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 Although enzootic icterus is well-known to most veterinarians in this country and has formed the subject of numerous unpublished reports and of voluminous correspondence, there is a remarkable void in the literature regarding the disease

until 1959. Since this date various aspects of the disease have been touched upon in publications of the author and his co-workers (Brown *et al.*, 1960; Pienaar & van der Merwe, 1966; Wagner & Brown, 1966a, b). The dearth of published information during the period mentioned is in no way due to lack of research. Numerous studies have been conducted during this time which served to narrow down the field of investigation. These studies included bacteriological and chemical pathological studies as well as a considerable amount of field toxicological work. The bacteriological work produced no results of significance to this discussion. The field work in connection with the disease has been fully described elsewhere (Brown & de Boom, 1966; Brown & de Wet, 1967) and the chemical pathological studies will be discussed in the appropriate places in this chapter. Mention has been made in Chapter 1 of how the author and his co-workers became involved in research into this syndrome. The studies on the epizootiology of the disease have recently appeared in print (Brown & de Boom, 1966). The reader is referred to this work for details on the distribution, incidence, precipitating factors, mortality and morbidity rates and the economic importance of the condition.

The acute form of the disease is unfortunately regarded as the typical form. This is largely because it is easily recognized and was the form most commonly seen by De Kock and his staff *at Onderstepoort* (Brown & de Boom, 1966; De Kock, 1928). It appears rather sporadically amongst individual animals in flocks in the affected areas throughout the year, sporadically amongst animals moved out of these areas and to a fair extent in any flock during severe extensive outbreaks of the disease in the affected areas. It is rather more common anongst groups of animals moved long distances in motor and rail transport and is typically an acute or explosive haemolytic crisis. Affected animals are severely icteric and anaemic and often show a fever of 104 to 106°F and severe haemoglobinuria. Malaise, apathy, anorexia and rapid loss of condition are prominent symptoms and are accompanied by hyperpnoea, a strong bounding pulse in the early stages and very often cardiac arrhythmia. There is nearly always some evidence of photosensitization in the form of *mild* swelling of the ears, eyelids and lips, rhinitis, blepharitis, conjunctivitis and often keratitis. The condition is always accompanied by an extremely severe gastro-intestinal stasis, the like of which is seen only in geeldikkop. As a rule these cases terminate fatally within one to five days of the onset of symptoms.

The commonest form of the disease is a chronic wasting syndrome which is seen most generally during extensive outbreaks. It is this form which strictly speaking should be regarded as the typical disease. The onset of symptoms is insidious and early cases are not easily recognized. Affected animals appear to look about for food with the rest of the flock but do not eat or drink (even though many have been seen to stand with their muzzles immersed in water). Others merely nibble at their food and wander off listlessly. The Griqua shepherds on the farms concerned have often been relied upon to pick out these cases from their flocks which they are able to do with unerring facility. These animals generally show a fever of 103 to 105°F; their visible mucous membranes are most often injected but sometimes anaemic, and in a few cases the typical chocolate brown colouration of a methaemoglobincythaemic case was seen. Gastro-intestinal stasis is invariably present.

The subsequent course of the disease may occupy three to five weeks before recovery or death occur. It is typically a steady decline in condition, affected animals rapidly becoming severely anaemic and cachectic. They are apathetic, disinclined to eat and fall readily if chased. There is invariably severe gastro-intestinal stasis,

a fever of 103 to 105°F, hyperpnoea, a bounding or else a weak thready pulse, rapid heartbeat, mild icterus and methaemoglobincythaemia or cyanosis. The wool of these animals is easily pulled out of their skin and they generally have a mangy appearance. Rhinitis, conjunctivitis, blepharitis and keratitis occur frequently and are often accompanied by some necrosis of the skin on the ears, eyelids and nostrils. This chronic form of the disease is subject to frequent exacerbations and remissions, the former being provoked by subjecting the animals to any stressful condition.

The disease is seen mainly in older animals and particularly in aged ones. Many cases in suckling lambs and weaners have been seen although they occur rather infrequently and generally only during severe outbreaks of the disease.

2. *Animals, materials and methods*

The cases used in the study of the haematology and chemical pathology of enzootic icterus are listed in Appendix 6. The most prominent symptoms observed in each instance and the place of origin of each case are also indicated in this appendix. The number of cases of different stages of the disease used in this work is indicated in Table 67. As in the case of the animals used in the studies on geeldikkop, various sheep were used to study different aspects of the disease.

TABLE 67.—*Number of cases of the different stages of enzootic icterus which were studied during this work*

(Details of these animals are to be found in Appendix 6)

Nature of cases	No. of cases
Early cases—(*a*) Mild cases when first seen	26
(*b*) severe cases	6
Chronic cases—(*a*) " post-haemolytic " cases	5
(*b*) mild cases	11
(*c*) severe cases	4
Total number of affected animals studied	52
Control animals from affected farms	3
Control sheep from Onderstepoort	9
Total number of controls used at Onderstepoort	12
Total number of animals used in this study	64

The majority of the animals used emanated from farms in the Fraserburg, Sutherland and Aberdeen areas and were obtained during the severe outbreaks of the disease in the late summers of 1957/1958 and 1961/1962. The animals emanating from Murraysburg and Rietbron were obtained during investigations into geeldikkop outbreaks in these areas. Those from Calvinia were examined on the farm " North Brabant ", in the Clarens district of the Orange Free State during a massive movement of small stock from drought-stricken areas in 1964. The early case, " Klopper No. 1", from Halseton, C.P., was sent ot the author for examination by the owner concerned.

Sheep 12223 to 12229, F6 to F12, and FB2 to FB14 inclusive were maintained under field laboratory conditions at Fraserburg and were fed and managed in exactly the same way as described in Chapter 2. The Sheep 2206 to 2208, 15064, 15065,

Klopper No. 1, Bekker 1 to Bekker 3, NB2 to NB5 and 5114 to 5132 inclusive were obtained as described and brought to Onderstepoort for study. All the cases except animals 5114 to 5132 were kept for no longer than 24 hours after arriving here before being slaughtered for histopathological studies.

Sheep 5114 to 5132 were brought from Fraserburg and Sutherland to Onderstepoort with the specific aim of precipitating acute attacks of the disease by first subjecting them to a long trip by rail bus from these two centres to Leeugamka Station, and then subjecting them to a three days' train journey to Onderstepoort. On arrival here they were fed teff hay and crushed maize *ad libitum*. This represented a diet completely foreign to them, the change being sufficient to induce a severe gastrointestinal disturbance. The majority of the animals were clinically normal when they left their places of origin. As will be seen from the discussion which follows, the combination of being transported and the introduction to a foreign diet proved highly successful in provoking acute attacks of the disease. The symptoms listed in Appendix 6 are those observed when the animals arrived at Onderstepoort. Sheep 5116, 5117, 5118, 5120 and 5127 were seen on their farms of origin during January of 1958 and were classified as chronic cases on the grounds of clinical and chemical pathological examinations. They left the farms concerned three weeks later for Onderstepoort and at this time were classed as recovered cases. The combination of subsequent stress factors provoked the exacerbations in these cases to be described in this chapter.

The cases listed as " post-haemolytic ", i.e. Group 1 of the chronic cases, were sheep in which the owners observed haemoglobinuria a few days before they were acquired. They had thus come through an acute haemolytic episode of fair severity and were in a period of apparent remission when examined.

Analytical methods used and other laboratory procedures of a routine nature were exactly as described for the studies on geeldikkop (Chapter 2.)

3. *The haematology of enzootic icterus*

Studies on the haematology of cases representing all stages of the disease are presented in Appendix 7. Normal values for these studies were as used for the studies on the geeldikkop cases, namely, red cell volume, 33 to 46 per cent; red cell count, 8 to 14 $\times$ 10^6/cu. mm; haemoglobin, 9 to 14·5 gm per cent; white cell count 4·9 to 9·15 $\times$ 10^3/cu. mm; MCHC, 24·1 to 38·6 per cent; MCV, 21·3 to 41·7 cu. μ; MCH, 6·5 to 16·1 $\mu\mu$ gm and differential leukocyte count, N=30 to 35 per cent; L=50 to 55 per cent; M=4 per cent; E=8 per cent; B=0 to 1 per cent.

It is evident from the data presented in Appendix 7 that in the mild early cases, apart from Sheep 2206 to 2208, the only constant changes are a definite neutrophilia and lymphocytopaenia. Very mild anaemic changes were found in some cases, e.g. Sheep 12224 and FB–2. The bloodsmears of these animals revealed nothing of note. Sheep 2206, 2207 and 2208 were severely anaemic. Bloodsmear examination revealed in these instances severe anisocytosis, polychromasia and the presence of numerous Jolly bodies and normoblasts. No evidence of a reduction in the number of platelets could be found. The severe anaemia in these cases was either normocytic, normochromic (Sheep 2207) or macrocytic hyperchromic (Sheep 2206 and 2208). All three animals showed a severe leukocytosis generally due to a neutrophilia. Lymphocytopaenia was evident in two of these cases (Sheep 2207 and 2208).

An anaemia, generally macrocytic hyperchromic of varying severity, was found in the severe early cases (Group 2) and it was generally accompanied by neutrophilia and severe lymphocytopaenia.

TABLE 68.—*Serial studies on the haematology of mild cases of enzootic icterus*
(The days indicated are days after arrival at Onderstepoort)

Sheep No.	Determination	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	
5114	RCV.....	45	36	25	21	19	16	MCHC= 26·87
	RCC.....	9·22	4·73	4·50	4·36	3·98	3·59	MCV= 44·56
	Hb.....	11·30	8·80	4·45	4·80	4·62	4·30	MCH = 11·97
	WCC.....	6,320	7,850	15,600	15,750	16,250	33,600	
5119	RCV.....	34	28	25	21	17	16	MCHC= 36·87
	RCC.....	7·61	6·68	6·53	3·71	3·00	3·61	
	Hb.....	7·90	7·12	7·12	5·95	5·20	5·90	MCV= 44·32
	WCC.....	2,370	4,300	5,200	11,700	7,750	15,650	MCH = 16·34
5121	RCV.....	28	20	12	12	12	DIED	MCHC= 34·91
	RCC.....	7·88	5·29	2·42	1·86	2·01		
	Hb.....	9·10	10·50	3·75	4·28	4·19		MCV = 59·7
	WCC.....	10,250	10,850	11,750	9,800	10,500	MetHb at death	MCH = 20·84
5128	RCV.....	29	30	27	22	20	DIED	MCHC= 35·00
	RCC.....	10·61	12·61	7·90	5·93	5·01		MCV = 39·92
	Hb.....	9·10	9·10	8·10	7·62	7·00		
	WCC.....	4,200	12,850	8,950	15,800	16,820		MCH = 13·97
5130	RCV.....	30	27	21	20	19	21	—
	RCC.....	8·20	6·35	4·56	4·25	4·62	4·32	—
	Hb.....	8·20	6·90	—	—	—	—	—
	WCC.....	4,200	6,250	7,950	7,450	6,850	2,500	
5131	RCV.....	37	30	25	22	18	16	—
	RCC.....	9·57	8·92	6·20	5·90	—	4·02	—
	Hb.....	9·10	—	6·20	5·50	5·00	4·17	—
	WCC.....	1,120	—	4,500	—	2,400	10,000	
5132	CV.....	38	36	16	12	11·0	DIED	MCHC= 34·09
	RCC.....	10·28	10·47	3·19	2·13	1·90		MCV= 57·89
	Hb.....	10·50	6·17	4·80	3·92	3·75		
	WCC.....	4,560	3,520	15,100	16,750	12,250		MCH = 19·73

N.B.—RCV = red cell volume (%); RCC = red cell count (10^6/cu. mm); WCC = white cell count (10^3/cu. mm); Hb = haemoglobin (gm%).

The post-haemolytic stage of enzootic icterus appears to be dominated by a normocytic normochromic anaemia of varying severity, as is evident from the data obtained from Sheep F–9, F–10 and F–11. In Sheep 12226 the anaemia appeared to be hypochromic microcytic and in Sheep 12227 of a severe hyperchromic macrocytic type. Severe leukopaenia was evident in Sheep F–10, F–11 and 12226, while Sheep F–9 and 12227 showed a neutrophilia and lymphocytopaenia. The severe anaemic changes described for Sheep 2206 to 2208 were seen in the bloodsmears of some of these animals as well.

A normocytic normochromic anaemia of varying severity was the general finding in the mild (Group 2) chronic cases. In some animals leukopaenia or a mild neutrophilia and lymphocytopaenia were also evident. The severely affected Group 3 chronic cases showed mainly a severe macrocytic normochromic anaemia.

The development of the haemolytic syndrome in enzootic icterus is illustrated by the data presented in Tables 68 and 69. These tables contain the results of daily serial studies on the haematology of some of the cases brought to Onderstepoort from Fraserburg and Sutherland, and illustrate some of the effects of severe stress (the journeys by rail bus and train and the drastic change of diet) on animals that are apparently asymptomatic cases of the basic disease entity. Day 1 in these tables is the day immediately after the arrival of these animals at Onderstepoort, and the studies were continued for a further five to six days.

The animals shown in Table 68 were not known to have had a history of a previous haemolytic episode and were clinically normal at the time they left their places of origin. As can be seen from this table the haemolytic episode developed rapidly after their arrival at Onderstepoort and in Sheep 5121, 5128 and 5132 terminated in death six days later. Sheep 5114, 5119, 5130 and 5131 all died during the course of the following week. The interval between the commencement of application of the combined stress influences and the appearance of the haemolytic syndrome was in all cases six to seven days. The absolute haematological indices given in Table 68 have been calculated from the data obtained on the last day of study (i.e. Day 5 or 6 as the case may be). It can be seen from these that the anaemia which developed in these animals was generally macrocytic hyperchromic. In the case of Sheep 5119 it was macrocytic normochromic and in the case of Sheep 5128, normocytic normochromic.

In five out of the seven cases a marked leukocytosis developed concurrently with the anaemia.

Methaemoglobincythaemia was clinically obvious in Sheep 5121 at the time of death.

The four animals shown in Table 69 were all classed as chronic cases of the disease when examined at their places of origin about a month before being sent to Onderstepoort. Just prior to this event they appeared to have recovered from the earlier attack. As can be seen from the data in Table 69 the effects of the combined stress (duration six to seven days) provoked a severe exacerbation of the subclinical haemolytic syndrome. Sheep 5116 died during the following week, Sheep 5118 and 5120 two weeks later, and Sheep 5117 apparently recovered, but died about two months later from a further attack. Haematological indices were calculated from the data of Day 5 in the case of 5117 and from the data of Day 7 in the case of Sheep

TABLE 69.—*Serial studies on the haematology of cases showing a known exacerbation of the haemolytic syndrome in enzootic icterus*

(The days indicated are days after arrival at Onderstepoort)

Sheep No.	Determination	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
5116	RCV	38	—	25	—	16	—	—
	RCC	—	6·38	—	3·86	—	2·57	—
	Hb	10·50	—	9·42	—	7·12	—	—
	WCC	4,200	6,820	—	30,100	—	20,600	—
5117	RCV	36	29	19	17	16	12	—
	RCC	9·12	9·23	6·49	4·97	5·24	5·00	—
	Hb	10·90	7·62	5·75	3·75	3·00	—	—
	WCC	9,450	15,800	5,550	14,000	16,350	15,000	—
5118	RCV	30	33	28	20	21	15	11
	RCC	8·22	7·97	7·79	5·60	4·95	3·87	2·80
	Hb	8·50	6·17	6·65	6·40	6·90	7·30	6·20
	WCC	4,150	5,500	3,700	2,500	7,700	11,950	4,350
5120	RCV	38	40	33	29	25	24	12
	RCC	13·32	9·01	10·40	8·90	6·63	6·30	5·80
	Hb	10·50	11·75	8·50	7·35	8·50	7·62	5·75
	WCC	4,025	8,150	7,000	10,150	8,200	12,550	8,600

N.B.—RCV = red cell volume (%); RCC = red cell count (10^6/cu. mm); WCC = white cell count (10^3/cu. mm); Hb = haemoglobin (gm%).

5118 and 5120. The anaemia in all three instances was found to be normocytic (hyperchromic in the case of Sheep 5118 and 5120 and hypochromic in Sheep 5117). The development of anaemia in these animals was once more accompanied by leukocytosis, neutrophilia and lymphocytopaenia.

No deviations from normal were seen in the erythrocyte sedimentation rate of any of the animals studied (Appendix 7).

Normocytic normochromic anaemias may result from sudden loss of blood, destruction of blood, lack of blood formation or dilution of blood with fluid. Under all these circumstances, other things being equal, the remaining red corpuscles were as normal in size or haemoglobin content as they were before blood loss, destruction or dilution had occurred. The total plasma protein figures of the animals examined are given in Appendix 7. These figures were obtained from the same blood used for the haematological studies indicated in each case and were generally within normal limits for this determination. It can therefore be assumed that haemodilution due to water retention does not contribute towards the low values for haemoglobin, haematocrit and red cell counts found during this work.

Blood regeneration of varying degrees occurs in acute posthaemorrhagic anaemia, in the haemolytic anaemias and sometimes even in aplastic anaemias. When the stimulus to new blood formation is great and the capacity for haemopoiesis is good, a large number of immature cells may pass into the circulation and the anaemias

may appear to be macrocytic and hyper- or hypochromic. Macrocytosis may be marked in the acute haemolytic anaemias and it may be long continued in the chronic forms (Wintrobe, 1947).

Enzootic icterus attacks are of rapid onset following stress, as shown, and are typically *hypocythaemic normocytic normochromic* or *hypocythaemic macrocytic hyperchromic* (*or normochromic*) in the early stages of the disease. The development of the anaemia is accompanied generally by a leukocytosis, which is due to an absolute neutrophilia and lymphocytopaenia in most cases. No evidence of thrombocytopaenia was observed in any of the cases reported here.

The " post-haemolytic " blood picture in enzootic icterus is that of a normocytic normochromic or macrocytic hyperchromic anaemia as before, which may be accompanied by a severe leukopaenia or relative neutrophilia and absolute lymphocytopaenia. These changes were observed by De Kock (1928) as well. The same changes are seen in mild and severe chronic cases of the disease.

Leukopaenia and lymphocytopaenia have already been discussed in connection with stress. The existence of adrenal insufficiency in enzootic icterus will be demonstrated later in this chapter. Absolute neutrophilias are generally associated with acute or localized infections. They are however very prominent findings in acute haemolytic episodes and intoxications associated with metabolic disorders such as uraemia (Wintrobe, 1947). It will be demonstrated later in this chapter that uraemia is a pronounced symptom in enzootic icterus. The neutrophilia in this disease is accompanied by a very marked " shift to the left ", as is seen in geeldikkop.

4. *Liver function in enzootic icterus*

(*a*) *Bile pigment and bile acid metabolism*: Total bilirubin and its glucuronides were determined on the plasma of all the animals listed in Appendix 6. Traces of bilirubin were present in the plasma of all the early cases. The highest total bilirubin figures found at the first examination of these animals were 3·4, 2·1 mg per cent and 1·8 mg per cent, from the plasma of Sheep 2207, 5116 and 5128 respectively. In the case of Sheep 2207 all the bile pigment circulating in the plasma was bilirubin, but in the case of 5116 and 5128, 1·5 mg per cent and 1·2 mg per cent was bilirubin glucuronide, and only 0·6 mg per cent in both instances was bilirubin. Total bilirubin levels varied in all the other Group 1 early cases from traces to 0·9 mg per cent and appeared to comprise mainly bilirubin. No bilirubinuria was found in any of the mild early cases apart from Sheep 5116 and 5128 in which it was very mild. Urobilinogen was present in the urine of all these cases, in traces only, and bile acid salts were either present in the same amounts or not at all.

The figures given in Table 70 are typical of those obtained from the animals in which haemolytic crises were induced by the factors already mentioned, i.e. the sheep shown in Tables 68 and 69. Although the anaemia in these animals became progressively very severe, clinical icterus was mild and total plasma bilirubin seldom exceeded 5·0 mg per cent. The tendency for the total plasma bile pigment to decrease as the anaemia increased in severity was seen in all these animals. Also seen in all cases was the presence of almost equal amounts of bilirubin glucuronide and bilirubin in fresh samples as indicated in Table 70.

TABLE 70.—*Serial studies on the plasma bile pigment levels and urine pigments of a typical mild case of enzootic icterus—Sheep* 5114

(See Table 68 for the haemotological data on this animal)

Item	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Total Bilirubin (mg%)....	1·9	5·0	3·9	2·9	2·2	2·0
Bilirubin glucuronide (mg%).....	0·9	2·1	1·8	1·3	1·0	0·8
Bilirubin mg%.....	1·0	2·9	2·1	1·6	1·2	1·4
Plasma creatinine mg%..	1·6	1·8	2·4	4·2	5·8	5·5
Urine bile pigments.....	0	±	+	±	0	0
Urine urobilinogen.....	±	+	2+	3+	5+	5+
Urine bile salts.....	±	0	0	0	0	0
Urine haem pigments....	0	5+	4+	0	0	0

Bilirubinuria was always very mild in these cases in spite of fair levels of the glucuronide in their plasma in the initial stages of the haemolytic syndrome. Bile salts tended in general to disappear from the urine of these cases entirely as the disease progressed. Plasma creatinine levels were in general as indicated in Table 70 and pointed to a rapidly developing renal insufficiency. This aspect will be discussed in detail later in this chapter. In spite of the presence of this lesion, urinary urobilinogen levels rose as the haemolytic syndrome progressed. Haemoglobinuria was seen in most of the cases during the first three or four days of the attack only. Considerable amounts of free haemoglobin were generally present in the plasma of most of these cases throughout the period of six to seven days of study. The amounts were not determined; the statement is made on the grounds of the distinct reddish-brown colour in the plasma of these animals during this time. Thus, apart from its appearance in the urine during the initial stages of this syndrome, this haemoglobin was generally not found in the urine. Haemoglobinuria was exceptionally severe during the first day or two of the attack in some of these cases, e.g. Sheep 5116, and then abruptly disappeared.

When Sheep 5116, 5117, 5118, 5120 and 5127 were first examined in the field and classed as chronic cases, total bile pigment levels in their plasmas were negligible. Bilirubinuria and haemoglobinuria were not seen in any of them.

Plasma bile pigment levels found in the severe early cases are indicated in Table 71. All the animals concerned were severely icteric. Sheep FB–12 was *in extremis* when examined and intensely icteric. This case is typical of the acute form of the disease in which there is an explosive haemolytic crisis followed very rapidly by death. The plasma bile pigment is almost exclusively bilirubin as can be seen from Table 71. Urine, obtained from the case at autopsy, contained some bilirubin glucuronide, traces of urobilinogen and considerable amounts of haemoglobin.

TABLE 71.—*Plasma bile pigment levels in severe early cases of enzootic icterus*

(Values are mg %)

Sheep No.	Total Bilirubin	Bilirubin glucuronide	Bilirubin
Bekker 1	15·1	9·0	6·1
Bekker 2	7·8	5·0	3·8
Bekker 4	6·1	3·0	3·1
FB-12	36·3	0·5	35·8
F-6	13·1	7·8	5·3
F-8	5·2	2·5	2·7

The other cases shown in the table were less severe acute cases of the syndrome, but cases which nevertheless would soon have had a fatal termination, had they not been sacrificed for obtaining specimens for tissue analyses and histopathological work. The presence of large amounts of conjugated bilirubin in the plasma of most of these cases is noteworthy. As will be seen later in this chapter some of these animals e.g. Sheep Bekker No. 1 and FB–12 showed evidence of renal insufficiency. This was however not a general finding. Bilirubinuria was negligible in these animals and bile salts were in general absent from the urine. It is evident from these data that although a haemolytic icterus dominates the haematological and clinical pictures, there is considerable interference in the biliary and secondary renal excretion of bilirubin.

Plasma total bilirubin levels in the chronic past-haemolytic cases ranged from traces to 0·4 mg per cent and in the Group 2 cases (mild cases) generally from traces to 0·6 mg per cent. In all these instances the plasma bile pigment was almost exclusively bilirubin. Urobilinogen was present in their urines in the usual small amounts, no bilirubinuria or haemoglobinuria were seen and bile acids were generally detectable in trace amounts. Sheep FB–9 and F–12 were slightly more severe cases and the following figures regarding plasma bile pigment levels were obtained form them:— Sheep FB–9: total bilirubin 2·5 mg per cent, bilirubin glucuronides 0·5 mg per cent and bilirubin 2·0 mg per cent. Sheep F–12: total bilirubin 2·3 mg per cent, bilirubin glucuronides 0·4 mg per cent and bilirubin, 1·9 mg per cent. These cases were thus typical of a low-grade haemolytic syndrome.

The severe chronic cases showed total plasma bilirubin figures ranging from 0·01 to 0·25 mg per cent, all of which was bilirubin. Urine analysis revealed no particular abnormalities of bile pigment metabolism.

Plasma bile acid levels were not determined in any of the cases being reported here.

(*b*) *Porphyrin metabolism*: No study of porphyrin metabolism in enzootic icterus was made during these investigations. It is obvious from the clinical symptoms described earlier in this chapter and in Appendix 6 that many acute or chronic cases of enzootic icterus are mildly photosensitive. The chronic cases particularly,

31610—10

often show considerable superficial necrosis of the exposed skin on the face, ears and limbs. Any disturbances of porphyrin metabolism which exist in the disease must be considerably milder than they are in geeldikkop.

(*c*) *Bromsulphalein* (*BSP*) *metabolism*: BSP tests were performed on a number of cases of different stages of the disease. The results are presented in Table 72.

TABLE 72.—*Bromsulphalein* (*BSP*) *retention in typical cases of all stages of enzootic icterus*

Sheep No.	Nature of case	% BSP retained 30 minutes after injection
2206	Early case: Group 1: Mild	5·0
2207	,, ,, ,, ,,	6·0
2208	,, ,, ,, ,,	4·6
12223	,, ,, ,, ,,	12·8
12224	,, ,, ,, ,,	19·2
12225	,, ,, ,, ,,	51·0
12228	,, ,, ,, ,,	10·4
12229	,, ,, ,, ,,	18·9
FB-12	Early case: Group 2: Severe	1·0
F-6	,, ,, ,, ,,	11·3
F-8	,, ,, ,, ,,	27·5
F-9	Chronic case: Group 1: " Post haemolytic "	0
F-10	,, ,, ,, ,, ,, ,, ,,	45·0
F-11	,, ,, ,, ,, ,, ,, ,,	50·0
12226	,, ,, ,, ,, ,, ,, ,,	100·0
12227	,, ,, ,, ,, ,, ,, ,,	25·0
F-7	Chronic case: Group 2: Mild	31·6
F-12	,, ,, ,, ,,	40·0
FB-14	,, ,, ,, ,,	3·0
FB-3	Chronic case: Group 3: Severe	25·0
FB-7	,, ,, ,, ,,	5·0
FB-8	,, ,, ,, ,,	10·0

The figures given in this table represent the percentage of the test dosed retained in the blood circulation at the end of the 30 minute test period. It is evident from these data that interference with BSP clearance from the blood is present from the earliest stages of the disease. The percentage retention was variable but very high in many instances. Unlike geeldikkop, enzootic icterus is characterized by interference with BSP *clearance* from the blood which may be equally severe in any of the forms or stages of the syndrome. Sheep 12226 is remarkable in that it failed to clear any of the injected BSP during the 30 minute test period. This finding is not consistent with either the chemical pathology or the histopathology of this case—in fact, as will be shown later, the high figures shown by many of these cases are not consistent with the rest of the pathological or chemical studies. Sheep FB–14 gave a figure of only 3 per cent retention of the injected dose. This sheep urinated during

the test period, passing dark red urine. Some of this was collected to examine it for haem pigments. It was found that the dark red colour was due to the presence of large amounts of BSP (pH of the urine was 8·2). It was evident that this mild chronic case eliminated what must have been the greater part of the BSP dose through the kidneys instead of through the liver.

(*d*) *Plasma protein levels and tests dependent upon protein metabolism*: Plasma protein levels were determined in the majority of cases studied. The results are presented in Tables 73 and 74. The values for albumins and globulins given in Table 73 were determined by the salt precipitation method used in the initial stages of this work (see Chapter 4 for a discussion on this point), while those given in Table 74 were determined by filter paper strip electrophoresis.

TABLE 73.—*Plasma proteins in cases representing all stages of enzootic icterus*

(Values are gm%. TPP=total plasma protein; Alb=albumins; Glob=globulins; A:G=albumin: globulin ratio)

Sheep No.	Nature of case	TPP	Alb	Glob	A:G
12223.....	Early mild case.....	7·00	—	—	—
12224.....	„ „	7·38	3·86	3·52	1·09
12225.....	„ „	7·63	3·23	4·40	0·73
5114.....	„ „	5·00	2·30	2·70	0·85
5119.....	„ „	5·10	2·00	3·10	0·64
5126.....	„ „	5·00	2·10	2·90	0·72
5131.....	„ „	4·50	2·20	2·30	0·95
5132.....	„ „	7·80	3·60	4·20	0·85
5117.....	„ „	5·50	1·80	3·70	0·48
5118.....	„ „	4·60	2·20	2·40	0·91
5120.....	„ „	5·20	1·90	3·30	0·57
5127.....	„ „	4·70	1·80	2·90	0·62
FB-12.....	Early severe case.....	6·60	—	—	—
F-6.....	„ „	6·75	2·85	3·90	0·73
F-8.....	„ „	7·05	3·39	3·66	0·92
F-9.....	Chronic "post-haemolytic".....	6·32	3·34	2·98	1·12
F-10.....	„ „ „ „	7·00	2·92	4·08	0·71
F-11.....	„ „ „ „	7·72	—	—	—
NB-2.....	Chronic mild case.....	7·00	4·00	3·00	1·33
NB-3.....	„ „	7·56	2·82	4·74	0·59
NB-4.....	„ „	7·90	4·40	3·50	1·25
NB-5.....	„ „	7·72	3·00	4·72	0·63
F-7.....	„ „	7·00	3·50	3·50	1·00
F-12.....	„ „	6·50	2·92	3·68	0·79
FB-9.....	„ „	5·20	—	—	—
FB-14.....	„ „	7·00	—	—	—
FB-16.....	Chronic severe case.....	7·80	—	—	—

TABLE 74.—*Plasma protein fractions determined by filter paper electrophoresis on plasma from cases of different stages of enzootic icterus*

(Values are gm%. TPP=total plasma protein; Alb=albumins; Glob=total globulins; $\alpha+\beta=\alpha$ and β-globulins; $\gamma=\gamma$-globulins; Und= undifferentiated fractions; A:G=albumin:globulin ratio)

Sheep No.	Nature of case	TPP	Alb	Glob	$\alpha+\beta$	γ	Und	A: G
12224.....	Early mild case.....	7·38	1·87	5·25	1·66	3·59	0·24	0·35
12225.....	,, ,, ...	7·63	2·60	4·46	1·43	3·03	0·55	0·58
12228.....	,, ,, ...	7·20	2·52	4·35	1·39	2·96	0·32	0·57
12229.....	,, ,, ...	7·50	2·26	4·66	1·40	3·26	0·57	0·48
Bekker 3..	,, ,, ...	7·47	2·92	4·55	2·17	2·38	0	0·64
F-6.....	Early severe case.....	6·75	2·54	3·38	1·51	1·87	0·81	0·75
F-8.....	,, ,, ...	6·39	3·14	2·61	1·34	1·27	0·64	1·20
Bekker 1..	,, ,, ...	9·62	3·29	6·33	3·46	2·87	0	0·51
Bekker 2..	,, ,, ...	7·42	2·42	5·00	2·14	2·86	0	0·48
Bekker 4..	,, ,, ...	7·56	2·52	5·04	2·00	3·04	0	0·52
F-9.....	Chronic " post-haemolytic "...	6·32	2·05	3·34	1·39	1·95	0·91	0·61
F-10.....	,, ,, ,, ,, ...	7·00	2·28	4·02	1·20	2·82	0·69	0·56
F-11.....	,, ,, ,, ,, ...	7·72	1·83	4·28	1·14	3·14	1·60	0·42
12226.....	,, ,, ,, ,, ...	6·75	2·46	4·14	1·48	2·66	0·14	0·59
12227.....	,, ,, ,, ,, ...	6·82	2·32	4·28	1·66	2·62	0·21	0·54
F-7.....	Chronic mild case.....	6·75	1·98	3·93	1·34	2·59	0·82	0·50
F-12.....	,, ,, ...	6·50	2·06	3·43	1·41	2·02	1·01	0·60

Plasma protein levels were below the normal limits for this blood constituent (Chapter 4) in Sheep 5114, 5119, 5126, 5131, 5117, 5118, 5120 and 5127. This finding as will be shown shortly, is due probably to water retention and is associated with the severe reaction to stress in these animals. As pointed out earlier under the haematology of enzootic icterus, total plasma protein concentrations were within normal limits in the majority of cases. Some of these animals presented slightly lowered albumin, and somewhat elevated globulins by the salt precipitation method. Apart from those of some of the stressed cases, A : G values were generally within normal limits or slightly lowered, according to this method.

Electrophoretic separation of the plasma proteins was performed on plasma samples from a number of the cases studied (Table 74). The results obtained with the samples from Sheep 12224, 12225, F6, F8, F9, F7, F10, F11 and F12 make an interesting comparison with those obtained by the salt precipitation method (Table 73). The two methods give quite different results for the albumins and globulins; those obtained by the electrophoretic technique are more reliable. The method showed a marked reduction in albumin levels in most cases of the disease with a corresponding increase in the globulin fraction. A: G ratios were thus in actual fact considerably below normal in most cases. The $\alpha+\beta$-globulin fraction was increased in many cases of all stages of the disease. Gamma globulins were markedly elevated in all the early mild cases, in most of the severe early cases and in nearly all the chronic cases. The attention of the reader is focused on the fevers shown by some of these cases when first examined (Appendix 6).

Typical electrophoretograms representing various stages of the disease are shown in composite Fig. 9. The clearly defined $\alpha+\beta$ globulin peaks and the marked increase in γ-globulins are quite obvious.

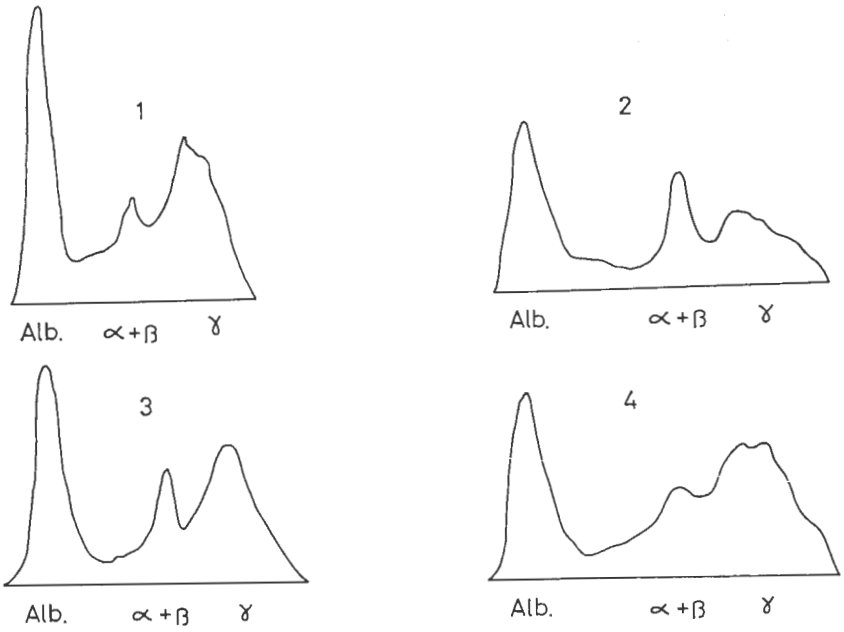

FIG. 9.—Plasma protein electrophoretograms found in typical cases of enzootic icterus:

1 = early prodromal case, sheep 12228;
2 = early acute case, sheep F6;
3 = chronic post-haemolytic case, sheep F10;
4 = mild chronic case, sheep F7.

Thymol turbidity and flocculation tests, zinc sulphate turbidity and colloidal gold flocculation tests were done on plasma samples from all the animals used in these studies. Thymol turbidity and zinc sulphate turbidity readings ranged from 0·2 to 3·8 and 0·65 to 5·5 respectively in all stages of the disease. The colloidal gold flocculation test gave similarly negative results in all the animals studied. Positive thymol turbidity tests were obtained in all of the severe chronic (Group 3) cases, but in none of the cases representing the other stages of the disease. The positive reactions were no more than one + in all instances.

(*e*) *Plasma total cholesterol levels*: These were determined on samples from a number of cases representing all stages of the syndrome. The results appear in Table 75. Moderately elevated levels of this plasma constituent were obvious in most of the early (mild) cases of the disease and in most of the chronic " post-haemolytic " cases. Normal values were apparent in the other cases studied.

(*f*) *Plasma alkaline phosphatase* (*AP*), *glutamic oxalacetic transaminase* (*GOT*) *and glutamic pyruvic transaminase* (*GPT*) *activity levels*: The relevant data are presented in Table 75. The levels of AP activity were generally within the normal limits given earlier (Chapter 4). Moderate elevations were seen in only two Sheep (F–11 and F–7).

TABLE 75.—*Plasma cholesterol* (*Chol*), *alkaline phosphatase* (*AP*), *glutamic oxalacetic transaminase* (*GOT*), *glutamic pyruvic transaminase* (*GPT*) *and iron* (*Fe*) *levels in cases of different stages of enzootic icterus*

(Cholesterol values are mg%, Fe values are mcg% and the enzyme activity levels are in the units defined in the methods used)

Sheep No.	Nature of case	Chol	AP	GOT	GPT	Fe
12223.....	Mild early cases (Group 1).....	223	7·8	132	86	113
12224.....	,, ,, ,, ,, ...	255	11·7	132	79	133
12225.....	,, ,, ,, ,, ...	273	7·4	108	89	96
12228.....	,, ,, ,, ,, ...	305	—	—	—	107
12229.....	,, ,, ,, ,, ...	259	—	—	—	120
Bekker 1..	Severe early cases (Group 2).....	—	—	254	73	—
F-6.....	,, ,, ,, ,, ...	250	6·7	267	42	299
F-8.....	,, ,, ,, ,, ...	246	10·9	311	98	407
F-9.....	Chronic " post-haemolytic " cases	227	19·1	212	39	172
F-10.....	,, ,, ,, ,, ,, ..	232	16·5	156	60	318
F-11.....	,, ,, ,, ,, ,, ..	300	32·2	282	60	220
12226.....	,, ,, ,, ,, ,, ..	268	—	—	—	356
12227.....	,, ,, ,, ,, ,, ..	282	—	—	—	133
NB-2.....	Mild chronic cases.....	86	—	220	42	220
NB-3.....	,, ,, ...	124	—	261	54	140
NB-4.....	,, ,, ...	124	—	89	36	120
NB-5.....	,, ,, ...	157	—	311	36	260
F-7.....	,, ,, ...	223	28·2	212	70	269
F-12.....	,, ,, ...	209	18·2	183	82	202

GPT levels were mildly elevated in one instance only (Sheep F–8) and within normal levels in the rest of the cases, GOT activity was elevated in the plasma of about half the cases studied only. None of the mild cases studied showed this abnormality, but it was seen in the three severe early cases: Sheep Bekker 1, F6 and F8; in one chronic post-haemolytic case (Sheep F11) and in half the mild chronic cases noted in Table 75.

(*g*) *Plasma iron levels*: These were determined in the cases shown in Table 75 where the results are also presented. Normal values were found in the mild early cases, but moderate to marked elevations were seen in the severe early cases and some of the " post-haemolytic " and mild chronic cases (Sheep F6, F8, F10, 12226, NB–5 and F–7, respectively). These elevated levels do not correlate particularly well with the severity of the haemolytic syndrome indicated by the data in Appendix 7, nor are they associated constantly with elevated GOT levels or severe BSP retention.

(*h*) *Blood sugar levels*: Determinations were done on samples from the animals listed in Table 76, in which the results also appear. A mild hyperglycaemic tendency is evident in at least half the early mild and severe cases and in about half the mild

chronic cases. Extremely high blood sugar levels were found in the three severe chronic cases, Sheep FB–3, FB–7 and FB–8. The first two of these were *in extremis* when examined and bled and the latter sheep was in a very weak condition. As mentioned earlier terminal hyperglycaemia is a common finding in many ovine diseases. Urine was obtained from all three of these animals at autopsy but in no instance was a glycosuria obvious. Marked elevations of blood sugar were seen terminally in some of the severely stressed animals, Sheep 5114 to 5132, but glycosuria was observed only in Sheep 5114 shortly before death.

Ketosis was a terminal complication in Sheep 5131 and 5127 as judged by the ketonuria found in these animals shortly before death. Sheep F12 showed a marked ketonuria in spite of its apparently normal blood sugar level.

Glucose tolerance experiments were not conducted on any of the enzootic icterus cases studied. The early and some of the chronic cases showed the same hyperglycaemic tendency as is seen in geeldikkop. The non-occurrence of glycosuria in severely hyperglycaemic animals is remarkable.

TABLE 76.—*Blood sugar and plasma ascorbic acid levels in cases of different stages of enzootic icterus (Values are mg%)*

Sheep No.	Nature of case	Sugar	Ascorbic acid
2206.....	Mild early case (Group 1).....	73·8	—
2207.....	,, ,, ,, ,, ...	85·0	—
2208.....	,, ,, ,, ,, ...	60·0	—
12223.....	,, ,, ,, ,, ...	61·5	0·63
12224.....	,, ,, ,, ,, ...	57·0	0·58
12225.....	,, ,, ,, ,, ...	44·0	0·62
12228.....	,, ,, ,, ,, ...	62·5	0·66
12229.....	,, ,, ,, ,, ...	74·5	0·66
FB-2.....	,, ,, ,, ,, ...	84·0	—
Bekker 1..	Severe early case (Group 2).....	85·0	—
F-6.....	,, ,, ,, ,, ...	57·0	6·0
F-8.....	,, ,, ,, ,, ...	76·5	1·44
F-9.....	Chronic " post-haemolytic " case.....	64·0	1·57
F-10.....	,, ,, ,, ,, ,, ...	38·0	0·39
F-11.....	,, ,, ,, ,, ,, ...	35·0	0·63
12226.....	,, ,, ,, ,, ,, ...	52·0	0·64
12227.....	,, ,, ,, ,, ,, ...	50·0	0·65
NB-2.....	Mild chronic case.....	26·0	—
NB-3.....	,, ,, ...	75·0	—
NB-4.....	,, ,, ...	48·5	—
NB-5.....	,, ,, ...	44·0	—
F-7.....	,, ,, ...	79·5	2·24
F-12.....	,, ,, ...	42·0	0·56
FB-9.....	,, ,, ...	82·5	—
FB-3.....	Severe chronic case.....	102·0	—
FB-7.....	,, ,, ...	205·1	—
FB-8.....	,, ,, ...	117·6	—

(*i*) *Plasma ascorbic acid levels*: These were determined on some of the cases studied. The results are also presented in Table 76. Values found for the control animals were in the same order as those shown in Table 52 for the control animals used in the studies on geeldikkop, i.e. 0·45 to 1·99. " Normal " values were found in most of the enzootic icterus cases shown in Table 76. The only elevations in plasma levels were seen in Sheep F6 (a severe early case) and Sheep F–7 (a mild chronic case).

5. *The erythrocyte in enzootic icterus*

Red cell fragility, methaemoglobin reduction and glutathione levels were studied in the erythrocytes of a number of cases representing different stages of the disease. The results are presented in Table 77. Fragility was found to be markedly increased

TABLE 77.—*Studies on red cell fragility, methaemoglobin reduction and glutathione levels in the erythrocytes of cases of various stages of enzootic icterus*

(Fragility is % fragility in 0·7% NaCl; MetHb reduction is % unreduced at end of test period)

Sheep No.	Nature of Case	Fragility %	MetHb reduction %	Glutathione mg%
2206	Early mild case (Group 1)	50·0	—	—
2207	" " " "	90·0	—	—
2208	" " " "	50·0	—	—
15065	" " " "	11·4	7·60	30·0
Klopper 1	" " " "	53·0	73·6	—
12223	" " " "	31·1	31·3	12·0
12224	" " " "	20·0	—	9·0
12225	" " " "	10·0	—	10·5
12228	" " " "	14·4	83·7	14·0
12229	" " " "	36·1	86·6	14·0
Bekker 3	" " " "	93·5	50·2	16·9
15064	" " " "	22·8	67·1	21·7
Bekker No. 1	Early severe case (Group 2)	98·7	64·7	11·3
Bekker No. 2	" " " "	85·7	34·4	16·6
Bekker No. 4	" " " "	96·0	36·4	19·7
F-6	" " " "	80·6	55·8	4·5
F-8	" " " "	88·4	47·9	12·0
F-9	Chronic " post-haemolytic " case	15·1	27·8	12·0
F-10	" " " " "	63·2	22·3	10·5
F-11	" " " " "	94·1	5·2	12·0
12226	" " " " "	21·8	20·8	14·0
12227	" " " " "	53·7	16·6	12·0
F-7	Chronic mild case	10·8	71·7	10·5
F-12	" "	17·1	59·9	12·0

in the severe early cases of the disease and in some of the apparently mild early cases as well. This is in accordance with earlier observations (Grosskopf, 1958). Elevated values or values in the upper part of the normal range (7·5 to 52·0 per cent for 80 per cent of Karoo animals) were more general in the " post-haemolytic " cases. Normal values were found in many of the early "mild" and the chronic "mild" cases.

Methaemoglobin reduction was found to be impaired in individual animals, mainly amongst the early mild cases (e.g. Sheep Klopper 1, 12228 and 12229). In general glutathione levels were within the range found for the control animals (viz. 3·5 to 17·5 mg per cent). Occasional high values were found amongst the early cases, e.g. Sheep 15065 and 15064.

6. *Kidney function in enzootic icterus*

Whole blood or plasma levels of the various non-protein nitrogenous substances of importance in this respect, inorganic phosphate and magnesium have been determined on samples from a number of cases representing all stages of the disease. The results are presented in Table 78. Normal blood levels of urea nitrogen were

TABLE 78.—*Levels of non-protein nitrogenous substances, inorganic phosphate and magnesium in the blood of cases of various stages of enzootic icterus*

(BUN=blood urea nitrogen; Cn=plasma creatinine; UA=plasma uric acid; AAN=plasma amino acid nitrogen; PO_4=plasma inorganic phosphate; Mg=plasma magnesium. All values are mg%)

Sheep No.	Nature of case	BUN	Cn	UA	AAN	PO_4	Mg
2206.....	Early mild case (Group 1).....	15·0	—	—	—	—	—
2207.....	,, ,, ,, ,, ...	14·8	—	—	—	—	—
2208.....	,, ,, ,, ,, ...	13·5	—	—	—	—	—
12223.....	,, ,, ,, ,, ...	18·4	0·8	8·0	7·8	2·4	2·5
12224.....	,, ,, ,, ,, ...	16·6	0·7	7·9	6·0	1·5	2·2
12225.....	,, ,, ,, ,, ...	20·2	0·7	8·0	6·5	3·2	2·0
12228.....	,, ,, ,, ,, ...	27·6	1·7	8·2	8·2	3·6	1·7
12229.....	,, ,, ,, ,, ...	36·8	1·6	7·6	6·5	1·9	3·5
FB-2.....	,, ,, ,, ,, ...	53·4	—	—	—	—	—
Bekker No. 1	Early severe case (Group 2).....	57·0	—	7·8	—	—	—
FB-12.....	,, ,, ,, ,, ...	47·8	—	—	—	—	—
F-6.....	,, ,, ,, ,, ...	23·9	0·9	2·4	9·5	4·6	1·3
F-8.....	,, ,, ,, ,, ...	26·7	1·5	2·8	9·8	3·4	1·1
F-9.....	Chronic " post-haemolytic ".....	16·6	0·9	3·9	9·3	4·0	1·3
F-10.....	,, ,, ,, ,, ...	33·1	0·5	7·8	9·1	3·5	2·5
F-11.....	,, ,, ,, ,, ...	27·6	0·6	13·5	8·7	3·9	2·4
12226.....	,, ,, ,, ,, ...	25·8	1·1	3·3	8·0	5·4	2·1
12227.....	,, ,, ,, ,, ...	20·2	1·2	3·7	7·4	7·2	2·1
NB-2.....	Chronic mild case (Group 2).....	37·0	0·8	1·8	6·0	7·0	3·5
NB-3.....	,, ,, ,, ,, ...	33·5	2·2	3·3	8·6	7·0	4·3
NB-4.....	,, ,, ,, ,, ...	24·5	1·2	2·5	6·8	7·3	4·3
NB-5.....	,, ,, ,, ,, ...	19·5	1·4	4·7	8·8	7·0	3·0
F-7.....	,, ,, ,, ,, ...	24·8	0·8	2·0	7·9	3·9	1·5
F-12.....	,, ,, ,, ,, ...	18·4	0·6	13·0	8·2	3·7	2·4
FB-9.....	,, ,, ,, ,, ...	20·2	—	—	—	—	—
FB-11.....	,, ,, ,, ,, ...	18·4	—	—	—	—	—
FB-14.....	,, ,, ,, ,, ...	12·0	—	—	—	—	—
FB-3.....	Chronic severe case (Group 3).....	112·2	—	—	—	—	—
FB-7.....	,, ,, ,, ,, ...	147·2	—	—	—	—	—
FB-8.....	,, ,, ,, ,, ...	51·5	—	—	—	—	—
FB-10.....	,, ,, ,, ,, ...	179·4	—	—	—	—	—

generally encountered in the mild early cases. Increased levels were apparent in two cases only: Sheep 12229 and FB–2. Fair to markedly elevated levels were found in two of the four severe early cases, in one chronic post-haemolytic case and two of the mild chronic cases (Sheep Bekker 1, FB–12, F–10, NB–2 and NB–3 respectively). Uraemia was a most prominent finding in all the severe (Group 3) chronic cases.

Blood creatinine levels were normal in all the cases shown in this table, but uric acid levels were moderately to markedly elevated in the majority of cases of all stages of the disease.

Total amino acid nitrogen levels were normal in all the early mild cases but elevated in the severe early cases and some of the chronic cases (Sheep F–9, F–10, F–11, NB–3 and NB–5).

An extremely severe uraemic syndrome was a characteristic feature of all the cases produced at Onderstepoort by stressful influences (Sheep 5114 to 5132). The development and severity of this syndrome is indicated by the data presented in Table 79. It is evident from these data that marked renal insufficiency began some

TABLE 79.—*The development of the uraemic syndrome in stress-induced cases of enzootic icterus*

(BUN=Blood urea nitrogen; Cn=Plasma creatinine; values are mg %; days are days after arrival at Onderstepoort)

Sheep No.	Item	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
5114.....	BUN.....	83·9	89·8	82·8	82·4	92·6	81·7	75·4
	Cn.....	1·8	2·0	2·4	2·6	4·2	5·8	5·5
5121.....	BUN.....	72·3	84·6	228·7	233·7	240·1	—	—
	Cn.....	4·7	8·2	9·9	15·6	15·6	—	—
5128.....	BUN.....	74·5	119·2	135·8	143·2	150·2	—	—
	Cn.....	2·4	8·2	6·9	15·6	15·6	—	—
5132.....	BUN.....	90·2	104·9	134·0	180·3	219·0	255·8	—
	Cn.....	2·4	4·2	7·1	8·2	13·2	16·7	—
5119.....	BUN.....	79·1	70·3	71·0	68·1	67·2	67·2	73·2
	Cn.....	2·8	3·4	3·4	—	4·2	4·2	5·2
5126.....	BUN.....	60·2	90·3	67·7	67·7	—	—	—
	Cn.....	1·8	—	—	3·4	—	—	—
5130.....	BUN.....	82·8	78·6	84·7	121·4	75·4	75·4	80·6
	Cn.....	3·2	2·3	3·5	4·5	2·6	1·8	2·9
5131.....	BUN.....	82·8	72·1	69·9	73·6	62·3	73·2	80·9
	Cn.....	2·3	—	3·2	3·4	—	—	4·7

time during the five day journey of these sheep to Onderstepoort, since urea retention was already marked on Day 1, the day after their arrival. The data obtained from Sheep 5121, 5128 and 5132 indicate the extreme severity which the uraemia and hypercreatininaemia can assume in enzootic icterus. The rapidity of onset of these symptoms is noteworthy.

When first examined in the field Sheep 5116, 5117, 5118, 5120 and 5127 presented blood urea nitrogen levels of 31·3, 23·9, 23·9, 27·6 and 25·8 mg per cent respectively. Table 80 portrays the results of the exacerbation of the haemolytic syndrome in these cases, as far as excretion of urea and creatinine is concerned. The rapid onset of uraemia and hypercreatininaemia is again obvious.

TABLE 80.—*The development of the uraemic syndrome in cases showing a known exacerbation of the haemolytic syndrome in enzootic icterus*

(BUN=blood urea nitrogen; Cn=Plasma creatinine; values are mg%; days are days after arrival at Onderstepoort)

Sheep No.	Item	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
5116.....	BUN.....	63·2	88·0	92·0	—	184·6	—	—
	Cn.....	2·2	—	5·6	—	14·8	—	—
5117.....	BUN.....	45·5	51·0	49·1	47·3	84·6	85·1	81·0
	Cn.....	2·4	1·4	2·9	2·4	3·7	2·9	3·4
5118.....	BUN.....	78·8	88·3	100·2	71·8	71·4	71·8	73·6
	Cn.....	1·4	2·2	5·8	3·9	3·7	10·4	10·4
5120.....	BUN.....	88·0	74·5	61·6	66·2	67·1	66·2	82·4
	Cn.....	1·4	2·2	2·2	3·7	2·9	4·2	4·6
5127.....	BUN.....	69·9	92·0	74·2	83·9	90·1	82·4	83·9
	Cn.....	1·8	3·0	—	2·5	3·7	4·2	3·8

Although the increased plasma creatinine levels followed in general increases in blood urea nitrogen the correlation is not a perfect one as can be seen from Tables 79 and 80. In some instances very high creatinine levels were associated with moderate increases of urea and in others very slight elevations of creatinine were found to accompany comparable blood urea concentrations (see for instance Sheep 5117 and 5118 in Table 80).

Plasma inorganic phosphate and magnesium levels were generally within normal limits in all the animals used in these studies.

Urine was examined whenever possible in the various cases studied. The position regarding urinary bile and haem pigments in these animals has already been discussed as also has the occurrence of glycosuria and ketonuria. Urinary specific gravities varied from 1.002 to 1.042 in the early mild cases with by far most of the samples showing specific gravities in the range 1·002 to 1·010. Urinary pH was generally 7·5 to 8·5 in these cases, but markedly acid in samples from most of sheep 5114 to 5132 just after their arrival at Onderstepoort. Severe albuminuria was seen in these cases during the first three or four days after their arrival at Onderstepoort, but rapidly decreased in severity as the haemolytic and uraemic syndromes became more severe. Albuminuria was seen inconstantly in the early cases examined in the field. Sheep 5118 developed a marked oliguria shortly after its arrival at Onderstepoort, which persisted for most of the first week of its illness.

Urinary specific gravities ranged from 1·020 to 1·026 in the chronic cases and urinary pH from 6 to 8·5. Severe albuminuria was seen in all the severe chronic cases and was invariably accompanied by the presence of desquamated tubule cells, leukocytes and red cell casts.

7. *Adrenal function in enzootic icterus*

Absolute eosinophile counts and plasma electrolyte studies were made on samples from a number of cases representing different stages of the disease. The results are presented in Table 81. Low eosinophile counts were found generally in most cases of the different stages of the disease. In some of the early mild cases (Sheep 5121, 5128 and 5130) these were accompanied by severe hyponatraemia. Mild to moderate hypochloridaemia was evident in some of these animals. Bicarbonate levels were

TABLE 81.—*Absolute eosinophile counts and plasma electrolyte levels in cases representing various stages of enzootic icterus*

(Eos C=eosinophile count per cubic millimetre; Ca values are mg%; other values are meq/L)

Sheep No.	Nature of case	Eos C	Ca^{++}	K^{+}	Na^{+}	Cl^{-}	HCO_3^{-}
12223.....	Early mild.....	20	13·1	5·6	162	93·7	36·0
12224.....	„	180	12·1	6·0	153	98·2	29·0
12225.....	„	280	12·6	5·6	144	89·3	22·0
12228.....	„	20	8·5	5·2	159	98·2	36·0
12229.....	„	40	8·8	8·6	144	89·3	—
5121.....	„	0	—	4·4	127	—	—
5126.....	„	275	—	4·9	148	—	—
5128.....	„	0	—	4·3	129	—	—
5130.....	„	50	—	5·1	126	—	—
F-6.....	Early severe.....	420	8·2	7·1	159	93·7	19·0
F-8.....	„ „	0	8·2	4·4	147	80·4	27·0
F-9.....	Chronic "posthaemolytic"	0	8·6	5·2	120	93·7	26·0
F-10.....	„ „ „ „	0	14·7	6·0	159	98·2	18·0
F-11.....	„ „ „ „	0	11·1	5·6	138	89·3	28·0
12226.....	„ „ „ „	0	8·4	5·4	156	107·1	21·0
12227.....	„ „ „ „	20	9·7	6·1	168	101·6	31·0
NB-2.....	Chronic mild case.....	0	10·0	5·9	144	98·2	28·0
NB-3.....	„ „	120	12·6	5·9	156	116·1	24·0
NB-4.....	„ „	440	12·1	6·3	159	98·2	25·5
NB-5.....	„ „	160	12·1	5·9	156	89·8	29·0
F-12.....	„ „	0	10·5	5·6	141	93·7	28·0
5115.....	„ „	50	—	6·0	148	—	—
5123.....	„ „	25	—	4·9	145	—	—

variable in the mild early cases, being elevated in some and low in one (normal levels for the sheep may be taken as being 24·6 to 29·0 meq/L with a mean value of 26·8 meq/L). Potassium varied within the extreme normal range for this plasma element in the mild early cases.

Hypochloridaemia was the only constant finding in the severe early cases and in Sheep F–6 was accompanied by low plasma bicarbonate and hyperkalaemia. Low chloride values and low plasma bicarbonate levels were also the most common

abnormalities in the chronic "post-haemolytic" cases. Potassium levels were generally within the extreme normal range in the various chronic cases of the disease. Sodium and bicarbonate levels were within normal limits in the chronic mild cases and in two of these animals only was the plasma bicarbonate low. Apart from a few slightly low values, probably of no significance, plasma calcium values were within normal limits in the different cases of enzootic icterus studied.

It will be noticed from the data presented in Table 81 that apart from Sheep F–9, hyponatraemia was found only in the stress-induced cases of the disease, Sheep 5121, 5128 and 5130. Serial studies on cases of this nature are presented in Table 82.

TABLE 82.—*Electrolyte disturbances in stress-induced cases of enzootic icterus*

(Eos=eosinophile counts/cu. mm; Na and K values are meq/L; TPP=total plasma proteins, gm%)

Sheep No.	Item	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
5114.....	Eos C.....	100	50	50	50	100	50
	Na.....	136	—	142	—	142	—
	K.....	4·2	—	4·1	—	4·0	—
	TPP.....	5·0	4·7	5·4	—	4·8	—
5119.....	Eos C.....	0	0	0	0	0	50
	Na.....	129	134	145	136	—	—
	K.....	4·4	4·3	4·5	3·9	—	—
	TPP.....	5·1	4·9	5·0	4·8	5·1	5·1
5131.....	Eos C.....	125	25	25	0	0	25
	Na.....	140	143	136	136	142	139
	K.....	4·8	4·5	5·5	4·4	5·4	5·0
	TPP.....	4·5	4·3	4·7	4·3	—	—
5132.....	Eos C.....	75	0	25	25	0	—
	Na.....	122	139	136	145	145	—
	K.....	5·0	4·3	4·3	4·3	4·3	—
	TPP.....	7·8	—	7·4	—	5·5	—
5117.....	Eos C.....	225	25	0	0	50	0
	Na.....	137	136	134	139	142	139
	K.....	5·2	5·1	5·1	5·2	5·2	5·1
	TPP.....	5·5	5·1	5·0	5·0	5·5	5·5
5118.....	Eos C.....	25	50	0	25	0	0
	Na.....	148	—	151	—	145	—
	K.....	4·9	—	4·8	—	4·6	—
	TPP.....	4·6	—	4·7	—	4·9	—
5120.....	Eos C.....	150	250	25	175	50	50
	Na.....	145	145	142	136	148	145
	K.....	4·6	4·9	6·8	4·8	5·3	5·5
	TPP.....	5·2	—	5·1	—	5·1	—
5127.....	Eos C.....	25	0	25	0	0	0
	Na.....	134	137	140	129	134	140
	K.....	4·7	5·0	5·0	5·1	4·7	4·4
	TPP.....	4·7	4·5	—	4·8	—	5·2

Low eosinophile counts are either present from the first day of study in these cases or develop rapidly thereafter. Hyponatraemia of varying severity is seen particularly on the first day of study and at variable times thereafter. Potassium levels were generally normal throughout the period of study in these animals. Total plasma protein values are constantly low in most of these cases and appeared to be associated with some degree of water retention.

Plasma electrolyte studies were performed on Sheep 5117, 5118, 5120 and 5127 when they were first examined in the field. Sodium values were in the lowest limits of the normal range in all cases and potassium levels were well within the normal range. Plasma protein concentrations were 7·4, 8·4, 7·8 and 7·2 gm per cent respectively. The exacerbation of the haemolytic syndrome was accompanied thus by a severer reaction to stress than when these animals were seen before.

8. *Copper metabolism in enzootic icterus*

The acute rapidly fatal form of enzootic icterus is very similar in appearance at autopsy to the acute haemolytic episodes seen in copper poisoning in the sheep and it is also very similar to the conditions known as " toxaemic jaundice " and " the yellowses " in Australia, Britain and elsewhere. Because of this similarity a tremendous amount of work at Onderstepoort was devoted to a study of copper metabolism in enzootic icterus during the early stages of research into the disease. None of this work has ever been published since no finality as regards the role of copper in the disease was obtained at that time. The author is indebted to Professor H. P. A. De Boom and G. J. Truter of this Institute for much of the information to be presented

TABLE 83.—*Liver copper levels in confirmed cases of enzootic icterus compared with those from apparently healthy Karoo sheep*

(Values are mg/100 gm wet liver tissue)

Case No.	Nature	Cu	Case No.	Nature	Cu
1	Normal sheep.....	21·4	8	Normal sheep.....	7·7
2	,, ,, ...	32·8	9	,, ,, ...	7·2
3	,, ,, ...	16·8	10	,, ,, ...	37·2
4	,, ,, ...	31·8	11	,, ,, ...	5·1
5	,, ,, ...	7·6	12	,, ,, ...	4·0
6	,, ,, ...	8·4	13	,, ,, ...	10·2
7	,, ,, ...	13·0	14	,, ,, ...	7·7

Range in these apparently normal sheep = 7·2–37·2 mg%
Mean value in these apparently normal sheep = 15·1 mg%

Case No.	Nature	Cu	Case No.	Nature	Cu
A	Acute enzootic icterus	86·0	E	Acute enzootic icterus	42·3
B	,, ,, ,,	90·0	F	,, ,, ,,	116·0
C	,, ,, ,,	60·0	G	,, ,, ,,	50·0
D	,, ,, ,,	70·0	H	,, ,, ,,	152·8

Range in these cases of enzootic icterus = 50·0–152·8 mg%
Mean value in these cases of enzootic icterus = 83·4 mg%

regarding these studies. The data which will be cited have been drawn from his notebooks and those of his former co-workers, G. de Kock, K. C. A. Schulz, J. W. Groenewald and C. W. A. Belonje who studied various aspects of the disease from 1928 to 1958.

De Boom found exceptionally high concentrations of copper and iron in the more vascular tissues of the bodies of cases of enzootic icterus. Concentrations of copper higher than normal were thought to occur in other body tissues as well, with the possible exception of the skeletal system. He found liver copper levels of 10 to 40 mg percent on a wet basis in normal Karoo sheep examined by him, and levels of 60 to 400 mg per cent in the livers of his acute cases of enzootic icterus. The figures shown in Table 83 have been selected at random from the data in his notebooks and are representative of his findings. Table 84 which follows contains data compiled from analyses done for him of the copper and iron content of the body tissues of nine typical cases of enzootic icterus collected on the farm " Witteklip " in the Murraysburg district. It is apparent from these data that copper levels in the livers and kidneys

TABLE 84.—*Combined results of the analyses of the body tissues of nine cases of enzootic icterus for copper and iron*

(The figures given represent the ranges and mean values of the combined data in mg/100 gm wet tissue)

Tissue	Copper	Iron
Liver	21·3–100·0 (64·4)	19·5–39·5 (28·2)
Kidney	7·4–29·5 (18·2)	75·0–126·1 (99·9)
Spleen	1·8–9·0 (4·1)	91·0–305·0 (197·0)
Heart	0·73–7·4 (3·2)	8·75–41·9 (20·7)
Lungs	1·2–4·9 (2·6)	29·0–118–0 (61·6)
Brain	0·85–2·3 (1·4)	2·60–4·5 (3·5)
Muscle	0·22–2·6 (0·75)	4·6–66·0 (17·0)
Pancreas	0·70–3·1 (1·5)	—
Adrenals	0·23–0·49 (0·35)	—
Lymph glands	0·16–1·0 (0·84)	—
Skeletal system (femur, rib, skull, vertebrae)	0·001–0·12 (0·019)	—
Tongue	0·20–0·84 (0·55)	2·5–3·8 (3·0)
Forestomach walls (rumen, reticulum, omasum, abomasum)	0·40–2·1 (0·76)	2·5–4·3 (3·4)
Small intestine (duodenum, jejunum, ileum)	0·25–1·78 (0·4)	1·6–3·0 (2·3)
Large intestine (caecum, colon, rectum)	0·35–2·6 (0·86)	1·6–2·4 (1·9)

were markedly increased as also were the iron levels in the parenchymatous organs of these cases. Copper levels in the rest of the tissues are also somewhat higher than those given for adult sheep tissues in general by Cunningham (1931) and Eden (1943). Plasma total copper levels varied in these cases from 125 to 570 mcg per cent with a mean value of 184 mcg per cent.

Acting on the assumption that enzootic icterus was a chronic copper poisoning, De Boom and his colleagues studied the copper content of the soils and vegetation of the affected farms. Topsoils on these farms were found to contain from 0·28 to 2·84 mg/100 gm depending upon the parent formations, while subsoils were found to contain 0·26 to 2·88 mg/100 gm. During 1958 specimens were taken by officers of the State Geological Survey Department of the various rocks and soils occurring on farms in the Fraserburg and Sutherland districts where the disease was annually severe and where it was unknown. These specimens were analysed spectrographically for copper, and the results were made available to De Boom and the author. No difference could be found between the two groups of farms as regards the copper content of soil or parent rock formations. Dolerite samples were regularly high in copper and contained 18·0 to 33·5 mg per cent of the element. Copper concentrations in sandstones, shale, mudstones and silt were 3·4 to 8·1, 2·3 to 9·6, 2·0 to 2·4 and 3·5 to 3·9 mg per cent, respectively. Soil samples taken from numerous different topographical localities on the farms concerned contained from 1·5 to 5·7 mg per cent. Abnormally high levels of copper were found in only three out of twenty-nine soil specimens, the values concerned being 14·0, 10·0 and 8·7 mg per cent, respectively.

The results of plant analyses for copper done at this time on material taken from affected farms are presented in Table 85. Some of this information was taken from De Boom's notes and some from a report by Walters (1959). The plants noted in the table were part of the dominant vegetation at the time on the farms concerned.

TABLE 85.—*The copper content of some common fodder plants from farms on which enzootic icterus is regularly severe*
(Values are mcg/100 gm dry plant material)

Species	Copper	
	Range	Mean
Tribulus terrestris	630–2500	1530
Phymaspermum parvifolium	1340–1550	1470
Pentzia incana var. *forma*	1380–1900	1750
Tetragonia arbuscula	1340–1730	1500
Salsola glabrescens	1000–2060	1390
Nestlera prostrata	1060–2230	1480
Chrysocoma tenuifolia	1200–3200	1740
Pteronia erythrochaeta	1400–2670	1870
Aridaria splendens	(n=1) 2533	—
Helichrysum pentzioides	(n=1) 2000	—
Osteospermum spinescens	(n=1) 2333	—
Pteronia glauca	(n=1) 1533	—

Walters (1959) has stressed that the copper content of Karoo grazing is dependent on the nature of the soil and its parent formations and seasonal factors. The copper content of some plants may drop by about one-third to half during dry seasons (which are the seasons in which most outbreaks of enzootic icterus occur).

The levels of copper in the natural grazing which can produce chronic copper poisoning in sheep are still the subject of considerable controversy (Russel & Duncan, 1956; Underwood, 1962). The development of intoxication is dependent upon many factors, not the least of which are the form in which the copper is ingested, and the amounts of molybdenum and inorganic sulphate present in the diet. An amount of 30 mg of copper daily produced typical symptoms of toxaemic jaundice *within six months* in experimental animals, but copper storgae was not excessive in sheep on mixed pastures containing 200 to 1040 mcg/100 gm of copper and 30 to 100 mcg/gm of molybdenum if the copper: molybdenum ratio was below about 10 (Russel & Duncan, 1956). Chronic copper poisoning in sheep occurs in Australia under three different sets of conditions:

(*a*) when the copper content of the soils and pastures are high. Under these conditions there is a straightforward high gross intake of copper by the sheep due to the abnormally high copper content of the pasture plants growing on the cupriferous soils of the affected area. Some plants growing on these soils contain as much as 50 to 60 ppm copper;

(*b*) when copper content of soils and pastures are normal. Under these conditions, intoxication is seasonal in occurrence, has only been observed on the more acid soils of the region and is favoured by dominance of the pastures with the clover, *Trifolium subterraneum* L. This plant usually contains 10 to 15 ppm copper and extremely low levels of molybdenum which rarely exceed 0·1 to 0·2 ppm. Such a level of copper in the grazing, coupled with the very low molybdenum, is believed to favour the development of a high copper status in the sheep and to lead to copper poisoning; and

(*c*) in association with liver damage caused by poisoning due to the pyrrolizidine alkaloids present in the plant *Heliotropium europaeum.* Copper retention in heliotrope damaged livers is extremely high due to an increased avidity of the cells for this element, thereby increasing the susceptibility of the sheep to death from chronic copper poisoning. It has been shown that this accumulation of copper in the liver is directly due to liver damage by the heliotrope alkaloids, heliotrine and lasiocarpine, and not just to the high copper content of the plant (Gallagher, 1964; Russel & Duncan, 1956; Underwood, 1962).

As far as is known, none of the conditions listed under (*a*), (*b*) and (*c*) above pertain in the areas where enzootic icterus occurs or are known to be operative in the pathogenesis of the disease. Thus in spite of the somewhat high levels of copper found in the Karoo vegetation, there is little evidence to indicate that this is in fact available *in toto* to the animal. De Boom's control sheep from the Karoo showed a liver copper range of 7·2 to 37·2 mg per cent with a mean value of 15·1 mg per cent (Table 83). The control animals used by the author in his studies on geeldikkop showed a liver copper range of 18·33 to 34·99 mg per cent with a mean value of 25·12 mg per cent (Table 26). In an earlier study the author found liver coppers in normal sheep maintained at Onderstepoort to range from 9·0 to 14·8 mg per cent (Brown, 1963). Liver copper levels are thus undoubtedly higher in Karoo sheep than those in animals raised elsewhere but there is no evidence as yet to indicate that these moderate elevations are in any way harmful to the sheep concerned.

In an earlier study (Brown, 1963) the author reported some figures for plasma copper levels in 23 acute cases and 54 chronic cases of enzootic icterus studied at Onderstepoort. These included studies made by De Boom and his earlier co-workers, and De Boom and the author. The values reported for the acute cases were 210 to

31610—11

790 mcg per cent (mean, 350 mcg per cent) and for the chronic cases, 110 to 250 mcg per cent (mean, 230 mcg per cent). Plasma copper levels found in the animals used for the present studies were (*a*) early cases—Group 1 (mild):— 62 to 83 mcg per cent; (*b*) early cases—Group 2 (severe):— 83 to 92 mcg per cent; (*c*) chronic " post-haemolytic " cases:— 62 to 87 mcg per cent; and (*d*) chronic mild cases:— 92·3 to 185·0 mcg per cent. No hypercupraemic state was thus apparently present in these latter animals at the time of examination.

The development of hypercupraemia during acute attacks of enzootic icterus is illustrated by the data presented in Table 86. It is evident from these figures that the hypercupraemia in enzootic icterus is variable in nature. The reader should compare the data given in Tables 68 and 69 with these figures. Hypercupraemia

TABLE 86.—*Plasma total copper levels in stress-induced cases of enzootic icterus*
(Values are mcg %)

Sheep No.	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
5114.....	210	320	410	300	270	—
5119.....	200	180	210	260	270	—
5128.....	820	790	510	360	—	—
5130.....	140	130	110	120	110	—
5117.....	230	260	180	220	150	160
5118.....	170	200	220	240	440	350
5120.....	130	160	180	170	180	200
5127.....	130	150	180	150	160	180

was not observed in many of these cases in spite of the presence of a progressively worsening haemolytic attack and of renal insufficiency, e.g. Sheep 5119, 5117, 5120, 5127 and 5130. It was present in Sheep 5128 from the first day of study to a most severe degree, but in this Sheep and in case 5114 plasma copper levels declined as the anaemia increased in severity. In only one instance was there an inverse relationship between the severity of the anaemia and of the hypercupraemia, viz. Sheep 5118.

Hypercupraemia in enzootic icterus appears thus to be a feature of the acute episodes of the disease. It can be extremely severe but is in general a transient and variable symptom.

The levels of copper in the livers of some of the cases studied during this work are presented in Table 87. The liver samples from Sheep 5116, 5121, 5123, 5128 and 5132 were taken just after they has succumbed to the stress-induced haemolytic syndrome. These values, and indeed those found by De Boom and his earlier co-workers (Table 83) are in the same general order *as those found in the advanced cases of geeldikkop*, namely 25·1 to 158·3 mg per cent (mean 62·5 mg per cent) (Table 24).

TABLE 87.—*Liver copper levels in various cases of enzootic icterus studied during these investigations*

Sheep No.	Nature of case	Liver Cu mg% (wet tissue)
15064.....	Early mild case.....	12·4
15065.....	„ „	66·8
5116.....	Stress-induced exacerbation.....	172·0
5121.....	„ „ acute case.....	78·8
5128.....	„ „ „	121·2
5132.....	„ „ „	201·5
5123.....	„ „ mild chronic case.....	63·2

In fact, although extremely high liver copper values have been encountered in some acute enzootic icterus cases in the past (as De Boom's figures of 60 to 400 mg per cent bear witness), disturbances in copper metabolism are more prolonged and more clearly defined in geeldikkop than they are in enzootic icterus.

9. *Other earlier chemical pathological studies of note*

It was believed by De Kock and Schulz during the course of their earlier studies that acute enzootic icterus attacks may be the consequence of an auto-intoxication following severe gastro-intestinal stasis developed as a result of transport of the animals concerned over long distances by rail. A considerable amount of time and energy was devoted to a study of the blood levels and urine concentrations of phenolic and indigoid compounds in affected animals. Although high blood levels of these compounds and increased urinary excretion were found in some animals with severe gastro-intestinal stasis, the correlation between these findings and the appearance of acute attacks of enzootic icterus was poor. (This information was supplied to me by Professor H. P. A. De Boom).

De Boom took this idea further and carried out an extensive study of the rumen micro-organisms in affected animals and the intra-ruminal production of volatile fatty acids in these cases. Many interesting results were obtained but there was a better correlation between them and the incidence of secondary complications like alkalosis or ketosis than between his findings and intravascular haemolysis.

Grosskopf's contributions regarding the increased erythrocyte fragility in the disease have already been mentioned. It remains to be mentioned here that G. C. S. Roets made a study of the haem pigments in the blood of cases studied by De Kock, Schulz and De Boom and recognized in 1943 the presence of methaemoglobincythaemia in some acute cases and the occurrence of fair amounts of free haemoglobin in the plasma of many of these animals [this information was contained in a note in De Boom's files on the disease and is probably a consequence of De Kock's earlier studies (De Kock, 1928)].

10. *General discussion*

The macroscopic pathology and histopathology of acute attacks of enzootic icterus were described by De Kock in 1928. The pathology of the acute syndrome has been studied extensively since then but apart from one recent paper (Pienaar &

van der Merwe, 1966) nothing further has appeared in print about it. The author is grateful to his colleagues, H. P. A. De Boom, R. C. Tustin and J. G. Pienaar for allowing him to present much of the new information contained in this discussion.

The description by De Kock (1928) of the autopsy findings in acute fatal episodes of the disease contains the following points: severe icterus and anaemia; methaemoglobincythaemia in some cases (this is deduced from his description of the blood in these instances); enlargement and pigmentation of the liver with greyish-yellow zones around the central veins in most cases; marked enlargement and dark reddish-purple pigmentation of the kidneys; tumor splenis, often marked in many cases, with the malpighian bodies and trabeculae of this structure often appearing indistinct; the lymph glands are generally swollen and oedematous and often pigmented; hyperaemia and oedema of the lungs is a general finding and severe impaction of the caecum and colon is seen frequently; haemoglobinuria is a fairly common symptom, while mild hydrothorax and hydropericardium are seen sometimes. In addition to these findings De Boom and the author have observed ulcerations of the abomasum in some cases and marked atrophy of the adrenals and lymphoid tissue in others. Enlargement and pigmentation of the kidneys and the extremely severe gastro-intestinal stasis were the autopsy signs which impressed most. The cortices of the affected kidneys are often intensely brownish-black in colour and the kidneys may be double their normal size. Icterus is generally most intense in these animals.

The most important changes found on microscopic examination of the organs of these cases were found by De Kock (1928) in the liver, kidneys and lymph glands. Extensive alterations, mainly in the centre part of the lobule were found in the liver. The most striking finding in this regard was the presence of large numbers of pigment-bearing macrophages in the affected areas and in Glisson's capsule. These large pigment cells are very characteristic of acute enzootic icterus and have been likened by De Kock (1928) to Gaucher cells. The pigment in them is not haemosiderin nor is it fatty material (De Kock, 1928). Fatty changes which varied in some cases from slight to widespread in others, were seen quite frequently. Many liver cells, particularly those around the central veins, were found to have enlarged nuclei. Bile pigment was generally obvious in the canaliculi.

Pienaar & van der Merwe (1966) re-examined some of De Kock's material during their search for intranuclear inclusions in the hepatocytes of enzootic icterus cases. They found in this material and in other collected subsequently, the presence of intranuclear inclusions in 24·3 per cent of the cases examined. The number of inclusion bodies found varied from very few per section, in most cases, to very numerous. Finely vacuolated, round to oval globules of varying size were also observed intranuclearly in the parenchymal cells of all cases in which inclusions were found. The globules were also present in the majority of cases in which inclusions were absent. Cytomegaly and karyomegaly were striking histological features in nearly every case examined, some of the hepatocytes attaining gigantic proportions. This enlargement of the liver cells was generally more pronounced in the periportal regions of the lobules but in many cases the lobules were found to be more or less uniformly affected. The occurrence of intranuclear inclusions and globules was mainly restricted to these abnormally large nuclei. Pienaar & van der Merwe (1966) concluded that although they could not establish it definitely, the inclusions were probably of a non-viral nature. They pointed out the similarity between the hepatic cyto- and karyomegaly of enzootic icterus and the " megalocytosis " first observed by Bull (1955) and later by others in chronic pyrrolizidine alkaloid intoxication (Peterson, 1965; Schoental & Magee, 1957, 1959). Similar changes have also been seen in aflatoxicosis (Pienaar & van der Merwe, 1966).

Tustin and Pienaar have in private communications to the author stressed that apart from the cytomegaly, pigmentation and macrophage invasion mentioned, the only changes of note in the livers of acute cases are mild centrilobular degeneration or necrobiotic changes which may result from anoxia consequent to the anaemia. An increase in the number of neutrophiles in the sinusoids is often observed in areas affected in this manner.

Thus in acute cases, the visible liver changes are, apart from the cell infiltrations, of a mild nature.

The kidney lesions described by De Kock (1928) included extensive pigmentation with only slight alterations in the parenchyma itself. These included changes of a fatty nature seen as the presence of a few fat or hyaline droplets in the epithelium of occasional tubules. Such changes were never extensive. The pigmentation was large, due to the presence of haemoglobin in the tubular lumens and Bowman's capsules and to haemosiderosis of the tubular epithelium.

Tustin and Pienaar (personal communications) have described the renal lesions in these cases as a nephrosis, in the sense of degenerative changes being present, without evidence of inflammatory reactions. The nephrotic changes found by them included severe degeneration and necrosis of the tubular epithelium and severe pigmentation.

De Kock (1928) was particularly struck by the presence of accumulations of the large pigment-bearing macrophages in the reticulum of the lymph glands of these cases. Haemosiderosis of the sinus cells was commonly observed. In many instances iron-free pigment was present in large amounts. Atrophy of lymphoid tissue was observed in the spleens of some cases. The most commonly seen change in this organ was however extensive haemosiderosis.

Extensive haemosiderosis of the lungs was also seen by De Kock (1928).

The histopathology of Sheep 15064, 15065, Klopper 1, 12223 to 12229, F6 to F12, Bekker 1 to 4 and NB2 to 5 has been studied for the author by R. C. Tustin. The mild early cases amongst these showed essentially the same liver and kidney changes as described above, but in all instances they were very mild. Atrophy of the lymph glands was a common finding. In one case (Sheep 12228) fairly numerous small disseminated foci of replacement fibrosis were observed in the myocardium of the animal. Pigmentation of the liver and kidneys dominated the histopathology of the severe early cases, which was in general as already described. Kidney lesions were in general mild and liver changes included mainly the presence of large numbers of pigment-bearing macrophages around the portal tracts, mild necrobiotic changes of liver cells around the central veins and in one instance mild portal cirrhosis with bile duct proliferation (Sheep F–6). Atrophy and pigmentation of the lymph glands were seen and in one case (Sheep F–6) numerous disseminated foci of degeneration were observed in the myocardium.

The autopsy findings in chronic cases have not been described before. The following are briefly the general changes observed by De Boom and the author during the course of field work on the disease: severe anaemia, cachexia, mild or negligible icterus, mild hydrothorax, hydropericardium and ascites, severe atrophy of the forestomachs and small intestines, impaction of the colon (which may be extremely severe), tumor hepatis and pigmentation of the liver, gall-bladders are generally distended with concentrated bile, mild to severe enlargement and pigmentation of the kidneys, atrophy of the spleen or, in many cases, marked tumor splenis,

atrophy of the cortex of the lymph nodes, with scattered deeply pigmented nodules being present in the cortices of many such glands, degeneration of the myocardium in some instances, pigmentation and oedema of the lungs (in at least 50 per cent of cases seen), degeneration of the adrenal cortex, hyperplasia of the red bone marrow and brownish pigmentation of the skeletal system. In longstanding chronic cases the pigment is often seen more in the cartilages than in osseous tissue. Ulceration of the pylorus was seen in one instance. Many cases have been seen in which there is apparently a hypertrophic cirrhosis present in the liver associated with localized areas of atrophic cirrhosis, particularly around the portal tracts. In early publications some extreme cases have been described in which the entire area around the portal fissure of the liver has been replaced with fibrous tissue giving this organ a dumb-bell appearance (Brown & de Wet, 1962). Such lesions were found in the liver of Sheep FB–14.

The histopathology of these cases is generally similar to that already described, except that all the lesions are much milder. Portal cirrhosis, pigmentation and macrophage invasion are seen mainly in the livers of affected animals, mild nephrotic lesions are present and mild atrophic changes coupled with an accumulation of pigment-bearing macrophages are generally seen in the lymph nodes.

The chronic " post-haemolytic " and mild cases showed essentially the histopathology described in the foregoing paragraph. Foci of degeneration of the myocardium with round cell infiltrations were observed in the heart muscle of sheep F–9, F–10 and 12227.

The liver histopathology of the severe chronic cases is dominated by varying degrees of portal cirrhosis and bile duct proliferation, and cell infiltrations into the portal tracts, the cells concerned being macrophages, lymphocytes or neutrophiles. Kidney lesions were surprisingly mild in these cases and myocardial lesions were not seen.

Although the results of some of the liver function tests used in this work are in keeping with the liver lesions seen in some of the cases studied, there are a number of inconsistencies as regards these tests in general. The BSP test indicated in many cases a marked interference in clearance of this dye from the bloodstream. There was no particular correlation between the degree of retention of the dye in the bloodstream and the stage of the disease. The marked failure to clear the dye from the systemic circulation in early cases like Sheep 12225 and most of the chronic " post-haemolytic " and mild cases was not at all consistent with the actual degree of liver damage seen in these cases, nor with the results of assay of plasma glutamic oxalacetic transaminase levels.

No evidence of interference with bilirubin conjugation is apparent in any of the studies reported here, but there is evidence that the biliary excretion of the conjugates may be considerably impaired in the early cases of the disease (Tables 70 and 71). Renal excretion of the pigment is definitely hindered in these cases. The ratio of bilirubin glucuronide to bilirubin as shown by the data in Tables 70 and 71 is more reminiscent of that seen in geeldikkop rather than of what one would expect to find in an acute haemolytic syndrome. The chronic cases of the disease show a bile pigment picture more typical of a low grade haemolytic syndrome.

Decreased plasma albumin values were associated with the same actual increases in $\alpha+\beta$ and γ-globulins as found in geeldikkop. That these elevated globulin levels may be present from the very start of an attack of enzootic icterus is demonstrated by the data given in Table 74. There is a poor correlation between the

albumin:globulin ratio and the severity of liver lesions in enzootic icterus and the various flocculation tests used are of little assistance in the laboratory diagnosis of the disease.

Moderate elevations of total plasma cholesterol were seen in the early mild and chronic post-haemolytic cases. Such elevations could be associated with an impairment of biliary excretion of cholesterol and an increased liberation of it consequent to the intravascular haemolysis. The same could be said of the plasma iron levels found in the cases studied. Elevated values were evident in all the severe early cases at almost half the chronic " post-haemolytic " and mild cases.

Plasma alkaline phosphatase and glutamic pyruvic transaminase levels showed in general no deviations from normal in the various stages of the disease. Although mild elevations of plasma glutamic oxalacetic transaminase activity were observed in about half the cases studied there was little correlation between this phenomenon and the degree of liver injury as shown by other tests and microscopic examinations.

The hyperglycaemic tendency seen in typical early cases of geeldikkop was observed in at least half the mild and severe early cases of enzootic icterus. Glycosuria was not seen in these cases and plasma ascorbic acid values were generally within normal limits. Ketosis was encountered as a complication in some cases.

Blood urea nitrogen levels in the cases studied in the field were not consistently elevated in the early cases, but the cases produced at Onderstepoort showed a rapidly developing severe uraemia and hypercreatininaemia. It is difficult to establish when the kidney insufficiency actually starts in enzootic icterus in terms of the sequence of symptomatological events, since early cases of this disease are by no means the easily recognizable clinical entities encountered in geeldikkop. It is however certain that embarrassment of kidney function is an early biochemical disturbance in enzootic icterus—its severity is apparently largely dependent on the degree of subsequent metabolic derangements. This is illustrated by general normal levels of plasma creatinine found in the cases studied in the field; the marked elevations of this plasma constituent on artificially induced cases and the unequal degree of urea or creatinine retention shown by these latter cases. Marked uraemia was a prominent symptom in the severe chronic cases.

Hyperuricaemia was a general finding in all stages of enzootic icterus and in many instances was extremely severe. Its severity was not related in many of the early cases to the degree of uraemia. The plasma uric acid elevations were generally as severe as those seen in the corresponding geeldikkop cases. Elevated plasma uric acid levels in the early cases of enzootic icterus may be a better indication of early kidney insufficiency than the plasma urea levels. On the other hand these elevated values may be equally a result of the markedly increased rate of red cell destruction.

Amino acid nitrogen levels were somewhat higher than normal in the blood of many of the early and chronic cases. This is an interesting finding and rather different from what is observed in geeldikkop. Marked disturbances of adrenal function were not as prominent in the early cases of enzootic icterus studied in the field as in the corresponding cases of geeldikkop. Since severe gastro-intestinal stasis is a general symptom in these cases, it may be assumed then that gluconeogenesis from protein sources is active, important and probably not seriously impaired. One wonders then whether these mild elevations of plasma amino acid nitrogen are due to increased formation as a result of this process, coupled with decrease utilization as a result of failure to clear these compounds from the blood at a fast enough rate.

Urine examinations do not generally reflect the onset of renal insufficiency in the early cases, apart from the abrupt cessation of haemoglobinuria in some of the severe cases. Changes in pH in the urine of the early cases are probably related to disturbances in electrolyte balance. Severe albuminuria accompanied by the presence of desquamated tubule cells and casts of different types is generally seen in the severe early and chronic cases and is a logical sequel to the extremely severe nephrotic lesions seen in these cases.

Although hypochloridaemia, changes in the plasma bicarbonate concentration, hyponatraemia and hypokalaemia were seen in *various cases of enzootic icterus examined in the field* (Table 81), these changes were not as general or as severe as those seen in geeldikkop. Normal plasma levels of sodium and potassium were almost general findings in these animals, the only evidence of an adrenal response to stress being in most cases a low absolute eosinophile count and hypochloridaemia.

Mild to severe hyponatraemia, water retention and low eosinophile counts reflected a more definite result of the severe stress imposed on *the cases produced at Onderstepoort* (Table 82).

Hypercupraemia in enzootic icterus is variable in nature and correlates poorly with the degree of haemolysis. Hyperferraemia is a more general symptom in the early and chronic cases of the disease. The marked elevations of the levels of both elements in the soft tissues of these cases indicate the rather sudden release of either (in all likelihood from the increased red cell destruction) and a simultaneous interference in their hepatic and renal excretion. Liver levels of copper are generally the same as found in geeldikkop cases, but the sequence of abnormal events in copper metabolism is more easily discernible in the latter syndrome than it is in enzootic icterus.

The same tetrad of symptoms as seen in geeldikkop, viz. intravascular haemolysis, impairment of hepatic excretion, renal insufficiency and adrenal dysfunction, are seen in enzootic icterus. Basically the disturbances of liver and kidney function commence by being mild in enzootic icterus. Careful consideration of all the results presented so far in this thesis will show that initially the same types of " biochemical blocks " to hepatic and renal excretion are present in both syndromes, but in the case of enzootic icterus they are very transient. If it was not for the severe degree of intravascular haemolysis seen in this condition it would be a very mild syndrome indeed. The severe haemolytic episodes complicate the entire picture and change the nature of the condition to that of a potentially lethal disease. Widespread liver cell death may follow the anoxia in severe cases and this in turn has as its sequelae the invasion of macrophages which is so prominent in this disease and eventual replacement fibrosis. Succeeding non-fatal exacerbations all add their quota of fibrous tissue to the injured liver and eventually the extreme cirrhotic forms described earlier are the result. These changes in themselves are relatively benign and in general hepatic excretory activity is restored early on in the course of an acute attack. Iron and copper excretion may be embarrassed for a longer time, although normally the hepatic excretion of these elements has its peculiar problems in the sheep (Brown, 1967a).

Death in enzootic icterus is largely due to a combination of anoxia as a result of the anaemia, the severe kidney lesions and adrenal insufficiency. The renal insufficiency changes very rapidly from the same type of block seen in geeldikkop, to

a malignant type of nephrosis in the severe cases of enzootic icterus. The transient nature of the renal block to excretion is best seen in the mild cases of this disease in which the subsequent kidney lesions are also generally very mild, as has been shown. Whereas the development of a biliary nephrosis is the general sequel to the renal excretory block in geeldikkop, the nephrosis in enzootic icterus is related directly to the renal ischaemia and the rapid accumulation of free haemoglobin in the tubules of the kidneys. The fact that hyperphosphataemia and hypermagnesaemia are not found in enzootic icterus indicates the transience of the initial block to renal excretion and the different nature of the subsequent nephrosis. Haemoglobinuric renal failure has been discussed in this context at the end of Chapter 7 (q.v.). It is this nephrosis more than anything else which changes the nature of the enzootic icterus syndrome, making it a different clinical entity from the very mild cases of geeldikkop. Seen in this context, the very severe Vosburg cases of the latter syndrome represent a condition falling between the mild cases of enzootic icterus and the typical cases of geeldikkop, which is very closely related biochemically and histopathologically to the acute fatal episodes of enzootic icterus. The sequence of events in these Vosburg cases was clearly defined as a severe haemolytic attack followed by failure of biliary excretion and then renal excretion—the same pattern seen in enzootic icterus.

The chemical pathology of adrenal function in enzootic icterus is important in understanding the pathogenesis of this condition and geeldikkop. In the first instance it is apparent from these studies that marked adrenal insufficiency follows the haemolytic crisis in enzootic icterus. It has been stated in many places in this paper that acute attacks of enzootic icterus can be precipitated by relatively mild stressful stimuli, whereas geeldikkop requires a severe stress for its appearance. The differences in the chemical pathology of adrenal function in the two syndromes illustrate this point very well. Marked adrenal embarrassment is already in evidence when the symptoms of geeldikkop appear.

In conclusion, it is desired to emphasize that two types of enzootic icterus cases with respect to the precipitating factors have been described in this chapter and there are some notable differences between them. In the first instance the cases examined in the field showed little evidence of severe stress, temperatures were observed in many of them, gastro-intestinal stasis was pre-existing and severe and hypergammaglobulinaemia was an almost constant finding from the start of the attack in the cases concerned. Evidence of myocardial lesions was found in many of these cases. The cases produced at Onderstepoort by transport and change of diet represent the second type of case characterized by an acute haemolytic episode and secondary nephrosis with subsequent adrenal failure and evidence of shock in some cases. Gastro-intestinal stasis was secondary to the stress applied and changes in plasma protein concentration were the result of disturbances in water and electrolyte balance. It seems possible from the evidence on hand that an infectious agent of viral but not bacterial nature may have been operative in precipitating the cases seen in the field.

CHAPTER 11

BILIRUBIN CONJUGATES FORMED BY THE SHEEP LIVER

1. *Introductory remarks*

One of the first studies made by the author with regard to geeldikkop was on the nature of the bile pigments retained in the tissues and systemic circulation of affected animals. Shortly before this work was commenced, Cole, Lathe and Billing (Cole & Lathe, 1953; Lathe, 1954; Cole, Lathe & Billing, 1954; Billing, 1955; Billing, Cole & Lathe, 1957) succeeded in isolating three forms of bilirubin from serum, bile and urine of patients with various forms of hepatic insufficiency. Schmid (1956, 1957) followed up this work with his demonstration that the pigments concerned were bilirubin, bilirubin nonoglucuronide and bilirubin diglucuronide. The contemporary studies of Overbeek, Vink & Deenstra (1955) on the coupling of bilirubin with diazonium salts and the spectral characteristics of the resulting azo-hydroxypyrromethene pigments were of considerable assistance in this respect. Shortly after Schmid's initial success Talafant (1956) announced the preparation of the lead salt of bilirubin glucuronide, naming it lead bilirubinyl-bis-(β-glucoside uronate). Reports of studies on the nature of the linkage between bilirubin and glucuronic acid and on the carriage of bilirubin by plasma proteins soon followed (Klatskin & Bungards, 1956; Schachter, 1957a, 1957b) and a method for the differential determination of the bilirubin conjugates and bilirubin in blood plasma soon followed (Schachter, 1959). Dutton and co-workers had successfully developed methods for studying and assaying glucuronic acid conjugating systems in tissues (Dutton & Storey, 1954; Dutton & Greig, 1957) and Grodsky & Carbone (1957) soon applied this work to a study of bilirubin conjugation in rat livers. It was not long before icteric states involving defects in bilirubin conjugation were being studied and reported (Axelrod, Schmid & Hammaker, 1957; Carbone & Grodsky, 1957; Lathe & Walker, 1957; Schmid, Axelrod, Hammaker & Swarm, 1958; Schmid, Hammaker & Axelrod, 1957). Other conjugates of bilirubin which were found to occur in small amounts in the bile of humans and small laboratory animals were sulphates, complexes with taurine, salt forms and other unidentified compounds of minor importance (Isselbacher & MacCarthy, 1959; Jirsa, Vecerek & Ledvina, 1956; Talafant, 1957; Watson, 1958). This exciting period of work on bilirubin was climaxed by the publication of a very elegant method for the preparation of C^{14}-labelled bilirubin and bilirubin glucuronide by Schmid's school (Ostrow, Hammaker & Schmid, 1961).

Using the techniques developed by Cole, Billing & Lathe (1957) and Schmid, (1956, 1957) it was possible to report in 1959 that at least two conjugates of bilirubin, thought to be glucuronides, as well as bilirubin itself, were present in the blood of geeldikkop cases (Brown, 1959). Almost simultaneously Cornelius, Kilgore & Wheat (1960) reported their chromatographic studies on the pigments present in the freshly secreted bile of several species. They could find no free bilirubin or biliverdin in the freshly secreted hepatic bile of sheep and concluded that the pigment present was all bilirubin glucuronide, on the basis of chromatographic data only. The positive identification of the conjugating groups present on ovine bile pigments by conventional chemico-physical techniques other than paper chromatography does not appear to have been reported yet. This chapter contains the results of work in this regard.

2. *Materials and methods*

Fresh liver bile was obtained from adult Merino sheep by cannulation of the common bile duct as described elsewhere in this report and earlier (Brown, 1959c; 1967a). These sheep were generally maintained on green lucerne hay, crushed maize and water given *ad libitum*. Bile samples were collected when required and treated immediately as described below. Samples collected over periods of one hour were used for preliminary identification of the pigments present by chromatographic analysis and spectral absorption techniques (described below). Samples collected in the interim from these animals (in all, seven sheep were used at various times) were deep frozen (—10°C) and used later for the preparation of larger amounts of pigment for the hydrolysis experiments described below.

The various methods used are either noted or described in the appropriate places in the text which follows. All reagents used were of analytical reagent or chromatographic grades and all solvents were redistilled. Pure bilirubin and biliverdin were obtained for comparative purposes from the Sigma Chemical Co., St. Louis, Mo. Chromatographically homogeneous material prepared as described below was used for spectral absorption studies and preparation of pure azobilirubin.

3. *The isolation and preliminary identification of the crude pigments of sheep bile*

Use was made of procedures described initially by Billing (1955), Billing, Cole & Lathe (1957) and Cole, Lathe & Billing (1954). Crude bile pigment was isolated by precipitation with ammonium sulphate (70 gm/100 ml of bile). The mixture was centrifuged and the tightly packed pellet of pigment lifted off the high density fluid below it by means of a glass rod. The pellet was extracted with successive small volumes of methanol; butanol (1:1 v/v) until the extracts were virtually colourless. The extracts were combined and the residue was extracted with 50 per cent v/v aqueous methanol until some turbidity developed in the extracts. These were combined with the previous extracts and the bulk taken just to apparent dryness in a Buchi " rotavapor " rotary evaporator *in vacuo* at 30 to 40°C. Final drying was completed overnight in the dark in an evacuated desiccator over $CaCl_2$ and paraffin wax chips. All operations up to this stage were performed in subdued light.

Preliminary separation of the bile pigments was carried out on columns of siliconized kieselguhr largely by the method of Billing (1955). " Hiflo supercel " (Johns Manville and Co.) previously dried at 110°C and then cooled was treated with

dichlorodimethylsilane vapour and made water repellent as described by Howard & Martin (1950). It was then repeatedly washed with methanol until the washings gave no acid reaction with brom-thymol blue. After drying at 100°C it was stored in airtight bottles until required.

Columns were packed as described by Billing (1955), but the pigment bands were eluted instead of being cut out of the expressed column mass. The following solvent system was employed: $CHCl_3$: CCl_4 : CH_3OH : H_2O : 0·05M phosphate buffer, pH6·0 :: 25 :25 : 38 : 6 : 6 v/v. The phases were separated after thorough equilibration. The columns were packed in 20 × 1·8 cm glass tubes drawn to a fine point at one end. These were fitted with short lengths of polythene tubing and small stopcocks to control the rate of flow. A small plug of glass-wool was placed at the bottom of the tube and covered with a 1 cm layer of washed fine quartz sand. Approximately 6 gm of siliconized kieselguhr were wetted, slurried and finally packed as detailed by Billing (1955). After light tamping down of the column of kieselguhr with a glass rod, the top was covered with a 1 cm layer of quartz sand. The apparatus was placed in a dark cupboard and 5 to 10 mg of dried pigment concentrate dissolved in about 0·2 ml of mobile phase was applied to the top of the column. The chromatogram was developed with the mobile phase at a flow rate of 1 ml/3 minutes and 1 ml samples were collected. Separation proceeded rapidly and sharp pigment bands were obtained. A typical fully developed chromatogram is shown in Fig. 10. The optical density of each 1 ml sample of eluate was determined spectrophotometrically

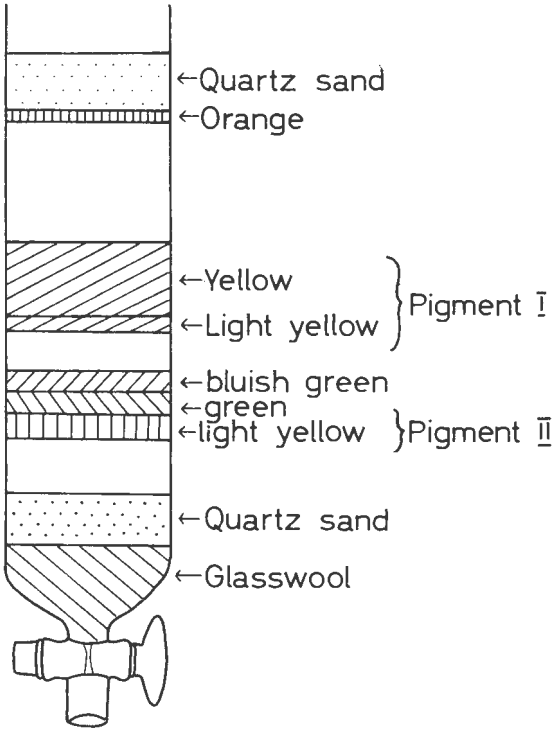

FIG. 10. —A typical fully developed bile pigment chromatogram on siliconized kieselguhr

at 430 m μ and plotted graphically against sample number. A typical result of this nature is shown in composite Fig. 11. Pigment II is obviously present in much greater amounts than Pigment I. To each 1 ml sample 0·5 ml of diazo reagent was added (Malloy & Evelyn, 1937) together with 2·5 ml of ethanol. The optical densities of each sample were determined thirty minutes later at 530 m μ, the spectrophotometer being set at zero with a reagent blank composed of 1 ml pigment free eluate and 0·5 ml of diazo blank solution (Malloy & Evelyn, 1937; Gray, 1953). Optical densities were plotted graphically against sample numbers as before. A typical result is shown in composite Fig. 11. The results of these experiments were in complete accord with

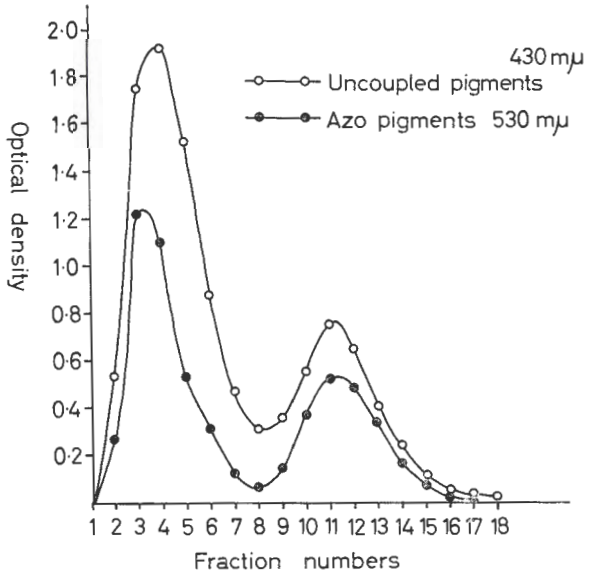

FIG. 11.—Spectral absorption curves of 1 ml eluates before and after diazotization

those obtained by Billing (1955) and Cole, Lathe & Billing (1954) with the pigments human necropsy bile. The same two polar pigments (designated Pigments I and II by these authors) can be demonstrated in sheep bile together with some nonpolar bilirubin which remains at the top of the column and some fastmoving bluish-green pigment which runs in close association with the fast moving Pigment II. This bluish-green component and bilirubin probably form as artifacts during the preliminary extraction procedure or on the column, since Cornelius, Kilgore & Wheat (1960) were unable to demonstrate any unconjugated bilirubin in fresh sheep liver bile, during the course of their chromatographic studies.

Cole, Lathe & Billing (1954) suggested that the polar pigments could be separated better on silane treated kieselguhr if the mobile phase of the system n-butanol: 0·005M phosphate buffer, pH 6·0 : : 1 : 1v/v was used instead of that already mentioned. The eluates containing the polar pigments collected from the chromatographic runs described above were pooled, taken to dryness *in vacuo* at less than 35°C and re-chromatographed on kieselguhr columns using the mobile phase of the system just mentioned. It was found almost impossible to free polar Pigment II from the fast-running greenish-blue material using either of the two systems. It appeared to

form on the column during passage of Pigment II down the column, in spite of performing the chromatographic runs in the dark. The amount of greenish pigment present could be reduced by developing the chromatogram in the refrigerator. This difficulty was also encountered by the earlier workers in this field (Cole, Lathe & Billing, 1954; Cole & Lathe, 1953). The greenish-blue material showed the same chromatographic behaviour and spectral absorption properties as the biliverdin samples obtained from the Sigma Chemical Co. Since it was also non-diazotizable it was assumed to be biliverdin.

Pigment I was obtained chromatographically homogeneous after careful rechromatography as described above.

The spectral absorption characteristics of polar Pigments I and II were examined as described by Cole, Lathe & Billing (1954) and Cole & Lathe (1953) and compared with those of a chromatographically pure sample of bilirubin dissolved in the same solvent. Readings were made at every 10 m μ over the spectral range of 250 to 600 m μ and at every 2 m μ at the peaks of maximum absorption. Absorption maxima found were : Pigment II, 420 m μ; Pigment I, 454 m μ and bilirubin, 450 m μ. These data compare favourably with those given in the earlier work cited with respect to bilirubin (450 m μ) and Pigment I (452 m μ) but differ considerably from these in the case of Pigment II where a peak of 448 m μ was found (Cole, Lathe & Billing, 1954). This discrepancy may be due to the contamination of our preparation by biliverdin.

4. *Paper and thin layer chromatographic separations of the sheep bile pigments*

The azo derivatives of Pigments I and II and pure bilirubin were prepared and chromatographed on paper according to the directions given by Schmid (1956, 1957). The solvent system used to develop the paper chromatograms was one proposed by Schmid (1956), viz. methyl-ethyl-ketone: pronionic acid: water :: 75 : 25: 30 v/v. The same azohydroxypyrromethene derivatives of the three pigments were found as did this author.

In a subsequent series of experiments the azo pigments were prepared directly from freshly secreted bile without any preliminary precipitation and purification of the bile pigments themselves (Schmid, 1957). Bile acids were removed from the reaction mixture subsequently by washing with n-heptane : n-butanol : 7 : 3 and chloroform as suggested by Schmid (1957). The azo pigments were then extracted into n-butanol at pH 4·0, and the extracts taken to dryness *in vacuo* in the dark in a rotary evaporator at $<35°C$. The residue was taken up in 1 ml of 1N acetic acid for paper and thin layer chromatography. Paper separations were done as described above. Thin layer plates were made from Merck's Kieselgel G Cat. No. 7731 ($\pm$ 13 per cent $CaSO_4$) and the solvent system used to develop these chromatograms was n-butanol : glacial acetic acid : 4 : 1. Typical paper and thin layer chromatograms are presented in composite Fig. 12. It is apparent from these chromatograms that besides the two major spots that could be attributed to the azo pigments of the dipyrryl-methenes derived from bilirubin monoglucuronide and bilirubin diglucuronide (Schmid, 1956) at least two other diazo-reacting compounds present in bile can be demonstrated by paper chromatography and from 2 to 4 additional diazo-reacting compounds can be demonstrated on thin layer chromatograms. Watson (1958) prepared bilirubin sulphate and postulated that it could form as a natural conjugate. Its presence in bile together with that of other minor fractions of diazo-reacting material was later demonstrated by Isselbacher & MacCarthy (1959). Jirsa, Vecerek & Ledvina (1956) have found bilirubin taurinide as a minor component of some bile specimens.

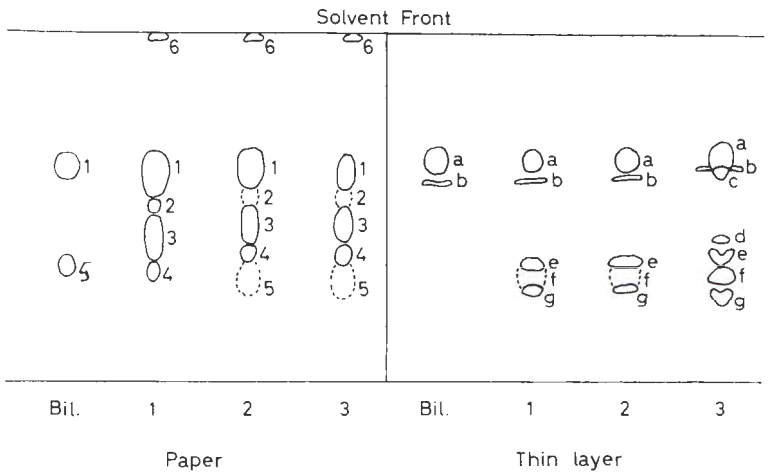

FIG. 12.—Paper and thin layer chromatograms of the azo pigments obtained after direct diazotization in bile without preliminary extraction. (Bil. = pure bilirubin; No. 1, 2, 3 below the chromatograms in both instances refer to different specimens of fresh liver bile. The colours of the spots marked on the chromatograms were 1, 2, 3, a and e = purple; 4, c, d, f and g = pink; 5 and b = yellow; 6 = green. Spots 5 and 6 are thus undiazotized pigments)

5. *Hydrolysis experiments*

Aliquots of the two major pigment fractions collected from the columns described earlier and their azopigments eluted from the paper chromatograms with 1N CH_3COOH or cut out of the thin layer chromatograms and eluted with 0·05N HCl were subjected to hydrolysis by β–glucuronidase (Sigma Chem. Co Type B-3, ex bovine liver) or 1N HCl (in sealed glass tubes at 100°C for 90 minutes) as described by Schmid (1956, 1957). The hydrolysates were decolourized and lyophilized as described by Schmid and tested for glucuronic acid by means of the carbazole reaction and by comparison with authentic glucuronic acid on paper chromatograms. Paper chromatograms were always run in duplicate by the ascending technique using the solvent system, n-butanol : pyridine : water :: 60 : 40 : 30. One chromatogram was sprayed after developing and drying, with a mixture of 0·5 gm benzidine in 20 ml glacial acetic acid and 80 ml absolute ethanol. The other was sprayed after developing and drying first with 0·5 per cent triphenyltetrazolium chloride in chloroform, dried and then sprayed with 0·5 N NaOH in 50 per cent aqueous ethanol. Both chromatograms were heated in a hot air oven for 5 minutes at 100°C to develop the colours concerned.

No difficulty was experienced in demonstrating the presence of material behaving like authentic glucuronic acid in the enzymic hydrolysates of either the Pigments I and II or their azo derivatives by the methods used. Acid hydrolysis however yielded a mixture which reacted poorly with carbazole and gave a spot on the paper chromatograms which differed in its *rf* value and reactions with the colour reagents from that given by glucuronic acid. The compound which formed on acid hydrolysis, judging from the colour reactions on the chromatograms did not condense with benzidine to give the brownish-coloured derivative typical of aldohexoses or hexuronic acids nor did it produce a red formazan with the tetrazolium spray, indicating thus the absence of an aldehyde group, as is present in glucuronic acid. Further chromatographic examination proved the compound which formed to be different from

glucuronolactone and gluconic acid, but identical in respect to *rf* value and colour reactions to glucosaccharic acid. When chromatographically pure glucuronic acid was subjected to heating with a small volume of 1N HCl in a sealed glass ampoule as was done in the case of the hydrolysates, it yielded three spots on paper chromatography with the system described. These corresponded in *rf* values and colour reactions with those produced by glucuronolactone, glucosaccharic acid and glucuronic acid. The lactone appeared to be produced in small amounts and the saccharic acid in larger amounts. Typical chromatograms illustrating these experiments are reproduced in

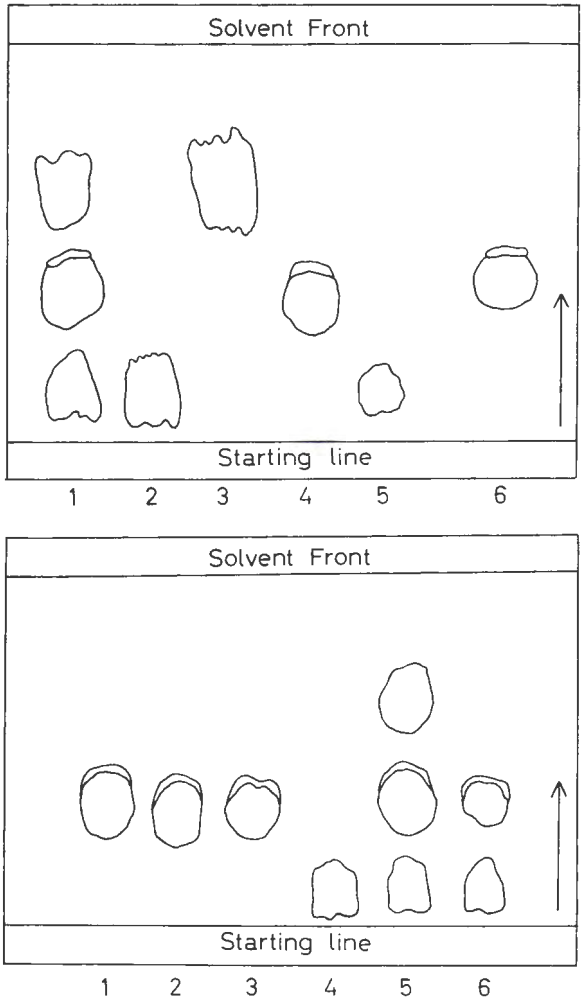

FIG. 13.—Top paper chromatogram is a comparison of reference substances with authentic saccharic acid (1 = glucuronic acid heated with 1N HCl in a sealed tube; 2 = glucuronic acid; 3 = glucuronolactone; 4 = glucosaccharic acid; 5 = gluconic acid; 6 = acid hydrolysate of azo pigment II). Lower paper chromatogram is a comparison between the spots obtained from acid hydrolysates of pigments I and II and reference substances (1 and 2 = fractions of pigment II from columns; 3 = similar fraction of pigment I; 4 = glucuronic acid; 5 = glucuronic acid heated with 1N HCl; 6 = mixture of authentic glucuronic and saccharic acids)

Fig. 13. It was concluded from these experiments that the conjugating group present in Pigments I and II is glucuronic acid. The hydrolysates were tested for the presence of amino acids (Moore & Stein, 1948; Wheeldon & Collins, 1957) and for sulphate (Isselbacher & MacCarthy, 1959; Spencer & Dodgson 1953), with negative results in both instances. Pure gluconic acid and glucosaccharic acid were prepared for this work by the methods described by De Moss, (1957), and Cohen, (1937).

6. *Conclusions*

Bilirubin is excreted by the sheep liver mainly as the glucuronide. At least two other conjugates are present in small amounts of freshly secreted liver bile. No work has been done as yet on the identification of the conjugating groups in these minor fractions. It is possible that sulphate or taurine may be present in these compounds, as is the case in the bile pigments of other species.

CHAPTER 12

Intrahepatic Cholestasis in the Sheep induced with the Icterogenic Pentacyclic Triterpene acids

1. *Introductory remarks*

The discovery of the icterogenic triterpene acids, the early work on their toxicology and the elucidation of their chemical structures have all been described briefly in the first chapter of this thesis. These compounds induce in experimental sheep a clinical syndrome of icterus and photosensitization outwardly similar to geeldikkop. It was therefore logical that a considerable amount of time was devoted to the study of the syndrome evoked by these compounds in experimental animals, and particularly in sheep. The pioneer work commenced by Quin, Rimington and later Roets as

well, was pursued for a while by Steyn and Louw (see Chapter 1) but nearly twelve years were to elapse before the next serious work on the effect of these compounds on the secretion of various components of bile appeared in print (Heikel, *et al.*, 1960). It was shown that the volume of biliary excretion diminished markedly some hours after administration of icterogenin and that the rate of elimination of bile pigment and porphyrins also fell to very low levels. Icterogenin was found to cause no discernible morphological changes which could throw light upon the disturbance in biliary excretory function which followed its administration. One of the highlights of this work was the development of a technique for the biological assay of the icterogenic potency of the triterpene acids. This involved the intra-peritoneal administration of these compounds to rabbits provided with cannulae into the common bile duct and analysis of bile samples collected at regular intervals after administration of the compound concerned (Heikel *et al.*, 1960).

The development of this assay procedure permitted a detailed study of the relationship between the chemical structure of the naturally occurring triterpene acids or their derivatives and their icterogenic activity. Four new icterogenic agents were discovered, namely 22β–angeloyloxyoleanolic acid, 22β–angeloyloxy–hedragolic acid, 22β–angeloyloxy–24–hydroxyoleanolic acid and 22β–angeloyloxy–24–oxo–oleanonic acid. The first two mentioned compounds were found to be extremely active, their potency far surpassing that of icterogenin. Rehmannic acid and Lantadene B were shown to be devoid of activity. Icterogenic potency was shown to be due to the presence of a β–equatorially orientated hydroxyl group at C (3) or a hydroxyl at C (24) and a 22β–angeloyl side-chain on the triterpene molecule. Stereoisomer specificity is shown in respect of icterogenicity of these compounds since the epimers of two of these substances carrying α–axially orientated hydroxyls at C (3) have been shown to have no such effect on bile flow or bilirubin excretion. Removal or saturation of the angelic acid side-chain, substitution of the hydroxyl groups or replacement of these with a ketone function and esterification of the C (28) carboxyl group were followed by loss of activity. In the case of the latter instance, loss of icterogenic potency was probably the result of a decrease in the solubility of the compound (Brown, Rimington & Sawyer, 1963; Brown & Rimington, 1964; Brown, *et al.*, 1963).

The author and his assistants have recently studied some of the biochemical effects produced by icterogenin on the liver of the rabbit. The studies were made at the height of the intoxication, when the effects on bile flow and bilirubin excretion were maximal. Some reduction in the ability of the liver to conjugate bilirubin following intoxication by icterogenin was evident in all the experimental animals. Uridine diphospho–glucose dehydrogenase was found to be either unaffected or increased at the end of the test period. The most noteworthy effects of the intoxication were found to be a marked decrease in the activity of succinic dehydrogenase and glyceraldehyde-phosphate dehydrogenase. Diaphorase, cytochrome C reductase and DPN- ase activities were not significantly affected although some reduction of ATP- ase activity was evident in most cases. It was concluded rather tentatively that the icterogenic triterpenes did not exert any significant effect on mitochondrial respiratory changes but appeared to exert a selective depressant effect on the activity of certain dehydrogenases (Brown, 1964). In a later study the effect of icterogenin on the polyethenoid fatty acid composition of liver cell membranes was followed and it was found to cause a fair to marked decrease in the amount of linolenic acid present particularly in the neutral lipids of the liver cell walls and associated structures. This was found to be associated with a rapid decline in bilirubin excretion (Brown, Wagner & Brink, 1966).

In this chapter the haematology and chemical pathology of icterogenin intoxication in the sheep is described for the first time and the condition is compared with the biochemical disturbances seen in geeldikkop and enzootic icterus.

2. *Animals, materials and methods*

Fifteen sheep were used in the work reported here. They were adult Merino wethers weighing 19 to 25 Kg, maintained on a diet of green lucerne, crushed maize and water all given *ad libitum*. Twelve of these sheep received single oral doses of icterogenin given at dosage levels varying from 100 mg to 600 mg/Kg. The icterogenin was generally dissolved in 25 to 100 ml of warm ethanol and immediately prior to dosing this was diluted with about 300 ml of water. Two animals received split oral doses of icterogenin—the regimens concerned will be detailed later. The remaining animal received the compound by intravenous injection.

The animals were introduced into metabolism cages about two weeks before dosing. Bleeding for baseline values was performed daily for one week before administration of the compound and daily thereafter until subsidence of the clinical symptoms. Urine was collected by means of a bottle placed beneath the sump of the cage and faeces collected in the standard faeces bags. The animals were tested for photosensitivity as described elsewhere in this chapter.

The methods used in this work are amongst those listed in Table 2, or have been described in the appropriate places elsewhere in this thesis.

The icterogenin used in the early stages of this work was either supplied to the author by his former co-workers Drs. P. R. Enslin and W. T. de Kock and Mr. L. A. P. Anderson, then all of the National Chemical Research Laboratories, Pretoria, or else it was prepared by the author himself and his assistants from the freshly collected root bark of *L. rehmanni* (Pears), according to the procedures outlined by Anderson, de Kock & Enslin (1961) and Anderson (personal communications).

3. *The haematology and general chemical pathology of icterogenin intoxication*

The results described in this section were obtained from the sheep given single doses of icterogenin as shown in Table 88. The animals were each studied for a week after administration of the compound.

TABLE 88.—*Details of single doses of icterogenin received by experimental sheep during this work*

Sheep No.	Dosage level mg/Kg	Amount received (gm)
7061	250	5·00
7054	280	7·00
16841	400	10·70
21513	600	12·00
19449	600	12·00
89091	100	2·36
1624	100	2·36
1726	100	1·45
88716	200	4·28
89551	200	3·90
1780	200	5·40
1780A	200	6·40

(*a*) *Haematology*: No changes were observed during the week following dosing in the red cell counts, white cell counts, packed cell volume, haemoglobin, sedimentation rate or absolute eosinophile counts in animals that received doses of icterogenin of less than 200 mg/Kg. Icterus and photosensitization were apparent in all these animals at some time during the test period.

The animals which received a dose larger than 200 mg/Kg developed a *hypocythaemic normocytic normochromic anaemia*, as can be seen from the typical data presented in Table 89. Haematological studies were performed each day for a week after dosing but only the results from the day immediately following dosing, the third day, and the final day of the experiment have been included in this Table to indicate the trend. Although the red cell count, packed cell volume and haemoglobin all

TABLE 89.—*Haematology of the animals that received doses of icterogenin higher than* 200 *mg/Kg*

(Day 1 refers to the day immediately after dosing and Day 7 to the last day of the experiment. RCC=red cell count (millions/cu mm), PCV= packed cell volume (per cent), Hb=haemoglobin (gm per cent), WCC=leukocyte count (thousands/cu mm.)

Sheep No.	Day	RCC	PCV	Hb	WCC	MCH ($\mu\mu$g)	MCHC (%)	MCV (Cuμ)
7061.....	1	7·60	28·0	8·20	5·30	10·78	29·27	36·8
	3	7·53	28·0	7·24	6·40	9·61	25·85	37·2
	7	6·44	21·0	5·55	6·30	8·61	26·42	32·6
7054.....	1	10·19	25·0	8·06	4·75	7·90	32·24	24·5
	3	6·17	24·0	8·20	4·75	13·30	34·16	38·9
	7	7·07	22·0	6·27	4·30	8·86	28·5	31·11
16841.....	1	11·80	33·0	9·79	10·10	8·28	29·66	27·9
	3	10·40	30·0	9·79	11·60	9·41	32·63	28·8
	7	7·10	26·0	9·79	10·00	13·80	37·65	36·6
19449.....	1	8·50	27·0	6·12	7·80	7·20	22·66	31·8
	3	7·50	24·0	5·12	7·70	6·82	21·33	32·0
	7	5·30	22·0	4·87	4·90	9·18	22·13	41·5

decreased proportionately as the experiment proceeded in these animals, no corresponding decrease in total plasma protein levels was generally apparent.

No white cell dyscrasias were observed in any of the animals given single doses of icterogenin, nor did the erythrocyte sedimentation rate show any deviations from normal at any time.

(*b*) *Liver function:* Plasma total bilirubin levels commence to rise from 24 to 48 hours after administration of icterogenin and clinical icterus is generally seen 48 to 72 hours after dosing. The figures presented in Table 90 are typical of the results obtained in this regard with various dosage levels. The *duration* of the block to bilirubin glucuronide excretion by the liver is related to the dosage employed as can be seen from these figures. The *severity* of bile pigment regurgitation and the *intensity* of the clinical icterus are related more to the duration of the effects of icterogenin than to the dosage level. This point is illustrated by the data obtained

TABLE 90.—*Plasma bilirubin levels in cases of icterogenin intoxication*
(Values are mg per cent. Days are days after dosing)

Sheep No.	Dosage level	Days	Total bilirubin	Bilirubin glucuronide	Bilirubin
89091.....	100 mg/Kg.....	1	0	0	0
		2	3·85	2·70	1·15
		3	2·95	1·35	1·60
		4	1·80	0·20	1·60
		5	1·10	0·90	0·20
		6	1·10	0·45	0·65
89551.....	200 mg/Kg.....	1	1·10	0·65	0·45
		2	5·90	3·85	2·05
		3	7·00	4·55	2·45
		4	8·20	6·75	1·45
		5	2·00	1·35	0·65
		6	1·35	0·65	0·70
16841.....	400 mg/Kg.....	1	0	0	0
		2	1·06	0·76	0·30
		3	3·30	2·20	1·10
		4	4·40	2·50	1·90
		5	3·40	2·00	1·40
		6	1·20	0·90	0·30
19449.....	600 mg/Kg.....	1	0	0	0
		2	1·04	0·76	0·28
		3	2·20	1·06	1·14
		4	4·00	2·60	1·40
		5	9·40	5·90	3·50
		6	7·60	6·90	0·70

from Sheep 89551 and 19449 and presented in Table 90. Sheep 88716, not featured in the table, developed the severest icterus seen in this series of experiments. This animal received 200 mg of icterogenin per Kg body weight. The plasma total bilirubin level in this case was 2·7 mg per cent 24 hours after dosing and it then rose steadily to reach a value of 22·3 mg per cent seven days after dosing, after which a slow return to normal over the following week was observed. At least two-thirds of the bile pigment circulating in the plasma of this animal during the experimental period was bilirubin glucuronide. It will be seen from Table 90 that although in most cases the greater part of the bile pigment being returned to the plasma is the water-soluble glucuronide, appreciable amounts of bilirubin are also retained. This is particularly striking in the case of Sheep 89091.

Bilirubinuria is always seen in icterogenin intoxication in the sheep, particularly at the height of the reaction. It is however generally mild and bears little relation to the high plasma levels of bilirubin glucuronide which may be found. The specific gravity of the urine fell in all cases from pre-dosing values of 1·015 to 1·045 to values of 1·010 to 1·015 when bilirubinuria occurred. This phenomenon has been noted earlier in experimental cases of common bile duct obstruction in the sheep (Brown, 1967c).

Urobilinogen disappeared from the urine in cases where the hepatic block to bilirubin glucuronide was sustained for a n umber of days, e.g. Sheep 88716, 1780A and 21513. The amount of bile acid salts exc reted in the urine generally increased

markedly within 36 hours of dosing, the excretion of these compounds following in general the excretion of bilirubin. Although blood levels of these compounds were not determined it is obvious that their hepatic excretion is blocked simultaneously with that of bilirubin.

Porphyrin excretion by the liver is markedly impaired during icterogenin intoxication. Photosensitivity is seen about two to four days after dosing, but marked coproporphyrinuria after about twenty-four hours. The figures presented in Table 91 are typical results. Baseline levels for plasma phylloerythrin and the 24 hourly urinary excretion of coproporphyrin were within the normal limits given earlier in this thesis for the various control animals used. The data given in this Table

TABLE 91.—*Plasma phylloerythrin levels and the 24 hourly urinary excretion of coproporphyrin in cases of icterogenin intoxication*

(Phyllo=phylloerythrin, mcg per cent; copro=coproporphyrin, mcg excreted/24 hr; days=days after dosing)

Sheep No.	Porphyrin	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
16841.....	Plasma phyllo.....	0·84	18	21	39	42	22	18	13
	Urine copro.....	96	180	312	80	324	358	121	101
19449.....	Plasma phyllo.....	0·78	12	17	25	28	32	30	27
	Urine copro.....	82	135	14	36	89	76	64	59

support the point made earlier in Chapter 4 regarding the probability of there being a critical level of phylloerythrin in the blood at which animals will become photosensitive. The presence of photosensitivity was determined in these two particular cases by using a high-energy emission Xenon lamp as source of the activating rays and a set of cut-out filters as used by Riemerschmid & Quin (1941) taped in place over depilated areas on the backs of the sheep. Only mild erythema was evoked in the areas covered by the filters corresponding to the action spectrum of phylloerythrin (Riemerschmid & Quin, 1941) on the second day of dosing. The animals showed no evidence of discomfort or erythema after exposure of their depilated heads and ears to solar radiation for half an hour. Marked erythema under the filters concerned, followed later by oedema, was produced by irradiation for a period of five minutes under the Xenon lamp on the third day of dosing while solar irradiation for half an hour produced the same effects. The animals were intensely photosensitive on the fourth day after dosing and both types of irradiation produced severe hyperaemia, pain and oedema in the exposed areas followed later by necrosis of the superficial layers of the skin. Neither animals were significantly photosensitive on the seventh and subsequent days.

Hypercoproporphyrinuria is present 24 hours after dosing icterogenin and in the two cases featured in Table 91, lasted for over a week after dosing.

In an earlier publication (Brown, 1967c) it is stated that common bile duct obstruction in the sheep is followed almost immediately by a precipitous fall in the levels of plasma iron in the cases concerned and that normal levels of this plasm a constituent were only regained about three to five days later. The same effect was seen in animals that received doses of icterogenin of 400 to 600 mg/Kg, but not in

those receiving lower doses. In Sheep 16841, 21513 and 19449 plasma iron levels fell from pre-dosing values of 140 to 250 mcg per cent to 77 to 80 mcg per cent twenty four hours after dosing, reaching 22 to 38 mcg per cent on the third day after dosing. Thereafter plasma iron levels slowly rose once more, reaching the pre-dosing values on the sixth to seventh day. The reason for this peculiar phenomenon is quite unknown to us at the moment. It is obvious from earlier work (Brown, 1967c) that it is related to the general impairment of biliary secretion.

Liver iron levels were determined on specimens from Sheep 7054 and 7061 seven days after administration of icterogenin. The values found were 14·8 mg per cent and 12·8 mg per cent (wet tissue) respectively, both within the normal limits stated earlier. Kidney iron values were 19·0 mg per cent and 11·2 mg per cent (wet tissue) respectively. The value found in the case of Sheep 7054 is somewhat higher than that found in the controls used throughout this work, but no particular significance is attached to it.

No changes of note in the various plasma protein fractions or the albumin : globulin ratio were observed in any of the sheep during the week following administration of icterogenin, except in the case of animal 21513. This case showed a gradual and sustained fall in total plasma protein levels from 7·97 gm per cent to 6·24 gm per cent over the week following dosing. This was associated with a similar decline in the albumin level from 3·98 to 3·28 gm per cent and in the globulins from 3·99 to 2·96 gm per cent. Haemoglobin levels fell in this animal from 15·2 to 9·3 gm per cent. Since this slow fall affected both major protein fractions and the red cells, it was related most likely to water retention and haemodilution.

The thymol turbidity, thymol flocculation, zinc sulphate turbidity and colloidal gold flocculation tests showed no deviations at any time after dosing from the pre-dosing (and " normal ") values in any of the experimental animals.

The results of BSP clearance tests in these cases are represented by the data presented in Table 92. These results show an increasing inability to clear the dye

TABLE 92.—*The results of bromsulphalein (BSP) tests in sheep poisoned with icterogenin*

(The figures given represent the % of the test dose retained in the blood stream 30 minutes after injection. Days refer to days after dosing)

Sheep No.	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
1780	7·1	—	—	67·1	—	—	20·0
1780A	—	79·0	—	—	—	—	5·3
1726	17·3	24·4	12·7	—	3·4	—	0
88716 (see also foot of table)	35·0	61·5	76·0	90·0	80·0	80·0	88·0
89091	6·2	44·8	56·0	9·2	0·8	0·5	0
89551	32·8	67·2	67·0	82·0	34·0	2·0	0
1624	34·4	60·3	58·4	59·4	41·8	1·2	0
7061	—	74·9	76·7	26·8	—	—	0
88716—Total Bilirubin mg%	2·70	8·85	10·80	12·15	12·15	13·80	22·30

from the bloodstream during the first three to four days after intoxication, and thereafter a return towards normal in this respect. These findings are in excellent accord with those for bilirubin glucuronide and porphyrins. In most cases normal clearances were found seven days after administration of the compound. Sheep 88716, which showed extremely high levels of total bilirubin throughout the entire test period, is remarkable in that the same phenomenon was observed in the case of BSP. The total bilirubin levels found in this animal are given at the foot of Table 92 for comparison. It will be remembered that this case received a dose of 200 mg/Kg and showed an exceptionally severe response to icterogenin at this level of administration. These data illustrate very nicely how the elimination of several substances normally excreted in bile may be affected simultaneously and to the same degree. The concentration of bile pigment found in the plasma of Sheep 88716 is almost identical to the figure which could be expected if the amount of bile pigment excreted during 24 hours in the bile was returned to the bloodstream daily for seven days. This hypothetical figure has been calculated to be 19·74 mg per cent (Brown, 1967c). The figure found at the end of the seven day test period in Sheep 88716 was 22·3 mg per cent. It is apparent thus that in this case at any rate the biliary excretion of conjugated bilirubin remained completely blocked over the whole test period.

Composite Fig. 14 shows the rate of clearance of injected BSP from the bloodstream of two of the animals after giving icterogenin, compared with similar data obtained from these animals before dosing. These clearance curves were constructed in the same way as those for glucose and pyruvate tolerance tests described earlier. BSP concentrations were determined on samples of blood drawn 1, 5, 10 and 30 minutes after injection; concentration of BSP was then plotted against time. It is obvious from the shape of the curves obtained from tests made during the intoxication with icterogenin, that clearance of BSP from the bloodstream by the liver is seriously impaired. By analogy with the work in this regard on the geeldikkop cases cited earlier, and taking into consideration the data found for Sheep 88716 discussed immediately above, it is likely that the excretion of BSP into the bile is *also* seriously imparied. This failure to clear the compound from the blood is very similar to that seen in early enzootic icterus.

Alkaline phosphatase activity was determined on plasma samples from the animals receiving doses of icterogenin of 200 mg/Kg and higher throughout the experiments. In no instance was any deviation from the " normal " range of 5 to 25 units found during the experiments.

Levels of activity of glutamic oxalacetic (GOT) and glutamic pyruvic (GPT) transaminases, phosphohexose isomerase (PHI), aldolase (Ald), isocitric dehydrogenase (ICD) and lactic dehydrogenase in blood plasma were determined only in the case of the sheep which received doses of icterogenin of 400 mg/Kg and higher, i.e. Sheep 16841, 21513 and 19449. GOT, PHI and Ald plasma levels were elevated in all three cases within two days of dosing and values for the activity of these enzymes remained high during the whole test period. The magnitude of the increase in activity of these enzymes in the plasma of affected animals was directly related to the dosage level employed, e.g. the highest recorded values for the plasma activity of each enzyme during the week following dosing were:

Sheep 16841 (400 mg/Kg): GOT 315, PHI 313, Ald 54;
Sheep 19449 (600 mg/Kg): GOT 515, PHI 408, Ald 142;
Sheep 21513 (600 mg/Kg): GOT 587, PHI 235, Ald 138.

Maximum levels of plasma activity of these enzymes were found four or five days after dosing.

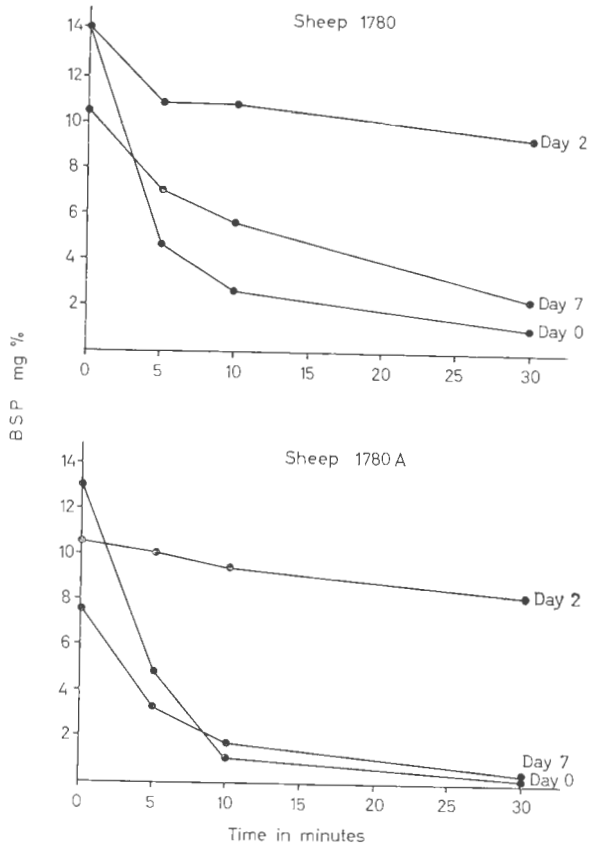

FIG. 14.—BSP clearance in cases dosed with icterogenin (Day 0 = the day before dosing was commenced; Days 2 and 7 are days after dosing; BSP mg% = plasma levels of BSP. Time in minutes – minutes after injection)

Plasma ICD levels remained unaltered during the week following dosing in all three cases. GPT levels remained similarly unaltered in Sheep 16841 and 19449, and plasma LD levels remained normal only in the sheep which received the lower dose, i.e. Sheep 16841. Plasma levels of this enzyme rose in the case of Sheep 19449 on the fifth and sixth day after dosing. This sudden rise in LD activity could be related to the severe photosensitization reactions evoked by the solar and Xenon emission irradiations described earlier. Plasma LD rose in this case from 350 to 610 during the first four days following dosing to 1020 to 1360 on the following two days.

Sheep 21513 (600 mg/Kg) was remarkable in that marked elevations of plasma activity of GOT, PHI, Ald and LD were present from 24 hours after dosing and persisted throughout the entire test period. LDH values rose from 850 on the day after dosing to 2310 three days later. This was the only sheep used during this work in which a marked and sustained rise in GPT levels have been found, viz. 137 twenty-four hours after dosing to 177 four days later. The results of these plasma enzyme

assays differed so from those obtained in the other two animals that the presence of muscle lesions in this case was suspected. Creatine phosphokinase activity was determined on plasma samples which had been deep frozen for later mineral analyses. Values of 6 to 7 units (i.e. well within the normal range for sheep; Wagner & Gray, 1967) were found in the samples from the first two days after dosing; the subsequent samples gave markedly elevated values of 35 to 41 units. The sheep was slaughtered at the end of the experiment and extensive focal muscle degeneration was found in the cadaver at autopsy examination.

(*c*) *The erythrocyte in icterogenin intoxication:* The detailed studies done in this regard in geeldikkop and enzootic icterus have not yet been carried out in icterogenin intoxication in sheep. Red cell fragility has been studied and it was found to remain unaltered throughout the entire experimental period at all the dosage levels employed. Red cell copper levels rose from pre-dosing values of 66 to 79 mcg per cent to values in the range 125 to 146 mcg per cent two to three days after dosing and remained in this range for the rest of the experimental period of one week in some of the cases. Such raised values for red cell copper were generally above the normal 80 per cent range found for sheep but were still within the upper 98 per cent limit (Brown, 1967c).

(*d*) *Kidney function:* Blood urea nitrogen, creatinine, uric acid and amino acid levels were studied and the usual routine urine analyses were performed before and during icterogenin intoxication. Urea nitrogen levels remained normal in the animals receiving less than 400 mg icterogenin per Kg, except in the case of Sheep 1780, where a rise occurred from the levels of 9·0 to 23·0 mg per cent found during most of the experiment to 29·0 and 31·0 mg per cent during the last two days of the experiment respectively. Elevations in the same order were observed during the entire week after dosing, in Sheep 16841 (400 mg/Kg) and 21513 (600 mg/Kg). Sheep 19449 (600 mg/Kg) also showed the same very mild uraemic disturbances but on the seventh day after dosing, blood levels of urea rose from 29·4 mg per cent to 82·8 mg per cent.

No alterations were observed in the blood levels of any of the other non-protein nitrogenous compounds mentioned after dosing icterogenin. It is interesting to note that although some of these sheep were allowed to develop extensive and severe lesions of photosensitization involving the depilated head and back (e.g. Sheep 7054 and 7061) following solar irradiation, no elevations in their blood uric acid levels occurred. These ranged in the first animal from 1·2 to 1·8 mg per cent and in the second from 0·6 to 1·0 mg per cent. The marked elevations of this blood constituent found in geeldikkop and enzootic icterus cases are thus probably not due to the ravages of photosensitization.

Plasma magnesium levels remained within the range 1·20 to 3·2 mg per cent in all animals after dosing except in the case of sheep 7061 (250 mg per Kg) when values of 5·6 to 6·0 mg per cent were found during the three days immediately following administration of the compound; thereafter normal values were obtained once more. Plasma inorganic phosphate levels similarly remained within normal limits in all the animals except those receiving 600 mg of icterogenin per Kg. Values of 10·7 and 11·1 mg per cent were found for plasma samples from each sheep respectively on the seventh day after dosing.

Apart from the mild bilirubinuria and disappearance of urobilinogen from the urine (of some cases) after dosing, no other abnormalities were detected in the daily samples from any of the experimental animals.

(*e*) *Adrenal function:* This was assayed as before by considering the results of determinations of plasma sodium, potassium, chloride, bicarbonate and absolute eosinophile counts. Hyponatraemia associated with hypochloridaemia was observed in only two of the experimental animals after dosing icterogenin. Both phenomena appeared two days after dosing the compound and were present for a further four to five days. Hypokalaemia associated with hyperchloridaemia was seen towards the end of the experimental period in Sheep 19449 (600 mg/Kg). Plasma bicarbonate levels fell gradually in the same case from pre-dosing levels of 24 to 26 meq/L to 16·0 meq/L at the end of the seven day experimental period. Absolute eosinophile counts remained within the normal limits in at least half the animals studied after giving icterogenin. The remainder showed a reduction of the eosinophile count of variable magnitude, figures of 20 to 80 cells/cu mm being found during the seven-day period after dosing icterogenin.

In general icterogenin intoxication is associated with a stress reaction of mild and variable nature. No definite pattern of electrolyte disturbances was found to be present, as for instance is the case in geeldikkop. All that can be said in this regard, of icterogenin intoxication, is that there is evidence of a successful adrenal response to the stress of the intoxication, the nature and magnitude of which is unrelated to the dosage level employed and the outcome of which is an uneventful recovery.

Plasma calcium levels were studied in two sheep only, 7054 and 7061. No deviations from the normal range of 9 to 12 mg per cent were noted at any time after giving icterogenin.

Blood sugar and ascorbic acid levels were studied in Sheep 88716, 89091, 89551, 1624, 1726, 1780A, 1780, 7054 and 7061. Pre-dosing blood sugar levels in these animals ranged from 33 to 55 mg per cent. A mild hyperglycaemic tendency was seen in sheep 1780 and 1780A, blood sugar levels ranging from 65 to 77 mg per cent, being regular daily findings in both cases for the entire seven-day period after dosing. This phenomenon was not observed in the other cases that received the same or higher doses. Plasma ascorbic acid levels remained unaltered in all these cases at the pre-dosing levels of 0·44 to 0·70 mg per cent.

(*f*) *Copper metabolism:* Total plasma copper levels were studied in Sheep 7061 (250 mg/Kg), 7054 (280 mg/Kg), 16841 (400 mg/Kg), 21513 and 19449 (both 600 mg/Kg), and the various plasma copper fractions in the last three mentioned sheep only. Total plasma copper levels ranged from 100 to 150 mcg per cent in these animals before giving icterogenin. A transient elevation in total plasma copper commencing two to three days after dosing and lasting for a further two to three days was seen in all the animals, bar sheep 19449 in which no change in total plasma copper was observed. These elevated copper levels ranged from 200 to 250 mcg per cent in all instances except sheep 16841 in which plasma levels ranging from 165 to 174 mcg per cent were found. The various blood copper fractions were determined on samples from Sheep 16841, 21513 and 19449. The results are presented in Table 93. Normal values for these fractions are as given earlier in this thesis. Dosing with icterogenin was followed twenty-four hours later by a rise in the loosely bound copper fraction in two out of the three animals. This fraction increased as the intoxication proceeded. In Sheep 21513 the ceruloplasmin fraction was markedly elevated instead. This latter fraction only rose above normal levels towards the end of the experimental period in the first two mentioned sheep. Blood copper fractions were found to be normal once more in all three cases ten days after dosing. Ceruloplasmin levels were down to 8·2 to 8·7 mg per cent and loosely bound copper ranged from 6·7 to 11·1 mg per cent.

TABLE 93.—*Blood copper fractions in animals poisoned with icterogenin*

(Values are mcg %, except in the case of ceruloplasmin where they are mg %. Days are days after dosing icterogenin)

Sheep No.	Fraction	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
16841	Total plasma Cu.	146·0	124·6	165·0	152·0	164·3	174·0	168·0
	Loosely bound Cu	8·2	0	1·4	2·4	10·0	25·0	11·3
	Ceruloplasmin....	10·3	6·0	5·1	7·6	19·6	19·9	15·1
	Red cell Cu.....	105·7	116·6	114·3	140·7	96·6	98·4	121·6
21513	Total plasma Cu.	158·1	—	206·6	—	168·0	—	118·1
	Loosely bound Cu	0	—	0	—	0	—	11·0
	Ceruloplasmin....	27·4	—	21·2	—	7·9	—	18·0
	Red cell Cu.....	79·4	—	145·8	—	182·2	—	147·8
19449	Total plasma Cu.	139·7	104·8	126·7	116·7	117·9	133·3	141·7
	Loosely bound Cu	6·8	0	0	1·2	8·8	11·3	12·6
	Ceruloplasmin....	6·6	5·7	4·5	5·7	12·2	18·3	15·7
	Red cell Cu.....	65·8	124·5	141·8	121·0	104·3	117·6	121·7

Red cell copper levels were found to rise above the normal 80 per cent levels on the second or third day after dosing. Elevated levels persisted throughout the experimental period in Sheep 21513 (in which the loosely bound plasma copper did not rise until near the end of the experiment, but ceruloplasmin levels rose soon after dosing). In the other two animals elevation of this copper fraction was transient.

The liver and kidney copper content was determined on samples of these tissues from Sheep 7054 and 7061 taken at the end of the experimental period. Kidney copper levels were within normal limits, 0·83 and 0·42 mg per cent being the respective values found. Liver copper values were 45·0 mg per cent and 33·34 mg per cent respectively. Sheep 7054 had an increased liver copper content and 7061 a liver copper in the same order as the control animals emanating from the Karoo that were used in the geeldikkop studies.

4. *The results of giving icterogenin by injection or in the form of successive small oral doses*

It will have been noticed from the above discussion that many of the abnormal events which occur after dosing icterogenin do so irrespective of the dosage level employed if this is above that sufficient to induce the intoxication. It seems that once *sufficient* icterogenin is given to induce the typical disturbances in biliary excretion its effect is maximal from the start, the only variable being the duration of the disturbances which is dosage dependent. The dose of 200 mg/Kg seems to be the lowest oral dose which gives repeatable results in the sheep. The total dose of icterogenin which must be employed even at this level to induce the intoxication in sheep, is large and a considerable amount of precious material must be used. If a higher dose is used to obtain a prolonged effect (see Table 88, for instance) the amount of material which has to be used is even greater. The author preferred to use fully-grown adult sheep for these studies, because of the volume of blood which must be drawn

daily to permit all the determinations described in the foregoing text and to facilitate the repeated performance of tests like BSP clearance which requires frequent bleeding for sample collection.

One of the major difficulties inherent in dosing icterogenin is its insolubility. Small doses may be dissolved in small volumes of ethanol and dosed by stomach tube, or aqueous solutions of the sodium salt may be easily given the same way. Single doses of 10 to 12 gm require considerable volumes of alcohol for solution, the dosing of which has its own particular well-known hazards. To keep the volumes as small as possible, the solutions must be given hot and a considerable amount of icterogenin will thus precipitate when the solution mixes with the rumen fluid. The same objections hold for the sodium salt of the compound.

It was decided therefore to give the total dose of icterogenin in the form of small single daily doses orally or by injection, and to see whether this method produced the required prolongation of the effects of the compound which were obtained with the single large doses given earlier. Three sheep were used for these studies and the following dosing regimens were employed: Sheep 89281 received a total of 8·2 gm of icterogenin given in the form of daily doses of 3, 2, 2 and 1·2 gm of the compound each dissolved in 40 ml alcohol and given by stomach tube; Sheep 1981 received 7 gm of the compound given in daily doses of 3, 2 and 2 gm in alcohol as above; Sheep 1541 received four daily doses of 0·25 gm icterogenin given by intravenous injection. The solution for injection was prepared by dissolving 1 gm of icterogenin in 22·6 ml of 96 per cent ethanol. To this was added 17·4 ml of 0·1 N NaOH and after mixing, 80 ml of isotonic sodium chloride was added. A dose of this solution equivalent to 0·25 gm of icterogenin was then given. It was found that this was the maximum dose which could be safely given by the intravenous route. Doses of 0·5 gm even if well diluted and injected very slowly caused death within minutes after injection. Generally observed autopsy findings included cyanosis, pulmonary oedema and hyperaemia, subepicardial haemorrhages and haemorrhages into the liver substance, tumor hepatis, tumor splenis, oedema of the gall-bladder and gastro-intestinal tract, and haemorrhages into the abomasum and small and large intestines. Death was attributed to shock, cardio-vascular collapse and generalized haemorrhagic diathesis.

Both ways of giving icterogenin produced the desired prolonged effect which resulted in the production of a clinically very severe icteric and photosensitivity syndrome comparable outwardly to severe early cases of geeldikkop. When the compound was dosed orally clinical icterus appeared about 48 hours after the first dose was given and photosensitivity 24 hours later. Both symptoms persisted for 5 to 7 days after the last dose was given. When the compound was given by injection severe regurgitation of bilirubin was apparent within 24 hours and photosensitivity appeared within 48 hours after giving the initial dose. The effects by this route are however far more transient than when icterogenin is given by mouth. Cessation of the daily injections of the compound resulted in a return to normal within 24 to 48 hours. This suggests that a considerable part of the prolonged action of orally given icterogenin is due to a slow and sustained rate of absorption from the digestive tract. The data pertaining to plasma bile pigment levels found in Sheep 89281 and 1541 during the experiments concerned illustrate these points. These data appear in Table 94. It is apparent from the data obtained from Sheep 1541 that injection of icterogenin may cause considerable haemolysis as judged by the large amounts of bilirubin in the plasma immediately after the injections. This statement can be supported by the fact that the packed cell volume and haemoglobin fell from pre-dosing values of

TABLE 94.—*The effects of icterogenin on plasma bile pigment levels when it is given by split daily oral doses or by daily injection of small doses*

(Values are mg%. Days are days after commencement of the experiment)

Sheep No.	Days	Dosing regimen	Total bilirubin	Bilirubin glucuronide	Bilirubin
89281.....	0	3 gm orally.....	0	0	0
	1	2 gm „	3·21	2·14	1·07
	2	2 gm „	4·29	2·86	1·43
	3	1·2 gm „	8·57	6·07	2·50
	4	—	5·75	2·89	2·86
	5	—	10·00	6·43	3·57
	6	—	8·93	5·70	3·23
	7	—	7·15	2·86	4·29
	8	—	3·57	1·79	1·78
	9	—	3·22	1·79	1·43
1541.....	0	0·25 gm injected.	0	0	0
	1	0·25 gm „ ..	8·13	3·13	5·00
	2	0·25 gm „ ..	10·00	5·62	4·38
	3	0·25 gm „ ..	13·12	7·51	5·61
	4	—	8·75	3·75	5·00
	5	—	1·25	0·31	0·94
	6	—	0·65	0	0·65
	7	—	0·25	0·20	0·05

38 per cent and 9·42 gm per cent to 25 per cent and 7·12 gm per cent on Day 4 and 20 per cent and 7·12 gm per cent on Day 7. Blood smears taken from this animal showed marked anisocytosis and a marked monocytosis and lymphocytosis on each of the seven days following the first injection. The monocytes were generally seen to be packed with phagocytosed red cells. Differential leukocyte counts done daily over this period revealed a relative neutrophilia as well. The following figures incorporate all the daily counts made on the seven days following the first injection:

neutrophiles, 5 to 32 per cent, eosinophiles, 1 to 3 per cent,
lymphocytes 20 to 60 per cent, basophiles, 0 to 1 per cent,
monocytes, 18 to 59 per cent.

These changes were not observed in the animals receiving split oral doses of icterogenin.

Total plasma protein, albumin and globulin levels remained unaltered throughout the experimental period in the animal which received icterogenin by injection. In the animals which received the compound orally total plasma protein levels rose about two days after the last dose was given, the rise being due to an increment in the total globulin fraction from pre-dosing levels of 3·16 to 3·44 gm per cent to 4·35 to 4·80 gm per cent.

Thymol turbidity and flocculation tests and the zinc sulphate and colloidal gold flocculation tests remained negative throughout the experiment in all three cases and plasma alkaline phosphatase values remained in the pre-dosing range of 5·0 to 13·3 units.

Plasma iron levels were not studied in the case of Sheep 1541, but remained unaltered throughout the experiments in the animals receiving orally dosed icterogenin. Total plasma copper levels remained within the pre-dosing range of 100 to 155 mcg per cent in all three animals.

Blood urea nitrogen and creatinine levels remained unchanged in Sheep 1541 at the pre-dosing ranges of 14·7 to 18·4 mg per cent, and 2·2 to 2·8 mg per cent respectively. In the case of Sheep 89281 a marked uraemia developed two days after the last dose was given (i.e. Day 5 indicated in Table 94). Urea levels rose from 11·6 to 18·3 mg per cent to 58·3 mg per cent at this time and finally to 73·3 mg per cent at the end of the experiment. Plasma creatinine levels varied in this animal from 3·4 to 4·4 mg per cent during the course of the intoxication.

Routine urine examinations were performed during the experiments on samples from all three cases. Bilirubinuria and bileaciduria were severe in the animals receiving icterogenin orally and negligible in Sheep 1541 in spite of the high levels of conjugated pigment in the blood of this animal. Urobilinogen disappeared from the urine of all three animals soon after commencing the dosing regimens, the urine from all three subsequently remaining urobilinogen-free for the duration of the experiment. Once bilirubinuria appeared, urinary specific gravity values fell from 1·015 to 1·030 before dosing to 1·010 for as long as bilirubinuria persisted in the animals concerned. Sheep 89281 developed a marked albuminuria and hematuria concomitantly with the uraemia mentioned earlier.

No changes of note were observed in plasma magnesium or inorganic phosphate levels in any of the animals as the experiments proceeded. Calcium levels remained likewise within normal limits in all instances.

BSP clearance from the blood was affected in the same manner as described earlier, but to a much severer degree in the animals receiving split oral doses of icterogenin. In Sheep 89281 for instance, 90·6 per cent retention of the dye at the end of the half hour test period was observed after the second dose had been given. On Day 7 indicated in Table 94, i.e. four days after the last dose had been given, 72·5 per cent retention of the test dose was observed at the end of the half hour test period. Traces of BSP were found in the urine 12 hours after the first test. The BSP from this dose continued to be excreted in the urine *over the next three days*. The blood plasma contained large amounts of BSP during these three days, as judged by the intense purple colour it developed on alkalinization. By the time the second test was done the plasma and urine were clear of the dye. The same phenomenon was observed once more after this second test; appreciable amounts of BSP remained in the plasma for the following three days, the dye appearing at the same time in the urine.

Injection of the first small dose of icterogenin into sheep 1541 produced within 24 hours 31 per cent BSP retention. After the third dose 65 per cent retention was found at the end of the test period and the dye appeared in small amounts in the urine. Three days after the last injection, 15 per cent of the injected BSP was still retained at the end of the test period.

The intravenous injections of icterogenin produced no significant changes in plasma electrolyte concentrations or in the absolute eosinophile counts during the entire test period in Sheep 1541. Oral dosing of the compound produced in Sheep 89281 a typical Addisonian syndrome as seen in early acute geeldikkop, two days after the last dose of the compound was given. This was manifested by a progressively worsening hyponatraemia, hypochloridaemia, hyperkalaemia, low plasma bicarbonate

levels and a very low absolute eosinophile count. The values found for the various blood constituents concerned five days after the last dose of icterogenin was given were: sodium, 118 meq/L; potassium, 8·2 meq/L; chlorides, 77·0 meq/L; bicarbonate, 13·9 meq/L; absolute eosinophile count, 20/cu mm. Sheep 1981, which received a lower total oral dose of icterogenin showed only a progressively worsening hyponatraemia and falling absolute eosinophile count as the intoxication progressed.

A hyperglycaemic tendency was observed in all three animals during the course of the intoxication. Blood sugar levels in Sheep 89281 varied between 34 to 53 mg per cent during the four days before icterogenin was dosed. On the day after the last (fourth) dose was given the blood sugar level was 70 mg per cent, on the following day 65 mg per cent and 86 and 90 mg percent on the next two days respectively. This tendency was not so pronounced in either Sheep 1981 or 1541 (which received the compound intravenously).

5. *General discussion*

Apart from icterus, lesions of photosensitization and severe bile pigmentation of the liver and kidneys, the only notable autopsy feature of uncomplicated icterogenin intoxication in the sheep is an extremely severe gastro-intestinal stasis. This is seen particularly in animals receiving small daily doses of the compound for a number of days and is characterized by some atrophy of the gastro-intestinal tract and considerable desiccation of the contents of the forestomachs, caecum and colon. This gut stasis was not observed in the sheep which received icterogenin in the form of repeated injections.

Microscopic examination of the liver and kidneys reveals only bile pigmentation and mild fatty infiltration of the cells of these organs. Cloudy swelling can be seen in some parenchymal cells of the liver and some of the kidney tubule cells. The lymphoid tissue of the body seems to be unaffected by the doses of icterogenin which have been given.

Seawright (1963) has studied the pathology of *Lantana camara* L. (Verbenaceae) intoxication in sheep and cattle, followed by dosing of dried powdered leaf of the plant, and Lantadene A (=rehmannic acid). The latter is now known to have no icterogenic properties but preparations of it are usually contaminated with the highly potent icterogenic agent, 22β–angeloyloxy–oleanolic acid (Brown, Rimington & Sawyer, 1963). Seawright (1963) found the same absence of significant changes in the liver cells in preparations from his animals but noted kidney lesions in his cases of protracted illness of three weeks duration. These lesions included massive fatty degeneration and necrosis of the tubular epithelium with occlusion by casts and consequent cystic dilation of the tubules. Such kidneys were associated with terminal uraemia, polyuria and albuminuria. As mentioned in the introduction to this paper, Heikel *et al.* (1960) found that icterogenin produced no discernible morphological changes in the liver of rabbits, which could explain the disturbances in biliary function which followed its administration.

As can be seen from the data presented in this paper the most obvious effect of icterogenin intoxication is that on the biliary excretion of bilirubin and BSP conjugates, bile acids and porphyrins. If sufficent icterogenin is given the block in the transfer of these substances into the bile is suddenly complete and of variable duration. The effects of icterogenin are however more profound than just a block in biliary excretion of certain compounds. In the first instances the studies with BSP show a failure on the part of the liver cells to clear the dye from the blood and what is more,

a decreased ability of the kidneys to eliminate the large amounts of the dye present in the blood. This embarrassment of renal function extends to bilirubin glucuronides, bile acids and possibly phylloerythrin as well as can be judged by the mildness of bilirubinuria and bileaciduria and the severity of photosensitization once sufficient levels of porphyrin have built up in the blood. The block in renal excretion is by no means as severe as that in biliary elimination.

Icterogenin appears to have a definite harmful effect on the erythrocytes of the animals dosed with it. This is seen particularly in animals receiving single doses of more than 200 mg/Kg and in those receiving repeated small doses of the compound. The evidence for this is the development of a hypocythaemic normocytic normochromic anaemia in these cases, the signs of active phagocytosis of degenerating cells by the markedly increased number of circulating monocytes and the presence of appreciable amounts of bilirubin in the plasma of these cases (although this latter finding may also be due to some interference of bilirubin conjugation). It is thought that the effects of icterogenin on the red cells of these cases are possibly different to the factors causing haemolysis in geeldikkop and enzootic icterus. Since no alterations in the red cell fragility have been seen, nor methaemoglobincythaemia in any of the animals studied, the effect of icterogenin on these cells is believed to be one which causes rather sudden death of the cells concerned, rather than the slower degenerative changes which are present in the erythrocytes of geeldikkop and enzootic icterus cases. This action of icterogenin is most likely a direct one on the red cell membrane.

Evidence has been presented in this chapter that when given in high doses icterogenin produces definite muscle lesions, which are manifested in the live animal by marked elevations in plasma GOT, PHI, Ald and CPK activity levels. These changes are not observed with lower dosage levels, but the presence of a distinct hyperglycaemic tendency in many of the cases studied may not be without significance in this regard. Elevations of the plasma enzyme levels mentioned are generally maximal about four days after the dose of icterogenin was given. When uraemia is seen, and it is more frequent in animals receiving high doses of icterogenin, it also appears some time after the compound was given. It seems as if the initial " biochemical " lesion in the kidneys and muscle membranes at any rate may progress to a more lethal one if the intoxication is sustained. Such factors as anoxia and biliary nephrosis may contribute towards the appearance of these secondary changes.

The presence of appreciable amounts of bilirubin as well as its glucuronide in the plasma of the experimental animals may indicate the existence of a haemolytic process or may be the result of some disturbance in conjugation as a result of a general decline in energy production in the liver cell as has been found in rabbits (Brown, 1964). The labile nature of bilirubin glucuronide no doubt accounts for some of this bilirubin as well.

The biliary excretion of copper is affected in the same way by icterogenin as that of other components of bile. This effect is seen as an increase in liver copper levels, a rapid and sustained increase in the loosely-bound copper fraction of plasma which occurs soon after dosing, an increase in red cell copper two or three days later and an increase in plasma ceruloplasmin some days after the compound was dosed. These effects have been observed in common bile duct obstruction in the sheep and the role of the ovine red cell in the emergency transport of copper has been mentioned (Brown, 1967b, 1967c).

31610—13

The time lag of 24 to 72 hours in the appearance of many of the typical biochemical effects of icterogenin is undoubtedly due to a slow but continuous rate of absorption of the compound from the digestive tract. Large doses prolong the duration of these effects, injected small doses bring about the effects much earlier and in such cases the effects are of short duration.

The action of icterogenin on the various body cell membranes when given in single doses, brings about an icterus and photosensitivity syndrome, which if the exposure of the animal to solar radiation is kept minimal, does not appear to constitute a severe stress in these animals. Repeated administration of the compound may induce, however, a typical Addisonian collapse in electrolyte balance such as seen in early geeldikkop cases. This follows the other biochemical disturbances induced by icterogenin. In geeldikkop it may precede such disturbances.

It is apparent from these studies and those cited earlier in the introduction that the main effects of icterogenin are on membrane permeability, the most powerful effects being on the liver cell membrane; those of the kidney and muscle cells and erythrocytes are affected to a lesser degree and under special circumstances. The typical disturbances of biliary excretion seen in geeldikkop and the hepatic pathology of this condition can be easily and repeatedly reproduced by small doses of icterogenin. Some of the other features of geeldikkop such as disturbances in renal excretion are reproduced as well in a very mild way, but features like myopathy are only produced by unnaturally high doses of icterogenin. Administration of the compound does not reproduce the typical haematology and in most instances the adrenal chemical pathology seen in classical geeldikkop.

CHAPTER 13

SELENIUM AS A POSSIBLE AETIOLOGICAL FACTOR IN GEELDIKKOP AND ENZOOTIC ICTERUS

1. *Introductory remarks*

At the end of the summer of 1960 the author found himself very much at the crossroads as regards work on the aetiology of geeldikkop and enzootic icterus. A lot of the work reported in this thesis had been completed; the epizootiology, symptomatology and histopathology of the conditions had been studied in detail; studies on *T. terrestris*, the plant traditionally associated with geeldikkop and the saponins and sapogenins isolated from it, had largely proved fruitless in this regard and all attempts to demonstrate a mycotic agent operative in the disease had also failed.

It was apparent from some of the studies reported in this thesis that the two conditions were related and very likely different manifestations of a single disease entity. There seemed little doubt that this entity was an intoxication of some kind and the epizootiology of enzootic icterus particularly pointed towards it being a cumulative intoxication. It was recognised that both conditions were precipitated by a number of severe non-specific and ill-defined stress conditions (Brown, 1959a, 1959b, 1959c, 1962, 1963, 1964, 1966a; Brown & de Boom, 1966; Brown & de Wet, 1962; Brown, *et al.*, 1960; Wagner, 1964).

During the course of the various field investigations into these two diseases, the author had been impressed by a number of vague syndromes which occurred in the Karoo from time to time amongst small stock. These conditions have been enumerated and described elsewhere (Brown & de Wet, 1962). Suffice it to say at this stage that they all embody many features of the sub-acute and chronic forms of selenium intoxication as it is described in the world literature. Since geeldikkop and particularly enzootic icterus also appeared to have some features in common with chronic selenosis, it was decided to investigate the levels of selenium in the tissues of cases of geeldikkop and enzootic icterus and of normal animals emanating from the Karoo as well as the levels of the element present in the vegetation of the Karoo.

The symptomatology of the various forms of selenosis have been reviewed in an earlier paper (Brown & de Wet, 1962). The geology and botany of the Karoo viewed in relation to the possible problem of selenosis in this area are discussed in this and a subsequent paper (Brown & de Wet, 1967). Four excellent reviews of the selenium problem elsewhere in the world are those of Moxon & Rhian (1943), Painter (1941), Rosenfeld & Beath (1964) and Russel & Duncan (1956). The author intends to discuss only his pertinent findings in this regard in this chapter; the bulk of the experimental work is presented elsewhere (Brown, 1962, 1963, 1964; Brown & de Wet, 1962, 1967).

2. *Selenium levels in tissues from cases of geeldikkop and enzootic icterus and from control animals*

The methods used for the determination of selenium in animal and plant tissues are described in Appendix 10. In cases of chronic selenosis the element may be found in appreciable amounts in all tissues, but the highest concentrations are encountered in liver, kidneys, spleen and heart muscle. It is present in the blood, mainly in the erythrocytes (Brown & de Wet, 1962; Rosenfeld & Beath, 1964). Liver and kidney tissue from the animals concerned in this work were selected for selenium analysis and specimens were taken from all the cases of geeldikkop and enzootic icterus described earlier which were slaughtered for biochemical or histopathological studies and from all the control animals sacrificed during this work. For comparative purposes specimens were taken from normal sheep slaughtered at Onderstepoort for staff rations. In addition to these specimens a large number of samples of liver and kidney from confirmed cases of either syndrome were submitted for selenium analyses by staff of the Department of Veterinary Field Services or by private practitioners. These results have also been included in this discussion.

All the tissues which were used for this work were preserved in 10 per cent formalin until the analysis could be completed. The method used for the analysis of the majority of the specimens made use of codeine phosphate as the colour reagent (see Appendix 10). This method is only suitable for detecting selenium concentrations of higher than 2 mcg/gm of material. Values indicated as 0 mcg/gm in the

text and tables which follow are thus in actual fact " less than 2 mcg/gm." Such concentrations of selenium were of little importance in this work, the primary object of which was to demonstrate the presence of dangerous levels of the element in the tissues of sheep emanating from the Karoo.

All the actual results obtained in these studies are reproduced in Appendix 8. The results are summarized in Tables 95 and 96. It can be seen from these tables that there was little real difference between the selenium content of the livers of

TABLE 95.—*Selenium levels in the livers of cases of geeldikkop and enzootic icterus and various groups of normal sheep*

(All results are expressed as mcg/gm of wet tissue)

Nature of cases	Number of cases	Mean Value	Range
Normal Onderstepoort sheep	11	±0·55	0–3·0
Normal Karoo sheep from farms on which the incidence of both syndromes is negligible	16	±0·84	0–5·5
Apparently normal animals from areas in which geeldikkop outbreaks were present	6	4·63	3·0–7·0
Geeldikkop cases (all stages)	47	5·10	1·0–14·0
Geeldikkop cases from the farm " Soutwater "	13	17·95	8·5–26·8
Enzootic icterus cases (all stages)	51	12·08	1·0–29·6

TABLE 96.—*Selenium levels in the kidneys of cases of geeldikkop and enzootic icterus and various groups of normal sheep*

(All results are expressed as mcg/gm of wet tissue)

Normal Karoo sheep from farms on which the incidence of geeldikkop is negligible	Number of animals.	16
	Mean value	2·75
	Range	0–28·0
Geeldikkop cases (all stages of the disease)	Number of animals.	8
	Mean value	6·50
	Range	0–25·5
Enzootic icterus cases (all stages of the disease)	Number of animals.	14
	Mean value	9·0
	Range	0–34·0

normal Onderstepoort sheep and clinically normal sheep taken from Karoo farms on which the incidence of the two syndromes has always been negligible. In the majority of cases in both groups selenium levels were less than 2 mcg/gm of tissue. In Table 95 is presented a summary of the results obtained from apparently normal animals taken from areas in which severe outbreaks of geeldikkop were present at the time. The mean value for selenium in the livers of this group of animals is for practical purposes the same as that obtained for the livers of geeldikkop cases and almost ten times that of the clinically normal sheep mentioned earlier. The thirteen

cases from the farm " Soutwater " noted in Table 95 were cases of geeldikkop slaughtered for specimens for the author at a time when large numbers of cases of both geeldikkop and enzootic icterus were present together on the farm. Liver specimens from some of these particular cases also showed typical features of enzootic icterus, described in an earlier chapter. It is obvious from the results summarized in Table 95 that very high levels of selenium are present in the livers of cases of geeldikkop and enzootic icterus, the highest values being found in specimens from animals on farms where the latter syndrome is prevalent. The generally higher levels of selenium in the livers of enzootic icterus cases is consistent with its nature of often being the more violently acute and rapidly fatal of the two syndromes. These data are also consistent with the epizootiology of enzootic icterus particularly with regard to its being found mainly in older animals (whereas geeldikkop occurs mainly amongst younger sheep) (Brown & de Boom, 1966) and suggest a cumulative intoxication.

A limited number of kidney specimens from typical cases of either syndrome and control animals were examined for the presence of abnormal levels of selenium. The results are presented in Appendix 8 and summarized in Table 96. The results follow the pattern established for the livers of the various groups of animals mentioned above. The fact that in some instances there appears to be a greater accumulation of selenium in these organs than in the liver of the same animal, might possibly be ascribed to the severe renal lesions discussed in the preceding chapters.

Fatal attacks of enzootic icterus in suckling lambs have been reported (Brown & de Boom, 1966). In earlier studies on abortion in Angora goats it was reported that possibly dangerous amounts of selenium ingested by the maternal animal could cross the placenta and be found in the liver of the foetus (Brown & de Wet, 1962, 1963). This topic has been reviewed in all its aspects by Rosenfeld & Beath (1964).

The attention of the reader is drawn to the values for selenium found in the livers and kidneys of the sheep designated Lamb E1 and Ewe E1 noted in Appendix 8. The latter was the maternal animal and the two were typical cases of enzootic icterus brought in for examination during one of the investigations at Victoria West. The lamb, which was still suckling, was *in extremis* at the time of examination and both were severely icteric. The liver of the lamb contained more selenium than is found in the average case of geeldikkop and both livers contained the amounts usual in typical cases of enzootic icterus. Since the lamb was still suckling it could only have acquired its dangerous load of selenium by placental transfer before birth or through its mother's milk. The excretion of dangerous amounts of the element in the milk of lactating females suffering from chronic selenosis is well known and important enough to consider as a hazard to human health (Rosenfeld & Beath, 1964).

The occurrence of enzootic icterus amongst Angora goats has been reported in an earlier paper (Brown & de Boom, 1966). The levels of selenium found in the livers of some of these cases are indicated in Appendix 8.

3. *Selenium levels in the vegetation of the Karoo*

Many hundreds of specimens of plants representative of the vegetation of the Karoo have been examined for the presence of potentially harmful amounts of selenium since this work was initiated. The results of these examinations made on specimens representing 45 families, 135 genera and 252 species of the Karoo vegetation have been presented in a separate report (Brown & de Wet, 1967). The composition of the Karoo vegetatiom, the veld types represented and the plant associations

are all reviewed in this report. The farming practices and the alarming deterioration of the natural Karoo pastures which have gone hand-in-glove with the increased incidence of geeldikkop and enzootic icterus in recent years, are described elsewhere (Brown & de Boom, 1966).

The reader is referred to the major work (Brown & de Wet, 1967) for full details of these particular investigations. It is quite apparent from these studies that most of the plants which are of importance on the Karoo pastures are capable of taking up fair to moderate amounts of selenium from the soil, given the appropriate conditions. The selenium content of the Karoo vegetation is however far lower than that in the seleniferous areas of America. Certain Karoo plants, which belong to widely separated genera, appear to be able to take up potentially or frankly dangerous amounts of the element under favourable conditions. A tentative classification of the Karoo vegetation according to the apparent uptake of selenium from the soil has been attempted. This has been modelled on an earlier one presented for American vegetation by Rosenfeld & Beath (1964), and is as follows:

Group 1 *plants* (selenium " converter or indicator " plants): These are plants containing 1000 to 10,000 ppm of the element and which apparently require it for their growth and development. No plants of this nature have been found in the Karoo.

Group 2 *plants* (" secondary selenium absorbers "): These rarely contain more than a few hundred ppm of the element (30 to 400 mcg/gm dry plant material, in our classification) and absorb these amounts of the element when they grow on soils containing high concentrations of freely available selenium. Nine species of Karoo plants have been placed into this group. Of these, *Chrysocoma tenuifolia* Berg, *Eriocephalus ericoides* Druce and *Nestlera prostrata* Harv. are invariably the dominant vegetation on farms where enzootic icterus is severe and are common on farms where geeldikkop occurs on a large scale.

Group 3 *plants*: These are accumulators of low but significant amounts of selenium under a variety of conditions and have been defined in our classification as plants containing 0 to 30 mcg/gm of dry plant material. A very large number of useful and inferior Karoo bushes fall into this group. Eleven species were found to contain 10 to 30 mcg of the element per gm of plant material regularly under a wide variety of circumstances. These plants include fodder plants like *Atriplex nummularia* Lindl. and *Tetragonia arbuscula* Fenzl as well as weeds like *Salsola kali* L. and known poisonous species like *Geigeria africana* Gr. These plants must all be regarded as potentially dangerous under most circumstances.

Sixty-one species have been listed by the author as containing selenium in amounts of 10 to 30 mcg/gm under probably special circumstances only. Although the majority of these plants are probably quite safe grazing under most circumstances, this particular property of theirs should be borne in mind. Many useful fodder plants and grasses appear on this list together with quite a few unpalatable and undesirable plants.

Forty-three species have been listed which appear to contain selenium in concentrations of 5 to 10 mcg/gm under circumstances which are probably exceptional, and 22 species which appear to take up neglible amounts of the element under normal conditions in the Karoo. Some of these latter plants like *Helichrysum* and *Mesembryanthemum* species do not appear to absorb significant amounts of the element even when growing in close proximity to very seleniferous types.

There seems to be little correlation between the different families of plants and selenium uptake in the Karoo, although some families like the *Gramineae*, *Chenopodiaceae* and *Ficoidaceae* appear to contain more seleniferous plants than other families. The findings as regards the *Gramineae* are on the whole the same as those of the American workers. It has also been found that the strong perennial species (e.g. *Cenchrus* sp., *Digitaria* sp., *Eragrostis* sp. and *Panicum* sp.) contain in general higher levels of selenium than the weaker annuals, almost all of which fall into the group of plants absorbing negligible amounts of selenium. It has also been demonstrated that the seeds of the perennial types mentioned contain more of the element than the leafy parts of the plant. It is of interest to note also that some of the American species of *Atriplex* (*Chenopodiaceae*) have been classified by the workers concerned as " secondary selenium absorbers ".

The primary object of this work was not only to establish the presence of potentially harmful amounts of selenium in the Karoo vegetation, but also to demonstrate a correlation between the amount of selenium present in general and the incidence of geeldikkop and enzootic icterus. Table 97 is a summary of the data in this respect (Brown & de Wet, 1967) presented in the form of comparisons between the data obtained from the vegetation of areas where the two syndromes occur, and those

TABLE 97.—*The percentage distribution of selenium in the vegetation of areas where geeldikkop and enzootic icterus are rife and areas where the diseases do not occur naturally*

Item	(1) Fraserburg Show-grounds	(2) Farms on which enzootic icterus is severe	(3) Farms on which geeldikkop is severe	(4) Farms on which geeldikkop appears sporadically	(5) Areas in which geeldikkop and enzootic icterus do not occur
% of plants examined containing more than 30 mcg Se per gm of dry material.....	2·5	4·2	4·3	0	0
% of plants examined containing from 10–30 mcg Se per gm of dry material	25·6	10·6	21·7	7·1	0
% of plants examined containing from 5–10 mcg Se per gm of dry material	28·2	19·1	21·7	14·2	22·2
% of plants examined containing less than 5 mcg Se per gm of dry material	43·5	65·9	52·1	78·5	77·7
Number of specimens examined.....	39	94	23	56	54

obtained from areas in which the occurrence of the syndromes is sporadic or unknown. The Fraserburg showgrounds referred to in this table are situated slightly to the west of the main enzootic icterus area in this part of the Karoo. The vegetation on these grounds is typical of the surrounding areas, i.e. *Arid Karoo*, and is allowed

to grow undisturbed for most of the year in contrast to that on the farms in this area which are in most cases heavily overgrazed. The areas in which neither geeldikkop nor enzootic icterus occur naturally are represented in this table by plants which have been collected from widely scattered points in South Africa, from markedly different veld types and on a variety of soil conditons, e.g. Onderstepoort, Rustenburg and Vaalwater (Transvaal); Harrismith and Brandfort (Orange Free State); Adelaide, Grahamstown, East London and Thornhill (Cape Province).

There appears to be a definite correlation between the selenium content of the vegetation and the occurrence of geeldikkop and enzootic icterus. A significantly higher percentage of the vegetation contains more than 10 mcg selenium/gm in areas where enzootic icterus occurs and where geeldikkop is severe, than in areas where the incidence of the latter conditon is sporadic or where both conditons are unknown. Similarly the percentage of specimens examined which contained less than 5 mcg/gm was significantly lower from farms where enzootic icterus and geeldikkop were severe than in those from the farms where the incidence of the disease was low or unknown.

A definite correlation has been found between *mismanaged* veld types, the selenium content of the vegetation and the incidence of enzootic icterus and geeldikkop. Under conditions of mismanagement the inferior, unpalatable and undesirable plants which are well represented in Groups 2 and 3 of the classification, invade and assume a dominant role in the plant associations concerned (Brown, 1959b; Brown & de Boom, 1966).

4. *The relevant biochemistry of selenium*

The biochemistry of selenium in general has been reviewed by Rosenfeld & Beath (1964) and Gallagher (1964).

In spite of the fact that selenosis has been recognized as such for the last three decades, work on the mechanism of selenium poisoning is still in its infancy. The basis of the toxic effect of selenium is largely due to its capacity to replace sulphur from biological compounds. The resulting compounds lack the biological acitvity of the original sulphur-containing progenitors. It seems to be generally accepted that it can replace sulphur in amino acids such as cystine and methionine, the toxicity of the seleno-analogues being comparable to that of selenite and the organic selenium present in toxic grass seeds. It has been found in crude glutathione isolated from the blood of cattle poisoned with selenium and it is known that it can replace the sulphydryl groups of dehydrogenases. Its activity as a systemic poison may well be due to this latter effect. The selenium content of hair, wool and hoof rises with chronic ingestion of selenium due probably to the replacement of sulphur in cystine and cysteine molecules (Gallagher, 1964; Rosenfeld & Beath, 1964).

Sulphate and selenate can be converted into " active sulphate " and " active selenate " by the same enzyme systems, i.e. AMP-selenate can be formed from ATP in the same manner as AMP-sulphate is formed. It is not known whether this occurs in animal tissues (Gallagher, 1964; Rosenfeld & Beath, 1964).

Although selenomethionine can serve as an efficient methyl donor as Se-adenosylselenomethionine for instance in creatine synthesis, selenite and selenate inhibit certain other transmethylations probably as a result of interference by selenium with sulphur metabolism.

Selenium is a potent inhibitor of enzyme systems, particularly those involving the action of respiratory dehydrogenases. It is known to inhibit succinic dehydrogenase probably by interference with sulphydryl metabolism. It is known to have an inhibitory effect on isocitric dehydrogenase, pyruvic oxidase, glyceraldehyde-phosphate dehydrogenase, choline oxidase, proline oxidases and tyramine oxidase. Most of the enzymes which require –SH groups for their function are effectively inhibited by selenium. Inorganic selenium salts have a marked inhibitory effect on glycolysis by yeast cells. Similar effects with inorganic selenium have not been obtained in work with slices of liver, kidney and tumour, although such effects were noticeable when rat liver homogenates were used. Selenocystine has been found to be a very potent inhibitor of succinic dehydrogenase in animal tissues (Gallagher, 1964; Rosenfeld & Beath, 1964).

Hirooka & Galambos (1966a, 1966b, 1966c) in some recent work on selenium metabolism have advanced the thought that dimethyl-selenide and other organic selenium compounds may be synthesized in the liver from inorganic selenium salts and that these may be transported in the lipid moiety of the serum proteins and/or by the erythrocytes to the lungs for excretion. After administration $^{75}SeO_4^{2-}$ the lipid fraction of serum lipoproteins was firmly labelled in both man and the rat. A higher association of ^{75}Se was found with the $\alpha 2$–globulins than with the other serum protein fractions. Subacute liver injury was found by them to be associated with increased uptake of selenium by the kidneys and lungs. In normal tissues much of the administered ^{75}Se was found to pass into the mitochondrial and supernatant cell fractions.

Other biochemical effects seen in selenium intoxication include a gradual decrease in the blood levels of vitamins A and C, a reduction in the rate of conversion of carotene to vitamin A, low liver storage levels of vitamins B_{12} and C, and a slowly developing hypoproteinaemia (Brown & de Wet, 1962). Rosenfeld & Beath (1964) state:

" The large number of sulphur compounds in the living system suggests that it would be fallacious to postulate that the toxic or nutritional effect of selenium is due to the inhibition or stimulation of one enzyme or to some aberration or change in a sulphur compound. A multitude of metabolic functions may be altered to some degree in intact animals and the response to selenium may be the manifestation of the total effects of selenium on metabolic activities of the animals ".

One of the most interesting aspects of the biochemistry of selenium compounds, with regard to their possible role in the pathogenesis of geeldikkop and enzootic icterus, is their effect on cell division and growth. It has been known for some time that various inorganic and organic derivatives of this element are powerful inhibitors of cell growth in low forms of life. Many of the organic compounds of selenium possess antimitotic activity and numerous ones have been tested for anti-tumour activity, e.g. 6–selenoquanidine. Selenomethionine inhibits cell division in *Chlorella vulgaris*, but not cell growth, with the result that many giant cells often appear in the test medium. This action has been described as " uncoupling growth from division " (Rosenfeld & Beath, 1964). The reader is reminded of the hepatic parenchymal cytomegaly and karyomegaly which has been described by Pienaar & van der Merwe (1966) as a prominent histopathological feature of enzootic icterus. The same phenomenon has been observed in chronic pyrrolizidine alkaloid poisoning and in aflatoxicosis in domestic animals (Pienaar & van der Merwe, 1966). Lasiocarpine N-oxide is known to reduce the capacity of the liver parenchymal cells for mitotic division with the result that regeneration of these cells was accomplished by

an increase in the size of the cells and the ploidy of their nuclei, and not by an increase in their number (Pienaar & van der Merwe, 1966). Megalocytosis has also been induced in rabbit livers in chronic tellurium poisoning (Pienaar, personal communication).

5. *Some studies on chronic selenosis in Sheep*

The findings reported earlier in this chapter make it imperative that studies be conducted on the effects of chronic lowgrade selenium intoxication in sheep. It is probable from this work and that done elsewhere (Rosenfeld & Beath, 1964) that the element is present in most of the Karoo plants concerned in an organic form. Supplies of various organic selenium compounds have been accumulated for this purpose, but in the meantime one preliminary experiment has been carried out in which four sheep were given inorganic selenium in their diet at a level of 20 ppm over a period of six months. The required amount of sodium selenite was dissolved in water and sprayed over teff hay, which was then allowed to dry before being baled. Samples taken at random from nine such bales gave on analysis selenium values of 19·8, 25·4, 22·6, 23·0, 19·8, 26·8, 18·7, 18·4, and 18·9 ppm Se (on a dry matter basis). This hay was fed *ad libitum* to the four sheep. The sheep were bled every two weeks and a full haematological and chemical pathological study made on the samples taken.

An extremely severe anaemia developed in these animals as the intoxication progressed. Red cell counts fell in the four animals from 9·65 to 9·77 $\times 10^6$/cu mm to 3·37 to 3·51 $\times$ 10^6/cu mm six months later. Packed cell volumes and haemoglobin values similarly fell from 31 to 33 per cent and 9·41 to 10·62 gm per cent to 21 to 23·5 per cent and 7·48 to 7·72 gm per cent respectively. The anaemia which developed was characterized as *macrocytic hyperchromic*. Mean values for the haematological indices in the four animals six months after dosing were MCH 21·73 $\mu\mu$ gm; MCHC 34·1 per cent and MCV 63·76 cuμ. Bloodsmears at this stage revealed marked anisocytosis, poikilocytosis and polychromasia. Severe anaemia is a wellknown feature of chronic selenosis in many animals (Rosenfeld & Beath, 1964). Red cell fragility increased in the four animals from baseline levels of 3·45 to 5·1 per cent to values of 96·6 per cent, 89·7 per cent, 71 per cent and 45 per cent in the four cases at the end of the experiment. Red cell glutathione varied within the range of 7·5 to 21 mg per cent in these animals during the intoxication. No abnormalities of methaemoglobin reduction were seen.

Total leukocyte counts remained normal within the ranges 3·65 $\times$ 10^3 to 8·80 $\times$ 10^3/cu mm in all four cases as the experiment progressed. All four sheep developed a severe lymphocytopaenia and relative neutrophilia by the end of the experiment, the respective differential counts ranging from 11 to 16 per cent lymphocytes, and 77 to 80 per cent neutrophiles respectively.

No alterations in erythrocyte sedimentation rate were seen. Eosinophile counts fell from 100 to 420/cu mm at the start of the experiment to 60 to 80 cu mm at the end of the six months of intoxication.

Total bilirubin levels at the end of the experiment ranged from 0 to 0·75 mg per cent; thymol turbidity and flocculation tests, and the zinc sulphate turbidity and colloidal gold flocculation tests remained negative throughout the experiment, and plasma alkaline phosphatase and total cholesterol levels similarly also remained within normal limits. Glutamic oxalacetic transaminase levels were ultimately raised above normal to 240 to 267 units in all cases but glutamic pyruvic transaminase rose in one case only to terminal values of 131 to 144 units.

A hyperglycaemic tendency was present towards the end of the experiment, blood sugar levels of 63 to 69·5 mg per cent being found with a terminal frank hyperglycaemia (126 mg per cent) appearing in one case only. Plasma ascorbic acid levels remained unchanged at 1·45 to 1·93 mg per cent.

No changes were found in plasma iron and total copper levels; plasma calcium, magnesium and inorganic phosphate levels similarly remained unchanged and no evidence of impairment of renal function could be seen from the blood urea nitrogen, uric acid creatinine and amino acid levels which all remained within normal levels as the experiment proceeded.

Although plasma protein levels fell from the pre-dosing ranges of 6·6 to 7·9 gm per cent to 5·1 to 5·3 gm per cent, the albumin: globulin ratios varied little from the range 0·75 to 0·80. A slowly developing hypoproteinaemia is a known feature of chronic selenosis in domestic animals (Brown & de Wet, 1962).

Apart from terminal hyponatraemia and hypochloridaemia, no particular changes were seen in the electrolyte balance of these animals as the experiment proceeded.

Two of the four sheep died in an emaciated state six months after commencing the intoxication and the remaining two were slaughtered at the same time. Liver selenium levels were 96·0, 67·5, 70·0 and 60·9 mcg/gm (wet tissue) in the four cases (mean value for normal Onderstepoort sheep is 0·55 mcg/gm wet tissue).

Autopsy findings, confirmed histologically on the four cases, included cachexia, oedema and congestion of the lungs, atrophy of all lymphoid tissue, gastro-intestinal stasis and severe anaemia. Marked atrophy of the liver with fairly severe fatty changes was observed on microscopic examination of this organ. Some atrophy of the kidneys and adrenal cortices was apparent in sections from these tissues. Bone marrow hypoplasia was a prominent finding.

6. *General discussion*

The fact that considerably higher levels of selenium were found to be present in the livers and kidneys of typical cases of geeldikkop and enzootic icterus than in the same tissues taken from animals emanating from areas where the diseases do not occur, or where their incidence is negligible, is an indication that this element might be concerned in the pathogenesis of these syndromes. This idea is supported by the facts that the Karoo vegetation contains potentially and in some instances frankly dangerous levels of the element, and that there appears to be a definite correlation between the selenium content of the vegetation and the occurrence of the two syndromes. This particular aspect is being investigated further by the author's co-workers in the field.

Chronic selenium intoxication is known to produce many biochemical effects which could lead to the appearance of the disturbances which characterize geeldikkop and enzootic icterus, e.g. inactivation of SH-group containing dehydrogenases, suppression of glycolysis and protein synthesis and a general decrease in the level of energy production in the cells of the body. By administering inorganic selenium in amounts sufficient to produce a chronic intoxication over a period of six months, some of the features which are seen in either geeldikkop or enzootic icterus have been produced, e.g. the severe macrocytic anaemia, markedly increased red cell fragility, atrophy of lymphoid tissue and a hyperglycaemic tendency which may have indicated an impending failure of carbohydrate metabolism. Since the selenium

ingested by animals grazing the natural pastures in the areas where geeldikkop and enzootic icterus occur is most likely organic selenium, it will be of great importance to see whether such compounds produce a syndrome even more closely akin to these diseases than does selenite.

The earlier work, cited frequently in this paper, has demonstrated that if selenium is concerned in the aetiology of geeldikkop and enzootic icterus, it is by no means the only factor which must be considered. Acute attacks of either disease appear to be precipitated by certain severe non-specific forms of stress. There is no doubt of this in the case of enzootic icterus which can be reproduced at will simply by moving animals from the areas concerned over long distances by rail or road. At present the author is of the opinion that both diseases are in fact different manifestations of the lowgrade selenium intoxication. Enzootic icterus represents cases of a more severe intoxication and therefore requires comparatively mild stress to induce its acute manifestations. Geeldikkop is a much less severe intoxication and one which requires considerably more violent forms of stress for the precipitation of its acute symptoms. This aspect of the disease and the stress factors concerned form the subject of the following chapter.

CHAPTER 14

THE POSSIBLE ROLE OF AN INFECTIOUS AGENT IN THE PRODUCTION OF THE ACUTE MANIFESTATIONS OF GEELDIKKOP AND ENZOOTIC ICTERUS

1. *Introductory remarks*

Many references have been made in the preceding chapters to the facts that the acute manifestations of geeldikkop and enzootic icterus appear to be precipitated by a variety of non-specific agents, best defined for the purposes of this paper as " stress factors ". Factors like sudden severe climatic changes, sudden changes in the nature of the natural pastures, e.g. lush growth followed by wilting and severe gastro-intestinal stasis which could for instance be induced by these changes or by the

saponins present in fast-growing invading annual plants like *T. terrestris*, appear to be operative in this regard in both instances (Brown, 1963). There is a considerable body of evidence, a lot of which has been presented in the preceding chapters, indicating that an infectious condition may be the main stressing agent which provokes the acute manifestations in the case of geeldikkop. The reader is reminded, for instance, of the severe haematological and metabolic upheavals present in the prodromal cases of the disease and of the hypergammaglobulinaemia which is present in typical cases from the onset of symptoms. The author has set out in an earlier paper (Brown, 1966a) a critical analysis of the symptomatology of geeldikkop and has indicated which symptoms are germane to this disease and also which symptoms belong properly to a relatively mild disease of probable viral origin.

It has been indicated in Chapter 10 that the cases of enzootic icterus which were studied fell into two categories as far as their manner of production was concerned, i.e. those which were precipitated by transport in confined spaces over long distances and subjected to drastic changes in diet and those which were collected during severe epizootics of the condition in which an infectious agent could well have been operative.

It was quite obvious from all our studies and those of previous workers that if an infectious agent was operative in precipitating outbreaks of either syndrome, it was not of bacterial nature. The severe epizootics of both conditions occur in the mid- and late summer months after the heavy annual rains. This is a period in which there is germination and rapid growth of a very large number of annual plants. It is also a period in which there is a marked upsurge in insect life as was found out through bitter experience during the many summer months spent working in the field. The epizootiology, symptomatology and haematology suggest an insect-borne virus infection. Repeated complaints from farmers in the areas concerned have been heard that the vaccine in current use against bluetongue does not give adequate protection in young animals. Some of these cases are undoubtedly due to negligence and careless use of the vaccine, but many have been shown to be geniune. The symptomatology of the disease suggests an infection which produces many symptoms reminiscent of a mild attack of bluetongue, an insect-borne disease (Brown, 1966a). For these reasons it was therefore decided to study the effects of mild bluetongue infections on sheep and to see whether any of the chemical pathology of geeldikkop or enzootic icterus could be reproduced by viruses of this nature. Since it was postulated that the infectious agent precipitates the acute outward manifestations of a largely sub-clinical disease entity (Chapter 13) it was obviously of importance to study the effect of bluetongue viruses on sheep which were newly obtained from areas where geeldikkop and enzootic icterus normally occur as well as on sheep born and raised elsewhere.

2. *Animals, infectious agents and methods*

A total of thirty-nine adult Merino wethers drawn from the pool of available sheep at Onderstepoort were used for this work, together with eight clinically normal Merinos obtained during a geeldikkop-free period from a farm on which the disease has occurred regularly. The data pertaining to plasma levels of certain enzymes were supplemented by similar data obtained from six typical early cases of geeldikkop (sheep 22860 to 22865) and two early cases of enzootic icterus (sheep E1 and E2) obtained from affected farms during severe recent outbreaks of the two syndromes.

The clinically normal Onderstepoort and Karoo animals were infected with a strain of bluetongue virus which produced in a number of cases a clinically very mild disease entity, some seven to ten days after infection by the intravenous route.

This disease entity consisted in most cases simply of a sudden fever reaction lasting two to three days, during which time body temperatures of over 104°F were found in affected animals. In the majority of affected cases no particular clinical symptoms of note were observed, not even anorexia being shown by many of the affected animals. In only a few instances were erosions seen on the external internasal septum and the dorsal and lateral aspects of the tongue and coronary band haemorrhages.

The virus strains used were supplied by Dr. P. G. Howell, of the Virus Research Section of this Institute, and were strains isolated in the field and designated by him " Type 1 B-Berg " and " Type 3 Cyprus ".

The animals were housed, maintained and fed as described earlier in this work for the experiments performed at Onderstepoort and the various methods employed in these studies were as used in all the work described previously. The animals were bled for the various studies before injection and thereafter every second day until the time of the expected fever reaction when daily bleedings were done until the fever had subsided. The animals were bled thereafter every second or third day for a further period of three weeks before being discharged from the experiments. Only the data obtained from animals which showed the typical fever reaction have been used for the discussion which follows.

3. *The haematology and chemical pathology of mild bluetongue infections*

(*a*) *Haematology*: Injection of the bluetongue virus strains produced in nine of the clinically normal Onderstepoort sheep a fair anaemia during the three weeks following the start of the reaction to the virus infection (which as has been said, lasted only two to three days itself). Haemoglobin and packed cell volume values and red cell counts fell in these sheep from 9·7 to 13·0 gm per cent, 28 to 43 per cent and 8·9 to 14·9 $\times$ 10^6/cu mm respectively before infection, to 6·6 to 8·6 gm per cent; 18 to 27 per cent and 7·0 to 7·2 $\times$ 10^6/cu mm three weeks after the start of the fever reaction. Calculation of the absolute haematological indices in these cases showed the anaemia to be *hypocythaemic normocytic and normochromic*.

White cell counts remained unchanged in all but two of the animals. These two cases developed mouth lesions, the appearance of which was followed by the development of a leukocytosis. Total leukocyte counts rose in these two instances from baseline values of 5,800 to 7·900/cu mm to 12,100 to 30,000 during the development of the lesions. Differential white cell counts showed this leukocytosis to be due to a severe neutrophilia, which was accompanied by a relative lymphocytopaenia and monocytopaenia.

Three of the eight normal animals emanating from the areas where geeldikkop is prevalent showed a temperature reaction and developed subsequently a similar mild anaemia, characterized in these cases as a *hypocythaemic macrocytic hypochromic* anaemia. This anaemia developed during the three weeks following the start of the fever reaction. No changes in the total leukocyte counts were noted in these cases.

The decline in haemoglobin values, red cell counts and packed cell volume percentages after the start of the fever reaction in both groups of animals was not associated with a similar change in the total plasma protein levels and was thus in all likelihood not due to water retention and consequent haemodilution.

Erythrocyte sedimentation rates remained unchanged in all the animals which reacted to the infection.

(*b*) *Liver function*! Most of the animals which showed a temperature reaction developed a very mild hyperbilirubinaemia; no values higher than 0·75 mg per cent of total bilirubin were recorded for plasma samples from any of the animals and the pigment present appeared to be mainly bilirubin.

The urinary excretion of coproporphyrin was studied in eight of the Onderstepoort sheep and all eight of the Karoo animals which were infected with bluetongue virus. The amount of coproporphyrin excreted daily by the individuals in the first group of animals ranged from 0·20 to 17·62 mcg/24 hours before infection. Five of these animals developed the typical fever reaction. In three of these five animals the coproporphyrin excretion slowly increased after the appearance of the fever to reach values of 30·40 to 40·36 mcg/24 hours six to seven days after the start of the fever reaction. Coproporphyrin excretion returned to the previous baseline levels after this. No change was observed in its excretion pattern in the remaining two animals which showed the typical fever of short duration. Three of the Karoo animals reacted to the infection and all three showed a similar transient increase in urinary coproporphyrin excretion as found in the three Onderstepoort sheep just mentioned.

Plasma phylloerythrin levels were not determined in any of these cases. All were however tested for photosensitivity by solar irradiation for about two hours each day for the two-week period following the start of the fever reaction, in the case of the Onderstepoort animals. The sheep emanating from the Karoo were kept in metabolism cages fully exposed to direct sunlight throughout the whole experiment. The latter animals which reacted to the infection and which showed increased urinary coproporhhyrin excretion became mildly photosensitive during the entire period over which the urinary excretion of this porphyrin was elevated. The animals concerned showed as symptoms of this phenomenon, hyperaesthesia, pruritus, hyperaemia and mild oedema of the lips, face, eyelids and ears and exposed shorn areas of the back. Marked hyperaemia, oedema and pain in the coronary bands of all four feet were also seen in these cases. The same symptoms were discernible in the Onderstepoort sheep which reacted but to a much less obvious degree. Composite Fig. 15 shows these symptoms in one of the Karoo animals at the time when its urinary coproporphyrin excretion was maximal.

No changes of any particular significance were found in the total plasma protein levels of any of the sheep which developed a fever reaction. In come of the animals studied, the γ–globulin fraction increased markedly very soon after the appearance of the fever reaction, maximum levels of this plasma protein fraction being attained about two to three weeks after the start of the fever reaction. Thereafter these γ–globulin levels slowly fell once more towards the pre-infection values. Albumin levels tended to decrease as the γ–globulin fraction rose and *vice versa* towards the end of the experiments concerned. The data from one of these animals are represented in Fig. 16 by tracings of cellulose acetate strip electrophoretograms made from plasma samples at the intervals indicated. The trends described above are obvious.

Plasma iron levels were studied in five of the Onderstepoort sheep and all of the animals emanating from Karoo farms. In general, no changes of any significance from the pre-infection range of 151 to 286 mcg per cent found for all these cases were noticed. Total plasma copper levels remained unaltered in the same animals, the range before and after infection being 110 to 200 mcg per cent for the whole group.

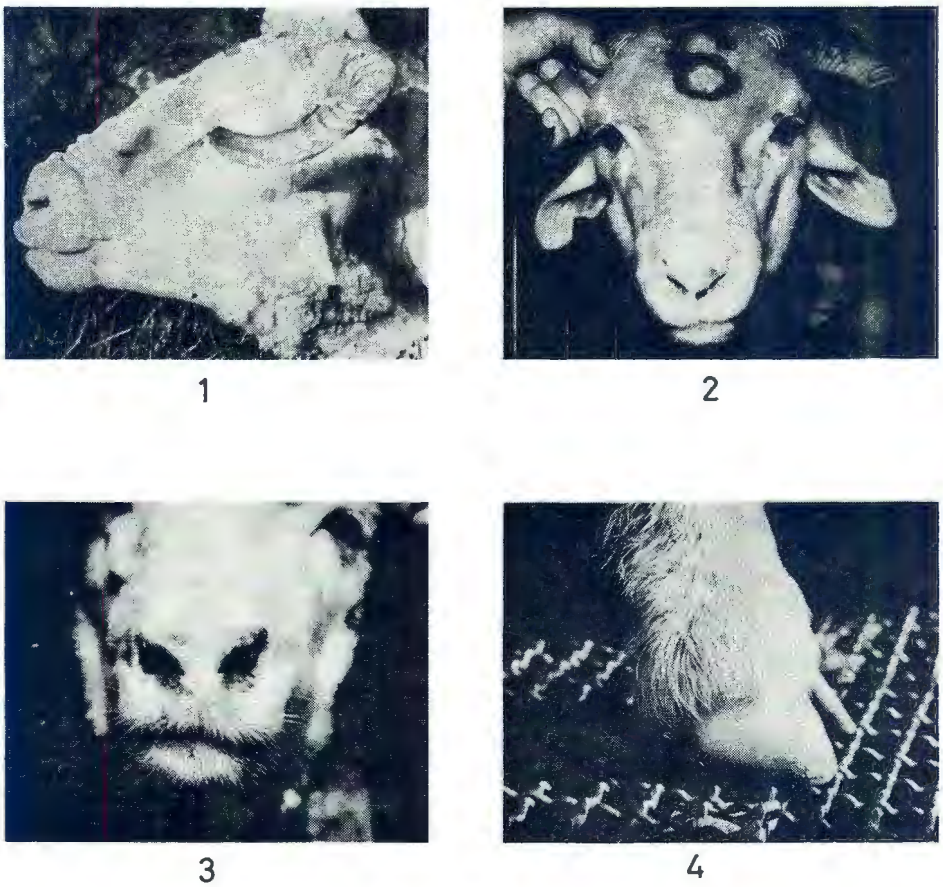

FIG. 15.—Photosensitivity reaction following injection of a mild strain of bluetongue virus into a sheep emanating from an area where geeldikkop is enzootic:

1. Swelling of the face, nostrils and lips
2. Front view of the same sheep. The angle at which the ears are held is typical of what occurs as a result of oedema during a photosensitization reaction
3. Close-up view of the nasal region of the same sheep. Note the erosion on the internasal septum
4. Swelling of the coronary band and surrounding tissues in the right forelimb of the same animal

Bromsulphalein clearance tests were performed on a number of the cases studied at various intervals throughout the fever reaction and during the two weeks which followed. In no instance was any failure to clear the dye completely from the blood within the thirty-minute test period noticed.

In three separate publications attention has been drawn to the fact that the levels of activity of certain enzymes in plasma may rise sharply some eight days after the height of the febrile reaction to bluetongue viruses and that such elevations may persist for a further week or so. The cause of the increase of these enzymes in

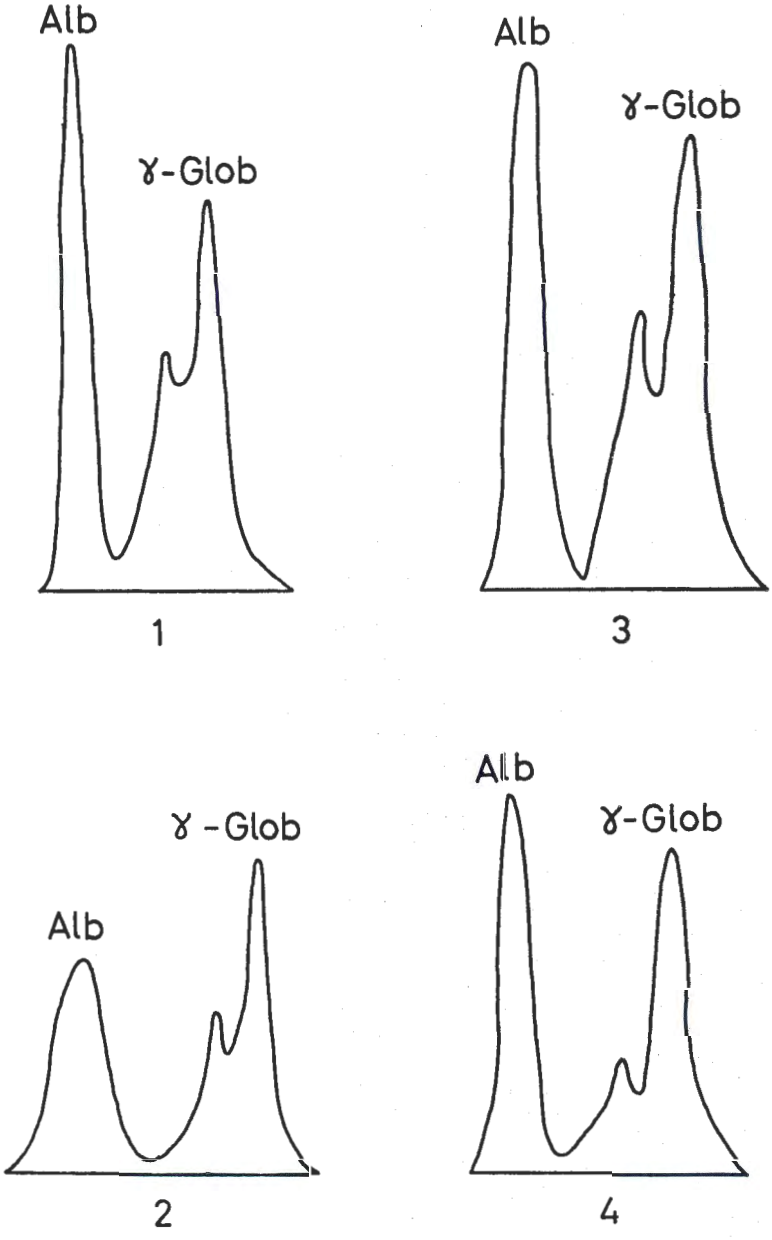

FIG. 16.—Electrophoretograms of plasma proteins in typical reactors, following injection of a mild strain of bluetongue virus:

1. Before infection
2. Two days after the appearance of the typical fever
3. Thirteen days after the appearance of the fever
4. Thirty-six days after the appearance of the fever

31610—14

the blood of affected animals was considered to be skeletal myopathy (Brown, 1966a; Clark, 1966; Clark & Wagner, 1967). The levels of activity of alkaline phosphatase (AP), isocitric dehydrogenase (ICD), phosphohexose isomerase (PHI), arginase (Arg), glutamic oxalacetic transaminase (GOT), glumatic pyruvic transaminase (GPT), aldolase (Ald), lactic dehydrogenase (LD) and creatine phosphokinase (CPK) were determined on the plasma of various experimental animals used during this work. Clark & Wagner (1967) have reported on the changes found in the levels of activity of GPT, GOT, Arg, Ald, CPK and LD. The interested reader is referred to their paper for the experimental details. Briefly, the following were the main findings of note: marked elevations of all these enzymes, except Arg were found in many of the cases studied during the post febrile period of the disease particularly. The most consistent rises were found in the case of GPT and Ald. CPK was found to be elevated in only about 50 per cent of cases during the post febrile period, although extremely high values were found in the plasma of fatal cases just after this period. High values for Ald and LD persisted for up to two weeks after the fever had passed off. GPT and LD were commonly elevated during the febrile period as well as after it, but the activity of the other enzymes only rarely so. Arginase levels were assayed on 32 plasma samples showing high activity of the other enzymes mentioned. In none of these samples was any increase in activity observed.

No changes were observed in ICD levels in any of the cases studied during the experimental periods concerned. Mild variable elevations of plasma PHI levels were found during and after the febrile period in most cases, while AP levels either remained unchanged or varied so widely before and during the experiments that no particular significance could be attached to them.

The animals emanating from the Karoo were not included in the study reported by Clark & Wagner (1967). The findings as regards the plasma enzyme levels mentioned were however the same in these particular cases, except that elevations of GPT activity were seen in only one of the three reacting animals. PHI activity was markedly elevated in all three reactors during the febrile period and for at least a week afterwards.

The six typical early cases of geeldikkop (sheep 22860 to 22865) followed the pattern described above, although markedly elevated plasma CPK was found only in sheep 22860, the most severely affected of the group.

During the writing of this particular chapter the author received two early cases of enzootic icterus from amongst a large number which were dying shortly after being transported by rail from Carnarvon to Kimberley, a distance of about 300 miles. These two particular animals were subjected to a further train journey of 350 miles to Onderstepoort. When examined by the author and his staff, the following levels of enzyme activity were found in their plasma: GOT, 620, 545; GPT, 36, 26; LD, 3750, 4480; PHI, 232, 221; Ald, 195, 88, and CPK 2·5 and 11 units respectively. Thus although extremely high values for GOT, LD and Ald may be found in acute enzootic icterus precipitated by transporting over long distances in a confined space, GPT and CPK values remain well within the normal limits set for these two plasma constituents, namely 7 to 88 and 3 to 17 units respectively. The reader should compare the values for plasma GPT found in these cases with those found in naturally-occurring cases of the syndrome examined during severe epizootics (e.g. Sheep 12223, 12224, 12225, Bekker 1, F7, F8 and F12, featured in Table 75) and those found in the bluetongue cases discussed above.

(*c*) *Carbohydrate metabolism*: Blood sugar levels were studied in seven of the animals which developed the typical fever reaction. Four of these animals were Onderstepoort sheep and the remaining three were those which came from the geeldikkop areas in the Karoo. Pre-injection blood sugar levels varied in all these animals

between the limits 29 to 59 mg per cent. In all four Onderstepoort sheep blood sugar levels rose towards the end of the fever reaction to 60 to 75 mg per cent and remained at these levels for about a week after the start of the fever. The same tendency was seen in the three Karoo sheep. Glucose tolerance tests performed on these animals showed that the hyperglycaemic tendency was associated with a decreased rate of clearance of a loading dose of glucose from the blood. Typical glucose tolerance curves from two of these animals and one which failed to react are reproduced in Fig. 17. The top set of curves in this figure shows the trend in one of

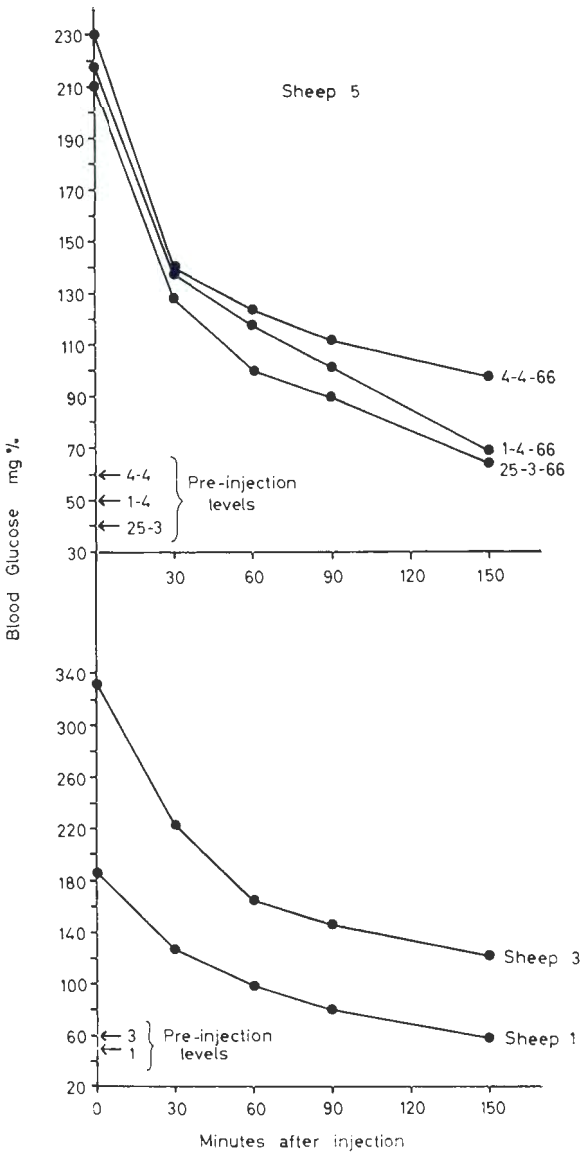

FIG. 17.—Glucose tolerance curves in cases of a mild bluetongue virus infection (sheep 3 and 5 and in a control animal (Sheep 1). Sheep 5 was infected with the virus on 26–3–66

these reactors, while the lower set is a comparison between glucose clearance in the other infected case at the height of its reaction and glucose clearance in a non-reactor on the same day.

Plasma lactate and pyruvate levels were studied in four different Onderstepoort sheep which reacted and in the three Karoo animals mentioned above. The febrile reaction was followed in one Karoo sheep by a sharp rise in plasma lactate up to 71·4 mg per cent during the hyperglycaemic period. No changes of note were seen in this respect in the other cases. Pyruvate levels remained within the pre-injection range in all instances, during and after the febrile reaction. Plasma ascorbic acid concentrations were studied in the same animals and similarly showed no deviations from the pre-injection range found for the two groups (viz., 1·20 to 2·00 mg per cent).

(*d*) *The state of the erythrocytes*: Clark (1966) reported that the post-febrile period of mild bluetongue reactions such as studied in the cases reported here, was associated with a marked and long sustained rise in red cell fragility. This phenomenon did not appear to be associated with a decrease in methaemoglobin reductase activity. Red cell fragility was studied in twenty-seven Onderstepoort sheep which reacted to the virus and in the three Karoo reactors. Pre-injection values found for this test in these animals ranged from 3·7 to 54 per cent rupture of the erythrocytes in 0·7 per cent NaCl solution. In ten of the Onderstepoort sheep red cell fragility increased markedly from four to eight days after the start of the febrile reaction, values in the order of 61 to 94 per cent rupture in 0·7 per cent NaCl being found for a number of days. In two of these cases a sustained progressive rise in red cell fragility was seen over the entire three-week period following the febrile reaction. Two out of the three Karoo sheep showed a transient rise in red cell fragility during the week following the fever reaction.

Methaemoglobin reductase activity in the erythrocytes was studied in the same animals used for the fragility studies. No abnormalities in this respect were noted in any of the animals during the course of the experiments.

Red cell glyceraldehyde phosphate dehydrogenase activity was studied during these experiments in seven of the Onderstepoort reactors and the three Karoo sheep mentioned above. Levels of activity of this enzyme ranged from 310 to 1120 mcg/ml of erythrocytes in the ten animals before infection. No change in this respect was observed during the month following the febrile reaction in the Onderstepoort sheep, but marked increases in the levels of activity of this enzyme in the red cells of all three Karoo sheep were observed during the week following the febrile reaction. Values in the range 1240 to 3990 mcg/ml were observed during this period. This rise in activity was followed by a return to normal in three instances during the second week of the post-febrile period.

(*e*) *Kidney function*: Urine analyses were performed at regular intervals throughout the experiments in most of the cases studied. No deviations from normal were found at any time in any of the cases. Blood urea nitrogen and creatinine levels were followed in seven of the sheep which reacted to the infection. No departures from the preinfection ranges of 9·2 to 21·1 and 0·05 to 0·9 mg per cent respectively were observed in any of the cases during the course of the experiments. Kidney function in these animals did not thus appear to be impaired as a result of the mild bluetongue infection.

(*f*) *Adrenal function and electrolyte balance*: This was studied in four of the typical reacting animals. In all four instances the onset of the febrile reaction was heralded by a fall in absolute eosinophile counts from pre-infection values of 200 to

760/cu mm to 0 to 20/cu mm. Such low counts persisted for two to three weeks after the febrile reaction had ended. No alterations of any significance were observed in the plasma concentrations of sodium, potassium, calcium, magnesium, chloride bicarbonate and inorganic phosphate in any of the animals during the fever reactions or the post-febrile period of three weeks. Blood and plasma volumes were determined at regular intervals throughout the experiments in two cases. No changes in either were noted as the experiments proceeded.

4. *General discussion*

Infection with the mild strains of bluetongue virus which were used for this work produced very few chemical pathological disturbances of note during the course of the febrile reaction itself. It was followed however by some very serious disturbances of metabolism during the post-febrile period. Although some of these disturbances appeared towards the end of the febrile period, most appeared four to eight days later and in some cases persisted for up to three weeks or more after the start of the fever reaction. This is exactly the type of biochemical upheaval which has been postulated as being operative in precipitating geeldikkop and which leaves its unmistakable imprint on the chemical pathology of the early cases of this disease.

The characteristic disturbances produced by the bluetongue virus strains used were a mild but progressively worsening aneaemia similar in nature to that seen in geeldikkop and enzootic icterus; a marked and in some cases very persistent increase in red cell fragility; marked disturbances in carbohydrate metabolism manifested by a distinct hyperglycaemic tendency and a retarded rate of clearance of injected glucose from the blood and skeletal myopathy which shows up as a marked and often sustained rise in the levels of activity of enzymes like GOT, GPT, Ald, LD and CPK in the blood. These are all prominent features of early geeldikkop cases.

The anaemia following infection with the bluetongue virus appears soon after the febrile reaction and becomes progressively worse during the three weeks which follow, while the increase in red cell fragility appears within four to eight days of the start of the fever. The hyperglycaemic tendency and reduced tolerance to injected glucose appear towards the end of the fever reaction and remain in general for a further week. This week is marked by the appearance of mild disturbances in biliary porphyrin excretion (associated with mild photosensitivity) and by the appearance of muscle lesions as reflected by the marked increase in the levels of the plasma enzymes mentioned. Although certain functions of the liver, like porphyrin excretion via the bile may be slightly embarrassed at this stage, the gross function and architecture of this organ appears to remain largely undisturbed as far as can be judged by BSP clearance from the plasma, plasma arginase and plasma bile pigment levels. The mild hyperbilirubinaemia seen in these cases is commonly encountered in mild stress conditions in sheep (Brown, 1963).

The disturbances described are primary effects of the bluetongue virus and do not follow any failure of adrenal function. The only apparent response to the stress of bluetongue infection that is evident from the chemical pathology of these cases is a marked fall in the levels of the circulating eosinophiles of these animals during and after the period of fever. No evidence of any adrenal hypocorticism has been found at any stage in bluetongue infections.

The fact that plasma levels of GPT are consistently elevated in the post-febrile period of bluetongue infections and that these elevations are not associated with detectable liver cell damage, indicates that this enzyme is leaking out of damaged muscle tissue. Elevations of the activity of the enzyme are seen in the plasma of

some geeldikkop cases and of naturally occurring enzootic icterus cases. Since similar rises in GPT concentration are not a feature of cases of enzootic icterus produced artificially by transport over long distances, this is taken as an indication that natural cases of this disease may be precipitated by the same possibly infectious agent which appears to be operative in the extensive annual geeldikkop outbreaks.

The presence of high plasma levels of GOT, GPT, Ald, LD and CPK in the blood of early geeldikkop cases has been shown in the earlier chapters to be due to myopathy. If this is the result of an infectious agent similar in nature to the bluetongue virus strains used, then by analogy with the work discussed in this chapter, the febrile reaction caused by this agent must have occurred about a week prior to the appearance of the typical symptoms of geeldikkop. The bluetongue cases produced by us, which showed a hypergammaglobulinaemia, did so soon after the start of the febrile period. Maximum plasma levels of γ-globulin were found in these animals about two to three weeks later. This would fit the fact that hypergammaglobulinaemia is already in evidence in geeldikkop cases at the moment the first typical symptoms appear.

The transient increase in erythrocytic glyceraldehyde-phosphate dehydrogenase activity which was found to follow the febrile reaction in the Karoo sheep given bluetongue is interesting and may be part of a compensatory mechanism for increased energy supply to these cells in a period of biochemical crisis.

It was hoped to produce a far more dramatic response to the bluetongue virus in the eight animals emanating from the farms where geeldikkop is generally severe, than was actually obtained. In terms of the hypothesis propounded at the end of the preceding chapter, the author would have liked to have seen these animals become typical acute cases of geeldikkop. There is a considerable amount of doubt however, that these sheep were in fact fully susceptible to the bluetongue virus with which they were infected—only three of these cases showed a mild fever reaction after infection, but these nevertheless did show a greater degree of photosensitivity than their Onderstepoort counterparts. Another point to be remembered is that if a virus infection is actually concerned in precipitating geeldikkop and enzootic icterus, it may be a myotropic one quite different in its various properties to the bluetongue virus strains. The author has never yet found a typical unequivocal case of bluetongue on an affected farm during a geeldikkop outbreak. All that can be said in this regard is that many symptoms in geeldikkop cases *reminiscent* of bluetongue (Brown, 1966a) have been seen.

CHAPTER 15

On the Relationship of Geeldikkop to Enzootic Icterus

In the foregoing chapter of this thesis many features of the epizootiology, symptomatology, histopathology, chemical pathology and general biochemistry which are common to both conditions have been pointed out. It remains now only to summarize the more important of these common features and to emphasize some particular points which may lead to the final elucidation of the aetiology of these diseases.

Geeldikkop and enzootic icterus are basically diseases which arise as a result of sudden aberrations in the selective permeability of the membranes or active transport across these of the cells of body tissues like the liver, kidneys, muscles and erythrocytes. The aberrations themselves are the consequence of a low-grade chronic intoxication, in which selenium may be of prime importance, and appear suddenly as the result of severe metabolic disturbances which are in turn induced by various severe forms of stress. The sheep is rendered susceptible to the various metabolic upheavals by its peculiar metabolic bias in favour of fatty acid metabolism and gluconeogenesis from non-carbohydrate sources. The latter processes may fail in the event of severe adrenal hypofunction, thus placing the full burden of energy supply on fatty acid metabolism. Should this be embarrassed by a failure of fatty acid supply as a result of severe gastro-intestinal stasis the consequences are bound to be very serious for the animal concerned. In a sheep maintained anywhere in the world other than the areas where geeldikkop or enzootic icterus occur the consequences would include largely the well-known signs of ovine ketosis and acidosis and as such would be no longer remarkable. If, however, selenium intoxication with its own particular set of biochemical effects is accepted as part of the aetiology of these diseases then it becomes easy to see why the traditionally expected ketotic syndrome is preceded, owershadowed or even completely modified by a highly specific set of constantly occurring symptoms which give rise to the clinical entities called geeldikkop and enzootic icterus and those vague intermediate or mixed syndromes for which there is as yet no name.

Both major syndromes are characterized in their early and advanced forms by lymphocytopaenia, relative neutrophilia and a striking anaemia, all of more or less severity. In geeldikkop the anaemia is generally hypocythaemic, hypochromic and macrocytic while in enzootic icterus it is hypocythaemic normochromic (or hyperchromic and normocytic (or macrocytic). Since geeldikkop is precipitated by more powerful forms of stress than enzootic icterus, the haematological disturbances which characterize it are often the manifestations of more prolonged demands on the haemopoietic tissues, hence the appearance of severe leukopaenia, thrombocytopaenia and the presence of large numbers of immature erythrocytes in the circulating blood in this condition.

The disturbances in hepatic cell membrane permeability are manifested in the early cases of both conditiohs by the presence of bilirubin glucuronides in the blood, bileaciduria, phylloerythrin retention, coproporphyrinuria, hyperferraemia, markedly increased liver copper storage, hypercholesterolaemia, and failure to excrete bromsulphalein via the bile. The further course of events is then largely determined by the nature of the syndrome itself, e.g. in severe geeldikkop the icterus is largely due to continued retention of bilirubin glucuronides whereas in enzootic icterus the markedly accelerated rate of red cell breakdown leads to a predominance of bilirubin in the plasma. Failure of the liver cell to excrete bromsulphalein in advanced cases of geeldikkop does not involve the marked failure to clear the dye from the blood as is seen in all forms of enzootic icterus, although this is seen in at least 50 per cent of *early cases* of the former syndrome.

Elevated plasma γ–globulin levels are seen in early geeldikkop cases from the moment the symptoms of this condition appear. The same finding is evident in *naturally occurring* early and chronic cases of enzootic icterus, and is associated in both *naturally occurring* syndromes with evidence of myopathy as judged by elevations of the levels of activity in the plasma of enzymes like GPT, CPK and Ald. The reader is reminded of the skeletal muscle and myocardial lesions which have been

detected histologically in individual cases of both syndromes. Plasma proteins occurring in increased amounts in early and advanced cases of both syndromes include the $\alpha 2$– and β–globulins and in particular ceruloplasmin.

Both conditions are marked by a hyperglycaemic tendency and reduced tolerance to loading doses of glucose in the early stages particularly. This is associated with an increase in plasma lactate levels in geeldikkop (not yet studied in enzootic icterus) and increased plasma ascorbate concentration in both syndromes.

Markedly increased red cell fragility is a feature of the early and more advanced cases of both conditions and is associated in individuals with decreased ability to reduce methaemoglobin. Reduction of glyceraldehyde-phosphate dehydrogenase activity in the erythrocytes is a feature of geeldikkop which has not yet been studied in enzootic icterus.

Both syndromes include a uraemia of more or less severity which is evident from the moment the symptoms appear and which is associated in the early stages with mild renal histopathology, mild bilirubinuria, mild albuminuria, failure to excrete bromsulphalein, phylloerythrin and uric acid. The initial disturbances in membrane permeability may be supplanted in both conditions by severe nephrosis which may possibly be biliary or haemoglobinuric in origin and which brings in train more pernicious symptoms such as oliguria, hypercreatininaemia, hypermagnesaemia phosphataemia and severe terminal albuminuria.

Although severe Addisonian electrolyte disturbances may precede the appearance of the symptoms of geeldikkop and dominate much of the biochemical picture thereafter, similar disturbances are seen in enzootic icterus generally to a lesser degree. The pathology of both diseases includes atrophy of lymphoid tissue throughout the body. In this context it should be recalled that iron-free pigment-laden macrophages are found in the lymph glands of cases of both conditions and even in these structures in apparently clinically normal animals in areas where the conditions are enzootic.

The administration of icterogenin to sheep induces many features of both syndromes particularly those attributable to disturbances in hepatic cell membrane integrity, e.g. regurgitation of bilirubin glucuronides, phylloerythrin, coproporphyrin, copper and bile acids into the blood circulation and failure to clear bromsulphalein from the plasma. Under special circumstances such as the administration of a number of consecutive small doses of the compound orally or single exceptionally large doses, the permeability of kidney, muscle and red cell membranes may be affected as well. This has as its consequences decreased renal abiltiy to excrete bilirubin glucuronides, bile salts and bromsulphalein, elevated plasma levels of GOT, Ald and CPK and uraemia, associated terminally with hypermagnesaemia and hyperphosphataemia. Adrenal hypofunction is seen only after prolonged administration of the compound. Icterogenin does not induce the red and white cell dyscrasias which are typical of the two diseases, nor does it induce the typical disturbances in carbohydrate metabolism. Its biochemical effects have as noted earlier, been shown to include alterations in the lipid components of hepatic cell membranes and induction of decreased levels of activity of hepatic succinic dehydrogenase, ATP-ase and glyceraldehydephosphate dehydrogenase.

Intoxication with inorganic selenium induces a macrocytic hyperchromic anaemia associated with increased red cell fragility, lymphocytopaenia, atrophy of all lymphoid tissue and the hyperglycaemic tendency typical of the two conditions.

The bluetongue viruses used induce the features of both diseases attributable to myopathy, namely increases in the plasma levels of GPT, CPK, Ald and LD. These elevations are associated with a marked hyperglycaemic tendency, slower rate of clearance from the blood of injected glucose and hyperlacticacidaemia. Simultaneous findings are marked increases in red cell fragility, increased plasma γ-globulin levels, coproporphyrinuria and a normocytic normochromic anaemia. Disturbances of porphyrin metabolism are most marked in animals emanating from the Karoo.

It is possible thus to reproduce most of the symptoms and disturbances which characterize the three components of the disease which manifests itself as geeldikkop or enzootic icterus depending on environmental circumstances, notably the basic underlying intoxication, the acute episodes and the disturbances directly attributable to the precipitating agent. It must be stressed, with regard to the latter, that the work on and discussion of the chemical pathology of mild bluetongue infections must not be construed as an attempt to incriminate these particular virus strains in the aetiology of the syndromes. There is however, much evidence to indicate that a similar type of myotropic virus may be operative in this regard and it is important to remember that the typical biochemical disturbances induced by the bluetongue virus only become evident during the post-febrile period of the infection. Herein may lie an explanation of the failure of previous workers to demonstrate the participation of an infectious agent in these diseases, since transmission experiments were done only with material obtained from cases showing unequivocal symptoms of geeldikkop or enzootic icterus.

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APPENDIX 1

DETAILS OF THE SHEEP USED IN THE STUDIES ON THE HAEMATOLOGY, GENERAL CHEMICAL PATHOLOGY, BIOCHEMISTRY AND HISTOPATHOLOGY OF GEELDIKKOP

A. Prodromal Cases

Sheep No.	Place of origin	Relevant clinical data
VB-P.....	" Werna ", Vosburg.....	Fever. No further symptoms
VB-Q.....	" Werna ", Vosburg.....	Fever. No further symptoms
VB-R.....	" Werna ", Vosburg.....	Fever. No further symptoms
VB-S.....	" Werna ", Vosburg.....	Apparently normal
VB-T.....	" Werna ", Vosburg.....	Apparently normal
VB-U.....	" Leeukopspan ", Vosburg.....	Apparently normal
VB-V.....	" Leeukopspan ", Vosburg.....	Fever? No further symptoms
VB-W.....	" Leeukopspan ", Vosburg.....	Apparently normal
VB-X.....	" Leeukopspan ", Vosburg.....	Apparently normal

B. Early Cases (1–7 *days standing*)

Sheep No.	Place of origin	Relevant clinical data
VB-A.....	" Werna ", Vosburg.....	Case of about 3 days standing. Severely icteric, highly photosensitive, severe lesions of photosensitization
VB-B.....	" Werna ", Vosburg.....	Case of about 3 days standing. Severely icteric, highly photosensitive, severe lesions of photosensitization
VB-C.....	" Werna ", Vosburg.....	Case of about 3 days standing. Severely icteric, highly photosensitive, severe lesions of photosensitization
VB-E.....	" Werna ", Vosburg.....	Case of about 3 days standing. Moderate icterus. Severe lesions of photosensitization
VB-F.....	" Werna ", Vosburg.....	Case of about 3 days standing. Severe icterus. Severe lesions of photosensitization
VB-G.....	" Tituspan ", Vosburg.....	Case of about 4 days standing. *In extremis*. Severely icteric. Severe lesions of photosensitization
VB-H.....	" Tituspan ", Vosburg.....	Case of about 1–2 days standing. Listless, apathetic, anorexia. Mild icterus and lesions of photosensitization

APPENDIX 1 (*continued*)

Sheep No.	Place of origin	Relevant clinical data
VB-I.....	" Tituspan ", Vosburg.....	Case of about 1–2 days standing. Listless, apathetic, anorexia. Mild icterus. Fair lesions of photosensitization. Still photosensitive
VB-J.....	" Tituspan ", Vosburg.....	Case of about 4–5 days standing. Moderate icterus. Moderate lesions of photosensitization
VB-K.....	" Tituspan B ", Vosburg.....	Case of 5–7 days standing. *In extremis*. Severely icteric. Severe sloughing lesions of photosensitization. Conjunctivitis. Panophthalmia
V1-1.....	" Slypfontein ", Victoria West.....	Case of about 1–2 days standing. Severe lesions of photosensitization. Mild icterus. Nervous symptoms. Dyspnoea
V1-2.....	" Slypfontein ", Victoria West.....	Case of 1–2 days standing. Mild icterus. Photosensitive. Severe lesions of photosensitization. Dyspnoea
V1-3.....	" Slypfontein ", Victoria West.....	Case of 1–2 days standing. No visible icterus. Highly photosensitive. Severe lesions of photosensitization. Intense pain
V1-11.....	" Hebron ", Victoria West.....	Case of about 5 days standing. Temp. 104° F. mild icterus, mild lesions of photosensiti zation
V1-13.....	" Hebron ", Victoria West.....	Case of about 3 days standing. Temp. 105 F. Icteric. Severe lesions of photosensitization. Necrosis of eyelids. Rhinitis. Dyspnoea. Severe acute coronitis. Tachycardia
V1-15.....	" Cellieria ", Teviot.....	Case of 3 days standing. Mild oedema of head with some necrosis of skin on nose and forelimbs. Apathy, weak, mild icterus. Acute coronitis
V1-16A.....	" Fonteintjie ", Teviot.....	Case of 3 days standing. Mild icterus and lesions of photosentitization
V1-17.....	" Dankie ", Hofmeyr.....	Case of 2 days standing. Icterus. Photosensitive. Severe lesions of photosensitization. Apathy, malaise
V1-18.....	" Dankie ", Hofmeyr.....	Case of 7 days standing. Icterus. Mild lesions of photosensitization. Severe panophthalmia. Severe malaise and apathy
V1-22.....	"Three Sisters ", Three Sisters station	Lamb. Case of about 3 days standing. Mild icterus, mild lesions of photosensitization

APPENDIX 1 (*continued*)

Sheep No.	Place of origin	Relevant clinical data
V1-23.....	"Three Sisters ", Three Sisters station	Lamb. Case of 2–3 days standing. Icterus. Mild lesions of photosensitization. Labiitis. Erosions on external nares. Acute coronitis
V1-24.....	"Three Sisters ", Three Sisters station	Lamb. Case of 2–3 days standing. Icterus. Photosensitive. Mild lesions of photosensitization. Rhinitis
V1-25.....	"Three Sisters ", Three Sisters station	Lamb. Case of 1–2 days standing. Mild icterus. Photosensitive. Severe lesions of photosensitization. Rhinitis
V1-26.....	"Three Sisters ", Three Sisters station	Lamb. Case of 3–5 days standing. Icterus. Oedema of face. Necrosis and sloughing of skin on eyelids, nostrils and plantal surface of limbs
F-1.....	" Mierfontein ", Vosburg.....	Case of 2–3 days standing. Severe icterus Mild lesions of photosensitization. Rhi nitis, conjunctivitis
F-2.....	" Mierfontein ", Vosburg.....	Lamb. Case of 2–3 days standing. Icterus. Mild lesions of photosensitization
F-5.....	" Mierfontein ", Vosburg.....	Lamb. Case of 2–3 days standing. Mild icterus. Temp. 104° F. Mild lesions of photosensitization. Coronitis
V3-6.....	" Rooikop ", Carnarvon.....	Lamb. Case of 4–5 days standing. Mild icterus. Severe lesions of photosensitization
V3-7.....	" Mierfontein ", Vosburg.....	Case of 4–5 days standing. *In extremis*. Severe icterus. Coronitis. Mild lesions of photosensitization
V3-10.....	" Vlakfontein ", Kromrivier station	Lamb. Case of 4–5 days standing. Severe lesions of photosensitization passing over into necrosis of facial skin. Mild icterus
V3-11.....	" Rietput ", Vosburg.....	Lamb. Case of 4–5 days standing. Severe sloughing lesions on face and eyelids. Mild icterus. Coronitis
V3-13.....	" Blouboskuil ", Beaufort West....	Lamb. Case of 2–3 days standing. Mild lesions of photosensitization. Mild icterus. Coronitis
V3-14.....	" Blouboskuil ", Beaufort West....	Lamb. Case of 2–3 days standing. Mild icterus. Mild lesions of photosensitization. Coronitis
V3-15.....	" Blouboskuil ", Beaufort West....	Lamb. Case of 2–3 days standing. Moderate icterus and lesions of photosensitization. Severe coronitis

APPENDIX 1 (*continued*)

Sheep No.	Place of origin	Relevant clinical data
V3-16.....	" Mierfontein ", Vosburg.....	Case of 4–5 days standing. Severe sloughing lesions on face, eyelids and ears. Mild icterus
V3-20.....	" Kanariesfontein ", Pampoenpoort	Case of 1–2 days standing. Icterus. Severe lesions of photosensitization. Coronitis
V3-21.....	" Kanariesfontein ", Pampoenpoort	Case of 1–2 days standing. Icterus. Highly photosensitive. Severe lesions of photosensitivity. Coronitis
V3-22.....	" Mimosa Lodge ", Beaufort West	Case of 1–2 days standing. Mild icterus. Highly photosensitive. Severe lesions of photosensitization
V3-23.....	" Mimosa Lodge ", Beaufort West..	Case of 1–2 days standing. Icterus. Photosensitive. Severe lesions of photosensitization. Coronitis. Rhinitis
V3-24.....	" Mimosa Lodge ", Beaufort West..	Case of 1–2 days standing. Icterus. Photosensitive? Mild lesions of photosensitization. Dyspnoea. Coronitis. Rhinitis
V3-25.....	" Mimosa Lodge ", Beaufort West..	Case of 1–2 days standing. Severe icterus. Mildly photosensitive. Mild lesions of photosensitization. Severe dyspnoea. Severe coronitis. Severe haemorrhages at base of horns. Rhinitis. Conjunctivitis
V3-26.....	" Mimosa Lodge ", Beaufort West..	Case of 1–2 days standing. *In extremis*. Severe icterus. Mild lesions of photosensitization. Coronitis. Rhinitis. Conjunctivitis
V3-27.....	" Mimosa Lodge ", Beaufort West..	Case of 1–2 days standing. Apathy. Malaise. Icterus. Severe lesions of photosensitization. Coronitis. Rhinitis

C. Advanced Cases—Group 1 (7–14 *days standing*).

Sheep No.	Place of origin	Relevant clinical data
VB-D.....	" Werna ", Vosburg.....	Case of 7 days standing. Mild icterus. Severe healing lesions of photosensitization
VB-L.....	" Werna ", Vosburg.....	Case of 7–8 days standing. *In extremis*. Moderate icterus. Severe sloughing lesions of photosensitization
VB-M.....	" Werna ", Vosburg.....	Case of 7–8 days standing. *In extremis*. Moderate icterus. Mild lesions of photosensitization

APPENDIX 1 (*continued*)

Sheep No.	Place of origin	Relevant clinical data
VB-N.....	" Werna ", Vosburg.....	Case of 7–8 days standing. *In extremis.* Severe icterus. Mild sloughing lesions of photosensitization
V1-4.....	" Slypfontein ", Victoria West.....	Case of 7–8 days standing. Mild icterus. Mild lesions of photosensitization. Severe panophthalmia and rhinitis. Severe dyspnoea. Apathy. Severe malaise
V1-5.....	" Slypfontein ", Victoria West.....	Case of 7–8 days standing. Mild icterus. Mild lesions of photosensitization. Severe dyspnoea. Apathy. Cachexia
V1-6.....	" Slypfontein ", Victoria West.....	Case of 7–8 days standing. *In extremis.* Cachexia. Mild icterus and lesions of photosensitization. Rhinitis. Panophthalmia
V1-6A.....	" Slypfontein ", Victoria West.....	Case of 7–8 days standing. Cachexia. Mild icterus. Lesions of photosensitization. Sloughing around eyelids and nostrils. Rhinitis. Panophthalmia
V1-7.....	" Slypfontein ", Victoria West.....	Case of 7–8 days standing. *In extremis.* Cachexia. Severe icterus. Mild lesions of photosensitization. Rhinitis. Labiitis
V1-8.....	" Hebron ", Victoria West.....	Case of about 10 days standing. Mild icterus. Severe lesions of photosensitization. Severe pain. Cachexia
V1-9.....	" Hebron ", Victoria West.....	Case of about 10 days standing. Mild icterus and lesions of photosensitization. Still photosensitive
V1-10.....	" Hebron ", Victoria West.....	Case of about 10 days standing. Mild icterus. Lesions of photosensitization still marked. Cachexia
V1-12.....	" Hebron ", Victoria West.....	Case of about 10–14 days standing. Mild icterus. Sloughing lesions of photosensitization. Severe acute coronitis
V1-14.....	" Hebron ", Victoria West.....	Case of 7 days standing. *In extremis.* Mild icterus and lesions of photosensitization Temp. 105° F. Tachycardia. Acute coronitis
V1-14A.....	" Hebron ", Victoria West.....	Case of 10–14 days standing. *In extremis.* No icterus. Very mild lesions of photosensitization
V1-20.....	"Three Sisters ", Three Sisters station	Case of 10–14 days standing. Cyanosis. Dyspnoea. Tachycardia. Severe sloughing lesions of photosensitization on eyelids, nostrils and plantar aspects of carpi. Acute coronitis

APPENDIX 1 (*continued*)

Sheep No.	Place of origin	Relevant clinical data
V1-21.....	"Three Sisters ", Three Sisters station	Lamb. Case of about 7 days standing. Oedema of ears. Erosions on external nares
V3-1.....	" Blikkraal ", Rietbron.....	Lamb. Case of about 8 days standing. *In extremis*. Severe icterus. Mild lesions of photosensitization. Cachexia. Coronitis
V3-1A.....	" Blikkraal ", Rietbron.....	Lamb. Case of about 8 days standing. Severe icterus. Mild lesions of photosensitization. Anaemia. Coronitis. Diarrhoea
V3-2.....	" Blikkraal ", Rietbron.....	Lamb. Case of 5–8 days standing. Mild icterus. Photosensitive. Severe sloughing lesions of photosensitization
V3-17.....	" Mierfontein ", Vosburg.....	Lamb. Case of about 7 days standing. Mild icterus. Sloughing lesions of photosensitization. Coronitis. Cachexia
V3-18.....	" Mierfontein ", Vosburg.....	Case of about 7 days standing. Severe icterus. Sloughing lesions of photosensitization. Cachexia. Coronitis
V3-8.....	" Sandaar ", Victoria West.....	Case of about 7–8 days standing. Mild icterus. Mild sloughing lesions on nose
V3-9.....	" Vlakfontein ", Kromrivier station.	Case of about 7–8 days standing. Mild icterus. Mild sloughing lesions on face. Coronitis
V3-12.....	" Rietput ", Vosburg.....	Case of about 7–8 days standing. Negligible icterus. Mild sloughing lesions on face. Coronitis

D. Advanced Cases—Group 2 (14–21 *days standing*)

Sheep No.	Place of origin	Relevant clinical data
VB-N1.....	" Werna ", Vosburg.....	Case of 14 days standing. *In extremis*. Severe icterus. Severe sloughing lesions of photosensitization
VB-N2.....	" Werna ", Vosburg.....	Case of 14 days standing. *In extremis*. Severe icterus. Severe sloughing lesions of photosensitization
VB-O.....	" Werna ", Vosburg.....	Case of 14 days standing. Severe icterus. Severe sloughing lesions of photosensitization
VB-Y.....	" Leeukopspan ", Vosburg.....	Case of 21 days standing. *In extremis*. Severe icterus. Sloughing lesions of photosensitization. Cachexia. Severe dehydration

APPENDIX 1 (*continued*)

Sheep No.	Place of origin.	Relevant clinical data
VB-Z1.....	" Leeukopspan ", Vosburg.....	Case of 21 days standing. Fair icterus. Healing lesions of photosensitization. Anorexia. Malaise. Apathy. Cachexia
V1-16.....	" Cellieria ", Teviot.....	Case of 21 days standing. Mild icterus. Severe sloughing lesions around eyes, nostrils and on nose and ears. Purulent conjunctivitis. Apathy
V1-16B.....	" Fonteintjie ", Teviot.....	Case of 14 days standing. *In extremis*. Mild icterus. Sloughing lesions of photosensitization

C. Recovered Cases

Sheep No.	Place of origin	Relevant clinical data
VB-Z.....	" Leeukopspan ", Vosburg.....	Case of about 21 days standing. Icterus negligible. Healed sloughs on exposed parts. Eating well
V1-17B.....	" Fonteintjie ", Teviot.....	Case of about 21 days standing. Icterus negligible. Healed sloughs on exposed parts. Eating well
V1-19.....	"Three Sisters ", Three Sisters station	Case of about 17 days standing. Slight oedema of limbs. Healed sloughs on face and ears. Eating well
F-3.....	" Mierfontein ", Vosburg.....	Lamb. Case of about 16 days standing. Faint icterus. Healed lesions of photosensitization
F-4.....	" Mierfontein ", Vosburg.....	Lamb. Case of about 16 days standing. Faint icterus. Healing sloughs on face, nostrils, eyelids, ears. Cachexia. Healing coronitis
V3-3.....	" Blikkraal ", Rietbron.....	Case of about 21 days standing. Lesions of photosensitization almost completely healed. Eating well
V3-4.....	" Blikkraal ", Rietbron.....	Case of about 21 days standing. Lesions of photosensitization almost completely healed. Eating well
V3-19.....	" Modderfontein ", Vosburg.....	Lamb. Mild healed lesions around the eyes. Eating well

APPENDIX 1 (*continued*)

D. Control Animals

Sheep No.	Place of origin	Relevant clinical data
V1-7054.....	" Kweekwa ", Victoria West.....	Apparently quite normal. Condition good
V1-7055.....	,, ,, ,, ...	,, ,, ,, ,, ,,
V1-7056.....	,, ,, ,, ...	,, ,, ,, ,, ,,
V1-7057.....	,, ,, ,, ...	,, ,, ,, ,, ,,
V1-7059.....	,, ,, ,, ...	,, ,, ,, ,, ,,
V1-7060.....	,, ,, ,, ...	,, ,, ,, ,, ,,
V1-7061.....	,, ,, ,, ...	,, ,, ,, ,, ,,
V1-7062.....	,, ,, ,, ...	,, ,, ,, ,, ,,
V1-7064.....	,, ,, ,, ...	,, ,, ,, ,, ,,
V1-7065.....	,, ,, ,, ...	,, ,, ,, ,, ,,
V1-7066.....	,, ,, ,, ...	,, ,, ,, ,, ,,
V1-7067.....	,, ,, ,, ...	,, ,, ,, ,, ,,
V1-7068.....	,, ,, ,, ...	,, ,, ,, ,, ,,
V1-7069.....	,, ,, ,, ...	,, ,, ,, ,, ,,
V1-7070.....	,, ,, ,, ...	,, ,, ,, ,, ,,
V1-7071.....	,, ,, ,, ...	,, ,, ,, ,, ,,
F-12221.....	" Klawervlei ", Beaufort West.....	Apparently quite normal. Condition good
F-12222.....	,, ,, ,, ...	,, ,, ,, ,, ,,
F-K1.....	" Klawervlei ", Beaufort West.....	Apparently quite normal. Condition good
F-K2.....	,, ,, ,, ...	,, ,, ,, ,, ,,
F-K3.....	,, ,, ,, ...	,, ,, ,, ,, ,,
V3-5.....	" Niekerksfontein ", Richmond.....	Apparently quite normal. Condition good
V3-28.....	" Montana ", Nelspoort.....	Apparently quite normal. Thin
V3-29.....	,, ,, ,, ...	,, ,, ,, ,,
V3-30.....	,, ,, ,, ...	,, ,, ,, ,,
V3-31.....	,, ,, ,, ...	,, ,, ,, ,,
V3-32.....	,, ,, ,, ...	,, ,, ,, ,,
V3-33.....	,, ,, ,, ...	,, ,, ,, ,,

APPENDIX 2

STUDIES ON THE LEUKOCYTES IN GEELDIKKOP CASES

(Abbreviations used in the right-hand column are: N = neutrophiles; L = lymphocytes; M = monocytes; E = eosinophiles; B = basophiles. Data regarding thrombocytes are expressed as " normal number of thrombocytes present, few thrombocytes, etc.". This refers to the number seen on each smear relative to the other cell types when compared with similar smears from the control animals.)

A. Prodromal Cases

Sheep No.	W.C.C. (10^3/cu mm)	Differential white cell count and remarks
VB-P.....	—	Hardly any leukocytes could be found in a numbre of stained smears after a most thorough search. Thrombocytes similarly absent
VB-Q.....	—	Definite leukopaenia present. It was very difficult to count the types of white cells. There were at least 40% of immature neutrophiles and a large number of immature lymphocytes. Very few thrombocytes present

APPENDIX 2 (*continued*)

Sheep No.	W.C.C. (10^3/cu mm)	Differential white cell count and remarks
VB-R.....	—	Too few leukocytes present to perform a proper count. Thrombocytes completely absent. A few odd coccoid and bacilloid organisms present
VB-S.....	—	Too few leukocytes present to perform a proper count. All the white cells which were present were highly immature. Most were immature neutrophiles. Lymphocytopaenia. The few lymphocytes present were large ones. Very few thrombocytes present
VB-T.....	—	Too few leukocytes present to perform a proper count. Those present were highly immature and identification was difficult. Most appeared to be immature neutrophiles. Very few thrombocytes present. Numerous small coccoid and bacilloid organisms present
VB-U.....	—	N 56%, L 24%, M 13%, E 7%, B 0%. All leukocytes very immature especially the neutrophiles. Normal number of thrombocytes
VB-V.....	—	N 50%, L 20%, M 12%, E 18%, B 0%. Nearly all the leukocytes were immature especially the lymphocytes and neutrophiles. Very few segmented neutrophiles present. Normal number of thrombocytes
VB-W.....	—	N 55%, L 20%, M 12%, E 11%, B 2%. Most leukocytes counted were immature. Very few properly segmented neutrophiles. Normal number of thrombocytes
VB-X.....	—	N 44%, L 26%, M 13%, E 15%, B 2%. All forms of neutrophiles present, from mature segmented types to unsegmented juveniles. Lymphocytes and monocytes highly immature. Normal number of thrombocytes

B. Early Cases

Sheep No.	W.C.C. (10^3/cu mm)	Differential white cell count and remarks
VB-G.....	—	All leukocytes present very immature and difficult to identify. At least 60% were immature neutrophiles. Normal number of thrombocytes
VB-H.....	—	Severe leukopaenia. All white cells present very immature and most difficult to identify with certainty. At least 80% were immature neutrophiles, with an almost complete absence of lymphocytes. Normal number of thrombocytes
VB-I.....	—	All leukocytes present were very immature and most difficult to identify. At least 70% were immature neutrophiles with relatively few lymphocytes. Normal number of thrombocytes
VB-J.....	—	All leukocytes present were very immature with at least 60% immature neutrophiles and very few identifiable lymphocytes. Normal number of thrombocytes
VB-K.....	—	Marked neutrophilia, mainly juvenile forms present. Lymphocytopaenia. Normal number of thrombocytes

APPENDIX 2 (*continued*)

Sheep No.	W.C.C. (10^3/cu mm)	Differential white cell count and remarks
V1-1.....	7·9	N 88%, L 0%, M 12%, E 0%, B 0%. Most neutrophiles very immature. Very few thrombocytes present
V1-2.....	4·75	N 90%, L 4%, M 6%, E 0%, B 0%. Most neutrophiles very immature. Very few thrombocytes present
V1-3.....	4·35	N 91%, L 4%, M 5%, E 0%, B 0%. Neutrophiles mainly mature segmented types. Normal number of thrombocytes
V1-11.....	3·55	N 54%, L 30%, M 14%, E 1%, B 1%. Neutrophiles mainly mature segmented types. Normal number of thrombocytes
V1-13.....	7·55	N 77%, L 5%, M 11%, E 6%, B 1%. A small percentage of immature neutrophiles. Normal number of thrombocytes
V1-15.....	4·65	N 54%, L 18%, M 14%, E 4%, B 0%. High percentage of immature neutrophiles. Normal number of thrombocytes
V1-17.....	3·8	All leukocytes present very immature and most difficult to identify At least 90% were juvenile neutrophiles. Numerous coccoid and bacilloid organisms present. Normal number of thrombocytes
V1-18.....	9·6	Almost 100% juvenile unsegmented neutrophiles. No thrombocytes could be found
V1-22.....	5·7	N 51%, L 24%, M 25%, E 0%, B 0%. Large percentage of immature neutrophiles. Normal number of thrombocytes
V1-23.....	8·75	N 79%, L 9%, M 11%, E 0%, B 1%. Large percentage of juvenile neutrophiles. Normal number of thrombocytes
V1-24.....	8·8	N 70%, L 14%, M 16%, E 0%, B 0%. Neutrophiles mainly mature segmented forms. Normal number of thrombocytes
V1-25.....	10·65	N 87%, L 7%, M 6%, E 0%, B 0%. Most neutrophiles mature segmented forms. Normal number of thrombocytse.
V1-26.....	8·3	N 88%, L 6%, M 6%, E 0%, B 0%. Most neutrophiles mature segmented forms. Normal number of thrombocytes.
F-2.....	10·2	N 78%, L 18%, M 3%, E 1%, B 0%. Neutrophiles all mature segmented forms. Normal number of thrombocytes.
F-5.....	5·2	N 55%, L 36%, M 5%, E 4%, B 0%. Nothing unusual. Normal number of thrombocytes

C. Advanced Cases—Group 1

Sheep No.	W.C.C. (10^3/cu mm)	Differential white cell count and remarks
VB-L.....	5·2	N 26%, L 30%, M 40%, E 4%, B 0%. Monocytosis? Neutrophiles all immature. Normal number of thrombocytes
VB-M.....	9·2	N 68%, L 9%, M 23%, E 0%, B 0%. A few immature neutrophiles. Normal number of thrombocytes

APPENDIX 2 (*continued*)

Sheep No.	W.C.C. (10^3/cu mm)	Differential white cell count and remarks
VB-N.....	9·4	N 81%, L 10%, M 9%, E 0%, B 0%. Nothing unusual. Normal number of thrombocytes
V1-4.....	6·4	N 94%, L 5%, M 1%, E 0%, B 0%. Mainly mature segmented neutrophiles. Normal number of thrombocytes
V1-5.....	7·8	N 83%, L 3%, M 13%, E 1%, B 0%. Mainly mature segmented neutrophiles. Normal number of thrombocytes
V1-6.....	11·15	N 80%, L 5%, M 14%, E 1%, B 0%. Mainly mature segmented neutrophiles. Normal number of thrombocytes
V1-7.....	3·35	N 90%, L 8%, M 2%, E 0%, B 0%. Large number of juvenile unsegmented neutrophiles. Normal number of thrombocytes. Numerous coccoid and bacilloid organisms
V1-8.....	3·75	N 48%, L 11%, M 41%, E 0%, B 0%. Nothing unusual. Normal number of thrombocytes
V1-9.....	4·9	N 36%, L 22%, M 40%, E 2%, B 0%. Nothing unusual. Normal number of thrombocytes
V1-10.....	7·1	N 56%, L 22%, M 22%, E 0%, B 0%. Nothing unusual. Normal number of thrombocytes
V1-12.....	7·3	N 63%, L 16%, M 14%, E 7%, B 0%. Neutrophiles mainly mature segmented forms. Normal number of thrombocytes.
V1-14.....	11·35	N 83%, L 6%, M 7%, E 0%, B 0%. Neutrophiles mainly mature segmented forms. Normal number of thrombocytes
V1-20.....	3·8	N 90%, L 4%, M 6%, E 0%, B 0%. Large percentage of juvenile neutrophiles. Normal number of thrombocytes
V1-21.....	3·9	N 70%, L 7%, M 23%, E 0%, B 0%. Most neutrophiles juvenile and unsegmented. Normal number of thrombocytes

D. Advanced Cases—Group 2

Sheep No.	W.C.C. (10^3/cu mm)	Differential white cell count and remarks
VB-O.....	4·2	N 70%, L 20%, M 9%, E 1%, B 0%. The leukocytes were in general mainly immature forms. Normal number of thrombocytes
VB-Y.....	4·3	N 80%, L 15%, M 5%, E 0%, B 0%. All leukocytes very immature. Scarcely any segmented neutrophiles. Reduced number of thrombocytes
VB-Z1.....	5·1	N 76%, L 20%, M 4%, E 0%, B 0%. All forms of neutrophiles present, mainly adult forms. Lymphocytes mainly large forms. Normal number of thrombocytes
V1-16.....	6·2	N 55%, L 19%, M 18%, E 8%, B 0%. Neutrophiles mainly mature forms. Normal number of thrombocytes

APPENDIX 2 (*continued*)

E. Recovered Cases

Sheep No.	W.C.C. (10^3/cu mm)	Differential white cell count and remarks
VB-Z.....	6·01	N 65%, L 25%, M 5%, E 5%, B 0%. All leukocytes very immature and difficult to identify with certainty. Neutrophiles were mainly unsegmented forms. Some myeloblasts present. Very few thrombocytes evident
V1-19.....	5·05	N 38%, L 50%, M 3%, E 9%, B 0%. Nothing unusual. Normal number of thrombocytes
F-3.....	8·35	N 78%, L 12%, M 10%, E 0%, B 0%. Neutrophiles mainly mature forms. Normal number of thrombocytes
F-4.....	3·50	N 86%, L 5%, M 7%, E 2%, B 0%. Neutrophiles mainly mature segmented forms. Normal number of thrombocytes

D. Control Animals

Sheep No. V1-7054–7057, V1-7059–7062, V1-7064–7071: White cell counts ranged from $4·9 \times 10^3$/cu mm to $9·15 \times 10^3$/cu mm. Differential white cell counts varied little around a mean of N 35%, L 50%, M 4%, E 8%, B 0%.

Sheep No. 12221 and 12222: White cell counts were $3·5 \times 10^3$ and $3·6 \times 10^3$/cu mm respectively, Differential white cell counts were N 54%, L 30%, M 8%, E 8 %, B 0% and N 56%, L 41%. M 3%, E 1% and B 0% respectively.

E. Normal Values for Leukocytes of the sheep taken from the literuture

Determination	Values	Sources
White cell count.....	4–12 × 10^3/cu mm.....	Schalm (1961, 1965)
	8–10 × 10^3/cu mm.....	Jackson (1948)
	6·1–12·2 × 10^3/cu mm (9·2)....	Boddie (1946)
Differential white cell count	N 30%, L 62%, M 2·5%, E 4·5%, B 0·5%	Schalm (Average values) (1961, 1965)
	N 30%, L 55%, M 4%, E 8%, B 0·5–1%	Jackson (1948)
	N 15–23 (24)%, L 58–78 (68)%, M 1·3-5·7 (3)%, E 0·5-8·5 (4)%, B 0%	Boddie (1946)
	N 30–40%, L 45–70%, M 2–5%, E 5–15%, B less than 1%	Scheunert & Trautmann (1965)

Note: (*a*) The values given by Jackson (1948) are compiled from figures on South African sheep. They have been used for comparison in the text together with those from our own control animals.

(*b*) Figures given in parenthesis in the middle column above represent mean values given by the authors concerned.

APPENDIX 3

STUDIES ON THE ERYTHROCYTES IN GEELDIKKOP CASES

[*Note.*—RCV = Red cell volume (%); Hb = haemoglobin (gm%); RCC = Red cell count (10^6/cu mm); ESR = Erythrocyte sedimentation rate (mm/hr); TPP = Total plasma proteins (gm%); MCHC = Mean corpuscular haemoglobin concentration (%); MCV = Mean corpuscular volume (cμ); MCH = Mean corpuscular haemoglobin ($\gamma\gamma$).]

A. Prodromal Cases

Sheep No.	RCV	RCC	Hb	TPP	ESR	MCHC	MCV	MCH	Blood and Bone Marrow Smear Examination—Remarks
VB-P	Not done	Not done	Not done	4·69	0	—	—	—	*Blood smear:* Very marked anisocytosis, large number of macrocytes. Anaemia
VB-Q	Not done	Not done	Not done	4·69	0	—	—	—	*Blood smear:* Fair degree of anisocytosis, large number of macrocytes. Anaemia
VB-R	Not done	Not done	Not done	4·59	1·0	—	—	—	*Blood smear:* Fair degree of anisocytosis, mild anaemia
VB-S	Not done	Not done	Not done	5·33	0	—	—	—	*Blood smear:* Severe anisocytosis with numerous large macrocytes. Numerous cells present with Jolly bodies
VB-T	Not done	Not done	Not done	4·69	1·0	—	—	—	*Blood smear:* Fair degree of anisocytosis with numerous large macrocytes. Numerous cells present with Jolly bodies
VB-U	Not done	Not done	Not done	5·01	0	—	—	—	*Blood smear:* Mild anisocytosis
VB-V	Not done	Not done	Not done	5·12	0	—	—	—	*Blood smear:* Pronounced anisocytosis with large number of macrocytes. Numerous cells with Jolly bodies.
VB-W	Not done	Not done	Not done	4·8	0	—	—	—	*Blood smear:* Marked anisocytosis with numerous large macrocytes. Numerous normoblasts and cells with Jolly bodies
VB-X	Not done	Not done	Not done	5·01	1·0	—	—	—	*Blood smear:* Fair degree of anisocytosis with large numbers of macrocytes. Large numbers of normoblasts and cells with Jolly bodies

APPENDIX 3 (*continued*)

STUDIES ON THE ERYTHROCYTES IN GEELDIKKOP CASES (*continued*)

B. Early Cases

Sheep No.	RCV	RCC	Hb	TPP	ESR	MCHC	MCV	MCH	Blood and Bone Marrow Smear Examination—Remarks
VB-G	Not done	Not done	Not done	6·83	0	—	—	—	*Blood smear:* Fair degree of anisocytosis
VB-H	Not done	Not done	Not done	5·33	0	—	—	—	*Blood smear:* Mild anisocytosis
VB-I	Not done	Not done	Not done	5·55	1·0	—	—	—	*Blood smear:* Nothing unusual
VB-J	Not done	Not done	Not done	5·97	0	—	—	—	*Blood smear:* Nothing unusual
VB-K	Not done	Not done	Not done	5·87	0	—	—	—	*Blood smear:* Nothing unusual
V1-1	46	10·03	11·1	7·60	0	24·1	45·86	11·06	*Blood smear:* Nothing unusual
V1-2	49	11·85	10·86	7·75	0	22·16	41·35	9·16	*Blood smear:* Nothing unusual
V1-3	41	12·38	10·13	7·95	0	24·7	33·11	8·18	*Blood smear:* Fair degree of anisocytosis *Bone marrow smear:* Apparent reduction in the number of erythrocyte and granulocyte precursors
V1-11	35·5	8·93	9·65	7·75	0·5	27·18	39·75	10·80	*Blood smear:* Mild anisocytosis with some macrocytes
V1-13	45	10·79	9·89	7·20	0	21·97	41·70	9·16	*Blood smear:* Mild anisocytosis
V1-15	46	6·72	10·62	8·3	0	23·08	68·45	15·80	*Blood smear:* Nothing unusual
V1-17	49·5	11·47	12·78	8·4	0	25·81	43·15	11·14	*Blood smear:* Nothing unusual
V1-18	45·5	9·69	11·10	8·4	0	24·39	46·95	11·45	*Blood smear:* Nothing unusual
V1-22	48·5	10·21	10·86	5·9	0	22·39	47·50	10·63	*Blood smear:* Nothing unusual
V1-23	41·0	10·12	9·65	5·6	0·5	23·53	40·51	9·53	*Blood smear:* Nothing unusual
V1-24	48·0	10·85	11·82	6·4	0·5	24·62	44·23	10·89	*Blood smear:* Nothing unusual *Bone marrow smear:* Bone marrow highly active

APPENDIX 3 (*continued*)

STUDIES ON THE ERYTHROCYTES IN GEELDIKKOP CASES (*continued*)

Sheep No.	RCV	RCC	Hb	TPP	ESR	MCHC	MCV	MCH	Blood and Bone Marrow Smear Examinatoin—Remarks
V1-25	48·0	12·50	11·10	6·9	0	23·12	38·40	8·88	*Blood smear:* Nothing unusual
V1-26	48·0	9·86	11·82	6·9	0·5	24·62	48·68	11·98	*Blood smear:* Nothing unusual
F-2	31·0	3·1	8·93	6·5	0	28·80	100·0	28·80	*Blood smear:* Nothing unusual
F-5	36·5	4·79	11·10	6·4	0	30·41	76·20	23·17	*Blood smear:* Nothing unusual
V3-6	39	Not done	9·15	8·42	0	23·46	—	—	*Blood smear:* Nothing unusual
V3-7	35	Not done	8·44	10·19	0	24·11	—	—	*Blood smear:* Nothing unusual
V3-10	27	Not done	5·70	8·08	0·5	21·11	—	—	*Blood smear:* Nothing unusual
V3-11	40	Not done	8·69	10·60	1·0	21·72	—	—	*Blood smear:* Nothing unusual
V3-13	37	Not done	10·14	6·82	0	27·4	—	—	*Blood smear:* Nothing unusual
V3-14	46	Not done	11·1	8·17	0	24·13	—	—	*Blood smear:* Nothing unusual
V3-15	46	Not done	10·6	7·90	0	23·04	—	—	*Blood smear:* Nothing unusual
V3-16	25	Not done	7·24	9·28	0·5	28·96	—	—	*Blood smear:* Nothing unusual
V3-20	42	Not done	8·3	8·60	0	19·76	—	—	*Blood smear:* Nothing unusual
V3-21	41	Not done	8·69	9·65	0	21·19	—	—	*Blood smear:* Nothing unusual
V3-22	41	Not done	9·65	7·29	0	23·53	—	—	*Blood smear:* Nothing unusual
V3-23	45	Not done	9·9	7·56	0·5	22·0	—	—	*Blood smear:* Nothing unusual
V3-24	46	Not done	9·65	8·08	0·5	20·97	—	—	*Blood smear:* Nothing unusual
V3-25	34	Not done	7·24	8·80	0	21·29	—	—	*Blood smear:* Nothing unusual
V3-26	49	Not done	10·8	8·80	0	23·47	—	—	*Blood smear:* Nothing unusual
V3-27	32	Not done	7·72	7·56	0	24·12	—	—	*Blood smear:* Nothing unusual

APPENDIX 3 (*continued*)

STUDIES ON THE ERYTHROCYTES IN GEELDIKKOP CASES (*continued*)

C. Advanced Cases—Group 1

Sheep No.	RCV	RCC	Hb	TPP	ESR	MCHC	MCV	MCH	Blood and Bone Marrow Smear Examination—Remarks
VB-L	Not done	Not done	Not done	7·47	0	—	—	—	*Blood smear:* Anisocytosis, polychromasia
VB-M	Not done	Not done	Not done	6·4	1·0	—	—	—	*Blood smear:* Nothing unusual
VB-N	Not done	Not done	Not done	7·47	0	—	—	—	*Blood smear:* Nothing unusual
V1-4	33	7·57	7·72	8·4	2·0	23·39	43·59	10·19	*Blood smear:* Slight anisocytosis
V1-5	41·5	11·47	9·65	7·95	0	23·25	36·18	8·41	*Blood smear:* Slight anisocytosis
V1-6	36	10·61	7·96	9·6	0·5	22·1	33·93	7·50	*Blood smear:* Slight anisocytosis *Bone Marrow smear:* Decrease of erythrocyte and granulocyte precursors. No normoblasts to be seen
V1-7	38	2·04	8·93	8·4	0	23·5	186·27	43·77	*Blood smear:* Nothing unusual *Bone Marrow smear:* Marked decrease of all cellular elements
V1-8	35	8·27	9·41	7·75	0·5	26·88	42·32	11·37	*Blood smear:* Nothing unusual
V1-9	28	9·1	8·2	7·6	0	29·28	30·77	9·01	*Blood smear:* Nothing unusual
V1-10	42	12·28	9·65	9·6	0	22·97	34·02	7·85	*Blood smear:* Nothing unusual
V1-12	22·5	7·18	6·27	8·1	0	27·86	31·33	8·73	*Blood smear:* Marked anisocytosis
V1-14	30	6·83	7·24	8·8	2	24·13	43·9	10·60	*Blood smear:* Nothing unusual
V1-20	34·0	10·65	9·17	7·3	1·0	26·97	31·92	8·61	*Blood smear:* Nothing unusual *Bone Marrow smear:* Very active haemopoiesis
V1-21	44·5	8·78	10·37	6·3	0	23·30	50·68	11·81	*Blood smear:* Nothing unusual
V3-1	31	Not done	6·75	9·84	0	21·77	—	—	*Blood smear:* Nothing unusual

APPENDIX 3 (*continued*)

STUDIES ON THE ERYTHROCYTES IN GEELDIKKOP CASES (*continued*)

Sheep No.	RCV	RCC	Hb	TPP	ESR	MCHC	MCV	MCH	Blood and Bone Marrow Smear Examination—Remarks
V3-1A	20	Not done	—	8·8	0	—	—	—	*Blood smear:* Nothing unusual
V3-2	43	Not done	9·65	8·60	0·5	22·44	—	—	*Blood smear:* Nothing unusual
V3-8	23	Not done	5·4	8·42	0	23·47	—	—	*Blood smear:* Nothing unusual
V3-9	40	Not done	9·65	7·34	0	24·12	—	—	*Blood smear:* Nothing unusual
V3-12	45	Not done	11·1	8·6	0	24·66	—	—	*Blood smear:* Nothing unusual
V3-17	24	Not done	5·3	5·46	0	22·08	—	—	*Blood smear:* Nothing unusual
V3-18	23	Not done	5·79	11·10	0	25·17	—	—	*Blood smear:* Nothing unusual

D. Advanced Cases—Group 2

Sheep No.	RCV	RCC	Hb	TPP	ESR	MCHC	MCV	MCH	Blood and Bone Marrow smear Examination—Remarks
VB-O	Not done	Not done	Not done	6·4	1·0	—	—	—	*Blood smear:* Nothing unusual
VB-Y	Not done	Not done	Not done	5·87	0	—	—	—	*Blood smear:* Nothing unusual
VB-Z1	Not done	Not done	Not done	5·33	0	—	—	—	*Blood smear:* Marked anisocytosis with numerous macrocytes and cells containing Jolly bodies
V1-16	31	7·3	7·24	6·5	1·5	23·35	42·46	9·92	*Blood smear:* Nothing unusual *Bone Marrow smear:* Markedly reduced number of cellular elements

APPENDIX 3 (*continued*)

STUDIES ON THE ERYTHROCYTES IN GEELDIKKOP CASES (*continued*)

E. Recovered Cases

Sheep No.	RCV	RCC	Hb	TPP	ESR	MCHC	MCV	MCH	Blood and Bone Marrow Smear Examination—Remarks
VB-Z	Not done	Not done	Not done	5·44	0	—	—	—	*Blood smear:* Nothing unusual
V1-19	47·5	13·55	10·62	7·3	0·5	22·35	35·05	7·83	*Blood smear:* Nothing unusual
F-3	36·5	12·4	11·58	7·5	0	31·72	29·43	9·33	*Blood smear:* Nothing unusual
F-4	36·1	2·68	9·65	7·26	2·0	26·73	134·70	36·00	*Blood smear:* Marked anisocytosis with large number of macrocytes
V3-3	51	Not done	9·1	8·26	0	17·84	—	—	*Blood smear:* Nothing unusual
V3-4	40	Not done	8·93	7·56	0	22·32	—	—	*Blood smear:* Nothing unusual
V3-19	32	Not done	7·7	5·30	0	24·06	—	—	*Blood smear:* Nothing unusual

F. Control Animals

Sheep No.	RCV	RCC	Hb	TPP	ESR	MCHC	MCV	MCH	Blood and Bone Marrow Smear Examination—Remarks
V1-7054	27	8·8	8·68	6·05	1·0	32·1	30·68	9·86	*Blood smear:* Nothing unusual
V1-7055	29	9·93	8·2	6·4	1·0	28·27	29·20	8·25	*Blood smear:* Nothing unusual
V1-7056	32	10·02	9·17	6·8	1·0	28·65	31·37	8·99	*Blood smear:* Nothing unusual
V1-7057	35	10·6	9·65	6·55	1·0	27·57	33·02	9·10	*Blood smear:* Nothing unusual
V1-7059	36	13·42	10·62	6·8	0	29·50	26·82	7·91	*Blood smear:* Nothing unusual
V1-7060	33	11·5	9·17	6·6	0·5	27·78	28·69	7·97	*Blood smear:* Nothing unusual
V1-7061	35	10·36	9·41	6·1	1·0	26·88	33·78	9·08	*Blood smear:* Nothing unusual
V1-7062	30	11·51	8·93	6·8	0·5	29·76	25·93	7·72	*Blood smear:* Nothing unusual
V1-7064	30	10·83	8·68	6·6	0·5	28·93	27·70	8·01	*Blood smear:* Nothing unusual

APPENDIX 3 (*continued*)

STUDIES ON THE ERYTHROCYTES IN GEELDIKKOP CASES (*continued*)

F. Control Animals

Sheep No.	RCV	RCC	Hb	TPP	ESR	MCHC	MCV	MCH	Blood and Bone Marrow Smear Examination—Remarks
V1-7065	33	13·46	9·17	6·6	0·5	27·78	24·51	6·81	*Blood smear:* Nothing unusual
V1-7066	28	10·24	8·2	6·3	2·0	29·28	27·34	8·01	*Blood smear:* Nothing unusual
V1-7067	31	10·68	8·68	6·4	1·0	28·0	29·02	8·12	*Blood smear:* Nothing unusual
V1-7068	31	Not done	9·65	Not done	1·0	31·12	—	—	*Blood smear:* Nothing unusual
V1-7069	32·5	Not done	9·89	Not done	1·0	30·43	—	—	*Blood smear:* Nothing unusual
V1-7070	29	Not done	10·13	Not done	0	34·93	—	—	*Blood smear:* Nothing unusual
V1-7071	30	Not done	9·17	Not done	0·5	30·56	—	—	*Blood smear:* Nothing unusual
F-12221	34·8	11·54	9·65	6·23	0	27·72	30·10	8·36	*Blood smear:* Nothing unusual
F-12222	25	6·00	9·65	6·0	0	38·60	41·66	16·08	*Blood smear:* Nothing unusual
F-K2	25·1	11·81	7·72	5·64	0	30·75	21·25	6·53	*Blood smear:* Nothing unusual
V3-5	40	Not done	9·65	7·0	0·5	24·12	—	—	*Blood smear:* Nothing unusual
V3-28	30	Not done	8·2	8·8	0	27·33	—	—	*Blood smear:* Nothing unusual
V3-29	34	Not done	8·69	9·84	0	25·55	—	—	*Blood smear:* Nothing unusual
V3-30	30	Not done	8·44	7·9	0	28·13	—	—	*Blood smear:* Nothing unusual
V3-31	35	Not done	9·65	8·6	0	27·57	—	—	*Blood smear:* Nothing unusual
V3-32	31	Not done	8·93	7·99	0	28·80	—	—	*Blood smear:* Nothing unusual
V3-33	19	Not done	5·79	9·28	0	30·47	—	—	*Blood smear:* Nothing unusual

APPENDIX 3 (*continued*)

STUDIES ON THE ERYTHROCYTES IN GEELDIKKOP CASES (*continued*)

G. *Normal Values for the Erythrocytes of the sheep taken from the literature*

RCV	RCC	Hb	ESR	MCHC	MCV	MCH	Source
—	11·0	—	—	—	—	—	Jackson (1948)
33–46	8·5–13·5	9–14·5	0	33–35	33·5–43	9–13	Coffin (1953)
25–33	9·7–13·3 (11·5)	11–13·8 (12·4)	—	37·1–45·1 (41)	23–31 (27)	—	Boddie (1946)
32	8–13 (10)	10–15 (12·6)	—	—	—	—	Scheunert & Trautmann (1965)
24–50 (38)	8–16 (12)	8–16 (12)	0	29–35 (32)	23–48 (33)	9–12 (10·7)	Schalm (1961, 1965)
—	9·24–11·9 (Lambs 2–12 months)	—	—	—	—	—	Reda & Hathout (1957)
—	9·02–10·45 (Young sheep 1–2 years)	—	—	—	—	—	,, ,, ,,
—	7·96–9·2 (Adult sheep)	—	—	—	—	—	,, ,, ,,

Note.—The average value for RCC given by Jackson (1948) has been taken for comparison in the text since it has been worked out on South African sheep. Values in parenthesis in the columns above represent mean values given by the authors concerned.

APPENDIX 4

PLASMA PROTEIN LEVELS IN GEELDIKKOP CASES AND CONTROL ANIMALS

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

Sheep No.	Stage of disease	TPP	Alb	Glob	A: G
VB-P	Prodromal Case.....	4·69	3·52	1·17	3·00
VB-Q	,, ,, ...	4·69	3·63	1·06	3·42
VB-R	,, ,, ...	4·59	3·55	1·04	3·41
VB-S	,, ,, ...	5·33	3·63	1·70	2·13
VB-T	,, ,, ...	4·69	3·63	1·06	3·42
VB-U	,, ,, ...	5·01	3·20	1·81	1·76
VB-V	,, ,, ...	5·12	3·41	1·71	1·99
VB-W	,, ,, ...	4·80	3·20	1·60	2·00
VB-X	,, ,, ...	5·01	3·41	1·60	2·13
VB-H	Early case: Group 1 (1–2 days)..	5·33	3·31	2·02	1·63
VB-I	,, ,, ,, (1–2 days)..	5·55	3·73	1·82	2·04
V1-25	,, ,, ,, (1–2 days)..	6·90	3·50	3·40	1·02
V1-3	,, ,, ,, (1–2 days)..	7·95	3·70	4·25	0·87
V1-2	,, ,, ,, (1–2 days)..	7·75	3·80	3·95	0·96
V1-23	,, ,, ,, (2–3 days)..	5·60	3·30	2·30	1·43
V1-22	,, ,, ,, (2–3 days)..	5·90	3·30	2·60	1·26
V1-24	,, ,, ,, (2–3 days)..	6·40	3·30	3·10	1·06
V1-17	,, ,, ,, (2–3 days)..	8·40	3·50	4·90	0·71
V1-1	Early cases: Group 2 (1–2 days)..	7·60	2·80	4·80	0·58
VB-A	,, ,, ,, (2–3 days)..	6·40	3·31	3·09	1·07
VB-E	,, ,, ,, (2–3 days)..	5·55	2·99	2·56	1·15
VB-F	,, ,, ,, (2–3 days)..	5·87	3·63	2·24	1·62
VB-C	,, ,, ,, (2–3 days)..	6·61	3·63	2·98	1·21
VB-B	,, ,, ,, (2–3 days)..	6·40	3·73	2·67	1·39
V1-15	,, ,, ,, (2–3 days)..	8·30	3·00	5·30	0·56
F-5	,, ,, ,, (2–3 days)..	6·40	1·84	3·84	0·71
V1-13	,, ,, ,, (2–3 days)..	7·20	2·80	4·40	0·63
F-2	,, ,, ,, (2–3 days)..	6·50	3·86	2·63	1·46
F-1	,, ,, ,, (2–3 days)..	—	—	—	—
VB-G	,, ,, ,, (3–5 days)..	6·83	3·41	3·42	0·99
VB-J	,, ,, ,, (3–5 days)..	5·97	4·28	1·69	2·53
V1-26	,, ,, ,, (3–5 days)..	6·90	2·65	3·25	0·81
VB-K	,, ,, ,, (5–7 days)..	5·87	3·31	2·56	1·29
V1-11	,, ,, ,, (5–7 days)..	7·75	3·80	3·95	0·96
V1-18	,, ,, ,, (5–7 days)..	8·40	2·80	5·60	0·50
V1-21	Advanced case: Group 1 (7–8 days)	6·30	2·80	3·50	0·80
V1-9	,, ,, ,, (8–10 days)	7·60	3·00	4·60	0·65
V1-10	,, ,, ,, (8–10 days)	9·60	2·80	6·80	0·41
V1-12	,, ,, ,, (10–14 days)	8·10	3·70	4·40	0·84

APPENDIX 4 (*continued*)

PLASMA PROTEIN LEVELS IN GEELDIKKOP CASES AND CONTROL ANIMALS (*cont*

Sheep No.	Stage of disease	TPP	Alb	Glob	A:G
VB-D	Advanced case: Group 2 (7–8 days)	5·55	3·41	2·14	1·59
VB-L	,, ,, ,, (7–8 days)	7·47	4·37	3·10	1·40
VB–M	,, ,, ,, (7–8 days)	6·40	3·09	3·31	0·93
VB–N	,, ,, ,, (7–8 days)	7·47	4·48	2·99	1·49
V1-5	,, ,, ,, (7–8 days)	7·95	3·50	4·45	0·78
V1-4	,, ,, ,, (7–8 days)	8·40	2·10	6·30	0·33
V1-6	,, ,, ,, (7–8 days)	9·60	3·50	6·10	0·57
V1-7	,, ,, ,, (7–8 days)	8·40	3·15	5·25	0·60
V1-14	,, ,, ,, (7–8 days)	8·80	2·30	6·50	0·35
V1-8	,, ,, ,, (8–10 days)	7·75	3·00	4·75	0·63
V1-20	,, ,, ,, (10–14 days)	7·30	3·30	4·00	0·82
VB-Z1	,, ,, ,, (14–21 days)	5·33	3·41	1·92	1·77
VB-O	,, ,, ,, (14–21 days)	6·40	3·41	2·99	1·14
VB-Y	,, ,, ,, (14–21 days)	5·87	3·09	2·77	1·11
V1-16	,, ,, ,, (14–21 days)	—	3·50	—	—
VB-Z	Recovered case (± 21 days).....	5·44	2·77	2·67	1·03
V1-19	,, ,, (± 17 days).....	7·30	3·30	4·00	0·82
F-4	,, ,, (± 16 days).....	7·26	2·25	4·39	0·64
F-3	,, ,, (± 16 days).....	7·50	2·82	4·22	0·67
F-K2	Control animal.....	5·64	1·94	3·70	0·52
F-12221	,, ,, ...	6·23	3·08	3·25	0·94
F-12222	,, ,, ...	6·00	3·00	3·00	1·00
V1-7054	,, ,, ...	6·05	2·30	3·75	0·61
V1-7055	,, ,, ...	6·40	2·45	3·95	0·62
V1-7056	,, ,, ...	6·80	2·45	4·35	0·56
V1-7057	,, ,, ...	6·55	2·65	3·90	0·67
V1-7059	,, ,, ...	6·80	2·75	4·05	0·67
V1-7060	,, ,, ...	6·60	2·75	3·85	0·71
V1-7061	,, ,, ...	6·10	2·40	3·70	0·64
V1-7062	,, ,, ...	6·80	2·70	4·10	0·65
V1-7064	,, ,, ...	6·60	3·70	2·90	1·27
V1-7065	,, ,, ...	6·60	3·80	2·80	1·35
V1-7066	,, ,, ...	6·30	3·15	3·15	1·00
V1-7067	,, ,, ...	6·40	3·60	2·80	1·28

APPENDIX 5

RESULTS OF FLOCCULATION OR TURBIDITY TESTS OF LIVER FUNCTION IN GEELDIKKOP CASES AND CONTROL ANIMALS

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

Nature of case	Sheep No.	TT	TF	CG	ZT
Control animals.....	F-K2	0·7	0	0	2·58
	F-12221	0·7	0	0	2·45
	F-12222	0·4	0	0	1·23
	V1-7054	0·5	0	0	1·5
	V1-7055	0·5	0	0	0·9
	V1-7056	0·8	0	0	0·9
	V1-7057	0·5	0	0	0·9
	V1-7059	0·4	0	0	1·5
	V1-7060	0·4	0	0	1·5
	V1-7061	0·4	0	0	1·5
	V1-7062	0·8	0	0	1·5
	V1-7064	0·4	0	0	0·8
	V1-7065	0·8	0	0	1·5
	V1-7066	0·8	0	0	1·5
	V1-7067	0·8	0	0	0·8
	V1-7068	0·8	0	0	0·8
	V1-7069	0·7	0	0	1·2
	V1-7070	0·3	0	0	0·9
	V1-7071	0·5	0	0	0·7
Prodromal cases.....	VB-P	1·2	0	0	0
	VB-Q	0	0	0	0
	VB-R	0	0	0	0
	VB-S	1·2	0	0	0
	VB-T	1·1	0	0	0
	VB-U	0	0	0	0
	VB-V	0	0	0	0
	VB-W	1·2	0	0	0
	VB-X	0	0	0	1·0

APPENDIX 5 (*continued*)

RESULTS OF FLOCCULATION OR TURBIDITY TESTS OF LIVER FUNCTION IN GEELDIKKOP CASES AND CONTROL ANIMALS (*continued*)

Nature of case	Sheep No.	TT	TF	CG	ZT
Early cases.....	VB-A	0	0	0	0
	VB-B	0	0	0	0
	VB-C	0	0	0	0
	F-1	0	0	0	0
	F-2	1·4	0	0	3·5
	F-5	2·0	0	0	2·75
	VB-E	0	0	0	0
	VB-F	0	0	0	0
	VB-G	0	0	0	0
	VB-H	0	0	0	0
	VB-I	0	0	0	0
	VB-J	0	0	0	0
	VB-K	0	0	0	0
	V1-1	1·8	0	0	3·0
	V1-2	1·4	0	0	3·0
	V1-3	1·0	0	0	2·2
	V1-11	1·4	0	0	0
	V1-13	1·4	0	0	3·0
	V1-15	4·0	0	±	8·5
	V1-17	0·8	0	0	1·5
	V1-18	0·9	0	0	3·0
	V1-22	0	0	0	0·8
	V1-23	0·8	0	0	0
	V1-24	0·8	0	0	1·5
	V1-25	0·8	0	0	0·8
	V1-26	1·4	0	0	2·1
Advanced cases: Group 1.....	VB-D	0	0	0	0
	VB-L	1·5	0	0	0
	VB-M	1·5	0	0	0
	VB-N	2·6	0	0	0
	V1-4	1·8	0	0	5·0
	V1-5	1·4	0	0	4·2
	V1-6	1·4	0	0	1·6
	V1-7	1·0	0	0	1·5
	V1-8	1·4	±	±	0
	V1-9	0	±	±	0
	V1-10	1·8	0	0	5·0
	V1-12	2·0	0	0	11·4
	V1-14	1·8	0	0	1·7
	V1-20	0·8	0	0	3·0
	V1-21	0·8	0	0	0·8
Advanced cases: Group 2.....	VB-O	1·6	0	0	0
	VB-Y	2·0	0	0	1·0
	VB-Z1	5·0	0	0	1·2
	V1-16	3·8	0	0	13·0
Recovered cases.....	VB-Z	1·2	0	0	1·0
	F-4	0·7	0	0	3·0
	F-3	1·4	0	0	3·5
	V1-19	0·8	0	0	2·2

APPENDIX 6

DETAILS OF THE SHEEP USED IN THE STUDIES ON THE HAEMATOLOGY, GENERAL CHEMICAL PATHOLOGY, BIOCHEMISTRY AND HISTOPATHOLOGY OF ENZOOTIC ICTERUS

A. Early clinical cases (1–5 *days standing*)
(1) *Group* 1: *Mild cases when first examined*

Sheep No.	Place of origin	Relevant clinical data
2206.....	Sutherland district.....	Listless, anaemic
2207.....	Sutherland district.....	Listless, anaemic
2208.....	Sutherland district.....	Fever, listless, anaemic
15065.....	" Rietpoort ", Murraysburg.....	Very thin, otherwise nothing specific
Klopper No. 1	" Eerstegeluk ", Halseton.....	Thin, listless
12223.....	" Klipfontein ", Fraserburg.....	Very mildly icteric, anaemic
12224.....	" Klipfontein ", Fraserburg.....	Listless, anorexia
12225.....	" Klipfontein ", Fraserburg.....	Anorexia
12228.....	" Avondrus ", Fraserburg.....	Nothing specific
12229.....	" Avondrus ", Fraserburg.....	Anorexia
Bekker No. 3.	" Soutwater ", Rietbron.....	Icterus, anorexia
15064.....	" Rietpoort ", Murraysburg.....	Icterus, anaemia, anorexia, listlessness
FB-2.....	" Avondrus ", Fraserburg.....	Very weak, anaemic, hyperpnoea, apathy
5114.....	" Bastersberg ", Sutherland.....	Weak, listlessness, anorexia
5119.....	" Bastersberg ", Sutherland.....	Weak, listlessness, anorexia
5121.....	" Avondrus ", Fraserburg.....	Weak, listlessness, anorexia
5126.....	" Celeryfontein ", Fraserburg....	Nothing unusual when first examined
5128.....	" Celeryfontein ", Fraserburg....	Ewe with suckling lamb, weak, listless
5130.....	" Bastersberg ", Sutherland.....	Nothing unusual when first examined
5131.....	" Bastersberg ", Sutherland.....	Nothing unusual when first examined
5132.....	" Bastersberg ", Sutherland.....	Nothing unusual when first examined
5116.....	" Avondrus ", Fraserburg.....	Exacerbation, severe anaemia, hyperpnoea
5117.....	" Avondrus ", Fraserburg.....	Nothing unusual when first examined, exacerbation later
5118.....	" Avondrus ", Fraserburg.....	Nothing unusual when first examined, exacerbation later
5120.....	" Avondrus ", Fraserburg.....	Nothing unusual when first examined, exacerbation later
5127.....	" Celeryfontein ", Fraserburg....	Exacerbation, anaemia, hyperpnoea

APPENDIX 6 (*continued*)

(2) *Group* 2: *Severe early cases*

Sheep No.	Place of origin	Relevant clinical data
Bekker No. 1..	" Soutwater ", Rietbron.....	Severely icteric, dyspnoea, lesions of photosensitization
Bekker No. 2..	" Soutwater ", Rietbron.....	Severely icteric, dyspnoea, lesions of photosensitization
Bekker No. 4..	" Soutwater ", Rietbron.....	Severe icterus, hyperpnoea, lesions of photosensitization
FB-12.....	" Bastersberg ", Sutherland.....	*In extremis*, severe icterus, anaemia, hyperpnoea, temp. 105° F, Cachexia, rhinitis-keratitis
F-6.....	" Kleinplaats ", Aberdeen.....	Severe icterus, anaemia, hyperpnoea, rhinitis, keratitis, cachexia
F-8.....	" Kleinplaats ", Aberdeen.....	Marked icterus, anorexia, listlessness, cachexia

B. Chronic cases (7–14 *days standing*)

(1) *Group* 1: " *Posthaemolytic* " *cases of* 7–14 *days standing*

Sheep No.	Place of origin	Relevant clinical data
F-9.....	" Kleinplaats ", Aberdeen.....	Anaemic, hyperpnoea, cachexia, keratitis, rhinitis, anorexia
F-10.....	" Kleinplaats ", Aberdeen.....	Anaemia, hyperpnoea, mild icterus, cachexia, keratitis, rhinitis, anorexia
F-11.....	" Kleinplaats ", Aberdeen.....	Cachexia, anorexia, gastro-intestinal stasis, hyperpnoea
12226.....	" Droogvoetsfontein ", Fraserburg	Cachexia, anaemia, hyperpnoea, anorexia
12227.....	" Droogvoetsfontein ", Fraserburg	Severe anaemia, hyperpnoea, rhinitis, keratitis, mild icterus, anorexia

APPENDIX 6 (*contined*)

(2) *Group* 2: *Mild chronic cases of* 7–14 *days standing*

Sheep No.	Place of origin	Relevant clinical data
NB-2.....	" Middelpos ", Calvinia.....	Weak, cachexia, anorexia, rhinitis
NB-3.....	" Middelpos ", Calvinia.....	*In extremis*, severe anaemia, hyperpnoea, rhinitis, keratitis
NB-4.....	" Middelpos ", Calvinia.....	Weak, very thin, anorexia
NB-5.....	" Middelpos ", Calvinia.....	Weak, very thin, anorexia, gastro-intestinal stasis
F-7.....	" Kleinplaats ", Aberdeen.....	Anaemia, hyperpnoea, cachexia, rhinitis, anorexia
F-12.....	" Kleinplaats ", Aberdeen.....	Icterus, anaemia, cachexia, gastro-intestinal stasis
FB-9.....	" Bakoondskraal ", Fraserburg...	Anaemia, hyperpnoea, rhinitis, temp. 105° F, icterus
FB-11.....	" Avondrus ", Fraserburg.....	Severe anaemia, hyperpnoea, cachexia
FB-14.....	" Bastersberg ", Sutherland.....	Weak, anorexia, gastro-intestinal stasis
5115.....	" Bastersberg ", Sutherland.....	Anaemia, anorexia, gastro-intestinal stasis
5123.....	" Rietfontein ", Fraserburg.....	Anaemia, anorexia, gastro-intestinal stasis

(3) *Group* 3: *Severe chronic cases of* 7–14 *days standing*

Sheep No.	Place of origin	Relevant clinical data
FB-3.....	" Avondrus ", Fraserburg.....	*In extremis*, severe anaemia, hyperpnoea, cachexia, gastro-intestinal stasis, dehydration
FB-7.....	" Achtersteland ", Hondefontein, Fraserburg dist.	Severe anaemia, hyperpnoea, cachexia, *in extremis*, temp. 104° F
FB-8.....	" Achtersteland ", Hondefontein, Fraserburg dist.	Anaemia, hyperpnoea, listless, anorexia, gastro-intestinal stasis
FB-10.....	" Avondrus ", Fraserburg.....	*In extremis*, cachexia, rhinitis, keratitis, temp. 104° F, anaemia, hyperpnoea

Control Animals:

The following animals, emanating from farms on which the disease was prevalent, were used to control the work done at Onderstepoort: 5124, 5125, 5129 (all from Celeryfontein, Fraserburg) and NB-6 from " Middelpos ", Calvinia. These animals were clinically normal in all respects.

This work was further controlled using the clinically normal sheep 1302, 1541, 3515, 3709, 3960, 89569, 8415, 12276, 10920 drawn from the pool of available animals at this Institute.

APPENDIX 7

STUDIES ON THE HAEMATOLOGY OF ENZOOTIC ICTERUS

Note: RCV = red cell volume (%); Hb = haemoglobin (gm%); RCC = red cell count (10^6/cu mm); TPP = total plasma proteins (gm%); ESR = erythrocyte sedimentation rate (mm/hr); MCHC = mean corpuscular haemoglobin concentration (%); MCV = mean corpuscular volume (cμ); MCH = mean corpuscular haemoglobin ($\gamma\gamma$); WCC = white cell count (10^3/cu mm); N = neutrophiles (%); L = lymphocytes (%); M = monocytes (%); E = eosinophiles (%); B = basophiles (%).

A. Early clinical cases: Group 1—mild cases when first examined

Sheep No.	RCV	RCC	Hb	TPP	ESR	MCHC	MCV	MCH	WCC	Differential white cell count (%)
2206.....	13·0	1·57	3·4	—	0	26·15	82·80	21·65	25,200	N 41, L 54, M 5, E 0, B 0
2207.....	14·5	3·49	5·05	—	0	34·82	41·54	14·46	18,300	N 57, L 39, M 4, E 0, B 0
2208.....	14·5	1·80	3·4	—	0	23·44	80·55	18·88	14,100	N 52, L 42, M 6, E 0, B 0
12223....	36·5	8·76	11·1	7·0	0	30·41	41·66	12·67	6,850	N 62, L 34, M 7, E 0, B 0
12224....	33·8	7·05	12·06	7·38	1	35·68	47·94	17·10	8,100	N 62, L 36, M 2, E 0, B 0
12225....	35·3	8·05	11·34	7·63	0	32·12	43·85	14·08	6,000	N 51, L 35, M 7, E 7, B 0
12228....	35·8	11·66	10·38	—	0	28·99	30·70	8·90	6,600	N 65, L 29, M 6, E 0, B 0
12229....	29·0	9·07	9·17	—	0	31·62	31·97	10·11	8,900	N 75, L 22, M 3, E 0, B 0
Bekker 3.	27·0	—	8·68	7·47	0	32·14	—	—	—	—
FB-2.....	—	4·66	7·00	—	0	—	—	15·02	12,600	—

APPENDIX 7 (*continued*)

STUDIES ON THE HAEMATOLOGY OF ENZOOTIC ICTERUS (*continued*)

B. Early clinical cases: Group 2—severe early cases

Sheep No.	RCV	RCC	Hb	TPP	ESR	MCHC	MCV	MCH	WCC	Differential white cell count (%)
Bekker 1.	28·0	—	10·14	9·62	0	36·21	—	—	—	—
Bekker 2.	24·0	—	7·80	7·42	0	32·50	—	—	—	—
Bekker 4.	32·0	—	10·62	7·56	0	33·18	—	—	—	—
FB-12....	—	—	8·00	6·60	1	—	—	—	—	—
F-6.....	35·0	6·28	6·03	6·75	0	17·22	55·73	10·56	8,500	N 72, L 13, M 9, E 6, B 0
F-8.....	35·0	8·59	9·41	7·00	0	26·88	40·74	26·88	7,050	N 85, L 10, M 4, E 1, B 0

C. Chronic cases: Group 1—"post-haemolytic" cases

Sheep No.	RCV	RCC	Hb	TPP	ESR	MCHC	MCV	MCH	WCC	Differential white cell count (%)
F-9.....	28·0	7·02	7·96	6·32	0	28·42	39·88	11·33	9,200	N 69, L 18, M 10, E 3, B 0
F-10.....	32·0	7·49	9·65	7·00	0	30·15	42·72	12·88	2,250	N 38, L 59, M 3, E 3, B 0
F-11.....	28·0	8·65	12·55	7·72	0	44·82	32·36	14·50	3,850	N 52, L 46, M 2, E 0, B 0
12226....	11·0	4·90	3·38	—	1	30·72	22·44	6·89	2,900	N 55, L 43, M 2, E 0, B 0
12227....	12·7	1·75	3·86	—	4	30·39	72·57	22·05	5,150	N 55, L 40, M 5, E 0, B 0

APPENDIX 7 (*continued*)

STUDIES ON THE HAEMATOLOGY OF ENZOOTIC ICTERUS (*continued*)

D. Chronic cases: Group 2—mild chronic cases

Sheep No.	RCV	RCC	Hb	TPP	ESR	MCHC	MCV	MCH	WCC	Differential white cell count (%)
NB-2.....	25·0	7·94	7·48	7·00	2	29·92	31·48	9·42	7,100	—
NB-3.....	19·5	4·03	5·55	7·56	0	28·46	48·38	13·77	8,850	—
NB-4.....	25·0	8·62	6·76	7·90	0	27·04	29·00	7·84	7,700	—
NB-5.....	31·0	8·55	8·20	7·72	0	26·45	36·25	9·59	8,650	—
5115.....	27·0	—	7·35	—	0	27·22	—	—	—	—
5123.....	28·0	11·37	7·90	—	0	28·21	24·62	6·94	3,800	—
F-7.....	14·0	5·10	4·34	7·00	0	31·00	27·45	8·50	6,800	—
F-12.....	37·9	8·33	8·44	6·50	0	22·26	45·49	10·13	7,050	N 54, L 42, M 4, E 0, B 0
FB-9.....	—	6·76	9·50	—	0	—	—	14·05	3,800	—
FB-11....	—	3·24	6·00	5·20	1	—	—	18·51	5,725	—

E. Chronic cases: Group 3—severe chronic cases

Sheep No.	RCV	RCC	Hb	TPP	ESR	MCHC	MCV	MCH	WCC	Differential white cell count (%)
FB-3.....	25·0	3·79	6·00	—	1	24·00	65·96	15·83	8,300	—
FB-7.....	—	4·03	6·50	—	—	—	—	16·12	6,250	—
FB-8.....	—	5·25	8·40	—	—	—	—	16·00	7,450	—

APPENDIX 8

SELENIUM LEVELS IN ANIMAL TISSUES
(Values are mcg/gm of wet tissue)

A. Normal healthy Onderstepoort sheep

Sheep No.	Liver Se	Sheep No.	Liver Se
5393	0	7303	1·5
9269	0	9814	1·5
7629	0	9725	0
5512	0	8634	3·0
8735	0	10382	0
7604	0		

B. Sheep used for experimental work in Victoria West

Sheep No.	Liver Se	Kidney Se	Sheep No.	Liver Se	Kidney Se
7054	3·0	0	7064	0	0
7055	0	11·5	7065	0	0
7056	0	1·5	7066	0	0
7057	0	0	7067	0	0
7059	0	0	7068	4·0	0
7060	0	28·0	7069	5·5	0
7061	0	3·0	7070	0	0
7062	1·0	0	7071	0	0

C. Apparently normal sheep taken from areas in which geeldikkop and enzootic icterus outbreaks were present

Sheep No.	Disease present	Liver Se
V3–28	Geeldikkop	7·0
V3–29	Geeldikkop	5·8
V3–30	Geeldikkop	4·5
V3–31	Geeldikkop	3·0
V3–32	Geeldikkop	3·0
V3–33	Geeldikkop	4·5
Celeryfontein No. 1	Enzootic Icterus	8·5
Celeryfontein No. 2	Enzootic Icterus	8·5

316:0—17

APPENDIX 8 (*continued*)

SELENIUM LEVELS IN ANIMAL TISSUES (*continued*)

D. Cases of geeldikkop studied during the various field investigations

Sheep No.	Stage of disease	Liver Se	Sheep No.	Stage of disease	Liver Se
V1-3	Early	0	V1-6	Advanced	3·0
V1-24	Early	0	V1-7	Advanced	0
V3-6	Early	5·8	V1-9	Advanced	0
V3-7	Early	5·8	V1-16	Advanced	6·0
V3-10	Early	10·0	V1-14	Advanced	11·0
V3-11	Early	1·0	V1-14A	Advanced	8·5
V3-13	Early	8·5	V1-20	Advanced	3·0
V3-14	Early	1·0	V3-1	Advanced	14·0
V3-15	Early	1·0	V3-1A	Advanced	22·6
V3-16	Early	7·0	V3-2	Advanced	11·5
V3-20	Early	0	V3-8	Advanced	4·5
V3-21	Early	8·5	V3-9	Advanced	0
V3-22	Early	10·0	V3-12	Advanced	1·0
V3-23	Early	7·0	V3-17	Advanced	0
V3-24	Early	5·8	V3-18	Advanced	7·0
V3-25	Early	10·0	V3-3	Recovered	7·0
V3-26	Early	8·5	V3-4	Recovered	4·5
V3-27	Early	3·0	V3-19	Recovered	0
F-1	Early	10·0	F-3	Recovered	3·0
F-2	Early	10·0	F-4	Recovered	3·0
F-5	Early	7·0			

E. Tissues from cases of geeldikkop submitted by veterinarians in the field

Sheep No.	Liver Se	Sheep No.	Liver Se
Jaagvlakte No. 1	0	Ratelfontein No. 2	8·5
Rooivlakte No. 1	8·5	Sheep Thornton	0
Ratelfontein No. 1	5·5	Sheep van den Bergh	5·8

F. Cases of geeldikkop from the farm "Soutwater"

Sheep No.	Liver Se	Sheep No.	Liver Se
17281	12·7	17288	18·4
17282	18·4	BW-1	10·0
17283	25·4	BW-2	8·5
17284	21·2	BW-3	12·7
17285	21·2	BW-4	18·4
17286	21·2	BW-5	18·4
17287	26·8		

APPENDIX 8 (*continued*)

SELENIUM LEVELS IN ANIMAL TISSUES (*continued*)

G. Cases of enzootic icterus studied during the various field investigations

Sheep No.	Liver Se	Sheep No.	Liver Se
Lamb E1	11·5	12228	10·0
Ewe E1	14·0	12229	11·5
Avondrus No. 1	5·5	F-6	10·0
Grootfontein No. 1	11·5	F-7	1·0
Grootfontein No. 2	10·0	F-9	3·0
Grootfontein No. 3	3·0	F-10	12·7
Irene No. 1	4·0	F-11	15·5
Albertsgraf No. 1	4·0	F-12	12·7
Albertsgraf No. 4	8·5	Klawervlei No. 1	29·6
FB-1	3·0	Klawervlei No. 2	28·6
FB-2	7·0	Rusoord No. 1	21·2
FB-3	17·0	12223	8·5
FB-10	8·5	12224	14·0
FB-13	10·0	12225	10·0
FB-21	3·0	12226	24·0
FB-23	1·0	12227	19·8

H. Confirmed cases of enzootic icterus submited by veterinarians in the field

Sheep No.	Liver Se	Sheep No.	Liver Se
Goat—Graham	11·0	Wessels No. 2	10·0
Visagie No. 1	11·5	Thornton 15064	14·0
Steenkamp No. 1	20·0	Thornton 40950	19·8
Steenkamp No. 2	1·0	Thornton 40951	10·0
Gertenbach No. 1	1·0	Thornton 41081	25·4
Gertenbach No. 2	17·0	Goat—Thornton	17·0
Gertenbach No. 3	11·5	Sheep 10863	24·0
Gouws No. 1	18·5	Sheep 10864	10·0
Thornton No. 1	21·2	Sheep 41081B	26·8
Thornton No. 2	8·5	Goat 13120	18·4
Wessels No. 1	14·0		

I. Angora goat foetal livers

Foetus No.	Nature of foetus	Liver Se	Foetus No.	Nature of foetus	Liver Se
3	Normal foetus	31·0	35	Aborted foetus	6·0
36	Normal foetus	14·0	40	Aborted foetus	0·0
38	Normal foetus	17·1	43	Aborted foetus	5·5

APPENDIX 8 (*continued*)

SELENIUM LEVELS IN ANIMAL TISSUES (*continued*)

J. Kidneys from cases of geeldikkop and enzootic icterus studied during the various field investigations

Sheep No.	Disease	Kidney Se
V1-6	Geeldikkop	11·5
V1-14	Geeldikkop	0
V1-14A	Geeldikkop	1·0
V1-16	Geeldikkop	25·5
V1-20	Geeldikkop	14·0
Rooivlakte No. 1	Geeldikkop	0
Ratelfontein No. 1	Geeldikkop	0
Ratelfontein No. 2	Geeldikkop	0
Lamb E1	Enzootic Icterus	3·0
Ewe E1	Enzootic Icterus	14·0
Goat—Graham	Enzootic Icterus	14·0
Avondrus No. 1	Enzootic Icterus	0
Visagie No. 1	Enzootic Icterus	11·0
FB-1	Enzootic Icterus	7·0
FB-2	Enzootic Icterus	8·5
FB-3	Enzootic Icterus	10·0
FB-6	Enzootic Icterus	34·0
FB-10	Enzootic Icterus	8·5
FB-13	Enzootic Icterus	0
FB-14	Enzootic Icterus	4·5
FB-21	Enzootic Icterus	11·5
FB-23	Enzootic Icterus	0

APPENDIX 9

THE DETERMINATION OF COPPER IN ANIMAL BODY FLUIDS AND TISSUES AND THE DETERMINATION OF IRON IN ANIMAL TISSUES

1. *Introduction:* Peterson & Bollier (1955) introduced bis-cyclo-hexanone oxalyl-dihydrazone as a reagent for determining copper in body tissues and fluids. Various studies on the condensation products of aldehydes and ketones with oxalyl hydrazide and the blue colours which the hydrazones gave with copper ions had been conducted previously (Peterson & Bollier, 1955). The introduction of this procedure was soon followed by the publication of numerous methods for the determination of copper in a variety of media using other reagents of this nature, e.g. oxalyldihydrazide and acetaldehyde (Stark & Dawson, 1958) or 1,5-diphenyl-carbohydrazide (Stoner & Dasler, 1960). Use of the Peterson & Bollier (1955) procedure was made for a while but it was found to be very sensitive to pH variations. The colour produced with copper ions at the concentrations encountered in blood also gave very low readings on the various photo-electric colorimeters then in use in the laboratory. The pink colour developed by combination of copper ions with oxalyldihydrazide and acetaldehyde was far more satisfactory and gave results of better reproducibility. Methods using these reagents however all suffered from the same serious disadvantage, notably the extremely volatile nature of acetaldehyde. This property made accurate work with this reagent most difficult during the hot summer months, particularly under the semi-desert conditions of the Karoo. It was decided therefore to develop the methods outlined below in which this objection is removed without decreasing the sensitivity of the colour reaction.

2. *Principle of the methods:* Copper forms a deep pink coloured complex with bis-acetaldehyde-oxalyldihydrazone at pH 8·4–9·1. The hydrazone is itself formed directly in the reaction medium by the interaction of acetaldehyde-ammonia in ammoniacal solution. Acetaldehyde-ammonia (1-amino-ethanol, α-amino-ethyl alcohol or " aldehyde ammonia ") is a crystalline solid which is conveniently stored at low temperature without appreciable decomposition and is easily handled at room temperatures. For use in the methods described below, fresh solutions of the compound

are made daily. Such solutions give all the reactions typical of acetaldehyde. The primary product of the reaction of a primary amine or ammonia with an aldehyde can be considered as the addition compound $R \cdot CH(OH) \cdot NHR$, which may lose the elements of water to give an azomethine or may undergo further condensation. When dry ammonia is passed through an ethereal solution of acetaldehyde, the well-known acetaldehyde-ammonia is formed. Although analytical figures correspond with the formula $CH_3 \cdot CH(OH) \cdot NH_2$, various investigations have shown that the product is in fact more complex. Its molecular weight in water corresponds with the formula, $3(CH_3 \cdot CH(OH) \cdot NH_2)$ (Hickinbottom, 1959). No general agreement on its structure has yet been reached. It appears that the formation of this type of compound from aromatic aldehydes like benzaldehyde and anisaldehyde is preceded by the production of an unstable additive compound of the general formula $(R \cdot CHO)_2NH_3$ (Hickinbottom, 1959). Whether this occurs with the aliphatic derivatives or whether such compounds form in solution is not known. Hydrolysis of the compound in aqueous solution might occur thus: $CH_3 \cdot CH(OH) \cdot NH_2 + H_2O = CH_3 \cdot CHO + NH_4OH$.

The overall reaction of acetaldehyde and oxalyldihydrazide with copper ions is however more complex than mentioned here. Formation of the pinkish-purple complex takes place best in the presence of ammonium ions (Peterson & Bollier, 1955). It was found that colour development is similar when acetaldehyde-ammonia is used instead of acetaldehyde in the presence of ammonia.

3. *Preparation of the colour reagents:* These are easily and rapidly prepared as follows:

(i) Oxalyldihydrazide: Two molecules of hydrazine react with one of diethyl oxalate to form one molecule of oxalyldihydrazide and two of ethanol (i.e. 64 gm of hydrazine and 146 gm of diethyl oxalate are required to produce 118 gm of the hydrazide). Add approximately 13·0 gm of 50 per cent hydrazine hydrate slowly to 20 ml absolute ethanol. In a separate container dissolve 14·6 gm diethyl oxalate in 20 ml absolute ethanol. Mix the two solutions. The hydrazide crystallizes out almost at once. Place the reaction flask in the refrigerator overnight to allow for complete precepitation. Separate the crystals by filtration with suction and wash them twice with ice-cold ethanol and twice with ice-cold water. Recrystallize the product twice by solution in the minimum of boiling water and rapid cooling in the refrigerator. Dry in a desiccator over sulphuric acid or phosphorus pentoxide.

(ii) Acetaldehyde-ammonia: This is conveniently prepared by passing dry ammonia gas through an ice-cold solution of the required amount of acetaldehyde in anhydrous ether as described by Cohen (1937). The reaction vessel is kept cooled by surrounding it with an ice-salt mixture. When the reaction is complete the crystals of aldehyde-ammonia are separated by filtration with suction (preferably in the dark), then washed with repeated small volumes of ice-cold ether, and finally dried *in vacuo*, over calcium chloride and paraffin chips in the dark. The finished product which is initially white, turns light yellow in time. It should be stored in an amber bottle in the refrigerator, in which case it will keep very well for at least eighteen months without appreciable deterioration. The reagent is still active even when yellowish-brown in colour.

4. *Outline of the methods:* (*a*) *General reagent solutions:* The following general reagents are required for the determination of the various copper fractions in blood and for the photometric part of the procedures for estimation of copper in urine, bile and body tisssues:—

(i) 2N HCl;
(ii) 60 per cent w/v aqueous trichloracetic solution;
(iii) concentrated ammonia ($\pm$ 28 per cent NH_3);
(iv) "Dilute ammonia" (Conc. NH_4OH 1: Water 2);
(v) oxalyldihydrazide solution: dissolve 0·25 gm of the hydrazide in 15 ml water with the aid of heat. The solution is a saturated one and much oxalyl hydrazide separates out on cooling. The supernatant solution is kept over the crystals and used when required. When it is exhausted, the same crystals are redissolved in another 15 ml water and after cooling, the supernatant is kept and used in the same way. Larger batches of the reagent may be made using the proportions given above. The crystals may be used repeatedly for making these solutions in the manner described above, until a saturated solution is no longer obtained;
(vi) acetaldehyde-ammonia solution: dissolve 0·5 gm acetaldehyde ammonia in 15 ml water. This reagent is generally made up fresh each day in the proportions given, although the solution keeps quite well for at least a week, if it is stored at 4° C;
(vii) stock copper standard solution, containing 100 mcg Cu/ml;
(viii) working copper standard solution, containing 100 mcg Cu/100 ml (i.e. 1 mcg/ml);
(ix) saturated aqueous solution of sodium pyrophosphate (5·4 gm $Na_4P_2O_7 \cdot 10H_2O$ dissolved in 100 ml water);
(x) saturated sodium citrate solution (64 gm $Na_3C_6H_5O_7 \cdot 2H_2O$ dissolved in 100 ml water);
(xi) 0·9 per cent w/v NaCl;
(xii) Acetate buffer for loosely bound plasma copper, pH 8·6.

Place 0·7708 gm ammonium acetate crystals in a 200 ml volumetric flask. Dissolve in about 100 ml distilled water, then add 1 ml of concentrated ammonia and 0·5 ml glacial acetic acid. Make to volume, mix and check the pH. Adjust to 8·6 if necessary.

(*b*) *Total plasma copper:* 2 ml plasma, 2 ml water for the reagent blank and 2 ml working standard solution are placed in separate clean centrifuge tubes. Add to each 2 ml 2N HCl. Let stand 10 minutes after mixing, then add 0·4 ml 60 per cent trichloracetic acid. Stir the contents of the tube containing plasma with a thin glass rod until the protein coagulum has been reduced to a fine slurry. Allow to stand for 10 minutes in the refrigerator and then centrifuge down the protein precipitate. Transfer 2 ml of the clear supernatant centrifugate and 2ml of the mixture from the " blank " and " standard " tubes to three clean test tubes. Add to each 0·65 ml of " dilute " ammonia. Adjust the pH to 8·6 if necessary with more ammonia or glacial acetic acid. Adjust the volume of each to 3·5 ml. Mix, then add to each tube 0·25 ml oxalyldihydrazide solution and 0·25 ml acetaldehyde-ammonia solution. Mix. Let stand for 1 hour and read the optical density at 544mμ.

$$\text{Total plasma copper (mcg\%)} = \frac{\text{OD Test}}{\text{OD Standard}} \times 100$$

(*c*) *Loosely bound* (*albumin*) *copper in plasma:* The standard used here is the " working " standard diluted one in ten to contain 10 mcg/100 ml copper. A reagent blank and standard are necessary for each batch of samples and for each individual sample a tube labelled " test " and one labelled " control " is required. Place in the various tubes concerned 1 ml water, 1 ml standard, and 1 ml plasma (this in both " test " and " control " tubes). Add to each tube 2·5 ml acetate buffer. The pH of each should be 8·6. Add to the control tube 0·25 ml water and to the rest of the tubes 0·25 ml oxalyldihydrazide solution. Add to all the tubes 0·25 ml acetaldehyde-ammonia solution. Mix. Let stand one hour. Read optical densities at 544 mμ.

$$\text{Loosely bound plasma copper} = \frac{\text{OD Test–OD control}}{\text{OD Standard}} \times 10$$
$$= \text{mcg\%}.$$

(*d*) *Total red cell copper:* Centrifuge the blood sample to obtain the erythrocytes and wash these twice with 0·9 per cent NaCl. After centrifuging following the second saline wash the packed red cells are suspended in an equal volume of saline. Mix thoroughly and withdraw some of the suspension to fill two Wintrobe haematocrit tubes. The packed cell volume of this suspension must be known. Let this value be C in the final calculation. Set up three centrifuge tubes labelled " Blank ", " Standard " and " Test " and in these place 2 ml water, " working " standard and cell suspension respectively. Add to each 2 ml $2NH_4Cl$. Mix. Allow to stand for 10 minutes, then add to each 0·4 ml of 60 per cent trichloracetic acid The contents of the tube containing the cell suspension are stirred with a thin glass rod until a smooth slurry is obtained. Allow to stand for 10 minutes in the refrigerator then centrifuge off the protein precipitate.

Into three clean tubes labelled as above, place 2 ml of the blank and standard mixtures and 2 ml of the clear centrifugate. To each tube add 0·4 ml saturated sodium pyrophosphate solution, 0·4 ml saturated sodium citrate solution and 0·65 ml of " dilute " ammonia. Adjust the pH if necessary to 8·6 and the volume of each solution to 4·25 ml (with water). To each tube add 0·25 ml oxalyldihydrazide and 0·25 ml acetaldehyde-ammonia solutions. Mix and let stand for 1 hour at room temperature. Read optical densities at 544 mμ.

$$\text{mcg Cu/100 ml red cells} = \frac{\dfrac{\text{OD Test}}{\text{OD Standard}} \times 10{,}000}{\text{C}}$$

(*e*) *Bile and urine copper:* Ten ml of bile or urine, 10 ml of working standard and 10 ml of water (for the blank) are placed into separate 100 ml Kjeldahl flasks together with 0·75 ml concentrated sulphuric acid, 5 ml concentrated nitric acid and some copper-free glass beads, porcelain chips or carborundum chips. Digest rapidly to charring. Cool and add to each flask 0·5 ml 60 per cent perchloric acid and a further 2·5 ml concentrated nitric acid. Digest until the solution is light yellow. Cool and add 5 ml water and 0·5 ml 100 vols. per cent hydrogen peroxide. Digest down to a volume of about 1·5–2·0 ml. If the digest is not colourless, add 2·5 ml water and 0·25 ml hydrogen peroxide. Digest again. Cool. Add 5 ml water only. Digest till white

fumes are evolved, and a residue of 0·5–1·0 ml of digest remains (the final addition of water and peroxide indicated above can be repeated until the digest is colourless, but the final strong heating with water only must be done to remove all traces of peroxide). Allow the digests to cool. To each add about 3 ml water, then transfer the contents to a 10 ml measuring cylinder. Rinse the flask with two 1 ml portions of water and add this to the main digest. Rinse the flask with 2 ml concentrated ammonia solution which is also carefully added to the main digest. Cool. Mix. Check the pH and adjust to 8·6 with " dilute " ammonia or glacial acetic acid. Adjust the volume to 10 ml with distilled water.

A 5 ml aliquot of this mixture is placed in a test tube for copper determination. The remaining 5 ml can be used for the determination of iron (see later) if so desired. Add to the 5 ml aliquot 0·5 ml oxalyldihydrazide and 0·5 ml of acetaldehyde-ammonia solutions. Mix. Let stand for 1 hour and read optical densities at 544 mμ.

$$\text{Urine or bile copper (mcg\%)} = \frac{\text{OD Test}}{\text{OD Standard}} \times 100$$

(*f*) *Tissue copper:* The standard used in this method is the "stock" standard containing 100 mcg Cu/ml. Place in separate Kjeldahl flasks 1 gm of tissue, 1 ml of standard and 1 ml water. To each add 5 ml conc. H_2SO_4 and 5 ml nitric acid. Digest to charring. Proceed with the digestion from this step onwards as outlined under (*e*) above. The final step includes strong heating to the evolution of white fumes after adding 5 ml of water and reduction of the volume of the digest to about 5 ml. The digests are transferred quantitatively to a 50 ml volumetric flask, the Kjeldahl flasks are thoroughly washed with small portions of distilled water and the washings are added to the digest, the volume of which is finally made up to the mark with distilled water. Mix. Take an aliquot of 5 ml for copper determination and reserve the remainder for the estimation of iron if desired (see later). To the 5 ml aliquots from the " Blank ", " Standard " and " Test " digests add 1 ml saturated sodium pyrophosphate, 1 ml saturated sodium citrate and 2 ml " concentrated " ammonia solutions. Adjust the pH of each to 8·6. Make all the tubes to the same volume with distilled water. Mix. Add 0·5 ml oxalyldihydrazide and 0·5 ml acetaldehyde-ammonia solutions. Mix. Let stand for an hour and read optical densities at 544 mμ.

$$\text{mg Cu/100g tissue} = \frac{\text{OD Test}}{\text{OD Standard}} \times 10.$$

5. *Experimental:* (*a*) *General remarks on the methods:* The choice of the quantities of plasma, erythrocytes, body fluids and tissue used in these methods, the use of 2N HCl and 60 per cent trichloracetic acid for liberation and extraction of bound copper and precipitation of plasma proteins and the use of sodium pyrophosphate and sodium citrate in the amounts specified here, to complex interfering ions are all based on principles embodied in earlier methods for the determination of copper in animal tissues and body fluids (Cartwright, Jones & Wintrobe, 1945; Gubler, Lahey, Ashenbrucker, Cartwright & Wintrobe, 1952). The methods of digestion of body fluids and tissues are likewise based on earlier work (Cartwright, Gubler & Wintrobe, 1954; Markowitz, Shields, Klassen, Cartwright & Wintrobe, 1961, and van Niekerk, 1937). The modifications introduced mainly concern the colour reaction with copper and the following sections are therefore devoted to experimental data concerning this aspect of the method only.

(*b*) *Spectral absorption of the copper complex:* The light absorption of the coloured complex as prepared by using 2 ml of working standard and subjecting it to the procedure outlined under " total plasma copper " was studied over the spectral range 200 to 600 mμ. The curve obtained by plotting optical density against wave length is a smooth bell-shaped curve with maximum absorption at 544 mμ. The compound absorbs very strongly over the range 535 to 555 mμ. If a photoelectric colorimeter is used for the estimation of copper by the methods outlined here, the light filter of choice would thus be an Ilford 625 or equivalent.

(*c*) *The influence of pH on complex formation and colour development:* A mixture of " working " standard, 2N HCl and 60 per cent trichloracetic acid was made up using the proportions described earlier under (*b*). Two ml aliquots of this were taken and dilute ammonia (NH_4OH 1: water 3) and water were added in the amounts shown in the table below. After measurement of the pH of the contents of each tube, 0·25 ml of oxalyldihydrazide and acetaldehyde-ammonia solutions were added, the contents of the tubes mixed and allowed to stand for one hour before reading optical densities against a reagent blank. The optical densities found are given in the table below. It can be seen from this table that the colour is maximal over pH range 8·4 to 9·1. Below pH 7·4

Tube No.	ml NH_4OH	ml water	pH	OD
1.....	0·05	1·45	1·1	0
2.....	0·10	1·40	1·2	0
3.....	0·15	1·35	1·2	0·001
4.....	0·20	1·30	1·2	0·002
5.....	0·25	1·25	1·2	0·001
6.....	0·30	1·20	1·3	0·001
7.....	0·35	1·15	1·35	0·001
8.....	0·40	1·10	1·40	0·001
9.....	0·45	1·05	1·6	0·002
10.....	0·50	1·00	1·9	0·006
11.....	0·55	0·95	1·9	0·006
12.....	0·60	0·90	7·46	0·058
13.....	0·65	0·85	8·46	0·071
14.....	0·70	0·80	8·75	0·072
15.....	0·75	0·75	8·95	0·072
16.....	0·80	0·70	9·00	0·071
17.....	0·85	0·65	9·10	0·068
18.....	0·90	0·60	9·15	0·069
19.....	0·95	0·55	9·25	0·055
20.....	1·00	0·50	9·35	0·055

complex formation is negligible and at pH values higher than 9·1, the intensity of the colour is also decreased. These experiments were repeated over the pH range of 8·4 to 9·1. Maximum colour development was found to occur at pH 8·6. The " dilute " ammonia solution described under (*a*) earlier was found to be the most satisfactory reagent for adjusting the pH.

(*d*) *Time taken for maximum colour development:* Two ml aliquots of the working standard 2N HCl: 60 per cent trichloracetic acid solution made up using the proportions given under (*b*) were treated with 0·25 ml of oxalyldihydrazide and acetaldehyde-ammonia solutions. After mixing, optical densities were determined at ten-minute intervals for the first hour and thereafter hourly for the next six hours. Colour develops within fifteen minutes and is maximal one hour after addition of the colour reagents. Very slight increases in optical density may be found over the next five hours, but thereafter the colour remains stable for at least three days if the tubes are corked and kept in the dark. Typical data are presented in the table which follows.

Tube No.	15 min.	30 min.	45 min.	60 min.	120 min.	24 hours
1.....	0·064	0·057	0·062	0·062	0·064	0·065
1A.....	0·064	0·057	0·062	0·062	0·065	0·064
2.....	0·065	0·063	0·064	0·066	0·065	0·065
2A.....	0·066	0·064	0·066	0·066	0·072	0·070
3.....	0·065	0·060	0·064	0·064	0·069	0·069
3A.....	0·064	0·060	0·064	0·064	0·068	0·067

For practical purposes optical densities of each batch of determinations are read at any time following one hour after addition of the colour reagents.

(*e*) *Reproducibility of results:* The table above shows values obtained from six different samples prepared simultaneously by three different operators. If the pH is carefully adjusted and the reagents are added using highly accurate micropipettes, the reproducibility is excellent as shown by these figures which are representative of the work.

(*f*) *Obedience to Beer's Law:* The method gives linear results over the copper concentration range of 40 to 600 mcg per cent, or 1 to 12 mcg in the test medium to which the colour reagents are added Dilutions of the stock standard were made to cover this range and each dilution was treated as described under (*b*) above. Optical densities were plotted against concentration and the resulting plot was a straight line throughout this range.

(*g*) *Tests for interfering substances:* The specificity of the reaction between oxalyldihydrazide, acetaldehyde and copper ions under conditions similar to those described here has been discussed by previous authors (e.g. Markowitz, Shields, Klassen, Cartwright & Wintrobe, 1961; Stark & Dawson, 1958). The following ions were tested for interference at the concentrations indicated in parentheses: Ca (10 mg%); Mg (2 mg%); Fe^{iii} (200 mcg%); Fe^{ii} (200 mcg%); Na (160 meq/L); K (6 meq/L); Cl (100 meq/L); HCO_3^- (27 meq/L) and $PO_4^{\equiv}$ (5 meq/L). No interference was noted in any of the experiments when these ions were added to " working " standard copper solutions at the concentrations indicated. Urea and glucose have been tested at concentrations of 60 mg% in each instance and have no effect on colour development.

(*h*) *Comparisons between the methods described here and other methods used:* The table below shows the results obtained in the determination of total plasma copper on a number of samples of sheep plasma by this method and those of Cartwright, Jones & Wintrobe, 1945 (which specifies sodium diethyl-dithiocarbamate as colour reagent), and Peterson & Bollier, 1955 (which is a bis-cyclohexanone oxalyldihydrazone method). It is apparent from this table that there is good agree-

Sample No.	Cartwright *et al.* mcg%	Peterson & Bollier mcg%	This method mcg%
9584	155	100	130
9596	144	80	130
9606	133	100	130
9613	133	67	124
1546	101	100	92
7888	111	100	117
9585	112	100	96
9586	73	67	71
9585B	100	95	92

ment between the results obtained with this method and that of Cartwright *et al.* (1945). The Peterson & Bollier (1955) procedure is inclined to give very variable results since colour development is weak if the pH of the reaction medium is not controlled very rigidly.

The results obtained by this method for copper in tissues similarly compare extremely well with those obtained by the older diethyl-dithiocarbamate procedures (e.g. Van Niekerk, 1937). No attempt has been made to adapt the Peterson-Bollier procedure to tissue work.

(i) *The effect of environmental temperature on colour development:* The method for total plasma copper outlined here has been used extensively during field work described earlier. A very large number of determinations have been performed under the bitterly cold winter conditions of the Cape Midlands and the extremely hot summer conditions in the semi-desert Karoo. Records have been kept of the optical density readings of the working standards used with every batch of samples analysed.

Although very low environmental temperatures retard *the rate* of colour development and high environmental temperatures markedly accelerate it, optical density readings obtained one hour after addition of the colour reagents were virtually identical under both sets of conditions and identical to results obtained under the more temperate summer and winter conditions of Pretoria.

6. *The determination of iron in animal tissues:* The method used was essentially a modification of those of Elvehjem (1930) and Kennedy (1927) cited by Hawk, Oser & Summerson (1954). Stock standard iron solutions were prepared by dissolving 1·4046 gm of ferrous ammonium sulphate (Mohr's salt) in 100 ml of 1 per cent v/v sulphuric acid solution and making up to 1 litre with distilled water. The iron content of these solutions is 200 mcg/ml.

3161)—18

One gram of tissue, 1 ml of the standard solution and 1 ml of water are digested in 100 ml Kjeldahl flasks as described under (*f*) above. The colourless digest is allowed to cool and is then diluted to 100 ml with distilled water. Take 10 ml aliquots of each of the blank, standard and test solutions. To each add 5 ml of 20 per cent potassium thiocyanate solution and 10 ml of iso-pentanol. Shake thoroughly until all the red ferric thiocyanate is extracted into the alcohol layer.

The alcohol layer is then drawn off with the aid of a separating funnel and the absorption of the solutions is determined in a photo-electric colorimeter using an Ilford 622 filter.

$$\text{mg iron/100 gm tissue} = \frac{\text{OD Test}}{\text{OD Standard}} \times 20.$$

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APPENDIX 10

THE DETERMINATION OF SELENIUM IN ANIMAL AND PLANT TISSUES

Introduction: At the time that the work on the selenium content of animal and plant tissues reported in this thesis was commenced, a number of procedures for the estimation of micro amounts of selenium were available. These included digestion of the selenium-containing material with acid and then: (i) distillation of the liberated selenium as the tetrabromide, reduction to elemental selenium and turbidimetric, gravimetric or colorimetric estimation of the element or redissolution of the precipitated selenium in a suitable solvent followed by titrimetric estimation; (ii) titrimetrically on the digest using permanganate; (iii) precipitation with mercurous chloride from the digests followed by gravimetry; (iv) application of a suitable colour reaction followed by colorimetric,

photometric or spectrophotometric estimation of the element. Such colour reactions included (*a*) the reaction between selenium and codein in concentrated sulphuric acid. A blue dye of unknown constitution is formed (Davidson, 1939; Gortner & Lewis, 1939; Schulz & Lewis, 1940); (*b*) oxidation of pyrrole in orthophosphoric acid to give a mixture of blue-green dyes of unknown constitution; oxidation of *asym*-diphenylhydrazine to give a mixture of unstable reddish-violet compounds; oxidation of 1,8-naphthalene diamine in acetic acid to give a mixture of brown compounds (Cheng, 1956) and (*c*) the formation of highly coloured piazselenoles in the appropriate media with 3′,3-diaminobenzidine or 4-dimethylamino-1,2-phenylene diamine and 4-methylthio-1,2-phenylene diamine (Cheng, 1956; Danzuka & Ueno, 1958; Sawicki, 1957).

In general most of the methods were not directly applicable to this work since they were too cumbersome, time-consuming or subject to too much interference by other elements. A serious objection to many of these methods was the large amounts of material used for the estimation of selenium. The distillation methods entailed some danger of loss of the element during the procedure and were in any case unpractical for handling the large numbers of samples available at the time. The reduction procedures associated with these methods were also subject to interference by other elements.

The methods depending on the formation of coloured piazselenoles were claimed to be highly specific and very sensitive for selenium, but at the time the reagents concerned were not generally available. More elegant procedures currently in use elswhere in the world included oxidation of the material in the Parr sulphur bomb and estimation of selenium by a variety of methods; spectrochemical, chromatographic and polarographic methods and cationic exchange. These methods were also not considered at the time since the necessary apparatus was not generally available or within means.

It was decided after much preliminary work with the distillation and reduction methods to make use of one of the known colour reactions for selenium and develop a photometric method which would suit requirements. Apart from the piazselenole methods the only colour reaction specific enough and which yielded an intense but stable colour with selenium was the reaction with codein in concentrated sulphuric acid.

Principle of the method: The reaction between codein and selenium in concentrated sulphuric acid is well known and was used extensively as a qualitative means of detecting selenium in sulphuric acid itself and in glass. Similarly the reaction was used by pharmacologists as a means of detecting codein in various preparations (U.S.P., 1942). A characteristic colour, varying from green to blue, due to a complex of unknown constitution, is produced. The reaction was first studied for use as a qualitative test for the presence of selenium in plants by Schmidt (1914). Horn (1934) studied the reaction in more detail and improved upon it considerably for qualitative purposes. while Davidson (1939), Gortner & Lewis (1939) and Schulz & Lewis (1940), put it to considerable use in work on various aspects of selenium intoxication.

The method as applied to animal tissues: One gm of tissue which may be fresh, preserved in ethanol or preserved in 10 per cent formalin is presssed dry between filter paper, cut up finely and placed in a 100 ml Kjeldahl flask. To this is added 0·4 gm yellow mercuric oxide, 10·0 ml distilled water, 10·0 ml conc. sulphuric acid, 2·0 ml of 30 per cent hydrogen peroxide ($\equiv$ 100 vols oxygen) and a few glass beads. These are mixed and digested over a low flame in a hood until the mixture assumes a brownish or yellowish brown colour. The contents of the flask are allowed to cool for a few minutes. Add a mixture of 10 ml water, 5 ml conc. sulphuric acid and 2 ml hydrogen peroxide. The mixture is once more digested over the low flame until it assumes a yellow colour. It is again allowed to cool slightly and then 2 ml water and 2 ml hydrogen peroxide (mixed) are very cautiously added. The digestion is repeated and if necessary more water and hydrogen peroxide are added in small amounts until the digest is colourless or at the most a faint yellow colour (which should disappear completely on cooling). It is essential that the small amounts of hydrogen peroxide and water be added to the digest while it is still hot. This brings about rapid discharging of the residual yellow colour, and allows the digestion procedure to be completed within about one-and-a-half hours over a low flame. It is essential that the digestion period is not prolonged, and that overheating does not occur, or selenium may be lost by volatilization.

The volume of the digest should now be about 14 to 15 ml. Digestion is then continued beyond this point to the evolution of copious fumes of sulphur trioxide, when the final desired volume of about 10 ml should be obtained.

The flask is allowed to cool until it can be handled, sealed at once with lead foil to prevent the absorption of water, and allowed to stand and cool thoroughly for about two hours. Most of the mercuric sulphate formed during the digestion will settle out at this stage.

The contents of the flask are now well mixed by lateral shaking, and poured rapidly into a centrifuge tube graduated at 15 ml. The volume of digest is noted. The amount of sulphuric acid required to make the volume up to 15 ml is taken and used to wash out the flask, and then transferred to the main digest in the centrifuge tube. Mix well and centrifuge at 3,500 rpm for half-an-hour to precipitate all the mercuric sulphate and any other insoluble sulphates formed from the tissue. The period of centrifugation must not be less than half-an-hour, since some finely divided mercuric sulphate, which usually settles out during this period is often present, and may give rise to troublesome turbidity if not removed properly.

A series of clean and dry test tubes is set up, each of which contains 0·15 ml of 5 per cent aqueous codein phosphate solution. The supernatant acid solution in the centrifuge tubes is carefully decanted into these tubes without disturbing the precipitate of insoluble sulphates. The contents of the tubes are carefully mixed by inversion, and the tubes are then tightly stoppered and allowed to stand in the dark for two hours. Photometric readings are made in a photometer at 600 mμ or with an Ilford 607 light filter.

Apparatus, Standards and Reagents: All the determinations and experimental work connected with the method were carried out on an E.E.L. Portable Model A Colorimeter, using the standard matched tubes for this instrument. All the reagents used were analytical grade reagents. The concentrated sulphuric acid used was that of Riedel de Haën A.G. (SG 1·83, Assay 95–97 per cent H_2SO_4, maximum limit of selenium impurity 0·0002 per cent). Other brands of sulphuric acid were tried but were rejected as they gave too high blanks.

A stock selenium standard solution, equivalent to 1 mg Se/ml, was made up by dissolving 333 mg of sodium selenite ($Na_2SeO_3 \cdot 5H_2O$) in distilled water and making up to 100 ml. Diluted working standards were prepared from this for construction of the standard calibration curve and for use with every batch of specimens. These working standards contained 5, 15, 25, 35, 50, 150 and 250 micrograms of selenium per 5 ml respectively. The standards were then put through the exact procedure outlined above, the digest being initially made up with one gram of " liver low in selenium " (see below) and 5 ml water plus 5 ml working standard instead of the 10 ml water used for unknown samples. Standards were also done throughout this research series, in duplicate and simultaneously with blanks and unknowns.

Reagent blanks for setting the photometer at zero were made up and treated exactly as detailed above, one gram of liver from freshly slaughtered Onderstepoort sheep being used. These livers were tested for selenium content as detailed above and those which gave no appreciable colour with the reagent in triplicate tests were designated as " livers low in selenium " and were preserved in 10 per cent formalin for use in making up blank and standard solutions. This being the case the method determines only selenium present in liver in amounts greater than those present in the livers of normal Onderstepoort sheep, i.e. more than 1 to 2 mcg/gm of wet liver tissue.

Experimental: (*a*) *The digestion procedure*: It has been stated that loss of selenium by volatilization during the Kjeldahl digestion procedure can be satisfactorliy prevented by the addition of a large excess of mercuric oxide and carrying out the whole procedure as rapidly as possible without overheating. It was said that mercuric oxide probably functions in preventing selenium loss by forming a double salt of selenium and mercury in the same way as mercuric chloride or zinc chloride form a double salt with the element, holding it in solution (Horn, 1934; Lyons & Shinn, 1902) Sreenivasan & Sadasivan (1939) who studied the nature of the action of selenium in the Kjeldahl method, held that when digestion is carried out in the presence of mercuric oxide, selenium in any form is converted into selenic acid. In the presence of reducing organic matter and in the absence of mercuric oxide, small amounts of selenious acid are formed which is partially reduced to elemental selenium, while selenic acid is completely reduced to selenious acid and to a small extent to elemental selenium as well. They concluded that the catalytic action of selenium in Kjeldahl reactions, in the presence of mercuric oxide, is due to the reversible reaction:

$$\text{selenium} \rightleftharpoons \text{selenious acid} \rightleftharpoons \text{selenic acid}$$

whereby the selenious acid acts as an efficient carrier of oxygen to the reducing organic matter, As long as there is unoxidized organic matter the forward reaction is more rapid than the reverse one. When oxidation is complete the reaction proceeds to completion in the forward direction. In the absence of mercuric oxide the forward reaction probably goes to a large extent no further than selenious acid.

Davidson (1939), Klein (1941) and Robinson (1933) have stressed the importance of keeping the selenium in a highly oxidized form during the digestion procedure to prevent loss by volatilization.

The official reviewers of the A.O.A.C. methods for the determination of selenium investigated the question of possible loss of the element during digestion with mercury in an open vessel. They concluded that there was little to choose between digesting in open vessels like beakers, and Kjeldahl flasks or in a closed system like the Soxhlet apparatus (Official Method, 1939; Klein, 1941).

In the A.O.A.C. methods the mercuric oxide is usually added in nitric acid solution. It was found that although the addition of nitric acid aids the digestion considerably, its use in the Codein method is undesirable. Although the frequent addition of water serves to decompose the nitrosyl sulphuric acid formed during the reaction, it is difficult to get rid of the last traces of nitrous acid. This can be accomplished by the addition of urea, but as this is best added in aqueous solution, its use is undesirable owing to the fact that during the final colour development the sulphuric acid must be kept as water free as possible. Traces of nitrous acid cause the formation of red, brown or yellowish-brown colours after the addition of Codein. This was also noted by Horn (1934). Similar objections pertain to the use of perchloric acid as an adjuvant to the digestion procedure for this method.

Davidson (1939) used 0·7 gm mercuric oxide for materials containing 50 to 200 mcg of selenium. The A.O.A.C. methods make use of 0·5 gm for similar material (Official Method, 1939; Klein, 1941) while Gortner & Lewis (1939) used 0·2 gm for every 1 gm of liver. It was decided on the basis of their data to make use of 0·4 gm for every 1 gm of liver since this appeared to be a sufficient excess for the amounts of selenium likely to be encountered in the materials to be dealt with.

(*b*) *The colour produced with codein*: Little is known of the chemistry of the reaction between selenium and codein, but it appears that selenium must probably be in the form of selenite before it participates in this reaction. Under the conditions of the digestion procedure outlined above the selenium is probably converted to selenic acid, but it is probable that on cooling after completion of the digestion and precipitation of mercury ions as the insoluble sulphate, it reverts to the more stable selenious acid.

Mention has been made of the interference by nitrous acid in proper colour development. It was also found that unless the hydrogen peroxide was completely removed during the final stages of the digestion, marked interference was produced. This was readily demonstrated by interrupting the digestion of samples of standard solutions at different stages before the evolution of oxygen had ceased, and adding codein after cooling and precipitation of the mercuric sulphate. Colours varying from lemon-yellow to brown or even red were obtained. In the method as outlined all residual hydrogen peroxide is removed during the final stage when the volume of the digest is reduced to 10 ml. If this step is carried out properly, there is no danger of interference by hydrogen peroxide.

In the early literature the colour produced in the reaction was variously described as green, blue or an immediate green colour turning to blue. This discrepancy was shown by Horn (1934) and Davidson (1939) to be due to varying amounts of water remaining in the digest or being absorbed by the acid after digestion was complete. Both authors stressed that for complete and proper colour development the acid must be as nearly anhydrous as possible. Horn showed that the colour produced after adding codein will gradually disappear if water is slowly added dropwise, while Davidson states that if much water is present, the colour will fail to develop. This is a serious disadvantage of the method, but can be satisfactorily overcome if the following precautions are observed: (i) the volume of the digest must be reduced to about 10 ml to expel any remaining water; (ii) the Kjeldahl flask must be sealed with lead foil as soon as it is cool enough to handle; (iii) during the centrifugation step and during the colour development the tubes must be firmly stoppered.

Davidson (1939) also noted that if the selenium present was in excess of the codein added, a green colour tended to develop. He concluded that the amount of codein added should be at least thirty times the amount of selenium expected to be present in the digest. The amount of codein solution is more than adequate for the amounts of selenium likely to be encountered in animal tissues, and most plant tissues, if 1 gm of tissue is used.

Davidson (1939) stressed the importance of allowing the digests to stand long enough for complete precipitation of mercuric sulphate before colour development. He found that varying shades of olive developed in samples in which the sulphate was incompletely precipitated. This objection is not mentioned by Gortner & Lewis (1939). In the present study it was found that incomplete precipitation of the mercuric sulphate has no effect on the colour, nor does mercury appear to contribute any blue colour with codein. Incomplete precipitation merely lends to a slowly developing and troublesome turbidity. This is especially so in the case of plant material (see later).

Gortner & Lewis (1939) noted that light was found to affect colour development, and that the blue colour turned to purple when exposed to sunlight. They recommended placing the tubes in the dark during colour development. Under those conditions it was found in this work that the colour remains stable for at least four days.

With regard to the time required for full colour development, Gortner & Lewis recommended seven hours. They found colour development to be erratic if the samples were allowed to stand for less than this period. Davidson (1939) did not comment on this aspect of the method other than to advocate a colour development of two hours. Klein (1941) in his report on methods for the estimation of selenium to the A.O.A.C. found that a period of two hours was quite sufficient.

31610—19

In the present study it was found that the two-hour period was quite satisfactory. During the first hour after addition of the codein reagent the colour develops rapidly, but by the end of the second hour it has reached full development and under the optimum conditions outlined above it remains stable for about four days. The erratic colour development mentioned by Gortner & Lewis was no doubt due to incomplete dehydration of the sulphuric acid during digestion.

Klein (1941) in his review states that the method is quite sensitive but lacks strict reproducibility and that the colour complex does not appear to follow Beer's Law. He obtained on a 5 mcg standard a 30 per cent variation and on a 50 mcg standard a 7 per cent variation. In order to investigate the reproducibility of the author's method, and to draw up a standard calibration curve, five working standards each done in quintuplicate were subjected to the entire procedure detailed above. The average reading for each set of standards was used to construct the calibration curve. The following table indicates the mean variation from the average values for each set of standards:—

Micrograms Se.....	5	15	25	35	50
Mean Variation.....	23·2%	20%	11·4%	10·6%	6·2%

These figures confirm Klein's findings that the method lacks strict reproducibility, although they are slightly lower than the average variations he obtained. The variation is greatest in the low concentrations of selenium and is directly due to the colour of the blanks used in each batch of determinations. The colour of a good blank should be a very faint lemon yellow colour. Often blanks are encountered which have a slightly stronger yellow colour, probably due to differences in the amounts of other inorganic substances present in the tissues used in the preparation of these. These differences in the colour of the blanks make a difference, albeit small, in the setting of the instrument for each batch of determinations and such differences are most apparent in the low concentrations of any substance being determined photometrically.

The sensitivity of the method is excellent for the present requirements of this work, 0·5 microgram of selenium giving a blue colour which is readily detectable by the eye and easy to differentiate from samples which give a negative test or in which only minute traces of the element are present. Better readings for these low concentrations of selenium are obtained on a spectrophotometer than on a photo-electric colorimeter and the figures thus obtained are more precise. In higher concentrations the colour is an intense blue, and well within the limits of sensitivity of the photo-electric colorimeter.

Contrary to Klein's findings and in accordance with those of Gortner & Lewis (1939) the colour adheres strictly to the Beer-Lambert law, the calibration curve being linear over the concentrations tested ranging from 0·5 to 150 mcg of selenium. Higher concentrations of selenium were not tested, since it was unlikely that these would be encountered in the present work.

The special absorption characteristics of the selenium-codein complex in concentrated sulphuric acid were studied on typical standard digests over the spectral range of 430 to 670 mμ in the E.E.L. photo-electric colorimeter. Two peaks of maximum absorption of almost equal intensity were found corresponding to the wavelengths covered by filters transmitting maximally at 530 and 600 mμ. The wavelength of 600 mμ (Ilford 607 filter) was selected as being the most appropriate for this method.

(*c*) *Recovery experiments*: The reproducibility of the method was further tested for recovery of known amounts of selenium added to the standard digests. The following table shows the results of these experiments:—

Micrograms Se present in Standard Digest	Micrograms Se added	Micrograms Se recovered	% Recovery
25	25	60	120
35	35	76	108·6
15	15	33	110
15	15	33	110
5	5	9	90
50	50	93	93
25	25	62	124

These recoveries are of the same order as those cited by Gortner & Lewis (1939). The mean value of the percentage recoveries is 107 per cent, i.e. 7 per cent above theoretical. A possible reason for these high recovery figures is given below.

When the digests were *overheated* during the final stages and the final volume taken right down to one ml, up to 72 per cent of the selenium added was lost. A number of experiments of this nature was performed using standards containing 50 mcg of selenium. Generally only 14 to 15 mcg could be demonstrated in the digest when it was made up to 15 ml before colour development.

(*d*) *Tests for interference by other elements*: Schmidt (1914) showed that ferric iron in quantities of one drop of strong ferric chloride solution in 10 ml of conc. sulphuric acid will interfere with the test. Horn (1934) however, digested two grams of ferric sulphate for one hour with 50 ml of sulphuric acid and mercuric oxide, and found that a test of this digest with codein gave no colour at all He concluded that iron does not interfere with the test. He further cited experiments in which the following elements were tested, 2 mg quantities of a salt of each element being added to a digest: chromium, nickel, titanium, beryllium, molybdenum, thallium, tellurium, vanadium, boron, antimony, bismuth, arsenic, iodine, manganese, iron, silicon and tungsten. Vanadium was the only one of these elements found to interfere with the test.

Gortner & Lewis (1939) made no mention of interfering ions, apparently having accepted Horn's findings. Davidson (1939) however, confirmed that vanadium interfered with the test, but stated quite emphatically that iron not only did not interfere but that it was essential to the proper development and stability of the colour. He even recommended the addition of iron to the digest, when materials low in this metal were being examined. He found that 10 ppm of vanadium give a colour equivalent to 1 ppm of selenium, and stated rather generally that other mineral elements present in the plant material may also affect the shade of the selenium-codein colour.

Klein (1941) in his review of the methods of selenium estimation quoted some experiments of his in which he showed that rather than stabilizing the colour of the codein-selenium complex, iron actually interfered. Ferrous sulphate present as 0·5 mg Fe prevented the normal development of the colour of 50 mcg of selenium. He stated further that codein reacts with trivalent iron to produce a blue colour, and that the intensity of colour is roughly proportional to the amount of iron present. He found that 0·46 mg of ferric iron would contribute an apparent selenium content of approximately 20 mcg, while 1·84 mg of the metal simulates the colour of approximately 40 mcg of selenium. He recommended that if the reaction was to be used for the determination of selenium in products containing iron, rigid precautions should be taken to keep the iron content of the samples and standards alike and that standard curves be constructed for all dissimilar products.

In view of the discrepancies in the literature regarding the effect of iron and since the statement made in regard to the other elements occurring in tissues of plants or animals are very vague, i was considered necessary to investigate this aspect much more fully than had been done by previous workers. If iron did interfere this would constitute a very serious objection to the method, since haemosiderosis is seen in many animal disease states and notably in geeldikkop and enzootic icterus. The elements tested in this regard and the amounts in which each was added to the contents of the test flasks are listed in the table below. Iron is not included in this table but is dealt with separately below.

The elements investigated were tested in groups and where one group was found to give a colour, the respective members were tested individually. All the salts used were of analytical reagent grade and were dried before weighing, where necessary. A convenient amount of each was weighed out and the various batches dissolved in dilute HCl and made up to 100 ml with distilled water. Ten ml of each batch or individual element was then taken, this amount containing the amount in micrograms of each element indicated in the table, and put through the entire procedure indicated earlier under " The Method ", including the addition of 0·4 gm mercuric oxide.

The only elements which were found to interfere are molybdenum and vanadium, each yielding a blue complex with codein. It was found that 110 mcg of vanadium simulate the colour of 25·5 mcg of selenium, while 240 mcg of molybdenum produce a colour equivalent to 56 mcg of selenium. This is not a serious objection in the case of vanadium since this element is not found to any extent in either plant or animal material. Molybdenum may attain such concentrations in plant or animal tissue in areas where the soil is rich in available molybdenum. However, 1 mcg of either element contributes a colour equivalent to only 0·23 mcg of selenium.

Element	mcg present in test	Element	mcg present in test	Element	mcg present in test
Na	440	W	204	Ce	200
K	217	Hg(2+)	370×10^3	Os	210
Li	294	Sb	203	Pd(2+)	214
Ca	200	As	250	Pt	200
Mg	203	Bi	209	U	238
Sr	200	V	110	B	216
Ba	229	Te	213	Si	281
Cd	335	Al	270	S	864
Cu(2+)	212	Ti(4+)	240	P	310
Co(2+)	295	Zn	218	Cl	284
Mn(2+)	275	Ag	216	I	706
Mo	240	Sn(2+)	237	Br	800
Cr	260	Pb	207	F	173
Ni(2+)	196	Au	197		

It is possible that the added mercuric sulphate suppresses any interfering effect other elements may have, and under the conditions of the method many of these would either escape in volatile form or precipitate out as insoluble sulphates.

Collectively the elements present in animal or plant tissues may alter slightly or enhance the selenium-codein complex colour. This is seen in the case of the blanks where varying shades of light yellow may be encountered. This factor may in part explain the slightly high recovery figures cited earlier (see also below, the effects of iron on the colour).

The effects or iron on the selenium-codein complex were studied in some detail. Amounts of iron ranging from 0 to 400 mcg were added to digests containing no tissue or selenium and these were then treated as detailed under " Method etc." earlier. The following table illustrates the results:—

Micrograms Fe	Colour produced	Selenium equivalent
0	Faint yellow	0
25	Light yellow	0
50	Light yellow	0
75	Yellow	0
150	Yellow	0
200	Olive to greenish blue	0
400	Blue	42 mcg

In the following experiment iron was added to standard digests containing both selenium and one gram of liver, the iron content of which was 266 mcg/gm. The results are presented in the table below:—

Sample	Added Iron (mcg)	Total Iron Present (mcg)	Added Se (mcg)	Recovered Se (mcg)
Blank	0	266	0	0
Blank	0	266	0	0
1st Standard	0	266	15	20
2nd Standard	0	266	25	33
3rd Standard	200	466	15	42
4th Standard	200	466	25	53
5th Standard	200	466	0	14
6th Standard	400	666	0	25

If any samples developed the olive to greenish blue colours indicated in the first table above after addition of codein, these samples were redigested and if the colour persisted, the entire digest was rejected. It will be seen from the table immediately above that total amounts of iron in the range 200 to 300 mcg give a value for selenium slightly higher than it should be, but amounts of iron in excess of 400 mcg interfere markedly.

This problem was studied further and simultaneous iron and selenium determinations were performed on a number of specimens taken from normal healthy Onderstepoort sheep, geeldikkop cases or other miscellaneous specimens. The results of these determinations are presented in the table which follows. It is apparent from these data that there is no correlation between the amounts of iron and selenium found in these tissues. The material used covered a very wide range of iron content.

Nature of case	Organ	mcg/gm Fe	mcg/gm Se
Normal Onderstepoort Sheep 1	Liver	332	0
" " " 2	Liver	340	0
" " " 3	Liver	346	0
" " " 4	Liver	266	0
" " " 5	Liver	148	3·0
" " " 5	Kidney	190	0
Normal Karoo Sheep A	Liver	92	0
" " A	Kidney	144	11·5
" " B	Liver	120	0
" " B	Kidney	210	1·5
" " C	Liver	110	0
" " C	Kidney	60	0
" " D	Liver	180	4·0
" " D	Kidney	160	0
" " E	Liver	496	5·5
" " E	Kidney	512	0
" " F	Liver	368	0
" " F	Kidney	232	0
" " G	Liver	560	0
" " G	Kidney	160	0
Geeldikkop case 1	Liver	106	3·0
" " 1	Kidney	90	11·5
" " 2	Liver	74	11·0
" " 2	Kidney	38	0
" " 3	Liver	124	6·0
" " 3	Kidney	344	25·5
" " 4	Liver	188	3·0
" " 4	Kidney	296	14·0
Normal Angora foetus	Liver	378	17·0
Aborted " "	Liver	160	5·5
" " "	Liver	36	14·0

From all these results it is concluded that when iron is present in amounts of less than 350 mcg any effect which it might have on the colour of the selenium-codein complex is to a large extent suppressed by the other inorganic salts present in the digest. Above 400 mcg iron may or may not contribute appreciably to the colour depending upon the relative amounts of other suppressing ions present. In the table above it is seen that two samples gave iron values of over 500 mcg. Neither of these developed any colour with the codein reagent.

In view of the fact that iron might interfere when present in large amounts, it is advisable to make up the blanks and standards for every batch of tissues with one gram of the same sort of tissue containing as nearly as possible the same amount of iron as would be expected to occur in the materials under examination, and further to control the selenium figures obtained, when and if possible by simultaneous iron determinations on the same material. A relatively simple and rapid qualitative test for excessive iron in animal tissues is the microscopic examination of frozen tissue sections stained for free iron or iron-containing pigments. If these appear to be present in excess an iron determination on these tissues should be done.

Although this method has certain inherent disagreeable features, not the least of which is the use of concentrated sulphuric acid throughout the entire procedure, and despite the fact that it lacks strict reproducibility, it has much to recommend it. It is sufficiently sensitive to use as a screening test for excessive amounts of selenium and for routine chemical pathological work; only one gram of tissue is required; it is quick and few manipulations are required—a trained assistant can comfortably work through a batch of twelve samples during a day and there is little danger of loss of selenium if the various conditions laid down here are strictly adhered to.

(*e*) *Preliminary treatment of the material to be tested*: Since selenosis is often accompanied by considerable fatty changes in the liver, and as much fat renders digestion difficult, Gortner & Lewis (1939) proposed preliminary extraction of the material with chloroform. There is no loss of selenium during this defatting process since the fat is selenium-free, most of the element being in protein combination. The author found this to be unnecessary in the case of the tissues he was working with since both undefatted and defatted specimens digested with equal facility, giving identical figures for selenium.

The Determination of Selenium in Plants.—Only the vegetative parts of the plants to be tested were used in this work. Since selenium is incorporated mainly into proteins and certain enzymes, in the place of sulphur, it is likely to be found in the highest concentrations in the leaves or seeds of these plants, if it is present in significant amounts.

The procedure is essentially the same as that for animal tissues. There are, however, certain difficulties attendent on the digestion of plant material which must be overcome and which necessitated a few minor modifications to the method. In the first instance considerable and troublesome foaming is invariably encountered in the initial stages of the digestion. To partially overcome this the plant material should be in the form of fairly coarse particles or pieces and not finely ground. It should be allowed to stand overnight in the sulphuric acid, with the addition of the mercuric oxide and only 5 ml of water at this stage. The remaining 5 ml of water and the hydrogen peroxide are added just prior to digestion. The first stage of the digestion must be carried out very carefully and the heating interrupted frequently to prevent excessive foaming. Once the contents of the digestion flask have become a smooth homogeneous brownish black slurry, the flask is allowed to cool and a further 10 ml of sulphuric acid instead of the 5 ml used for animal tissues is added at this stage together with 10 ml of water. Digestion is then commenced cautiously once more.

Digestion of plant material takes considerably longer than does that of animal tissues. It is essential not to try and hurry the procedure by too much heating. Frequent interruptions of heating with the addition of small amounts of water and hydrogen peroxide are required.

The entire procedure takes about four hours before digestion is complete. The volume of digest is then again reduced to about 10 ml, and then after sealing the flask with lead foil, the flask is allowed to stand overnight to allow for complete precipitation of the mercuric sulphate. This takes very much longer in the case of plant digests and if this precaution is not observed, very troublesome turbidity may be encountered during colour development.

The procedure is further identical to that for animal tissues.

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APPENDIX 11

THE ISOLATION OF BSP CONJUGATES FROM SHEEP BILE

1. *Materials and methods*: Six fully grown Merino wethers were provided with common bile duct cannulae to the exterior, as described earlier in this work. These sheep were maintained and fed as described earlier (Brown, 1967).

Pure disodium-bromsulfalein (Fluka AG; puriss. p.a. grade) was used for this work. Doses of 0·5 gm of the compound dissolved in 50 ml of physiological saline were given slowly intravenously and bile samples were collected hourly as described earlier and elsewhere (Brown, 1967) until the biliary excretion of the dye declined to negligible levels. These sheep received intravenous injections of 0·5 gm of the dye, on a number of consecutive days, until sufficient stocks of the various conjugates being excreted had been prepared for peliminary chemical studies.

2. *Isolation of the water soluble conjugates of BSP*: To 2 ml of bile from each hourly sample collected, 6 ml of acetone was added, the precipitate of proteins and pigments removed by centrifugation and the aqueous acetone phase taken to dryness in a rotary evaporator at $< 30°C$ in the dark. The crude pigment residue, which contained most of the BSP present in the original aliquot was stored in the deep-freeze at $-10°$ C until it could be handled further. The remainder of the bile collected during each hourly period was bulked; acetone added in the ratio of 3 acetone: 1 bile, the precipitate removed by filtration and the aqueous-acetone extract was taken to dryness as described above. The residue was washed with three successive 100 ml portions of chloroform. The first wash removed much green and yellow bile pigment, the second removed only yellow pigment and the final wash the last traces of yellow pigment. The residue was then washed with 100 ml of ice-cold n-heptane, 7: n-butanol, 3. After this was removed the residue was stored in the deep-freeze at -10 C.

3. *Paper chromatography of the isolated conjugates*: This was carried out by the procedure of Grodsky, Carbone & Fanska (1959), using an ascending chromatographic run of 15 hours, with tert-butanol, 30: water, 17·3 v/v as the developing solvent. After drying the chromatograms, the purple colour of the conjugate spots was brought out by spraying with 0·05N NaOH solution.

In 1963 the author reported that three major-conjugates of BSP appeared in sheep bile after giving the loading doses of the dye mentioned above. These conjugates were designated BSP's A, B and C respectively (Brown, 1963). Traces of free BSP, probably formed as an artefact during the isolation were found to be present as well. Typical paper chromatograms made from the 2 ml bile aliquots mentioned above are shown in Fig. A. The fractions on these chromatograms designated " conc. bile extract " represent the acetone extract of bile before evaporation to dryness.

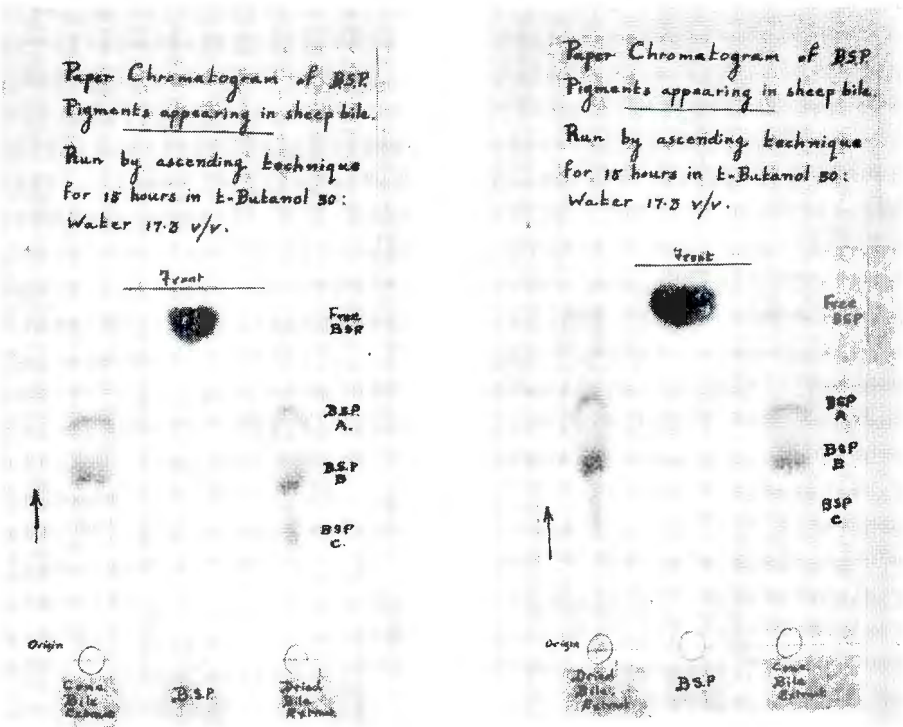

FIG. A.—Paper chromatograms of BSP conjugates isolated from bile after injection of the dye.

The dry residues from this procedure are represented by the fractions named " dried bile extracts ". The large spots appearing above the designation " BSP " were obtained from samples of the material injected into the animals concerned which were chromatographed as reference standards concurrently with samples of the bile extracts.

The dried and washed extracts from the bulked bile samples yielded identical chromatograms.

It is obvious from these chromatograms that conjugate B is present in bile in the largest amounts. Conjugate A is present in appreciable quantities and C is present in low concentration.

4. *Thin layer chromatography*: Samples of each conjugate were prepared for subsequent chemical work by chromatography of the bulk material on a large number of thin layers made from a mixture of 35 gm Kieselgel G (Merck's " nach Stahl " containing 13 per cent of $CaSO_4$ and particle size 10 to 40μ) and 55 ml of water mixed immediately before use. The chromatograms were developed with a mixture of tert-butanol, 30: concentrated NH_4OH, 1·0: water 16·3. The same three conjugates mentioned above were present together with traces of two or three other slower running compounds reacting purple in the alkaline developing medium. After the chromatograms had become almost dry, the coloured spots were scooped off with small stainless steel spatulae into suitably labelled containers and stored in the dry state at −10°C until required for later work, when elution of the conjugates from the Kieselgel residues was achieved with minimal amounts of acetone 3: water 1 made alkaline with a drop or two of concentrated ammonia in order to visualise the completeness of elution.

5. *Preliminary identification of the conjugates*: The pure pigments eluted from thin layer chromatograms as described above were dissolved in 0·1 N NH_4OH and the spectral absorption of the solutions was determined in the ultra-violet and visible regions of the spectrum. Pure BSP in

0·1 N ammoniacal solution absorbs very strongly at 577·5 mμ and shows small secondary peaks at 366·5, 308 and 277 mμ. All three major conjugates were found to absorb strongly in alkaline solution at 577 mμ but showed none of the secondary peaks of BSP at concentrations of 0·02 per cent. Conjugate B absorbs at 214 mμ as well as at the major peak.

Further work on the spectral properties of the conjugates and their chemical nature will form the subject of a separate paper.

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