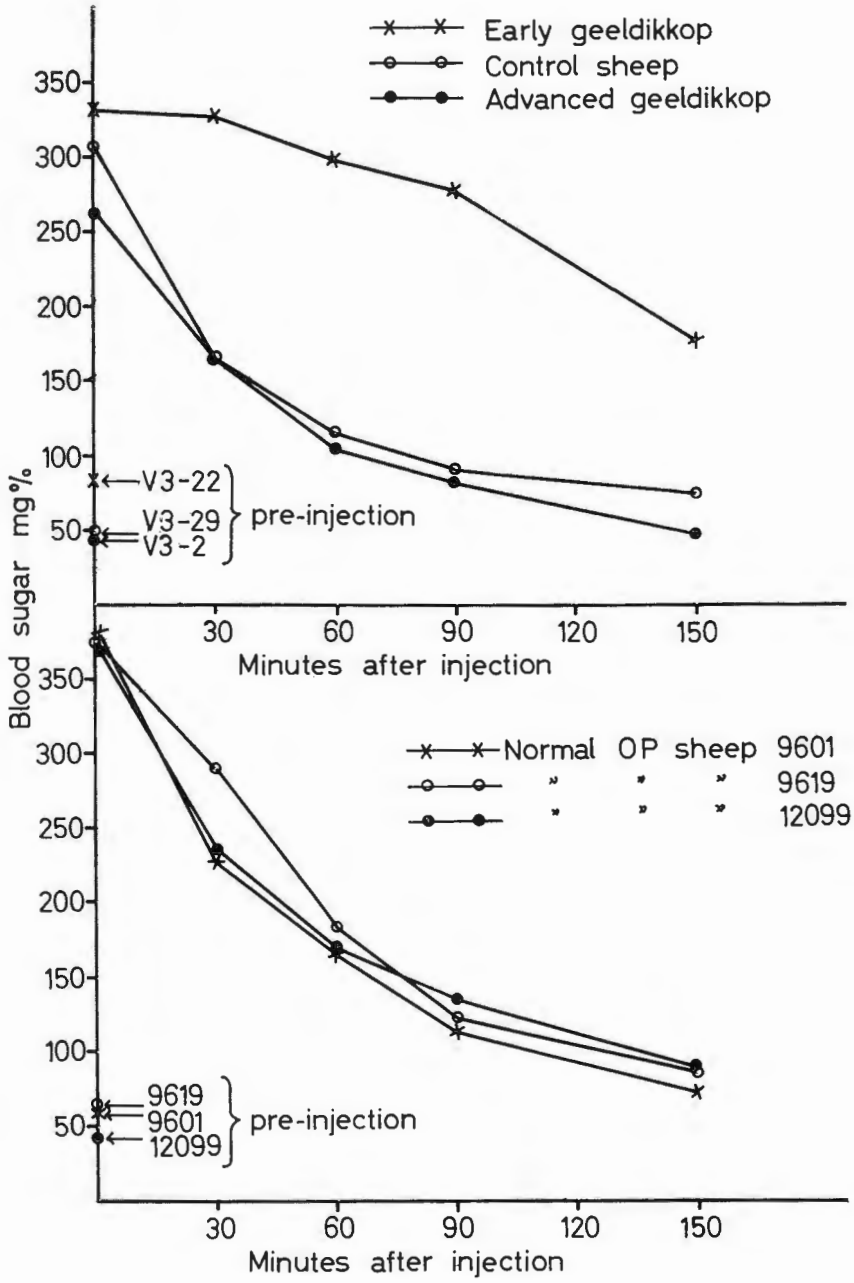

FIG. 6.—Glucose tolerance curves in geeldikkop cases and clinically normal sheep. (Note: OP in the lower graph means Onderstepoort).

Fig. 6, where plasma clearance curves are presented for an early, advanced and control case. Similar curves for three clinically normal sheep drawn from the pool of available animals are presented for comparison in the same figure. The test consisted of administering glucose intravenously at the rate of 0·25 gm/Kg body weight (dissolved in sufficient 0·9 per cent NaCl) and taking blood samples before injection and at intervals of 1, 30, 60, 90 and 150 minutes after injection. Blood sugar levels were determined on each sample and the glucose concentrations were plotted against time to give the curves shown in Fig. 6. Typical data are presented in Table 46.

TABLE 46.—*Typical data from which the glucose tolerance curves were constructed*

Sheep No. and details	mg% before injection	mg% 1 min after	mg% 30 min after	mg% 60 min after	mg% 90 min after	mg% 150 min after
V3–20 Early case (Group 2) 2–3 days standing.....	75	348	330	281	244	137
15582 Normal Onderstepoort sheep.....	54	464	290	184	130	64

A markedly decreased tolerance to intravenously administered glucose was shown by all the early cases during the first two or three days of illness. Thereafter tolerance to the loading dose of glucose becomes greater until normal curves are obtained in many of the advanced cases and in the recovered cases. Abnormally low tolerance curves are still a feature of many of the seriously ill advanced cases. The decreased tolerance curves in the early cases particularly are essentially similar to those shown by diabetic patients (Bodansky & Bodansky, 1957; Cornelius & Kaneko, 1963; Forsham & Mortimore, 1959).

The glucose tolerance test is of particular value when the fasting blood sugar appears to be at the upper limit of normal (Cornelius & Kaneko, 1963) and reflects the inability of the animal to dispose of a test dose of glucose. It has long been held that the diabetic curve is a reflection of the inability of the pancreas to provide additional insulin. More recent postulates delegate a primary role to the homeostatic mechanisms of the liver. The liver can supply as well as remove glucose and therefore occupies a central position in the regulatory mechanisms of blood sugar levels. The metabolic activities of the liver are primarily directed towards supply rather than utilization of glucose. The enzyme glucose-6-phosphatase occupies a key position in this function of the liver. Its levels in this organ can be increased two-and-a-half fold in laboratory animals made diabetic by administration of alloxan (Forsham & Mortimore, 1959). An increase in the activity of this enzyme will cause an increased production of glucose by the liver if precursors are freely available (Forsham & Mortimore, 1959). Little is still known of the levels of activity of sheep liver enzymes. Glucose-6-phosphatase activity is normally only about two-thirds of the activity found in rats. Increased production of glucose by the ruminant liver is also known to be accompanied by an increase in glucose-6-phosphatase activity. The ruminant is an animal well adapted to a carbohydrate economy based on the

endogenous synthesis of glucose from non-carbohydrate sources, but glucose oxidation by individual tissues as well as by the intact animal is lower in ruminants than in non-ruminants. Although overall oxidation may be different, the pathways by which this oxidation is accomplished are essentially similar to those of other animals (Cornelius & Kaneko, 1963).

Liver levels of glucose-6-phosphatase activity found in control animals and cases of all stages of geeldikkop are reproduced in Table 47. It is obvious from this table that the levels of activity are markedly elevated above those of the control

TABLE 47.—*Glucose-6-phosphatase activities in the livers of control animals and cases of geeldikkop*

Sheep No. and details	Units/100 mg liver	Sheep No. and details	Units/100 mg liver
V3–33 Control.....	22·2	V3–25 Early case 2–3 days.....	14·0
V3–31 „	20·0	V3–16 „ 3–5 days	74·0
V3–28 „	26·0	V3–10 „ „	40·0
V3–29 „	24·0	V3–11 „ „	20·0
V3–32 „	21·2	V3–6 „ „	19·2
V3–30 „	24·0	V3–7 „ „	14·0
V3–22 Early case 1–2 days.....	20·0	V3–8 Advanced case 7–8 days	32·0
V3–23 „ „	33·6	V3–12 „ „ „	36·0
V3–21 „ „	30·0	V3–17 „ „ „	24·0
V3–24 „ „	14·8	V3–9 „ „ „	36·0
V3–14 „ 2–3 days.....	74·0	V3–2 „ „ „	40·0
V3–13 „ „	56·0	V3–18 „ „ „	14·0
V3–15 „ „	62·8	V3–1 „ „ 8–10 days	34·0
V3–27 „ „	21·6	V3–4 Recovered case.....	45·0
V3–20 „ „	38·0	V3–3 „ „	40·0

animals in more than half of the geeldikkop cases studied. The greatest elevations (up to three times the control values) are to be found in cases of two to five days standing. Although the levels of activity were somewhat lower during the advanced stages of the disease (generally one-and-a-half to twice the control level) significantly raised levels of activity apparently persist right into the stage of recovery.

The primary biochemical lesions of diabetes lead to secondary changes in many phases of intermediary metabolism. It is for instance a demonstrated fact that fructose undergoes glycolysis in the diabetic and can rectify the abnormalities in carbohydrate, fat and presumably protein metabolism in liver slices from diabetic animals, and to a limited extent in the whole diabetic organism as well. This is so because of the presence of an insulin-independent fructokinase in the liver, and to a lesser extent in muscle and brain (Forsham & Mortimore, 1959). Since its transformation into glucose proceeds rather rapidly, the intravenous administration of fructose will eventually lead to hyperglycaemia in the diabetic not given insulin (Forsham & Mortimore, 1959).

A fructose tolerance test was done on one early case only because of time considerations, i.e. sheep V3-6, an early case of three to five days standing. The curve is reproduced in Fig. 7. The test was done in the same way as the glucose tolerance test, a dose of 0·25 gm/lb body weight being used. It is apparent from this curve, that in contrast to the lowered tolerance to glucose in the early cases of geeldikkop, fructose was utilized rapidly in this instance.

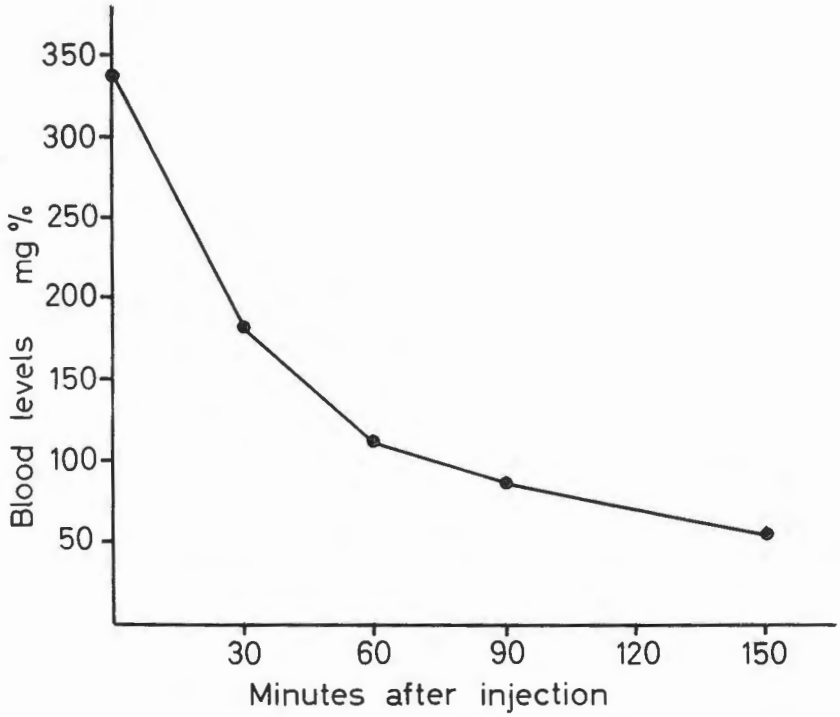

FIG. 7.—Fructose clearance, sheep V3–6

The levels of lactic acid found in control animals and cases of all stages of geeldikkop are given in Table 48. Under the laboratory conditions pertaining at the time, virtually no lactic acid could be determined in the blood of the control sheep

TABLE 48.—*Plasma lactic acid values in control sheep and cases of all stages of geeldikkop*

Sheep No. and details	mg%	Sheep No. and details	mg%
V3–22 Early case 1–2 days.....	0·64	V3–8 Advanced case 7–8 days	2·94
V3–23 ,, ,, ...	0·07	V3–12 ,, ,, ,,	0·93
V3–21 ,, ,, ...	0·91	V3–17 ,, ,, ,,	1·00
V3–24 ,, ,, ...	0	V3–9 ,, ,, ,,	3·35
V3–26 ,, ,, ...	0·20	V3–2 ,, ,, ,,	2·84
V3–14 ,, 2–3 days.....	11·00	V3–18 ,, ,, ,,	0
V3–13 ,, ,, ...	10·36	V3–1 ,, ,, 8–10 days	3·55
V3–15 ,, ,, ...	11·25	V3–19 Recovered case.....	1·17
V3–27 ,, ,, ...	0·15	V3–4 ,, ,, ...	1·60
V3–20 ,, ,, ...	1·31	V3–3 ,, ,, ...	3·06
V3–25 ,, ,, ...	0·17	V3–33 Control.....	0·64
V3–16 ,, ,, ...	10·32	V3–31 ,, ...	0
V3–10 ,, ,, ...	2·58	V3–28 ,, ...	0
V3–11 ,, ,, ...	1·61	V3–29 ,, ...	0
V3–6 ,, ,, ...	2·32	V3–32 ,, ...	0
V3–7 ,, ,, ...	3·08	V3–5 ,, ...	0·91
		V3–30 ,, ...	0

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by the method used. It became generally determinable once symptoms of geeldikkop had appeared and in many instances high values were found during the second to third days of illness. Thereafter the levels tended to return to normal (in this case the range 0 to 0·9 mg per cent shown by the controls) although moderate elevations persisted through into recovery. The highest values were encountered in the severely ill Group 2 early cases of two to three days standing, e.g. Sheep V3-14, V3-13, V3-15, and V3-16.

Blood pyruvate levels were determined in cases representing all stages of the disease. Values in the control animals ranged from 0·06 to 0·17 mg per cent, in the early cases from 0·05 to 0·86 mg per cent, in the advanced cases from 0·05 to 0·15 mg per cent and in the recovered animals from 0·05 to 0·12 mg per cent. No significant differences were thus seen between any of the groups of animals. Pyruvate clearances were determined on three of the early cases of one to three days standing, viz., V3-7, V3-15 and V3-23, using a technique similar to the intravenous sugar tolerance tests. A dose of 0·25 gm Na pyruvate/Kg body weight was dissolved in sufficient 0·9 per cent NaCl and given in travenously. Blood specimens were taken as in the sugar tolerance tests and the results plotted in the same way. The curves obtained are presented in Fig. 8. Essentially similar curves were found in

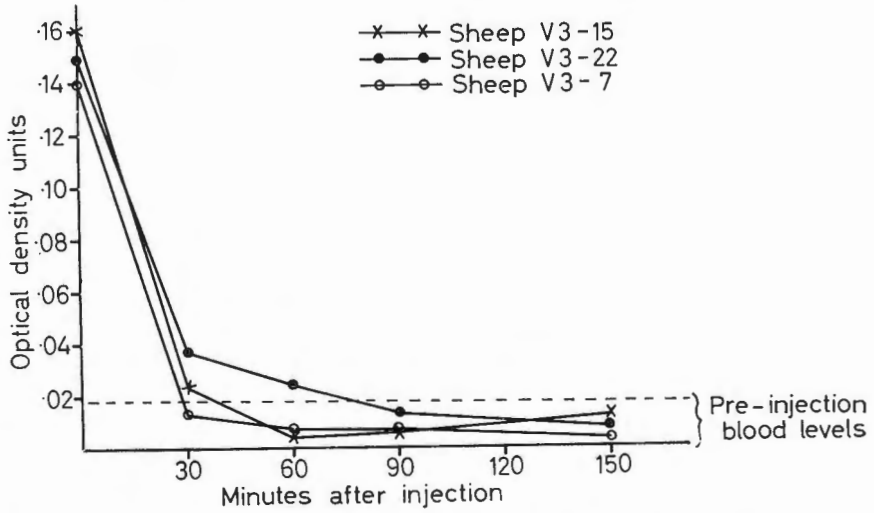

FIG. 8.—Pyruvate clearances. Optical density units have been used here for convenience in plotting, instead of mg per cent

normal animals. Thus although lactate tends to accumulate in the blood of early cases of two to three days standing, additional pyruvate is rapidly and efficiently disposed of.

No specific study of fatty acid metabolism in geeldikkop has been possible so far because of all the other work which had to be done in the time available. The frequency of ketosis amongst the early and advanced cases of the disease has therefore *not* been established with any certainty. Whenever possible urine samples were collected from the animals being studied and examined according to standard routine procedure. Judging by the frequency with which positive qualitative reactions were obtained for ketones in these samples, latent ketosis must be a fairly common complication, particularly in fat animals, although no clinical symptoms have ever been observed which could be attributed to it (Brown, 1962, 1963, 1966a).

4. *The tricarboxylic acid cycle*

Plasma oxalacetate, citrate and ∝-ketoglutarate levels were determined on samples from all the animals designated V-3. No significant differences were observed between the control animals and the various groups of geeldikkop cases as regards oxalacetate and citrate. The levels of ∝-ketoglutarate were if anything slightly higher in the early cases of the disease than in the other groups of animals.

The levels of activity of succinic dehydrogenase and isocitric dehydrogenase in the livers of cases of various stages of geeldikkop and of the control animals were determined. The results are presented in Table 49. Results obtained from the livers of clinically normal Onderstepoort sheep are presented in Table 50.

TABLE 49.—*Levels of activity of succinic dehydrogenase (SD) and isocitric dehydrogenase (ICD) in the livers of control animals and cases of the various stages of geeldikkop*

(The results are expressed as units per 100 mg of liver tissue)

Sheep No.	SD	ICD	Sheep No.	SD	ICD
1. Early cases—			2. Advanced cases—		
V3–22	162	6	V3–8	275	14
V3–23	171	6	V3–12	307	12
V3–21	178	10	V3–17	200	6
V3–24	129	5	V3–9	204	12
V3–26	226	2	V3–2	226	4
V3–14	291	26	V3–18	64	2
V3–13	265	16	V3–1	259	3
V3–15	226	12	3. Recovered cases—		
V3–27	152	8	V3–4	194	8
V3–20	210	12	V3–3	226	8
V3–25	184	18	4. Controls—		
V3–16	239	12	V3–33	207	6
V3–10	291	8	V3–31	265	6
V3–11	64	6	V3–28	194	8
V3–6	236	2	V3–29	162	6
V3–7	129	8	V3–32	262	4
			V3–30	149	6

TABLE 50.—*Levels of activity of succinic dehydrogenase (SD), isocitric dehydrogenase (ICD) and glutathione reductase (GR) in the livers of clinically normal Onderstepoort sheep*

(The results are expressed as units per 100 mg of liver tissue)

Sheep No.	SD	ICD	GR
15141	259	4·0	139
15692	226	4·0	206
15698	226	4·0	186
14745	161	4·0	133
15822	226	4·0	193
15837	129	4·0	213
14499	350	6·0	106
14489	162	4·0	133
Mean values	215	4·25	164

The mean value for succinic dehydrogenase found in the livers of the control Karoo sheep is 190. This is somewhat lower than the mean value of 215 found for the livers of Onderstepoort sheep. Such differences between Karoo and Onderstepoort have been established statistically for other enzymes in plasma and erythrocytes (Wagner, 1964; Wagner & Brown, 1966a, 1966b). Although a few low values for liver succinic dehydrogenase were found amongst the early cases, the mean value for this group is 197, i.e. in the same order as that for the control animals. The levels of activity found in the advanced cases (mean value = 205) were generally higher than in the early cases, but were in the same general order as the control animals. It seems from these results that there might be some suppression of succinic dehydrogenase activity in the livers of the early cases, but many more figures will be needed to establish this point with any certainty. The variation amongst the control animals in this respect is quite considerable, which makes attempts to interpret the figures in Table 49 rather hazardous and unrewarding.

Isocitric dehydrogenase levels are on the other hand unequivocally increased in the early cases and particularly amongst the cases of two to three days standing The mean value found for Onderstepoort sheep was 4·25 and that for the Karoo controls 6·0 units/100 mg liver. The early cases yielded activity levels ranging from 2 to 26 with a mean value of 9·81 units/100 mg of liver. High values are still found amongst the advanced cases of seven to eight days standing (mean value 7·6, range 2 to 14 units/100 mg liver). Recovery seems to be accompanied by a return to normal levels of activity.

5. *Glutathione reductase, plasma ascorbic acid and plasma catalase levels*

The levels of glutathione reductase in the livers of control animals and cases of various stages of geeldikkop are presented in Table 51. The reader should refer back to Table 50 for the levels of activity found in normal Onderstepoort sheep. Some interesting points emerge from the data presented in Tables 50 and 51.

TABLE 51.—*Levels of activity of glutathione reductase (GR) in the livers of control animals and cases of the various stages of geeldikkop*

(The results are expressed as units per 100 mg of liver tissue)

Sheep No. and details	GR	Sheep No. and details	GR
V3–22 Early case 1–2 days.....	186	V3–8 Advanced case 7–8 days.	502+
V3–23 „ „	500+	V3–12 „ „ „	825+
V3–21 „ „	233	V3–17 „ „ „	233
V3–24 „ „	159	V3–9 „ „ „	300
V3–26 „ „	176	V3–2 „ „ „	206
V3–27 „ 2–3 days.....	510+	V3–1 „ „ 8–10 days.	61
V3–20 „ „	306	V3–4 Recovered case.....	133
V3–25 „ „	520+	V3–3 „ „	213
V3–10 „ 3–5 days.....	386	V3–33 Control animal.....	86
V3–11 „ „	266	V3–31 „ „	93
V3–6 „ „	193	V3–28 „ „	86
V3–7 „ „	150	V3–29 „ „	66
		V3–32 „ „	66
		V3–30 „ „	86

Where figures are accompanied by a + sign, the values were so high that they could not be measured accurately.

The liver activity levels of glutathione reductase are considerably lower in the control sheep from farms in areas where geeldikkop is prevalent than in clinically normal sheep studied at Onderstepoort. With the appearance of the first symptoms of geeldikkop there is a dramatic rise in glutathione reductase activity which is sustained throughout the first week of the illness, declining only slowly as the animals recover. Consistently elevated levels are present during the first three days of illness. In a few instances (marked with a + sign in Table 51) glutathione reductase was so increased that it could not be measured accurately with the method used. Extremely high levels of the enzyme were still to be found in the advanced cases, while the recovered cases yielded values similar to those found in Onderstepoort sheep.

Since the sulphydryl (SH) group-containing enzymes are only active in the thiol (SH) or reduced state, it has been suggested that an important function of glutathione (GSH) is to keep these enzymes in the reduced form (Dixon & Webb, 1958). A further important function of GSH in animals is to protect ascorbic acid from oxidation. Recent work (West & Todd, 1952) seems to indicate that GSH-ascorbic acid systems may have considerable influence in controlling redox potentials in cells. Above pH 6·5 the reaction between GSH and the oxidized form of ascorbic acid, viz. dehydroascorbic acid is instantaneous and probably not reversible in tissues (Burns, 1960):—

dehydroascorbate + 2GSH → ascorbate + GSSG (GSSG = oxidized glutathione).

This reaction occurs spontaneously, is non-enzymatic and could under certain conditions act as an oxidizing system for reduced TPN (reduced triphosphopyridine nucleotide or TPNH), this latter reaction being linked to glutathione reductase. The latter is a GSSG specific TPNH-linked dehydrogenase first found in animal tissues by Meldrum & Tarr (1935) and catalyses the following reaction:—

TPNH + H + GSSG → TPN + 2GSH.

It reacts with reduced DPN (reduced diphosphopyridine nucleotide or DPNH) in the same way but at a slower rate (Kun, 1961). Glutathione reductase is present mainly in the cytoplasmic sap, where most but not all of the cell's glycolytic enzymes are located (Gallagher, 1964).

There is a good correlation between the increments of glutathione reductase activity in the livers of geeldikkop cases and the elevations of plasma ascorbic acid also seen in the disease. The figures for this blood component presented in Table 52

TABLE 52.—*Levels of ascorbic acid in the plasma of control animals and cases of the various stages of geeldikkop*

Sheep No. and details	mg%	Sheep No. and details	mg%
V1–7054 Control	0·66	V3–23 Early case	3·01
V1–7055 „	0·53	F–5 „	2·03
V1–7056 „	0·66	F–2 „	3·11
V1–7057 „	0·49	F–1 „	3·88
V1–7059 „	0·53	V1–5 Advanced case	2·50
V1–7060 „	0·84	V1–8 „	2·21
V1–7061 „	0·47	F–4 Recovered case	2·02
V1–7062 „	0·63	F–3 „	3·07
Sheep 12291 „	1·94		

are representative. Marked increases are seen in the early stages of the disease. Lower values are found as the disease progresses, but values well above normal may persist throughout the course of the disease to be found even in recovered animals.

Sheep liver has a disproportionately high catalase activity as compared with other enzyme systems and other species (Gallagher & Buttery, 1959). The metabolic significance of catalase in the sheep is unknown, but it has been suggested that the high liver levels develop as a consequence of the absence of regulatory levels of adreno-cortical steroid usual in non-ruminant species—the levels of adrenal steroids in the plasma of normal sheep are reported to be very low (Gallagher & Buttery, 1959). It has been found that sheep plasma is very rich in catalase and marked differences have been demonstrated in this respect between sheep bred in the Karoo, where geeldikkop occurs and those raised elsewhere (Wagner, 1964). Plasma catalase levels are approximately eight times higher in the latter group than in animals emanating from the Karoo, i.e. a normal (80 per cent) range of 8·63 to 9·1 units compared with 0·2 to 1·65 units for Karoo sheep.

Plasma catalase levels were studied in the geeldikkop cases during the latter field investigations. The results obtained from control animals and typical cases of the disease are presented in Table 53. The upper 10 per cent limit of the normal range

TABLE 53.—*Levels of catalase in the plasma of control animals and cases of the various stages of geeldikkop*

Sheep No. and details	Units/ 0·1 ml	Sheep No. and details	Units/ 0·1 ml
V3-22 Early case 1–2 days.....	9·03	V3-8 Advanced case 7–8 days.	2·05
V3-23 „ „	9·79	V3-12 „ „ „	4·37
V3-21 „ „	8·21	V3-17 „ „ „	6·00
V3-24 „ „	8·63	V3-9 „ „ „	2·31
V3-26 „ „	4·64	V3-2 „ „ „	6·40
V3-14 „ 2–3 days.....	5·88	V3-18 „ „ „	9·80
V3-13 „ „	5·63	V3-1 „ „ 8–10 days	8·70
V3-15 „ „	9·75	V3-19 Recovered case.....	5·58
V3-27 „ „	6·31	V3-4 „ „	8·00
V3-20 „ „	8·34	V3-3 „ „	9·01
V3-25 „ „	8·19	V3-33 Control.....	4·33
V3-16 „ 3–5 days.....	7·2	V3-31 „	4·55
V3-10 „ „	1·55	V3-28 „	4·11
V3-11 „ „	9·89	V3-29 „	4·88
V3-6 „ „	8·12	V3-32 „	4·22
V3-7 „ „	8·00	V3-30 „	4·55

established for Karoo sheep is 2·875 units (Wagner, 1964). The controls used in this work gave somewhat higher values. Plasma catalase was found to be increased by up to or more than two-fold in many of the early cases, while moderate elevations over the levels shown by the control animals were observed in most. High values were found to persist in many of the advanced cases and in the recovered animals.

6. *General discussion*

The findings outlined in this chapter underline two important conclusions reached in the preceding chapters, namely that profound biochemical disturbances may precede the actual appearance of the clinical symptoms of geeldikkop and that in typical cases these disturbances reach a maximum in the first two or three days of illness. In such cases a rapid return to normal can be expected during the subsequent five to seven days as far as most of the biochemical systems studied are concerned. Some of the biochemical disturbances may persist for a long time in the severer type of case and still be noticeable in recovered cases. In those cases which will have a fatal termination, many of the disturbances continue to increase in severity until death supervenes.

The most serious biochemical defects uncovered in these investigations are those which relate to carbohydrate metabolism. In the first instance a severe hypoglycaemia may mark the prodromal stage of the disease and in very severe cases this may be a most persistent symptom. In the more typical type of case there is a hyperglycaemic tendency instead of the low blood-sugars seen in the very severe cases. During the first two or three days of illness this hyperglycaemic tendency is accompanied by a markedly decreased tolerance to intravenously administered glucose, but possibly not to fructose. This may indicate a decreased utilization of glucose in this stage of the disease.

Before proceeding with the discussion, some known facts are quoted which are of importance in regard to the role of the liver in carbohydrate metabolism in general, and in the sheep in particular. The principal site of the action of insulin has been localized as being prior to glucose-6-phosphate in the metabolic events of glucose (Kaneko, 1963). In order for extracellular glucose to gain entry into the metabolic pathways, it must first enter the cell and then be phosphorylated. Whether insulin acts on the first or second of these steps has been a debatable subject. The bulk of current evidence favours the initial entry of glucose into the cell as the principal site of insulin's action and not on the hexokinase reaction which brings about phosphorylation. The effect of insulin is postulated as being the facilitation of the transfer of glucose across the cell membrane (Kaneko, 1963). This membrane transfer system is rate-limiting. In peripheral tissues like muscle it is insulin sensitive, but this is not the case in liver tissue where glucose diffuses freely across the liver cell membrane (Cahill, Ashmore, Earle & Zottu, 1958). It has been demonstrated (Soskin, Essex, Herrick & Mann, 1938; Cahill *et al.*, 1958) that at a blood sugar level of approximately 150 mg per cent the liver ceases to take up or supply glucose to the circulation. This level might then be termed the " steady state " at which those mechanisms of importance in supply and removal are operating at equal rates (Kaneko, 1963). Above this level glucose removal is greater than supply, and the balance is reversed when the blood glucose level is below 150 mg per cent (in humans and non-ruminants). This effect may be partially due to a mass action effect but a greater degree of control appears to be exercised by the rate-limiting reactions in the liver.

The membrane transfer of glucose into the liver cell of the non-ruminant may be independent of insulin but the glucokinase reaction in the intact liver cell is readily demonstrated to be insulin sensitive (Chernick & Chaikoff, 1951). The opposing reaction is catalyzed by the enzyme glucose-6-phosphatase, found mainly in the liver. The concentration of this enzyme increases in the liver after fasting, a change which would favour the production of glucose by the liver (Kaneko, 1963). An even

greater increase is to be found in insulin deficiency, i.e. diabetes mellitus, in spite of the hyperglycaemia. The increase in glucose-6-phosphatase activity together with decreased utilization offers an explanation for the excessive production of glucose by the diabetic liver. The effect of insulin upon hepatic glucokinase activity in the non-ruminant also suggests an anatomical separation for the action of insulin between muscle and liver (Kaneko, 1963). This dual role for insulin, affecting membrane transport in muscle and glucokinase in liver, has been suggested by Cahill, Ashmore, Renold & Hastings (1959).

Although sheep brain homogenates catabolize glucose very actively, sheep liver preparations do not do so unless hexokinase is added to the homogenate. On the other hand liver homogenates can readily make use of hexose phosphates (Gallagher & Buttery, 1959). These results suggest an inability on the part of the sheep liver to use free glucose because of a lack of phosphorylation by glucokinase. Furthermore this deficiency in glucose utilization by sheep liver homogenates is present from birth (Gallagher & Buttery, 1959). Sheep liver mitochondria can oxidize glucose but only in the presence of added hexokinase. TPN has a stimulating effect on this metabolism which suggests that part at least of the oxidation occurs via glucose-6-phosphate dehydrogenase and the phosphogluconate shunt (Gallagher & Buttery, 1959).

On the other hand sheep liver mitochondria oxidize fatty acids very actively and sheep liver is very largely dependent upon the oxidation of fatty acids for the maintenance of tricarboxylic acid cycle activity. Under normal conditions volatile fatty acids are supplied in abundance via the portal circulation (Gallagher & Buttery, 1959). The supply of glucose for utilization by peripheral tissues, e.g. the central nervous system, and by the liver cells themselves, must come from gluconeogenesis and the liver will be the main site of glucose production from non-carbohydrate sources. Consequently, liver metabolism in the sheep is biased towards the formation of glucose rather than its destruction. The glucokinase reaction is the most effective point for such bias to be applied (Gallagher & Buttery, 1959).

Ruminant nervous tissue is similar to that of other animals in being an obligatory glucose-utilizing tissue, but the utilization is lower in ruminants than in other animals. Ruminant muscle tissue also utilizes less glucose than muscle tissue of other species (Reid, 1950a, 1950b).

The inability of sheep liver homogenates to catabolize glucose anaerobically is not peculiar to the sheep, but because of the dependence of this animal upon liver gluconeogenesis to supply blood glucose and liver glycogen, failure to catabolize glucose may assume a very great importance in the sheep. Unavailability of glucose as an alternative source to fatty acids of acetyl-coenzyme A, leaves the sheep particularly prone to liver failure, since this leaves it dependent on only one of the two usual sources of energy. Any inhibition in fatty acid oxidation or a fall in the supply of fatty acids to the liver will lead to a directly proportional failure of liver metabolism. The liver glycogen reserve will, under conditions like this, soon be exhausted in supplying blood glucose or acetyl-coenzyme A *and the glycogenic amino acids which may be available cannot much affect the issue* (Gallagher & Buttery, 1959. The italics are mine; the wording is theirs).

With this brief survey of glucose metabolism in the non-ruminant and the sheep in mind, let us return to the position in geeldikkop. Since sheep liver cannot utilize free glucose, the markedly reduced tolerance to this compound must be the result of decreased peripheral utilization. The muscles, because of their large mass in proportion to the rest of the body, must be directly concerned. The reader is

reminded of the indications for the existence of a myopathy in geeldikkop mentioned in the previous chapter. The markedly increased liver levels of glucose-6-phosphatase must mean an attempt to raise glucose production by the liver. The markedly increased blood levels of lactic acid in the early cases can mean either—

(*a*) an inhibition in the conversion of pyruvate (coming from various sources as the result of gluconeogenesis) to glucose-6-phosphate. The most likely site for such inhibition is at the level of glyceraldehyde-phosphate dehydrogenase. The accumulating pyruvate would then be shunted into lactate by lactic dehydrogenase; or

(*b*) a failure somewhere in the respiratory pathways of the liver cell for the oxidation of hydrogen. This would result in an immediate fall in the energy level of the cell, and increased gluconeogenesis in an attempt to supply more energy and the use of any pyruvate which is being formed to dispose of embarrassing hydrogen ions by formation of lactate. The rapid rate at which injected pyruvate was cleared from the blood of these early cases suggests equally rapid conversion to glucose or lactate.

The markedly increased levels of activity of UDPG-dehydrogenase, isocitric dehydrogenase and glutathione reductase suggest an attempt on the part of the liver parenchyma to raise the general energy level of its cells by increasing oxidations in the major metabolic pathways. This in turn suggests a prior serious breakdown in the supply of energy to those cells.

Although the efficiency of bilirubin conjugation was lowered in about half of the early cases, particularly those of one to two days standing and in five out of seven of the advanced cases, there is no evidence to suggest any impairment of actual enzymic mechanisms concerned. The available evidence suggests rather an increase in the conjugating potential of this system (as judged by the increase in UDPG-dehyrogenase levels). The efficiency of this system may have been impaired by a lack of ATP, which in turn resulted from an energy failure in the hepatic cells.

If the markedly reduced tolerance to glucose which was observed in the early cases is due to extrahepatic failure of the utilization of glucose, e.g. in the muscles, then either or both of the following mechanisms may be concerned:

(*a*) Impairment in the extrahepatic membrane transport of glucose, or

(*b*) impairment in the phosphorylation of glucose by glucokinase, due once again to a deficiency of ATP. Whatever the mechanism is that is involved, the essential point to bear in mind is that this is a membrane transfer which is concerned and as such is an indication that interference with membrane transfers is not confined to the liver in geeldikkop but may involve other tissues in the body. An attempt will be made to demonstrate in the next two chapters that the erythrocytes and kidney cells are affected in a similar manner.

The marked increases in glutathione reductase that were observed in the livers of the early and advanced cases and the increased plasma levels of ascorbic acid in the same animals may have a more profound significance than forming a part of a general compensatory increase in vital metabolic factors consequent to the postulated energy failure. On the assumption that glucose metabolism is inhibited at the level of glyceraldehyde phosphate dehydrogenase (Brown, 1964), the idea is put forward that the increase in glutathione reductase, ascorbic acid and lactic acid may be due to the opening of a " by-pass " from glyceraldehyde-3-phosphate via methylglyoxal and lactic acid to pyruvate. The existence of this mechanism is yet

to be proved. On the other hand, the glucuronate (uronate or C6 oxidative) pathway from UDP-glucose via UDP-glucuronic acid, gulonate, xylulose, xylulose-5-phosphate and the pentose cycle to glucose-6-phosphate, if it exists in sheep liver could become important as an alternate pathway for energy supply (Kaneko, 1963). Ascorbate could form from l-gulonate and since the step from glucuronate to l-gulonate involves TPN as hydrogen carrier, ascorbate, glutathione and glutathione reductase would become necessary in larger amounts than usual.

The administration of hydrocortisone or cortisone depresses liver catalase activity considerably. It has been suggested that the high catalase activity of sheep liver is a result of very low levels of adrenal steroids in the plasma of this animal (Gallagher & Buttery, 1959). If this is the case then the high plasma levels of catalase seen in geeldikkop may be consequent to the severe adrenal hypofunction which is a prominent feature in the disease. This aspect will be discussed in one of the following chapters. On the other hand the marked increase in plasma catalase may reflect an attempt on the part of the tissues of the affected animals to deal with large amounts of hydrogen peroxide being formed as a result of the severe metabolic derangements present.

CHAPTER 6

The General Chemical Pathology and Biochemistry of Geeldikkop

C. *Erythrocyte Lesions in Geeldikkop and Glucose Metabolism in the Ovine Erythrocyte*

1. Introductory remarks
2. Erythrocyte fragility
3. Methaemoglobin reduction
4. Glyceraldehyde phosphate dehydrogenase activity
5. Glutathione and pyridine nucleotide levels
6. General discussion

1. *Introductory remarks*

One of the first observations that was made when the author and his colleagues started work on geeldikkop and enzootic icterus was that erythrocytes from cases of the latter syndrome were exceedingly fragile and in many instances ruptured even in physiological saline (Grosskopf, 1958). This work was soon confirmed and similar studies conducted on geeldikkop cases demonstrated the presence of markedly increased fragility of the erythrocytes in these animals as well (Brown, 1962, 1963, 1964). It had also been known for some time that methaemoglobincythaemia was frequently encountered in typical acute cases of enzootic icterus (Brown, 1963). Subsequent studies on the erythrocytes of cases of geeldikkop and enzootic icterus demonstrated the presence of marked disturbances of methaemoglobin reduction in cases of both diseases (Brown, 1962, 1963, 1964). It was then soon established that a

statistically significant difference, with regard to erythrocyte fragility and methaemoglobin reduction, existed between sheep born in areas where these syndromes were prevalent and those born and raised elsewhere. The methaemoglobin reductase systems of the erythrocytes of animals emanating largely from the Karoo appeared to be considerably less efficient than those of animals raised in the Transvaal. Furthermore the red cells of the former group of animals were markedly more fragile in 0·7 per cent saline than those of the Transvaal sheep (Wagner, 1964). The results of all these studies and of subsequent work form the subject of this chapter.

2. *Erythrocyte fragility*

The fragility of ovine erythrocytes was measured in 0·7 per cent saline since any red cells which rupture in this medium are almost certainly abnormal. The normal (80 per cent) range found for Transvaal animals is 2·5 to 31·0 per cent fragility in 0·7 per cent NaCl with an upper 10 per cent limit of 52·5 per cent. The corresponding figures for sheep emanating from the Karoo are 7·5 to 52·0 per cent and 72·5 per cent (Wagner, 1964). The results obtained from cases of all stages of geeldikkop and from the control animals are presented in Table 54. It is evident

TABLE 54.—*Percentage fragility in 0·7 per cent NaCl of erythrocytes from cases of all stages of geeldikkop and from control animals*

Sheep No. and details	% fragility	Sheep No. and details	% fragility
V3-22 Early case 1–2 days.....	7·9	V3-8 Advanced case 7–8 days.	36·4
V3-23 „ „	5·4	V3-12 „ „ „	3·0
V3-21 „ „	85·7	V3-17 „ „ „	33·0
V3-24 „ „	24·4	V3-9 „ „ „	48·6
V3-26 „ „	86·0	V3-2 „ „ „	26·3
V3-14 „ 2–3 days.....	80·0	V3-18 „ „ „	97·0
V3-13 „ „	84·7	V3-1 „ „ 8–10 days	75·0
F -5 „ „	21·8	V3-19 Recovered case.....	54·9
V3-15 „ „	55·3	V3-4 „ „	10·2
V3-27 „ „	9·9	V3-3 „ „	46·8
V3-20 „ „	86·6	F -3 „ „	33·7
F -2 „ „	42·7	F -4 „ „	34·7
V3-25 „ „	85·0	V3-33 Control.....	0·2
F -1 „ „	43·0	V3-31 „	2·5
V3-16 „ 3–5 days.....	54·4	V3-28 „	51·6
V3-10 „ „	47·8	V3-29 „	0·4
V3-11 „ „	10·0	V3-32 „	6·9
V3-6 „ „	61·0	V3-5 „	14·0
V3-7 „ „	84·0	V3-30 „	0·8

from these data that a very marked increase in red cell fragility is present from the moment the symptoms of geeldikkop appear. Most high values were found in the second and third days of illness although instances of extremely high fragility were still obvious up to ten days after the onset of symptoms. High values within the normal range are a feature of most of the recovered cases.

3. *Methaemoglobin reduction*

The methaemoglobin (MetHb) reduction test devised by Brewer, Tarlov & Alving (1960) for detecting cases of primaquine sensitivity in humans has been used by the author for assaying the activity of the ovine erythrocyte MetHb reductase system. The test is an *in vitro* method which actually measures glucose-6-phosphate dehydrogenase (G-6-PD) activity indirectly in the case of the human red cell. In the case of the ovine erythrocyte it is probable that it is actually an indirect measurement of glyceraldehyde phosphate dehydrogenase (GAPD) (see Section 4 below). The test depends upon the *in vitro* formation of MetHb by adding sodium nitrite to a red cell suspension in a glucose containing medium, and then measuring the amount of MetHb remaining unreduced at the end of a three-hour incubation period.

The human syndrome commonly known as " primaquine sensitivity " was described by Brewer *et al.* (1960), Carson, Flanagan, Ickes & Alving (1956), and others, as an hereditary G-6-PD deficiency. Affected individuals experience acute haemolytic episodes after the ingestion of certain drugs like primaquine and plant foods such as fava beans (*Vicia faba*, L. Papilionaceae). In individuals where the trait is fully expressed the erythrocyte lifespan is shortened even in the absence of drug administration Bruwer *et al.*, 1960). These authors cite 5 per cent or less of total haem pigments remaining as MetHb at the end of the test as being indicative that the patient does not suffer from G-6-PD deficiency. Values of 5 to 80 per cent MetHb remaining are regarded as an intermediate expression and values above 80 per cent as a full expression of the hereditary trait. Normal sheep erythrocytes do not reduce MetHb as efficiently as human erythrocytes do under the conditions of this test. The ability of the sheep red cell to reduce MetHb varies considerably from sheep to sheep. The normal (80 per cent) range has been established for Transvaal sheep as 19·5 to 49·5 per cent with a 10 per cent upper limit of 75 per cent. The corresponding values for sheep emanating from areas where geeldikkop and enzootic icterus are prevalent are 22 to 61·5 per cent and 75·0 per cent respectively (Wagner, 1964). A significant difference exists between the two sheep populations in this respect.

The results of the test in cases representing all stages of geeldikkop and in control animals are presented in Table 55. Isolated high values were found amongst the early cases of the disease, e.g. Sheep F-1, F-2, and V3-11. This is consistent with the symptomatology of the condition. Methaemoglobincythaemia is much more common in enzootic icterus than it is in geeldikkop. Abnormally high values for this test are consequently found more commonly in the former syndrome than in geeldikkop (see later). No correlation was found between the high values for this test in geeldikkop and the markedly increased red cell fragility described above.

In an earlier publication, some of the difficulties inherent in this test were mentioned (Brown, 1963) and the use of a figure which was called the " fragility index " was proposed as a screening test to detect, particularly, incipient cases of enzootic icterus. This figure was the product of the percentage of unreduced MetHb at the end of the test described above and the percentage fragility in 0·7 per cent NaCl. Normal values were accepted as being less than 1,000; values lying between 1,000 to 2,000 were regarded with suspicion and values above 2,000 were taken as indicative of incipient enzootic icterus. During the subsequent field investigations many of the early cases of geeldikkop were found to give values ranging

TABLE 55.—*Results of the MetHb reduction test in cases representing all stages of geeldikkop and in control animals*

Sheep No. and details	% MetHb unreduced	Sheep No. and details	% MetHb unreduced
V3-22 Early case 1–2 days.....	48·8	V3-8 Advanced case 7–8 days.	38·1
V3-23 „ „	52·4	V3-12 „ „ „	59·2
V3-21 „ „	52·7	V3-17 „ „ „	41·3
V3-24 „ „	48·0	V3-9 „ „ „	40·5
V3-26 „ „	49·0	V3-2 „ „ „	40·5
V3-14 „ 2–3 days.....	9·9	V3-18 „ „ „	4·8
V3-13 „ „	46·4	V3-1 „ „ 8–10 days	28·8
F5 „ „	34·5	V3-19 Recovered case.....	36·0
V3-15 „ „	9·1	V3-4 „ „	47·5
V3-27 „ „	33·5	V3-3 „ „	41·2
V3-20 „ „	42·0	F-3 „ „	61·4
F-2 „ „	70·8	F-4 „ „	44·6
V3-25 „ „	48·0	V3-33 Control.....	39·5
F-1 „ „	78·1	V3-31 „	47·4
V3-16 „ 3–5 days.....	25·1	V3-28 „	52·6
V3-10 „ „	36·5	V3-29 „	45·6
V3-11 „ „	74·6	V3-32 „	32·9
V3-6 „ „	46·1	V3-30 „	46·2
V3-7 „ „	59·0	V3-5 „	43·9

between 2,806 to 4,503; values in the upper part of this range were common and far above the figures previously found for enzootic icterus. The statistically established normal ranges for both fragility and MetHb reduction are so wide in the Merino sheep in South Africa that accurate interpretation of the " fragility index " as originally proposed is difficult and beset with more pitfalls than that of either figure alone.

4. *Glyceraldehyde-3-phosphate dehydrogenase* (*triose phosphate dehydrogenase, phosphoglyceraldehyde dehydrogenase*)

When methaemoglobin reduction in the ovine erythrocyte was first studied it was thought that the reductase may have been coupled with glucose-6-phosphate dehydrogenase as it is in the human and some other species (Brown, 1963). Further work soon convinced us that the levels of activity of glucose-6-phosphate were negligible in the ovine erythrocyte and that glycolysis in this cell proceeded mainly via the direct Embden-Meyerhoff pathway (Brown, 1964; Wagner & Brown, 1966b). This being the case, the DPN linked enzyme gyceraldehyde phosphate dehydrogenase would be responsible for the provision of hydrogen from glycolysis for the MetHb reductase system and in this role was of major importance in the studies.

The normal (80 per cent) level of this enzyme in ovine erythrocytes has been established as 425 to 730 units, the 10 per cent lower and upper limits being 400 and 900 units respectively (Wagner & Brown, 1966b). It was not possible to establish any statistical differences between Karoo and Transvaal sheep in this regard. Levels of the enzyme were studied in cases of all stages of geeldikkop and in control

animals during the last field investigations. The results are presented in Table 56. Some remarkably low levels of activity of this enzyme were found in the red cells of geeldikkop cases. Low levels of activity may be present from the onset of symptoms (e.g., as in Sheep V3-26) but become more frequent from the second day of illness onwards, and may persist into the recovery stage of the disease.

TABLE 56.—*Glyceraldehyde phosphate dehydrogenase levels in the erythrocytes of cases of the various stages of geeldikkop and of control animals*

Sheep No. and details	mcg/ml	Sheep No. and details	mcg/ml
V3-22 Early case 1–2 days.....	425	V3-12 Advanced case 7–8 days.	250
V3-23 „ „	610	V3-17 „ „ „	620
V3-21 „ „	300	V3-9 „ „ „	180
V3-24 „ „	650	V3-2 „ „ „	340
V3-26 „ „	200	V3-18 „ „ „	390
V3-14 „ 2–3 days.....	375	V3-1 „ „ 8–10 days	350
		V3-1A „ „ „	130
V3-13 „ „	325	V3-19 Recovered case.....	290
V3-15 „ „	250	V3-4 „ „	190
V3-27 „ „	425	V3-3 „ „	180
V3-20 „ „	180	V3-33 Controls.....	325
V3-25 „ „	300	V3-31 „	425
V3-16 „ 3–5 days.....	400	V3-28 „	425
V3-10 „ „	120	V3-29 „	300
V3-11 „ „	280	V3-32 „	500
V3-6 „ „	525	V3-5 „	530
V3-7 „ „	250	V3-30 „	375
V3-8 Advanced case 7–8 days..	475		

5. *Glutathione and total pyridine nucleotide levels*

The reaction catalyzed by glyceraldehyde phosphate dehydrogenase is particularly interesting because it couples phosphorylation to dehydrogenation, producing an ester with a high $-\triangle$ °F of hydrolysis. Coupling may be carried out by at least four different systems; in animal tissues the lactic dehydrogenase system is primarily concerned (Axelrod, 1960). It has been known for some time that the sulfhydryl groups of the mammalian enzyme had to be maintained in the fully reduced state for enzyme activity. The oxidized form can be readily changed to the reduced state by exposure to suitable SH reagents, e.g. glutathione and cysteine (Axelrod, 1960). The enzyme was the first dehydrogenase found to have a pyridinonucleotide (in this case, DPN) strongly attached to the protein. Two molecules of DPN are tightly bound to the enzyme. It has a multifunctional activity, but regardless of the nature of the reactions it catalyzes, DPN is an essential component. Glutathione is known to be bound to the enzyme as well (Axelrod, 1960).

In view of the marked reductions in the levels of activity of glyceraldehyde phosphate dehydrogenase in the erythrocytes of many cases of all stages of geeldikkop, the levels of glutathione and DPN present in these cells become of immediate importance. The results of assays of both are presented in Table 57. The levels

TABLE 57.—*Glutathione and total pyridine nucleotide levels in the erythrocytes of cases of the various stages of geeldikkop and of control animals*

Sheep No. and details	Glutathione mg%	Total pyridine nucleotides mg%
V3-22 Early case 1–2 days	10·5	2·29
V3-23 „ „	27·2	2·13
V3-21 „ „	7·0	1·80
V3-24 „ „	18·5	2·30
V3-26 „ „	7·0	1·71
V3-14 „ 2–3 days	8·8	2·00
V3-13 „ „	9·8	2·72
V3-15 „ „	13·0	2·47
V3-27 „ „	19·3	2·71
V3-20 „ „	14·1	1·90
V3-25 „ „	17·5	2·50
V3-16 „ 3–5 days	7·0	2·60
V3-10 „ „	11·9	2·66
V3-11 „ „	3·5	2·37
V3-6 „ „	15·7	3·97
V3-7 „ „	10·8	3·17
V3-8 Advanced case 7–8 days	3·7	2·17
V3-12 „ „ „	18·5	2·44
V3-17 „ „ „	9·7	2·70
V3-9 „ „ „	14·3	1·40
V3-2 „ „ „	22·9	3·44
V3-1 „ „ 8–10 days	16·3	3·77
1A „ „ „	17·2	5·30
V3-19 Recovered case	13·6	2·34
F-4 „ „	19·5	—
V3-4 „ „	35·4	3·70
F-3 „ „	23·0	—
V3-3 „ „	31·8	2·25
V3-33 Control	3·5	3·21
V3-31 „	17·5	2·51
V3-28 „	3·5	3·56
V3-29 „	10·5	3·02
V3-32 „	12·2	3·06
V3-5 „	17·5	3·57
V3-30 „	10·5	3·00

of glutathione found in the red cells of the control animals showed considerable variation, i.e. 3·5 to 17·5 mg per cent. The variations in the early and advanced cases are equally great. The two high values found in the recovered Sheep V3-4 and V3-3 are associated with low values of the dehydrogenase. In general the wide variations found here preclude the drawing of valid conclusions.

The values for red cell total pyridine nucleotides found in the geeldikkop cases are in general lower than those found for the red cells of the control group. The normal range (80 per cent) for these compounds in the erythrocytes of Karoo sheep has been established as 2·37 to 3·72 mg per cent with a lower 10 per cent limit of 2·25 mg per cent and an upper 10 per cent limit of 5·25 mg per cent. Most of the

low values were found in the erythrocytes of cases of one to five days standing. In general these low values are associated with low levels of activity of glyceraldehyde phosphate dehydrogenase.

6. *General discussion*

Evidence was presented in Chapter 3 for the existence of a haemolytic syndrome as part of the general geeldikkop disease picture. The very marked increase in red cell fragility which is present in many cases from the onset of clinical symptoms is further support for the existence of this condition. The marked differences which exist in this regard between animals raised in the areas where geeldikkop is prevalent and those born and raised elsewhere, lend some support to the contention that geeldikkop as such is merely an acute manifestation of a more profound condition which remains largely latent for most of the year. The author will endeavour to show in one of the following chapters that enzootic icterus represents another acute manifestation of the basic disease condition, in the typical cases of which the animal succumbs to an explosive haemolytic crisis before it can develop lesions of frank photosensitivity. It is apparent from the figures presented in Table 54 that many of the cases of geeldikkop are virtually on the brink of a haemolytic crisis and may remain so throughout the acute course of the disease. It is clear from earlier studies on the gross pathology and histopathology of the disease that cases of geeldikkop showing many of the features of enzootic icterus which can be attributed to the severe intravascular haemolysis typical of this disease, are by no means uncommon (Brown, *et al.*, 1960). Such features include general haemochromatosis (uncommon), " gunmetal " greyish-blue to even blackish-brown pigmentation of the kidneys (common, particularly in advanced cases), severe anaemia (common) and severe hypoplasia of the bonemarrow (common).

The biochemical studies on the erythrocytes point once more to extremely severe intracellular metabolic disturbances being present from the moment of onset of clinical symptoms. These disturbances are once more maximal during the first one to three days of illness. The severe lowering of glyceraldehyde phosphate dehydrogenase activity which might, from the evidence presented, be associated with a lowering of DPN levels, must have a profound effect on carbohydrate metabolism and thus on energy metabolism as a whole. This must in turn have a profound effect on the permeability and integrity of the red cell membrane. It is possible that cells in this state may become more permeable to ions like copper with further deleterious results.

It is apparent from the data presented that MetHb reduction in the sheep cannot be dependent on hydrogens from glycolysis alone. If this was so, methaemoglobincythaemia should be a far more common symptom than it actually is. The red cell, like the liver cell of the sheep, seems to be one that makes use of compounds other than glucose as its prime sources of energy. Leng & Annison (1962a) have demonstrated the glycolysis proceeds in sheep blood at 20 to 30 per cent of the rate observed in human blood. Glycolysis could be stimulated by addition of methylene blue to the system, and if this occurred then 80 to 100 per cent of the glycolysis was found to proceed via the pentose phosphate pathway. In the absence of methylene blue the pentose phosphate pathway accounted for only approximately 15 per cent of glycolysis. Sheep red cells were found to have a low permeability to glucose, which was not affected by insulin (Leng & Annison, 1962a). The sheep red cell is permeable to lactate which it can produce in considerable amounts. Tricarboxylic acid cycle activity was found to be too low to be measured (Leng & Annison, 1962a). Formate, added to washed sheep erythrocytes was oxidised at a low rate,

but the rate of oxidation was found to be considerably enhanced by the addition of methylene blue, glutathione or cysteine under aerobic conditions. Similar stimulation was also observed after the addition of a hydrogen peroxide generating system, e.g. glucose and glucose oxidase (Leng & Annison, 1962b).

Comparisons have been made of the levels in the erythrocytes of different species of some of the enzymes concerned in glycolysis. Glucose-6-phosphate dehydrogenase is virtually absent from the erythrocytes of sheep and goats, but is present in fair to large amounts in the red cells of other species (Brown, 1964; Wagner & Brown, 1966b). Typical data pertaining to some other metabolic enzymes in the red cells of sheep and other species are presented in Table 58. Aldolase levels are considerably lower in the sheep red cell than in those of other species; glyceraldehyde

TABLE 58.—*The levels of activity of some metabolic enzymes in the erythrocytes of the sheep compared with similar levels in the red cells of other species*

(Hex = hexokinase, PHI = phosphohexose isomerase, Ald = aldolase, GAP = glyceraldehyde phosphate dehydrogenase, G6P = glucose-6-phosphatase, LD = lactic dehydrogenase, KBD = β-ketobutyric dehydrogenase, GOT = glutamic oxalacetic transaminase, GPT = glutamic-pyruvic transaminase and ICD = isocitric dehydrogenase. The figures given are the ranges of activity found in the erythrocytes of different species. Units are as defined in the original procedures except unless stated otherwise in the footnote below. Figures in parentheses are mean values of activity found)

Species	Hex	PHI	Ald	GAP	G6P	LD	KBD	GOT	GPT	ICD
Sheep	0	480–670 (611)	45–108 (73)	725–920 (853)	0	4,800–6,700 (6,150)	3,700–7,000 (5,283)	0–174 (68)	122–420 (240)	0
Bovine	0–2·4 (1·0)	458–662 (554)	137–320 (202)	800–1,310 (998)	0–8·4 (4·7)	2,400–3,800 (4,267)	8,700–29,400 (3,183)	48–328 (182)	106–218 (125)	0–840 (223)
Horse	2·4–6·0 (4·1)	480–620 (561)	149–205 (177)	860–1,240 (1,026)	0·8–11·3 (5·3)	4,100–7,300 (5,250)	3,600–6,200 (5,217)	36–248 (134)	340–430 (370)	0
Dog	0–8·0 (4·7)	540–600 (563)	230–610 (435)	925–1,530 (1,121)	2·8–16·0 (8·4)	6,400–17,000 (10,683)	8,700–29,400 (14,967)	86–352 (179)	0–34 (11)	550–1,140 (813)

Note.—(*a*) Hexokinase, phosphohexose isomerase, aldolase, glucose-6-phosphatase and the two transaminases were determined on a 50 per cent haemolysate of red cells in distilled water. (*b*) In the case of canine red cells the 50 per cent haemolysate was diluted twice or five times for the determinaton of aldolase. (*c*) GOT and GPT were determined as for blood serum using 0·2 ml of 50 per cent haemolysate instead. Calculatinos were adjusted accordingly. (*d*) LD, KBD and ICD were determined on a 10 per cent haemolysate in distilled water. In the case of LD 0·1 ml of haemolysate was used. One unit of activity is defined as a decrease inoptical density of the test solution of 0·001/min/ml red blood cells. KBD was determined by the procedure of Elliot & Wilkinson (1961) using 0·1 ml of a 10 per cent haemolysate of red cells. In the case of ICD, the method used was that for blood serum, except that 0·25 ml of 10 per cent haemolysate was used instead and the calculations adjusted accordingly.

phosphate dehydrogenase activity is in general slightly lower, while glucose-6-phosphatase and hexokinase appear to be completely absent. The sheep erythrocyte on the other hand is very well endowed with phosphohexose isomerase, lactic acid dehydrogenase and glutamic pyruvic transaminase when compared with those of other species.

If the relative importance of fatty acid metabolism in the erythrocytes of various species can be judged by the levels of activity of β-hydroxy-acyl dehydrogenase then the figures given for ketobutyric dehydrogenase in Table 58 show that the sheep erythrocyte is no better off than its counterparts in other species. The absence of

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isocitric dehydrogenase and the low values of glutamic-oxalacetic transaminase activity in the sheep red cell indicate a negligible tricarboxylic acid cycle when compared with similar activities in the erythrocytes of other species. The ovine red cell on the other hand is well supplied with glutamic-pyruvic transaminase.

The absence of glucose-6-phosphate dehydrogenase, the low levels of aldolase and the generally high levels of phosphohexose isomerase indicate a possible link with the pentose phosphate pathway and the glucuronate pathway through fructose-6-phosphate and not through glucose-6-phosphate as suggested by Leng & Annison (1962a).

The presence of large amounts of lactic dehydrogenase and glutamic pyruvic transaminase in the ovine erythrocyte suggests that the reactions: alanine $\rightleftharpoons$ pyruvate $\rightleftharpoons$ lactic acid are of considerable importance in these cells. Leng & Annison (1962a) have established that ovine erythrocytes are able to form lactic acid in fair amounts. The data presented here suggest the ability to metabolize it efficiently, given the presence of active systems which will clear pyruvate (GPT activity may be important here) and re-oxidize DPN satisfactorily. Herein may lie the answer to many of the problems in connection with the pathogenesis of geeldikkop e.g. the accumulation of so much lactate in the blood of affected animals during the first two days of illness.

All things considered the ovine red cell, because of its peculiar and rather inefficient glycolytic mechanism, starts off with a distinct disadvantage when compared with the erythrocytes of other species which can make better use of glucose as a source of energy. Should any interference in its glycolytic mechanism take place (e.g. lowered activity of glyceraldehyde phosphate dehydrogenase) and should its source of energy in the form of fatty acids be diminished in any way, then the results may be disastrous as they apparently are in geeldikkop and enzootic icterus.

The apparently good correlation which exists between low glyceraldehyde phosphate dehydrogenase and total pyridine nucleotide levels is interesting and important. The pyridine nucleotides were estimated chemically by means of a fluorimetric method which depends on the formation of a fluorescent compound between nicotinamide and acetone in strongly alkaline medium (Levitas, Robinson, Rosen, Huff & Perlzweig, 1947). The method is claimed to be specific for N-methyl-nicotinamide, DPN and TPN. The actual amount of these compounds present in red cells and not their hydrogen-carrying ability was thus determined. If DPN is as firmly bound to ovine erythrocytic glyceraldehyde phosphate dehydrogenase, as it is in other preparations of the enzyme studied (Axelrod, 1960) then the low levels of pyridine nucleotides found in the red cells of geeldikkop cases may represent a lowered formation of the dehydrogenase in these cells and not merely some type of inhibition or suppression of its activity.

CHAPTER 7

The General Chemical Pathology and Biochemistry of Geeldikkop

D. Kidney Function in Geeldikkop

1. Introductory remarks
2. Blood levels of non-protein nitrogenous compounds
3. Blood levels of inorganic phosphate and magnesium
4. Urine examinations in geeldikkop cases
5. General discussion

1. *Introductory remarks*

It was demonstrated in Chapter 4 that bilirubinuria and bileaciduria are not features of the chemical pathology of geeldikkop in spite of extremely high plasma levels of bilirubin glucuronides and bile acids. Similarly phylloerythrin does not appear to be excreted in the urine of early cases in spite of high plasma levels and hypercupriuria does not occur in spite of frank hypercupraemia. It seems thus that a block to the secondary renal excretion of many of the regurgitated biliary constituents may co-exist in geeldikkop with the hepatic block already discussed. The block in hepato-biliary secretion or excretion is thought to be a biochemical lesion involving either the selective permeability of the liver cell membranes or the systems supplying energy for active transport in these membranes. In Chapter 5 it was indicated that interference in membrane transfers might be widespread in the body tissues of the affected animals, involving e.g. muscle, and in the previous chapter it was indicated that the erythrocytes were by no means excluded from the severe metabolic disturbances which make up the disease picture of geeldikkop. This chapter will be devoted to an attempt to demonstrate that the kidneys are affected in a similar way to the liver and possibly other tissues as well, in the early stages of the disease.

2. *Blood levels of non-protein nitrogenous compounds*

Urea, creatinine, uric acid and total amino acids were determined in the blood of cases representing all stages of the disease. The results are presented in Tables 59, 60 and 61.

TABLE 59.—*Blood urea nitrogen and creatinine levels in the prodromal cases and severe Vosburg cases*

Sheep No. and details	Urea nitrogen mg%	Creatinine mg%
VB-P Prodromal case	18·4	3·0
VB-Q „ „	23·9	2·6
VB-R „ „	20·2	3·2
VB-S „ „	23·9	3·1
VB-T „ „	20·2	2·8
VB-U „ „	18·4	2·6
VB-V „ „	20·2	2·8
VB-W „ „	12·9	2·2
VB-X „ „	20·1	3·0
VB-H Early case 1–2 days	82·8	9·3
VB-I „ „	69·9	8·1
VB-A „ 3 days	66·2	5·6
VB-E „ „	40·5	5·2
VB-F „ „	64·4	6·2
VB-C „ „	55·2	5·9
VB-B „ „	68·1	4·4
VB-G „ 4 days	92·0	7·5
VB-J „ 4–5 days	46·0	5·0
VB-K „ 7 days	33·1	4·5
VB-D Advanced case 7–8 days	20·2	3·8
VB-L „ „ „	88·3	7·9
VB-M „ „ „	104·9	11·1
VB-N „ „ „	160·1	12·8
VB-Z1 „ „ „	29·4	4·0
VB-O „ „ „	66·2	8·5
VB-Y „ „ „	35·0	4·0

TABLE 60.—*Blood urea nitrogen (UN), uric acid (UA), creatinine (CN) and amino acid nitrogen (AAN) in early typical cases of geeldikkop*

Sheep No. and details	UN mg%	UA mg%	CN mg%	AAN mg%
V1-25 Early case 1–2 days	44·2	0·8	2·3	3·1
V1-3 „ „	79·1	8·6	1·1	2·5
V1-2 „ „	68·1	7·0	0·8	3·1
V1-1 „ „	130·6	6·0	1·4	4·2
V1-23 „ 2–3 days	29·4	2·8	1·6	3·1
V1-22 „ „	27·6	1·0	1·5	3·1
V1-24 „ „	33·1	2·4	2·0	3·2
V1-17 „ „	27·6	1·4	2·2	3·4
V1-15 „ „	27·6	2·6	1·8	3·5
F-5 „ „	25·8	8·9	1·5	7·6
V1-13 „ „	60·7	5·6	1·1	3·7
F-2 „ „	46·0	3·8	2·5	7·1
F-1 „ „	99·4	7·3	3·5	9·0
V1-26 „ 3–5 days	46·0	2·0	2·4	3·2
V1-11 „ 5–7 days	36·8	7·0	0·6	3·2
V1-18 „ „	38·6	1·4	2·6	3·7

TABLE 61.—*Blood urea nitrogen (UN), uric acid (UA), creatinine (CN) and amino acid nitrogen (AAN) in advanced and recovered cases of geeldikkop and control animals*

Sheep No. and details	UN mg%	UA mg%	CN mg%	AAN mg%
V1-21 Advanced case 7–8 days.....	27·6	2·2	1·6	3·2
V1-5 „ „ „	46·0	7·6	0·3	2·4
V1-4 „ „ „	46·0	3·4	1·6	3·8
V1-14 „ „ „	46·0	4·4	0·6	4·0
V1-7 „ „ „	239·0	8·0	13·1	4·6
V1-6 „ „ „	189·5	9·4	10·0	6·0
V1-9 „ „ 8–10 days.....	27·6	4·0	0·1	2·9
V1-10 „ „ „	73·6	11·8	3·4	2·9
V1-8 „ „ „	27·6	4·2	0·4	3·3
V1-12 „ „ 10–14 days.....	29·4	5·2	0·1	2·2
V1-20 „ „ „	29·4	2·4	1·4	3·1
V1-16 „ „ „	29·4	0·8	2·2	4·1
V1-19 Recovered case.....	31·3	2·8	1·4	3·3
F-4 „ „	35·0	5·9	1·4	6·3
F-3 „ „	42·3	9·6	1·5	7·5
VB-Z „ „	14·7	1·6	3·2	6·3
V1-7054 Control.....	16·6	1·6	0·7	4·3
V1-7055 „	19·3	1·5	0·6	4·6
V1-7056 „	16·6	1·2	0·6	4·3
V1-7057 „	21·0	1·4	0·6	4·6
V1-7059 „	28·5	2·4	0·8	4·2
V1-7060 „	22·1	1·8	0·6	4·2
V1-7061 „	23·9	0·6	0·7	4·1
V1-7062 „	20·2	1·4	0·4	6·8
V1-7064 „	23·9	1·4	0·6	6·3
V1-7065 „	23·9	1·0	0·7	6·7
V1-7066 „	24·8	1·8	1·4	6·4
V1-7067 „	23·9	2·0	1·8	4·0
V1-7068 „	23·9	1·0	1·4	6·2
V1-7069 „	22·4	0·9	1·6	6·2
V1-7070 „	20·2	1·1	1·4	6·4

Although the normal ranges for these plasma components in Karoo sheep have not been established statistically by the methods used, the reader may accept the following as being these ranges for the purposes of this discussion:

Urea nitrogen..... 7 to 25 mg per cent.
Creatinine..... 0·5 to 2·7 mg per cent.
Uric acid..... 0·5 to 2·0 mg per cent.
Amino acid nitrogen..... 5·5 to 8·5 mg per cent.

The figures are based on earlier work at Onderstepoort by Graf (1933) and Hamersma (1934).

It will be seen from Table 59 that no disturbances of note as regards blood urea and creatinine are present in the prodromal cases. A severe uraemia and hypercreatininaemia were present from the moment of onset of symptoms in the severe Vosburg cases. High levels of both blood constituents were found in at least half the advanced cases of this group.

The same trends are obvious in the more typical early cases (Table 60) as far as blood urea and uric acid were concerned. Creatinine levels were normal in this group of animals and amino acid levels generally below normal.

Severe uraemia, hypercreatininaemia and hyperuricacidaemia are prominent features in a number of advanced cases (Table 61) but blood amino acid levels were again found to be on the low side of the normal range.

In three out of the four recovered cases blood urea nitrogen levels were found to be still mildly elevated, as were uric acid levels. Creatinine values were within normal limits in all the recovered cases and total blood amino acids within the normal range in three of these cases.

Values for the four blood constituents were generally well within the normal limits cited above in the case of the control animals, although the figures for total amino acids were found to be slightly on the low side in half these animals (Table 61).

3. *Plasma inorganic phosphate and magnesium levels*

The values found for these blood constituents in the plasma of control animals are given in Table 62, while those for cases of the different stages of geeldikkop are presented in Table 63. The normal ranges for these constituents of sheep plasma

TABLE 62.—*Plasma inorganic phosphate and magnesium levels in control animals*

Sheep No.	Phosphate mg%	Magnesium mg%
V1-7054	5·47	2·4
V1-7055	6·32	1·6
V1-7056	7·37	1·6
V1-7057	6·32	3·0
V1-7059	3·58	3·1
V1-7060	5·47	3·1
V1-7061	4·20	2·8
V1-7062	7·02	2·4
V1-7064	5·38	3·1
V1-7065	4·07	1·2
V1-7066	6·91	1·6
V1-7067	4·51	1·2
V1-7068	6·31	2·6
V1-7069	5·61	2·4
V1-7070	6·67	2·0
V1-7071	5·61	2·8
F-K2	5·57	1·7

have not been statistically established for the methods in use, but for practical purposes they may be accepted as being:—

Total inorganic phosphate..... 3 to 9 mg per cent.
Magnesium..... 1·5 to 3·5 mg per cent.

These ranges are also based on the earlier studies by Graf (1933) and Hamersma (1934). The figures given for these blood constituents in Table 62 for the control animals are well within these limits.

TABLE 63.—*Plasma inorganic phosphate and magnesium levels in geeldikkop cases*

Sheep No. and details	Phosphate mg%	Magnesium mg%
V1-25 Early case 1–2 days	8·70	4·8
V1-3 „ „	3·74	4·0
V1-1 „ „	7·02	3·3
V1-23 „ 2–3 days	4·21	4·4
V1-22 „ „	7·02	5·2
V1-24 „ „	5·61	4·8
V1-17 „ „	5·97	6·0
V1-15 „ „	7·90	6·8
F -5 „ „	8·94	2·5
V1-13 „ „	8·42	5·6
F-1 „	8·32	4·0
V1-26 „ 3–5 days	5·61	5·2
V1-11 „ 5–7 days	9·62	6·4
V1-18 „ „	7·02	8·0
V1-21 Advanced case 7–8 days	7·02	4·0
V1-5 „ „ „	5·97	4·4
V1-4 „ „ „	6·32	6·0
V1-14 „ „ „	5·19	4·4
V1-7 „ „ „	9·10	8·8
V1-6 „ „ „	14·73	2·8
V1-9 „ „ 8–10 days	7·02	4·8
V1-10 „ „ „	6·32	7·6
V1-8 „ „ „	11·23	4·8
V1-12 „ „ 10–14 days	7·02	6·0
V1-20 „ „ „	5·47	5·2
V1-16 „ „ „	11·93	5·2
V1-19 Recovered case	4·63	5·6
F-4 „	10·20	4·03
F-3 „	8·94	3·91

Hypermagnesaemia is a common feature in early and advanced geeldikkop (Table 63). Plasma inorganic phosphate values were found to be generally on the high side of the normal range during the first week of illness. Hyperphosphataemia was seen in some of the seriously ill advanced cases, and in one recovered case.

4. *Urine examinations in geeldikkop cases*

Whenever possible urine was collected from the various cases studied and subjected to the usual routine laboratory examination. This was easily achieved in wethers, using the collecting bottle described earlier (Brown, 1959b). Samples were collected from ewes generally when these animals urinated while being handled first thing in the morning. Bladder specimens were taken at autopsy from the cases listed as *in extremis* in Appendix I. The examination of urine specimens for bile pigments, bile acid salts and porphyrins has already been described in Chapter 4. It remains now to discuss only a few other abnormal constituents or phenomena which are of immediate interest.

Urinary specific gravity was generally low (in the order of 1·010) in the mild early cases. The pH of these urines ranged from about 7·5 to 8·5 and traces of albumin were detected in the urine of a few individuals only. The same mild abnormalities were noted in the urine of the more severe type of early cases and the mild advanced cases.

The pH of the urine of the severe advanced cases varied from animal to animal in being acid (pH 4·0 to 7·0) in some and very alkaline in others (pH 8·5 to 9·0). Specific gravity was generally low, 1·010 to 1·025, the urine samples were generally pale limpid yellow and contained in most cases traces of albumin. A severe albuminuria was seen in two cases only (Sheep V1-6A and VBO). A mild glycosuria was seen in Sheep V1-6A only.

A mild to severe albuminuria was seen in all the cases *in extremis*. The urine of these animals was generally very acid (pH 3·0 to 6·0) and specific gravities ranged from 1·015 to 1·045. Frank glycosuria was seen in a few of these cases, the presence of glucose being confirmed chromatographically (King & Wootton, 1956).

Mild ketonuria was observed in some of the advanced cases, particularly in fat animals.

The urine of early and advanced cases of geeldikkop is typically light in colour, of low S.G. and generally, virtually bile pigment and bile salt free. In severely affected animals the pH abnormalities described reflect the severe electrolyte imbalances which will be discussed in the following chapter. Albuminuria is generally negligible and when severe was always a terminal change. Glycosuria is a well-known terminal phenomenon in many fatal ovine diseases and has thus no particular significance in these cases. Apart from the coproporphyrinuria seen in cases of one to three days standing the urine of geeldikkop cases gives a very poor indication of the severe metabolic disturbances taking place in the animal.

Mention was made in Chapter 4 of the oliguria present in the early cases of geeldikkop. This is often severe in the first three days of illness and is also seen in many of the advanced cases, particularly those *in extremis*.

5. *General discussion*

No evidence of impairment of renal function was found in the prodromal cases. It is evident from the data presented, however, that extremely severe disturbances of kidney function are present from the moment the symptoms of geeldikkop first appear. These disturbances which were most pronounced in the severe Vosburg cases, but which are also present in many of the typical early cases, appear to take the form of a severe uraemic syndrome. This includes oliguria and markedly increased blood levels of urea, creatinine, uric acid, inorganic phosphate and magnesium. The same changes were observed in a number of the advanced cases, particularly the critically ill ones. It is also evident from the data presented for the recovered cases that in many animals disturbances of kidney function may persist into this stage of the disease.

Urea, the most abundant solute in urine, is freely diffusable across all cell membranes and is completely filtered at the glomerulus. A variable proportion then diffuses back through the tubule cells. The concentration of urea in the blood varies with the rate of protein catabolism, the rate of urine excretion and the efficiency of the kidneys as measured by glomerular filtration. High blood levels are found in, amongst other conditions, excessive protein catabolism, dehydration and advanced renal insufficiency particularly where back diffusion is aggravated by tubular degeneration. Blood urea levels rise in early nephritis to about 30 to 40 mg per cent but are very high in terminal chronic nephritis. Low figures are usually encountered in nephrosis (Bodansky & Bodansky, 1957; Grollman, 1957, 1963; Hawk, Oser & Summerson, 1954; King & Wootton, 1956; Milne, 1959).

Creatinine is the least variable nitrogenous constituent of blood. Exogenous creatinine is excreted in man by filtration supplemented by secretion, a most efficient combination. It is little affected by diet and activity and its plasma levels increase only slowly when kidney function is impaired. It is claimed that the clearance of endogenous creatinine is a measure of filtration in man. In early nephritis plasma values are encountered of 2 to 4 mg per cent and in chronic nephritis with uraemia, blood levels may rise to as high as 35 mg per cent. Since creatinine is more readily excreted by the kidney than urea or uric acid, an increase of plasma creatinine over 5 mg per cent is evidence of marked renal function impairment and indicates an unfavourable prognosis in man. When such a rise in creatinine occurs less than 25 per cent of the kidney tissue may still be functional (Bentinck-Smith, 1963; Hawk, Oser & Summerson, 1954; King & Wootton, 1956; Milne, 1959).

Uric acid is excreted by filtration and active (selective) reabsorption from the kidney tubules. Blood levels of this compound are more sensitive than those of creatinine to variations of diet, and it is also excreted less efficiently than creatinine. Its concentration in the plasma rises more rapidly than that of the latter compound when renal function is impaired, values of up to 10 mg per cent being seen in early nephritis, and up to 25 mg per cent in advanced nephritis. Blood levels can, however, be markedly raised during extensive tissue destruction (Bywaters & Glynn, 1959; Grollman, 1957, 1963; Hawk, Oser & Summerson, 1954; King & Wootton, 1956).

The majority of the plasma amino acids seem to be filtered through the glomeruli and reabsorbed to various degrees from the renal tubules. Low plasma levels are encountered in a number of infectious diseases, the nephrotic syndrome and some reactions to injury (Grollman, 1957, 1963).

Phosphate is reabsorbed in a relatively specific manner and has a definite maximal reabsorption rate. Hyperphosphataemia is seen in severe nephritis and may bear some relation to the acidosis found in cases of this nature. It develops progressively with hypocalcaemia as renal failure advances and is associated with a decreased glomerular filtration rate. In severe renal insufficiency phosphate excretion may almost cease entirely (Bland, 1956; Bodansky & Bodansky, 1957; Bentinck-Smith, 1963; Hawk, Oser & Summerson, 1954; King & Wootton, 1956; Robinson, Murdaugh & Peschel, 1959; Simeson, 1963; Walser & Mudge, 1960).

Hypermagnesaemia is most frequently seen in renal insufficiency, particularly during the oliguric or anuric phases of this condition, and during extensive dehydration. It is seen in both acute and chronic renal failure and its appearance is closely related to the degree of reduction of the filtration rate. The ionized fraction of the plasma magnesium is most generally increased in uraemic conditions. High plasma levels of the element follow adrenalectomy and are seen in Addison's disease, while in severe catabolic illness there occurs a release of intracellular magnesium that is normally combined with cell protein (Bland, 1956; Robinson, Murdaugh & Peschel, 1959; Wacker & Vallee, 1964).

While the severe photosensitization in geeldikkop and consequent extensive skin damage undoubtedly contribute towards the elevation of the levels of uric acid, particularly and to a lesser extent those of urea and magnesium in these animals, such tissue destruction is not widespread enough to induce the severe uraemic state seen in some of the early cases. The extremely high levels to which urea nitrogen and creatinine may rise in these early Vosburg cases suggest a failure in renal filtration and secretion from the moment the symptoms of geeldikkop appear. The renal histopathology of geeldikkop is thus of considerable importance in explaining this aspect of the disease.

The following is a brief description of the renal pathology of the cases studied during the earlier investigations, i.e. all sheep prefixed VB- and V1- (Brown *et al.*, 1960). The kidneys of the early and advanced Vosburg cases appeared deeply pigmented at autopsy; their cortices were generally dark-brown in colour. On microscopic examination nephrotic changes were found to be present particularly in the advanced cases. Such changes included dilatation of the tubules, the tubular cells showing fatty infiltration, hyaline degeneration and bile pigmentation. Bile pigment could be demonstrated in both the convoluted and straight parts of the tubules but the pigmentation tended to be confined to focal groups of tubules. The tubular cells concerned appeared swollen and were either packed with pigment granules or showed varying degrees of fatty infiltration of the cytoplasm together with the presence of hyaline droplets and pigment granules. In some cases desquamation of the affected epithelial cells had taken place into the tubular lumens which also contained bile pigment. The long-standing cases in this group of animals showed severe gun-metal greyish-blue pigmentation of the kidney cortices. The same microscopic picture as described above was seen in these instances.

The kidneys of the more typical early cases, i.e. sheep designated V1-, were found at autopsy to be slightly enlarged and pigmented with bilirubin. They revealed on microscopic examination mild to moderate nephrotic changes involving especially the proximal convoluted tubules which also contained small granules of bile pigment and albuminous material in their lumens.

The advanced V1- cases showed essentially the same changes as their early counterparts. The tubular cells of some individual animals contained some iron-containing pigment as well as bile pigment. Some of these cases, particularly those *in extremis* (e.g. Sheep V1-6, V1-7 and V1-14) showed pathological features of both geeldikkop and enzootic icterus. Their kidneys were found at autopsy to be extremely enlarged, with dark gun-metal grey cortices. Numerous small pin-point size reddish areas were scattered throughout the cortical tissue. These kidneys were found on microscopic examination to show moderate to severe nephrotic changes, some of their tubules being distorted in appearance. The epithelial cells of such tubules showed hyaline degeneration or fatty changes with much granular debris present in the lumens of the tubules themselves. Granular iron-free pigment was present both in and outside the tubular cells. Some of the capsules of Bowman contained pigment casts and their glomeruli appeared atrophic. In quite a few of the tubule cells a fair amount of iron-containing pigment was present.

The pathology of the kidneys of the more recent cases studied, i.e. the sheep designated V3- and F-, differed little from that already mentioned. Pigmentation of the kidneys was a pronounced macroscopic finding in most of the early cases. In general the kidneys appeared yellowish-brown to dirty greyish-brown in colour and were normal in size. In the more severely ill early cases the kidneys were generally enlarged and often light to dark greenish-brown in colour. In many instances the structure of the kidney cortices, particularly, appeared indistinct. The kidneys of a few of these cases appeared quite normal. On microscopic examination mild early nephrotic changes were apparent in the kidneys of nine out of nineteen of these cases studied. In some of these cases and a further four, iron-free pigmentation of the tubule cells was prominent. Iron-containing pigment was seen in the tubule cells of two cases only and in five of those showing nephrotic changes some albuminous material was present in the degenerating tubules. No obvious changes were seen in the kidneys of six of these nineteen cases.

The pathology of eight advanced cases was studied during the more recent investigations. No changes were detected by macroscopic or microscopic examination in the kidneys of two of these cases, Sheep V3-8 and V3-9. The macroscopic appearance of the kidneys of five of the remaining six animals was similar to that of the mild early cases just described. Mild nephrotic changes were visible on microscopic examination in the kidneys of three of these animals. Such changes included the presence of fat droplets in a few of the epithelial cells of some tubules, pycnotic nuclei in the cells of others and mild cloudy swelling of the cells of some tubules. Mild to moderate accumulations of iron-containing pigment were observed in the tubule cells of three of these five cases. The remaining animal in this group, Sheep V3-1A was severely icteric and gravely ill (see Appendix 1). The kidneys of this animal were light grey-brown in colour with numerous small round white areas and infarcts visible in the cortex. Microscopic examination established the presence of a disseminated focal purulent nephritis. Coccoid bacteria were present in the lesions. Severe iron-pigmentation of the tubule cells was also present. The attention of the reader was drawn in Chapter 3 to the presence of a bacteriaemia in many severely affected geeldikkop cases. The purulent nephritis in this case could have followed such a condition.

Some very mild nephrotic changes and iron-pigmentation were still visible in the kidneys of the recovered Sheep F3, F4, V3-3, V3-4 and V3-19. The kidneys of the control Sheep V3-28 and V3-33 were found to be normal in all respects.

The lesions seen in the kidneys of the typical early cases (i.e. those sheep designated V1-, V3- and F-1) are characteristically very mild and bear no relation to the nitrogen retention indicated in Table 60. In quite a few instances no obvious changes, apart from pigmentation, were present at all. Such degenerative changes as there are are often focal in distribution. Bile pigmentation of these kidneys is characteristically severe; in other words, bilirubin conjugates accumulate in the tubule cells of these kidneys. Why then is there no excretion of these pigments which are freely water soluble over a wide pH range? The lesions seen in the typical advanced cases are again very mild apart from severe pigmentation and are once more not reconcilable with the severe nitrogen retention seen in some of these cases.

The severest changes were seen amongst this group of animals in the few cases *in extremis* which showed pathological features of both geeldikkop and enzootic icterus, e.g. Sheep V1-6 and V1-7. These two cases were also the most severely uraemic in this group.

The Vosburg cases once more illustrate the severity which the lesions in geeldikkop can attain. Even though the nephrotic changes in the early cases of this group of animals were severer than in the cases just mentioned, they were not severe enough to induce the uraemia and hypercreatinaemia seen in these cases. It is only in the advanced cases of this group that there is some good correlation between the histopathology and chemical pathology.

It is apparent from this discussion that although the early stages of geeldikkop are distinguished by very mild renal histopathological changes, a severe block to the excretion of many of the substances normally appearing in urine may exist from the moment the first clinical symptoms appear. It is difficult to believe from the clinical, pathological and chemical pathological evidence presented that such a block involves the glomerular filtration mechanism. It is more likely to involve a breakdown in the mechanisms of secretion of substances like creatinine and reabsorption of substances like urea, uric acid and phosphate. It is known for instance

that the increased blood urate levels in gout may be due to increased tubular reabsorption of uric acid (Bywaters & Glynn, 1959). It has also been known for a long time that uricosuric agents like caronamide and probenecid increase the urinary excretion of uric acid and lower its blood levels by inhibiting tubular reabsorption.

It is not known how bilirubin glucuronides are excreted by the sheep kidney. It is believed that conjugated bilirubin accumulates in the tubule cells of human nephrons during obstructive jaundice by filtration through the glomerulus into the tubular lumen, concentration and reabsorption into the tubule cells (Popper & Schaffner, 1957). It is very likely that it accumulates in the tubule cells of the sheep in the same way except that in this instance there is probably an unchecked reabsorption due to a breakdown in the selective permeability of the tubule cell membrane. This would probably also apply in the case of substances like urea, uric acid and phosphate in geeldikkop, the breakdown in selective permeability being due once more to a failure in the local energy supply.

It is also evident from the data presented in this chapter that the nephrotic changes in the kidneys during geeldikkop may be progressive particularly in severely affected animals. In cases of obstructive jaundice in humans the epithelium of Bowman's capsule and of the proximal convoluted tubules contains bile pigment granules as already mentioned, and pigment casts appear in the tubular lumen, particularly in the lower nephron. This is followed by degenerative and even necrotizing changes of the pigmented epithelial cells, especially near casts. Vacuoles and hyaline droplets are frequently seen in the epithelial cells and evidence of regeneration is noted. The basement membrane is intact, and involvement of glomeruli is not part of the process (Popper & Schaffner, 1957; Sherlock, 1958). This impressive morphological picture has been called " cholaemic nephrosis " or " biliary nephrosis " and is apparently a direct effect of the pigment reabsorbed into the tubule cells. Biliary nephrosis is conspicuous in cholestasis of any type. It is not found in haemolytic jaundice in which bilirubinuria does not occur and is not severe in fulminant viral hepatitis in which the urinary bilirubin excretion is low (Popper & Schaffner, 1957). Biliary nephrosis is however the renal lesion best correlated with the degree and duration of human obstructive jaundice.

Another renal lesion seen in human liver disease is acute nephrosis which is often accompanied by azotaemia and oliguria. Features of acute nephrosis may accompany the severe biliary nephrosis of obstructive jaundice. In such cases elevations of the blood urea are frequent although glomerular filtration is not reduced. The creatinine level increases but not necessarily to the same degree as that of urea. In the majority of cases the elevation of blood urea is due to an abnormal reabsorption of urea through the damaged tubules (Popper & Schaffner, 1957).

It is possible that the progressive nephrosis seen in severe geeldikkop cases is essentially similar to the biliary nephrosis and acute nephrosis of human hepatic disease. The presence of demonstrable amounts of iron-containing pigment in the tubule cells of geeldikkop cases is yet another indication of the operation of a haemolytic syndrome in this disease. It is likely that the nephrosis could be aggravated by this low-grade intravascular haemolysis. Haemoglobinuric renal failure is associated in humans with various conditions which predispose to renal ischaemia, e.g. surgical shock, anaemia, incompatible blood transfusions and black-water fever. If haemolysis is accompanied by a fall in renal blood flow or renal vasoconstriction then severe renal damage may be produced by a quantity of free haemoglobin which would be innocuous in the normal subject (Milne, 1959). Frank haemoglobinuria is

not a feature of any stage of geeldikkop, as far as is known. It is a prominent symptom, however, in enzootic icterus and may be of considerable importance in the pathogenesis of the renal lesions in this syndrome.

The very low plasma amino acid levels encountered in the early and advanced cases of geeldikkop may have a special significance of their own. Hyperaminoaciduria is not a feature of geeldikkop as far as is known. There is thus no reason to suppose that excessive urinary loss is responsible for lowering the plasma amino acid level. Geeldikkop is a disease in which a very severe gastro-intestinal stasis is present from possibly before the onset of the typical clinical symptoms (Brown, 1966a). This being the case, the absorption of volatile fatty acids from the rumens of affected animals must be minimal. Since carbohydrate metabolism is seriously disturbed during the initial stages of the disease, the animal must obtain energy for its life processes by increased lipolysis and gluconeogenesis, the latter from protein sources. If there was efficient gluconeogenesis from protein sources one would expect slightly raised or at the best normal amino acid levels. The low levels actually found might indicate that extrahepatic amino acid release for gluconeogenesis is not keeping pace with intrahepatic use of these compounds. This particular aspect will be mentioned again in the following chapter.

CHAPTER 8

The General Chemical Pathology and Biochemistry of Geeldikkop

E. Adrenal Function in Geeldikkop

1. Introductory remarks
2. Absolute eosinophile counts
3. Plasma electrolytes and water balance
4. General discussion

1. *Introductory remarks*

The role played by various severe non-specific stress conditions in precipitating attacks of geeldikkop was recognized early in the field work on these diseases (Brown, 1959c, 1962, 1963; Brown *et al.*, 1960). A considerable amount of work has been devoted since then towards defining more closely the nature of these non-specific stress conditions and studying their effects on sheep in general and Karoo sheep in particular (Brown, 1962, 1963, 1964, 1966a, 1966b; Brown & de Boom, 1966). One of the concluding chapters of this thesis is devoted to a discussion of this aspect of the pathogenesis of these diseases. It is, however, necessary to note a few pertinent points in this regard at this stage. Enzootic icterus, which is the more severe of the two syndromes, can be readily precipitated in animals which are candidates for acute attacks by relatively mild forms of stress, e.g. digestive disturbances following changes of diet, movement of the animals concerned over long distances by road or rail, inoculation with live vaccines and dosing with many of the common vermicidal preparations (Brown & de Boom, 1966). Many of the cases described later in this thesis were produced merely by transporting the sheep over a distance of 20 to 50

miles by lorry from their farms of origin to the laboratory at Fraserburg. Geeldikkop on the other hand, a comparatively mild condition by contrast, requires considerably severer forms of non-specific stress to precipitate acute attacks of the disease. These appear to include a severe gastro-intestinal stasis induced by the saponins present in the rapidly growing annual plant, *T. terrestris* L. and extreme changes in environmental temperature, gastro-intestinal disturbances following sudden desiccation or wilting of the natural grazing and possibly infectious diseases (Brown, 1959a, 1962, 1963, 1966a). It is highly likely that an infectious agent is most important in this regard (Brown, 1966a, 1966b).

The reader is reminded of the following important points which have emerged from the discussion so far:

(*a*) the severe haematological disturbances seen in the prodromal cases, e.g leukopaenia, thrombocytopaenia and anaemia, and the severe hypoglycaemia encountered in these cases;

(*b*) when the symptoms of geeldikkop first appear they do so accompanied by many biochemical disturbances which are either maximal from the onset or reach their peak within the first three days of illness. The most important of these disturbances to bear in mind for the purposes of the present discussion are the hypergammaglobulinaemia, the marked elevations of plasma GOT, LD, PHI and aldolase activity levels in the absence of obvious hepatic pathology, the high levels of plasma catalase activity, the low levels of plasma amino acids and the explanations offered in connection with the latter two phenomena; and

(*c*) the lymphocytopaenia and neutrophilia which are part of the haematology of the latter stages of the disease.

Adrenal function was assayed in cases representing all stages of the disease by careful consideration of the results from the following tests or determinations: the absolute eosinophile count, plasma sodium, potassium and chloride levels and blood sugar. The results of these analyses must be interpreted with caution since sheep do not always follow the classical human patterns of hypo- or hyperfunction of the adrenals. Blood sugar levels were discussed in Chapter 5. The reader should bear in mind the discussion already given.

The normal values for tests which must still be discussed are: absolute eosinophile count, 200 to 500 per cu. mm (Brown, 1963); plasma sodium, 138 to 150 meq per litre and plasma potassium, 4·1 to 5·5 meq per litre (Clark, 1959); and plasma chlorides, 95 to 105 meq per litre (Brown, 1963).

Because of the large amount of work which had to be done during these investigations it was not possible to do complete electrolyte balance studies on these animals. Plasma bicarbonate determinations had for instance to yield priority to more important studies. In any event the presence of marked kidney lesions in many of these cases would make attempts to interpret electrolyte balance studies in terms of adrenal pathology a most difficult and probably unrewarding task. The reader is, however, referred to the sections dealing with total plasma protein values and inorganic phosphate and magnesium levels which have been discussed in the previous chapters in a different context. Total plasma calcium levels are presented in the tables in this section. No attempt was made to partition the plasma calcium.

Values found for the absolute eosinophile counts and determinations of plasma calcium, sodium, potassium and chloride on the blood of control animals are given in Table 64; the corresponding values for the prodromal and severe Vosburg cases are given in Table 65 and those for the more typical cases of all stages of geeldikkop in Table 66.

TABLE 64.—*Absolute eosinophile counts and plasma electrolyte levels in the control animals*

Sheep No.	Eos. C. per cu. mm	Ca++ mg%	Na+ Meq/L	K+ Meq/L	Cl− Meq/L
V1-7054	200	13·3	144	5·3	98·2
V1-7055	247	12·0	147	5·1	91·1
V1-7056	364	10·6	144	6·0	93·7
V1-7057	244	10·0	144	5·8	98·2
V1-7059	183	12·3	153	5·3	98·2
V1-7060	247	12·3	147	4·5	98·2
V1-7061	181	11·8	141	4·9	100·0
V1-7062	400	10·6	150	5·1	103·6
V1-7064	247	11·2	147	5·0	102·7
V1-7065	267	11·8	147	5·8	102·7
V1-7066	247	10·0	147	4·9	104·5
V1-7067	240	11·2	150	5·0	102·6
V1-7068	200	10·6	144	5·0	91·2
V1-7069	210	11·8	144	5·0	98·2
V1-7070	218	10·6	141	5·0	97·7
V1-7071	222	11·2	156	5·3	102·6
F-K2	240	10·8	159	5·6	102·7
F-12221	220	10·2	144	5·4	98·2
F-12222	240	11·1	153	5·2	100·9

TABLE 65.—*Absolute eosinophile counts and plasma electrolyte levels in the prodromal and severe Vosburg cases*

Sheep No. and details	Eos. C. per cu. mm	Na+ meq/L	K+ meq/L	Cl− meq/L
VB-P Prodromal case	140	139	8·4	85·8
VB-Q ,, ,,	50	132	6·0	71·5
VB-R ,, ,,	150	132	4·2	82·2
VB-S ,, ,,	140	129	4·2	87·2
VB-T ,, ,,	100	151	4·2	85·0
VB-U ,, ,,	150	127	3·8	97·2
VB-V ,, ,,	290	132	4·0	82·9
VB-W ,, ,,	180	136	4·2	87·2
VB-X ,, ,,	260	139	3·2	78·6
VB-H Early case 1–2 days	20	157	5·9	62·9
VB-I ,, ,,	10	157	5·6	77·2
VB-A ,, 3 days	0	148	4·8	78·6
VB-E ,, ,,	10	154	4·8	77·9
VB-F ,, ,,	60	57	1·6	76·5
VB-C ,, ,,	130	160	4·0	78·6
VB-B ,, ,,	20	160	4·8	80·7
VB-G ,, 4 days	2400	136	3·8	69·3
VB-J ,, 4–5 days	20	139	4·8	74·31
VB-K ,, 7 days	0	145	5·0	68·6
VB-D Advanced case 7–8 days	20	148	4·4	81·5
VB-L ,, ,, ,,	10	145	3·2	108·6
VB-M ,, ,, ,,	0	145	5·2	100·0
VB-N ,, ,, ,,	10	154	6·0	90·0
VB-Z1 ,, ,, ,,	40	180	7·6	88·6
VB-O ,, ,, ,,	10	148	5·0	87·2
VB-Y ,, ,, ,,	0	122	3·4	72·9

TABLE 66.—*Absolute eosinophile counts and plasma electrolyte levels in typical cases of all stages of geeldikkop*

Sheep No.	Details	Eos. C. per cu. mm	Ca^{++} mg%	Na^{+} Meq/L	K^{+} Meq/L	Cl^{-} Meq/L
V1-25	Early case 1–2 days.....	20	11·8	105	6·9	80·4
V1-3	,, ,, ...	0	10·0	135	4·5	80·4
V1-2	,, ,, ...	0	10·2	147	4·5	80·4
V1-1	,, ,, ...	100	10·2	138	5·4	71·4
V1-23	,, 2–3 days.....	60	11·2	115	4·8	89·6
V1-22	,, ,, ...	40	11·6	136	5·3	94·3
V1-24	,, ,, ...	20	10·6	126	4·9	89·3
V1-17	,, ,, ...	20	10·0	122	6·3	93·7
V1-15	,, ,, ...	25	11·8	124	5·0	93·7
F-5	,, ,, ...	60	13·6	127	4·9	89·3
V1-13	,, ,, ...	360	10·6	144	4·8	74·1
F-2	,, ,, ...	62	11·2	166	6·4	89·3
V1-26	,, 3–5 days.....	20	10·0	118	6·1	87·5
V1-11	,, 5–7 days.....	80	11·2	132	4·6	89·3
V1-18	,, ,, ...	0	11·8	120	5·0	84·8
V1-21	Advanced case 7–8 days.	20	13·0	120	5·0	91·1
V1-5	,, ,, ,,	0	10·6	135	3·8	80·4
V1-4	,, ,, ,,	0	10·0	132	4·0	89·3
V1-14	,, ,, ,,	40	8·5	118	4·3	84·8
V1-7	,, ,, ,,	40	12·3	122	4·1	71·4
V1-6	,, ,, ,,	20	10·0	141	5·3	80·4
V1-9	,, ,, 8–10 days	140	10·6	132	5·1	89·3
V1-10	,, ,, ,,	20	9·0	147	6·6	86·6
V1-8	,, ,, ,,	120	10·0	141	5·8	80·4
V1-12	,, ,, 10–14 days	480	11·2	144	5·1	89·3
V1-20	,, ,, ,,	0	10·0	115	4·8	91·1
V1-16	,, ,, ,,	0	10·0	110	3·5	92·9
V1-19	Recovered case.....	700	11·2	122	5·1	95·5
F-4	,, ,, ...	230	12·0	162	5·8	89·3
F-3	,, ,, ...	252	13·4	159	5·1	82·2
VB-Z	,, ,, ...	230	12·2	148	4·9	83·9

2. *Absolute eosinophile counts*

The absolute eosinophile counts found in the control animals were in general well within the normal limits given. A fair eosinopaenia was observed in the prodromal cases, and a marked eosinopaenia was found in all the early and advanced severe Vosburg cases except in Sheep VB-G which showed a marked eosinophilia. Severe eosinopaenia was similarly prominent and almost general in the more typical early and advanced cases. Normal counts were found in the recovered cases except in the instance of Sheep V1-19 which showed a marked eosinophilia.

3. *Plasma electrolytes and water balance*

Plasma calcium levels in the sheep range normally from 9 to 12 mg per cent (Dukes, 1955). The values found in the control animals fall generally within this range. With one exception the typical cases of geeldikkop showed no deviations from this range. The exception, Sheep V1-14, gave a value of 8·5 mg per cent for this blood constituent. No particular significance need be attached to this slightly low figure. Calcium levels were not determined on the plasma of the prodromal and Vosburg cases.

Plasma, sodium, potassium and chloride levels were generally within the normal ranges for these constituents in the control animals. A few isolated high values for plasma sodium and potassium were found in these cases, but such values were within the extreme ranges given by Clark (1959).

Severe hyponatraemia was found in two of the prodromal cases, and in most of the rest of these cases, plasma sodium levels were within the lower part of the extreme range (132 to 156 meq/L, Clark, 1959). Potassium values were normal in six of these cases, markedly elevated in two and lower than normal in one case. [The extreme range for this plasma constituent is given by Clark (1959) as 3·8 to 6·0 meq/L]. Hypochloridaemia was a general finding in this group of animals. Low sodium values were associated with either elevated or lowered potassium levels.

Normal plasma sodium levels (within the extreme limits) were found in five out of the ten early Vosburg cases, hypernatraemia in four of these animals and extremely severe hyponatraemia in one case. In this animal (Sheep VB-F) the hyponatraemia was associated with an extremely severe potassium depletion. Apart from this case potassium levels were generally within the normal plasma levels for this element and all within the extreme ranges in this group of animals. Severe hypochloridaemia was a constant feature of these cases.

Severe hypernatraemia accompanied by hyperkalaemia was found in one advanced case in the Vosburg group of animals (Sheep VB-Z1) and severe hyponatraemia together with hypokalaemia in another (Sheep VB-Y). Sodium values in these animals were otherwise within the normal range. Hypokalaemia was present in a further case (Sheep VB-L). Values within the extreme limits for this plasma constituent were found in the other cases. Chloride levels were normal in the plasma of two of these seven cases and although below normal in the rest, were generally higher than in the early cases.

It is evident from Table 66 that the same patterns of electrolyte disturbances were present in the typical early and advanced cases. Severe hyponatraemia was a fairly general feature in both of these groups of animals. In some cases it was accompanied by hyperkalaemia, and in most by normal potassium values or values within the extreme limits for this element. Hypochloridaemia was present in all the early and advanced typical cases.

The recovered cases generally showed some residual disturbances of the plasma electrolyte balance. Potassium values were generally quite normal but most of the chloride values found were still low. Sodium values were either normal, elevated or lower than normal.

Much as the author would have liked to, it was not possible to fit determinations of blood and plasma volumes and total body water into the study programme. Marked dehydration is a prominent feature in many cases of geeldikkop and was noted particularly in the severe Vosburg cases. This phenomenon was observed particularly in advanced cases *in extremis*, e.g. Sheep VB-F, VB-Y and VB-Z1. The former, it will be recalled, showed extremely severe hyponatraemia and hypokalaemia and the latter markedly elevated plasma levels of both elements. Dehydration was also pronounced in many of the advanced cases showing features of both geeldikkop and enzootic icterus, e.g. Sheep V1-6. The carcases of the severely dehydrated animals were typically markedly emaciated, the flesh appeared dry and had a peculiar pungent odour. The blood was extremely concentrated and very

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dark red in colour. The skin was dry, parchment-like and tore apart easily. There was virtually no subcutaneous tissue and the animals were in fact skinned by merely pulling the skin off the carcases. Extremely severe atrophy of the gastro-intestinal tract was a constant feature in all these cases (Brown *et al.*, 1960).

4. *General discussion*

The triad of lymphocytopaenia, eosinopaenia and neutrophilia following stressful stimuli is seen in many serious disease conditions in animals (Gordon, 1955; Schalm, 1961). In human patients with chronic adrenal insufficiency a leukopaenia with a relative lymphocytosis and eosinophilia is generally seen and neutropaenia is by no means uncommon. Human adrenal hyperfunction is generally accompanied by lymphocytopaenia and eosinopaenia (Bland, 1956; Bodansky & Bodansky, 1957; Grollman, 1963; Jailer & Longson, 1959). Leukopaenia is encountered in the sheep in severe adrenal cortical deficiency states and this is generally due to lymphocytopaenia as a result of atrophy of lymphoid tissue throughout the body (Clark, 1941). Various tests based on the response of circulating eosinophiles to the administration of ACTH have been introduced in medicine as measures of adrenal function (Bodansky & Bodansky, 1957; Kaneko, 1963; Jailer & Longson, 1959). It has been found that the eosinophile count, in consideration with the plasma electrolyte values, is very useful for this purpose in the sheep. Although a rise in the absolute eosinophile count is sometimes encountered in sheep showing apparent adrenal hypofunction, there is more generally a marked fall in the level of these cellular elements, very low counts being the rule until the animal recovers or death supervenes (Brown, *et al.*, 1960).

The typical picture of chronic adrenal insufficiency in the human is seen in Addison's disease. It is characterized by marked hyponatraemia, hypochloridaemia, low levels of plasma bicarbonate and hypoglycaemia. Plasma potassium levels may be increased or normal; calcium is generally within normal limits, but the plasma inorganic phosphate generally increases in many cases. The non-protein nitrogen levels in blood are generally increased, the elevated levels being mainly due to an increase in the blood urea nitrogen fraction. In the early stages of the disease there is a marked loss of water through the urine, but as the disease progresses the urinary loss becomes less and more fluid is lost through vomition and diarrhoea. Typical cases actually show a decreased ability to excrete water. A reduced glomerular filtration rate and renal blood flow is often present in these cases. Hypoglycaemia is due to an impaired capacity to form glucose and glycogen from intermediate products of carbohydrate and protein metabolism, that is mainly due to impaired gluconeogenesis. An increased tolerance is exhibited towards administered glucose (Bland, 1956; Bodansky & Bodansky, 1957; Grollman, 1963; Jailer & Longson, 1959).

Basically similar findings are seen in acute adrenal insufficiency and in the adreno-genital syndrome in humans. In the latter condition, diabetes and decreased tolerance to glucose may be present (Bodansky & Bodansky, 1957). Hyperfunction of the adrenals in human patients (e.g. those with Cushing's disease) is characterized by a " hypokalaemic, hypochloraemic alkalosis ". Sodium levels in plasma are frequently normal; bicarbonate levels are elevated and there is hyperglycaemia, glucosuria and a decreased glucose tolerance. Although plasma calcium levels are frequently normal, there is an excessive loss of calcium in the urine. A negative nitrogen balance is the rule in these cases (Bodansky & Bodansky, 1957).

It is obvious from that data presented here that geeldikkop in its typical form is accompanied by severe adrenal hypofunction manifested by a characteristically Addisonian type of plasma electrolyte imbalance. Hyponatraemia and hypochloridaemia are general findings in the typical early and advanced cases and are usually accompanied by normal potassium levels, although numerous cases were found to be mildly hyperkalaemic. These disturbances are still evident in recovered animals.

Evidence of marked disturbances of adrenal function was found in the prodromal cases. Hypoglycaemia and hypochloridaemia were general findings. Sodium levels were generally on the low side of normal or less commonly below the normal limits. Potassium levels in the plasma were also generally normal but raised or lowered in odd cases. The same patterns are seen in the early and advanced Vosburg cases. Although adrenal function is obviously seriously impaired in these extreme cases, the chemical pathological picture is not as well defined as in the typical cases and apart from that of individuals in which complete adrenal collapse is evident (e.g. Sheep VB-F, VB-Y, VB-Zl), it seems to represent a phase in which hyper- and hypofunction alternate—a phase which results either in adaptation and recovery or in total adrenal exhaustion (as in Sheep VB-F, VB-Y, VB-Zl) in accordance with Selye's concept of the general adaptation syndrome (Selye, 1946). Marked sodium depletion together with hypokalaemia generally heralded a fatal outcome in these, cases, and as mentioned earlier was generally accompanied by dehydration.

In view of the interpretation of the data given in Tables 64 to 66 which has just been presented, it is essential that the gross and microscopic pathology of geeldikkop relevant to this discussion be reviewed briefly.

The following is extracted from one of the earlier studies (Brown *et al*, 1960):

" Autopsies of the severe Vosburg cases revealed fair to marked atrophy of the spleen with a marked atrophy of splenic lymphoid tissue; marked atrophy of all the lymphoid tissue in the body, the lymph nodes being severely involved and often difficult to find—even the large mesenteric lymph glands were affected in this way. The parotid, submaxillary and prescapular lymph nodes which drain the areas involved in the photosensitization reactions were found to be often enlarged and very oedematous. The adrenal glands appeared to be severely affected, showing a marked atrophy or marked enlargement, their colour varying from normal to light yellowish-brown, and their structure on section being often very indistinct. On microscopic examination evidence of atrophy of the lymphoid tissue was seen, together with a diminution in the cellular content of the Malpighian bodies of the spleen and the cortical nodules of the lymph nodes. The adrenals did not appear to show any particular changes".

The adrenals of the early typical (Sheep V1–) cases showed obvious degenerative changes at autopsy. There appeared also to be a marked atrophy of all lymphoid tissue; the lymph nodes were very small and difficult to find with a concomitant marked decrease in splenic lymphoid tissue. Atrophy of the lymphoid nodules of the spleen and lymph nodes was confirmed microscopically. In the lymph nodes the reticulo-endothelial cells were loaded with a finely granular iron-free pigment. In one of these cases the lymph nodes contained numerous macrophages loaded with iron-free pigment, which resembled the " giant pigment cells " seen in enzootic icterus. Once more, nothing specific was seen in the adrenal glands.

The same changes as seen in the early cases were found in the advanced cases of the disease, prefixed V1- and the cases showing features of both geeldikkop and enzootic icterus. Amongst the latter cases, one animal showed in addition to marked atrophy of the cortex and lymphoid nodules of the lymph nodes, an accumulation in the latter structures of some very large macrophages loaded with iron free pigment resembling the " giant pigment cells " of enzootic icterus.

The pathology of the most recently studied group of cases (Sheep prefixed V3- and F-) was basically similar to that of the V1- cases as regards the adrenal glands, spleen and lymphoid tissue. The adrenals of the early and advanced cases appeared generally on microscopic examination to be enlarged with a rather indistinct structure. Microscopic examination of sections of these glands revealed little of note in most cases. Some pyknosis of cortical cell nuclei was evident in a few sheep. In most of these animals evidence of considerable phagocytic activity was found on microscopic examination of spleen sections. This took the form of a general reactive hyperplasia of the red pulp. There appeared in many instances to be a fair to moderate increase in iron-containing pigment in many of these cases. Atrophy of the lymphoid tissue was a general finding in both the early and advanced cases; many of the lymph glands examined contained numerous macrophages laden with iron-containing pigment.

Nothing of particular note regarding the adrenals and lymphoid tissue was evident in the pathology of the recovered cases. The histopathology of the lymph glands of the control animals is of interest in that macrophages laden with iron-containing pigment were found to be present in these structures in all instances. This phenomenon, indicating an increased rate of erythrocyte destruction, is further evidence of the existence of a haemolytic process in the general run of geeldikkop cases. Its presence in the control animals indicates that the haemolytic syndrome is present and of a low grade nature in animals showing no clinical symptoms of either geeldikkop or enzootic icterus.

The general severe atrophy of lymphoid tissue is a prominent feature of stress conditions in the sheep and was first described in this animal by Clark (1941) during his studies on ovine pregnancy toxaemia. The results of macroscopic examination of the adrenals of geeldikkop cases point to obvious involvement of these structures in the pathogenesis of the syndrome. The singular lack of microscopic changes in these organs indicates however that the adrenal insufficiency which is present in geeldikkop is due to a functional failure of the adrenal cortex and not to a destructive process.

Geeldikkop is a disease in which a severe gastro-intestinal stasis is present from before the time at which the first symptoms of the disease appear (Brown, 1966a; Brown *et al.*, 1960). This means a drastic reduction in the supply of volatile fatty acids to the animal for energy purposes. Evidence has been produced of the severe disturbances which exist in carbohydrate metabolism in these cases. This makes these animals entirely dependent on fatty acids obtained by lipolysis of reserve fat and gluconeogenesis from protein sources for their energy supply. It is obvious from the data presented in Tables 65 and 66 (and particularly the latter table) that adrena insufficiency is severe from before the onset of symptoms and extremely severe particularly during the first week of illness. This means in effect a complete withdrawal of the hormonal stimuli for gluconeogenesis, the results of which can only be catastrophic to the animal concerned, even if its endogenous fatty acid supply remains unimpaired.

CHAPTER 9

The General Chemical Pathology and Biochemistry of Geeldikkop

F. General Discussion on the Histopathology of the Disease in Relation to the Chemical Pathology

One of the most intriguing aspects of geeldikkop has always been the fact that the violent clinical symptoms are accompanied in typical cases by such mild histopathological changes. The chemical pathological studies on the disease, as outlined in the foregoing chapters, make it considerably easier to understand why this should be so and help to explain the development of phenomena which must be regarded as secondary changes, e.g. degeneration of the kidney tubules. When the gross pathology, histopathology and chemical pathology of the disease are considered together very critically, it is apparent that the syndrome of geeldikkop consists of three definite components, namely:

(*a*) a pre-existing and for the greater part sub-clinical disease condition;

(*b*) an acute episode with its characteristic sequence of biochemical events and clinical symptoms, and

(*c*) a condition which precipitates the acute episodes.

It is possible to separate these components from one another, and as will be demonstrated in the subsequent chapters, also possible to reproduce the most important features of these components. These facts bring us even closer to understanding the pathogenesis of this rather complex disease.

Mention has been made earlier of the very marked differences which exist between sheep born and raised in areas where geeldikkop is prevalent and those born and raised elsewhere. The most important differences are those concerning red cell fragility, methaemoglobin reduction and red cell glutathione (Wagner, 1964). Mention has also been made of the ease with which an acute haemolytic attack can be induced in enzootic icterus and of various pointers to the fact that such attacks apparently occur on a subclinical scale in animals from areas where geeldikkop is more common, e.g. demonstrably increased amounts of iron-containing pigment in the spleens of these animals and the presence of large numbers of macrophages laden with iron-containing pigment in their lymph glands.

These phenomena are all part of the pre-existing subclinical disease condition, which will be discussed at greater length in one of the following chapters.

The acute episode results directly from an alteration in the selective permeability of cell membranes in the liver, kidneys, muscles and possibly other tissues as well. This alteration in permeability is coupled with a marked decrease in energy production in the tissues concerned and with failure of vital hormonal control in the form of adrenal cortical insufficiency. In typical uncomplicated cases the acute episode is brief and recovery can be expected. In the more severe cases particularly those in which the lesions of photosensitization have run their full course unchecked, adrenal cortical collapse is present, the alteration in cell membrane permeability is persistent, the cells of the affected tissues commence to undergo secondary degenerative changes and death is the inevitable conclusion.

The main biochemical consequences of the altered membrane permeability are regurgitation of the constituents of bile into the systemic blood circulation, failure to excrete them through the kidneys, retention of non-protein nitrogenous waste products normally excreted by the kidneys and decreased tolerance to glucose. These phenomena can be reproduced in part by administration of icterogenic triterpene acids to experimental animals (see Chapter 13).

The course of the typical acute episode can be complicated or markedly altered by the lesions of photosensitization and accelerated rate of intravascular haemolysis, the appearance of nephrotic lesions and the adrenal insufficiency, depending upon their severity and duration. Any of these factors alone can cause the death of the case concerned, the most important in this regard being the kidney lesions coupled with the adrenal insufficiency.

Mention has been made in the preceding chapters of the marked biochemical and haematological disorders which precede the appearance of the clinical symptoms, and indications have been given that these disturbances may be due to the action of an infectious agent. This agent and its effects constitute the third component of the geeldikkop syndrome and are probably the most important factors in precipitating the acute episodes. The marked elevations of plasma GOT, LD, PHI and aldolase activity are part of this component, and as will be demonstrated in a later chapter, can be reproduced most effectively by infecting susceptible sheep with strains of a myotropic virus, which produce a clinically insignificant febrile reaction.

Since the author has only recently begun to appreciate the possible role of a myotropic virus in the pathogenesis of geeldikkop, no particular study was made of the muscles in the earlier (Sheep prefixed VB– and V1–) cases. The presence of myocarditis was noted in individual animals but no significance was attached to it at the time (Brown, *et al.*, 1960).

Muscle tissue was collected for examination from the most recent cases (Sheep V3– and F–). Specimens of myocardium, longissimus dorsi, rhomboideus and of the vastus group were taken at random during autopsy examination of these cases. Small scattered foci of lymphocytic myocarditis were found in the heart muscle specimens from Sheep F–4, F–5, V3–15, V3–23, V3–25 and V3–26. Areas of Zenker's degeneration were clearly visible in the skeletal muscle tissues from Sheep V3–2.

The marked atrophy of the gastro-intestinal tract which has been observed in quite a few cases has been described earlier (Brown *et al.*, 1960). The gastro-intestinal stasis and the atrophy which can result from it are in themselves peculiar since they are of a particular severity not observed in any other ovine disease apart from enzootic icterus.

Severe jaundice following regurgitation of conjugated bilirubin into the systemic blood circulation in the absence of significant hepatocellular damage is not confined to geeldikkop. Hanger & Gutman (1940) reported that arsphenamine could produce a type of jaundice in humans resembling obstructive jaundice but without inducing significant parenchymal injury. This same type of injury has now been shown to occur following therapy with methyl testosterone, chlorpromazine and other drugs. Such responses are individual and unpredictable and are often associated with commonly accepted manifestations of hypersensitivity, such as fever and skin eruptions (Drill, 1958). These conditions have been called the *intrahepatic cholestases* and include hypersensitivity reactions, direct effects of certain drugs not associated with hypersensitivity and functional intrahepatic cholestasis, which is commonly associated with viral hepatitis or cirrhosis in human patients (Drill, 1958; Popper & Schaffner, 1957).

The term hypersensitivity denotes a mechanism for the production of lesions which differs from the usual pharmacological action or effects of overdosage. Such reactions are not related to dosage and are individual and unpredictable (Drill, 1958). A drug producing hypersensitivity is believed to act as a haptene and the antibodies are thought to remain attached to the tissue cells. Under such circumstances, the re-administration of the drug may cause an antigen antibody reaction on the surface of the cells to which the antibody is fixed and so produce injury within a specific organ. The incidence of such reactions in the liver is generally low and the hepatic injury may be expressed as parenchymal changes with or without jaundice or as cholestasis with jaundice (Drill, 1958). Drug reactions of the latter type have an excellent prognosis as there is no, or at most, a minimal parenchymal reaction. Upon withdrawal of the drug the jaundice will slowly disappear despite the fact that it may have existed previously for a period of months. Liver biopsies from the patients at the height of jaundice induced by methyl testosterone show stasis and accumulation of bile within the bile capillaries of the central portions of the lobules. Arsphenamine jaundice is accompanied by lesions in the portal triads, swelling of the bile ducts and cholangiolitis. Chlorpromazine induces a characteristic picture of stasis in the bile canaliculi with varying degrees of inflammatory infiltration e.g. eosinophilic infiltration in the portal zones (Drill, 1958; Popper & Schaffner, 1957).

The outstanding examples of drugs which induce cholestasis directly in the absence of parenchymal lesions demonstrable by ordinary light microscopy are icterogenin, 22β-angeloyloxyoleanolic acid and other icterogenin pentacyclic triterpenes (Brown, *et al.*, 1963; Brown & Rimington, 1964; Brown, Rimington & Sawyer, 1963; Heikel *et al.*, 1960). These compounds produce a functional depression of the secretion of bile pigments unassociated with any hepatocellular damage or obstruction of the bile passages. The result in sheep is a syndrome clinically very similar to geeldikkop and embracing many of the biochemical features of the acute episode of this disease. These compounds and their effects will be fully discussed in Chapter 13. Their effects on experimental animals are predictable and highly reproducible.

Functional intrahepatic cholestasis is a term used by Popper & Schaffner (1957) to cover the condition seen frequently in viral hepatitis and cirrhosis. It is characterized by the presence of exudate in the portal tracts or around the intra-lobular ductules, and cell infiltrations in these regions. Bile plugs are found in the bile ductules up to but not beyond the intra-lobular and periportal ductules. The condition is said to be due to increased permeability of the ductules permitting bile to flow back through the ductular epithelium into the surrounding tissue with subsequent regurgitation into the blood. More water than biliary substances may flow back resulting in inspissation of the biliary solids (Popper & Schaffner, 1957). A similar condition can be produced in experimental animals using cholangiotoxic agents like toluylene diamine, manganous chloride, α-naphthyl-thiourea (ANTU) and ethionine (Popper & Schaffner, 1957).

In terms of this discussion and its liver pathology, geeldikkop is an intrahepatic cholestasis. It resembles very closely the type of cholestasis induced by the icterogenic pentacyclic triterpene acids, but is quite different from the hypersensitivity cholestases or functional disturbances following increased permeability of the bile ductules. The cholestasis of geeldikkop is due to a decreased permeability of the liver cell membranes and a failure to secrete most of the components of bile. It has not yet been possible to establish with certainty whether the parenchymal cells, the bile duct epithelium

or both are involved in the decreased permeability phenomena. The cholestasis of geeldikkop is aggravated by the presence of a co-existing haemolytic syndrome and thus differs from the human types in that a considerable amount of the bile pigment circulating in the systemic blood is unconjugated bilirubin.

Geeldikkop is also not unique in being a photosensitivity disease of which the acute attacks are precipitated by various forms of severe non-specific stress. Acute attacks may be precipitated in human acute intermittent porphyria and in the cutaneous hepatic porphyrias (even the hereditary types) by a variety of stressful stimuli which have at various times been said to include menstruation, pregnancy, drinking bouts, infections and the prolonged ingestion of barbiturates and other drugs (Goldberg & Rimington, 1962).

CHAPTER 10

THE HAEMATOLOGY AND GENERAL CHEMICAL PATHOLOGY OF ENZOOTIC ICTERUS

1. *Introductory remarks*

In its typical form enzootic icterus is an acute or chronic haemolytic syndrome precipitated as already mentioned by various non-specific forms of stress. The acute episodes, which are often fatal, are characterized by intense icterus, severe haemolytic anaemia, severe renal pathology, severe gastro-intestinal stasis and many biochemical disturbances which will be described in this chapter. Chronic forms of the disease are common, often dominating any particular large-scale outbreak and are characterized by marked anaemia and renal lesions.

The disease has been known from the earliest times of intensive farming in the central and south-western areas of the Cape Province, under the name of " geelsiekte " (literally, " yellow disease "). Numerous aged inhabitants in the enzootic areas recount outbreaks of the disease covering three generations on their particular farms.

The first record of the disease is that of De Kock (1928a, 1928b) in two papers on other topics. Although enzootic icterus is well-known to most veterinarians in this country and has formed the subject of numerous unpublished reports and of voluminous correspondence, there is a remarkable void in the literature regarding the disease