RATE OF HAEMOGLOBIN SYNTHESIS AFTER BLOOD LOSS
IN THE SHEEP. INFLUENCE OF DIETARY PROTEIN.

By

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SUMMARY

Experiments have been carried out to determine the capacity of the sheep to synthesize haemoglobin and the effect of dietary protein intake on the rate of haemoglobin synthesis.

An effect of intravenous adrenalin on jugular haemoglobin concentration was measured, and a good agreement was found between the Evans-blue method of determining blood volume and the ^{51}Cr-tagged red cells method of estimating blood volume.

Splenectomized sheep responded to single massive bleeding and prolonged daily bleeding with an increase in the rate of haemoglobin synthesis and in the retention of dietary nitrogen.

In intact sheep a moderate degree of experimental anaemia (6 gm Hb%) maintained by daily bleeding provoked a marked increase in the rate of haemoglobin synthesis. The maximum rate of haemoglobin synthesis achieved was about 3½x the normal rate which was equivalent to the production of 0.25 gm Hb/day/kg body weight. Haemoglobin concentration was maintained at 6 gm Hb% partly at the expense of the reserve haemoglobin in the spleen. Haemodilution was observed during bleeding followed by a haemoconcentration during the recovery period.

Comparison of the rate of haemoglobin synthesis on two levels of protein intake (65 gm and 145 gm crude protein per sheep per day) suggested that the rate of haemoglobin synthesis was independent of dietary intake within the limits examined, but the level of the circulating mass of haemoglobin after recovery from

bleeding was probably influenced by the protein intake.

The implications of the results from these experiments are discussed in relation to the factors which influence the rate of haemoglobin synthesis.

Preface

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University, and to the best of the author's knowledge contains no published or written material from another person, except where due reference has been made in the text.

I. INTRODUCTION

A. Factors Influencing the Rate of Haemoglobin Synthesis

Haemoglobin synthesis is intimately associated with erythropoiesis, the process whereby red cells are produced by the bone marrow and released into the circulation. Any discussion therefore of the factors which influence the rate of haemoglobin synthesis, must of necessity include those factors which determine the production of erythrocytes from precursors in the bone marrow.

The presence of haemoglobin in the erythrocyte is a characteristic of vertebrate blood, and the functional significance of the blood pigment in relation to the transportation of oxygen to the tissues was investigated a century ago by Hoppe-Seyler (1864) who demonstrated the spectral variations which occur when haemoglobin undergoes oxidation and reduction.

During the latter half of the nineteenth century and the first quarter of the twentieth century there were only spasmodic observations on the factors influencing the formation of the blood cells. Most workers were concerned with a descriptive and morphologic approach. Then in 1925 Whipple and Robscheit-Robbins made an important contribution to the study of hematopoiesis by initiating an investigation into the dietary factors which influence blood protein formation in the dog, and the dynamic function of the blood proteins in protein metabolism.

1. Nutritional Requirements for Red Cell Formation

Cartwright (1947) and Dinning (1962) in their reviews

on the dietary factors concerned in erythropoiesis have indicated the
particular importance of amino acids, protein, certain essential
vitamins and minerals. Cartwright has mentioned the difficulty
of separating those factors which are necessary for the formation
of the red cell from those factors which are directly concerned
in the formation of haemoglobin, and Aschkenasy (1960) has pointed
out that the various nutritional factors are metabolically inter-
related. It is difficult therefore to define with precision the
degree of responsibility which should be attributed to a deficiency
of one or another dietary factors when blood cell anomalies occur.

a) _Protein and amino acids_.- Protein forms a major part
of the blood and blood forming tissues, and haemoglobin is
quantitatively an important protein in vertebrates. Compared with
estimates of the amounts of many other specific proteins in man,
haemoglobin is present in the body in a relatively large amount,
and accounts for about 1% of the body weight (Pauling, 1955).

Whipple and Robscheit-Robbins (1925) established standard
techniques for assaying the influence of dietary protein and other
food factors on blood regeneration in the dog. They used standard
anaemic dogs which were bled until their haemoglobin concentration
was 40-50 per cent normal and then maintained at that level by
daily or 2-3 weekly bleedings, to ensure a constant stimulus to
red cell formation. A maintenance diet consisting of a standard
bread was given to the dogs which were able to synthesize 2-3 gm
haemoglobin a week above the maintenance level. Additional food,

extracts, or specific food factors, added to, or given in place of
the basal diet could then be assayed for their ability to stimulate
the synthesis of haemoglobin and plasma protein. The amount of
haemoglobin and plasma protein removed by daily bleeding to
maintain the dogs at a standard level of anaemia would then indicate
the rate of blood protein synthesis. The recovery from a single
massive bleeding and the effect of dietary intake on the recovery,
was also observed.

The importance of an adequate supply of dietary protein
for haemoglobin synthesis was reported by Daft, Robscheit-Robbins
and Whipple (1933), who showed that acute protein starvation in
the anaemic dog stimulated a breakdown of tissue protein and a
conservation of dietary nitrogen, in an attempt to maintain a
limited haemoglobin synthesis. This work was continued by Hahn
and Whipple (1939) who studied the increased haemoglobin synthesis
in standard anaemic dogs when a limited protein intake was
supplemented by additional food protein, and calculated that each
additional 7-8 gm food protein would contribute to the synthesis
of 1 gm haemoglobin.

An interesting development to the understanding of the
role of protein in blood formation was reported by Alt (1938) who
found that a deficiency of an essential amino acid in the diet
of young rats suppressed growth and reduced the rate of red cell
formation. If the young rats were bled however, an increased
erythropoietic response indicated that the reduced rate of red cell

formation was not due specifically to a nutritional deficiency.
From these results Alt concluded that growth and bleeding produce
a similar stimulus to erythropoiesis. Alt's work also suggests
that an increase in erythropoiesis in response to acute bleeding
may be independent of the diet.

Further effects of protein, amino acids, haemoglobin and
serum digests on haemoglobin and plasma protein production were
reported by Robscheit-Robbins, Miller and Whipple (1943) using the
standard anaemic dog technique. They found that when the dietary
supply of protein was adequate haemoglobin and plasma protein were
synthesized simultaneously. In the event of protein depletion
however, haemoglobin appears to be synthesized preferentially at the
expense of the tissue protein. From these observations Whipple
(1948, 1956) developed the concept of a dynamic protein pool with
plasma protein acting as an intermediary between blood and tissue
proteins, and suggested that the synthesis of the various proteins
is determined in accordance with a priority system.

An example of the dynamic nature of the body proteins
can be seen in the experiments of Yuile et al. (1959). Yuile
et al. used plasma proteins labelled with ^{14}C-lysine to demonstrate
the shift which occurred after plasmapheresis, in the ratio of
protein in the interstitial fluid to protein in the plasma. A
change from the normal ratio of 1:1 to greater than 2:1 was measured
in the dog, and this transfer of protein favoured the maintenance
of the plasma protein concentration. When the depletion of protein
from the total exchangeable pool exceeded 50 per cent, the removal

of additional plasma protein was associated with a loss in body weight.

Bethard et al. (1958) gained support for the concept of a priority system for protein synthesis from their studies on the aberrations in protein metabolism and associated anaemia in neoplastic diseases. The authors have suggested that the rapid decline in erythropoesis found in rats following protein deprivation, is an adjustment of the organism to utilize protein in accordance with a priority system. The reduction in erythropoiesis following protein deprivation however, may be related to a decreased demand for oxygen (Jacobson et al., 1957); erythropoiesis can be stimulated in a protein depleted rat by bleeding or cobalt administration. Bethard et al. (1958) also suggest that rapidly growing tumors in debilitated patients can preferentially utilize protein, which results in a marked reduction in erythropoiesis.

Dinning (1962) has summarized the effects of dietary protein on blood cell formation. He suggests that the effects of a deficiency may be complex and lead not only to a deficiency of building materials but also produce an hormonal imbalance, and a depletion of other hematopoietic factors. In conclusion he proposes that the requirement for protein is basically a requirement for the "essential" amino acids.

The requirement for the "essential" amino acids in the ruminant however, is modified by the intervention of the ruminal micro-organism. A considerable proportion of dietary protein is

converted into microbial protein before leaving the rumen, and microbial protein provides a significant proportion of the digestible protein which is available to the ruminant. The solubility of the dietary protein is one important factor in determining the extent of the conversion of food protein into microbial protein (McDonald, 1954, and McDonald and Hall, 1957). The microorganisms in the rumen can supplement the amino acid composition of the food protein, and it has been shown by Loosli et al. (1949) and Duncan et al. (1953) that the amino acids which are known to be dietary essentials for the rat can be synthesized from urea in the rumen. Urea as a nitrogen source does not completely satisfy the requirements of the rumen microflora, although satisfactory growth in lambs has been noted by Starks et al. (1954) when urea constituted 90 per cent of the nitrogen in the ration. However, a natural ration of hay and grain has been shown by Duncan et al. (1953) to be superior to urea as a source of nitrogen in the calf. It appears therefore, that the basic advantage of the rumen is related to the synthetic activities of the microorganisms which can synthesize the essential amino acids which are not represented in the diet.

b) Vitamins.- Although vitamins are required for reactions which are qualitatively similar to other tissues, experimental evidence on the specific influence of deficiencies on red cell formation suggests that certain reactions are quantitatively different. In particular vitamin B_{12}, folic acid, vitamin C and

vitamin B_6 appear to have a significant influence on red cell
formation (Dinning, 1962).

It is recognized now that the beneficial effects of some
of Whipple's diets (Whipple and Robscheit-Robbins, 1925) on blood
protein formation were due to the presence of certain essential
vitamins. For example, the ability of liver to stimulate
erythropoiesis in anaemic dogs was almost certainly related to the
vitamin B_{12} content of the liver. Rachmilewitz et al. (1960) have
shown that B_{12} is stored in the liver, kidney and intestines, and
that bleeding stimulates mobilization and transfer of the B_{12} to
the bone marrow.

The "extrinsic factor" which Castle (1929) postulated
formed a substance effective in the treatment of pernicious anaemia
when incubated with an "intrinsic factor" in normal gastric juice,
was subsequently identified with vitamin B_{12}, the growth factor
described by Shorb (1948), and was isolated independently by
Rickes et al. (1948), and Smith et al. (1948). Recent research on
the isolation of "intrinsic factor" would support the view that
"intrinsic factor" is probably a protein (Smith, 1960). In
particular the isolation of a fraction from pig pylorus with
"intrinsic factor" activity has been reported by Holdsworth (1961);
this fraction was found to be a high molecular weight glycoprotein
whose amino acid composition and physical chemical properties were
determined. Chosy and Schilling (1963) have also attempted to
isolate "intrinsic factor" from human gastric juice with a procedure

used for the purification of protein.

Bishop et al. (1955) have shown that vitamin B_{12} must be combined with intrinsic factor to be absorbed, and the physiological mode of vitamin B_{12} absorption mediated by intrinsic factor has been studied, for example, by Glass, Boyd and Stephenson (1954), Doscherholmen et al. (1957, 1959), and Holdsworth and Coates (1960, 1961). Glass, Boyd and Stephenson postulated the existence of an "intramural intestinal-B_{12} acceptor" and Doscherholmen et al. suggests that slow absorption under certain conditions may be due to a temporary storage of vitamin B_{12} in the intestinal wall. These results are not incompatible with the more recent studies by Holdsworth and Coates who have shown that the most active site of vitamin B_{12} absorption in the baby pig and rat is just distal to the small intestine.

Of interest, although in need of confirmation, is the work of Wagle, Mehta and Connor Johnson (1958) who have shown that vitamin B_{12} is associated with the 'pH5 enzymes' fraction from the differential centrifugation of liver cells. The 'pH5 enzyme' fraction is responsible for catalysing the activation of amino acids with ATP, and the incorporation of the amino acids into microsomal protein, which suggests that vitamin B_{12} may have a direct influence on blood protein synthesis. This work was not confirmed by Fraser and Holdsworth (1959) who studied the uptake of vitamin B_{12} in the deficient chick liver and found that only about 6 per cent of the vitamin taken up by the liver was contained in the pH5 enzymes. The

majority of the vitamin was found in the cell supernatant fraction. Fraser and Holdsworth also compared the pH5 fraction from deficient liver with the pH5 fraction from normal liver and were unable to find a direct influence of the vitamin B_{12} content of the fractions in the activation of amino acids or in the rate of incorporation into microsomal protein. In addition, when vitamin B_{12} was added to cell free systems from chick liver and rat liver it did not effect the incorporation of amino acids into protein (Fraser and Holdsworth, 1959; Arnstein and Simkin, 1959).

It has been proposed by Block (1962) that one of the primary causes of megaloblastic anaemia, which implicates vitamin B_{12}, folic acid and vitamin C, is an interference with nucleic acid metabolism. The synthesis of nucleic acid involves two stages, first the synthesis of purines and purimidines from CO_2, formate and glycine and secondly the anabolism of purines and purimidines to nucleic acid. Block suggests that folic acid and vitamin C (the latter acting to convert folic to folinic acid) are catalysts of the first stage, and vitamin B_{12} catalyses the second stage, which implies that megaloblastic anaemia can be caused by a nutritional deficiency of vitamin B_{12}, folic acid or vitamin C. In the case of vitamin B_{12} a decreased absorption of the vitamin from a lack of "intrinsic factor" secreted by the gastric mucosa may also be involved.

Dinning (1962) considers that a primary role of vitamin B_6 in haemoglobin formation appears to be related to the requirement for pyridoxal phosphate in the biosynthesis of haeme. Vitamin B_6

probably acts as a co-decarboxylase in the decarboxylase step in the synthesis of haeme from glycine and "active" succinate, in the nucleated red blood cells.

Vitamin deficiency in the ruminant however is modified by the synthetic activities of the ruminal micro-organisms. It was observed [Theiler et al. (1915) quoted by Kon and Porter (1954)] that ruminants could be maintained for long periods on a diet low in certain vitamins, and it was suggested that the extensive bacterial flora in the rumen might synthesize the vitamins. It was pointed out however by Savage and McKay (1942), that the young ruminant might require an exogenous supply of the vitamin B complex, until the rumen function develops when the animals start eating roughage. This was confirmed by Johnson et al. (1950) and Draper and Johnson (1952) who fed young calves on a synthetic milk diet deficient in various B complex factors.

The effect of the composition of the diet on the synthesis of thiamine, riboflavin, nicotinic acid, pantothenic acid, pyridoxine, biotin, folic acid and vitamin B_{12}, in the fistulated steer, has been reported by Kon and Porter (1953). A substantial synthesis of all the vitamins measured occurred on the low vitamin diets, which were composed of straw and a nitrogen source such as casein. By comparison, except for vitamin B_{12} and related compounds, the vitamin concentration in the rumen did not change appreciably on a normal diet of hay and grain.

The ruminant has the advantage therefore, of synthesizing vitamins of the B complex in the rumen before the food enters the

digestive tract, so that utilization is more favourable than in
other animals where intestinal synthesis of vitamins occurs.

c) Minerals.- Three minerals, iron, copper and cobalt are
outstanding for their fundamental function in erythropoiesis.

(1) Iron. Bethard (1962) states that as early as 1680
Thomas Sydenham prescribed iron filings steeped in wine for anaemia,
and in 1831 Pierre Blaud advocated 1 gm ferrous sulphate daily for
anaemia: a prescription which it would be difficult to improve
on today, for iron deficiency anaemia. Coleman et al. (1953) have
also discussed the effectiveness in man of ferrous sulphate on
haemoglobin synthesis, when added as an iron supplement to the diet
of blood donors.

In 1925 Whipple and Robscheit-Robbins were beginning to
differentiate between the limiting effects of iron and protein on
the rate of haemoglobin synthesis, and subsequently Hahn and
Whipple (1939) showed that when the reserve stores of iron in the
body are depleted, the uptake of iron is an absolute limiting
factor on haemoglobin synthesis. The availability of radioactive
iron facilitated studies on the utilization of iron, and radioactive
iron was used by Hahn et al. (1939) to demonstrate an increased
absorption of iron from the small intestine in the dog after the
stores of iron were depleted by bleeding. A three fold increase
in the absorption of ferrous iron in the duodenum of man and of the
rat after bleeding, has been found by Bothwell et al. (1958), who
have also shown that the status of the iron stores and the rate of
erythropoiesis influence iron absorption.

The effect of protein deprivation in the young rat on the distribution of injected ^{59}Fe has shown that there is a reduction in ^{59}Fe incorporated into the red cells and an increased accumulation of ^{59}Fe in the liver (Bethard et al., 1952; Aschkenasy, 1962) which parallels the decreased growth rate and suppression of erythropoiesis.

The normal distribution of iron in man has been discussed by Bethard (1962) and in the ruminant by Underwood and Morgan (1963). In man 3 gm iron is associated with haemoglobin and about 1.5 gm iron is stored as ferritin and haemosiderin. A further 0.5 gm is present in transferrin, myoglobin, cytochrome, and iron - dependent enzymes such as catalase and succinic dehydrogenase. In the ruminant Underwood and Morgan (1963) have demonstrated that the distribution of iron is similar to that in man.

(ii) Copper and cobalt. The influence of copper and cobalt deficiencies in the sheep has been very thoroughly investigated, (Marston et al. 1938; McDonald, 1942; Bennetts and Beck, 1942). In relation to red cell production Marston et al. (1938) have observed that in sheep grazing certain coastal areas in South Australia a severe anaemia was present, which only responded when both copper and cobalt supplements were provided. The occurrence of an uncomplicated cobalt deficiency in sheep in South Australia has been noted however by McDonald (1942); this condition was associated with a variable degree of anaemia and responded to cobalt supplement alone. Bennetts and Beck (1942) have

investigated a condition called "enzootic ataxia" in Western Australia, associated with a severe anaemia in sheep grazing copper deficient pastures. They found that some haemoglobin synthesis was still possible provided the sheep were not called upon to produce and rear progeny. The anaemia responded to a supplement and a return to a normal blood picture occurred within four weeks.

The function of copper in erythropoiesis has been studied by Cartwright (1947) and Wintrobe, Cartwright and Gubler (1953). They have shown that when iron is fed to animals deficient in iron and copper the total iron content of the liver and spleen increases in proportion to the amount of iron fed. This indicates that iron is absorbed and stored normally in the absence of copper. If copper is then fed to the animals, haemoglobin synthesis is stimulated with a subsequent reduction of the iron stores to a level found in severely anaemic animals. The authors conclude that copper is essential for the mobilisation of iron from the tissues, and for its conversion into haemoglobin.

Cartwright (1947) has also pointed out that copper is essential for the cytochrome oxidase activity of the liver, kidney and blood, which emphasizes the role of copper in erythrogenesis and in basic metabolic processes.

The function of cobalt in erythropoiesis is very important. A deficiency results in anaemia, whilst the administration of small amounts produces erythrocytosis, and the administration of large

amounts depresses erythropoiesis (Goldwasser et al., 1958). In the
sheep the anaemia associated with cobalt deficiency can be cured
when the sheep are given a dose of 300 mg vitamin B_{12} per sheep
per week (Marston and Lee, 1952; Anderson and Andrews, 1952).
Vitamin B_{12} was found to contain cobalt when it was isolated
independently by Rickes et al. (1948) and Smith et al. (1948), and
Hale et al. (1950) showed that vitamin B_{12} is synthesised in greater
amounts in the rumen of normal than of cobalt deficient sheep. Ford
and Porter (1952) have isolated other cobalt containing compounds
related to vitamin B_{12} from rumen contents and consider that these
compounds may be necessary for normal microbial function. In
general however, animal tissues contain mainly vitamin B_{12}, whilst
other natural materials and bacteria contain a preponderance of the
cobalt containing compounds related to vitamin B_{12} (Ford et al.,
1953). It would appear therefore, that although cobalt may fulfil
a dual function in the ruminant, its main function in relation
to erythropoiesis is as a constituent of vitamin B_{12}.

2. Fundamental Stimulus and Control of Erythropoiesis

The last ten years have seen the development of new ideas
on the fundamental stimulus for erythropoiesis with emphasis on
a humoral control. Grant and Root (1952) in their comprehensive
review on the primary stimulus for erythropoiesis, regard Miescher
(1893) as the first person to propose a mechanism of control.
Miescher was probably influenced by the observations of Bert (1882)
and others, on the polycythemia which occurs in people living at

high altitudes. He proposed that anoxia of the bone marrow was the primary stimulus for erythropoiesis.

Miescher's hypothesis was widely accepted until Grant (1948) tested the hypothesis experimentally, and was unable to demonstrate any difference in the oxygen saturation in the bone marrow of anaemic and control dogs. Evelev and Hughes (1959) have also shown, that in a suspension of rabbit bone marrow, cell multiplication increased with an increased oxygen saturation.

Interest in the humoral control of erythropoiesis developed from the observations of Carnot and Deflandre (1906) who found that after injecting the serum from anaemic rabbits into normal rabbits, there was a significant increase in the concentration of red blood cells. From these observations they postulated the existence of an erythropoietic stimulating factor in the plasma, which they named hemopoietine. Very little interest was shown in Carnot and Deflandre's observations until Krumdieck (1943) and Bonsdorff and Jalavisto (1949) presented impressive experimental evidence in support of a humoral factor in the plasma of anaemic rabbits. Bonsdorff and Jalavisto named their factor erythropoietin.

The next few years marked the accumulation of a large amount of experimental data in support of a erythropoietic stimulating factor (ESF) much of which has been summarized by Jacobson et al. (1959, 1960), Gordon (1959) and Borsook (1959). Jacobson and co-workers have also attempted to standardize the assay procedures for erythropoietin, and establish a unit of erythropoietin in terms

of the response in the starved rat to 5 μ mole of cobaltous chloride, as measured by the appearance of ^{59}Fe in the circulating red cells after incorporation of ^{59}Fe in the bone marrow.

Jacobson (1957) has also suggested that the body's oxygen supply and demands could determine erythrocyte production. He found lowered ESF levels in the plasma following hypophysectomy, and also in cases of acute starvation, where the overall metabolic oxygen requirement was reduced, and increased levels of ESF when there was a demand for oxygen following exposure of an animal to lowered barometric pressures, or after blood loss.

The possibility that the products of haemolysed blood may be a stimulus to red cell production and provide another mechanism of erythropoietic control has been examined by Brown et al. (1963) who suggests that the control of erythropoiesis may involve porphyrin-like compounds in a feedback mechanism.

Goldwasser and White (1959, 1962) and White et al. (1960) have used ion exchange procedures to purify erythropoietin, and a highly active fraction has been obtained containing the hormone, which has the physico-chemical properties of a small molecular weight glycoprotein.

Linman and Bethell (1957), Linman (1962) and Oley and Delor (1955) have presented evidence for a second humoral factor controlling erythropoiesis, which is soluble in fat solvents. Other workers however have been unable to verify their findings and the majority of workers now favour a single entity.

Meineke and Crafts (1957) and Fisher (1958) have reported
a specific effect of corticoids and growth hormone on erythropoiesis.
This has been supported by Weinstein (1962), who suggests that
certain hormones may have a direct effect on erythropoiesis, although
in general the endocrine system influences erythropoiesis by its
control of basic metabolic processes.

The acceptance of a humoral factor controlling erythro-
poiesis has stimulated a search for the site of production and an
investigation of the mode of action of the hormone. There is strong,
but not conclusive evidence, that the hormone is produced or
activated by the kidney. Jacobson et al. (1957), Naets (1960) and
Warter et al. (1962) have demonstrated some activity in kidney
extracts and in saline washes from kidney infusions. Prentice
and Mirand (1957) and Gordon (1959) provide evidence that damage
to the liver increases the life span of the circulating ESF, which
suggests that the liver may play a role in the metabolism of
erythropoietin.

Regarding the mode of action of the humoral factor Erslev
(1959) and Erslev and Hughes (1959) have demonstrated a direct
effect of erythropoietin on bone marrow suspensions in vitro, and
on cellular proliferation in the bone marrow in vivo. Erslev
recognizes three main stages in the transformation of stem cells
to mature red cells in the bone marrow. First a differentiation of
multipotential stem cells into pronormoblasts, a non-mitotic process
which is probably controlled by erythropoietin, then the nucleated

red cells go through a number of mitotic divisions, and finally maturation and haemoglobin synthesis takes place.

Alpen and Cranmore (1959), Lajtha and Oliver (1960), and Filmanowicz and Gurney (1961) support Erslev and Hughes (1959) observations, and present experimental evidence which suggests that erythropoietin accelerates the differentiation of stem cells into pronormoblasts. Maturation and multiplication then proceeds at a rate which is determined by the nutritional status and the endocrine balance of the animal, but is independent of the primary stimulus.

Lirman (1962) maintains however, that a second humoral factor can alter the mitotic rate, and has produced evidence that a thermostable factor which is probably a lipid determines the mitotic rate. This discrepancy in the general trend of thought has not been resolved as yet, although most workers do not subscribe to Lirman's hypothesis.

3. The Life Span of the Red Cell

The life span of the erythrocyte in the normal mammal is well documented. It has been shown that there is a relationship between body mass and the potential life span of the red cell, which ranges from 30 days in the mouse to 120 days in the sheep and man (Allison, 1960). This suggests that the average daily turnover of red cells in the sheep is approximately 1% per day or about 10 ml cells per day, which means that 1% of the total circulating red cell volume is removed daily by the reticulo-endothelial system, and replaced by newly formed red cells from the bone marrow.

After extrusion of the nucleus, shortly before entering the circulation, the red cell has lost its capacity to synthesise protein. Butler (1962) has shown however, that the red cell retains the capacity to metabolize glucose to form the high energy phosphate compound ATP, which is probably important for the maintenance of the integrity of the membrane. By the end of four months the human red cell has lost many of its metabolic activities, due to a reduction in certain enzyme systems. The aged cell is then destroyed by the reticulo-endothelial system.

The methods that have been used for the measurement of the life span of the red cell have been described by Berlin et al. (1959) and Blahd (1962). Measurements of the life span of a mixed population of red cells in man have been made using the Ashby differential agglutination method, in which the disappearance of a sample of universal donor cells in a suitable recipient can be followed by taking blood samples at intervals, agglutinating the recipient cells using a suitable antiserum, and counting the remaining donor cells.

A useful check on Ashby's method can be made by tagging a sample of erythrocytes in vitro with ^{51}Cr, reinjecting the cells, and plotting the disappearance curve of the ^{51}Cr, from samples of blood taken at convenient intervals.

The potential life span of the red cells in the sheep has been estimated by labelling the red cells in vivo with ^{59}Fe. The ^{59}Fe was utilised by the bone marrow, incorporated into new

red cells, and the potential life span of the ^{59}Fe in the circulating red cells was found to be 130 days (Tucker, 1959).

4. The Spleen

No evidence of a direct influence of the spleen on the rate of red cell formation in the dog and rabbit could be found by Paton et al. (1902), who showed that after splenectomy dogs and rabbits recovered from haemorrhage as quickly as normal controls. Jacobson et al. (1957) and Lorber (1958) have also been unable to demonstrate an effect of splenectomy on blood regeneration after blood loss.

The spleen is involved in erythropoiesis in man during the first five or six months of intrauterine life. It has also been demonstrated that the spleen retains a latent capacity to form red cells. When normal spleen tissue is injected into animals whose bone marrow has been irradiated, the injected cells pass into the bones and form the nucleus of new bone marrow. Filmanowicz and Gurney (1961) and Kurtides et al. (1963) have shown that in the mature mouse and rat the spleen continues as an erythropoietic organ and forms erythrocytic elements from primitive reticular cells.

Barcroft (1930) regarded the spleen essentially as a reservoir for red cells, which could make a fine adjustment for varying temporary demands. In the sheep, the dynamic storage function of the spleen in relation to fluctuations in the red cell concentration in the jugular vein has been shown by Turner and Hodgetts (1959) to be very important. The spleen in the sheep may contain

1/7th of the total blood volume and 1/4th of the red cell volume.

Crosby (1959) and Craddock (1962) have suggested that the spleen is a specialized part of the vascular system, and acts as a master filter. The authors also consider that the primary function of the spleen is the removal of aged or deformed cells and cell debris from the circulation. After the catabolism of the red cell in the spleen, iron is either stored or transport to the bone marrow, globin is degraded to form part of the pool of available amino acids, and haeme is converted to bilirubin and excreted in the bile.

B. Purpose of the Present Investigation

This investigation was undertaken to determine the capacity of the sheep to synthesize haemoglobin under various experimental conditions.

The classical studies of Whipple and his associates, summarized by Whipple (1956), on the effect of dietary restrictions on haemopoiesis have shown clearly the importance of protein intake in erythropoiesis. Whipple's group were able to establish the capacity of the dog to synthesize haemoglobin on different dietary intakes. The dogs were bled until their haemoglobin concentration was 40-50 per cent normal and then maintained at that level by daily bleeding. The "Standard Anaemic Dogs" (Whipple, 1960), were then used for haemoglobin regeneration studies. Aschkenasy (1959) has also examined the effects of protein deficiency on erythropoiesis and found that a reduction in tissue oxidation within the bone marrow may be involved in the maturation block in the anaemia of protein-depleted rats.

Coleman et al. (1953) have measured the rate of haemoglobin regeneration in man, and shown the importance of an adequate supply of iron. Rates of 2-3x normal were observed following blood loss, and Crosby and Akeroyd (1952) showed that in haemolytic disease, haemoglobin synthesis may increase to seven times the normal rate.

A search of the literature however, has shown that information is lacking concerning the capacity of the sheep to synthesize haemoglobin, or the effects of protein intake on ruminant

erythropoiesis. Richards et al. (1954) have shown that lambs subjected to a heavy infection of the stomach worm (Haemonchus contortus) developed an anaemia which was equivalent to the loss of 200 ml blood per day in uninfected lambs. This suggests that under certain conditions sheep may be subjected to a sustained loss of blood. Experiments were therefore designed to determine the capacity of the sheep to synthesize haemoglobin after blood loss, and the influence of dietary protein on ruminant erythropoiesis. The results from those experiments are reported in this thesis. See also Shutt and McDonald (1965).

II.   MATERIALS AND METHODS

1.   Estimation of Haematocrit Values

Hodgetts (1959) confirmed the earlier findings of Kennedy and Millikan (1938), that the amount of plasma trapped by the red cells of sheep blood is greater than that trapped by the red cells of human blood when both are centrifuged under similar conditions, to determine the relative red cell volume in the blood by the haematocrit method.   Gregersen (1951) and Leeson and Reeve (1951) estimated that the packed cell volume of human blood contained 3-5% trapped plasma, when centrifuged in 2mm x 10cm haematocrit tubes at 3000 r.p.m. equivalent to 1500 g for 30 min when the radius of gyration measured to the base of the haematocrit tube was 15 cm.

Hodgetts (1959) used the ^{131}I-labelled serum method of Leeson and Reeve (1951) for estimating % trapped plasma in the packed cell volume of sheep blood, and showed that under the standard conditions used for human blood the trapped plasma in sheep packed cells may be as high as 14%.   This agreed with the findings of Jennings et al. (1955) who made a comparative study of the amount of plasma trapped in the packed cell volume of the blood from several species.   To reduce the trapped plasma in sheep haematocrits, Hodgetts recommends centrifuging the samples at speeds up to 5000 r.p.m. equivalent to 4800 g for 60 min when the radius of gyration to the base of the tube is 17 cm.

In this investigation samples of well-mixed heparinised or oxalated sheep blood were centrifuged on an M.S.E. centrifuge

at 3000 r.p.m. (20 cm radius of gyration) equivalent to 2000 g
for 40 min, in 3 mm x 100 mm haematocrit tubes. When samples
of blood from the same sheep were centrifuged at 4000 r.p.m.
equivalent to 3600 g for 60 min, the packed cell volumes obtained
from both methods differed by less than 2%. This suggested that
in the method used the trapped plasma would be about 6%, as determined
by extrapolation from a graph (Hodgetts, 1959 p.102), and subsequent
haematocrits were corrected accordingly.

2. Anticoagulants

Heparin (1 mg/10 ml blood) was used for small samples,
and oxalate (120 mg NH_4 oxalate + 80 mg K oxalate/100 mg blood) was
used for large samples.

3. Estimation of Plasma Volume

Gregersen (1951) reviewed the Evans Blue (T-1824) method
of estimating plasma volume, and indicated that extrapolation of
the disappearance curve to the time of injection on a semilog plot
has been the standard practice over the last fifteen years.

The reason for the early decline of the dye curve is
still controversial and may be due to either mixing and/or a rapid
loss of the dye. Standardisation of the experimental procedure
can lead to accurate reproducible results however, as shown by
Constable (1958) who has used a modified method for the estimation
of Evans Blue in the plasma of the guinea pig, rat, pig, rabbit and
human. Macfarlane et al. (1959) has also found Evans Blue suitable

for measuring relative changes in the plasma volume in sheep.
Macfarlane et al. (1959) and Hix et al. (1959) have both used a
single sample taken 5 and 10 min respectively after injection of
the dye for their calculations of the plasma volume of sheep.

For the initial exploratory work on haemoglobin synthesis
it was decided to use a single sample taken 10 min after injection
of the dye for estimating plasma volume but in subsequent work
extrapolation of the disappearance curve on a semilog plot to
the time of injection was used.

Evans Blue (T-1824) is a blue azo dye that has been shown
to be non-toxic at the level used in these experiments, i.e. on the
average, 30 mg injected each week, as 5 ml of an 0.6% solution of
T-1824 in 0.9% NaCl. In aqueous filtered solutions T-1824 has been
shown to be stable for several months. The disappearance rate
in vivo is of the order of 5-8% per hour, and the dye is removed
from the blood partly by diffusion and partly by phagocytosis.
It has also been shown (Kennedy and Millikan, 1938; Falholt, 1958)
that the dye circulates bound to plasma protein.

The method used in this investigation was essentially
the method of Chinard (1951). An initial sample of blood was taken
from a jugular vein of the sheep for use as a blank. Then 30 mg
of T-1824 in 0.9% NaCl was injected quantitatively via a cannula
introduced into a jugular vein. Samples of blood were then taken
from the other jugular vein either 10 min after injection of the
dye or at 5 min intervals up to 30 min after injection. The

samples were spun down at 1500 g for 15 min and if necessary the plasma was respun for a further 15 min to increase clarification, as Chinard (1951) has shown that turbidity from lipemia in the plasma can lead to error, and in extreme cases an acetone extraction might be necessary.

Blanks were prepared from 3 ml plasma + 1 ml distilled water, and a standard, equivalent to 9 μg T-1824/ml, was prepared for each run from 3 ml plasma + 1 ml dye (36 μg T-1824/ml). Test plasma-dye samples were diluted with 1 ml distilled water and the optical densities of the blanks, standards and test solutions were obtained from readings taken on a Unicam spectrophotometer SP.600 using a red filter at a wavelength of 620 mμ.

Plasma volume (PV) was calculated from the formula $PV = \frac{A}{C_o}$ where A is the amount of dye injected in mg and C_o is the concentration of dye in the plasma as measured by the concentration of the dye in the 10 min sample or the zero extrapolated sample.

4. Calculation of Blood Volume

Initial work on haemoglobin synthesis using splenectomized sheep simplified estimations on blood volume, for Turner and Hodgetts (1959) have shown that the spleen of sheep may contain more than 25% of the total red cell volume.

Blood volume was calculated from plasma volume estimations and jugular haematocrit corrected for the ratio of $\frac{\text{Total Haematocrit}}{\text{Jugular Haematocrit}}$ (F_{cells} ratio). The correction is required because the concentration of red cells is higher in the large vessels than in the capillary

beds. Hodgetts (1961) has shown the ratio to be 0.81 for splenectomized sheep and for intact sheep $2\frac{1}{2}$ min after adrenalin (30 μg per kg body weight) was injected to contract spleen. Total blood haematocrit was determined from the formula: $\frac{RCV}{RCV + PV} \times 100$ where RCV is the red cell volume determined by the ^{51}Cr-labelled red cells method and PV is the plasma volume calculated from the Evans Blue method. Therefore blood volume (BV) $= \dfrac{PV \times 100}{100 - \text{Jugular Haematocrit} \times 0.81}$

5. Measurement of Plasma Protein Concentration

Yuile et al. (1959) found that there is a high exchange rate between the plasma proteins and the proteins of the interstitial fluid. Consequently the rate of synthesis of plasma protein cannot be determined by the techniques used for estimating haemoglobin synthesis.

The amount of protein removed as plasma protein in the blood samples was measured by the method of Phillips et al. (1945). Plasma protein concentration [PP] in gm/100 ml was calculated from the formula [PP] = 360 (PSG - 1.0070) where:

PSG = plasma specific gravity as determined from measuring the specific gravity of the plasma in a graded series of copper sulphate solutions.

1.0070 = the specific gravity of protein-free plasma ultra-filtrate.

360 = a factor derived from the determination of plasma protein by the Kjeldahl method.

Total circulating plasma protein (cPP) can then be calculated from PV x [PP].

6. <u>Haemoglobin Estimation</u>

The direct photometric method of oxyhaemoglobin estimation described by Hawk et al. (1954) was used.

To 20 ml of 0.2% NH_4OH in a 25 ml McCartney bottle was added 0.1 ml oxalated or heparinised thoroughly mixed whole blood. The solution was then shaken well to lyse the cells and oxygenate the haemoglobin. NH_4OH prevents turbidity which can precipitate the blood proteins, whilst too much ammonia can convert haemoglobin to methaemoglobin and must be avoided (Ssigeti 1940).

The optical density of the oxyhaemoglobin was read on the Unicam spectrophotometer SP.600 (blue filter) at a wavelength of 540 mμ and compared with the optical density of a standard blood supplied by the N.S.W. Red Cross Blood Transfusion Service, Sydney. The blood from the Red Cross was standardized by the National Standards Laboratory, Sydney, and the haemoglobin concentration was based on the iron content of the blood. This would make the haemoglobin concentration approximately 2-3% higher than if it had been based on the CO capacity of the blood. That is partly because c. 2% of the iron is in the plasma, and a small percentage of the haemoglobin is in a non-functional form as carboxyhaemoglobin and methaemoglobin.

Comparison with a reliable standard reduces most of the errors usually associated with inadequate standards, and King et al. (1948) and Nicholas (1952) have shown that the oxyhaemoglobin

method is probably one of the most useful methods of measuring relative changes in haemoglobin concentration.

Total circulating haemoglobin (cHb) can then be calculated from BV x [Hb] in the jugular blood x 0.81.

7. Effect of Adrenalin on Haematocrit

Turner and Hodgetts (1959) have demonstrated that an intravenous injection of adrenalin will contract the spleen, which may contain more than 25% of the circulating red cells, and thus increase the circulating red cell volume.

In these experiments adrenalin (1: 1000) as the hydrochloride (Parke Davis & Co.) was used, diluted to 1: 10,000 with 0.9% NaCl before injecting. Adrenalin (30 $\mu g/kg$ body weight) was injected into four Merino ewes on several occasions, and in all cases a peak for jugular haematocrit was reached after 2-3 min accompanied by a transient tachycardia and hyperpnoea. Increases in jugular haematocrit ranged from 15-35%, and there was a proportionate increase in haemoglobin concentration. A small increase in plasma protein concentration was also observed; this could be accounted for by a slight haemolysis.

8. Estimation of Red Cell Volume Using ^{51}Chromium

Na_2 $^{51}CrO_4$ in isotonic saline with a half life of 27.8 days was obtained from the Radiochemistry Centre, Amersham, U.K. The chromium content was 18.2 $\mu g/ml$ and the specific activity was of the order of 75 $\mu c/\mu g$ Cr. Dilute solutions containing 20 μc

^{51}Cr/ml were made up in saline. This was found to be more suitable than the acid-citrate-detrose-^{51}Cr(ACD-^{51}Cr) solutions used as an incubation medium by Mollison and Veall (1955) or Sterling (1960), as incubation of sheep red cells with ACD-^{51}Cr solution was followed occasionally by haemolysis.

It was also found that a stock solution containing about 20 μc ^{51}Cr/ml in a ACD medium would have negligible available activity after five days. This may be because the ACD solution reduces the chromate ion from the hexavalent to the trivalent state, which reduces the available activity for "tagging". Gray and Sterling (1950) have suggested that the trivalent ion may not be able to penetrate into the erythrocyte. Gray and Sterling propose that penetration into the erythrocyte is a prerequisite for the labelling process, and that the chromium is probably bound to the globin portion of the haemoglobin molecule by co-ordinate covalent bonds.

In the technique used, which was essentially the method described by Sterling (1960), 20 ml samples of fresh heparinized sheep blood were spun down at 1500 g for 15 min. The plasma was removed and stored in the cold, as plasma proteins will also bind the chromate ion (Sterling 1960). To 6 - 9 ml packed red cells (p.r.c.) was added 60-80 μc ^{51}Cr in 10 ml 0.9% NaCl, and the red cell ^{51}Cr suspension was then allowed to incubate for 40-60 min on a rotating wheel at room temperature. The cells were then washed three times with saline, resuspended in the plasma, and

made up to a set volume with saline. After incubation it was found that 50-70% of the ^{51}Cr was taken up by the red cells.

Suspensions of 4-5 ml labelled cells with a specific activity of 2.1-7.5 μc/ml p.r.c. containing about 0.2 μg Cr/ml p.r.c. were injected intravenously, and $2\frac{1}{2}$ min after injection of the ^{51}Cr labelled red cells, adrenalin (30 μg/kg body weight) was injected. The adrenalin contracts the spleen and thus allows the ^{51}Cr to mix rapidly with the blood from the spleen in the general circulation. A sample of blood was taken for the determination of jugular haematocrit and jugular haemoglobin concentration $2\frac{1}{2}$ min after injection of the adrenalin. Subsequent samples at 10 min intervals were taken from the jugular vein and the specific activity of the blood was measured. The specific activity of the plasma was found to contain about 2% of the total activity in the whole blood. Haematocrits were determined on the injected red cell suspensions, and subsequent samples, so that the activity could be recorded in μc/ml packed red cells.

Red cell volume (RCV) was calculated from the isotope dilution formula:

$$\text{RCV in ml} = \frac{\text{Total radioactivity}}{\text{Radioactivity/ml p.r.c.}}$$

where total radioactivity = volume of reconstituted blood injected x radioactivity/ml, and radioactivity/ml p.r.c. =

$$\frac{\text{radioactivity in whole blood x 100}}{\text{volume of sample counted x haematocrit}}$$

In this method the reproducibility of the samples taken 10, 20, and 30 min after injection of ^{51}Cr should be within 5%.

An Ecko well-type scintillation counter was used to measure the activity of the samples. [51]Chromium emits about 8% radiations, and the instrument counts about 55% of all emissions. Maximum sensitivity was obtained by counting 5 ml samples of whole blood, when sedimentation of the red cells was not appreciable. All counts were corrected for background, and the stability of the instrument from day to day was determined by counting a uranium standard. A vial containing 5 ml distilled water was used as a blank, and 5 ml samples of the blood before injection, the injected blood, and the ^{51}Cr-saline solution were counted also.

Blood volume (BV) can also be calculated from the ^{51}Cr determination of red cell volume from the formula: $BV = \frac{RCV \times 100}{H+ \times 0.61}$ where H+ is the haematocrit $2\frac{1}{2}$ min after an injection of adrenalin (30 μg/kg body weight).

9. Calculation of the Rate of Haemoglobin Synthesis after Blood Loss

Tucker (1959) using ^{59}Fe-labelled red cells has estimated the potential life span of sheep erythrocytes to be 130 days. As the average life span of the red cells may be nearer 100 days, it is convenient to use a 1% daily turnover rate for comparison purposes when calculating rates of haemoglobin synthesis.

Finch et al. (1950) have pointed out, that when bleeding is maintained over several weeks there is a decrease in the age of the red cell population, and the % breakdown of red cells would be expected to decrease. For comparison purposes therefore, age

changes in the red cell population have been accounted for by
estimating breakdown to be equal to 1% of the final circulating
mass of haemoglobin at the end of a five week bleeding schedule;
this is consistent with the data of Finch et al. (1950).

The rate of haemoglobin synthesis can then be calculated
as follows:

Let physiological haemoglobin synthesis = 1%/day of the initial
circulating mass of haemoglobin.

Then the net haemoglobin synthesis/day =

$$\frac{\text{gm haemoglobin removed by bleeding} - \text{gm haemoglobin deficit}}{\text{No. of days}}$$

and the rate of haemoglobin synthesis/day = net haemoglobin/day +
the average breakdown/day.

10. Total Nitrogen Determinations

The Kjeldahl method of McKenzie and Wallace (1954) was
used.

Nitrogen balances were derived by estimating the total
nitrogen intake in the diet and determining the total nitrogen output
in the faeces and urine. Essentially Total N_{Diet} = Total N_{Faeces} +
Total N_{Urine} when the sheep is in nitrogen balance. The nitrogen
utilised for wool growth amounts to about 1 gm N/day, but was
disregarded in the calculations of nitrogen balance. Aliquots
equal to 1/10th of the daily urine output were bulked over
consecutive 3-4 day periods, then diluted so that 1 ml samples
taken for micro digestion before distilling would contain 0.5-1.5 mg N.

Similar aliquots of faeces were also bulked over 3-4 day periods. The urine samples were preserved by adding 50 ml 4N H_2SO_4 to the daily samples.

Samples of chaff and bulked samples of faeces, as shed, were macerated, and then duplicated 1 gm samples of the faeces and food were used in a macro digestion, and made up to volume after digestion. Aliquots containing 0.5 to 2.0 mg N were distilled in a Markham steam distillation apparatus, and a $(NH_4)_2SO_4$ solution (1 ml equivalent to 1 mg N) was used as a standard.

III. EXPERIMENTAL RESULTS

A. Effect of Daily Bleeding, and Massive Bleeding on Haemoglobin Synthesis, Plasma Protein Synthesis, and Nitrogen Balance

This initial experiment was carried out with splenectomized sheep to avoid the variable effect of the spleen on blood volume. Two five-year-old Merino wethers, 581 and 586, were splenectomized and placed in metabolism pens on a low protein diet, and allowed to accustom themselves to the pens, and the diet. Sheep 581 was allowed 600 gm, diet R9 (equal parts lucerne chaff and wheaten chaff) per day, and 586 was allowed 500 gm lucerne chaff per day (as R9 was refused), and subsequently 800 gm lucerne chaff per day; no residues were left. A salt mix containing 10 gm $NaCl$, 30 mg Fe as $FeSO_4.7H_2O$, 10 mg Cu as $CuSO_4.5H_2O$ and 1 mg Co as $CoCl_2.6H_2O$ was given daily to both sheep throughout the experimental period. Coleman et al. (1953) have emphasized the requirement for a dietary supplement of iron, to facilitate haemoglobin synthesis after blood loss, and the amount provided was equal to approximately three times the daily loss from bleeding.

Both animals were drenched with phenothiazine prior to the experiment to counteract Haemonchus infestation, as Richard et al. (1954) have demonstrated that the worms are capable of consuming up to 200 ml blood/day. Blood films were also examined for the blood parasite Eperythrozoon ovis which Littlejohns (1960) has found may cause anaemia.

Nitrogen balances were determined from the nitrogen of
the food intake and the nitrogen excreted in the urine and faeces,
and the total circulating mass of haemoglobin and plasma protein
were determined before bleeding and then at weekly intervals,
during and after bleeding.

1. Response to Massive Bleeding

Sheep 581 was bled twice from the jugular vein: 450 ml
in one bleeding, and after recovery approximately 6 weeks later,
960 ml was bled over a period of 4 days. On both occasions there
was a marked decrease in nitrogen excretion in the urine during the
first three weeks following bleeding (Fig. 1), and an increase in
the rate of haemoglobin synthesis.

The rate of haemoglobin synthesis increased to $2\frac{1}{2}$ times
the normal rate. Major recovery appeared to occur over a period
of approximately three weeks. The plasma protein concentration in
the blood returned to normal before haemoglobin, but as it is
possible that the exchange rate between the plasma proteins and the
interstitial proteins is high (Yuile et al., 1959), the rate of
plasma protein synthesis could not be determined by the methods
used in this investigation for estimating the rate of haemoglobin
synthesis.

2. Response to Daily Bleeding

Sheep 586 was bled 50 ml blood/day for eleven weeks.
This was equivalent to a loss of 6 gm protein/day. After six weeks

Fig. 1.- Response of a splenectomized sheep to massive bleeding: 450 ml was bled in one bleeding, and about six weeks later 960 ml was bled over a period of four days. The relationship between the recovery of the circulating mass of haemoglobin and a retention of dietary nitrogen is shown.
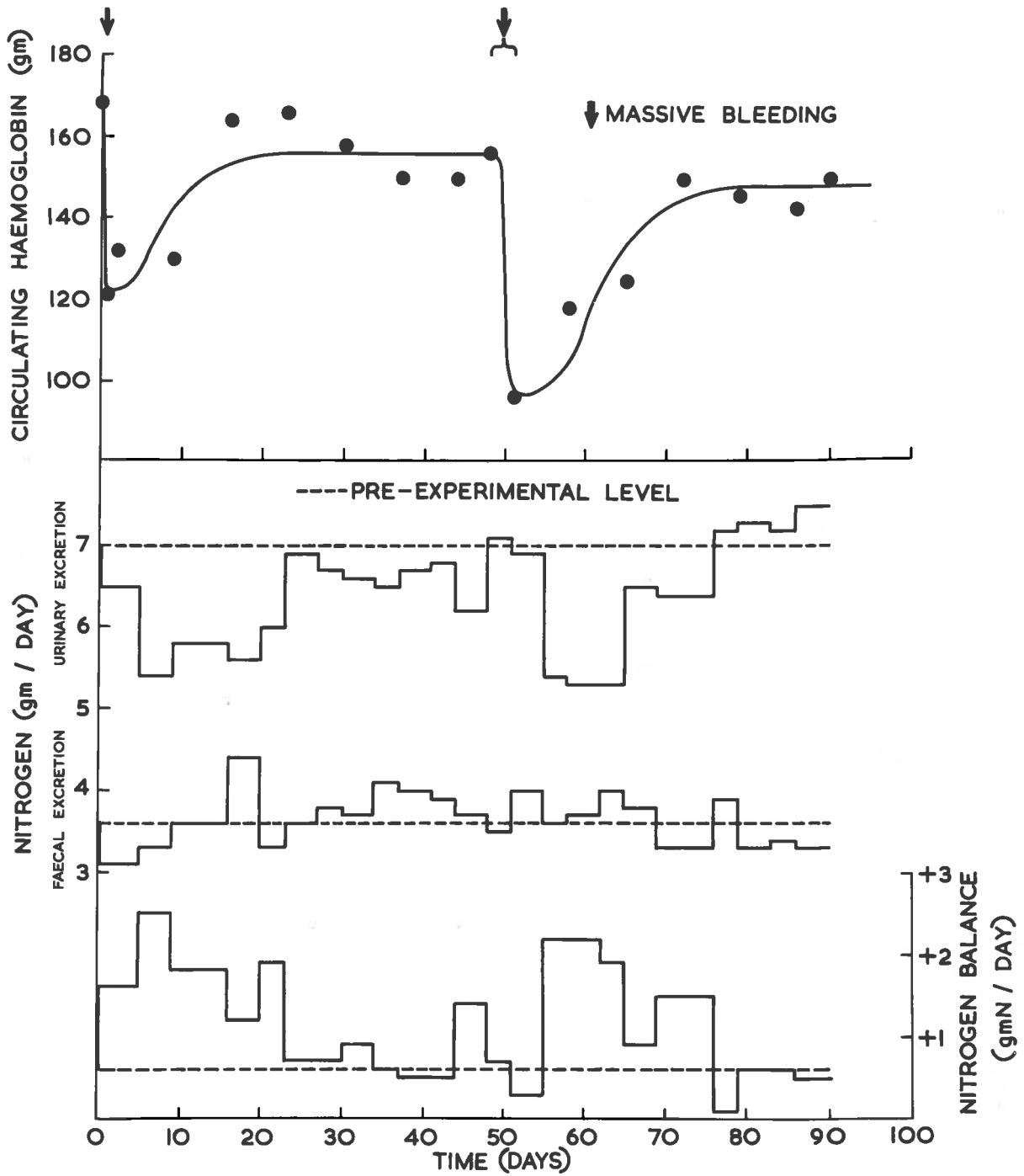
37a.

Fig 1.

of bleeding the diet was increased from 500 to 800 gm lucerne chaff.
Daily bleeding provoked an increased retention of nitrogen, and
an increased rate of haemoglobin synthesis. (Fig. 2).

The circulating mass of plasma protein returned to normal
when the diet was increased from 500 gm to 800 gm lucerne chaff.
The effect of increased protein intake on haemoglobin synthesis
however, was not measurable. Body weights remained constant
throughout the experimental period, and egg counts on the faeces
indicated that the sheep remained virtually free of intestinal
parasites (< 200 eggs/gm faeces), and blood smears showed no sign
of the blood parasite Eperythrozoon ovis.

Fig. 2.- Response of a splenectomised sheep to daily bleeding. The circulating mass of haemoglobin was maintained at a lower level during bleeding by an increase in the rate of haemoglobin synthesis and a retention of dietary nitrogen. The calculated level of nitrogen excretion is based on the proportion of the dietary nitrogen which would have to be excreted to maintain the nitrogen balance at the pre-experimental level.
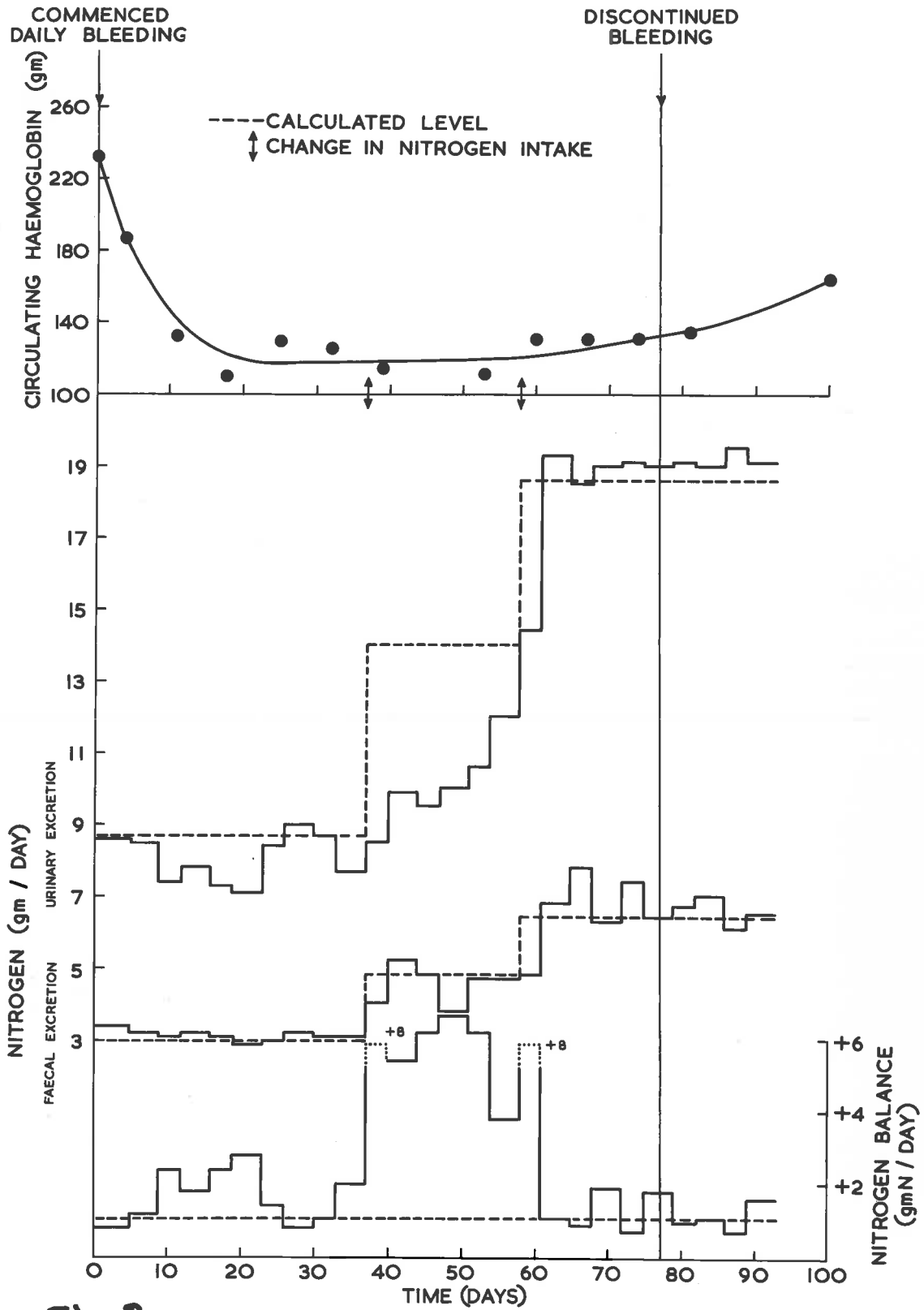
38 a.

Fig 2.

B. <u>Determination of F_{cells} Ratio for Sheep, and a</u>
<u>Comparison of Methods for the Estimation of Blood Volume</u>

Four Merino ewes were selected to determine the ratio of
<u>total blood haematocrit</u> (F_{cells} ratio) in the sheep. The sheep
<u>jugular blood haematocrit</u>
were also used to compare the Evans blue - adrenalin method of
estimating blood volume with the ^{51}Cr-labelled red cells method
of determining blood volume. Table 1 shows the F_{cells} ratio
obtained for the four sheep, and Fig. 3 illustrates the rise in
jugular haemoglobin concentration which occurred after an injection
of adrenalin (30 $\mu g/kg$ i.v.). Using the procedure described
previously a good agreement was found between the two methods of
estimating blood volume. This agrees with the experimental data
of Hodgetts (1961). A comparison of the values obtained by the
two methods of determining blood volume is summarized in Table 2.

It was decided therefore to use the Evans blue - adrenalin
method for the determination of blood volume in subsequent experi-
ments, as there are technical disadvantages in the use of ^{51}Cr,
viz, a long biological half life, the time factor involved in
labelling, and the care that is necessary when using radio-active
material.

TABLE 1

F_{CELLS} RATIO IN FOUR MERINO EWES

Sheep No.	Total Blood Haematocrit (a)	Jugular Blood Haematocrit (b)	F_{cells} Ratio a/b
874	33.0	42.5	0.78
871	38.5	48.0	0.80
881	28.5	34.0	0.84
619	34.0	42.0	0.81
		Mean	0.81

Fig. 3.- Changes in jugular haematocrit, haemoglobin concentration and plasma protein concentration following intravenous injection of adrenalin (30 μg/kg body weight).
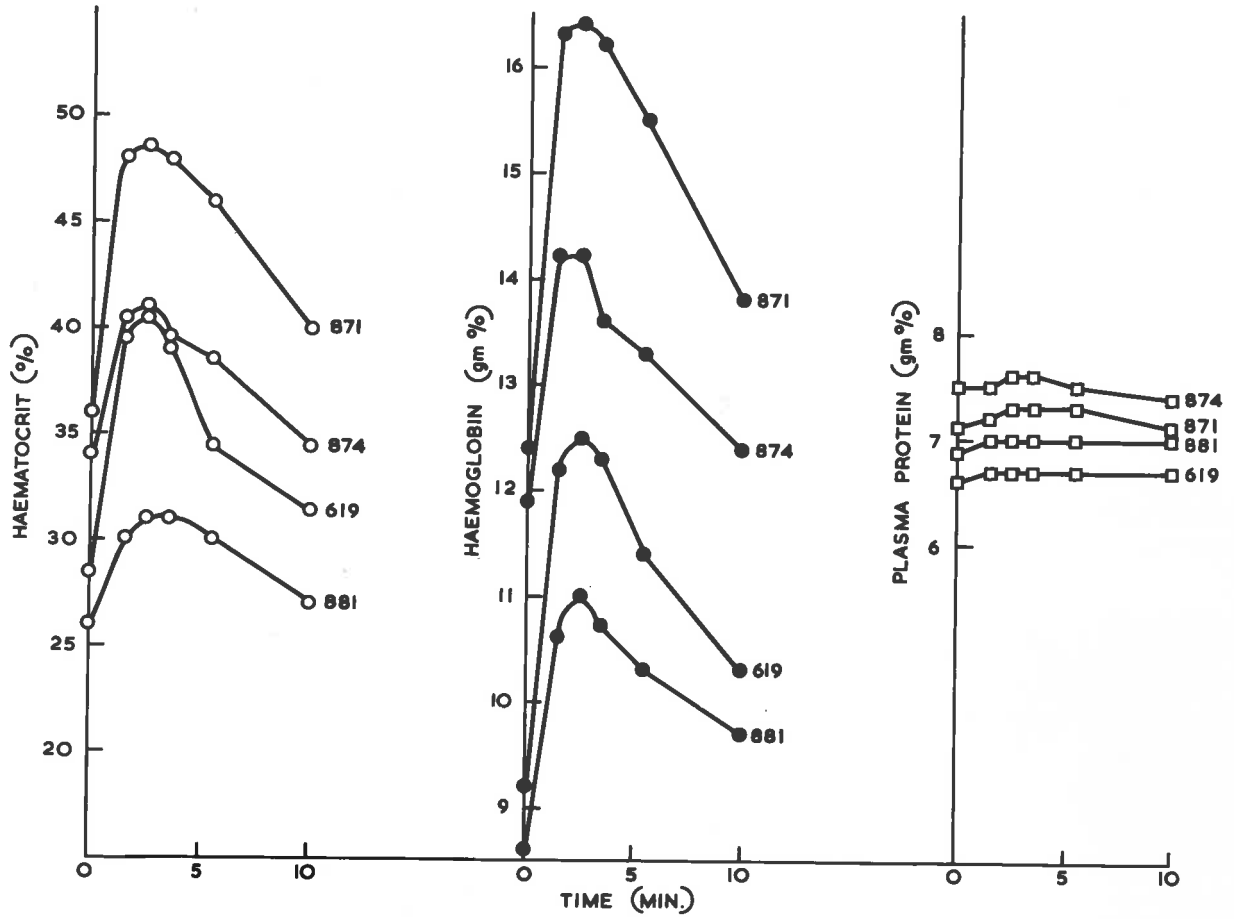
39b.

Fig 3.

TABLE 2

VALUES FOR RED CELL VOLUME (RCV), PLASMA VOLUME (PV)

AND BLOOD VOLUME (BV)

Sheep No.	Body Weight kg	$RCV_{51_{Cr}}$ ml/kg	$PV_{Evans\ blue}$ ml/kg	$BV^{(1)}$ ml/kg	$BV^{(2)}$ ml/kg
874	40.6	22.2	45.2	68.2	65.2
871	44.4	23.7	38.2	62.8	60.9
881	31.2	22.3	56.6	78.0	80.1
619	35.0	25.5	50.0	75.6	74.5
Mean	37.8	23.4	47.5	71.2	70.2

Note: $BV^{(1)}$ and $BV^{(2)}$ were calculated from the following formulae:

$$BV^{(1)} = \frac{PV_{Evans\ blue} \times 100}{100 - Ht \times 0.81}$$

$$\text{and } BV^{(2)} = \frac{RCV_{51_{Cr}} \times 100}{Ht \times 0.81}$$

C. Selection of a Level of Experimental Anaemia Which Would Provoke a Marked Increase in the Rate of Haemoglobin Synthesis

It has been shown in the experiment with two splenectomized sheep that daily bleeding will provoke an increase in the rate of haemoglobin synthesis.

To determine the capacity of the normal sheep to synthesize haemoglobin however, it was necessary to devise an experimental procedure which would maintain a marked stimulus for haemoglobin synthesis. It appeared that this could be achieved by producing a state of experimental anaemia in the sheep, by bleeding the sheep down to a desired level of anaemia, and then maintaining the anaemia by daily bleeding. The amount of haemoglobin removed by daily bleeding would then indicate the rate of haemoglobin synthesis. (Cf. The "Standard Anaemic Dog" (Whipple, 1960).)

Three five-year-old Merino ewes were chosen to determine the level of experimental anaemia which would provoke the maximum rate of haemoglobin synthesis. The sheep were allowed to accustom themselves to single pens and were fed, ad libitum, diet R9 (equal parts lucerne chaff and wheaten chaff). Total circulating haemoglobin (OHb) was then determined before bleeding the sheep down to the selected levels of 8, 6 and 4 gm Hb/100 ml blood, identified as mild, moderate and severe anaemia respectively.

Four to seven days were taken to bleed the sheep down to the required levels, and the level of anaemia was then maintained by daily bleeding for approximately four weeks. Bleeding was then discontinued and an estimate of the circulating mass of haemoglobin obtained. Blood volume and haemoglobin concentration were then determined at convenient intervals to follow the rate of recovery.

Throughout the experimental period a daily salt mix was given to the sheep containing 200 mg Fe as $FeSO_4$ which was equivalent to about six times the daily loss of iron from bleeding. Total circulating haemoglobin was also estimated at intervals throughout the experimental period on a control group of three sheep, and it was found that any variation in the circulating haemoglobin in the control group could be accounted for by the error of the experimental method which is of the order of 5%.

In the experimental group of sheep, haemoglobin synthesis increased to a maximum of 2.8 times the normal rate at both moderate (6 gm Hb%) and severe (4 gm Hb%) degrees of anaemia. A mild anaemia (8 gm Hb%) does not appear to stimulate maximum haemoglobin synthesis, and severe bleeding affects the appetite of the sheep. Therefore for practical purposes a moderate anaemia (6 gm Hb%) appears to be the most suitable for provoking a marked stimulus for haemoglobin synthesis.

The results from the experiment are outlined in Table 3.

TABLE 3

HAEMOGLOBIN SYNTHESIS AT VARIOUS LEVELS OF EXPERIMENTAL ANAEMIA

Sheep No.	Degree of Anaemia	Initial Circulating Haemoglobin gm Hb/kg	Food Intake Ad Libitum diet R9* (11.9% Crude Protein) gm	Bled ml blood/ day	Hb bled gm/day	Hb bled gm/day/ kg	Rate of Haemoglobin Synthesis		
							Rate x normal	gm Hb /day	gm Hb/ day/kg
21	Mild (8 gm Hb%)	6.8	720	90	7.7	0.22	2.3	6.0	0.18
25	Moderate (6 gm Hb%)	7.6	600	150	10.5	0.32	2.8	7.5	0.23
38	Severe (4 gm Hb%)	7.8	620	250	14.6	0.38	2.8	8.8	0.24

* Diet R9 is composed of 50% lucerne chaff and 50% wheaten chaff

41a.

D. Haemoglobin Synthesis Provoked by Moderate

Anaemia

The general procedure outlined in the previous experiment was repeated with three more sheep, fed ad libitum, diet R9, except that all three sheep were bled down to 6 gm Hb% and maintained at that level for four weeks by daily bleeding.

The response of this group to a moderate anaemia appeared to be related to their appetite and general "vigour". The youngest sheep increased its haemoglobin synthesis to 3.8 times the normal rate. The two older sheep however were less "vigorous" in their response, their food intake was much less than the other sheep, and their rate of haemoglobin synthesis only increased to 2.3 - 2.9 times the normal rate (Table 4).

TABLE 4

HAEMOGLOBIN SYNTHESIS PROVOKED BY MODERATE ANAEMIA

Sheep No.	Degree of Anaemia	Initial Circulating Haemoglobin gm Hb/kg	Food intake ad libitum diet R9* (11.9% Crude Protein) gm	Bled ml Blood/ day	Hb bled gm/day	Hb bled gm/day/ kg	Rate of Haemoglobin Synthesis		
							Rate x normal	gm Hb /day	gm Hb/ day/kg
604	Moderate (6 mg Hb%)	7.0	1220	160	10.3	0.27	3.8	10.4	0.27
874	"	7.1	860	140	10.3	0.24	2.9	8.8	0.20
871	"	7.8	500	130	9.8	0.23	2.3	7.7	0.19

* Diet R9 is composed of 50% lucerne chaff and 50% wheaten chaff

42a.

E. Effect of Protein Intake on the Rate of Haemoglobin

Synthesis

To examine the effect of protein intake on the rate of
haemoglobin synthesis, six ewes were divided into two groups of
three. One group was fed a low protein diet and the other group
was fed a high protein diet. The group of sheep on the low protein
diet were allowed 15 gm R9/kg body weight/day which amounted to
500-600 gm R9/day (equivalent to 65-78 gm crude protein/day). The
high protein diet (RI) consisted of 50% lucerne chaff and 50%
cracked corn (equivalent to 14.5 gm crude protein %), and the sheep
on the high protein intake were allowed 1000 gm RI/day. An iron
supplement of 200 mg Fe/day was also supplied to each sheep.

After a ten week equilibration period the sheep were bled
down to a moderate level of anaemia (6 gm Hb%) as described before,
and maintained at that level for four weeks by daily bleeding.
The changes which occurred in the concentration and circulating
mass of haemoglobin can be seen in Fig. 4.

The response of the two groups of sheep was found to be
similar, and the results are compared in Table 5.

Haemoglobin synthesis increased up to a maximum of $3\frac{1}{2}$
times the normal rate, equivalent to 0.25 gm Hb synthesized/day/kg
body weight. The values represent the average rate of haemoglobin
synthesis throughout a five week experimental period of bleeding,
and may therefore be slightly less than the maximum rate of
haemoglobin synthesis for the period, as White et al. (1960) have

Fig. 4.- Comparison of the changes which occur in the circulating mass and concentration of haemoglobin during and after bleeding. A group of sheep on a low protein diet are compared with a group of sheep on a high protein diet. Adrenalin (30 $\mu g/kg$ body weight) was injected intravenously to contract the spleen, for the determination of the maximum total circulating haemoglobin.
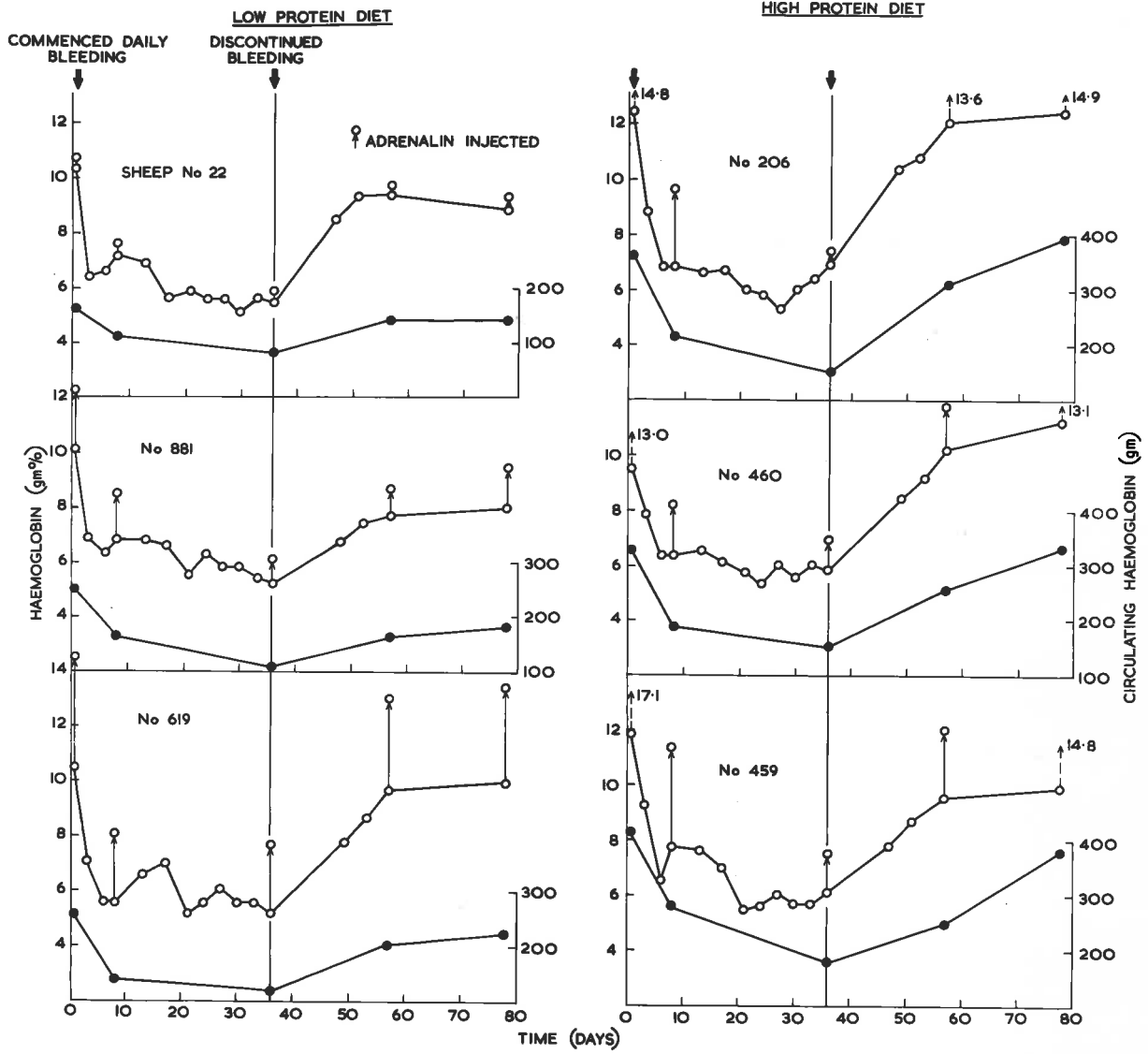
43a.

Fig 4.

TABLE 5

COMPARISON OF THE RATE OF HAEMOGLOBIN SYNTHESIS ON A LOW PROTEIN DIET, AND ON A HIGH PROTEIN DIET

Sheep No.	Degree of Anaemia	Initial Circulating Haemoglobin gm/Hb/kg	Feed Intake (Restricted) Diet R9* (13% crude protein) or Diet R1* (14.5% cr. Protein)	Bled ml blood /day	Hb Bled gm/day	Hb Bled gm/day /kg	Rate of Haemoglobin Synthesis								
							During Bleeding Period 5 Weeks			1st 3-Week Recovery Period			2nd 3-Week Recovery Period		
							Rate x Normal	gm Hb /day	gm/Hb/ day/ kg	Rate x Normal	gm Hb/ day	gm Hb/ day/	Rate x Normal	Hb/ day	gm Hb /day/ kg
Low [22	Moderate (6 gm Hb%)	6.2	500 gm R9	120	7.5	0.31	3.5	5.9	0.25	2.2	3.9	0.16	0.7	1.1	0.05
Protein [881	"	7.5	520 " "	140	9.7	0.36	2.7	6.7	0.25	1.6	4.0	0.15	0.9	2.1	0.08
Diet [	"	7.0	600 " "	150	10.3	0.33	3.0	7.7	0.25	2.1	5.4	0.17	1.0	2.7	0.09
High [206	"	8.0	870 gm R1	220	16.2	0.27	3.3	11.7	0.27	2.6	9.4	0.21	1.7	6.1	0.14
Protein [406	"	7.2	1000 " "	190	13.3	0.29	3.0	10.0	0.22	2.0	6.7	0.15	1.6	5.4	0.12
Diet [459	"	7.5	1000 " "	230	17.2	0.31	3.0	12.4	0.22	1.3	5.5	0.10	2.0	8.3	0.15

* Diet R9 is composed of 50% lucerne chaff and 50% wheaten chaff and diet R1 is composed of 50% lucerne chaff and 50% cracked corn.

shown that the maximum stimulation for blood regeneration may not occur over the first 4-5 days of anaemia.

It was also observed as bleeding continued (Fig. 5), that there was a marked reduction in the reserve haemoglobin released from the spleen after adrenalin injection. This indicated that although the haemoglobin concentration was being maintained in the circulating blood, the total mass of haemoglobin was being reduced. The reduction in reserve haemoglobin however, in the form of the red cells concentrated in the spleen, was largely replaced during the recovery period.

Changes in plasma volume were also measured throughout the experimental period, and it was found that haemodilution during bleeding was followed by haemoconcentration during the recovery period (Fig. 6).

The sheep maintained their body weight throughout the experimental period, and remained virtually free from intestinal parasites as determined by egg counts on the faeces. A slight loss of appetite did occur in two sheep, but this did not appear to influence the rate of haemoglobin synthesis.

Fig. 5.- Haemoglobin released from the spleen in response to adrenalin injection (30 μg/kg i.v.) during and after daily bleeding. The sheep were bled 120-230 ml blood/day for 36 days.
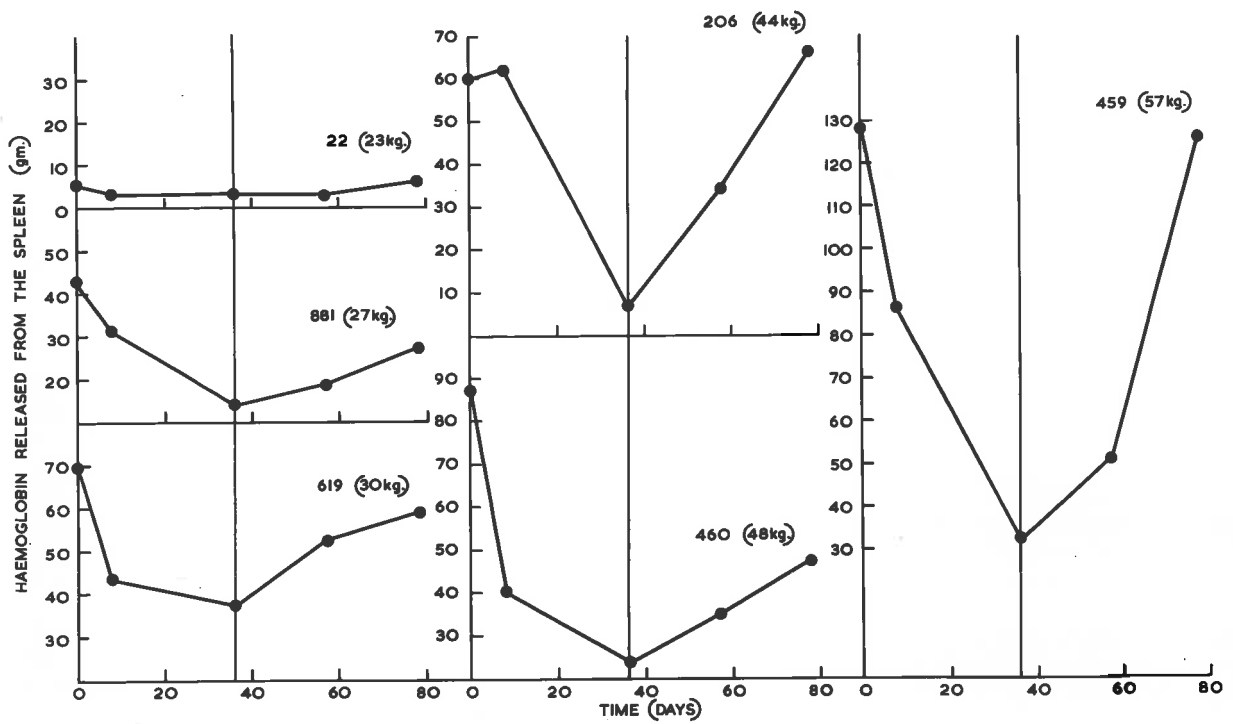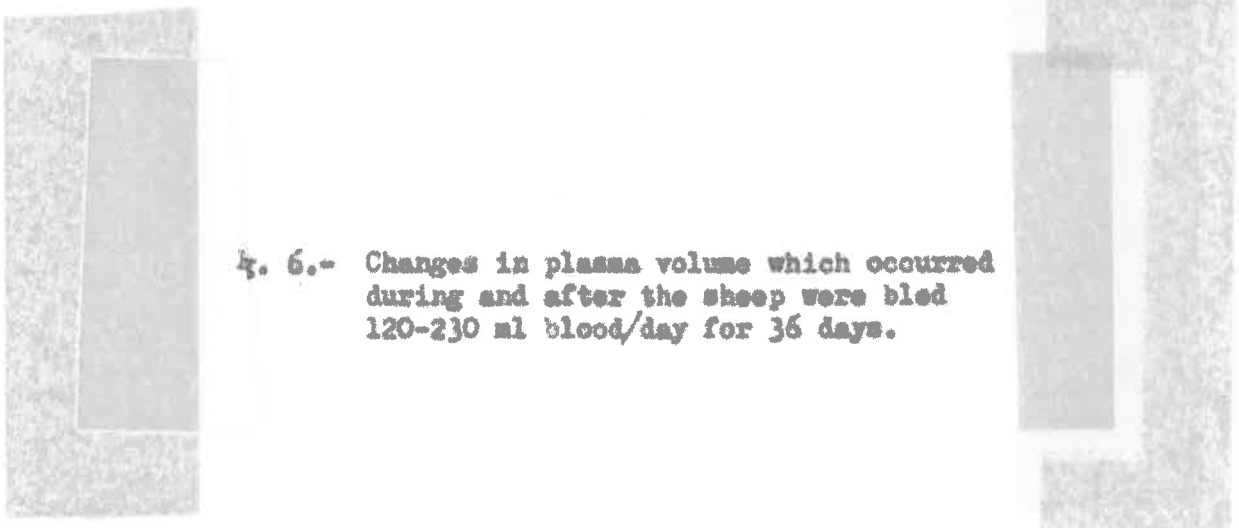
44a.

Fig 5.

446.

Fig. 6.- Changes in plasma volume which occurred
during and after the sheep were bled
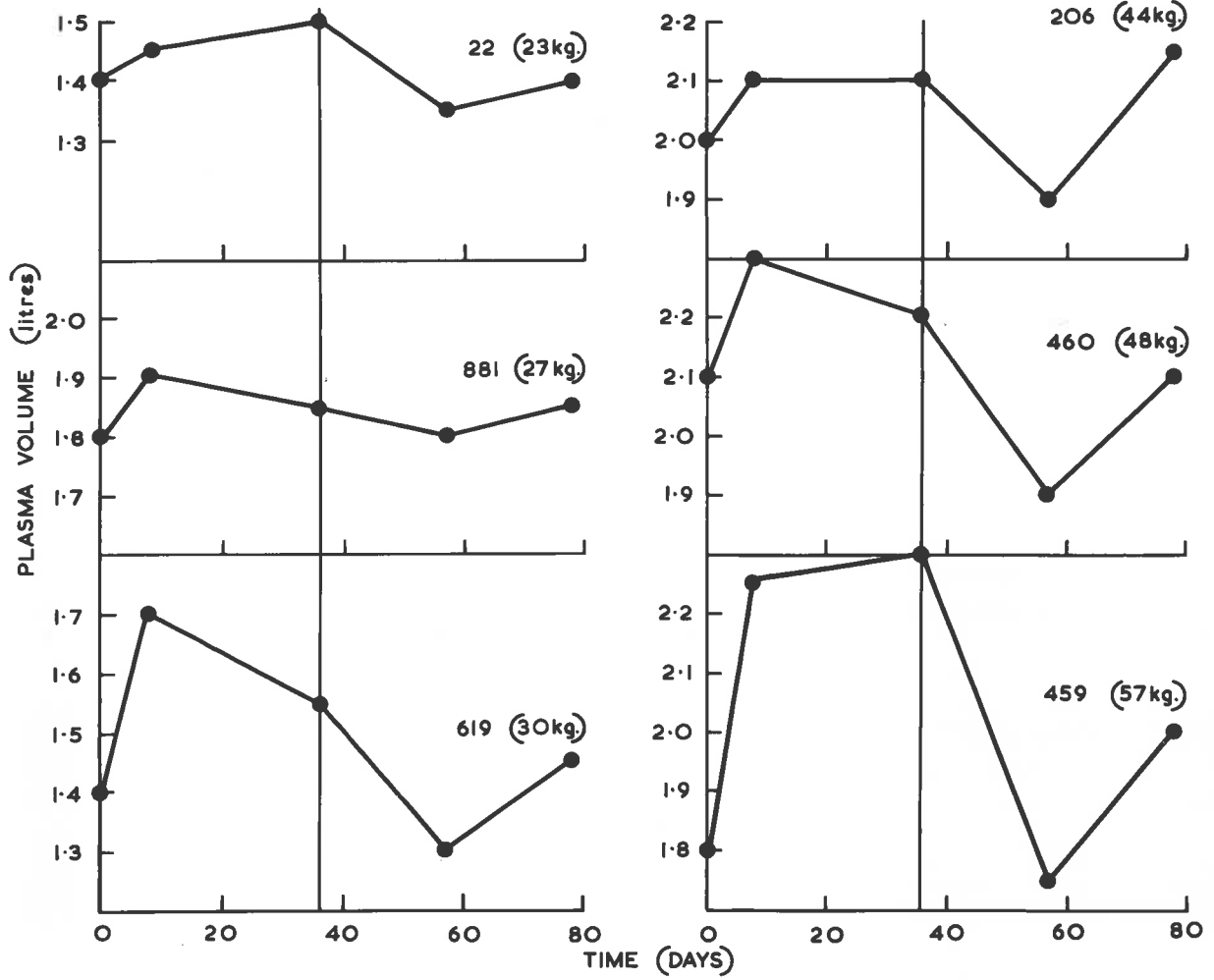120-230 ml blood/day for 36 days.

44c.

Fig 6.

IV. DISCUSSION

A. Response of the Splenectomised Sheep to Massive Bleeding and Daily Bleeding

The ability of the splenectomised sheep to respond to massive bleeding and prolonged daily bleeding was demonstrated by an increased rate of haemoglobin synthesis and a retention of nitrogen. It was interesting to note however, that after massive bleeding the sheep was unable to restore the circulating mass of haemoglobin to its initial level at the end of a six week recovery period.

Paton et al. (1902) have shown that the dog and the rabbit after haemorrhage or haemolysis are able to regenerate erythrocytes as rapidly without the spleen as with the spleen. Whipple et al. (1920) also found that splenectomy did not retard the rate of haemoglobin synthesis after bleeding in the dog, and also showed that the curve of haemoglobin regeneration can be influenced by the dietary intake. More recently Jacobson et al. (1957) have shown that rats subjected to splenectomy retain their capacity to respond to bleeding with an increase in plasma erythropoietin, the erythropoietic stimulating factor.

The rate of haemoglobin synthesis was not appreciably affected by increasing the protein intake of the sheep, that was bled daily. Although this suggests that protein was not rate limiting in that sheep, a more likely explanation may be derived from the observation that during the latter period of bleeding the

sheep became infected with corynebacteria, which formed abscesses
in the lymph node regions of the neck. It is well known that
the splenectomized animal is more susceptible to infection,
probably because the spleen is implicated in the production of
antibody globulin (Craddock, 1962). Robscheit-Robbins (1947)
and Whipple (1956) have also found that infection has a marked
inhibitory effect on red cell formation in the dog, which is
associated with a leucocytosis.

The mechanism underlying the decrease in urinary nitrogen
excretion in response to bleeding has not been described in the
sheep. However, Schmidt-Nielson, Osaki, Murdaugh and O'Dell (1958),
during studies on the regulation of urea excretion in sheep, found
that a normally fed sheep, from which "a considerable number of
blood samples had been taken", showed a tendency towards the
characteristic urea conservation observed in sheep on a low-protein
diet. Loss of blood protein may stimulate the secretion of growth
hormone which could bring about a retention of nitrogen. De Bodo
and Altszuler (1957) have reported a retention of nitrogen
following the administration of purified growth hormone to dogs,
rats and mice, and recently Wheatley, Wallace and Bassett
(unpublished data, 1963) have demonstrated a retention of nitrogen
in the sheep after subcutaneous injections of growth hormone.
Conservation of nitrogen in the anaemic dog has also been reported
by Robscheit-Robbins (1947).

B. Haemoglobin Synthesis Provoked by Experimental

Anaemia

Robscheit-Robbins and Whipple (1941) have found that the
rate of haemoglobin synthesis in the dog, stimulated by an experi-
mental anaemia is related to the severity of the anaemia. A mild
anaemia will not provoke maximal haemoglobin synthesis and a
severe anaemia will impair appetite and normal activity. It
has also been shown (White et al., 1960) in sheep made anaemic
with phenylhydrazine, that the level of erythropoietin in the blood
is proportional to the degree of anaemia.

The data outlined in Table 3 from the experiment with
the three sheep which were subjected to different levels of
experimental anaemia, indicates that a mild anaemia (8 gm Hb%)
did not provide a maximum stimulus for haemoglobin synthesis in
the sheep. A moderate anaemia (6 gm Hb%) however, and a severe
degree of anaemia (4 gm Hb%) did provide a marked increase in the
rate of haemoglobin synthesis. The severe level of anaemia
could only be maintained by bleeding 250 ml blood/day from the
sheep. This would impose considerable stress on the sheep and
probably impair the appetite of the sheep.

The results from the second group of sheep (Table 4) fed
ad libitum and subjected to a moderate anaemia indicate that
appetite, age and general "vigour" may influence the rate of
haemoglobin synthesis. The youngest sheep of the group, No. 604,
had the best appetite and increased its rate of haemoglobin synthesis

to 3.8 x the normal rate. This was about 30% more than the
synthesis achieved by either of the other two sheep.

C. Influence of Protein Intake on Haemoglobin Synthesis

The experiment comparing the rate of haemoglobin synthesis on a low protein diet, and on a high protein diet suggests that the rate of haemoglobin synthesis is not influenced by the protein intake on 'ordinary' rations. Hahn and Whipple (1939) have found that the dog requires 7-8 gm of good quality protein to synthesize 1 gm of haemoglobin, and as the sheep on the low protein diet were fed 65-80 gm protein/day and synthesized 6-8 gm Hb/day, the protein intake may have been adequate for the requirements of the sheep for haemoglobin synthesis, at least for a limited experimental period of anaemia.

Whipple and Robscheit-Robbins (1925) have shown that if a dog is placed on a deficient protein diet, the dog has usually sufficient reserve protein to regenerate large amounts of haemoglobin for 2-3 weeks, after which the rate of haemoglobin synthesis is reduced. The dog can then be used for assaying dietary supplements, and an increase in the rate of haemoglobin synthesis will then reflect the quality and quantity of globin-building protein in the supplement. The synthesis of the pigment radicle is not usually a limiting factor, and if an adequate supplement of iron is provided then the rate of haemoglobin synthesis is mainly dependent on the protein intake and the protein reserves of the animal (Hahn and Whipple, 1939).

It appears that the sheep on the low protein diet were not depleted to an extent where a reduction in the rate of haemo-

globin synthesis would occur. If the sheep were placed on a deficient protein diet however, the amount of blood protein one would have to bleed before the rate or haemoglobin synthesis was reduced, would probably reflect the protein reserves for haemoglobin synthesis; this reserve would presumably be related to the previous history of the sheep (diet, exercise, infection, etc.).

Haemoglobin concentration in all sheep was maintained partly at the expense of the haemoglobin contained in the spleen, during the experimental bleeding period, and it was significant that the total circulating mass of haemoglobin had not returned to the pre-experimental level six weeks after the bleeding was discontinued in the group of sheep on a low protein diet.

D. General Conclusions

The maximum rate of haemoglobin synthesis achieved by the sheep in these experiments was 3.8 x the normal rate. This was in response to an acute moderate experimental anaemia, and is comparable to the findings of Coleman et al. (1953), and Chapman and Block (1998) in man, and of Robscheit-Robbins and Whipple (1941) in the dog, who observed increases in the rate of haemo-globin synthesis up to 3 x the normal rate in response to repeated bleeding. In a chronic haemolytic anaemia, Crosby et al. (1952) have shown that the hyperplastic bone marrow of man is capable of producing 7 x the normal output of haemoglobin, so that the potential maximum capacity of the bone marrow of the sheep was probably not achieved from an acute moderate experimental anaemia. Robscheit-Robbins and Whipple (1941) and Robscheit-Robbins (1947) have also reported that values as high as 8x the normal rate of haemoglobin synthesis can be achieved by extending the period of anaemia, which produces a hyperplastic condition of the bone marrow, and by providing a large protein intake and increasing the available iron by intravenous injections.

Changes in the rate of haemoglobin synthesis, and the level of the total circulating haemoglobin, can be thought of as responses to changes in the dynamic equilibrium of the erythron. (The erythron is defined as the body organ consisting of the red blood cells and the precursors in the bone marrow.) Jacobson et al. (1957) propose that the dynamic equilibrium of the erythron is controlled

by the amount of circulating erythropoietin, which is produced by
the kidney in response to the oxygen supply - demand relationships
of the body. The dynamic equilibrium of the erythron adjusts
itself automatically to changes in the metabolic pattern of the
animal, and is chiefly influenced by the endocrine glands. The
erythron will also respond to physiological stresses that affect
the oxygen supply - demand relationships, or factors that interfere
with the production or utilization of erythropoietin. Starvation,
hypophysectomy and transfusion reduce erythropoiesis as the oxygen
supply is in excess of the demand, whilst triiodothyronine, high
altitude, haemorrhage etc. increase erythropoiesis as the demand
for oxygen in these cases is greater than the supply.

There is ample evidence in the literature that iron
(Coleman et al., 1953; Finch et al., 1950) or protein (Hahn and
Whipple, 1939; Robscheit-Robbins, 1947) can limit the response to
a demand for an increase in haemoglobin production. In these
experiments with the sheep however, as neither iron or protein
were apparently limiting, the rate of haemoglobin synthesis must
have reflected the capacity of the bone marrow to produce red
cells in response to an increase in production of erythropoietin.
Individual variations in bone marrow capacity probably modified the
response, and the rate of haemoglobin synthesis in individual sheep
was probably also influenced by the age, health and appetite of the
sheep.

The level of protein intake on 'ordinary' rations does not appear therefore to influence the rate of haemoglobin synthesis in an acute experimental anaemia, but it is probably a factor in determining the level of the circulating mass of haemoglobin after recovery from bleeding, by the influence of food intake on the potential metabolic level of the animal. This emphasizes the importance of haemoglobin which is preferentially synthesized (Whipple et al., 1947), in comparison to the more labile proteins of the intestinal mucosa which have been investigated by Platt (quoted from Morton 1962), or the labile enzyme systems of the liver (Williams, 1961), both of which have been shown to be depleted following a reduction in dietary protein.

V. ACKNOWLEDGMENTS

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