DIAGNOSIS AND EFFECTS OF COBALT DEFICIENCY
IN THE PREGNANT EWE.

A thesis submitted for the Degree of Doctor of Philosophy in the Faculty of Science of the University of Glasgow.

By

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"... in the morning, going down the lane, she heard the ewe call and the lambs came running, shaking and twinkling with new born bliss. And she saw them, stooping, nuzzling, groping to the udder, to find the teats, whilst the mother turned her head gravely and sniffed her own. And they were sucking, vibrating with bliss on their little, long legs, their throats stretched up, their new bodies quivering to the stream of blood-warm, loving milk."

D.H. LAWRENCE.
From The Rainbow.

DEDICATION.

For the past, Mary and David.

To the future, Daniel and Georgia.

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SUMMARY.

The literature relating to the diagnosis and effects of cobalt (Co) deficiency, particularly in the pregnant ewe, was reviewed.

Two housed experiments, both with 60 hill ewes, were undertaken with the aim of characterising the effects of Co deficiency on reproductive performance. The diagnosis of sub-clinical Co deficiency was also studied. Three treatment groups of 20 ewes each were established in both experiments. The NS (non-supplemented) groups of both experiments were maintained on a Co-deficient intake for the whole of pregnancy. Sheep in the HS (half-supplemented) group of Experiment 1 received a Co-sufficient intake until mid-pregnancy after which they were maintained on a Co-deficient diet. The HS group of Experiment 2 received a Co-deficient intake until mid-pregnancy, after which it was replenished with Co. The FS (fully-supplemented) group of both experiments received a Co-sufficient intake for the whole of pregnancy.

Co status of the ewes was monitored by fortnightly sampling and analysis for serum vitamin B12 and methylmalonic acid (MMA) concentrations. Serum B12 was determined by either microbiological assay or radio-immuno assay (RIA).

In Experiment 1 the NS and HS ewes were sub-clinically Co-deficient before lambing. In Experiment 2 the NS and HS sheep were sub-clinically Co-deficient at the start of tupping and the clinical disease was detected in the

NS ewes at the start of lambing. After Co repletion from mid-pregnancy, serum B12 and MMA concentrations in the HS sheep returned to normal levels and they were Co-sufficient before lambing. The FS ewes of both experiments were Co-sufficient throughout the investigations. In Experiment 1 and 2 the differences amongst treatments for serum B12 and MMA concentrations were significant ($P<0.001$).

Concentrations of MMA in serum were a more accurate and precise marker of sub-clinical and clinical Co deficiency, than serum B12 levels. Within treatments, serum B12 concentrations were more variable than serum MMA levels and could imply false positive diagnoses. In contrast, there were no problems from diagnosing false negatives with serum MMA concentrations. However, there was evidence to suggest that liver damage could lead to incidences of very high levels of MMA in serum. Concentrations of B12 in serum provided an effective indication of Co intake and responded to declining Co status before serum MMA levels. Thus, the joint analysis of serum B12 and MMA concentrations was necessary for the prognostic, as well diagnostic, detection of Co deficiency.

The microbiological assay and RIA of serum B12 both provided effective diagnostic data. However, for ewes fed a diet high in concentrates, mean serum B12 concentrations determined by the RIA were 20 per cent

lower than those measured by the microbiological assay (r = 0.97, P<0.001). The diagnostic criteria applied to RIA data therefore required alteration in this situation. For the microbiological assay, values between 200 and 400 ng/l were regarded as indicative of sub-clinical disease. If ewes were fed a high concentrate ration, the range 160 to 340 ng/l applied to data from the RIA provided the same diagnostic inference.

A small housed experiment with wethers was undertaken to determine the presence of diurnal fluctuations in serum B12 and MMA concentrations. Endogenously stimulated fluctuations, of either marker, were not detected. However, serum levels of both B12 and MMA in Co-deficient and sufficient sheep showed inconsistent but marked rises in response to the exogenous stimulation of twice daily feeding. Serum MMA levels also fell just before feed intake. The effect on serum B12 could be enhanced by delaying feeding for 2 hours. These fluctuations could render serum B12 and MMA data useless in the diagnosis of Co deficiency. Therefore, it was recommended that blood sampling for diagnostic purposes should be undertaken outwith the time period of 1 hour before and 2 hours after the provision of supplementary feed to sheep.

The sub-clinical Co deficiency imposed on the NS and HS ewes at tupping in Experiment 2, did not adversely affect mating performance. Neither was there any evidence of an effect, in either experiment, on conception rate, the number of barren ewes, the number of lambs born or lamb

birthweights. However, in Experiment 2 there were significanlty more stillbirths in the NS group, compared to the HS and FS treatments (4 vs 1 vs 0, respectively, $P<0.05$). In both experiments, there was a tendency for the NS ewes to have lower lambing rates to 4 weeks post partum, relative to the FS controls, and in Experiment 1 this effect was significant ($P<0.05$).

In Experiments 1 and 2, a study was made of the effect of Co deficiency on immune cell function in the sheep and the transfer of passive immunity from ewe to lamb. Sub-clinical Co deficiency in the HS and NS ewes resulted in a 50 per cent reduction in the killing activity of isolated neutrophils, against phagocytosed Candida albicans yeast cells, compared to FS controls ($P<0.001$). Co repletion of the HS ewes from mid-term in Experiment 2 resulted in a rapid return of neutrophil function to normal levels.

Lambs from sub-clinically Co-deficient ewes were less vigorous and acquired lower levels of passive immunity than those from Co-sufficient dams. In Experiment 2 lambs from the NS and HS ewes took at least twice as long to commence suckling post partum, compared to those from FS dams ($P<0.001$). In both experiments the NS and HS group lambs also tended to have lower concentrations of immunoglobulin G ($P<0.001$), B12 ($P<0.001$) and ZST values ($P<0.05$) in serum, at 2 and 4 weeks post partum, relative to FS group controls. Normal concentrations of vitamin

B12 in the serum of pre-weaned lambs appeared to be in the range 200 to 400 ng/l.

These observations were related to decreased post-natal viability in lambs from NS and HS ewes, compared to those from FS dams. In Experiment 1 there were significantly more neonatal mortalities in NS group lambs, relative to the HS and FS groups (9 vs 1 vs 1, respectively, $P<0.05$) and more lambs in the HS group required treatment for ill-health, compared to NS and FS group controls ($P<0.05$). These incidences were associated with low serum immunoglobulin G concentrations and ZST values in lambs with depressed viability.

The consequences of the results for the diagnosis and effects of Co deficiency in pregnant ewes in the field situation were discussed. A survey of Co status in pregnant sheep on 15 farms in Scotland with a history of Co deficiency, was also undertaken. However, the use of one sampling during pregnancy was not adequate to indicate the Co status of flocks during tupping, pregnancy and lactation.

CHAPTER 1.

LITERATURE REVIEW.

1.1 INTRODUCTION.

The literature relating to the diagnosis and effects of cobalt (Co) deficiency in the pregnant ewe will be reviewed. To gain a full understanding of the results to be presented, particularly those concerned with the effects of Co deficiency on immune status, a wider area of work will be discussed. This may not relate directly to Co deficiency, but is relevant to the original work of this project.

The condition in ruminants, especially sheep, which arises as a result of a dietary deficiency of the trace element Co, must have been recognised as a disease in ancient times. An early reference to the occurrence of Co deficiency in south west Scotland was made by Hogg (1831) in his report on common diseases in sheep. Although the 'Ettrick Shepherd' had no knowledge of the causative agent, he did state that the problem was not contagious, but was related to diet.

Hogg (1831) also noted grazing and land types on which the disease most frequently occurred. He reported that the severity of this 'pining' varied from a marked wasting disease, to a mild ill-thrift and that the best curative measure was the periodical shifting of sheep to different pastures.

Reports of the same or similar conditions appeared over the years from many parts of the World. The names applied to the syndrome were wide and varied, for example, moor sickness, Moiroa dopiness, Morton Mains disease, bush

sickness, Denmark wasting disease, coast disease and coastiness. Other names derived from the clinical symptoms of the deficiency, such as pine, vinquish, daising, enzootic marasmus and neck ail.

A century after Hogg made his observations, Australian workers pioneered the investigation of the cause of the disease. Initial experiments led to the speculation that the problem was a deficiency of iron in the diet (Aston, 1932). It was found that doses of limonite, a naturally occurring hydrated oxide of lime (Fe_2O_3), could cure the weight loss and anaemia associated with the clinical syndrome in sheep. Such treatment was widely used. However, that iron was the curative agent was challenged by Filmer (1933), who stated that the "...disease is due to some mineral which is present in the iron compounds found to be successful in prophylaxis and treatment."

The search for this mineral continued apace and fractionation studies on limonite (Underwood and Filmer, 1935) and the dosing of diseased penned sheep with Co salts (Lines, 1935 and Marston, 1935), finally ascertained that the disease was due to a dearth of Co in the diet. In field situations, the syndrome was often associated with a complicating copper deficiency, but Askew (1939) and McDonald (1942) reported cases of unequivocal Co deficiency in southern Australia.

In Scotland, early work on defining Co deficiency was undertaken by McGowan and Smith (1922); Corner (1939) in

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Berwickshire, Roxburghshire and Selkirkshire; Stewart, Mitchell and Stewart (1941 and 1942) in Ross-shire, Inverness-shire and Sutherlandshire; Stewart, Mitchell, Stewart and Young (1946) in Kirkcudbrightshire; Dunlop (1946a) in Dumfries and Galloway and Ayrshire and by Boddie (1947) in the Hebrides. These workers recognised the syndrome in both clinical and sub-clinical forms.

1.2 THE DISCOVERY OF VITAMIN B12.

The biological form of Co in animal tissues was not apparent until Smith (1948a), simultaneously with workers in North America, isolated an anti-pernicious anaemia factor from liver. Four tons of material yielded one gram of a red substance, which contained two red pigments. The compound was named vitamin B12 and the presence of Co in its molecular structure was quickly recognised (Smith, 1948b).

When sufficient supplies of this newly discovered vitamin became available, Anderson and Andrews (1952) were able to show that injection of small amounts of B12 were fully effective against Co deficiency disease in sheep. However, relatively large amounts were needed to show any effect if the vitamin was given orally (Andrews and Anderson, 1954 and Kercher and Smith, 1955). It was then a short step to postulate and determine that Co was active in preventing deficiency, because it was incorporated into B12 between ingestion and absorption into circulation (Phillipson and Mitchell, 1952 and

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Kercher and Smith, 1956).

A further link in this story was made when notice was taken of the earlier studies of Tosic and Mitchell (1948). These workers reported that rumen microorganisms in sheep could concentrate Co. Thus, it became clear that dietary Co was incorporated into vitamin B12 by the rumen microbes (Smith and Loosli, 1957). These organisms synthesise B12 which is stored in the liver of the host animal (Andrews, Hart and Stephenson, 1958). Ford and Hutner (1955) reported that B12 was essential for rumen microbial metabolic processes, while Dryden, Hartman, Bryant, Robinson and Moore (1962) found that these microbes produced different forms of the vitamin, some of which were physiologically active and some inactive in the host animal.

The processes and inter-relations of the rumen microorganisms that produce vitamin B12 are little known, but that both ciliates and bacteria perform this de novo synthesis, has been established (Bonhomme, Durand, Quintana and Halpern, 1982 and Jetera, Olivera and Roth, 1984). Further, the absorption and transport mechanisms for vitamin B12 have also been researched and documented, particularly in humans (Smith, 1965 and Ellenbogen, 1979).

1.3 THE METABOLIC ROLE OF COBALT.

To the limit of present knowledge, Co has no physiological role in higher animals, except via incorporation into vitamin B12. Different forms of this vitamin, the 'cobalamins,' exist (Dryden et al, 1962), but only two are physiologically active in mammalian tissues (Millar and Penrose, 1980). These are adenosylcobalamin and methylcobalamin. The molecular structure of the former is shown in Figure 1. The 'corrin' ring of this molecule, with four positions A, B, C, and D, has a Co atom as its centre. This is the case for all the cobalamins (Castle, 1975).

1.3.1 Vitamin B12 and Methylmalonyl CoA Mutase.

The function of adenosylcobalamin in mammalian tissues is as a co-factor in the reversible conversion of methylmalonyl CoA to succinyl CoA. This conversion takes place on the pathway of degradation of propionyl CoA, as shown in Figure 2. In this sequence, propionyl CoA is carboxylated to D-methylmalonyl CoA in a biotin dependent reaction. D-methylmalonyl CoA then undergoes an enzyme catalysed racemization; a step which is necessary because the B12 dependent mutase is specific for the L-enantiomer. L-methylmalonyl CoA is then converted to succinyl CoA by the B12 dependent enzyme methylmalonyl CoA mutase, EC 5.4.99.2 (Babior, 1975).

This reaction is of vital importance to the ruminant. The volatile fatty acid propionate is produced by the
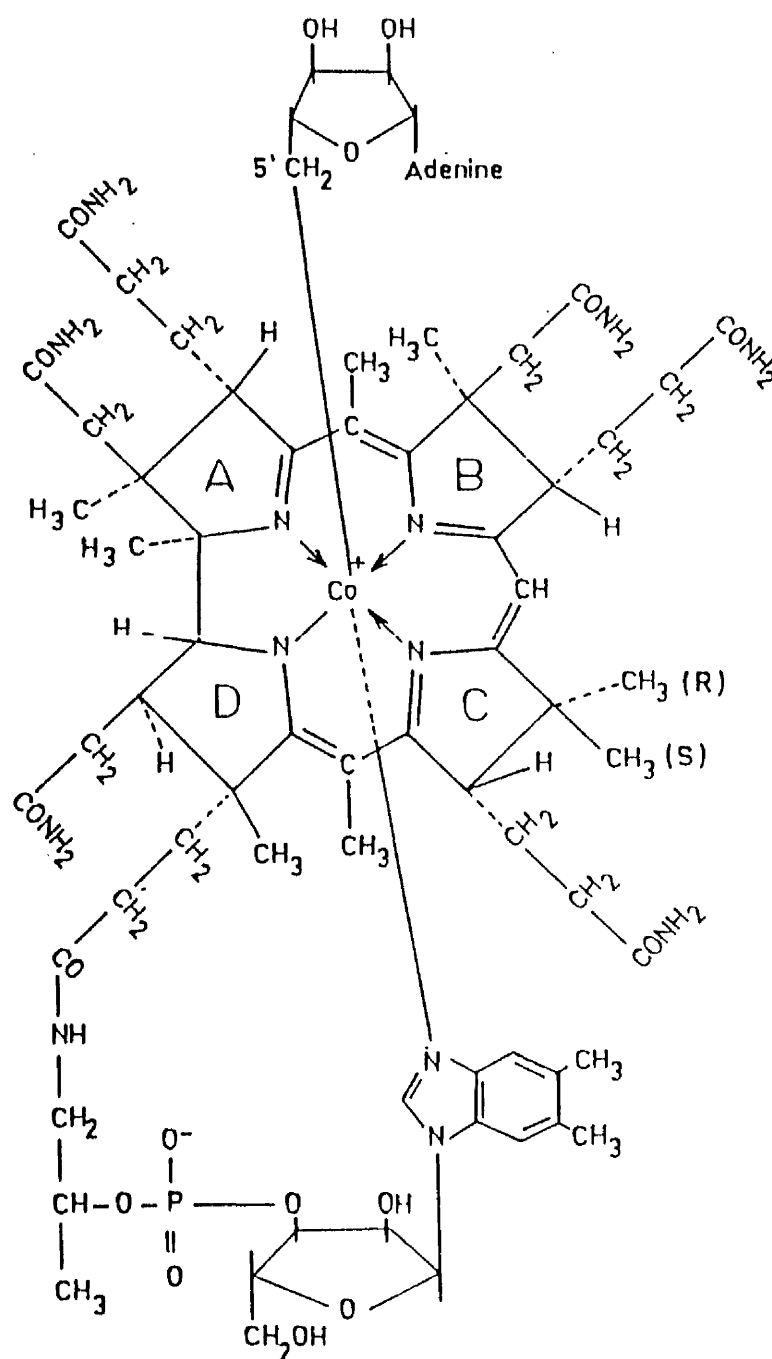
Figure 1. The molecular structure of adenosylcobalamin (vitamin B12), showing the A, B, C and D positions of the corrin ring. (After Schneider and Stroinski, 1987).

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Propionyl CoA

$CH_3CH_2COSCoA$

ATP, CO_2

propionyl CoA
carboxylase

D-Methylmalonyl CoA

CH_3—CH
 | \
COSCoA COOH

racemase

L-Methylmalonyl CoA

CH_3—CH
 | \
COOH   COSCoA

methylmalonyl CoA mutase
(EC 5.4.99.2)

+ adenosylcobalamin

Succinyl CoA

CH_2—CH_2
 | \
COSCoA COOH
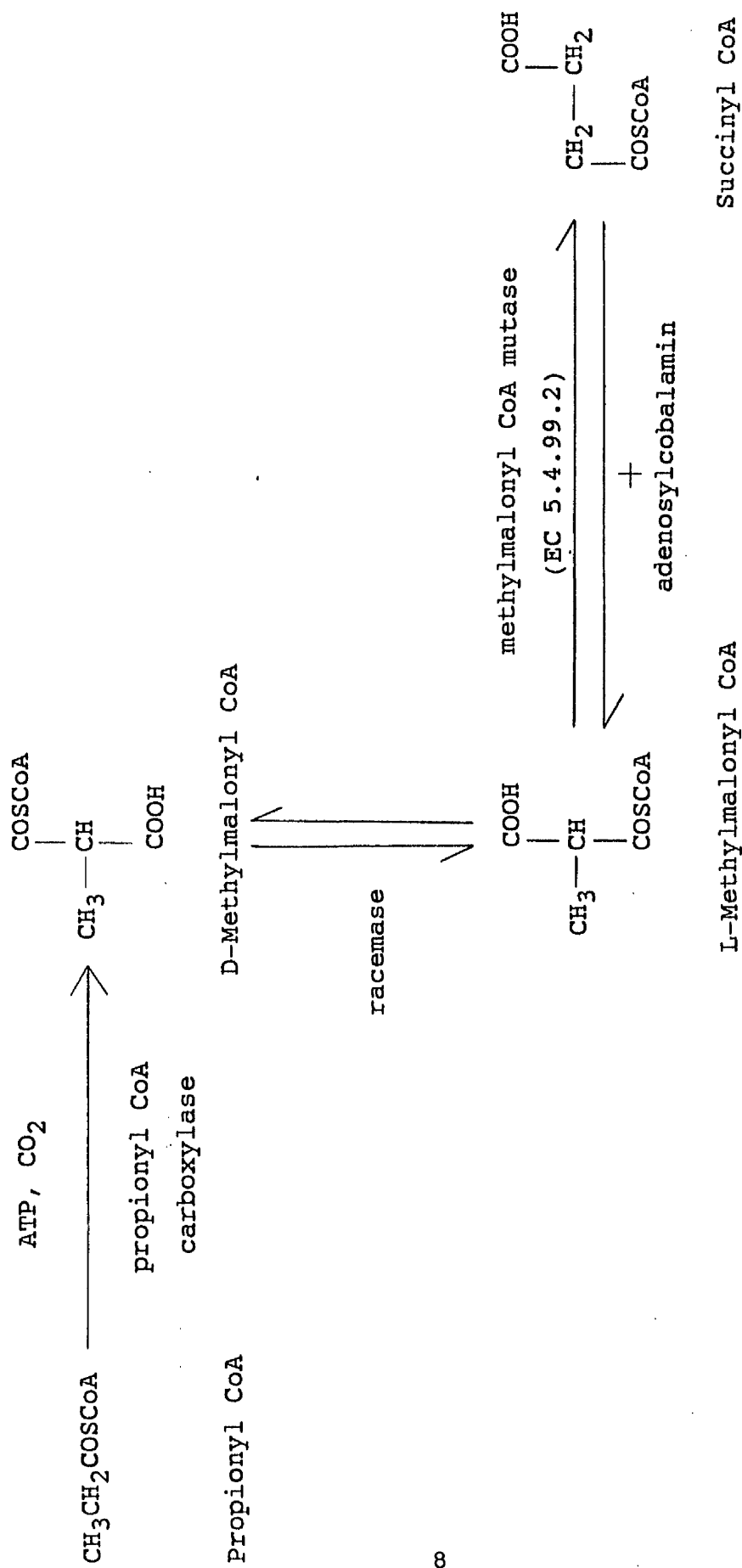
Figure 2. The conversion of propionyl CoA to succinyl CoA, catalysed by the vitamin B12 dependent enzyme, methylmalonyl CoA mutase. (Adapted from Babior, 1975).

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rumen microbes and is a major energy source to the host animal (McDonald, Edwards and Greenhalgh, 1981), which is catabolised within the liver cells. Succinyl CoA thus produced is an intermediate of the Kreb's tricarboxylic acid cycle. Propionate can be used via this pathway (linked with the electron transport chain) as a substrate for the production of energy. Excess can also be employed in the production of glucose, when necessary.

The impairment of propionate catabolism is the primary metabolic defect, supervening Co/vitamin B12 deficiency in sheep. This was established by Marston, Allen and Smith (1961) and was also reported to occur in the rat by Smith and Monty (1959). More detailed work at the liver cell level, investigating the role of B12 as a co-factor for this enzyme, was reported in monogastrics by Cardinale, Dreyfus, Auld and Abeles (1969) and in the ovine by Peters and Elliot (1984).

1.3.2 Vitamin B12 and Methionine Synthetase.

The function of methylcobalamin in mammalian tissues is as a co-factor to the enzyme homocysteine: N^5-methyltetrahydrofolate methyltransferase, EC 2.1.1.13 (Schneider and Stroinski, 1987). The reactions in which this enzyme system is involved are shown in Figure 3. The end point of the pathway is the production of the amino acid methionine.

The importance of this reaction to ruminants is not entirely clear. The sequence was first studied in sheep

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Histidine

histidase

NH$_3$

Urocanic acid

urocanase

H$_2$O

4-Imidazolone-5-propionic acid

4-I-5P hydrolase

Homocysteine

Methionine

$$
\begin{array}{c}
\text{COOH} \\
| \\
\text{H}-\text{C}-\text{NH}-\text{CH}=\text{NH} \\
| \\
\text{CH}_2 \\
| \\
\text{CH}_2-\text{COOH}
\end{array}
$$

Tetrahydrofolic acid

Formimino-L-glutamic acid

Vitamin B12
dependent enzyme
(EC 2.1.1.13)

5-Methyl
tetrahydrofolate

5-Formimino
tetrahydrofolate

L-Glutamic acid
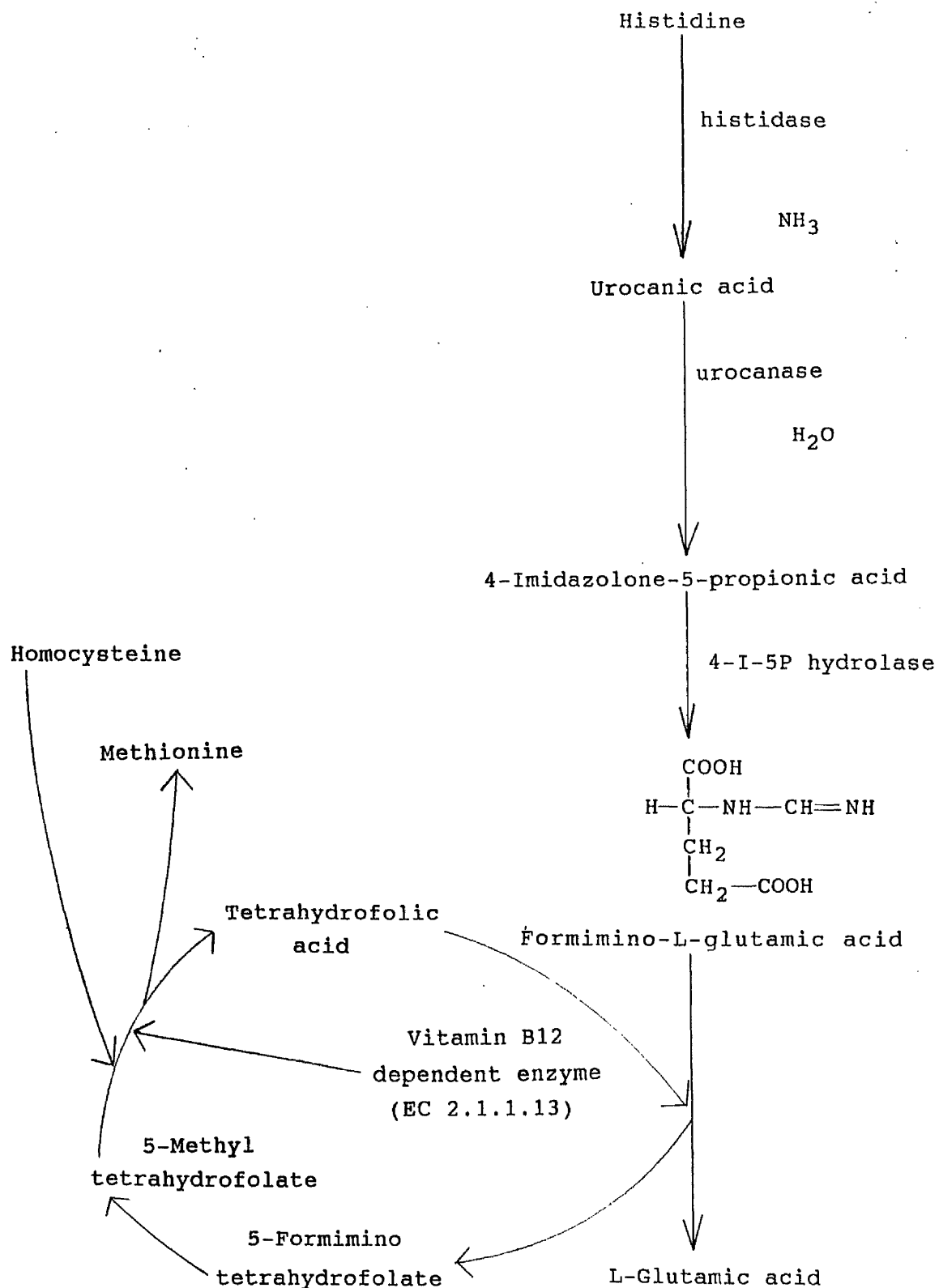
Figure 3. The role of Vitamin B12 in the homocysteine: N^5-methyltetrahydrofolate methyltransferase (EC 2.1.1.13) reaction, in the 'methionine synthetase' pathway. (Adapted from Givens, 1978).

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by Gawthorne (1968) and has been investigated in more detail since (Smith, Osborne-White and Gawthorne, 1974).

1.4 NUTRITIONAL ASPECTS.

Much work has been published regarding the physiologically active and inactive forms of vitamin B12, synthesised by the rumen microorganisms. The effects of Co intake and dietary composition on this production and subsequent absorption in the host amimal, have also been widely studied. This section will review the literature pertaining to these influences.

1.4.1 Synthesis of Vitamin B12 in the Rumen.

In terms of 'true' vitamin B12 supply to an animal, the relative proportions and rates of production by rumen microbes of the true forms and inactive analogues, are important factors. Bigger, Elliot and Rickard (1976) stated that the proportionate production of B12 forms in sheep was influenced by the dietary characteristics of Co content and roughage:concentrate ratio. Further, these workers proposed that the levels and proportions synthesised influence the absorption of true B12 by the host animal and hence affect B12 status.

Smith and Marston (1970) reported that the efficiency of Co incorporation into B12 was higher when Co was depleted in the rumen than when it was present in excess. These workers also stated that the efficiency of Co incorporation by rumen microbes was greater when Co

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originated from the diet, than when Co was provided via
an oral drench.

More specifically, Bigger et al (1976) investigated the
ruminal production rates of three inactive analogues of
B12 in sheep, in relation to the synthesis of true
vitamin. These data are shown in Table 1.

Table 1. Rumen microbial production of true vitamin B12
and three inactive analogues.

FORM OF B12	ug/day ± S.D.	ug/g DDMI ± S.D.
Pseudovit. B12	324 ± 63	0.52 ± 0.10
Factor A	1309 ± 112	2.15 ± 0.19
Factor B	757 ± 86	1.24 ± 0.14
True vit. B12	1725 ± 101	2.84 ± 0.17

DDMI = Digestible dry matter intake.
[Adapted from Bigger et al (1976)]

In comparison with this, Smith and Marston (1970)
presented ranges of ruminal production rates from >400
ug/d in Co sufficient sheep to <100 ug/d in deficient
animals. Hedrich, Elliot and Lowe (1973) reported total
B12 production levels in sheep given 0.047 (Co-
deficient), 0.41 and 0.83 mg Co/d in the diet, of 37,
1006 and 1553 ug/d, respectively.

Sutton and Elliot (1972) also studied various
nutritional aspects using duodenal and ileal re-entrant
canulated sheep. These workers concluded that increasing

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the amount of concentrate feed given, relative to roughage, from zero to 60 per cent ground corn:40 per cent ground hay, reduced the rate of microbial total B12 production from 1.6 to 0.9 ug/g DDMI and also decreased the amount of true vitamin as a proportion of the total synthesised.

In dairy cattle, Walker and Elliot (1972) also reported that increasing the concentrate:roughage ratio in the diet resulted in a higher proportion of inactive B12 analogues being produced by the rumen microflora.

Gawthorne (1970a) studied the influence of Co intake on microbial B12 synthesis. His investigations showed that true vitamin as a proportion of the whole produced, increased from 35 to 65 per cent, as Co in the diet was reduced from 0.34 to 0.04 p.p.m. More recently, McDonald and Suttle (1986) reported that Co intake per se, as well as dietary form, could affect the type of rumen fermentation present. In studies with model in vitro rumens, these workers concluded that decreasing Co intake lead to reduced growth of acetate producing microorganisms and increased growth of propionate producing microbes. The overall effect of declining Co intake was therefore to depress acetate:propionate ratio in rumen fluid, with a consequent propionate type fermentation.

1.4.2 Absorption of Vitamin B12.

Smith and Marston (1970) and Hedrich et al (1973) both stated that the site of absorption is the small intestine. The former showed that over 90 per cent of B12 was associated with the fibrous phase of rumen digesta, due to adherence of vitamin containing bacteria to solid material. These microbial cells are lysed in the acid conditions of the abomasum, releasing the B12, which is largely unharmed as it passes to the lower tract.

The efficiency of absorption is low, with the majority of the vitamin lost in the faeces. Efficiency values for the absorption of total B12 are quoted at circa 5 per cent (Smith and Marston, 1970) and circa 3 per cent (Gawthorne, 1970a).

By tracing the destination of ^{57}Co labelled B12 analogues, Rickard and Elliot (1978) reported that the efficiency of absorption of cyanocobalamin and factor B in sheep was 8 to 38 per cent and 7 to 22 per cent respectively. These authors also noted that these efficiencies were "...higher with a diet which would result in predicted synthesis of B12 of 113 ug daily, than with those with predicted synthesis of over 1000 ug daily."

Once absorbed, Smith and Marston (1970) attempted to show the fate of B12, by tracing an injection of 30.4 ug of [^{60}Co] cobalamin. They recovered 5.7 per cent from faeces, 2.6 from urine and 25.6 in the liver. Of the vitamin deposited in the liver, 52 per cent was

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associated with mitochondria. The remainder of the vitamin was distributed throughout the body organs and interstitium.

Whether or not there is preferential absorption of true vitamin B12 over inactive analogues, is not entirely clear. Sutton and Elliot (1972) reported significant levels of inactive analogue forms as part of the total vitamin determined in sheep serum. On the contrary, Gawthorne (1970a), Marston (1970) and Halpin, Harris, Caple and Patterson (1984) found no or negligible amounts of inactive analogues in sheep serum, suggesting that true B12 is preferentially absorbed in the ovine.

1.5 THE COBALT REQUIREMENTS OF SHEEP.

Many reports in the literature have attempted to define the Co requirements of sheep. Such experiments have usually taken the form of 'dose/response' trials. Table 2 reviews some of this data. From this table and the assertions of Andrews (1970a), young rapidly growing sheep have the greatest requirement for Co, followed by lactating ewes and finally mature ovines.

Table 2.   The requirement of sheep for cobalt.

ANIMAL	REQUIREMENT	OBSERVATIONS
Young lamb	0.20 mg Co/d[a]	Optimum liveweight performance, maintained blood haemoglobin.
Mature ewe	0.08 mg Co/d[a]	Optimum liveweight and maintained blood haemoblobin.
Mature ovine	0.07 - 0.08[b] mg Co/kg DM	Normal clinical condition, serum B12 marginal.
Mature ovine	0.10 mg Co/d[c]	Optimum liveweight and serum B12.
Mature Ovine	0.11 - 0.13[d] mg Co/Kg DM	Normal liveweight gain, serum and liver B12.
Ovine	0.11[e] mg Co/Kg DM	Optimum liveweight gain and serum B12.
Lactating ewe	0.15[f] mg Co/d	Normal milk yield and neonatal lamb growth rate.

REFERENCES:
a - Lee and Marston (1969)
b - Somers and Gawthorne (1969)
c - Gawthorne (1970)
d - Marston (1970)
e - MacPherson (1982)
f - Quirk and Norton (1987)

1.6 MANIFESTATIONS OF COBALT DEFICIENCY.

The effects of Co deficiency in ruminant livestock can
be split into two broad areas, namely clinical and sub-
clinical. The consequences of clinical Co deficiency are
produced by prolonged periods of severe Co deprivation
and are manifested by animal ill-health. Sub-clinical Co
deficiency can occur during a development into clinical
disease, before animal health is affected, or as a result

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of prolonged periods on a marginal Co intake (Latteur, 1962).

The greatest loss, in terms of animal production, occurs through the sub-clinical syndrome (Andrews, 1965). Farmers may not realise that a sub-clinical Co deficiency problem exists, but livestock fail to attain maximal productive performance. With clinical deficiency, acute animal suffering is evident and investigation of its cause usually leads to its diagnosis.

It is important to note however, that these two degrees of Co deficiency are not static forms. In a Co-deficient area the situation is usually in a state of flux, with animals experiencing sub-clinical developing to clinical disease; or recovering from clinical symptoms to sub-clinical deficiency, or further to a sufficient state.

These changing circumstances will be affected by soil and herbage factors, along with climatic and seasonal influences. For example, it has long been established that severe Co deficiency is most prevalent in the grass growing season (Hogg, 1831) and some remission is afforded in the autumn and winter, when sward length is low and increased soil ingestion provides an improvement in Co status (MacPherson, 1982). Further, clinical deficiency can be more apparent in the field situation, where poor nutrition, inclement weather and parasitic attack may contribute as additional stressors. In such circumstances, a clinical deficiency may be precipitated more rapidly and with greater effect on animal health,

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than if the syndrome develops in a controlled housed situation (MacPherson, 1981).

1.6.1 Clinical Cobalt Deficiency.

The detrimental effects of Co deficiency arise from a dearth of Co/vitamin B12 for metabolic functions. As such, the development and existence of clinical disease is not only characterised by outward symptoms, but also by changes in the concentrations of metabolites in tissues. Thus, as the primary role of Co/B12 is in the catabolism of a major ruminant energy source, propionate, deficiency results in a lack of energy supply to the animal. This was shown by Marston, Allen and Smith (1972), who demonstrated a clear inability of Co-deficient sheep to remove propionate from circulation. The liver catabolism and gain of energy from this volatile fatty acid, is therefore reduced. These observations were confirmed more recently by Peters, Bergman and Elliot (1983a and 1983b).

Prolonged high levels of propionate in blood lead to a reduction in voluntary feed intake, mediated by the nervous mechanisms which contol appetite and respond to concentrations of metabolites in circulation (McDonald, Edwards and Greenhalgh, 1981). Therefore, the main symptoms of clinical Co deficiency arise from a dearth in energy supply and inappetence and are mainly indistinguishable from those of starvation. Thus, animals

appear lethargic and emaciated, with the musculature wasted (marasmus). The mucous membranes and skin are pale and fragile, from a progressive normochromic, normocytic anaemia (Smith and Loosli, 1957), with depressed packed cell volume (PCV) and red cell counts (Gawthorne, Somers and Woodliff, 1966). In addition, animals may be photosensitive, with profuse lacrymation.

These visual symptoms are accompanied by internal tissue and metabolite changes. The time taken to develop clinical disease will depend on Co history, individual animal variation and the degree of deficiency in the diet. However, MacPherson, Moon and Voss (1976) reported a loss of appetite in housed sheep after 11 weeks of Co deficient intake. Plasma glucose levels fell after 34 weeks implying an impaired propionate metabolism, with a fall in whole blood haemoglobin concentrations due to progressive anaemia, after 44 weeks. In serum, ascorbic acid levels were depressed and glutamic-oxalacetic transaminase (GOT) concentrations elevated, indicating liver damage (MacPherson and Moon, 1974). Low serum pyruvate kinase and blood pyruvate levels were also evident, implying an induced thiamine deficiency.

These studies demonstrate that clinical Co/B12 deficiency leads to a series of complex biochemical changes, where primary and secondary causes and effects are difficult to characterise. For example, that cerebrocortical necrosis (CCN) is a consequence of thiamine deficiency is well documented (Edwin and Lewis,

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1971). However, Hartley, Kater and Andrews (1962), MacPherson et al (1976) and MacPherson, Moon and Voss. (1977) described the occurrence of CCN in severely Co deficient sheep. Although the numbers found suffering were not large, it was positively demonstrated that Co supplementation prevented the appearance of CCN in situations where prolonged and severe disease existed. Such findings have been refuted (Edwin, 1977), but the complex aetiology of clinical symptoms is thus demonstrated.

Further effects of clinical Co deficiency on the central nervous system (CNS) have been documented. Garton, Duncan and Fell (1981) reported atrophy and degeneration of neurones in the cerebral cortex and brain stem of clinically Co-deficient ewes. Lambs from these sheep showed reduced numbers and broadening of the cerebral gyri. These workers have also reported the occurrence of astrocytosis in the brain and spinal cord, with severe status spongiosus along the junction of the grey and white matters in clinically Co-deficient animals (Fell, Hesketh, Lough, Duncan and Mackie, 1985). Both of these papers concluded that such CNS changes were probably secondary to the clinical effects of Co deficiency on the liver and could, therefore, be described as resulting from hepatocerebral disease.

It may be surmised from these discussions that the symptoms of clinical Co deficiency occur mainly as a

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result of liver dysfunction. This observation is further enhanced by reports of white liver disease (WLD) in ovines suffering from the clinical condition. At post mortem the liver may appear pale, swollen and fatty. This may be a consequence of propionate and methylmalonyl CoA being used as precursors for the synthesis of triglycerides. Duncan, Morrison and Garton (1981) reported elevated levels of odd numbered and branched chain fatty acids in the livers of Co-deficient lambs. The former indicate the employment of propionate as a precursor for fatty acid anabolism; the latter implies that excess methylmalonic acid (MMA) replaces malonate as a chain extension unit during fatty acid production in the liver. These observations may be linked with the occurrence of ovine WLD, reported in New Zealand (Clark, Cornforth, Jones, McKnight and Oliver, 1978 and Sutherland, Cordes and Carthew, 1979), Northern Ireland (McLoughlin, Rice and Taylor, 1984) and Norway (Ulvund and Overas, 1980).

Other symptoms of clinical Co deficiency in the ovine, such as hyperplasia of the bone marrow (Ibbotson, Shirley and Gurney, 1970) have not received much attention in the literature. However, the seriousness of the condition is well illustrated by the evidence which has been presented above.

1.6.2 Sub-Clinical Cobalt Deficiency.

As no outward symptoms are evident in marginal, or sub-clinical Co defciency, this form of the disease is harder to characterise than the clinical syndrome. However, dose/response trials have demonstrated that ruminants of marginal Co/B12 status, show sub-optimal production. This was recognised by Dunlop (1946b), who reported that non-supplemented sheep in "...sub-minimal Co-deficient areas" had depressed fertility and reduced lamb liveweight gain, compared to Co-supplemented controls.

Many other such reports can be cited. For example, a detailed investigation by Andrews (1965) on the thrift of young sheep on marginally Co-deficient pasture at Wallaceville, Wellington, New Zealand, demonstrated the fluctuating state of deficient animals. In some years no response by growing lambs to Co supplementation could be reported and sufficiency was presumed. However, in other seasons, Co dosed animals showed lower death rates from parasitic infection and greater (though not statistically significantly different) liveweight gains, than undosed controls.

1.7 THE EFFECTS OF COBALT DEFICIENCY ON PREGNANCY AND LACTATION.

1.7.1 Present Knowledge.

The possibility that Co deficiency might have a detrimental effect on ewe and lamb performance, received early recognition by Dunlop (1946b). Working throughout

Scotland, he reported that on one farm, the administration of 100 mg Co/head per os on three occassions (before tupping, before lambing and at the summer dipping) resulted in a reduction in the number of barren ewes and an increase in lambing percentage, compared to untreated controls (Table 3).

Table 3. The effect of 100 mg/head oral doses of Co before tupping, before lambing and at summer dipping on ewe mean reproductive performance, over four years on a Scottish hill grazing.

TREATMENT	% BARREN EWES (± SED)	% EWES WITH LAMBS AT 8 WEEKS (± SED)
-Co	7.1 ± 1.5	76.3 ± 2.6
+Co	4.3 ± 0.5	89.4 ± 2.4

(Adapted from Dunlop, 1946b).

In another experiment the administration of 100 mg Co/head per os 14 days before tupping, had a similar effect of improving reproductive performance. An additional observation in this trial was that treated ewes lambed earlier than untreated controls.

This pioneering work provided circumstantial evidence for an effect of Co deficiency on the presence of oestrus, blastocyst implantation and foetal development. The appearance of late lambs from ewes not dosed with Co, could be explained by deficiency affecting the timing of the first fertile seasonal oestrus. A delay in this first

oestrus in Co-deficient sheep would result in the observed late lambing. The increase in the number of barren ewes in deficient compared to Co dosed sheep could be explained by anoestrus behaviour, or by failure of blastocyst implantation, with subsequent _in utero_ reabsorption. Further, the reduced lambing percentage might provide circumstantial evidence for decreased ovulation rates, increased numbers of abortions or depressed neonatal lamb viability in deficient ewes, compared to sufficient controls.

There is a lack of evidence in the literature which would explain Dunlop's findings in detail and whether or not these effects might be widespread in practice. However, some work can be cited which might provide clues to the explanations required.

For example, Mgongo, Gombe and Ogaa (1985) found that goats in the early stages of clinical Co deficiency, showed irregular oestrus (22.6 $\pm$ 0.8 vs. 18.0 $\pm$ 0.3 days between cycles) and lower cyclic progesterone and luetinising hormone concentrations in blood, than Co-sufficient controls. In a later experiment, these same workers found evidence of a greater number of anovulatory cycles in Co-deficient compared to sufficient East African short-horned goats (Mgongo, Ogaa and Gombe, 1986). They concluded that the site of action in this disruption of hormonally contolled oestrus, was the hypothalamus-pituitary axis.

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The Aberdeen workers Duncan et al (1981), attempted to characterise the effect of Co deficiency on ewe reproduction and lamb viability and relate observations to serum concentrations of vitamin B12 and urinary MMA levels. In a housed trial, a Co-deficient ration was fed to two groups of Scottish Blackface sheep. Group A, comprising 9 animals, were introduced to the deficient diet at tupping and two ewes received a Co supplement in the drinking water. The 8 sheep of group B fed on the Co-deficient ration from 16 weeks before mating and three of these animals were provided with the Co supplement.

Due to the small numbers of sheep used, differences between Co treated and control ewes were not statistically significant. However, these workers reported increased numbers of perinatal mortalities and stillbirths in lambs from deficient dams, compared to sufficient controls. These findings could be related to serum B12 and urinary MMA status, which indicated varying degrees of Co deficiency from mid-pregnancy to parturition. Three of the group B ewes and one from group A were clinically deficient by lambing time, as judged by serum B12 concentrations, although urinary MMA levels were more varied and difficult to relate to reproductive performance.

In a similar experiment, Garton et al (1981) reported comparable results and concluded that "...perinatal mortality and morbidity can be high amongst lambs born to Co-deficient ewes when they have received an inadequate

intake of Co for some time before, and during pregnancy. It may be more than coincidental that enhanced production of MMA... is apparently associated with failure of the ewes to produce viable lambs."

The Australian investigators Quirk and Norton (1987), reported that clinically Co-deficient ewes produced fewer lambs, with depressed birthweights and post natal liveweight gains, compared to sufficient controls. Clinically deficient ewes also yielded less milk than sufficient counterparts. This work was published after the completion of the studies to be reported in this project. Therefore, the results of Quirk and Norton (1987) will be discussed later in Chapter 5.

The detrimental effects of Co deficiency on milk yields and milk B12 content, have been studied by other investigators. Peters and Elliot (1983) could not find evidence of an influence of Co deficiency on milk yield. However, in a trial where ewes were rendered clinically Co-defciient, voluntary feed intake, liveweight change and daily milk protein production were lower in a group of deficient dams than in sufficient controls. In addition, O'Halloran and Skerman (1961) also reported a depression in colostrum and milk protein content in Co-deficient ewes, as well as low lipid levels.

O'Halloran and Skerman (1961) also reported evidence of decreased B12 concentrations in milk from Co-deficient ewes, while lambs from these animals had low liver B12

levels, compared to sufficient controls. Further, lambs born to deficient sheep were lighter at birth and had slower initial liveweight gains, than those from sufficient dams, although these effects were not statistically significant.

While studying the use of intra-ruminal cobalt oxide pellets as a cure for Co deficiency, Hart and Andrews (1959) found that providing ewes on marginally Co-deficient pasture with this form of supplementation, raised their milk B12 levels at 3 months <u>post partum</u> above those in non-supplemented controls (10.3 vs. 2.5 ug B12/l milk, respectively). Although these workers did not relate their results to lamb liveweight gain, this evidence suggests that a response to Co supplementation in terms of milk B12 content, can be achieved in a sub-clinical as well as in a clinical disease situation.

1.7.2 <u>Future Research.</u>

Mills (1981) identified the area of Co deficiency and reproductive performance as one which warranted further and close investigation. The study of Quirk and Norton (1987) contributed greatly to this requirement, but questions still remain. For example, the exact mechanisms of cause and effect between Co/B12 deficiency and reduced ewe fertility and lamb viability, are still largely unknown.

The influence of sub-clinical disease is unclear. Most of the evidence in the literature is

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derived from experiments where animals were rendered clinically Co-deficient. However, Dunlop (1946b) recognised that ewe fertility was depressed by the sub-clinical, as well as the clinical syndrome. Thus, the influences of sub-clinical Co deficiency on ewe reproductive performance await clarification.

In the clinical situation, it is difficult to differentiate between the direct effects of B12 deficiency and those mediated indirectly, via an induced reduction in energy supply to the reproducing ewe. In marginal deficiency cases, energy supply to the ewe and lamb can be presumed to be normal, as voluntary feed intake is unaffected and clinical symptoms do not exist. Under these circumstances, direct effects of Co/B12 deficiency may be assumed and at present, such influences need investigation and explanation.

1.8 IMMUNOLOGICAL ASPECTS OF COBALT DEFICIENCY IN RUMINANTS.

In reviewing the literature concerned with Co deficiency in ruminant livestock, Andrews (1970b) stated that, "...we know little of what effects deficient states may have on immunity to common pathogens." Eighteen years on, characterisation of the effects of Co or vitamin B12 deficiency on immune response and the resistance to pathogenic attack is not much further forward. This not only applies to the ruminant species, but to all other domesticated animals and man.

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This section will review evidence in the literature which does indicate a role for Co in host animal immune response to infection. Three areas will be discussed: a) The interaction of Co deficiency and immune status with respect to gastrointestinal parasitic worm infection, b) The effect of Co deficiency on immunocompetent cell function and c) The possible influence of Co deficiency on the transfer of passively acquired immunity from the ewe to the neonate lamb.

1.8.1 Immunity to Parasitic Worm Infestation.

That Co or vitamin B12 might be essential factors in the efficient immune response to pathogenic attack, was first implied by observations on interactions between the deficient state and gastrointestinal parasitic worm burdens. Investigative trials have taken the form of employing animals either sufficient or deficient in the trace element and free from worm burdens or given a measured parasitic infestation. The influence of anthelmintics on top of a combination of these treatments, has also been studied.

A degree of confusion has arisen, where the evidence from some trials has suggested that supplementation of deficient animals with Co enhances worm infestation, compared to unsupplemented controls (Threlkeld, Price and Linkous, 1956, Downey, 1965, Nicol, Smith, Dimmock, Green, Murphy and Barry, 1983). Other experiments have

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concluded the opposite and indicate that Co-deficient ruminants are more susceptible to parasitism, compared to sufficient counterparts (Downey, 1966, Andrews, Hogan, Stephenson, White and Elliot, 1970 and MacPherson, Gray, Mitchell and Taylor, 1987).

The results of these conflicting reports, suggest that the worms themselves may require Co or vitamin B12 to be maximally infective. The number of worm eggs from established adults appearing in the host faeces and the number of adult worms present in the gut at post mortem examination are generally used as indicators of the degree of parasitic infestation and the level of immune resistance in the host animal.

In reviewing the literature which supports the contention that dosing deficient animals with Co actually enhances the establishment of parasitic worms, it is important to note the degree of deficiency imposed and the size of the worm burden given. Threlkeld et al (1956) took lambs weaned at one week of age and fed a Co-deficient ration, which eventually produced clinical symptoms. A large worm burden of 70,000 infective larvae, being a mixture of the three abomasal parasites Haemonchus contortus, Ostertagia circumcincta and Trichostrongylus axei, was administered in a single oral dose. Although at necropsy all treatments showed similar numbers of established worms in the abomasal mucosa, lambs recieving Co supplementation and given a worm infestation showed higher rates of mortality due to

helminthosis than those clinically deficient in Co and also infected. Dosing with Co had a significant positive effect on liveweight gain compared to deficient controls, but seemed to encourage a more vigorous establishment.

In contrast, Andrews et al (1970) investigated the effects of Co and anthelmintic (thiabendazole) therapy, on parasitic worm burdens in marginally Co-deficient grazing sheep. In animals where no anthelmintic was given, Co supplementation significantly reduced faecal worm egg counts compared to deficient controls (Table 5).

Table 5. Mean faecal worm egg counts (No:/g) in Co-deficient and sufficient sheep.

	September	December
-Co	290	1230
+Co	70	100
Sign.	P<0.05	P<0.01

(After Andrews et al, 1970)

The work of Downey (1966) supports these results. This investigator studied lambs given a mild to moderate infestation of O. circumcincta, on top of a long standing T. axei burden. The lambs were maintained in a sub-clinically Co-deficient state and were infected with 15,000 larvae, followed by 17,000 more 24 hours later. After 69 days, a further 32,000 O. circumcincta larvae were administered to these animals.

For infested lambs, those dosed with Co had lower mean faecal O. circumcincta worm egg counts (4920 vs. 9400); lower mean worm numbers recovered from the abomasum at necropsy (3906 vs. 6150) and higher mean whole blood haemoglobin levels 56 days post-infection (8.6 vs. 7.2 g/100ml), compared to controls left in the deficient state. Although none of these results were statistically significant due to the small numbers of animals used, an enhancement in immune response against the parasite was inferred.

Similar evidence was reported by Ferguson, Mitchell and MacPherson (1988) with lambs. From 6 weeks of age, housed animals were given 2,000 infective L_3 larvae of O. circumcincta per week, split into 3 doses. This was repeated for 16 weeks. Co-deficient lambs showed higher mean worm egg counts and greater plasma pepsinogen levels (1500 to 2000 u units/l at 37 ^{O}C), compared to sufficient controls (1000 u units/l at 37 ^{O}C). Pepsinogen leaks into plasma as a result of structural damage to the cells of the abomasal mucosa, caused by the parasites and is thus a measure of worm establishment. In this trial the mortality rate in the infected and Co-deficient group was much higher than in the Co supplemented and infected treatment, and significantly more worms were recovered at necropsy in the former animals.

In order to provide an explanation for these discrepancies in the literature, it must be presumed that

Co and/or vitamin B12 are essential to the developing parasites in the abomasum, as well as to the host. Threlkeld et al (1956) concluded that "...the ability of H. contortus larvae to establish themselves within the host animal appears to be dependent upon the presence of Co in the diet of the host animal." Further, Richard Schumard, Pope, Phillips, Herrick and Bohstedt (1954), speculated that abomasal worms require Co for the efficient production of eggs.

Assuming that worms do use rumen microbially produced vitamin B12, Downey (1966) postulated that in situations where Co intake is very low and liable to precipitate a clinical deficiency in the host, along with the imposition of a large helminth burden, the parasites do not receive their Co/B12 requirement and perform a sub-optimal parasitic function. Thus, in trials where such a situation exists, dosing the host with Co effectively treats the worms for a Co deficiency and they are able to be normally infective. Hence, in such cases Co therapy seems to result in a more highly infective parasitic attack. This could account for the high mortality rates in Co treated and infected lambs, for example in the studies of Downey (1965).

In contrast to this situation, where host Co intake is marginal and the deficiency sub-clinical and the imposed worm burden is mild to moderate, then the Co/B12 requirement of the parasites is met. Dosing with Co to

the host does not therefore enhance worm establishment. In this situation, the detrimental effect of Co deficiency on immune response to the parasite is evident and Co supplementation appears to offer some protection against an overtly vigorous establishment.

If this is indeed the case, it could be presumed that wherever a Co deficiency existed, immune dysfunction against the parasite would occur. Whether or not Co supplementation resulted in a more effective establishment in the host would then depend on the size of the worm burden imposed and the level of Co deficiency evident. Downey (1966) further pointed out that different worm species could have varying Co/B12 requirements and this would add to the complexity of the situation.

1.8.2 Immunocompetent Cell Function.

The immune system in higher animals is immensely complex and involves the independent and interdependent functions of mucosal barriers, cellular and humoral factors. The specific effects of Co deficiency on immune function could be in any or all of these areas. The delineation of cause and effect thus delves into the fields of immunology and cellular biology, not just that of trace element nutrition. However, there is some evidence in the literature which suggests possible mechanisms for a detrimental effect of Co and other trace element deficiencies on immunocompetent cell function.

Neutrophils are roaming polymorphonuclear (PMN) immune

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cells, which phagocytose and destroy foreign cells and particles. Phagocytosis is dependent on cell receptor recognition of a pathogen (antigen) and this can be mediated and enhanced by humoral factors such as antibodies (immunoglobulins - Ig) and complement, via the processes of opsonisation and agglutination. The 'killing' or cellular digestion of engulfed cells and particles is dependent on many enzymic mechanisms within the neutrophil, which collectively serve as powerful intracellular reducing agents for antigen lysis and destruction.

Boyne and Arthur (1979) demonstrated that selenium (Se) deficiency in Friesian steers did not affect the ability of isolated neutrophils to phagocytose Candida albicans, but the ability of the cells to kill the yeast was significantly reduced in Se-deficient animals, compared to sufficient controls ($P < 0.001$). These workers also found impaired nitroblue tetrazolium (NBT) reduction, depressed peroxidase activity and no glutathione peroxidase (GSH+Px) in neutrophils from deficient steers. Peroxidase is involved with hydrogen peroxide production in the cell. Hydrogen peroxide can act through myeloperoxidases to help destroy ingested particles in the neutrophil. Hence, the decrease in neutrophil peroxidase activity would contribute to the fall in candidacidal activity.

Further, the antioxidant GSH+Px is important in

protecting the enzymes of the hexose monophosphate shunt (HMPS) pathway in neutrophils. This pathway produces nicotinamide adenosine dinucleotide phosphate (NADPH), which is used in the production of the reducing and killing agent, superoxide (O_2^-). Thus, a lack of GSH+Px in neutrophils from Se-deficient steers would result in a depressed superoxide output, accounting for the impaired ability to reduce NBT and impaired microbicidal activity.

Similar effects on neutrophil and leucocyte activity have been reported for deficiencies of copper(Cu). Boyne and Arthur (1981) demonstrated that neutrophils isolated from Cu-deficient steers showed only a 10 per cent kill of C. albicans after 28 weeks on a low Cu diet, compared to 40 per cent for Cu-sufficient controls. Jones and Suttle (1981) reported that while Cu deficiencies in ewes, lambs and cattle did not impair blood leucocyte viability or phagocytic activity against C. albicans, a depression in killing capacity was evident. In ewes, there was a direct relationship (r = 0.75, P<0.001) between plasma Cu levels over the range 1 to 17 umol/l and per cent kill of yeast cells over the range 7 to 21 per cent.

The effects of Co deficiency on immunocompetent cell function are less clear. In humans, there is much evidence that vitamin B12 is essential for nucleic acid synthesis. Deficiency (pernicious anaemia) can result in reduced dividing of cells which normally show great mitotic or meiotic activity. An example is in the

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haematopoietic tissue of the bone marrow, where immunocompetent lymphocytes and leucocytes are produced (Stinnett, 1983). Such changes due to vitamin B12 deficiency have been shown to affect cell mediated and humoral immunity adversely in rats and mice (Gershwin, Beach and Hurley, 1985). However, Stinnett (1983) stated that in humans, these changes only produce moderate and probably insignificant effects on the immune system.

Kaplan and Basford (1976) found that leucocytes isolated from pernicious anaemic patients had a reduced killing capacity for engulphed Staphylococcus aureus cells, compared to those from healthy humans. However, Stinnett (1983) speculated that due to the 'overkill' of PMN activity, only a depression in per cent kill of more than one-half the log of normal levels would lead to a drop in actual resistance to usual pathogenic attack.

The differences between treated and control groups in the study of Kaplan and Basford (1976) were certainly not of this magnitude. It is interesting to note however, that the reduced per cent kill of leucocytes from these pernicious anaemic patients was associated with a reduced ability to initiate the HMPS pathway in these cells. Activated leucocytes from B12 deficient patients had a mean HMPS turnover that was 36 per cent of that evident in cells isolated from normal humans (P<0.005). This pathway produces NADPH, which is oxidised by a flavoprotein oxidase to produce the killing agents

superoxide (O_2^-) and hydrogen peroxide (H_2O_2).

Returning to ruminants, Ibbotson et al (1970) reported hypoplasia of haematopoietic bone marrow tissue of Co-deficient ewes, with resultant reductions in white cell counts. These findings would appear to be similar to those already discussed in pernicious anaemic humans (Stinnett, 1983). However, this evidence came from a group of only three low Co intake sheep and only occurred in severe clinical deficiency.

The Russian worker Nuriev (1986) reported improvements in circulating levels of immunoglobulins M and G, leucocytes, properdin and lysozymes, by supplementing sheep with Cu, Se and Co. Fellow Russians Koval and Medvetskii (1986) reported shown increased white cell bacteriacidal activity, lysozyme levels and non-specific immune reactivity in calves dosed with Co, Cu, iodine and vitamins A and E. However, these latter two workers did not address the question of which combination of supplements resulted in such apparently beneficial effects.

In Scotland MacPherson et al (1987), working with cattle, reported a depression in neutrophil function due solely to a deficiency in Co. Per cent kill of C. albicans by neutrophils isolated from deficient steers was half that of cells from sufficient animals (P<0.001). These results were repeated in similar animals by Paterson and MacPherson (1987), where Co repletion rapidly restored neutrophil candidacidal activity to

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normal levels. However, these two investigations made no suggestions as to the mechanisms of such an effect.

1.8.3 The Transfer of Passively Acquired Immunity.

The transfer of immunity from ewe to lamb in the first day of life is of great importance to the survival of the neonate. Ungulates do not secrete immunoglobulin (Ig) across the placenta and are born agammaglobulinaemic. During the first 12 to 24 hr. post partum, the neonate lamb is able to non-selectively absorb a variety of macromolecules from ingested colostrum (Halliday, 1965), including beta lactobulin and all the Ig's (antibodies).

The Ig's absorbed in this way are specific for pathogens encountered by the dam in the enviroment in which gestation took place. Therefore, the lamb obtains passive immune resistance to the infective agents it is most likely to encounter in the early stages of life. This is designed to give the lamb protection until its own immune system develops sufficient immunocompetence at approximately 4 to 6 weeks of age onward.

Absorption of Ig's across the intestinal mucosa and into circulation in the newborn ovine, is by pinocytosis and intracellular transport of the proteins by pinocytotic vesicles to large supranuclear vacuoles. The contents of these vacuoles are discharged into the extracellular fluid at the basolateral membranes of gut epithelial cells (Mayrhofer, 1984). This is the 'leaky

gut' syndrome.

Campbell (1974) reported that 80 per cent of lambs given colostrum and an infection of _Escherichera coli_ in a housed situation, survived the first week of life. Of those given no colostrum, only 50 per cent survived and in a group given no colostrum and a pathogenic challenge of _E. coli_, 80 per cent died within a week of birth.

A measure of Ig levels in lamb serum can be obtained by using the zinc sulphate turbidity test (ZST). It is generally considered that an adequate level for serum ZST in lambs is >14 units. Harker (1974) reported that in an on-farm trial in lowland Scotland, the mean ZST value in lambs that died before two weeks of age was 5.7 ± 1.9 (arbitrary units). For those that survived. the corresponding value was 43.4 ± 4.6.

The importance of passively absorbed Ig's to the immune status of neonate ruminants has also been demonstrated in cattle. Nocek, Braund and Warner (1984) showed that calves deprived of colostrum had lower liveweight gains ($P<0.05$), more incidence of scouring ($P<0.01$) and higher mortality rates than those fed colostrum. Further, calves given high levels of Ig's in colostrum gained weight faster ($P<0.05$), scoured less frequently ($P<0.01$) and had scours of shorter duration ($P<0.05$) than those fed colostrum but with a low Ig content.

Returning to lambs, Shubber, Doxey, Black and Fitzsimons (1979b) concluded that the "amount of Ig

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available to the lamb decreases much in the first 24 hr. and lambs which fail to feed in this time will have difficulty in getting enough Ig." These neonates would be more susceptible to infection and consequent morbidity and mortality. Indeed, Halliday (1974) observed that in a housed trial with lambs, neonatal mortalities showed lower serum IgG concentrations at 48 hr. post partum, than survivors, although no statistical significance was given.

This crucial absorption of maternal Ig's from colostrum may be influenced by many factors, including colostrum Ig concentrations, absorptive function and ewe/lamb behavioural interactions. Normal concentrations of Ig in pre-suckled colostrum vary between breeds (Halliday, 1965). Typical values for total IgG are 8000 mg/100 ml for Border Leicester X Merino ewes (Dawe, Husband and Langford, 1982), 9000 to 9600 mg/100 ml in the Scottish Blackface (Halliday, 1978a) and 11,500 mg/100 ml in the Columbia breed (Hunter, Reneau and Williams, 1977). IgG is the major maternal Ig in the colostrum of all ruminant species, and further varies in concentrations according to age of the dam and the number of previous lactations (Devery-Pocius and Larson, 1983).

Although Ig levels can easily be determined, many variables combine so that no correlation between ewe colostrum IgG concentrations and amounts of this Ig in lamb serum have been found (Hunter et al, 1977 and Halliday, 1978b). Further, Shubber et al (1979a) found no

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correlation between lamb total serum Ig and the amount of colostrum consumed for Greyfaced ewes and their lambs. One factor may be that colostral Ig concentrations are meaningless unless whey proportion is taken into account (Shubber et al, 1979b). Taking an over-view of the literature in general, within breed variation in the proportion of whey in colostrum and the amount of Ig's contained in that whey, are large. Taking into account the possibility of variation in absorptive function between lambs (Halliday, 1978b), the absence of simple correlations between ewe and lamb serum concentrations is not surprising.

Large inter-breed differences in the degree of non-selective absorption of immunological proteins also gives rise to great variation in the normal concentrations of lamb serum Ig's between breeds. Further, while some investigations have shown significant differences in serum Ig levels between lambs of the same breed due to litter size and sex of twins (Halliday, 1974 and Shubber et al, 1979a), other trials have shown no such influences (Hunter et al, 1977 and Halliday 1971a). In addition, ewe and lamb weight seems to have no effect on lamb serum Ig concentrations at 48 hr. post partum (Halliday, 1974), but more vigorous lambs show increased suckling frequency and thus greater levels of Ig in circulation (Shubber et al, 1979a).

With these influences noted, typical values for lamb

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serum Ig concentrations quoted in the literature are shown in Table 6.

Table 6. Typical serum immunoglobulin levels in lambs.

BREED	Ig	TIME post partum (hr.)	CONCENTRATION (mg/100 ml)		REFERENCE
Finnish Landrace X Dorset Horn	Total	48	2950		Halliday 1971a
Scottish Blackface	Total	18-21	2500 $\pm$ 190	S	Halliday 1971b
			1960 $\pm$ 150	T	
Columbia	G	0	7 $\pm$ 0		Hunter et al 1977
		8	2534 $\pm$ 270		
		24	3556 $\pm$ 327		
Scottish Blackface	Total	168	1560 $\pm$ 119		Halliday 1978a
Greyface X Suffolk	Total	54	3140 $\pm$ 100	S	Shubber et al 1979a
			2770 $\pm$ 99	T	
(Border Leicester X Merino) X Dorset	G1	24	900 $\pm$ 75		Dawe et al 1982
	G2	24	35 $\pm$ 8		
	M	24	135 $\pm$ 30		
	A	24	115 $\pm$ 40		

S = Singles
T = Twins

When the many factors influencing the transfer of passive immunity are considered, the possible mechanisms of a direct or indirect effect of Co deficiency on this process may be varied and complex. To add to the picture, Newby (1984) stated that neutrophils and macrophages as well as Ig's are present in colostrum. Thus in terms of

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the gut lumen only and pathogenic attack on the lamb in this area, opsonisation of bacteria and polymorphonuclear phagocytosis within the intestine by colostral Ig's and cells may be a viable protective mechanism. The detrimental effect of Co deficiency on neutrophil microbicidal activity has already been reviewed. The possibility that this area of the passive transfer system could be affected by Co deficiency and thus render the neonatal lamb more susceptible to intestinal infection, particularly leading to scouring, can not be ruled out. However, no investigations pertaining to this theory can be found in the literature.

The work of this project includes investigation of the possibility that Co deficiency in the ewe, has a detrimental influence on the transfer of passive immunity to the new born lamb. The only evidence of a possible effect to date of trace element deficiency on the transfer of passive immunity, comes from Nuriev (1986), working in the Kazan region of the Soviet Union. Lambs from ewes dosed with Co, Cu and Se during gestation showed higher gamma and beta globulins in serum and higher total blood protein levels, than controls from undosed ewes. However, statistical significance was not given.

1.9 THE DIAGNOSIS OF COBALT DEFICIENCY IN SHEEP.

Work concerned with the metabolic changes which occur as a result of Co deficiency and investigations designed to ascertain the ruminant requirement for Co and vitamin B12, have provided basic data and potential methods for use in diagnosis of the disease. Other work has sought to predict when ruminant livestock might suffer production loss due to Co deficiency, as well as to establish the degree or severity of a suspected problem. Thus, while the aim of earlier experiments was to define techniques and criteria for diagnosis of the clinical syndrome, later investigations have concentrated more on employing new and existing methods for the prognosis of Co deficiency. This has attempted to define the sub-clinical form of the disease, where the greatest loss in production is incurred (Latteur, 1962).

Techniques used in the prognosis and diagnosis of Co deficiency, ranging from soil/herbage analysis to sophisticated determination of blood parameters, will be discussed. The literature concerned with the problems of different approaches will be critically reviewed, with special reference to the diagnosis of the sub-clinical condition.

1.9.1 A Regime for Diagnostic Techniques.

No diagnostic technique for any disease of veterinary importance is useful unless it can be practically and economically accommodated in the routine of a relevant

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laboratory.

A proposed method should be accurate and precise in the diagnosis of acute (clinical) and chronic (sub-clinical) disease, to permit the dissemination of reliable advice on the basis of the data produced. Of increasing importance given reduced financial support for scientific work, especially in the field of agriculture, is the need for a method to have a quick turn-round time and to be cost effective. Finally, a more challenging criterion may be added. Great advantage in terms of time and financial saving on the farm would be gained if a technique could be used prognostically, as well as diagnostically.

1.9.2 Soil and Herbage Analysis.

An approach to diagnosis which focuses on the cause of deficiency, is the determination of Co levels in the soil and herbage on which livestock graze. Many techniques and modifications have been developed in an attempt to ascertain representative values of Co content. These most notably include colourimetric (Kidson and Askew, 1940), spectrophotometric (McNaught, 1948a) direct current arc spectrographic (Scott, Mitchell, Purves and Voss (1971) and atomic absorption (Gelman, 1972) methods.

The use of soil and herbage Co contents as diagnostic indicators of Co deficiency may, in theory, be appropiate. Particularly when consideration is given to the fact that pasture improvement on British upland and hill farms in recent times, especially the practice of

liming, has reduced the amount of Co in such lands, or rendered it unavailable to the herbage. For example, Burridge, Reith and Berrow (1983) working in the north east of Scotland, reported that liming of soils, thereby changing the pH from 5.6 to 6.9, reduced the available levels of Co in ryegrass and clover from 0.07 to 0.06 and 0.11 to 0.08 mg Co/Kg DM, respectively.

The level of Co in forage is not only dependent on soil type and pH, but also on the type of herbage present (Burridge et al, 1983). Clover (Trifolium spp.) usually contains 0.15 mg Co/Kg DM and ryegrass (Lolium perenne) 0.08 mg Co/Kg DM. Hence the use of nitrogen fertilizers to improve pastures may tend to reduce Co content by virtue of increasing the proportion of grass to clover, particularly where the soil Co level is intrinsically low. Further, the use of large quantities of sulphur and phosphates (superphosphates) to improve grazing land, may reduce the availability of herbage Co to ruminant species (Halpin, Caple, Shroder and McKenzie, 1981).

The interaction of factors affecting soil/herbage Co content and availability is complex. In addition, the techniques used to determine levels are too lengthy for them to be ideal for the diagnosis of animal Co deficiency. However, the continued characterisation of soils in respect of Co status should be encouraged, as such work has proved successful in identifying areas at highest risk from deficiency (Andrews, 1970a and Clark,

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Fraser, Mortleman, Bithell and Williamson, 1985).

Further, where Co deficiency disease is suspected a farmer may wish to sample and obtain values for pasture Co content, particularly if Co salt top-dressing of the land is envisaged as a curative treatment. In this respect MacPherson (1982) listed typical herbage Co contents, which might be found on Co-deficient and borderline soils. These are shown in Table 7. It is worthy of note that sward Co contents are also influenced by season, with levels tending to be lower in the summer than in the winter.

Table 7. Typical values for herbage Co content on different soils and swards.

SWARD TYPE	Co CONTENT (mg Co/Kg DM)	COMMENTS
Mixed	<0.08	On Co-deficient soils.
Mixed	<0.10	On soils of borderline Co content.
Grass	<0.08	On freely drained soils of borderline Co content.
Clover	>0.10	On soils of borderline Co content.
Clover	<0.10	On freely drained soils of borderline Co content, in the summer months.

(Adapted from MacPherson, 1982).

Interestingly, Jones and Anthony (1970) found a significant correlation between faecal vitamin B12

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concentration and dietary Co content. They devised the equation:

$$y = 0.0779x - 0.0757$$
$$r^2 = 0.73 \quad n = 12$$

where, y = Co intake (mg/Kg DM) and x = mg vitamin B12/g dry faeces. These workers recommended the use of this equation for surveying areas grazed by sheep, for potential Co deficiency problems.

In practice, many difficulties have arisen with employing soil and herbage Co contents in the diagnosis of ruminant Co deficiency. This is partly due to problems with methodology and also from confusion over the influence of various factors on availability, from soil to plant and from plant to animal. Early recognition of these disadvantages was provided by Dunlop (1946c), working in the south west of Scotland. This worker described the determination of adequate Co contents in soil and herbage, from areas where animals were suffering from pine.

Recently, in a survey of the north east Roramia territory of Brazil, Sousa and Darsie (1985) found herbage contents of 0.06 mg Co/Kg DM, but could provide no evidence of deficiency in free grazing ruminants. Further, McLaren, Lawson, Swift and Purves (1985) reported that current methods of determining soil and herbage Co content produced unreliable results on soils above pH 6.0, with no correlation between soil and

pasture Co levels at any pH. Additionally, out of twelve sites where animals were suffering from Co deficiency, only five could be predicted by soil and herbage Co analyses.

1.9.3 Animal Parameters.

While it is useful to gain as broad a view as possible in a Co-deficient situation, it is perhaps more appropiate for routine analysis and diagnosis to concentrate directly on the animals concerned. This is particularly so when doubts exist as to the accuracy and precision of soil and herbage Co content data and the difficulty of their interpretation. To this end, many approaches to the prognosis and diagnosis of Co/vitamin B12 deficiency in ruminant animals have been investigated.

1.9.3.1 Animal response trials.

A definitive technique in diagnosis is to use animal response trials, such as those reported by Andrews (1970a and 1970b). In such experiments, the presence and severity of deficiency can be deduced by the degree of animal response, usually in liveweight gain, to Co supplementation.

The animal response trial approach is costly, time consuming and not practical for routine use. Although it provides an accurate and (if designed carefully) precise diagnosis, it is inherently not prognostic. However, such

work must form the basis against which other techniques should be compared. Indeed, subsequent methods have been widely developed as diagnostic parameters in controlled dose/response trial type experiments.

1.9.3.2 Liver cobalt/vitamin B12 analysis.

The primary storage organ and site of metabolic action for vitamin B12 in ruminants is the liver. It is in the liver cells that the main function of B12, in the catabolic handling of propionate, takes place (Marston et al, 1961). It is therefore logical to measure liver Co content, or vitamin B12 concentration, as a diagnostic marker of deficiency.

Liver Co and vitamin B12 levels have been measured in many Co dose/response trials and related to animal performance to give diagnostic criteria. For example, Andrews, Grant and Stephenson (1964) attempted to define the relationship between liver/kidney B12 concentrations and wool growth/body weight, as response parameters to doses of Co. By such work, ranges of concentrations have been established for sheep of adequate, sub-clinical and clinical deficient Co status. Examples of these diagnostic ranges are shown in Table 8. For this data, measurement of Co was by accepted techniques of soil and herbage analysis. Determination of vitamin B12 was by microbiological, or radio-immunoassay.

Table 8. Liver Co and vitamin B12 concentrations in the diagnosis of Co deficiency in sheep.

TYPE	LIVER B12 (ug/g wet wght.)	LIVER Co (mg/Kg DM)	STATUS	REFERENCE
Lamb <3 mnth.		>0.08 <0.04	A D	McNaught (1948b)
Lamb >3 mnth.		>0.10 <0.06	A D	"
Lamb	>0.19 0.11 - 0.19 0.07 - 0.11 <0.07		A M D Very D	Andrews, Hart and Stephenson (1960)
Lamb		>0.09 0.07 - 0.09 <0.07	A M D	Andrews (1965)
Ewe	>0.20 0.10 - 0.20 <0.10		A M D	Millar and Penrose (1980)
Mature sheep	>0.19 0.11 - 0.19 <0.11	>0.10 0.06 - 0.10 <0.06	A M D	MacPherson (1982)

KEY:
A = Adequate
M = Marginal
D = Deficient

There is general agreement on the range for liver B12, although opinions on the diagnostic values for liver Co are more varied. Andrews et al (1960) stated that liver B12 concentrations were more closely related to the degree of deficiency and less variable in a group of sheep receiving the same Co intake, than liver Co contents. Whether this was due to differences in the sensitivity of analytical methods used for determining these two liver parameters, or is an inherent

characteristic of liver levels in different deficient situations, is unclear. However, it is generally considered that liver B12 is a more accurate and reliable diagnostic marker of Co deficiency, in both clinical and sub-clinical forms, than liver Co.

The analysis of liver vitamin B12 concentration has been used routinely in diagnostic laboratories. For example, in New Zealand the measurement of total B12 in liver, as cyanocobalamin, was widely used in diagnosis until about 1980 (Millar and Penrose, 1980). A standard of <0.10 ug B12/g wet liver was employed as indicative of Co deficiency.

The sampling of liver in live animals by surgical biopsy is not practical in many on-farm situations and the sampling of liver post mortem is of no prognostic use. Therefore, much effort has been expended in finding other parameters, which are more amenable to routine use.

As the liver is the primary site of metabolic action of vitamin B12 in ruminants, the determination of B12 in this tissue may be considered the 'ideal' for diagnosis of Co deficiency and severity of disease. Thus, as liver B12 levels have been related to animal performance in dose/response trials, alternative diagnostic techniques should in turn be compared to liver B12 analysis, as well as animal response (Sutherland, 1980).

1.9.3.3 Serum vitamin B12 analysis.

If the collection of liver samples by biopsy is impractical on farms, then obtaining blood samples and the determination of vitamin B12 in serum or plasma, is an alternative and more practical technique. Much work and argument has surrounded the use of serum vitamin B12 as a diagnostic marker for Co deficiency in sheep. There is substantial evidence in the literature, both for and against its employment.

The problems involved with serum B12 are based on confusion over methodology and the measurement of physiologically inactive analogues of the vitamin, by different analytical techniques (McMurray, Rice, McLoughlin and Blanchflower, 1985). Further, it seems debatable whether or not this parameter can be related to animal response and liver B12 concentrations, especially in the sub-clinical region of deficiency (Findlay, 1972). However, serum B12 has been positively developed and diagnostic criteria are given in Table 9.

The confusion which has arisen over different methods for the determination of vitamin B12 in serum and the possible presence of inactive analogues, still remains. The vitamin can be measured by microbiological assays, which depend on the turbidimetric estimation of microbial growth. Culture growth is allowed to occur in nutrient broths, where vitamin B12 is the only limiting factor and test and standard amounts of the vitamin have been added. Examples are the use of the bacterium

Table 9. Serum vitamin B12 in the diagnosis of Co
deficiency in sheep.

TYPE	SERUM VIT. B12 (ng/l)	STATUS	REFERENCE
Lamb #	>450	N	Andrews and
	300 - 450	Indefinite D	Stephenson
	200 - 300	Probable D	(1966)
	<200	D	
Mature @ sheep	1000 - 3000	N	Findlay (1972)
	>700	A	
	300 - 700	M	
	<300	D	
Mature $ sheep	>1000	A	Halpin (1979)
	500 - 1000	M	
	<500	D	
Ewe $	>300	A	Millar and
	200 - 300	M	Penrose
	<200	D	(1980)
Mature * sheep	>400	A	MacPherson (1982)
	200 - 400	M	
	<200	D	

KEY:
N = Normal
A = Adequate
M = Marginal
D = Deficient

* = Microbiological assay with Lactobacillus leichmannii.
\# =   "   "   "   Ochromonas malhalmensis.
@ =   "   "   "   unknown microbe.
$ = Radio-immuno assay.

Lactobacillus leichmannii in the methods of Thompson,
Dietrich and Elvehjem (1950) and Skeggs, Nepple,
Valentik, Huff and Wright (1950) the employment of the
bacterium Ochromonas malhamensis by Ford (1953) and the
utilisation of the protozoan Euglena by Hutner, Bach and
Ross (1956). These techniques convert all B12 analogues

into cyanocobalamin before measurement and thus detect 'total B12'. However, the use of different microbes on the same samples, can produce markedly different results (Millar and Penrose, 1980).

An alternative technique is the radio-immuno assay (RIA), which is based on the principle of competitive protein binding. This method was developed for the diagnosis of pernicious anaemia (vitamin B12 deficiency) in humans by Lau, Gottrieb, Wasseman and Herbert (1965), Green, Newmark, Musso and Mollin (1974) and Buchanan, McIntyre, Scheffel and Wagner (1977). A blocking agent can be employed, so that only 'true B12' and no inactive analogues are measured. According to Millar and Penrose (1980) the RIA technique is cheaper, faster, more specific and suffers less from contamination than all of the microbiological methods.

Confusion over methodology still exists, because different techniques are employed (often with the same diagnostic criteria applied to them) and perhaps not surprisingly, contradictory results are evident in the literature. Further, it is clear from Table 9 that different techniques and situations result in dissimilar diagnostic ranges being set.

For example, it is generally accepted that the use of O. malhamensis in the microbiological assay of serum B12, is more specific to the true vitamin than methods employing L. leichmannii. However, Carlos, Givens,

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Johnson, Slade and Telfer (1985) reported that in cattle, O. malhamensis yielded higher results for B12 than an assay employing L. leichmannii on the same sera. In addition, for some cases the RIA technique gave B12 values over 50 per cent less than the L. leichmannii method. Such discrepencies cast doubt on the use of such data for diagnostic purposes, or at least point out that correct diagnosis depends on criteria being appropiate to the assay employed.

Superimposed on this picture is the presence in serum of inactive analogues of vitamin B12. While Gawthorne (1970a and 1970b), Marston (1970) and Halpin et al (1984) found that these were in negligible amounts in the blood of sheep, other workers have presented opposing evidence. For example, Sutton and Elliot (1972) reported significant levels of inactive forms (i.e. those other than hydroxycobalamin and methylcobalamin) in sheep serum.

From such work, it seems that the RIA technique provides more accurate and precise diagnostic data, as it can be employed to determine true, physiologically active forms of the vitamin only.

However, that this is the case has been disputed by Millar and Penrose (1980) and Wright, Taylor and Greer (1982). These workers reported inaccuracies in the RIA technique in measuring true B12, due to the phenomenon of residual protein binding in the method. Slater, Harkins, Suttle, Herbert and McDonald (1985) attempted to define

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exactly which forms of vitamin B12 the RIA method measured and how consistently they were detected. They compared RIA data with cobalamin determination using a specific and sensitive high performance liquid chromatography (HPLC) technique. The results obtained were confusing, with many inconsistencies. These workers concluded that in some cases the RIA determined forms of B12 other than the physiologically active ones, even though inactive analogues were apparently not present by HPLC analysis.

Assuming that serum vitamin B12 can be and is used as a diagnostic marker of Co deficiency in ruminants, the question of how it relates to animal response and storage B12 levels in the liver must be addressed (Mills, 1980). Initial work found a close correlation between serum B12 concentrations and Co status in terms of sufficiency, sub-clinical and clinical deficiencies (Andrews and Stephenson, 1966). Marston (1970) reported a statistically significant regression between serum and liver levels of the vitamin in sheep. However, continued research has questioned these findings. For example, Millar and Albyt (1984) and Millar, Albyt and Bond (1984) found no relationship between B12 concentrations in serum and liver in the ovine. In growing cattle, Givens and Simpson (1983) and Judson, McFarlane, Riley, Milne and Horne (1981) could not relate serum B12 levels to the incidence or otherwise of Co deficiency and effects on

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performance.

Sutherland (1980) attempted to clear up this state of obvious confusion. He argued that for sheep, although most work in the literature reported cases where liver and serum B12 levels did not correlate, to conclude that serum B12 concentrations were therefore an unreliable index of Co status was simplistic. Serum vitamin levels do not always correspond to liver concentrations, because the biological function of B12 in these two tissues is different. He concluded that serum B12 is a useful diagnostic and prognostic indicator of Co deficiency, but only if the data are interpreted correctly.

The arguments that Sutherland (1980) put forward can be supported by evidence in the literature. It should be assumed that serum B12 is a sensitive indicator of Co intake (Somers and Gawthorne, 1969 and MacPherson et al, 1976), while liver B12 is a marker of storage reserves and responds more slowly to changes in Co intake (Underwood, 1977). Thus, serum can be viewed as having a transport function for the vitamin, while liver has a utilisation and storage role. In this sense, serum should be used as a test of Co intake and not vitamin reserve, although animals with low liver B12 levels invariably also show low serum B12 concentrations. If this is the case, then the fact that serum and liver B12 data do not correlate statistically becomes irrelevant to the aim of diagnosis. To illustrate these points, Sutherland (1980) presented a model of Co deficiency and liver/serum B12

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status. This model is reproduced in Figure 4.

The four phases represented in this model, describe all aspects of the development of Co deficiency and repletion and the application of serum vitamin B12 to the diagnosis of Co status. In phase 1, Co sufficiency exists and marked diurnal fluctuations in serum B12, observed by Somers and Gawthorne (1969), make mathematical correlation between serum and liver vitamin concentrations impossible.

In phase 2, which describes the development of Co deficiency, B12 levels fall in serum, before liver concentrations decline. Thus, vitamin B12 in serum is utilised before stores in the liver. This is in agreement with the work of Frenkel and White (1973), who evolved a similar model for B12 depletion in the rat. During this phase, mathematical correlation between serum and liver B12 concentrations is still impossible. This period of ensuing deficiency would account for the confusing results of Millar and Penrose (1980) and Millar and Albyt (1984).

In phase 3, the animals are in a state of functional Co deficiency and both liver and serum B12 levels remain consistently low. Such situations have been described by Andrews et al (1960), Marston (1970), MacPherson et al (1976) and Sutherland et al (1979).

In phases 2 and 3, the variation in serum B12 levels within and between animals declines, with no
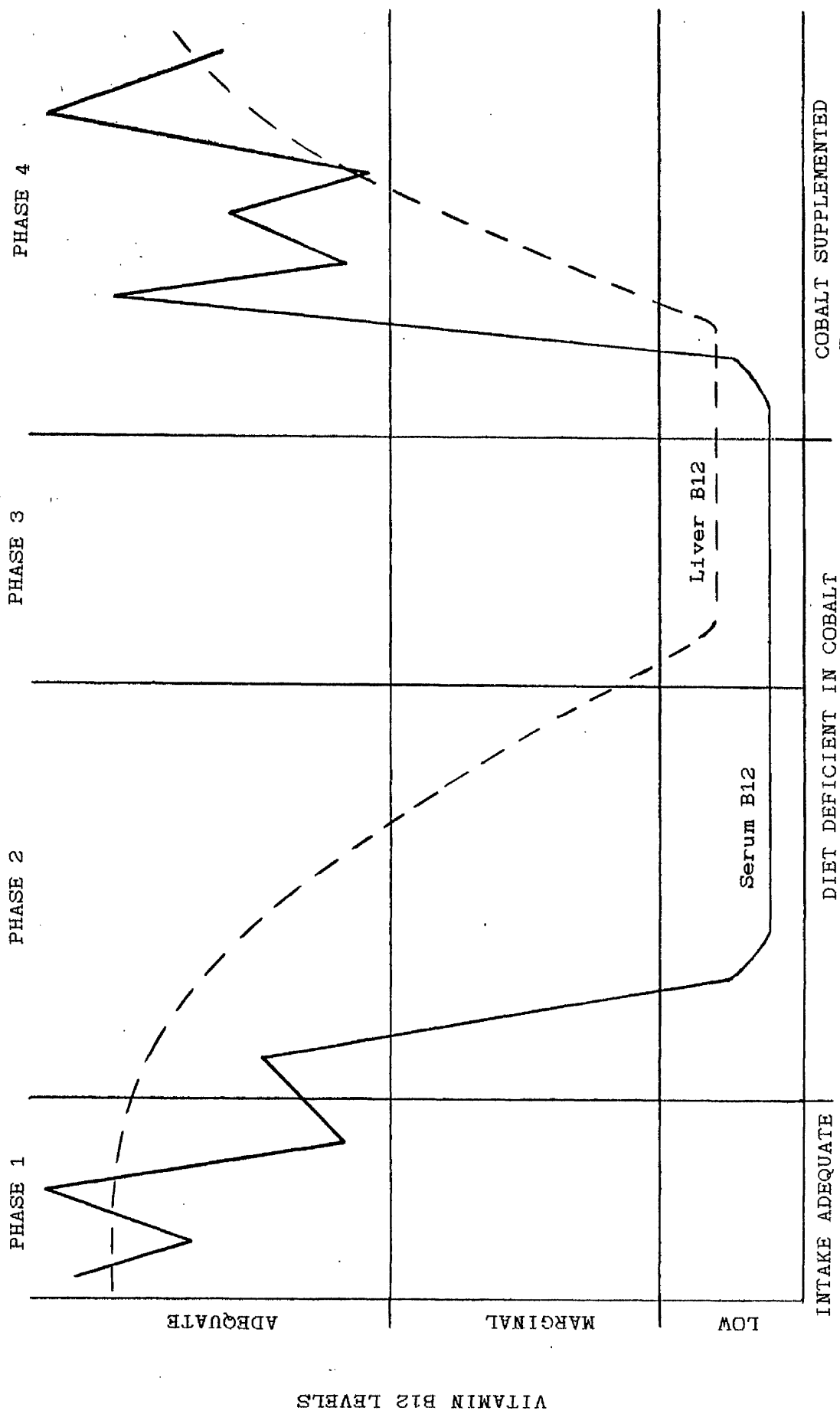
Figure 4. A Model for the Inter-Relationship Between Liver and Serum Levels of Vitamin B12 and Dietary Cobalt.

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influence of diurnal fluctuations. Within the third phase, correlation between serum and liver B12 concentrations is possible, as found by Marston (1970). However, the absence of diurnal fluctuations in levels is not a valid argument according to Millar and Albyt (1984), who could find no evidence of such daily variations in sheep serum B12 concentrations.

In phase 4, serum B12 levels respond more rapidly to Co supplementation than liver concentrations. This sharp response in serum B12 has been reported by Tressol and Lamand (1979) and MacPherson (1987). Diurnal fluctuations in serum vitamin levels again become more apparent in this phase and no correlation between serum and liver B12 concentrations is possible. This model is further supported by the conclusions of Gardiner (1977), in his review of Co deficiency in ruminant nutrition.

However, Suttle (1986), while accepting the scientific basis of such arguments, proposed that dicussions for and against serum and liver B12 "...are irrelevant, unless one or the other can be indicative of functional deficiency." He suggested that serum, as well as liver, has a storage role for vitamin B12. As such, both indicators show a classic pattern of decline in the development of deficiency, but serum stores are used before liver B12 so that concentrations of the vitamin in blood provide a prognostic, as well as diagnostic marker for Co deficiency in the liver.

On the basis of Suttle (1986) the only relevant

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question is, below which concentration of B12 in serum does functional deficiency occur? While this may depend on the definition of 'functional', Suttle (1986) proposed that the threshold between marginal and functional Co/vitamin B12 deficiency, occurs at a serum B12 level of approximately 190 ng/l.

In a final analysis, there is much evidence in the literature to suggest that serum B12 is an accurate and precise prognostic and diagnostic marker of clinical Co deficiency in sheep. This applies as long as data are interpreted properly and care is taken to note the analytical techniques employed. The ability of serum B12 levels to indicate sub-clinical deficiency is more questionable. For example, although Sutherland (1980) stated that the approach is reliable in a marginally deficient situation, Millar and Penrose (1980) and Findlay (1972) suggested that in the sub-clinical area, great within- and between-animal variations mean that the determination of serum B12 is of little use in diagnosing the Co status of a flock of sheep.

Millar and Albyt (1984) asserted that serum B12 should not be used as an indicator of liver B12 concentrations in sheep, but it is reasonable to conclude from the literature that serum B12 is a useful indicator of Co intake (MacPherson et al, 1976). It is also a prognostic marker of deficiency at the liver (Sutherland, 1980) and can be used as evidence of functional (clinical)

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deficiency, when concentrations are very low.

1.9.3.4 Urinary and serum Formiminoglutamic acid analysis.

Due to the apparent confusion surrounding the use of serum vitamin B12 in the diagnosis of ruminant Co deficiency, some investigators have explored the possibility of employing other metabolic parameters as markers for the syndrome. Among these attempts is the determination of formiminoglutamic acid (FIGLU) in urine. This metabolite is excreted as a result of dysfunction in the B12 dependent methyltetrahydrofolate-homocysteine transmethylase reaction, due to vitamin B12 and/or folic acid deficiency (Givens, 1978).

The use of urinary FIGLU as a diagnostic marker for vitamin B12 or folic acid deficiencies, was developed in humans. The analytical technique is an enzyme linked spectrophotometric method, devised for pernicious anaemic patients by Tabor and Wyngarden (1958).

Pioneering investigations into the use of urinary FIGLU in the diagnosis of Co deficiency in sheep, were carried out by Gawthorne (1968). The promising results of this work were reiterated by Russel, Whitelaw, Moberly and Fawcett (1975). These workers found that the detection of 0.05 to 0.31 umol FIGLU/ml urine in Scottish Blackface lambs, facilitated the diagnosis of both clinical and sub-clinical Co deficiency. On repletion with oral doses of Co, levels of this metabolite in the urine of these

lambs fell to virtually zero.

Contrary to these reports, Givens (1978) and Givens, Cross, Shaw and Knight (1978) found that levels of FIGLU in the urine of lambs were too variable and appeared too late in the development of deficiency, to be of any practical prognostic or diagnostic use. Stebbings and Lewis (1983) supported these findings. These latter investigators provided evidence that FIGLU was only detectable in the urine of lambs when they were severely Co-deficient and some weeks after serum B12 concentrations had fallen below 200 ng/l. Further, one Co-sufficient animal in this trial showed small amounts of the metabolite in urine, during a period of weight loss. They concluded that urinary FIGLU was of no diagnostic use for Co deficiency and only appeared to be excreted during periods of weight loss, whether this was induced by Co deficiency or not.

Urinary FIGLU estimations are not used routinely as a diagnostic marker for Co deficiency. Besides the problems of applicability, this may also be due to the fact that sampling urine in an on-farm situation is not as practical on a routine basis as blood sampling.

1.9.3.5 Urinary and serum methylmalonic acid analysis.

The initial investigations of Gawthorne (1968) into the appearance of FIGLU in the urine of Co deficient sheep, also included some original work on the excretion of methylmalonic acid (MMA) by the same animals. MMA rises

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in serum and urine due to dysfunction of the vitamin B12 dependent enzyme methylmalonyl co-enzyme A mutase (EC 5.4 99.2). Reduced activity of this enzyme, particularly in the liver, results in a large increase in the excretion of MMA.

The detection of this metabolite in urine was first proposed as a screening technique for the diagnosis of pernicious anaemia in humans (Barness, 1967). The analytical methods employed were colourimetric (Giorgio and Plaut, 1965) and by thin layer chromatography (TLC), (Bashir, Hinterberger and Jones 1966). However, Williams, Spray, Newman and O'Brien (1969) demonstrated in rats that while these techniques were useful for screening surveys, they were not sensitive enough to be employed in detecting the extent of a B12 deficiency.

In ruminants, investigations proceeded using these techniques, mainly on the determination of MMA in the urine of sheep (Andrews et al, 1970; Hogan, Lorentz and Gibb, 1973 and Lough and Calder, 1976). Early experiments proved promising. Smith, Osborne-White and Russell (1969) demonstrated in pair-fed sheep, that MMA levels in the livers of clinically B12-deficient animals, determined at post mortem by ^{14}C isotope dilution analysis, were over twice those shown by sufficient animals.

At this stage in the development of MMA determination as a diagnostic marker, the analytical methods used were not specific or sensitive enough to employ the technique

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in the detection of sub-clinical deficiency. To overcome this problem, more sensitive packed column gas chromatographic anlyses were developed (Millar and Lorentz, 1974 and Elliot, Haluska, Peters and Barton, 1979).

Subsequent investigations proved more successful in the accurate diagnosis of deficiency. Millar and Lorentz (1979) suggested that the determination of >30 ug MMA/ml urine in grazing sheep was indicative of clinical Co deficiency. However, these workers did not recommend the employment of this approach in the diagnosis of sub-clinical disease. Judson, Hannam, Benson and Reuter (1981), found that serum MMA was raised in grazing Co-deficient sheep when serum B12 concentrations fell below 300 ng/l. In addition, serum MMA levels were less variable than urinary concentrations and these investigators concluded that MMA in serum was a more sensitive marker of Co deficiency than urinary MMA. Further, they postulated that serum MMA was probably preferable to serum vitamin B12 in the diagnosis of the sub-clinical form of the syndrome.

The application of MMA analysis to the detection of clinical Co deficiency was therefore established, but its use as a marker of sub-clinical disease was still questioned (Mills, 1981 and Elliot et al, 1979). However, further development using a capillary gas chromatographic technique for the determination of MMA in plasma and urine by McMurray, Blanchflower, Rice and

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McLoughlin (1986) provided further scope for the employment of this metabolite as a prognostic, as well as diagnostic, marker (McMurray, Rice, Kennedy, Blanchflower and McLoughlin, 1985).

McMurray, Rice, McLoughlin and Blanchflower (1985) presented criteria for employing plasma MMA in conjunction with plasma B12, for prognostic and diagnostic usage. These ranges are shown in Table 10.

Table 10. Possible diagnostic criteria for plasma vitamin B12 and MMA in sheep.

VIT. B12 (pg/ml)	MMA (umol/l)	COMMENT	STATUS
>300 *	<4.6	High B12/Normal MMA	Normal
<300	<4.6	Low B12/Normal MMA	Normal
<300	<15.0	Low B12/Moderate MMA	Sub-Clinical
<300	>15.0	Low B12/High MMA	Clinical

* Analysed by RIA, as 'true vitamin B12.'
(After McMurray et al, 1985).

This Irish team also stated arguments in favour of the diagnostic employment of MMA in addition, or as an alternative to serum B12. They viewed serum B12 "...as a passive marker of Co status, because it has no direct functional role in serum, being bound to the transcobalamins." For MMA, this alternative approach was regarded as having "...the potential of being used as an active marker. A raised concentration in serum or urine

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is a direct reflection of abnormal metabolism, since it results from degradation of accumulated levels of methylmalonyl CoA, a metabolic event directly associated with Co or vitamin B12 deficiency." (McMurray et al, 1985).

These positive remarks are balanced by the caution of other investigators. Andrews and Hogan (1972), although working with a less sensitive technique, stated that "...there may be considerable limitation as to the precision with which serum B12 values can be used to predict the vitamin B12 status of grazing sheep and this could be of special significance when liver reserves are close to, or below, deficient levels." However, these authors went on to point out that, "Present results indicate that, in a non-Co-deficient flock, there will be a few individuals of apparent adequate vitamin B12 status that will excrete measurable amounts of MMA." Further caution is added to this possibility of MMA analysis producing false positives, when these workers suggested that, "...the amount of MMA found may depend upon the amount of feed consumed during the recent past and the time elapsed between ingestion and sampling. These points require investigation."

In addition to these more negative comments, Suttle (1986), while recognising that the use of serum MMA increases the precision of diagnosing Co deficiency compared with the employment of serum B12 alone, offered

a note of warning. He proposed that high MMA levels only reflect a deficiency in adenosylcobalamin and will not show a deficiency of the other physiologically active form of vitamin B12, methylcobalamin. Thus, if situations exist where deficiency of the methylcobalamin form predominate, then analysis of MMA will not diagnose this.

Further to these points, there is also a possibility that a deficiency in selenium or vitamin E may affect MMA levels, as well as a deficiency in Co/vitamin B12. Working with rats, Pappu, Fatterpaker and Sreenivasan (1978) found increased concentrations of MMA in the urine of Vitamin E-deficient/vitamin B12-sufficient rodents. They postulated that vitamin E, as well as vitamin B12, may have an effect on the methylmalonyl CoA mutase enzyme. In vitamin E deficiency, adenosylcobalamin may not have its usual activity as a co-factor for this enzyme.

In contrast to these sceptical views, Rice, McLoughlin, Blanchflower, Goodall and McMurray (1987) reported that concentrations of MMA in the plasma and urine of sheep, were accurate indicators of Co status. These workers published their results after the completion of the studies to be reported in this project. Therefore, their findings which relate to the work of this project will be discussed later in Chapter 3.

More work is needed on factors affecting levels of MMA in blood and urine before this apparently accurate and precise prognostic and diagnostic technique can be

advocated for use in sheep. Firstly, Rice et al (1987) stated that whereas urinary MMA "...represents the total MMA excreted from plasma over a period of hours," plasma MMA is a reflection of the liver's ability to convert propionate to succinate, over a shorter time period. Thus, study of the relative merits of both should continue. It may prove that blood MMA levels are a more accurate indicator of Co/B12 deficiency in the liver, as Judson et al (1981) postulated.

Secondly, Co-deficient inappetence may reduce the levels of MMA excreted in clinical disease (Rice et al, 1987). In this situation, propionate load on the liver would be markedly reduced with a subsequent drop in the amount of excess methylmalonyl CoA present. Thus, it may be that in severe deficiency, the measurement of MMA in blood or urine is irrelevant to diagnosis. This point requires clarification.

Thirdly, Andrews and Hogan (1972) postulated that there might be an effect of feeding time and period from feeding to sampling on MMA concentrations. These investigators also raised the possibility of diurnal fluctuations in serum MMA levels. The influence of such factors on the use of MMA as a diagostic marker, also need investigation.

1.9.4 Diagnostic Strategy.

From this review of the literature, it seems that the single most accurate, precise and reliable way of diagnosing Co/vitamin B12 deficiency in sheep, is by the measurement of the indirect marker, MMA (Suttle, 1986).

The use of soil and herbage analysis is inaccurate (McLaren et al, 1985) and difficult to relate to animal trace element status. Determination of liver B12 is perhaps the ideal strategy in terms of accurate diagnosis, but is not practical for routine employment. The measurement of serum B12 is a widely used approach, but suffers from problems of variability and data interpretation (Sutherland, 1980). The detection of FIGLU in urine, as a routine diagnostic marker, now seems to have been abandoned.

Much work is needed before the analysis of MMA alone can be used as a diagnostic approach with full confidence. Even if this is a possiblity for the future, perhaps the most accurate, precise, reliable and routinely applicable diagnostic strategy, which also meets the challenge of prognosis, is the joint use of serum B12 and MMA concentrations in a similar manner to that advocated by McMurray, et al (1985).

CHAPTER 2.

ANALYTICAL TECHNIQUES.

2.1 INTRODUCTION.

The analytical techniques used in this project will be described in the order in which they appear in the text. Methods which were integral to the work will be detailed in full. Techniques which yielded additional, but secondary information, will be given in outline only.

2.2 ANALYSIS OF COBALT CONTENT IN FEEDS AND LIVER SAMPLES.

The Co contents of feeds and liver samples were determined by a DC arc spectrochemical technique, using the method of Scott et al (1971).

2.3 BLOOD SAMPLING.

On all occassions blood samples were collected from sheep by jugular-venipuncture, employing 7 ml silicone coated and 10 ml lithium heparin containing re-evacuated sampling tubes ('Monoject,' Sherwood Medical, St.Louis, Mo., USA). For the neutrophil function test (section 2.9) blood was collected in 10 ml re-evacuated silicone coated tubes, which contained 1 ml of EDTA/saline solution (pH 6.8).

2.3.1 Preparation of Sera.

Blood samples in 7 ml silicone coated sampling tubes were placed in a water bath at 30 $^{\circ}$C and allowed to clot for 1 hr. The clots were removed and the sera were centrifuged at 1000 g for 20 min. The supernatant sera were then pipetted off into plastic storage tubes and

stored at -20 ^{O}C.

2.3.2 Preparation of Plasma.

Blood samples in 10 ml lithium heparin containing sampling tubes were centrifuged at 1000 g for 20 mins. The supernatant plasma were pipetted off into plastic storage tubes and stored at -20 ^{O}C.

2.4 ANALYSIS OF SERA FOR VITAMIN B12.

Serum vitamin B12 concentrations were determined either by microbiological assay using Lactobacillus leichmannii as the test organism, or by radio-immuno assay (RIA). The microbiological assay and RIA were based on the work of Skeggs et al (1950) and Lau et al (1965), respectively. The RIA employed a commercial analytical kit, produced by Becton and Dickinson (Orangeburg, New York, USA).

2.4.1 Microbiological Assay.

2.4.1.1 Summary.

Test sera were diluted 1 to 9 in an acetate buffer (pH 4.85), containing 20 mg/l potassium cyanide. After heating at 100 ^{O}C for 30 min., the serum proteins were precipitated and the vitamin B12 content converted to stable cyanocobalamin. 2 ml of the protein free supernatant were removed and added to 8 ml of an assay broth. The broths were sterilised and inoculated with a L. leichmannii suspension. After incubation for 18 to 24

hr. at 40 ^{O}C, growth levels in the assay tubes were measured to determine spectrophotometric optical density and compared with standards of known vitamin B12 content. There was a direct relationship between vitamin B12 concentration and growth of <u>L. leichmannii</u>, and hence optical density of an inoculated broth.

2.4.1.2 Preparation of sera.

Sera stored at -20 ^{O}C were allowed to thaw and mixed thoroughly. Acetate buffer was distributed in 4.5 ml aliquots into 100 x 16 mm conical bottomed polypropylene tubes. Test sera were added to each tube in 0.5 ml amounts. The tubes were capped and sterilised by heating at 100 ^{O}C in a covered boiling bath, or 108 ^{O}C in an autoclave, for 30 min. After cooling, the tubes were 'ringed' to remove adherent protein deposits and centrifuged for 15 min. at 1000 g. The supernatant contained the vitamin B12 extract.

2.4.1.3 Preparation of assay broth.

A.   A vitamin B12 free assay medium was prepared by reconstituting a powdered formula (Difco B12 assay medium USP, Difco Laboratories, Detroit, Michigan, USA) in deionised water, heating to 100 ^{O}C and simmering for 2 to 3 min.

B.   2 ml of test sera vitamin B12 extract were added to pyrex glass tubes, along with 8 ml of the medium. The

tubes were stoppered and sterilised by autoclaving at 15 pounds per square inch for 5 min. Longer heat exposure would result in destruction of media constituents and subsequent poor bacterial growth.

C.   Once cool each tube was inoculated with a standard drop (approximately 0.025 ml) of prepared culture suspension. The tubes were again stoppered and incubated in a water bath at 40 $^{\circ}$C for 18 to 24 hr.

2.4.1.4 Preparation of inoculum.

A. $\underline{L.}$ $\underline{leichmannii}$ (ATCC 7830) was grown in continuous culture at 37 $^{\circ}$C, with daily subculturing using Difco B12 culture broth (Difco Labs.). The culture was shaken to resuspend the deposit. A sterile Pasteur pipette was used to transfer 2 ml to a sterile plastic universal bottle.

B.   This aliquot was centrifuged at 1200 g for 15 min.

C.   The supernatant was discarded and the deposit resuspended in 10 ml of sterile Hank's balanced salt solution (BSS). The suspension was gently and thoroughly mixed, then recentrifuged as above. This procedure was repeated four times.

D.   After the final wash, 0.2 ml of the suspension was diluted in 20 ml of BSS. This dilution was used as the inoculum strength.

E.   Vials of this final strength were stored for use in a 15 per cent glycerol/BSS (v/v) solution, and kept at -20 $^{\circ}$C.

By this procedure a culture of pure, viable $\underline{L.}$

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leichmannii in a smooth phase, with no contaminants, could be maintained at a satisfactory concentration to achieve adequate growth levels within the set incubation time and temperature.

2.4.1.5 Standards.

Two sets of standards were employed. The first set was prepared with and incorporated into each run, using a sera of known vitamin B12 value (Wellcomtrol abnormal K3072) and calibrated against the IRBI reference serum (British standard secondary). The standard B12 concentrations in these sera were 143, 285, 360, 567 and 1,250 ng B12/l.

The second set of 'aqueous' standards were prepared separately and used on every third run as an overall check on the method. These were made up using a commercial formula of cyanocobalamin, as follows:

A.   One 250 ug ampoule of vitamin B12 standard (Cytamen, Glaxo Group Research Ltd., Greenford, Middlesex) was made up to 250 ml in a volumetric flask with 30 per cent v/v ethanol. This 1 ug/l vitamin B12 solution was kept in a dark bottle at 4 $^{\circ}$C for up to two weeks.

B.   1 ml of 1 ug/l concentration was made up to 100 ml in a volumetric flask with deionised water, to give a 10 ng/ml solution.

C.   1 ml of 10 ng/ml concentration was made up to 200

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ml in a volumetric flask with deionised water, to give a
50 pg/ml solution.

The protocol, which was used to prepare two replicates
of each aqueous standard, is shown in Table 1. When the
aqueous standards were employed, they were inoculated and
incubated at the same time as a test run.

Table 1. Protocol for preparing aqeous standards.

A(ml)	B(ml)	C(ml)	STANDARD CONC. (ng/l)
0	5.00	5.00	Blank
0.25	4.75	5.00	63
0.50	4.50	5.00	125
0.75	4.25	5.00	188
1.00	4.00	5.00	250
1.00	4.00	5.00	250
2.00	3.00	5.00	500
3.00	2.00	5.00	750
4.00	1.00	5.00	1000
5.00	0	5.00	1250

KEY:

A - Standard solution (50 pg/l)
B - Fresh deionised water
C - Double strength vitamin B12 media

2.4.1.6 Calculation of results.

Using water as a blank at 540 nm, the top standard
(1250 ng/l) recorded an optical density of 1.4 to 1.8
units on a Philips/Pye Unicam PU 8610 UV kinetics
spectrophotometer, when satisfactory growth had been
attained. A standard curve was obtained by plotting

optical density (y axis) against known vitamin B12 (ng/l)
content in prepared standards (x axis). Determination of
vitamin B12 in test sera was by interpolation from this
curve.

2.4.2 <u>Radio-immuno Assay (RIA).</u>

2.4.2.1 Summary.

The principle used in this assay was that of
competitive protein binding (CPG), which is summarised
below:

$$S* \quad\quad\quad\quad\quad\quad\quad S* - B$$
$$\quad\quad\quad + B$$
$$S \quad\quad\quad\quad\quad\quad\quad S - B$$
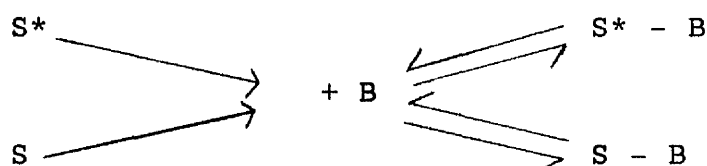
Where 'S' is unlabelled vitamin B12, 'S*' is
radioactively labelled vitamin B12 and 'B' is a binder.
The binder had equal affinity for prepared and test serum
vitamin B12. Labelled (prepared) and unlabelled (test
serum) vitamin B12 competed for a limited number of
available specific binding sites, until an equilibrium
was established. The level of radioactive bound vitamin
B12 was therefore inversely related to the concentration
of non-radioactive vitamin in test sera. After adequate
incubation the bound and free fractions were separated
using charcoal, and the amount of bound radioactivity
quantitatively measured by gamma scintillation.

The commercial kit (Becton and Dickinson) was employed
to measure only physiologically active ('true') vitamin

B12. Inactive analogues were not included in the determination. This was achieved by altering the specificity of the binder used. The binder had sites for reception of true vitamin (intrinsic factor - IF) and inactive B12 analogues (non-intrinsic factor - NIF). A NIF blocking agent, derived from inactive analogues, was used to bind all NIF sites on the binder. Thus the binder, only having IF sites available which were specific for true B12, would react and bind with only labelled and unlabelled true vitamin B12 in the assay mixture.

2.4.2.2 Reagents.

A.   **Dithiothreitol solution:** Dithiothreitol (5 per cent) and stabiliser (2 ml/vial).

B.   **NIF blocking agent:** Analogues derived from vitamin B12 (10 ug/ml) in borate buffer with preservatives (2.0 ml/vial).

C.   **Vitamin B12 tracer:** Less than 1.5 uCu [^{57}Co] true vitamin B12 in borate buffer (pH 9.3) with human serum albumin, dextran, potassium cyanide, dye and preservative (100 ml/bottle).

D.   **Working tracer/buffer solution, with NIF blocking agent:** One vial NIF blocking agent and one vial dithiothreitol were added to one bottle of vitamin B12 tracer, to make up this working solution.

E.   **Vitamin B12 standards:** Formulated in a synthetic

matrix with sodium chloride and preservatives.

F.   **Vitamin B12 binder:**   A lyophilised preparation, containing porcine intrinsic factor, human serum albumin, dextran and preservatives. The intrinsic factor was designed to have a trace binding (Bo) of 55 $\pm$ 15 per cent. The binder contained R-protein, which when used with NIF blocking agent, contributed no vitamin B12 binding activity.

G.   **Dextran – charcoal tablets:**   Contained charcoal dextran and inert fillers.

2.4.2.3 Procedure.

A.   Polypropylene tubes were numbered 1 to 16 for the standards. Beginning at 17, two tubes for each test serum were used.

B.   Standards and test sera were added as shown in Table 2.

Table 2. Organisation of tubes, standards and test samples.

TUBES	STANDARD OR SAMPLE	VITAMIN B12 CONC. (ng/l)
1,2 (Totals)	---	0
3,4 (Blank)	200 ul	0
5,6 (Bo)	"	0
7,8	"	100
9,10	"	200
11,12	"	400
13,14	"	1000
15,16	"	2000
17,18 etc.	" Test serum	Unknown

C.	1 ml of working tracer/buffer solution with NIF blocking agent was added to each tube. All were mixed gently and covered with aluminium foil.

D.	All tubes (except 1 and 2) were heated in a water bath at 100 ± 2 $^{\circ}$C for 15 min. The tubes were then removed and cooled to between 20 and 25 $^{\circ}$C in a running water bath.

E.	The binder was then added in 0.1 ml amounts to tubes 5 to 16 and all test sera and mixed gently by hand. Tubes 3 to 16 and all sample sera were incubated at room temperature for 30 min. from the time of the last addition of the binder. The rack of tubes was covered throughout this period with aluminium foil to exclude light.

F.	One charcoal tablet was added to each tube, except tubes 1 and 2. The tubes were vortexed for 5 seconds each and kept at room temperature for 10 min., from the time of the last addition of charcoal. In order to pellet the charcoal, all tubes were then centrifuged at 1000 g for 10 min.

G.	The supernatant of each tube, which contained the bound vitamin B12 fraction, was decanted into fresh polypropylene tubes. Maximum transfer was achieved by hitting the rims of the two tubes together, taking care not to include any of the charcoal in the supernatant transfered.

H.	The radioactivity of the supernatants and tubes 1

and 2 were counted in sequence for one minute each, using a scintillation (gamma) counter (LKG, Sweden).

2.4.2.4 Calculation of results.

A.   The counts of the blank tubes 3 and 4 (no binder added) were averaged. This blank value was subtracted from all other tube results to obtain 'corrected counts.' The corrected counts were used in the following calculations.

B.   The corrected counts of tubes 1 and 2 were averaged to give a 'total count' for the assay.

C.   The average of the corrected counts for tubes 5 and 6 (binder added, but no B12 present) was divided by the corrected total count to give the trace binding, Bo. This value was greater than 40 per cent.

Bo = Trace binding =

$$\frac{\text{Average corrected counts, tubes 5 and 6}}{\text{Total Count}} \times 100$$

D.   The corrected counts of each tube were divided by the average corrected counts for tubes 5 and 6, to give the per cent of trace binding for each tube:

Per cent of Trace Binding =

$$\frac{\text{Corrected count}}{\text{Average corrected counts tubes 5 and 6}} \times 100$$

E.   A standard curve was plotted, using log graph paper, of per cent trace binding in standards (y axis)

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against vitamin B12 (ng/l) of the standards (x axis). The concentration of vitamin B12 in test sera was determined by interpolation from this curve.

2.5 ANALYSIS OF SERA FOR METHYL MALONIC ACID.

2.5.1 Summary.

Methyl malonic acid (MMA) was extracted from serum by shaking with ethyl acetate under acidic conditions. The butyl ester was formed using butanol acetyl chloride and was dissolved in hexane. The butyl ester of MMA in hexane was quantified by capillary gas chromatography (GC) using a Hewlett Packard 439 GC. Ethyl malonic acid (EMA) was used as an internal standard (McMurray et al, 1986).

2.5.2 Reagents.

All the solvents and reagents used were 'Analar' grade and obtained from BDH Ltd. (Poole, Dorset), unless otherwise stated.

A.   **MMA** (Stock standard) 10 umol/ml. 1.18 mg/ml of MMA (Sigma Chamicals Co., Poole, Dorset) were dissolved in acetone. A working standard of 0.5 umol/l was prepared by diluting the stock standard 1 to 9 in acetone.

B.   **EMA** (Stock standard) 10 umol/l. 1.32 mg/ml of EMA (Aldrich Chem. Co., Gillingham, Dorset) were dissolved in acetone. A working standard of 1.0 umol/l was prepared by diluting the stock standard 1 to 9 in acetone.

C.   **Saturated sodium chloride solution.** Approximately

150 g of sodium chloride dissolved in 500 ml of distilled water.

D.   **10 M sulphuric acid.** 38 ml of concentrated sulphuric acid were mixed with 200 ml of distilled water in a conical flask, with cooling. To avoid boiling, the acid was added to the water slowly and carefully while swirling the flask. This solution was then made up to 250 ml with distilled water.

E.   **0.5 M sulphuric acid/ saturated sodium chloride.** 10 M sulphuric acid was diluted 1 to 9 with saturated sodium chloride solution.

F.   **Butanol/acetyl chloride reagent.** 0.5 ml of acetyl chloride was added slowly and with swirling to 5.0 ml of butan-1-ol ('Aristar' grade) in a 30 ml glass vial. This reagent was freshly prepared before use.

2.5.3 Gas Chromatography Conditions.

The GC technique employed a flame ionisation detector, with a 25 m X 0.22 mm capillary column (Hewlett Packard CP-SIL 5B). Split injections were made onto the column, using a 2.5 ul injection capacity. The split ratio was 10:1 (hexane:sample) using hydrogen as the carrier gas. The injection port temperature was maintained at 200 ^{o}C, with the detector temperature held at 280 ^{o}C.

Oven temperature profile.

1. 120 ^{o}C for 2 min.

2. Rise of 5 ^{o}C/min. to 150 ^{o}C.

3. Rise of 40 °C/min. to 230 °C.

4. 230 °C for 5 min. to flush the column.

2.5.4 Procedure.

A.   500 ul aliquots of sera were placed in small quickfit tubes. The samples were tested singly. Standards containing 100 ul of 0.5 umol/l MMA were also set up. The volume of these was made up to 500 ul with distilled water.

B.   100 ul of 0.1 umol/l EMA were added to each tube as the internal standard. 500 ul of acetone were then added to each tube, while vortexing, followed by 2.0 ml of 0.5 M sulphuric acid/saturated sodium chloride solution.

C.   1.0 ml of ethyl acetate was added and each tube was shaken vigorously for 20 seconds. The tubes were then centrifuged at 1000 g for 10 min. and the ethyl acetate layers transfered to 1 ml capacity microtubes. The ethyl acetate in each microtube was evaporated off in a dry block heater set at 60 °C, under a constant flow of oxygen free nitrogen.

D.   A further 1.0 ml of ethyl acetate was added to each tube and the extraction, centrifugation and evaporation stages were repeated, with the ethyl acetate layers again being transfered to the corresponding microtubes.

E.   100 ul of the butanol/acetate chloride reagent were added to each microtube. These tubes were then

stoppered and placed in a water bath at 70 $^{\circ}$C. After 2 min. the microtubes were vortexed and returned to the water bath for a further 20 min.

F.   After cooling, 300 ul of hexane and 600 ul of distilled water were added to each microtube. These tubes were then shaken for 5 seconds and the layers allowed to separate. The upper layers were then transfered into GC autosampler vials for analysis.

2.5.5 Calculation of Results.

Examples of chromatograms of standard and test samples are shown in Figure 1. For each standard in a run, the ratio of MMA to EMA was calculated from the chromatograms as follows:

$$\text{Standard ratio} = \frac{\text{MMA peak area}}{\text{EMA peak area}}$$

An average standard ratio for a run was then obtained. A test ratio for each sample was calculated in the same manner; by dividing the test MMA peak area by the internal standard EMA peak area. For each sample, the concentration of MMA in serum was then calculated as follows:

$$\text{Serum MMA concentration} = \frac{\text{Test ratio}}{\text{Average standard ratio}} \times 100$$

88
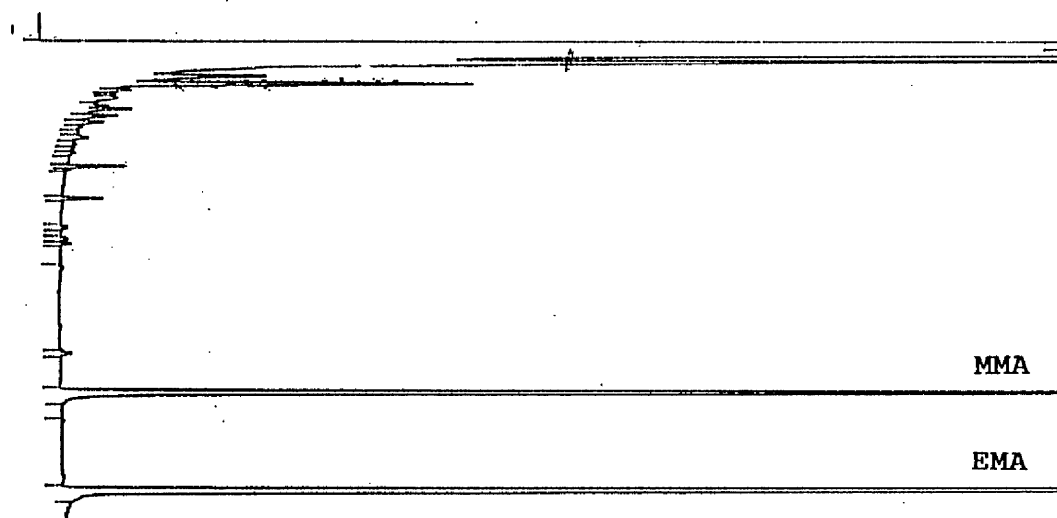
a)
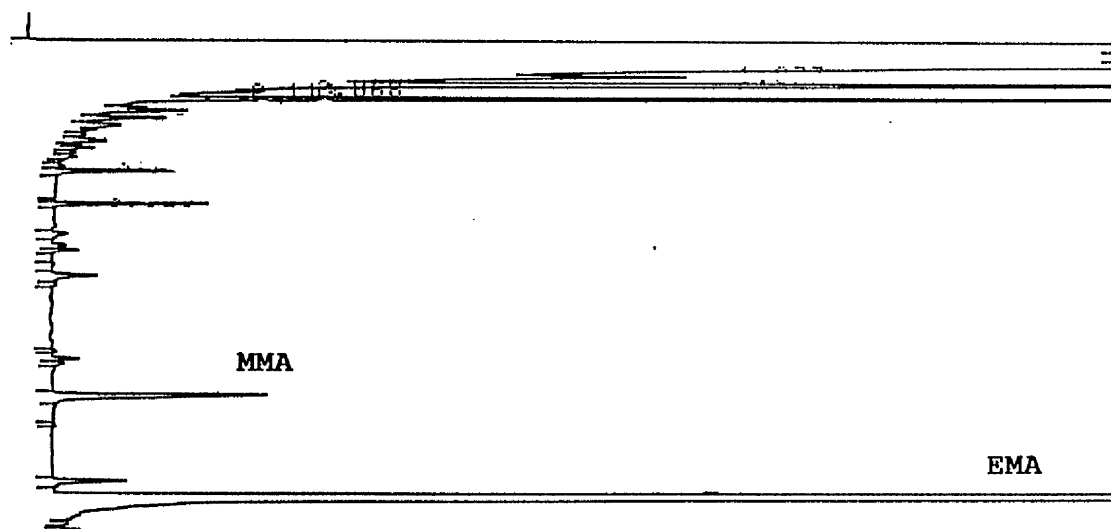
b)

Figure 1. Two chromatograms showing the butyl esters of MMA and EMA in a) a standard preparation and b) a test serum preparation.

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Serum MMA concentrations in the test sera were thus calculated in umol/l.

2.6 ANALYSIS OF WHOLE BLOOD FOR HAEMOGLOBIN.

Whole blood collected in the lithium heparin containing sampling tubes, was analysed for haemoglobin (Hb) content. The procedure used was that detailed by Sigma Chem. Co. (1980), employing a Philips/Pye Unicam SP8 - 500 UV/VIS spectrophotometer.

2.7 ANALYSIS OF WHOLE BLOOD FOR GLUTATHIONE PEROXIDASE.

Whole blood collected in the lithium heparin containing sampling tubes, was analysed for glutathione peroxidase (GSH-Px) activity by the enzymic method of Paglia and Valentine (1967), as modified by Anderson, Berrett and Patterson (1978).

2.8 ANALYSIS OF PLASMA FOR COPPER, CALCIUM AND MAGNESIUM.

Plasma samples were analysed for copper (Cu), calcium (Ca) and magnesium (Mg) content by an atomic absorption technique.

2.9 THE NEUTROPHIL FUNCTION TEST.

2.9.1 Summary.

Neutrophils were isolated from blood and incubated with a suspension of Candida albicans. After staining for dead yeast cells, the phagocytic and killing activity of neutrophils from sheep could be determined.

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2.9.2 Isolation of Neutrophils.

A.   Within two hours of collection, blood samples were centrifuged at 1000 g for 20 min. The plasma portion and uppermost part of the red cell column (mono-fraction) were then removed by aspiration. The remaining sample, which contained the neutrophils, was resuspended in 4ml of 0.8 per cent buffered saline.

B.   This fraction was transfered to 50 ml conical based tubes. To each sample 20 ml of distilled water were added with gentle mixing, to lyse the red blood cells. After 45 seconds, 10 ml of 2.7 per cent buffered saline were added to restore isotonicity.

C.   Each tube was centrifuged at 500 g for 10 min. and the supernatant carefully removed by aspiration. The white cell buttons were resuspended in 25 ml of 0.8 per cent buffered saline to facilitate washing. The tubes were then recentrifuged for a further 10 min. at 500 g. This procedure was repeated for a further two washes. Finally, The white cell buttons were resuspended in 1.0 ml of Hank's balanced salt solution (BSS).

2.9.3 Assay of Candidacidal Activity.

A.   Fresh ovine serum and the suspensions of neutrophils were pre-warmed at 37 ^{o}C. Neutrophils (1.0 X 10^6), C. albicans (2.0 X 10^6) and Hank's BSS were added to plain siliconised vials to give a total volume of 0.6 ml. To this was added 0.15 ml serum and the test was incubated in a pre-set oven for a total of 75 min. at 37

^{O}C, with mild agitation.

B.   A control to candidacidal activity was prepared by adding all the normal constituents to a vial, except the neutrophils.

C.   After 60 min. of the incubation period, dead C. albicans cells were stained by adding 0.2 ml of 0.4 per cent methylene blue to each tube.

D.   The relative concentrations of neutrophils and C. albicans were determined by use of a Coulter counter ('Model Ta 2'). In general, employing 10 ml of blood initially and a three day old culture of the yeast, 0.25 ml of neutrophil suspension, 0.20 ml of C. albicans and 0.15 ml of Hank's BSS were required. The yeast was grown continuously at 30 ^{O}C, centrifuged and resuspended in 5ml Hank's BSS, then diluted 1 in 4, again in Hank's BSS.

E.   After full incubation the preparation was examined using a Neubauer chamber under a light microscope at a magnification of X 550. One hundred isolated neutrophils were counted and the number containing killed (blue stained) C. albicans recorded. Clumped neutrophils were not used in counting as this bunching made viewing of individual cells difficult and inaccurate. To avoid excessive clumping the vials were shaken vigorously before microscopic examination. Dead yeast cells took up the methylene blue stain and appeared blue on examination. Phagocytosed, but still viable Candida, were not stained and appeared translucent.

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F.   Neutrophils containing no, or viable candida, were scored negative. Those containing at least one killed yeast cell were scored positive. Thus, a percentage kill for each sample was obtained. The control was considered satisfactory if it contained less than 1.0 per cent dead <u>C. albicans</u>. On no occassion while using this technique did the control show a non-viable cell population above this level.

G.   That Co deficiency does not affect the ability of neutrophils to phagocytose the yeast cells has already been established repeatedly at these laboratories (Taylor, pers. comm.). Consequently, a measure of phagocytic activity was not taken.

2.10 <u>ANALYSIS</u> <u>OF</u> <u>SERUM</u> <u>AND</u> <u>COLOSTRUM</u> <u>FOR</u> <u>IMMUNOGLOBULIN</u> <u>G.</u>

2.10.1 <u>Summary.</u>

The single radial immunodiffusion (SRID) technique used to determine IgG in colostrum whey and serum was that described by Mancini, Carbonara and Heremans (1965) and modified by Fahey and McKelvey (1965). IgG_1 and IgG_2 were measured together. All statements concerning IgG relate to these two immune proteins. In ovines, the IgG_2 form is present in negligible amounts, relative to total IgG.

The method relied on the formation of a precipitate due to an antigen/antibody reaction. An unknown amount of protein antigen, in this case colostrum whey or blood serum, was allowed to diffuse radially from a point application, in a uniformly thin layer of antibody

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(antiserum) containing agar, for a sufficient time to allow most of the antigen to combine. The antigen/antibody reaction took place in the agar and formed a precipitin ring. The area reached by the precipitate was directly proportional to the amount of antigen (Cag) employed and inversely proportional to the concentration of antibody.

A plot of ring diameter squared (d^2) against known standard antigen concentration, produced a straight line after complete diffusion. This linear relationship was expressed by the equation:

$$d^2 = K[Cag] + SO$$

The intercept (SO) was a function of Cag and of the diameter of the well cut into the agar, in which the antigen was placed.

Mancini et al (1965) described Cag for equal volumes of test fluids and their corresponding precipitin ring diameters after complete diffusion. Fahey et al (1965) discovered that ring diameter could be measured well before the end point of diffusion. These latter workers also showed that the logarithm of Cag was proportional to precipitin ring diameter after just 18 hr. incubation. This relationship was linear when plotted on semi-log chart paper, and was time and temperature dependent.

2.10.2 Reagents and Materials.

All chemicals were reagent grade and obtained from BDH Ltd., unless otherwise stated.

A.   **Agar Noble:**   Low ash agar for use in electrophoretic and immunodiffusion techniques. (Difco Labs). 1.8 g of agar noble were dissolved in 150 ml of 0.1 M barbitone acetate buffer, pH 8.6. The agar was allowed to cool in a water bath maintained at 56 ^{O}C. This yielded a 1.2 per cent agar solution.

B.   **Sodium acetate:**   ('Analar' grade).

C.   **Sodium barbitone:**

D.   **0.1 N Hydrochloric acid:**

E.   **0.1 M Barbitone acetate buffer, pH 8.6:**   5.0 g sodium barbitone and 3.25 g sodium acetate were dissolved in 32.2 ml of 0.1 N HCl. This mixture was diluted to 500ml with distilled water.

F.   **Standard ovine IgG:**   A lyophilised, essentially salt free preparation. No: 1-5131 (Sigma Chemicals Co., P.O. Box 14508, St. Louis, Mo., USA). 10 mg reconstituted in 0.25 ml distilled water gave a concentration of 4000 mg/100 ml.

G.   **Antibody (Antiserum to ovine IgG):**   Anti-sheep IgG, heavy and light chains. (ICN Immuno-biologicals, P.O. Box 1200, Lisle, Illinois, USA). Code 65-130. Lot No: R962. Control R100. Produced in a lyophilised form and raised in rabbits. Titre was 2.9 mg antibody/1.0 ml, determined by quantitative precipitin analysis, using sheep serum in the test. Specific for ovine IgG only.

Each vial was reconstituted with 2 ml distilled water.

H.   **Plastic calibrated plates and well cutter:** Plates (95mm X 46mm) and 2mm well cutter, with cutting template. (Miles Laboratories Ltd., Stoke Poges, Slough).

2.10.3 Preparation of Plates.

A.   In a water bath maintained at 56 ^{O}C, the agar was dispensed in 5.6 ml aliquots into pre-warmed glass bijou bottles, using a pre-warmed glass pipette. Each aliquot represented the amount of agar needed to fill one plate.

B.   For each 5.6 ml of agar, 0.2 ml of antiserum was added to 0.8 ml of sodium barbitone buffer, with gentle mixing. This 1.0 ml suspension was added to the agar immediately before plate pouring. The agar and antiserum contained in each bijou were mixed thoroughly and gently in the water bath.

C.   The concentration of antiserum required was calculated from the following equation:

$$P = \frac{4\ V_{ag}\ C_{ag}}{T\ \pi\ h\ (D^2 - d^2)}$$

where P = antiserum concentration required (ul/ml gel); V_{ag} = volume of antigen (ul); C_{ag} = maximum concentration of antigen (g/l); T = antiserum titre; h = depth of gel; D = maximum precipitin ring diameter (mm) and d = well diameter (mm).

D.   The agar was poured into plates (Figure 2) directly from the bijou bottles. To ensure even agar

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TOP VIEW

Base

Water moat

Agar

Millimeter Calibration
(continuous on three lines)

2mm Diameter wells
(cut into agar)

SIDE VIEW

Raised floor of plate
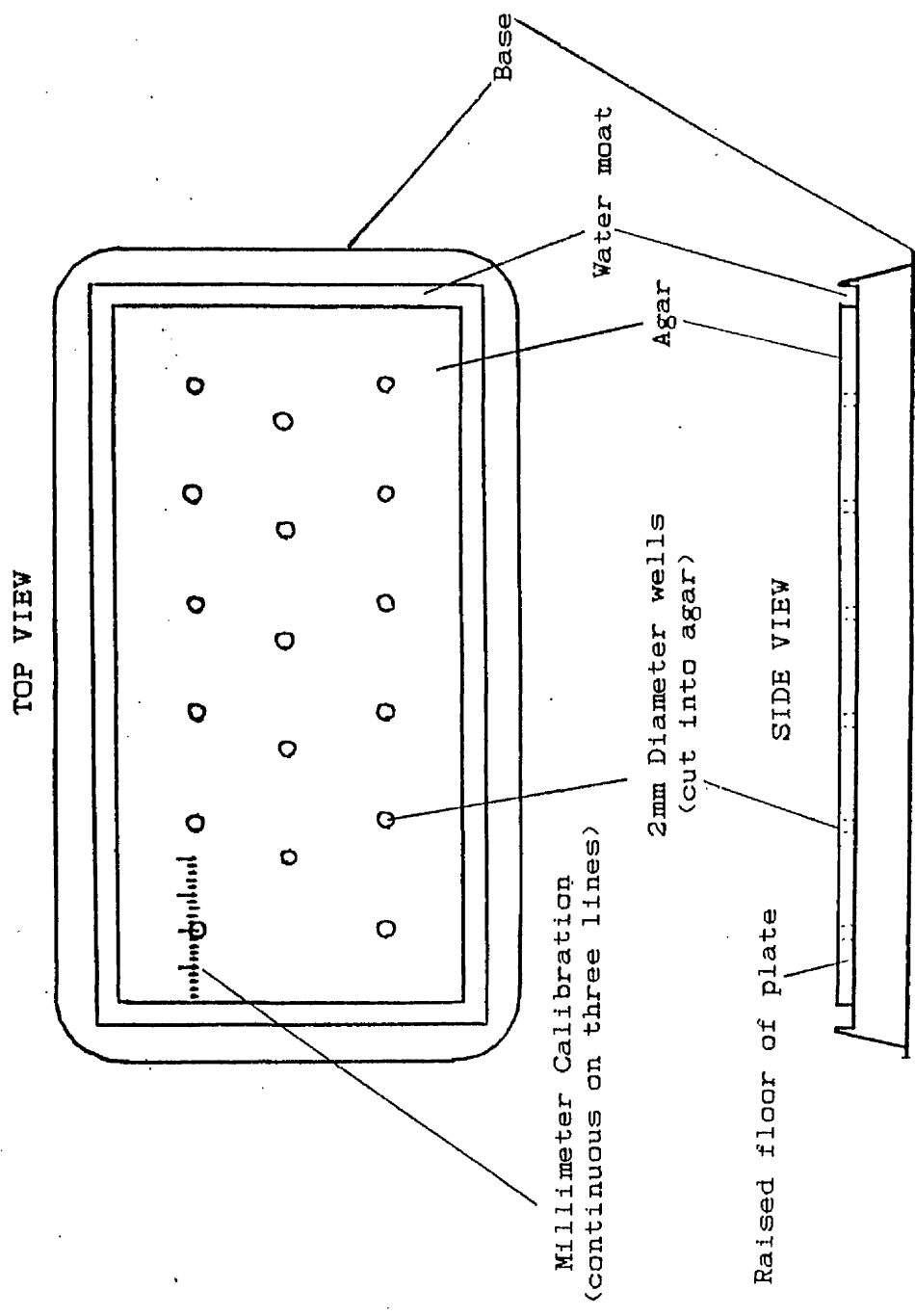
Figure 2. Plastic calibrated plate used in the SRID determination
of IgG in ewe colostrum and lamb serum.

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thickness, this was carried out on a perfectly flat surface. Once the agar had set the plates were stored at 4 °C before use.

E.   Immediately before use seventeen 2 mm diameter, 5 ul capacity wells were cut from the agar of each plate, employing a template and 2 mm well cutter.

2.10.4 <u>Preparation</u> <u>of</u> <u>Standards.</u>

2.10.4.1 Experiment 1.

The standard suspension of ovine IgG was 3000 mg IgG/100 ml. This was diluted with normal saline to produce standards of 3000, 1500, 750, 375, 187.5 and 93.75 mg IgG/100 ml.

2.10.4.2 Experiment 2.

The standard suspension of ovine IgG was 4000 mg IgG/100 ml. This was diluted with normal saline to produce standards of 4000, 2000, 1000, 500, 250 and 125 mg IgG/100 ml.

In both experiments, standards were replicated in triplicate and distributed among several plates in a batch. This was in order not to give any bias to the standard results due to possible inacurracies in plate pouring. Standards were added to the wells in 4.0 ul amounts.

2.10.5 Preparation of Whey.

Whey was extracted from colostrum samples by the method of Sharp (1965). 0.25 ml of rennet (BDH Ltd.) was added to every 10 ml of colostrum in centrifugation tubes. After gentle mixing and leaving to stand at room temperature for 2 hr., the tubes were centrifuged at 100 g for 10 min. The supernatant whey was pipetted off into 2 ml sample storage tubes.

2.10.6 Procedure.

A.   Samples of colostrum whey or serum were diluted to half concentration by mixing 0.2 ml with 0.2 ml normal saline. In some cases, the colostrum whey samples were diluted further to a third of the initial concentration, to ensure that the resultant precipitin ring diameters were within the range of the standards.

B.   The test solutions were added to the agar wells in 4 ul amounts. The water trough of each plate was filled to ensure that the agar did not dry out during incubation. Tests and standards were run together in the same batch, with 18 hr. incubation at room temperature.

C.   After incubation, all precipitin ring diameters were measured directly from the millimeter calibration marks on the plates.

2.10.7 Calculation of results.

A standard curve was obtained by plotting IgG concentration (mg/100 ml) on the logarithmic y axis of

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semi-logarithm chart paper, against ring diameter (mm) (x axis). The amount of IgG in test colostrum whey or serum was interpolated from this graph. The results were multiplied by 2 or 3, depending on initial dilution, to give concentrations in mg IgG/100 ml.

2.11 ANALYSIS OF SERUM BY THE ZINC SULPHATE TURBIDITY TEST.

2.11.1 Summary.

A turbidimetric method for the detection and estimation of increases in blood gamma-globulin levels was first described for human serum. The technique measured the degree of turbidity developed when highly dilute salt solutions, such as copper or zinc sulphate, were allowed to react with serum. Relative turbidity in standards and test samples was determined spectrophotometrically. The immune globulins are similar in structure and reaction behaviour to gamma-globulins. It was therefore possible to apply the zinc sulphate turbidity (ZST) test as an indirect measure of lamb immune status, according to the method of McEwan, Fisher, Selman and Penhale (1970).

2.11.2 Procedure.

A.   A stock solution of zinc sulphate (BDH Ltd.) was prepared by dissolving 250 mg $ZnSO_4$. $7H_2O$ in 1 litre of distilled water. The distilled water was boiled for 15 min. immediately before use, to remove dissolved carbon dioxide.

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B.   This stock solution was diluted by adding one part distilled water to five parts stock. The pH of this solution was adjusted to pH 7.0. If such precautions were taken, it was unnecessary to buffer the system.

C.   For each test serum, 6.0 ml of diluted zinc sulphate solution were dispensed into disposable plastic tubes. 0.1 ml of serum was added to each tube, making sure that all serum had been expelled from the pipette tip. Sera were tested singly.

D.   The tubes were shaken vigorously, covered and allowed to stand for 1 hr. at room temperature. The mixture was again shaken vigorously before reading.

E.   The degree of turbidity in each tube was determined spectrophotometrically by measurement of optical density (OD) at 620 nm, in a 1 cm plastic cuvette. Distilled water was used as a blank.

F.   The spectrophotometer (MSE, Spectro-plus) was calibrated with a solution of barium sulphate. To make this standard, a solution of 3 ml of barium chloride (BDH Ltd.), containing 1.15 g $BaCl_2 \cdot 7H_2O$ per 100 ml, was made up to 100 ml with 0.2 N sulphuric acid. When measured under the same conditions as the ZST test, this solution gave an OD of 0.60.

In accordance with the work of McEwan et al (1970), this calibrating solution, giving an OD of 0.60, was assigned an arbitrary ZST value of 20 units. A direct linear relationship existed between OD and ZST value.

Thus, any test serum OD could be given a value relative to the barium sulphate calibration standard. A straight line plot of OD against ZST value, passing through the origin and the coordinates 0.60 (OD) and 20 (ZST units), sufficed in obtaining ZST values from test OD results.

G.   Red blood cells in haemolysed samples interfered with the passage of light to the light sensitive detector of the spectrophotometer. This resulted in falsely high OD readings being obtained from haemolysed test sera. In these cases, 6.0 ml of boiled distilled water and 0.1 ml of serum were mixed and used as a blank. Twice the OD of the blank was subtracted from the test OD, to obtain an estimate of the true OD of the sample. This correcting procedure for haemolysed samples was that recommended by Fisher and Martinez (1975).

CHAPTER 3.

THE DIAGNOSIS OF COBALT DEFICIENCY IN THE PREGNANT EWE.

3.1 INTRODUCTION.

Much work has been reported in the literature which relates to the diagnosis of ovine Co deficiency. However, surprisingly little has been directed specifically at pregnant sheep.

Sub-clinical Co deficiency has been shown to have detrimental effects on ewe fertility and neonatal lamb growth (Dunlop, 1946b). Clinical disease has also been reported to lead to an increase in the incidence of stillbirths and neonatal lamb mortalities (Duncan et al, 1981). With this background, it would be pertinent to develop methods for the efficient diagnosis of Co deficiency in reproducing sheep (Mills, 1981), particularly for the sub-clinical syndrome. The Sub-clinical disease is more difficult to detect than the clinical form (Andrews, 1970b) and losses in production due to marginal deficiency may pass unnoticed by the farmer (Latteur, 1962). Thus, the ability to detect Co deficiency in pregnant ewes, both prognostically and diagnostically, would help to reduce animal production losses.

The role of serum vitamin B12 and MMA concentrations in the prognosis and diagnosis of Co deficiency in sheep, was discussed in the literature review. For the purpose of the work to be reported here, it is important to note that serum B12 is an essential Co-factor in the methylmalonyl CoA mutase system in liver cells. Limiting concentrations of B12 in the liver due to Co deficiency

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lead to the appearance of elevated levels of MMA in blood (McMurray et al, 1985).

The chronology of developing deficiency is an important influence on the employment of these two diagnostic markers. As the appearance of MMA in blood is dependent on a limiting concentration of liver B12 in the Co-deficient state, it may be expected that a fall in B12 levels in serum will precede a rise in MMA concentrations (Mills, 1981).

This apparent advantage of serum B12 over MMA as a diagnostic marker may be balanced by other factors. McMurray et al (1985) regarded plasma B12 as a 'passive' marker of the condition and MMA as an 'active' marker. Serum MMA concentrations may also provide a more reliable indication of sub-clinical Co deficiency, than serum B12 levels (Judson et al, 1981 and Suttle, 1986). Further, difficulties have been reported with the interpretation of serum B12 data (Millar and Penrose, 1980, Sutherland, 1980 and Millar et al, 1984).

Another problem with the use of serum B12 concentrations in the diagnosis of Co deficiency is the confusion which exists over the different analytical methods available (Slater et al, 1985). A further complication is added by the possible presence of inactive analogues of the vitamin in sheep serum (Sutton and Elliot, 1972 and Halpin et al, 1984).

The objectives of this investigation were to assess the

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application of serum B12 and MMA concentration to the prognosis and diagnosis of Co deficiency in pregnant ewes. Particular reference was made to the detection of the sub-clinical form of the disease. A comparison of serum B12 analysis by microbiological and radio-immuno (RIA) assays was also undertaken, which aimed to clarify the employment of these two techniques for routine diagnostic work.

3.2 MATERIALS AND METHODS.

The data presented were obtained from two experiments undertaken at the Brickrow Farm Unit, The West of Scotland College, Auchincruive, Ayr, Scotland, in 1985/86 (Experiment 1) and 1986/87 (Experiment 2). These investigated the effect of Co deficiency in pregnant hill ewes on reproductive performance and lamb viability. Results were derived from sheep of high and low Co status and from animals experiencing a progressive development into, as well as recovery from, the deficient state.

3.2.1 Animals and Treatments.

3.2.1.1 Experiment 1 (1985/86).

Sixty Co-sufficient Scottish Blackface X Swaledale ewes (Plate 1) were housed in four pens and bedded on sawdust (Plate 2). A Co-deficient ration (Table 1) was fed from 30/9/85. This was 17 days before Suffolk rams were introduced to the sheep. All ewes were initially blood sampled on day zero (10/10/85) and for the last time on

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Plate 1. Scottish Blackface X Swaledale ewes employed in Experiment 1.
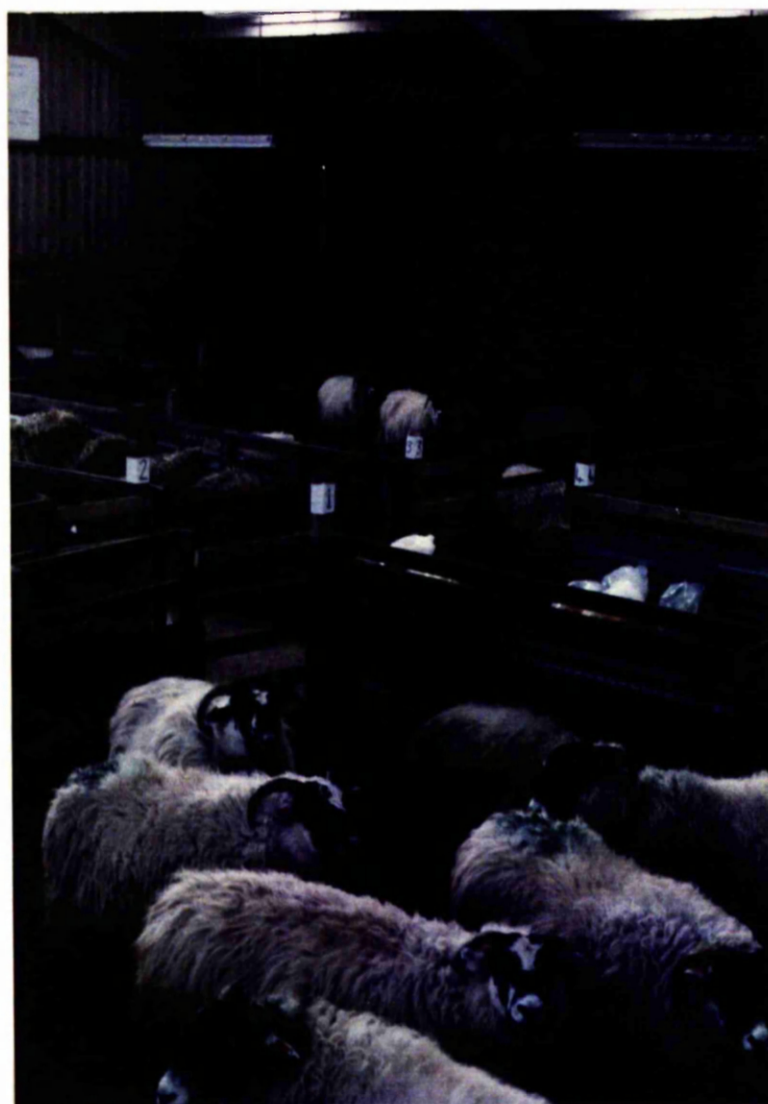
Plate 2. The shed and pens used in both Experiment 1 and Experiment 2.

Table 1. Co-deficient diet fed to all ewes in Experiments 1 and 2.

FEED	Co CONTENT (mg Co/Kg DM)	MAX. FRESH WEIGHT FED (Kg/head/day)
Timothy hay	0.06 ± 0.01	ad libitum
Micronised maize	0.026 ± 0.005	0.64
Maize gluten	0.03 ± 0.009	0.25
Skimmed milk powder	0.015 ± 0.007	0.10
Dicalcium phosphate	—	0.03
Na Cl	—	0.005
Water	—	ad libitum

day 189. This period included 2 weeks pre-mating, mating, pregnancy and the first 4 weeks of lactation.

According to weight and condition score on 30/9/85, the sheep were randomly assigned to three treatment groups, of 20 animals each:

1. Non-supplemented - 'NS' - Co-deficient intake throughout pregnancy.

2. Half-supplemented - 'HS' - Co-sufficient intake for the first half of pregnancy, but deficient from mid-term.

3. Fully- supplemented - 'FS' - Co-sufficient intake for the whole of pregnancy.

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The NS ewes were maintained on the basal Co-deficient ration throughout the experiment. The HS group received a weekly Co supplement of 0.7 mg Co/head, given as an oral dose of cobalt sulphate ($Co_2SO_4 . 7H_2O$), from 30/10/85 (day 20) until mid-pregnancy on 23/12/85 (Day 74), inclusively. These animals were then maintained on the basal diet for the remainder of the experiment. The FS sheep were given the weekly Co supplement from day 20, until the end of the investigation.

3.2.1.2 Experiment 2 (1986/87).

Thirty Scottish Blackface X Swaledale and thirty pure Scottish Blackface ewes, all Co-sufficient, were housed and bedded on sawdust, in the same four pens used in Experiment 1. A Co-deficient ration (Table 1) was introduced on 10/10/86. This was 57 days before Blue-Faced Leicester rams were introduced to the sheep. All ewes were blood sampled on day zero (10/9/86) and fortnightly thereafter, until day 260. This period included 8 weeks pre-mating, mating, gestation and the first 4 weeks of lactation.

According to weight and condition score on 2/9/86, the sheep were randomly allocated to three treatment groups, of 20 animals each:

1. Non-supplemented - 'NS' - Co-deficient intake throughout pregnancy.

2. Half-supplemented - 'HS' - Co-deficient intake for the first half of pregnancy, then Co-supplemented from mid-term.

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3. Fully-supplemented - 'FS' - Co sufficient intake for the whole of pregnancy.

The ewes were maintained on the basal Co-deficient ration throughout the experiment. The HS group also received only this diet until mid-term (18/2/87 - day 161), when they were replenished with weekly oral doses of 0.7 mg Co/head as cobalt sulphate, until the end of the experiment. The FS sheep were given the weekly Co supplement from day 50, (37 days before the start of tupping), until the end of the experiment.

3.2.2 Blood Sampling and Analysis.

All ewes were bled fortnightly by jugular-venipuncture. In both experiments, sera were analysed for vitamin B12 content by the microbiological assay using Lactobacillus leichmannii as the test organism (Chapter 2, section 2.4.1). In Experiment 2, B12 was also determined in sera by the RIA method (Chapter 2, section 2.4.2). MMA concentrations in the sera from both experiments were measured by a capillary gas chromatographic method (Chapter 2, section 2.5).

3.2.3 Statistical Analysis.

In both experiments, one way analysis of variance was used to test for significant difference between mean serum vitamin B12 or MMA concentrations among treatments at all the fortnightly samplings. Where an analysis of

variance for a particular sampling was significant, the least significant difference test was used to determine significant differences between the means of individual treatments.

A regression analysis of treatment mean serum B12 levels, determined by the microbiological assay in Experiment. 2, on RIA concentrations was performed. Thus, the mean serum B12 concentrations of each treatment at each sampling, analysed by the microbiological assay, were regressed on the corresponding results from the RIA technique.

3.3 RESULTS AND DESCRIPTION OF TRENDS IN THE DATA.

This section will present the serum B12 and MMA results of Experiments 1 and 2. A description of trends in the data will also be included. This will be derived directly from, and will contain no interpretation of, the results. For the purpose of data analysis and discussion, the following diagnostic criteria will be applied to the results:

Serum vitamin B12 >400 ng/l - Co adequate status.
                                 (Lower normal limit - LNL)

         200 - 400 ng/l - Marginal Co deficiency.
                          (Sub-clinical)

            <200 ng/l - Functional Co deficiency.
                        (Clinical)

(After MacPherson, 1982).

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Serum MMA   <5.0 umol/l - Co adequate status.
                             (Upper normal limit - UNL)

          5.0 - 15.0 umol/l - Marginal Co status.
                             (Sub-clinical)

          >15.0 umol/l - Functional Co deficiency.
                         (Clinical)

(After McMurray et al, 1985).

3.3.1 Serum Vitamin B12 by Microbiological Assay
- Experiment 1.

3.3.1.1 General trends.

Treatment mean serum B12 results are presented in
Figure 1 and Appendix 1. Serum B12 concentrations in the
NS ewes fell to be consistently below the LNL from day 55
onward, which was 65 days after the introduction of the
Co-deficient diet.

The serum B12 status of the HS animals was high and
above the LNL until mid-pregnancy, when these sheep were
taken off the Co supplement (day 74). Serum B12
concentrations in this treatment then fell below the LNL
on day 133 (59 days after the introduction of a Co-
deficient intake) and remained low for the rest of the
experiment.

Serum B12 levels in the FS sheep remained above the
LNL for the whole of the experiment.

3.3.1.2 Statistical evaluation.

There were no statistically significant differences in
mean serum B12 concentrations among treatments until day

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Figure 1. Mean serum vitamin B12 concentrations, analysed by the microbiological assay, for the ewes in Experiment 1. (|————| = Tupping period, |-----| = Lambing period, ↓ = Point at which the HS sheep were taken off the weekly Co supplement).

41. At this time, the NS ewes had significantly lower serum B12 levels compared to the HS and FS sheep (P<0.001). Serum B12 concentrations in the NS animals then remained significantly below the FS controls for the remainder of the experiment (P<0.01 to P<0.001).

After removal of the Co supplement from the HS ewes on day 74, serum B12 levels in this group became significantly lower than those in the FS sheep (P<0.001), but still higher than the NS treatment (P<0.01), on day 90. Serum B12 concentrations in the HS animals were then significantly lower than the FS controls for the rest of the experiment (P<0.01 to P<0.001). No statistically significant differences were found for serum B12 levels between the HS and NS ewes on day 133, or for the remainder of the experiment.

3.3.2 Serum MMA – Experiment 1.
3.3.2.1 General trends.

Treatment mean serum MMA results are presented in Figure 2 and Appendix 2. Serum MMA concentrations in the NS ewes rose to be consistently above the UNL, from day 133 onward. This was 143 days after the introduction of a Co-deficient intake.

Serum MMA concentrations in the HS animals rose above the UNL on day 161; 87 days after the introduction of a Co-deficient intake. However, MMA levels in this group fell below the UNL on day 189, after being above this threshold on day 175. This unexpected result may have
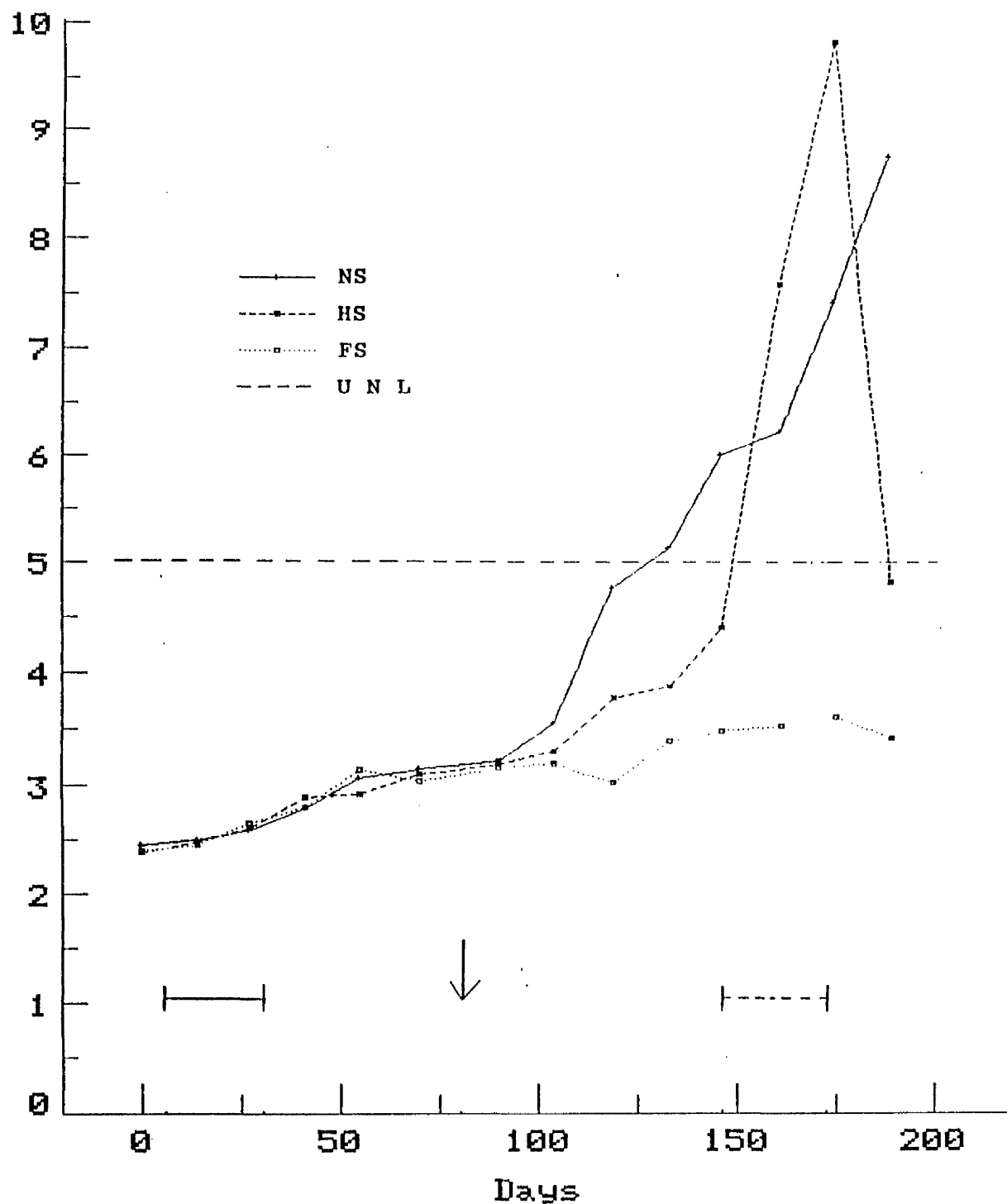
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Figure 2. Mean serum MMA concentrations for the ewes in Experiment 1. ($\vdash$———$\dashv$ = Tupping period,$\vdash$----$\dashv$= Lambing period, $\downarrow$ = Point at which the HS sheep were taken off the weekly oral Co supplement).

been due to the small number of ewes available for bleeding at this last sampling date, producing an unrepresentative value.

Serum MMA Levels in the FS sheep remained below the UNL throughout the experiment.

3.3.2.2 Statistical evaluation.

There were no statistically significant differences in mean serum MMA concentrations among treatments until day 104, when all groups differed from one another ($P < 0.05$). A significant difference was again detected among all treatments on day 119 ($P < 0.01$ to $P < 0.001$). On days 133 and 146, mean serum MMA levels in HS and FS sheep were not significantly different, but were significantly lower than those in the NS animals ($P < 0.001$).

After removal of the Co supplement from the HS sheep on day 74, serum MMA levels in this group became significantly higher than those in the FS treatment on day 161 ($P < 0.001$) and remained so for the rest of the experiment. From day 161 onward, serum MMA levels in the HS and NS ewes were not significantly different.

MMA concentrations in the serum of NS and FS ewes differed significantly from day 104, to the end of the experiment ($P < 0.05$ to $P < 0.001$).

3.3.3   Comparison of Serum B12 and MMA Data
                    - Experiment 1.

Serum MMA concentrations in the NS ewes rose above the
UNL 77 days after serum B12 levels in the same animals
had fallen below the LNL. Similarly, MMA concentrations
in the serum of the HS group rose above the UNL 28 days
after serum B12 levels in the same sheep had fallen below
the LNL.

A plot of treatment mean serum B12 concentrations,
determined by the microbiological assay, against
treatment mean serum MMA levels for Experiment 1, is
presented in Figure 3. The points enclosed within the box
in this figure represent occasions where the mean serum
B12 concentration in a treatment, at a particular
sampling, was <400 ng/l, with a corresponding mean serum
MMA level <5.0 umol/l.

3.3.4 Serum Vitamin B12 by Microbiological Assay   and
                    RIA - Experiment 2.

3.3.4.1 General trends.

Treatment mean data for the microbiological and RIA
determination of serum B12 in Experiment 2, are presented
in Figures 4 and 5 and Appendices 3 and 4, respectively.
In the section below, details of the RIA results are
given in brackets, following those for the
microbiological assay.

Serum B12 concentrations in the NS and HS ewes fell to
be consistently below the LNL from day 84 (42) onward.
This was 54 (12) days after these sheep were placed on a
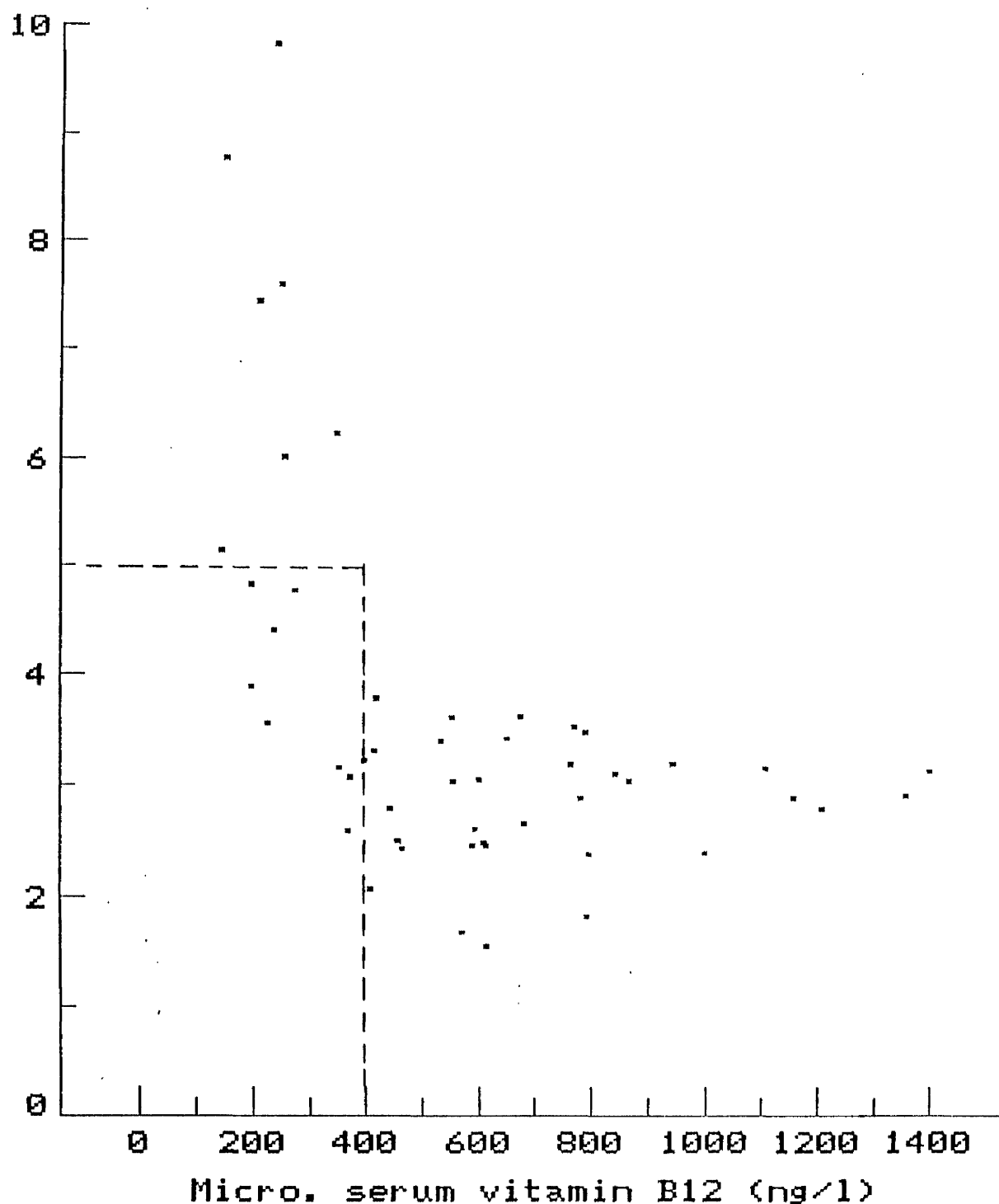
Figure 3. Scatter diagram showing the relationship between treatment mean serum MMA and serum vitamin B12 concentrations, at each sampling, for the ewes in Experiment 1. (Serum B12 analysis by microbiological assay).
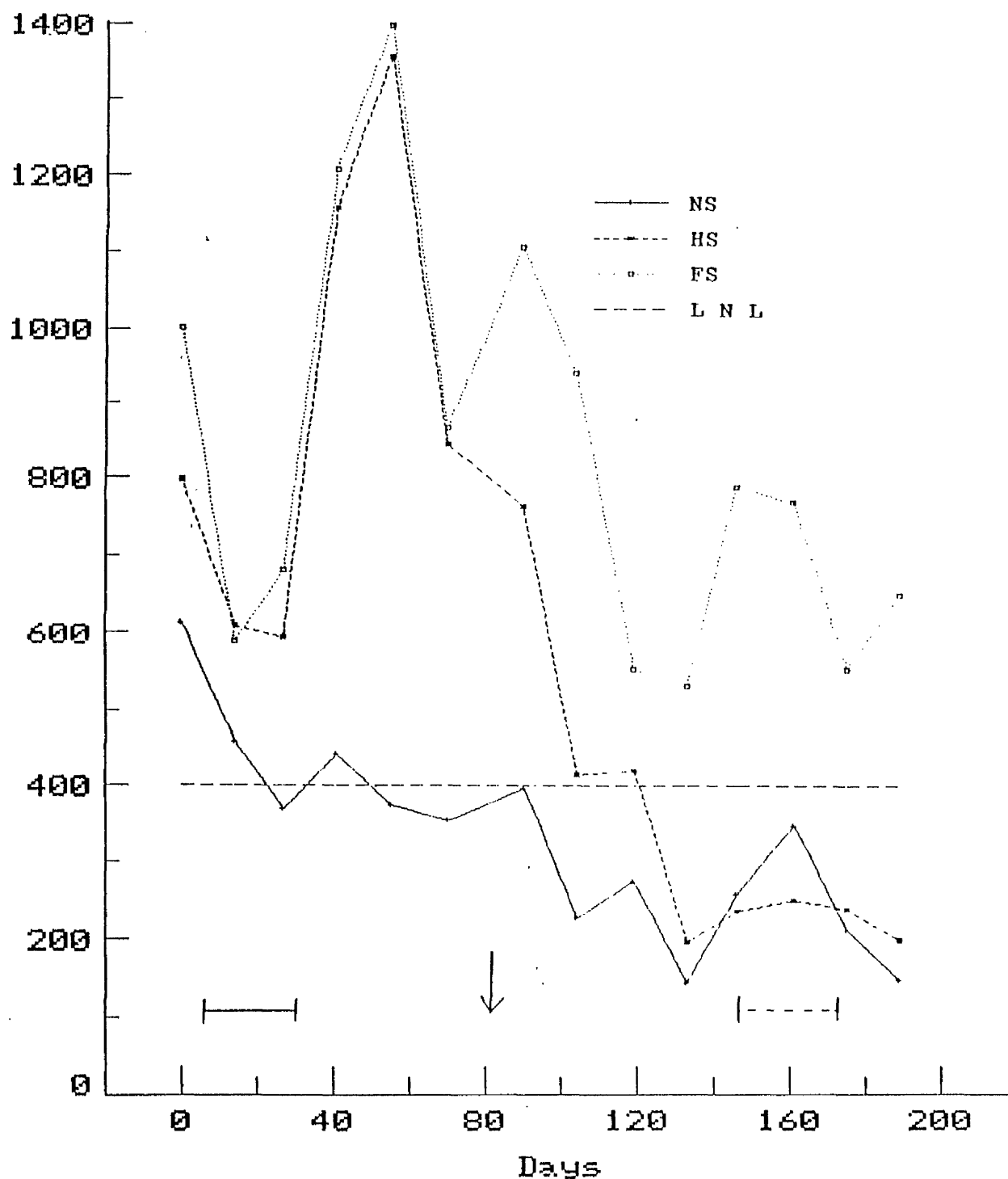
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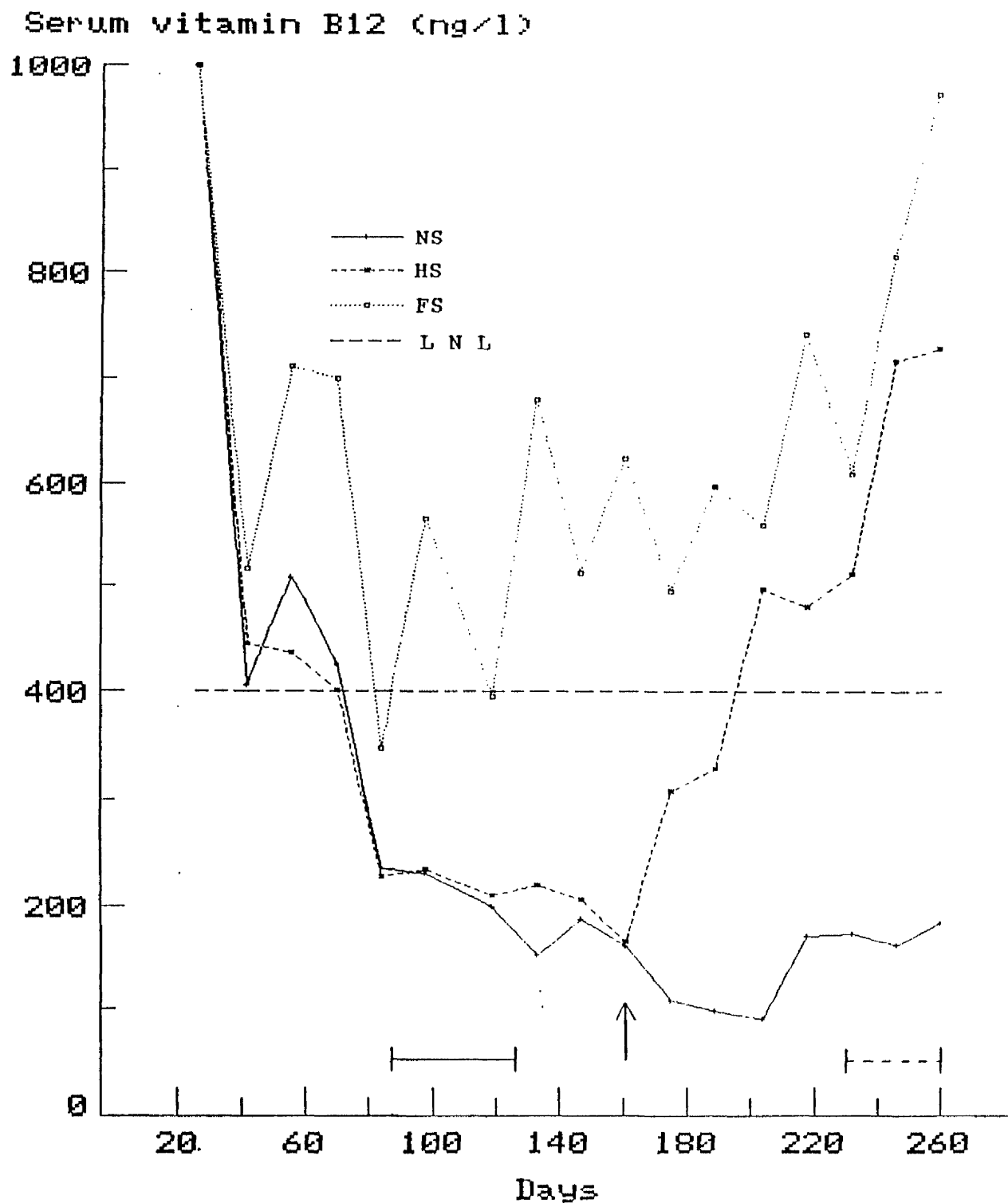
Figure 4. Mean serum vitamin B12 concentrations, analysed by the microbiological assay, for the ewes in Experiment 2. (├─────┤ = Tupping period, ├───┤ = Lambing period, ↑ = Start of weekly oral Co supplementation of the HS sheep).
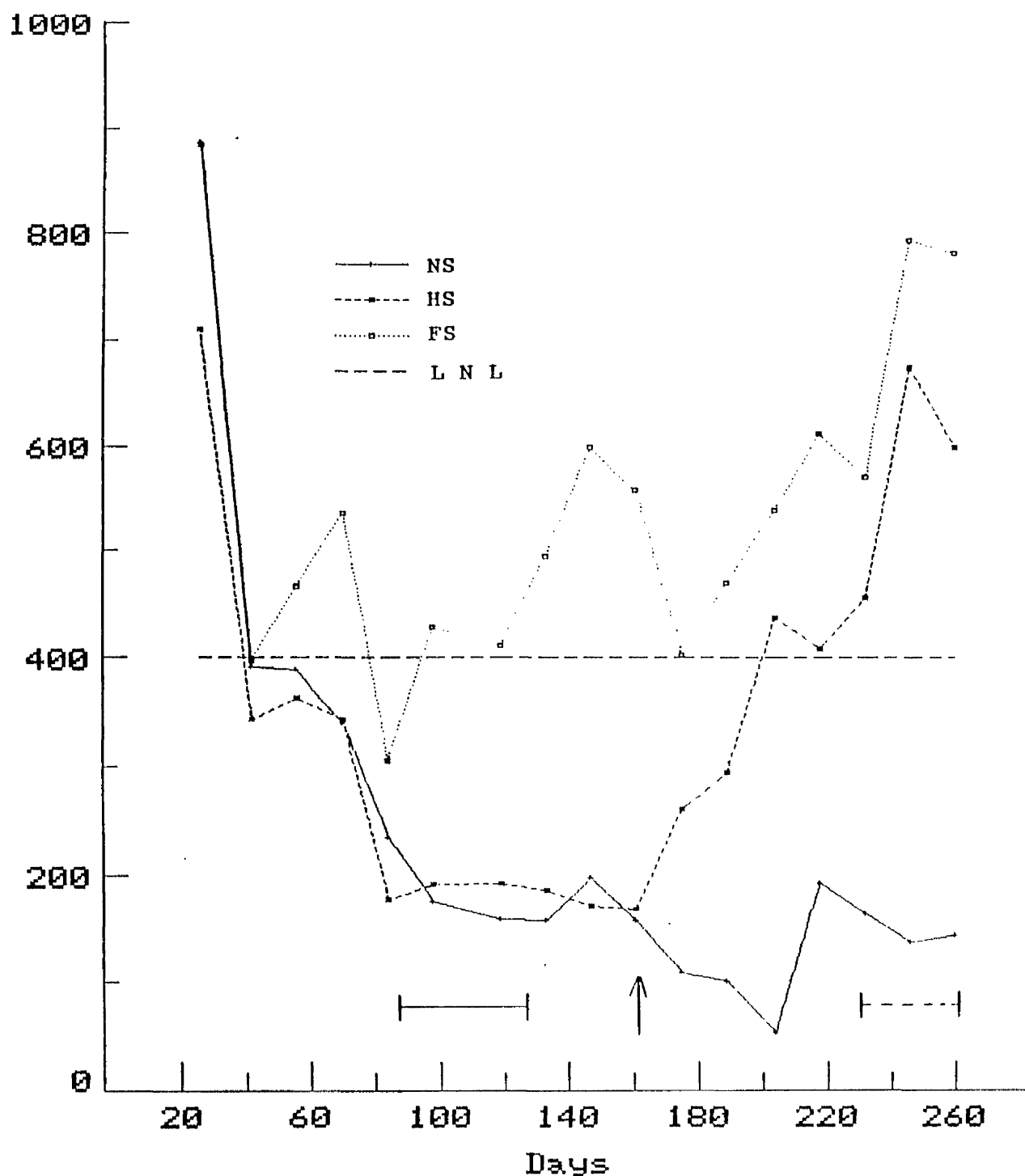
Figure 5. Mean serum vitamin B12 concentrations, analysed by the RIA, for the ewes in Experiment 2. (⊢———⊣ = Tupping period,⊢----⊣= Lambing period, ↑ = Start of weekly oral Co supplementation of the HS sheep).

Co-deficient intake. The serum B12 status of the NS group fell further, with mean values consistently below 200 ng/l from day 133 (98).

Serum B12 concentrations in the HS treatment fell until the introduction of the weekly oral supplement on day 161. Levels in these animals then increased and rose above the LNL, 43 (43) days after the start of Co repletion.

Serum B12 levels in the FS animals remained generally above the LNL throughout the experiment.

3.3.4.2 Statistical evaluation.

There were no statistically significant differences in mean serum B12 concentrations among treatments, until after the introduction of Co supplementation to the FS ewes on day 50. On day 56 (70), serum B12 levels in the FS sheep were significantly above those in the NS and HS animals (P<0.01). Serum B12 concentrations in the FS and NS animals then remained significantly different for the rest of the experiment (P<0.001).

Serum B12 concentrations in the HS ewes were significantly below those in the FS sheep, from day 56 (70) to 189 (218) inclusive (P<0.01 to P<0.001). On day 204 (232), 43 (71) days after the start of Co repletion, levels in HS animals were not significantly different from the FS treatment and remained so for the rest of the experiment. Serum B12 levels in the HS group rose to be significantly above those in the NS ewes on day 175 (175)

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(P<0.001). This was 14 (14) days after the start of Co
repletion and this difference between the HS and NS
groups was maintained for the remainder of the
experiment.

3.3.5 Serum MMA - Experiment 2.

3.3.5.1 General trends.

Treatment mean serum MMA data are presented in Figure 6
and Appendix 5. Serum MMA concentrations in the NS ewes
rose to be consistently above the UNL from day 119
onward. This was 149 days after the introduction of the
Co-deficient ration. MMA levels in the NS sheep continued
to rise and were consistently above a mean of 15 umol/l
from day 232 to the end of the experiment.

Mean serum MMA levels in the HS ewes rose above the UNL
on day 133 (163 days after the introduction of the Co-
deficient diet) and remained above this threshold until
after the initiation of Co repletion on day 161. Levels
in this treatment then fell below the UNL on day 189, 28
days after the start of Co supplementation.

Serum MMA levels in the FS animals remained below the
UNL throughout the experiment.
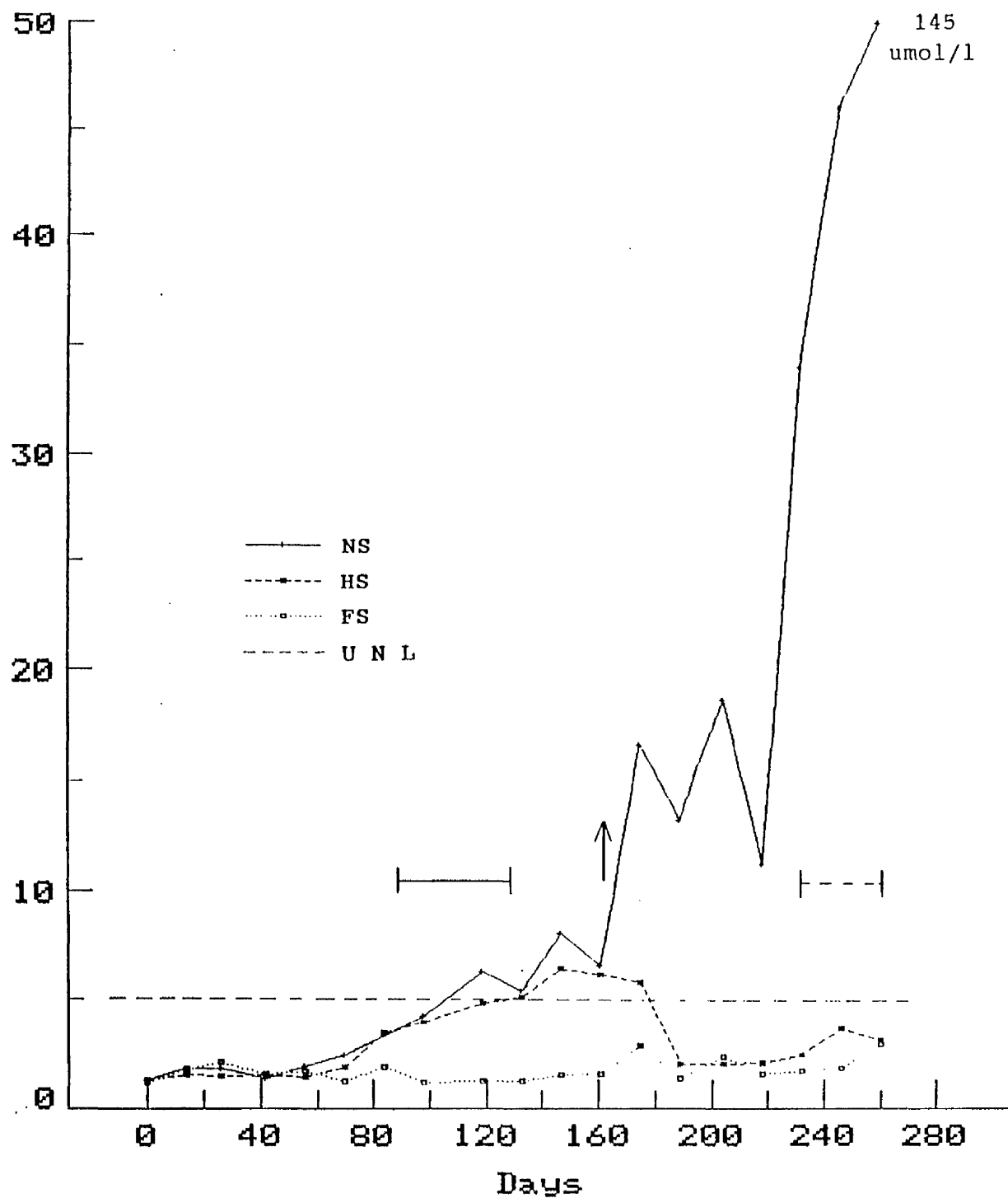
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Figure 6. Mean serum MMA concentrations for the ewes in Experiment 2. (├────┤ = Tupping period,├ ---┤ = Lambing period, ↑ = Start of weekly oral Co supplementation of the HS sheep).

Table 1a. Serum MMA and Vitamin B12 concentrations in ewes 3, 4 (HS) and 24 (NS) compared with treatment means in Experiment 2.

DAY OF EXP.	EWE No:	SERUM MMA	TREATMENT MEAN	SERUM VIT. B12	TREATMENT MEAN
0	3	ND	1.3umol/l	865ng/l	2383
	4	ND	1.3	985	2383
	24	8.0umol/l	1.3	630	2152
14	3	20.5	1.5	840	1559
	4	56.8	1.5	510	1559
	24	7.2	1.8	625	1545
26	3	62.4	1.4	668	1449
	4	ND	1.4	940	1449
	24	6.0	1.8	560	1398
42	3	39.5	1.5	ND	444
	4	25.8	1.5	ND	444
	24	8.3	1.3	455	406
56	3	97.4	1.4	290	436
	4	31.0	1.4	280	436
	24	9.8	1.9	390	509
70	3	78.4	1.9	165	425
	4	65.0	1.9	150	425
	24	14.9	2.4	170	401
84	3	180.5	3.4	110	235
	4	223.2	3.4	130	235
	24	33.4	3.3	165	228
89	3	165.9	3.9	80	231
	4	180.1	3.9	140	231
	24	85.0	4.2	120	234
119	3	446.2	4.8	75	199
	4	325.9	4.8	140	199
	24	163.4	6.3	110	210
133	3	464.4	5.0	90	220
	4	ND	5.0	180	220
	24	145.8	5.4	80	152
147	3	384.0	6.4	ND	206
	4	496.4	6.4	ND	206
	24	271.8	8.0	ND	187
161	3	610.9	6.2	180	165
	4	506.2	6.2	190	165
	24	280.8	6.6	ND	161

3.3.5.2 Statistical evaluation.

Serum MMA concentrations in ewes 3, 4 (HS) and 24 (NS) were much higher than the mean levels for the respective treatments (Table 1a). Elevated serum MMA concentrations (>15 umol/l) were evident in these sheep when corresponding serum B12 levels were in the sufficient region (>400 ng/l). For these reasons, the data from these three ewes was not included in the statistical analysis of serum MMA concentrations. This anomaly will be discussed later in section 3.4.2.4.

There were no statistically significant differences in mean serum MMA concentrations among treatments until day 98, when levels in the NS and HS groups rose to be significantly above those in the FS animals (P<0.01). A statistically significant difference in serum MMA levels between the NS and FS groups was then maintained for the rest of the experiment (P<0.01 to P<0.001).

Serum MMA concentrations in the HS sheep were significantly above those in the FS treatment from days 98 to 161, inclusively (P<0.01 to P<0.001). On day 175 (14 days after the start of Co repletion), MMA levels in the HS ewes were not significantly different from those in the FS group and remained so for the remainder of the experiment. Also on day 175, serum MMA concentrations in the HS ewes fell to be significantly below those in the NS treatment (P<0.001). This difference was maintained for the rest of the experiment.

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3.3.6 Comparison of Serum B12 and MMA Data - Experiment 2.

Mean serum MMA concentrations in the NS and HS ewes rose above the UNL 35 (77) and 49 (91) days respectively, after serum B12 levels in the same sheep had fallen below the LNL.

From the start of Co repletion of the HS treatment, mean serum B12 concentration took 15 (15) days longer to return to a normal value than serum MMA levels.

A plot of treatment mean serum B12 concentrations, determined by the microbiological assay, against treatment mean serum MMA levels for Experiment 2 is presented in Figure 7. The points enclosed within the box in this figure represent occasions where the mean serum B12 concentration in a treatment, at a particular sampling, was <400 ng/l, with a corresponding mean serum MMA level <5.0 umol/l.

3.3.7 Comparison of Serum B12 Data as Determined by Microbiological Assay and RIA - Experiment 2.

A statistically significant regression was found between B12 concentrations analysed by the microbiological and RIA techniques, in the sera of ewes from Experiment 2 (Figure 8). On average, the mean results for each treatment at each sampling differed between the two methods by 20 per cent, when this difference was expressed as a percentage of the microbiological assay data. Thus, the microbiological technique gave serum B12 concentrations 20 per cent

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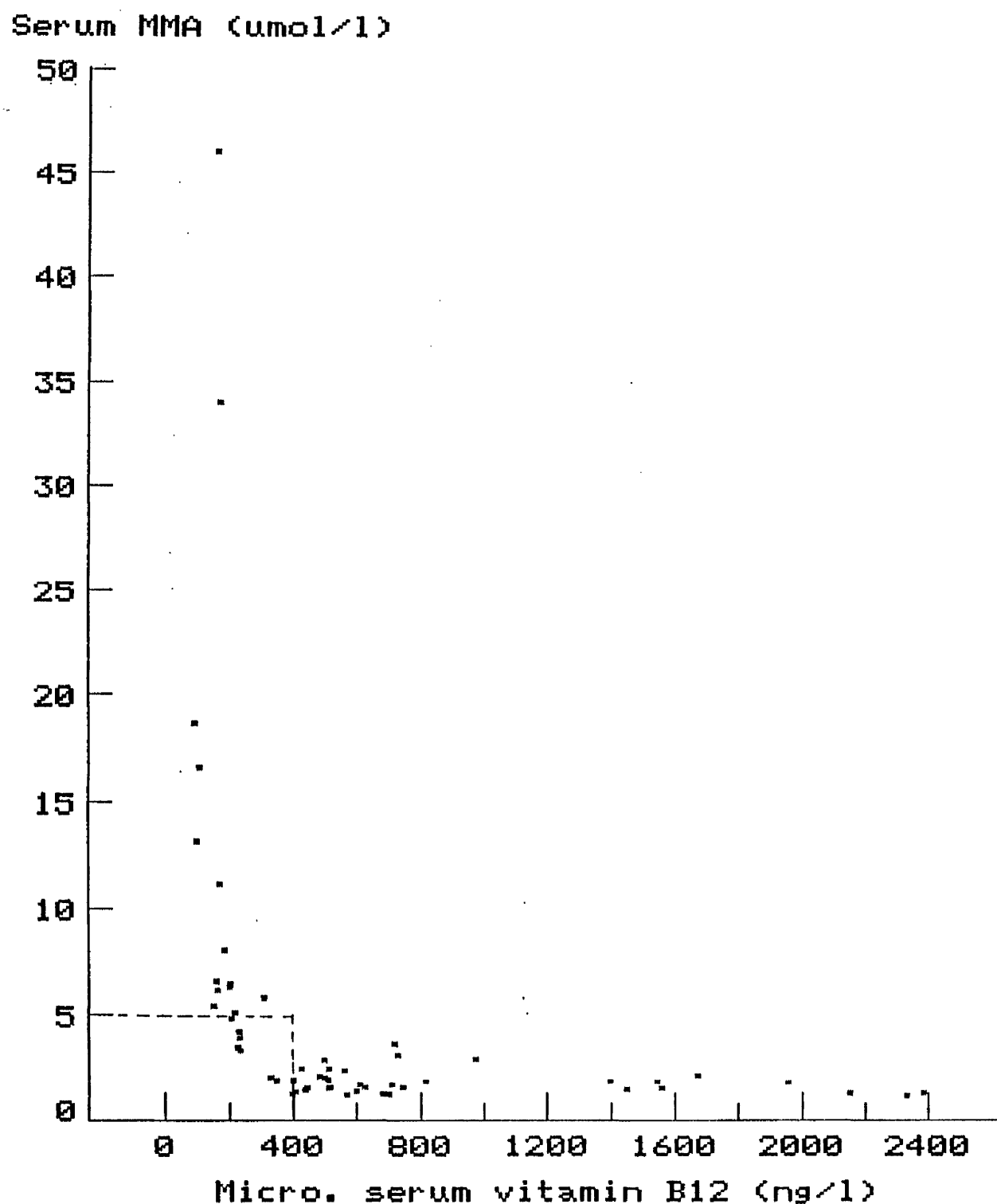
Figure 7. Scatter diagram showing the relationship between treatment mean serum MMA and serum vitamin B12 concentrations, at each sampling, for the ewes in Experiment 2. (Serum B12 analysis by microbiological assay).
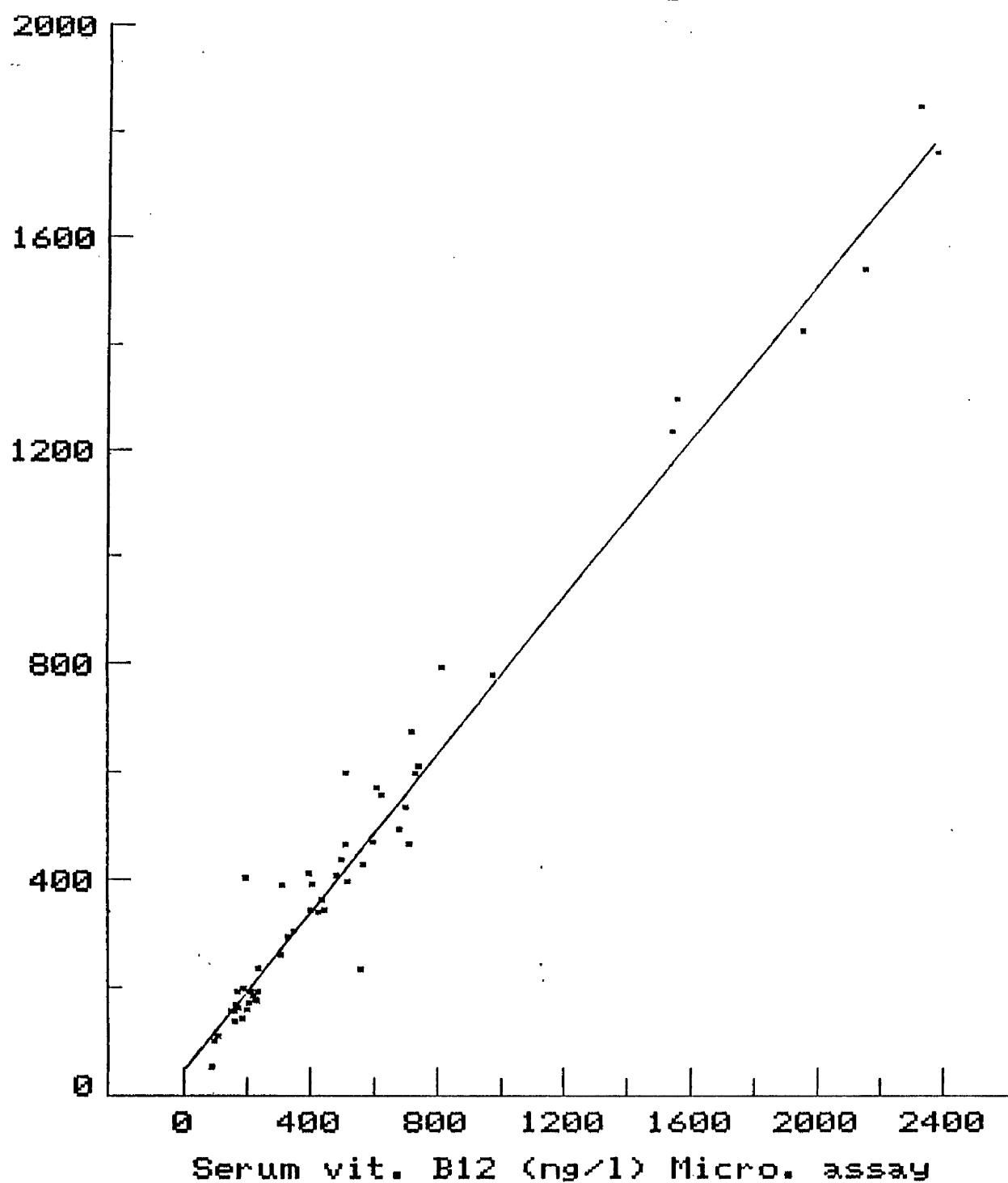
Figure 8. Scatter diagram showing the relationship between serum vitamin B12 concentrations analysed by the microbiological assay or the RIA, at each sampling, for the ewes in Experiment 2.

$y = 0.71x + 57.11$ ($P<0.001$, RSD = 77.7, n = 54, r = 0.97)

higher than the RIA method, on the same sera. The regression equation is shown below:

$$y = 0.71x + 57.11$$

RSD for variation about y = 77.71

$$r = 0.97 \quad n = 54 \quad (P<0.001)$$

Where, y = serum B12 concentration by RIA and x = serum B12 by microbiological assay.

3.4 DISCUSSION.

3.4.1 Levels of Cobalt Deficiency in Ewes - Experiments 1 and 2.

3.4.1.1 Experiment 1.

Using the diagnostic criteria stated at the beginning of the results section, both the NS and HS ewes of Experiment 1 were sub-clinically Co-deficient before the start of lambing. As deficiency progressed, mean serum B12 concentrations in these treatments fell to between 200 and 400 ng/l (Figure 1) and mean serum MMA levels increased to within the 5.0 to 15.0 umol/l range (Figure 2). Mean serum B12 and MMA levels in the FS ewes indicated Co sufficiency throughout the experiment.

Mean serum B12 concentrations in the NS group did fall to 150 ng/l on days 133 and 189. However, the appearence of such values in the clinical range were not consistent and serum MMA levels did not indicate clinical Co deficiency at these samplings. Between days 133 and 189, there was a peri-parturient rise in serum B12

concentrations in all treatments.

These conclusions were supported by observations of animal health. All animals were clinically normal throughout the experiment, with no symptoms of clinical Co deficiency disease evident.

3.4.1.2 Experiment 2.

Both the serum B12 and MMA data from this experiment indicated that Co deficiency had progressed in the NS ewes during pregnancy and that by the end of the investigation these sheep had experienced sub-clinical, developing to clinical, disease. However, the clinical deficiency evident from the start of lambing was not severe. Mean serum B12 concentrations in the NS animals (Figure 4) were within the sub-clinical range from approximately day 70 to day 133, and in the clinical region thereafter. Mean serum MMA levels for this treatment (Figure 6) were in the sub-clinical range from day 119 until day 232, after which they were consistently in the clinical region. Mean serum B12 and MMA concentrations in the HS ewes indicated sub-clinical Co deficiency from tupping until mid-pregnancy, when these sheep were supplemented with Co. Mean levels of B12 and MMA in the serum of the FS group implied Co sufficiency throughout the investigation.

As in Experiment 1, these conclusions were supported by observations of animal health. Ewes in the HS and FS treatments were clinically normal throughout Experiment

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2. The NS sheep appeared to suffer from clinical Co deficiency at the start of lambing, although the symptoms were not clear. These ewes were lethargic at lambing time and this continued until the end of the experiment. However, no other symptoms such as pale and sensitive skin and mucous membranes, lacrymation, photosensitivity, loss of appetite or emaciation were evident.

3.4.2 Comparison of Serum B12 and MMA as Diagnostic Markers.

3.4.2.1 Variability in serum B12 and MMA data.

In both experiments, mean serum B12 concentrations in pregnant ewes were more variable than serum MMA levels (Figures 1, 2, 4 and 6). For example, in Experiment 2 the mean serum B12 concentrations in the FS sheep fell briefly below the LNL on days 70 and 119 (Figure 4). This would supposedly indicate sub-clinical Co deficiency in these Co supplemented animals. However, serum MMA levels in these same ewes did not rise above the UNL at any time.

Another example occurred in Experiment 2 where mean serum B12 concentrations in the NS sheep were variable around the LNL between days 14 and 90 (Figure 1). This made it difficult to decide whether or not these animals were sub-clinically Co-deficient at this time. In contrast, mean serum MMA levels in the same ewes were less varied between samplings and when concentrations rose above the UNL on day 133, thus inferring sub-

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clinical disease, this was decisive with concentrations remaining above this threshold thereafter.

3.4.2.2 Serum B12 and MMA in the diagnosis of cobalt deficiency in pregnant ewes.

Serum B12 concentrations indicated Co deficiency in pregnant ewes before the same conclusions could be drawn from the serum MMA data. This was evident in Experiment 1, where mean serum B12 levels in the NS and HS ewes indicated sub-clinical disease, 77 and 28 days respectively, before mean serum MMA concentrations in the same sheep implied this degree of deficiency. The same conclusion could also be derived from the NS and HS treatment data of Experiment 2. Further, mean serum B12 levels in the the NS sheep consistently indicated clinical Co deficiency from day 133, while serum MMA concentrations did not consistently imply this degree of disease until 99 days later, on day 232.

These results suggest that serum B12 levels reflected a fall in Co status in pregnant ewes some weeks before the same diagnostic inference could be drawn from the serum MMA data. This finding is in agreement with the theoretical discussions concerning the biochemistry of Co deficiency in section 3.1.1. The appearance of MMA in blood is dependent on a limiting concentration of B12 in the liver (McMurray et al, 1985). Thus, it is not surprising that serum B12 levels fall before a rise in serum MMA concentration is detected.

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3.4.2.3 Implications for the diagnosis of cobalt deficiency in pregnant ewes.

These observations on the variability and chronological characteristics of serum B12 and MMA concentrations, in relation to Co deficiency, may have implications for the use of these two markers in the diagnosis of the disease in pregnant ewes. These effects will be discussed in this section.

That serum B12 concentrations declined before serum MMA levels increased means that there were inevitably a proportion of cases where the use of these two markers did not coincide in terms of diagnosis. These instances of disagreement occurred at serum B12 concentrations of between 200 and 400 ng/l, in the sub-clinical range.

In the field situation, the chronological characteristics of these two markers in a developing Co deficiency may not be important, as a diagnosis would be made on samples taken on only one or two occasions. However, it is possible to envisage a proportion of cases where these differences in the diagnostic interpretation of serum B12 and MMA concentrations could affect the advice given to farmers. For instance, where a sub-clinical deficiency problem on a farm might be diagnosed by analysis of serum vitamin B12, measurement of serum MMA on the same samples may not lead to the same conclusion. If the diagnostic criteria employed in these experiments are applied, then a number of such situations will arise.

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This point is well illustrated by the scatter plots of Figures 3 and 7. These represent mean serum vitamin B12 (microbiological assay) and MMA data from both experiments. Without exception, for all samplings where mean serum B12 concentrations for a treatment were above the LNL (i.e. Co-sufficient), the corresponding MMA levels were in the sufficient range below the UNL. However, in Experiment 1 on 15 occasions where mean serum B12 levels for a treatment were in the deficient range below 400 ng/l, 8 (53 per cent) also showed concentrations of MMA in the sufficient region. Similarly, in Experiment 2, on 26 occasions where levels of the vitamin were below the LNL, 11 (42 per cent) had corresponding serum MMA concentrations below 5 umol/l. The anomalous values are enclosed in the boxes in Figures 3 and 7. Similar results to these were reported by Rice et al, (1987). Thus, a deficient serum vitamin B12 concentration may not be reflected by a serum MMA level which would yield the same diagnostic conclusion. There are three possible reasons for this anomaly.

Firstly, the ranges set as diagnostic criteria for either marker may be wrong, at least in the context of pregnant ewes. Those employed for the microbiological determination of serum B12 using L. leichmannii, have been tested and found to be useful in many animal response experiments, both in tightly controlled housed experiments (MacPherson et al, 1976) and in the field (MacPherson, 1982). The ranges set for the employment of

serum MMA in the diagnosis of ovine Co deficiency, were studied by McMurray et al, (1985) and Rice et al, (1987) and were found to correspond to animal response in terms of Co/B12 status.

Assuming that the two markers did broadly reflect response of the sheep to Co status, a second explanation for the anomaly might be inconsistency in analysis. It has long been postulated that spurious low values ('false positives') can occur in serum B12 analysis (Findlay, 1972 and Mills, 1981). This possibility of false values and the problems associated with great variability of serum B12 data in the diagnosis of Co deficiency, has led some workers (Findlay, 1972, Millar and Penrose, 1980 and Judson et al, 1981) to conclude that this parameter is of little use as an indicator of sub-clinical Co deficiency in sheep. Variability in mean serum B12 concentrations between samplings for the same treatments, was certainly evident in the two experiments reported here.

The charges of inconsistency and variability can not be levelled at the serum MMA data of Experiments 1 and 2. Indeed, levels of MMA in serum were less variable than concentrations of B12. Further, Rice et al (1987) concluded that there were no problems from the diagnosis of false positives or negatives with the analysis of MMA in the blood of grazing sheep.

The third possible explanation is that as the imposed deficiency developed, voluntary feed intake was reduced,

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or the deficient animals stopped feeding completely. This would have the effect of lowering the propionate load on the liver. The production of MMA would subsequently decline leaving little in serum, while serum B12 remained low. This possibility was highlighted by Andrews and Hogan (1972) and Rice et al (1987). However, the ewes in these experiments were housed and under close supervision, and inappetence due to Co deficiency did not occur.

The correct interpretation of the anomaly perhaps lies with the second explanation and with the consideration that serum B12 is a passive marker of Co deficiency, while serum MMA is an active indicator. For these reasons, a decline in serum B12 concentration is not necessarily an accurate indication of functional vitamin levels and stores in the liver. At some point during B12 depletion the amount of vitamin in the liver becomes limiting to the methylmalonyl CoA mutase enzyme. The difficulty in relation to diagnosis is to determine the B12 concentration in serum which reflects such a situation in the liver.

Comparison of the results from these two experiments with evidence in the literature suggests that it may be impossible to define a concentration of B12 in serum, which would accurately and precisely make such a prediction. Vitamin B12 has no functional role in serum and blood may simply act as a transport mechanism (McMurray et al, 1985) and store (Suttle, 1986) for B12.

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This store, if such it be, has to be used before there is any possibility of deficiency at the liver level.

If this is the case, low concentrations of B12 in serum would not be caused by a dearth of functional vitamin levels and stores in the liver. Rather, a serum B12 decline would precede a deficiency in the liver. When serum stores are utilised and concentrations fall, then at some point, a deficiency in the liver would ensue. A direct cause and effect relationship between serum and liver B12 levels can not then be inferred and serum concentrations would have a purely passive role in the diagnosis of Co defciency.

With this scenario, a low level of vitamin B12 in serum would not necessarily indicate metabolic deficiency in the liver. In comparison, it is postulated that MMA does not rise in serum until B12 actually becomes limiting to the methlmalonyl CoA enzyme in liver cells. Therefore, MMA is a more positive and active marker of Co deficiency than serum B12, especially in the grey area of sub-clinical disease (McMurray et al, 1985 and Rice et al, 1987). Further, there should be no possibility of diagnosing false negatives with serum MMA analysis (Rice et al, 1987). That is, when serum MMA concentrations are below the UNL, B12 is not deficient in the liver. When serum MMA levels are high, functional concentrations of the vitamin in liver must be limiting.

Rice et al (1987) compared the use of plasma B12 and

MMA concentrations for the diagnosis of Co deficiency in grazing sheep. They concluded that it "...should be possible to make a positive diagnosis of Co deficiency on the basis of a high plasma MMA result from one animal." However, to avert any possibility of false negatives, "...it would be best to assess the status of a flock using a small group of animals."

The results from these Experiments 1 and 2 were consistent with these arguments. That Co deficiency was indicated in the pregnant ewes by increased MMA concentrations in serum weeks after serum B12 levels had fallen below the LNL, is therefore not relevant to accurate diagnosis (Suttle, 1986). It is postulated here that such 'early' low concentrations of vitamin in serum, during the development of disease, are false in terms of indicating a sub-clinical B12 deficiency in the liver.

It may therefore be argued that the diagnostic ranges applied to the serum B12 data should be changed, in order to increase their accuracy and precision in diagnosing ovine Co deficiency. If this was undertaken, the problem of variability in the serum B12 results would not be overcome, but the data would be more applicable to the positive identification of Co deficiency disease in the liver.

However, it should be remembered that in the pregnant ewes of both experiments the fall in serum B12 concentrations before the observed rise in serum MMA levels, was a consistent phenomenon. Further, serum B12

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concentrations are reported to be a reliable indication of Co intake (MacPherson et al, 1976 and Sutherland, 1980). For these reasons, serum B12 concentrations may provide an acceptable indication of ensuing deficiency (Sutherland, 1980) and the diagnostic ranges applied to serum B12 data should therefore not be changed. Thus, while serum MMA levels may be a more accurate and precise diagnostic marker of Co deficiency at the liver in reproducing sheep, concentrations of B12 in serum may be used in the prognosis of the condition. The results reported here suggested that only serum B12, and not serum MMA, could be used as a prognostic marker of sub-clinical and clinical Co deficiency.

The most accurate and precise means of detecting Co deficiency in pregnant ewes, both prognostically and diagnostically, may thus be to determine the concentrations of both of these markers in serum and use them as joint indicators of disease. Such an approach was described by McMurray et al (1985). For this purpose, proposed criteria for the joint use of serum B12 and MMA concentrations in the diagnosis of Co deficiency in pregnant ewes, are presented in Table 2.

Table 2. Proposed diagnostic criteria for serum B12 and MMA in pregnant ewes.

VIT. B12* (ng/l)	MMA (umol/l)	COMMENT	STATUS
>400	<5.0	High B12:Normal MMA	Co-sufficient.
200 - 400	<5.0	Moderate B12:Normal MMA	Co-sufficient, but deficient intake.
200 - 400	5.0 - 15.0	Moderate B12 and MMA	Sub-clinical Co deficiency.
<200	>15.0	Low B12:High MMA	Clinical Co deficiency.

* Serum B12 determination by microbiological assay, using <u>L. leichmannii</u> as the test organism.

3.4.2.4 A possible problem with serum MMA.

In Experiment 2, three ewes showed consistently high concentrations of MMA in serum throughout the experiment. Recorded levels in these animals reached as high as 600 umol/l. Concentrations were generally around 15.0 umol/l when corresponding mean levels in their counterparts were in the Co-sufficient range, below 5.0 umol/l.

Two of these sheep (No:3 - HS and No:24 - NS) were found to be barren at scanning. The other (No:4 - HS) gave birth to a small lamb weighing only 2.4 Kg (the mean birthweight of the lambs in the HS treatment was 4.8 Kg). Ewes 3 and 24 were culled and the Co content of their livers analysed. Deficient liver Co values of 0.02 mg/Kg fresh weight were evident in both these animals. Their

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livers also appeared fatty on gross inspection and No:3 was diagnosed as suffering from white liver disease.

These observations provide a note of caution in relation to the use of serum MMA in the diagnosis of ovine Co deficiency. Where liver damage is present, this metabolite may 'leak out' into circulation in much higher concentrations than normal. This could produce situations where the analysis of serum for MMA would yield a false positive diagnosis of Co deficiency in individual animals.

In these cases, the liver damage was probably caused by the Co deficiency. In other circumstances, liver damage may result from different causes, but where Co status is adequate. Further work is needed to establish whether or not leakage of MMA from the liver would occur in such situations, resulting in abnormally high serum MMA concentrations in Co-sufficient animals.

3.4.2.5 The diagnosis of cobalt deficiency in flocks.

The results presented suggested that serum vitamin B12 concentrations were variable and somewhat inconsistent over time, compared to serum MMA levels. Thus, the diagnosis of Co deficiency in a whole flock of reproductive ewes, can not be based on the B12 analysis of serum samples from just one or two sheep. Determination of the more accurate and precise marker, serum MMA, may render such a diagnosis possible on the basis of a few samples. However, the possibility of

obtaining false positives due to liver damage and the general lack of background research, lead to the conclusion that such confidence can not yet be placed in the diagnostic efficiency of serum MMA.

The best possible approach to take in the prognosis and diagnosis of Co deficiency in a flock of reproducing ewes, would be to sample a larger proportion of the animals. This is particularly the case when it is necessary to detect the sub-clinical disease. A guideline proportion to sample, to enable an accurate diagnosis to be made, might be 10 per cent of a flock.

3.4.2.6 Cobalt repletion of the HS ewes in Experiment 2.

There is no evidence in the literature of any investigations which studied the effect of Co repletion on high serum MMA concentrations in Co-deficient ewes. In Experiment 2, the HS sheep were rendered sub-clinically Co-deficient and were then replenished from day 161.

In terms of mean serum concentrations, both B12 and MMA took several weeks to return to normal levels, after the start of Co supplementation (Figure 4 and 6). The fortnightly samplings were too infrequent to determine exactly when serum B12 and MMA levels indicated Co sufficiency in the HS sheep, but mean serum MMA concentrations seemed to fall below the UNL approximately 2 weeks before serum B12 levels rose above the LNL. These results suggested that both serum B12 and MMA

concentrations were sensitive indicators of Co intake in deficient ewes.

The fact that the two markers responded decisively to Co supplementation of the HS sheep, increases confidence in their use as diagnostic indicators of Co deficiency in pregnant ewes. Further, there were no marked fluctuations or variations in the response of either serum B12 or MMA concentrations. Both markers did take weeks, and not days, to return to normal levels, but the level of Co repletion provided was not great.

That serum MMA levels returned to the normal range in advance of serum B12 suggested that functional concentrations of the vitamin in liver were restored to adequate levels before this was reflected by B12 levels in circulation. This is in disagreement with the assertions of Sutherland (1980). The model for ovine Co depletion and repletion presented by this worker (Chapter 1, Figure 4), implied that in response to Co supplementation, serum B12 concentrations should rise before liver B12 levels. The results of Experiment 2 indicated that liver B12 status increased (leading to a decline in serum MMA concentrations) before serum B12 levels.

It should be noted, however, that comparison between the model of Sutherland (1980) and the results from Experiment 2, is difficult. If the HS sheep had been sampled more frequently, then serum B12 concentrations may have been shown to rise before serum MMA levels fell.

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This would fit Sutherland's model. The results that were obtained suggested that increased rumen microbial production of B12 after repletion was not reflected by rapid rises in serum B12 concentrations from fortnightly samplings.

Weekly oral dosing of the deficient HS ewes with Co, may have led to transient increases in serum B12 concentrations. This extra vitamin would have been used or stored in the liver. Such an effect would tend to increase B12 levels in the liver, with a consequential fall in serum MMA concentrations, while serum B12 status fluctuated according to time of sampling in relation to the last weekly Co supplement. The increase in B12 status of the liver was therefore not reflected in serum B12 concentrations from fortnightly samplings. The exact sequence of events could only have been determined by collecting blood more frequently from the HS ewes during the period of Co repletion.

These arguments imply that the model of Sutherland (1980) for Co repletion, is simplistic. The smooth, graded fashion in which this model envisaged the reponse of serum B12 to Co repletion, was not reflected in this investigation. Rather, weekly Co supplementation probably led to a more staggered response, which was not reflected by the sampling regime until several weeks after the start of Co repletion. Such a pattern is supported by the work of MacPherson (1987).

3.4.3 Comparison of the Microbiological Assay and RIA Techniques for the Determination of Serum Vitamin B12.

Due to the confusion which exists in the literature over the methodology of determining serum vitamin B12 (Millar and Penrose, 1980 and Slater et al, 1980), provision was made in Experiment 2 to study two analytical techniques. Serum vitamin B12 data measured by the microbiological assay method were compared with those determined by the RIA technique. Both methods provided serum B12 results which revealed the same trends in ewe vitamin status, in the three treatments of Experiment 2. However, the mean serum B12 concentrations for each group at each sampling differed between the two techniques by (on average) 20 per cent. In this way the microbiological assay consistently yielded mean serum B12 levels which were higher than those evident from the RIA. This was shown by the regression analysis performed on the two techniques, presented in Figure 8.

This phenomenon meant that confusion could arise in interpreting the results from these two methods. If the diagnostic criteria applied to the microbiological assay results were imposed on the serum B12 data from both techniques, they suggested different timings in the chronology of developing Co deficiency. Unfortunately, in advisory work, the same diagnostic ranges are applied to both techniques, but the RIA B12 data implied the presence of progressive Co deficiency in NS and HS ewes some days before the microbiological assay results.

The reasons for this discrepancy arise from the analytical methods employed. In sample preparation for the microbiological assay, all analogues of B12 were converted to cyanocobalamin. This form of the vitamin was then measured indirectly by the growth of the test bacterium, <u>L. leichmannii</u>. Thus, this technique determined 'total' B12 in serum, which included both physiologically active and inactive forms of the vitamin.

The RIA employed a blocking agent to inactive B12 analogues, hindering their ability to bind to the added binder. These analogues were therefore not measured by this technique. Hence, the RIA determined only 'true' B12 and yielded lower values for serum concentrations, than the microbiological assay measurement of 'total' B12.

The relationship between the data produced by these two analytical techniques was linear, as presented in Figure 8. The regression analysis was calculated and the graph was drawn from the means of over 1000 sera samples. The regression also had a high 'r' value of 0.97, which gives a high degree of confidence in the derived equation.

3.4.4   Implications for the diagnosis of cobalt deficiency in pregnant ewes.

The data presented here suggested that a change in the diagnostic criteria applied to the RIA (Becton and Dickinson) technique, may be appropiate. This might be achieved by simply reducing the ranges applied to the RIA method, which were established for use with the

microbiological assay, by 20 per cent. Thus, when this RIA method is used in the diagnosis of Co deficiency in pregnant ewes, serum B12 concentrations between 160 and 340 ng/l should be considered as indicative of sub-clinical (marginal) deficiency. Values less than 160 ng/l, would indicate clinical disease.

Before these new criteria can be employed, two factors must be clarified. Firstly, the diagnostic ranges superimposed on the data from either method, should enable accurate diagnosis. Taking the limitations of serum B12 concentrations in the diagnosis of Co deficiency into account, the two methods used did indicate the occurrence of the disease in the pregnant ewes of Experiments 1 and 2. Thus, in terms of diagnosis, the new criteria applied to the RIA technique would be effective.

The second factor to be noted is that these results were obtained from sheep fed a high concentrate type ration. There is evidence in the literature to suggest that high concentrate intakes reduce the amount of rumen microbially produced true vitamin B12, as a proportion of the whole, compared to high roughage intakes (Sutton and Elliot, 1972 and Walker and Elliot, 1972). This phenomenon may result in an increased proportion of inactive analogues being present in serum. Thus, for this data, the microbiological assay gave higher values for serum B12, because it measured all forms of the vitamin. On the contrary, the RIA technique did not determine such

increased levels of inactive analogues and in consequence produced lower serum B12 values than the microbiological assay.

An opposing situation to this might occur at pasture, where rations are high in roughage. In such cases it may be expected that the proportion of true B12 in serum will be more and that of inactive analogues less than that present in this experiment. In this situation, the difference between the two methods in serum B12 analysis would not be as great.

Work carried out at the Veterinary Investigation Centre, Auchincruive, supports this argument (Taylor and Greer, 1982). When analysing sera from 29 sheep at grass for serum B12 content, using exactly the same two techniques employed in Experiment 2, the following regression equation was obtained:

$$y = 0.91x + 47.7$$
$$r = 0.97 \quad n = 29$$

Where. 'y' = serum B12 by RIA and 'x' = serum B12 by microbiological assay. This equation implies only a negligible difference in serum B12 concentrations between the two methods. This is in strong disagreement with the consistent 20 per cent discrepancy found in the Experiment 2 data of this project.

For this reason, changing the diagnostic criteria

applied to serum B12 data from the RIA technique, may not be appropriate in the field situation, where sheep have a high roughage intake. Therefore, the results from this investigation can at present only be related to reproducing ewes on high concentrate diets.

More work is needed to clarify the position in different practical circumstances. For example, it is not clear how these arguments would apply to RIA determined serum B12 levels in gestational ewes at grass, when up to 1 Kg/ewe of concentrates per day are fed in the latter stages of pregnancy. It may be that in such circumstances, significant concentrations of inactive analogues would be present in serum and differences between RIA and microbiologically analysed B12 data might exist.

It is also important to note that some workers have found no evidence of the presence of inactive analogues in sheep serum, or have detected them in only negligible amounts (Gawthorne, 1970, Marston, 1970 and Halpin et al, 1984). If this is the case, then the above arguments used to explain the differences in microbiological assay and RIA B12 data would not be valid.

In addition, it has been postulated that the RIA method may not measure only 'true' B12 in serum (Millar and Penrose, 1980 and Wright, Taylor and Greer, 1982). Residual binding of inactive analogues to the added binder may occur in this technique, even in the presence of inactive analogue blockers. This would result in the

measurement of 'true' B12, plus any analogues that were residually bound.

It is clear from this discussion that the situation is confused and complex and that this investigation of two analytical techniques for serum B12, can not answer all the questions posed. Therefore, more work is necessary to establish whether or not inactive analogues are present in sheep serum and exactly what forms of the vitamin are measured by the RIA method.

Millar and Penrose (1980) reported that microbiological and RIA methods gave good agreement on serum B12 concentrations in sheep, except in situations where analogue production was significant. Thus, it seems that the differences in the two techniques used in this investigation were probably due to varying rates of rumen microbial B12 analogue production and absorption into serum. In which case, the correct diagnostic criteria must be imposed on corresponding serum B12 data, if accurate and reliable prognosis and diagnosis is to be achieved. The 'correct' criteria will depend on the interaction between the physiological state of the sheep and the type of ration fed. These factors must be considered before diagnosis is attempted.

Having discussed the results from Experiments 1 and 2 and compared them with evidence in the literature, there is a need to decide on the most effective strategy for diagnosing Co deficiency in pregnant ewes. However,

before such statements can be positively made other factors, such as the effect of feeding on serum B12 and MMA concentations, require investigation. Such influences will be studied in Chapter 4.

CHAPTER 4.

DEVELOPMENT OF SERUM VITAMIN B12 AND METHYLMALONIC ACID AS DIAGNOSTIC MARKERS OF COBALT DEFICIENCY IN SHEEP.

4.1 INTRODUCTION.

The use of serum vitamin B12 and MMA concentrations in the diagnosis of Co deficiency in pregnant ewes, was described in Chapter 3. Three aspects require further investigation: a) The possible presence of diurnal fluctuations in serum B12 and MMA levels. b) The effect of feeding and changing feeding times on the concentrations of B12 and MMA in serum. c) The effect of starvation on serum B12 and MMA levels.

4.1.1 Diurnal Flucuations and Feeding Pattern.

The possibility that concentrations of vitamin B12 in the blood of sheep might fluctate in a diurnal pattern was first proposed by Somers and Gawthorne (1969). In a trial with 10 individually housed Merino wethers fed controlled diets of graded Co content, these workers found marked fluctuations in plasma B12 levels during a day of intensive sampling, when blood was collected by jugular-puncture every 4 hours. Plasma B12 concentrations varied over a 24 hr. period by up to 130 per cent.

Such fluctuations could have been related to the exogenous stimulation of feeding frequency, or endogenous stimuli associated with the time of day. However, fluctuations in blood B12 levels only occurred in some sheep. In other wethers there seemed to be no relationship between time of feed intake, or time of day and the rise and fall in plasma B12 levels. Where variations did exist, they were more marked in animals

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with generally high plasma B12 concentrations and given a Co-sufficient intake, than in those showing low levels and receiving a Co-deficient diet.

Somers and Gawthorne (1969) concluded that plasma B12 concentrations did not appear to be affected by animal handling, but were dependent upon feed and thus Co intake. After a period of feeding, microbial production of vitamin B12 would increase in the reticulorumen of Co-sufficient sheep, followed by a rise in plasma concentrations. Liver stores would have been adequate and thus have had no buffering effect on large fluctuations in plasma B12 levels. On the contrary, in Co-deficient animals a rise in microbial production of B12 after a period of feeding on a diet deficient in the trace element, would not be as marked. In this case, liver stores would have been low and any vitamin transported in the blood would have been quickly used or stored in the liver and thus fluctuations in plasma B12 levels were buffered. Although this scenario explains the variations found by Somers and Gawthorne (1969) these investigators made no attempt to define any innate daily fluctations in plasma B12 concentrations, other than those associated with twice daily feeding.

The findings of these earlier workers are in sharp contrast to those of Millar et al (1984), who took six sheep at pasture, housed them for one day, fed cut grass ad libitum and bled at hourly intervals. Although serum

B12 concentrations in individual sheep ranged from 400 to 2500 ng/l, no diurnal fluctations were evident. Further, in six sheep fed at pasture and bled every three hours over a two day period, there were no diurnal fluctuations in serum B12 levels. From this later work, it would appear that the diurnal flucuations found by Somers and Gawthorne (1969), were solely related to a twice daily feeding pattern and not to time of day per se.

Investigations into the possibility that diurnal or other fluctuations in the concentration of MMA in blood might exist, have not been reported in the literature. Such characterisation is necessary if this parameter is to be used in the diagnosis of Co deficiency. This requirement for further work was identified by Andrews and Hogan (1972) who observed that "...the amount of MMA found in a sample is likely to vary according to the amount of propionate (from rumen fermentation) presented for metabolism, since propionate is a precursor for methylmalonyl CoA; that is , the amount of MMA found may depend upon the amount of feed consumed during the recent past and the time elapsing between ingestion and sampling. These points require investigation."

4.1.2 The Effect of Starvation.

The effect of starvation on blood levels of vitamin B12 and MMA is not clear. Inappetence due to Co deficiency is a classic clinical symptom of the disease (Underwood, 1977). Therefore, the effect of such an occurrence on the

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role of these two diagnostic markers needs verification. Although many investigations documenting clinical Co deficiency have been reported in the literature (Andrews and Anderson, 1955; Andrews Hart and Stephenson, 1958 and MacPherson et al, 1976), there is no clear evidence of what effect starvation might have on serum B12 concentrations.

That grossly depressed feed intake might result in a large decrease in the circulating levels of MMA in Co-deficient sheep was proposed by Andrews and Hogan (1972). This possibility was highlighted more recently by Rice et al (1987) who proposed that Co-deficient induced inappetence would depress propionate load on the liver. In consequence, the abnormal production and secretion of MMA might sharply decline, or cease. In such circumstances, the application of MMA analysis to the diagnosis of clinical deficiency would fail.

Williams, Spray, Newman and O'Brien (1969) found that 16 hours starvation of B12-deficient rats resulted in a marked decrease in the amount of MMA excreted in urine. After 24 hours starvation, an intraperitoneal injection of sodium propionate lead to the resumption of MMA excretion.

The objectives of this investigation were to characterise the effects of diurnal fluctuations and feeding pattern on serum concentrations of B12 and MMA. The implications of the results for the use of B12 and

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MMA as diagnostic markers for Co deficiency were considered. The effects of starvation were also studied.

4.2 MATERIALS AND METHODS.

4.2.1 Experimental Animals and Diet.

Eight Co-sufficient mule type wethers of various ages, were housed in one pen and bedded on sawdust. They were fed twice daily between 08:45 and 09:00, and 16:30 and 16:45. All animals were fed the same Co-deficient ration (Table 1). The hay fed was treated with urea to provide the sheep with the required daily RDP intake (ARC, 1980). To achieve this a 40 per cent urea solution was allowed to soak into baled hay and provided an intake of 17g urea/sheep/day.

Table 1. Basal Co-deficient diet.

FEED	Co CONTENT (mg/Kg DM $\pm$ S.E.D)	AMOUNT FED (g FW/head/day)
Flaked Maize	0.026 ± 0.005	660
Timothy Hay	0.06 ± 0.01	650
Water	___	ad libitum

4.2.2 Experimental Design.

The experiment ran for 101 days. The Co-deficient diet was introduced to all sheep on day zero. On day 53, the four wethers with the highest serum vitamin B12

concentrations were dosed orally with 0.7 mg Co/head, as cobalt sulphate ($CoSO_4$. $7H_2O$). These animals were designated the numbers 1, 2, 3 and 4 and were given a further dose of 0.7 mg Co/head on day 58, and a final drench containing 0.35 mg Co/head on day 88.

The remaining four sheep, 5, 6, 7, and 8, were not dosed with Co at any stage during the experiment. Thus, half of the wethers (1 to 4) were maintained on a 'high' Co/B12 status (+Co), whilst the other four (5 to 8) were kept on a 'low' Co/B12 level (-Co). Treatment allocation was thus not random. This was expedient in terms of the time taken to achieve a high and low Co status in the two groups of wethers.

Blood was collected from the sheep weekly. Such bleeding always took place before the morning feed. On day 50 all the wethers were bled hourly, between 08:45 and 18:15. This day of intensive sampling was used to check the system of blood collection and analysis and to acclimatise the animals to frequent sampling and restraint.

On day 98, the sheep were bled hourly between 08:45 and 22:30, with feed offered at 09:00 and 16:30. On day 99, the wethers were again sampled hourly between 08:45 and 22:30, with feed offered two hours later than on day 98, at 11:00 and 18:30.

On days 100 and 101 the sheep received no feed at all, but had access to water ad libitum. All eight animals were bled at 09:45 and 17:15 on day 100 and at 11:00 and

17:00 on day 101. These sampling times corresponded to approximately 14.75, 22.25, 40.00 and 46.00 hours starvation (time since the end of the last feed).

4.2.3 Blood Analysis.

Concentrations of vitamin B12 in serum were determined by the microbiological assay (Chapter 2, section 2.4.1). Levels of serum MMA were determined by solvent extraction and capillary GC (Chapter 2, section 2.5).

4.2.4 Statistical Analysis.

The means for serum B12 and MMA in the +Co and -Co sheep, were tested for significant difference at each weekly sampling and at each time of sampling, during days of intensive blood collection. One way analysis of variance was used to test for significant differences.

When variation within a treatment on one particular day was great, the means for both treatment groups were analysed for significant differences, between sampling times, within treatments. One way analysis of variance was also used to test for significant difference on these occasions.

4.3 RESULTS AND DESCRIPTION OF TRENDS IN THE DATA.

The results are presented in Figures 1 to 12. The B12 and MMA data from the weekly samplings will be related, followed by the results from the days of intensive

sampling. A description of the data will also be included. This will be derived directly from, and will contain no interpretation of, the results.

4.3.1 <u>Serum</u> <u>Vitamin</u> <u>B12.</u>

The mean serum vitamin B12 results from weekly samplings are presented in Figure 1 and Appendix 6. The serum B12 concentrations in all sheep showed a tendency to decline throughout the experiment, with marked variations between samplings. Serum B12 levels in the -Co animals were significantly less than those in the +Co group on day 58 ($P<0.001$). This was 5 days after the +Co sheep received their first oral dose of Co. The -Co wethers did however show mean serum B12 concentrations significantly lower than the +Co group before this time, on days 10 ($P<0.025$) and 44 ($P<0.01$). This was not surprising as the basis of group selection was serum B12 status. After day 58, serum B12 levels in the -Co sheep remained significantly below those of the +Co treatment for the remainder of the experiment ($P<0.05$ to $P<0.001$).

Mean serum B12 concentrations in the -Co wethers fell below the lower normal limit (LNL) of 400 ng/l, at approximately day 70 and remained below this threshold for the rest of the experiment. This occurred 10 weeks after the introduction of the Co-deficient ration. Serum B12 levels in the +Co animals were above the LNL throughout the experiment.
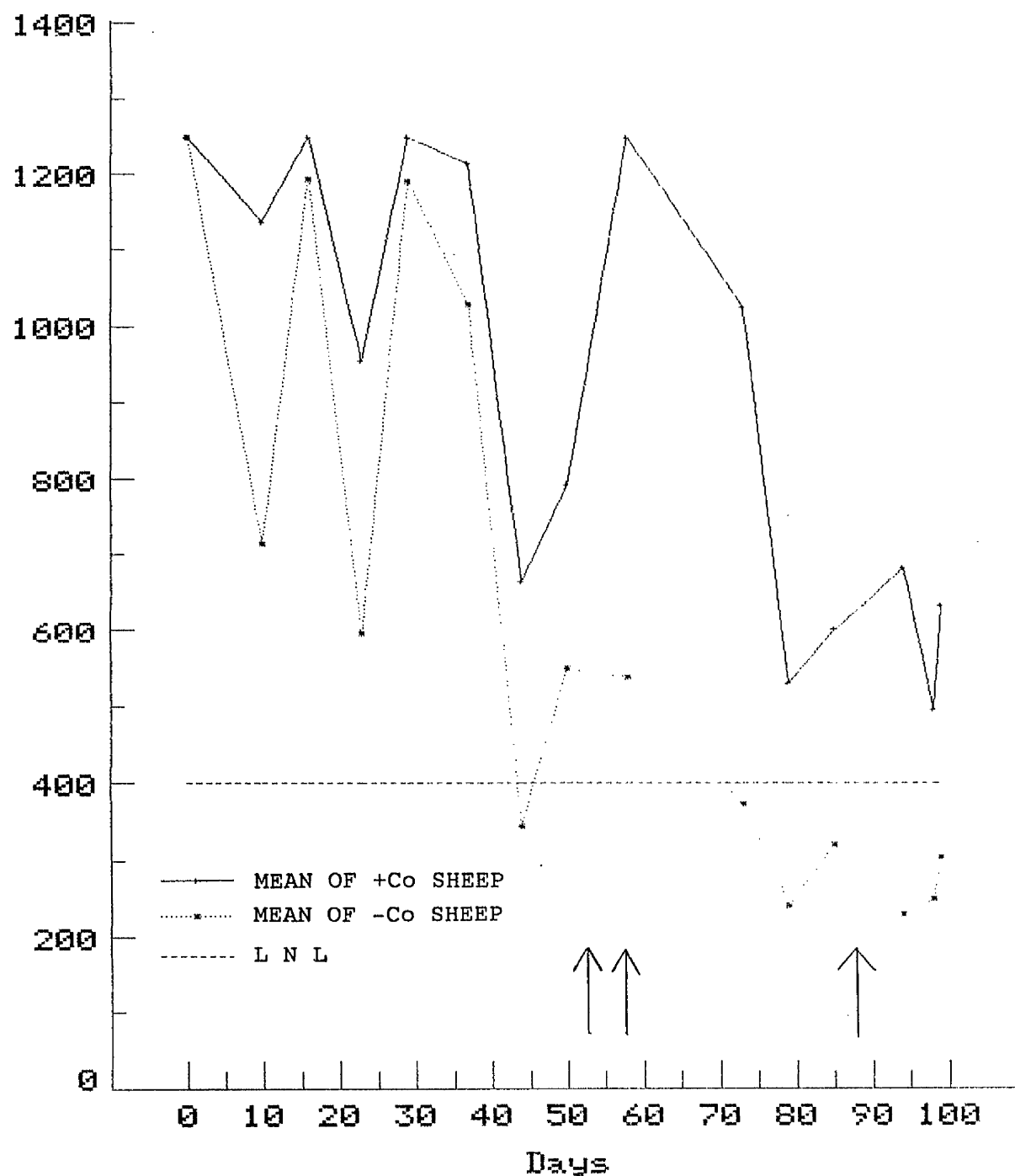
Figure 1. Mean serum vitamin B12 concentrations in Co-supplemented (+Co) and non-supplemented (-Co) wethers, throughout the experimental period. (↑ = +Co sheep dosed with Co).

4.3.2 Serum MMA.

The mean serum MMA results from weekly samplings are presented in Figure 2 and Appendix 7. Although large differences in serum MMA levels between treatments were evident, they were only statistically significant on day 94 ($P < 0.05$). The general lack of significant difference in this data could have been due to the small numbers of animals used.

Mean serum MMA in the +Co group remained below the upper normal limit (UNL) of 5 umol/l throughout the experiment. Concentrations of serum MMA in the -Co treatment rose above the UNL at approximately day 75. This was only some 5 days after serum B12 levels in the same animals had fallen below the LNL. After day 75, serum MMA levels in the -Co wethers remained above the UNL for the remainder of the experiment.

4.3.3 Day 50 - Serum Vitamin B12.

Treatment mean serum B12 data for day 50 are presented in Figure 3. The raw data are presented in Appendix 8. At all samplings on this day, each sheep had serum B12 concentrations above the LNL, with no significant differences between treatments.

Serum B12 levels in both groups dropped slightly after the morning feed. However, there was a marked rise after the evening feed. In the +Co animals, this rise seemed to start before the evening feed was given, with B12 concentrations gradually increasing in serum betwen 14:00
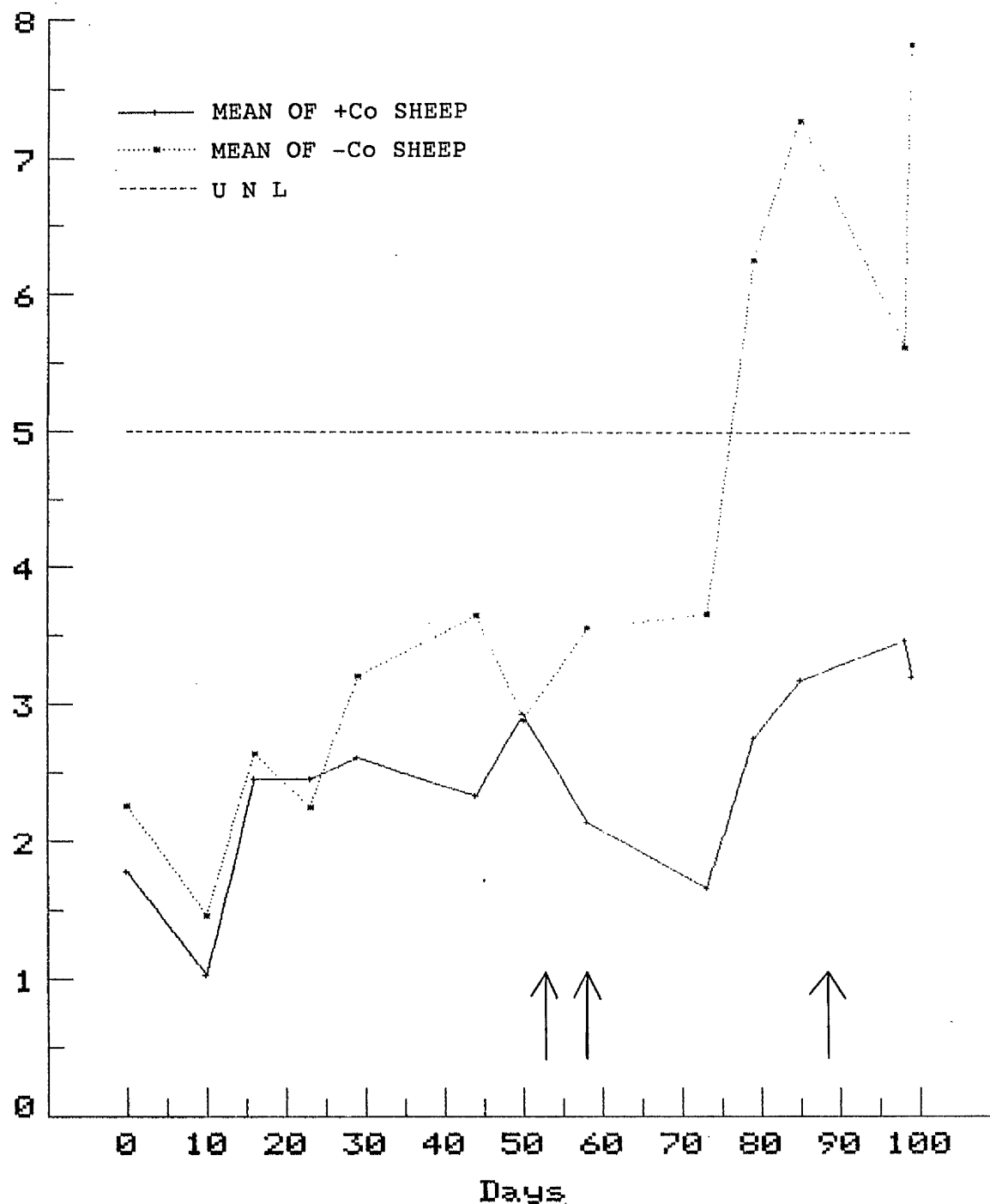
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Figure 2. Mean serum MMA concentrations in Co-supplemented (+Co) and non-supplemented (-Co) wethers, throughout the experimental period. (↑ = +Co sheep dosed with Co).

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Figure 3. Mean serum vitamin B12 concentrations in Co-supplemented (+Co) and non-supplemented (-Co) wethers, between 08:45 and 18:15 on day 50. (⊢⊣ = feeding).

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and feeding time at 16:30.

The dramatic rise in serum B12 levels after the evening feed was not a consistent occurrence. Figures 4 and 5 represent plots for individual animals on day 50. The post-evening feed rise in serum B12 was most marked in sheep numbers 4 (+Co), 7 and 8 (-Co). The rise in these three wethers denoted an increase in serum B12 concentrations between the pre and post-feeding samplings, of some 91 (sheep 4) to 193 per cent (sheep 8).

4.3.4 Day 50 - Serum MMA.

Mean treatment serum MMA data for day 50 are presented in Figure 6. The raw data are presented in Appendix 9. At all sampling times on this day, each sheep had serum MMA levels below the UNL, with no significant differences between treatments.

A marked increase in the concentration of MMA in serum occurred in both groups after the morning feed. Levels in all animals then declined gradually throughout the day, with a second rise in response to the evening feed. There was also a sharp fall in concentrations just before the evening feed.

4.3.5 Day 98 - Serum Vitamin B12.

Mean serum B12 data for day 98 are presented in Figure 7 and Appendix 10. Serum B12 concentrations in the +Co wethers declined throughout the day and were
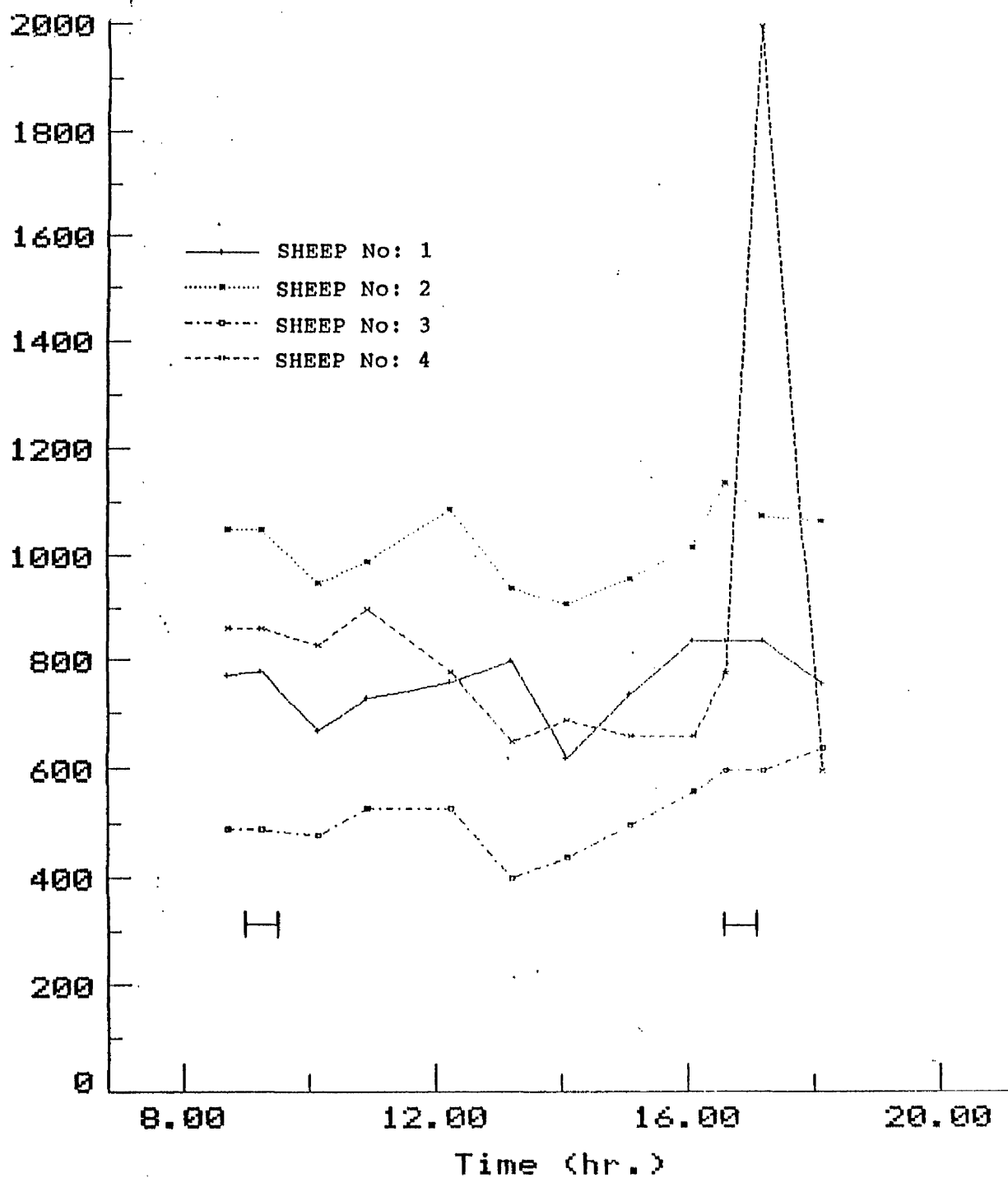
Figure 4. Serum vitamin B12 concentrations in the four Co-supplemented (+Co) wethers, between 08:45 and 18:15 on day 50. (⊢⊣ = feeding).

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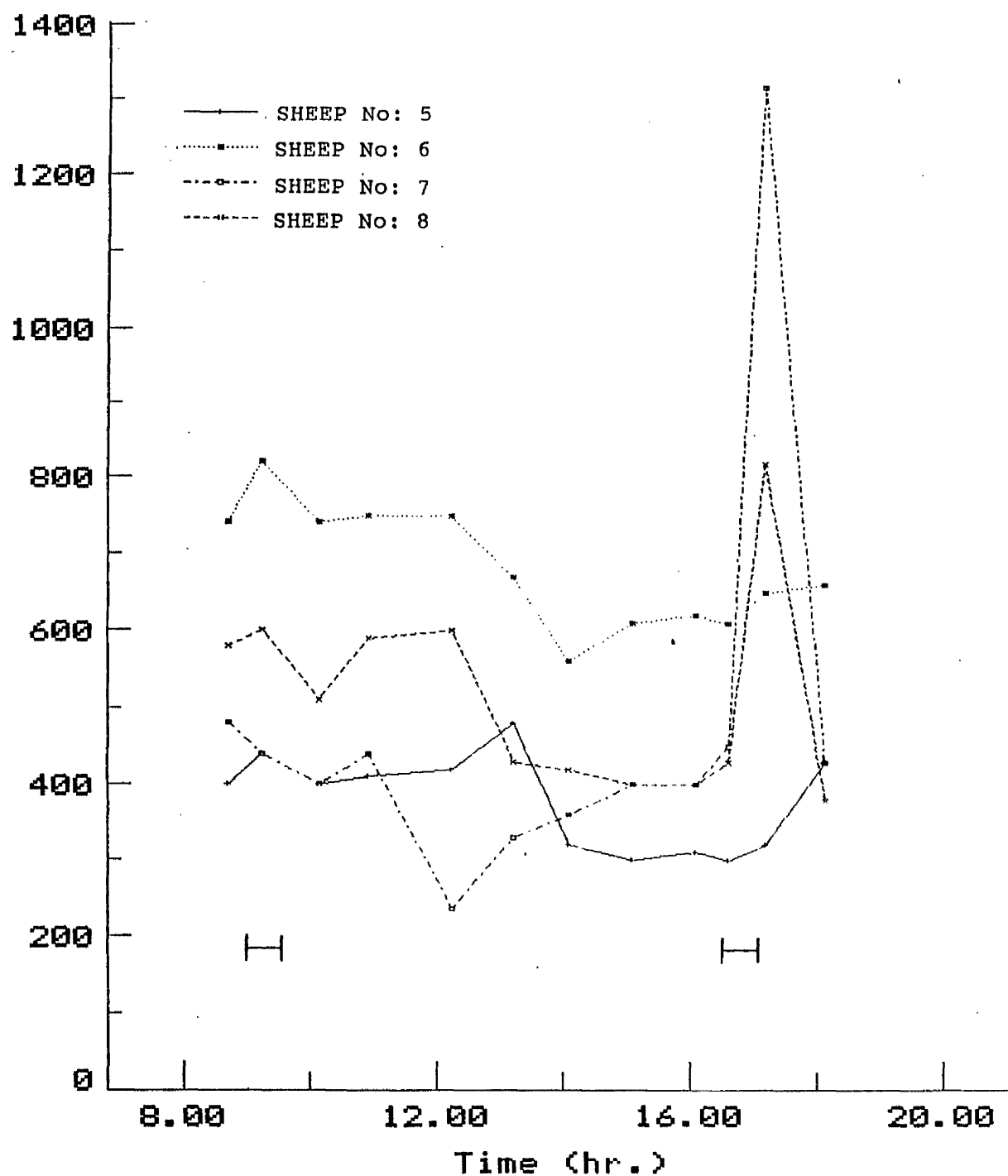
Figure 5. Serum vitamin B12 concentrations in the four non-supplemented (-Co) wethers, between 08:45 and 18:15 on day 50. (⊢⊣ = feeding).

Figure 6. Mean serum MMA concentrations in Co-supplemented (+Co) and non-supplemented (-Co) wethers, between 08:45 and 18:15 on day 50. (⊢⊣ = feeding).

Figure 7. Mean serum vitamin B12 concentrations in Co-supplemented (+Co) and non-supplemented (-Co) wethers, between 08:50 and 22:15 on day 98. (⊢⊣ = feeding).

significantly higher than levels in the -Co sheep at all samplings (P<0.05 to P<0.01). Serum B12 concentrations in the -Co animals remained below the LNL on this day, and ranged between 203 and 328 ng/l.

There were no apparent responses in serum B12 levels in the -Co group to the two feeds. Further, there was only a small rise in serum B12 in the +Co animals after the morning feed, with no response to the evening feed. However, there was a general rise in B12 concentrations in both treatments between the 20:15 and 22:30 samplings.

4.3.6 Day 98 - Serum MMA.

Mean serum MMA data on day 98 are presented in Figure 8 and Appendix 10. Levels in the -Co wethers were above the UNL throughout the day, while those in the +Co animals were below this threshold. There were no statistically significant differences between the two treatments, except at the 16:15 sampling.

As on day 98, concentrations of MMA in serum rose in response to the morning feed. This was most marked in the -Co group, where the increase denoted a rise of 91 per cent in mean serum MMA levels between 08:55 and 10:50 (non-significant). After the 09:00 feed a plateau in MMA concentrations in the -Co sheep was reached and maintained until just before the evening feed, when serum MMA concentrations in both treatments showed a small fall. After the evening feed, MMA levels in both treatments demonstrated a responsive rise.

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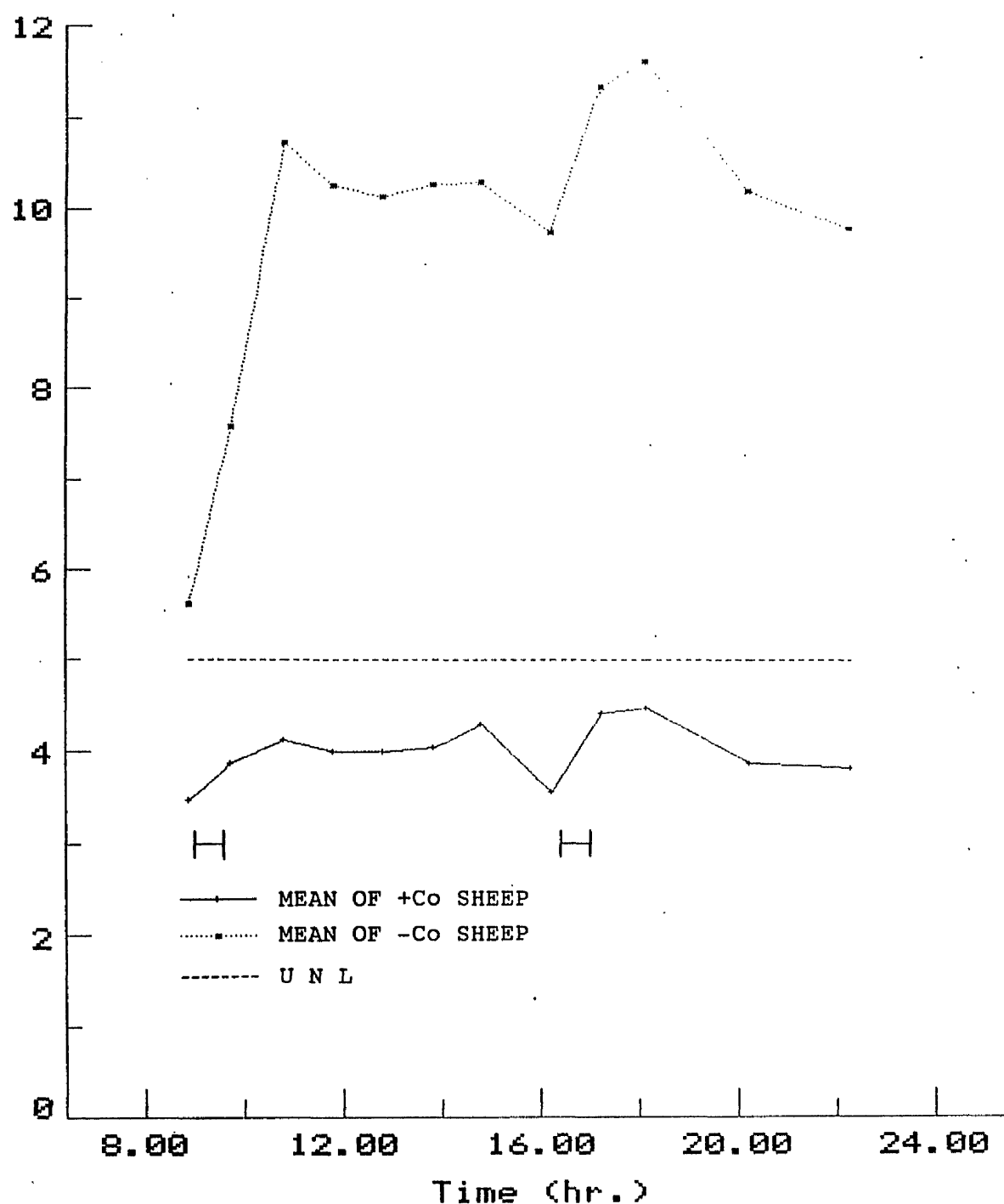
Figure 8. Mean serum MMA concentrations in Co-supplemented (+Co) and non-supplemented (-Co) wethers, between 08:50 and 22:15 on day 98. (⊢⊣ = feeding).

Serum MMA in all animals tended to fall at the end of the sampling period (20:15 to 22:30), which corresponded to the rise in serum B12 levels at this time.

4.3.7 Day 99 - Serum Vitamin B12.

Mean serum B12 data for day 99 are presented in Figure 9 and Appendix 11. The pattern of serum B12 concentrations in both treatments on this day was markedly different from that evident on day 98. On day 99, the morning and evening feeds were given two hours later than on day 98.

Serum B12 levels in the -Co wethers were below the LNL throughout the day. Those in the +Co animals remained above the LNL, until the 19:05 sampling, when they fell below this threshold and remained below 400 ng/l for the remaining two samplings. Serum B12 concentrations in the +Co sheep were significantly above those in the -Co animals at all samplings ($P<0.05$ to $P<0.001$).

In all sheep, serum B12 levels fell until after the morning feed. The increase in response to this feed intake was dramatic and denoted a rise in mean serum B12 concentrations in the -Co wethers between 13:05 and 15:05, of 207 per cent ($P<0.01$). For the +Co animals, the rise represented a mean increase in serum B12 levels between 14:05 and 16:05 of 172 per cent ($P<0.01$). This response to the morning feed occurred more rapidly in the -Co than in the +Co group. However, the response in all the sheep was not as quick as that shown to a previous
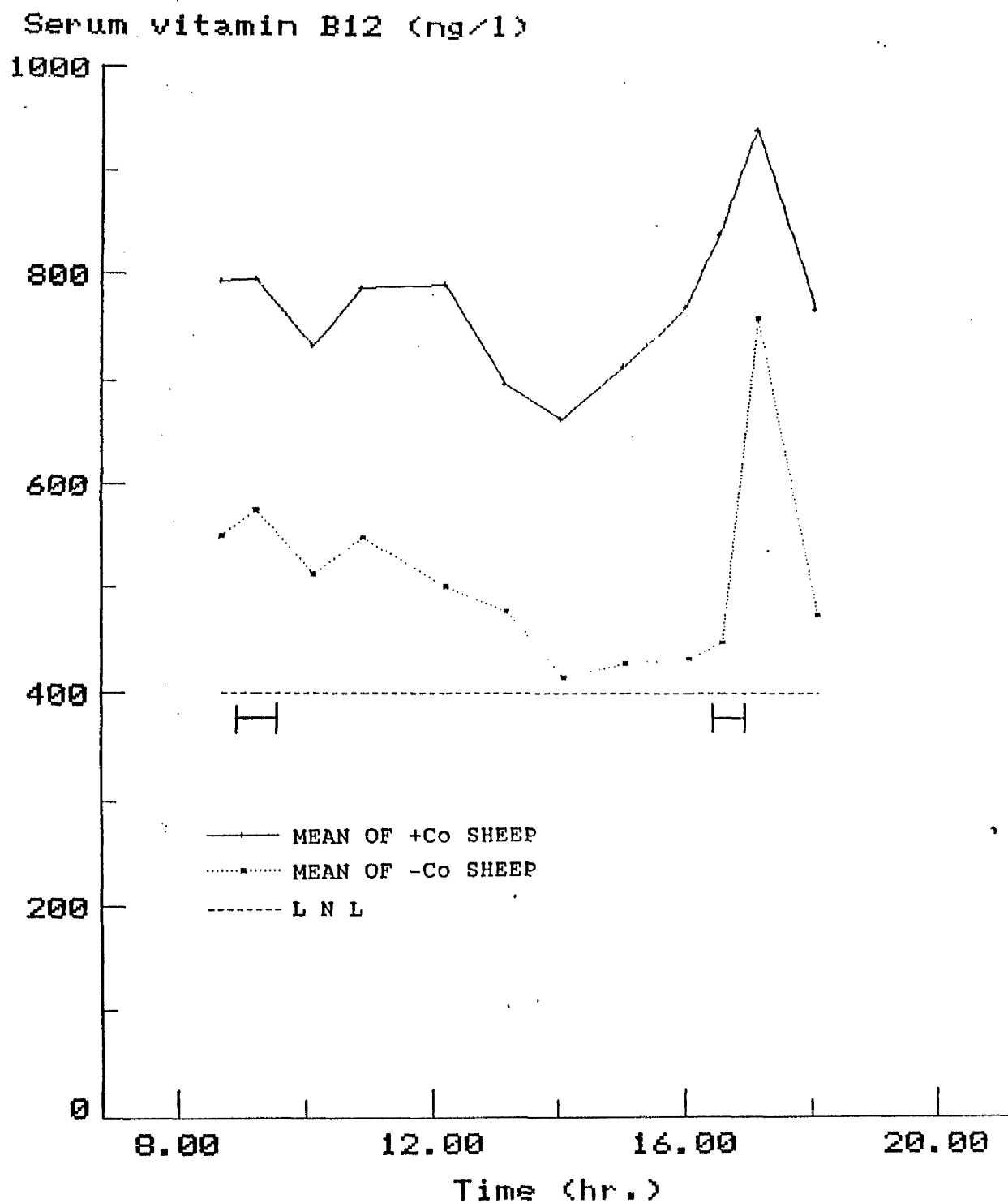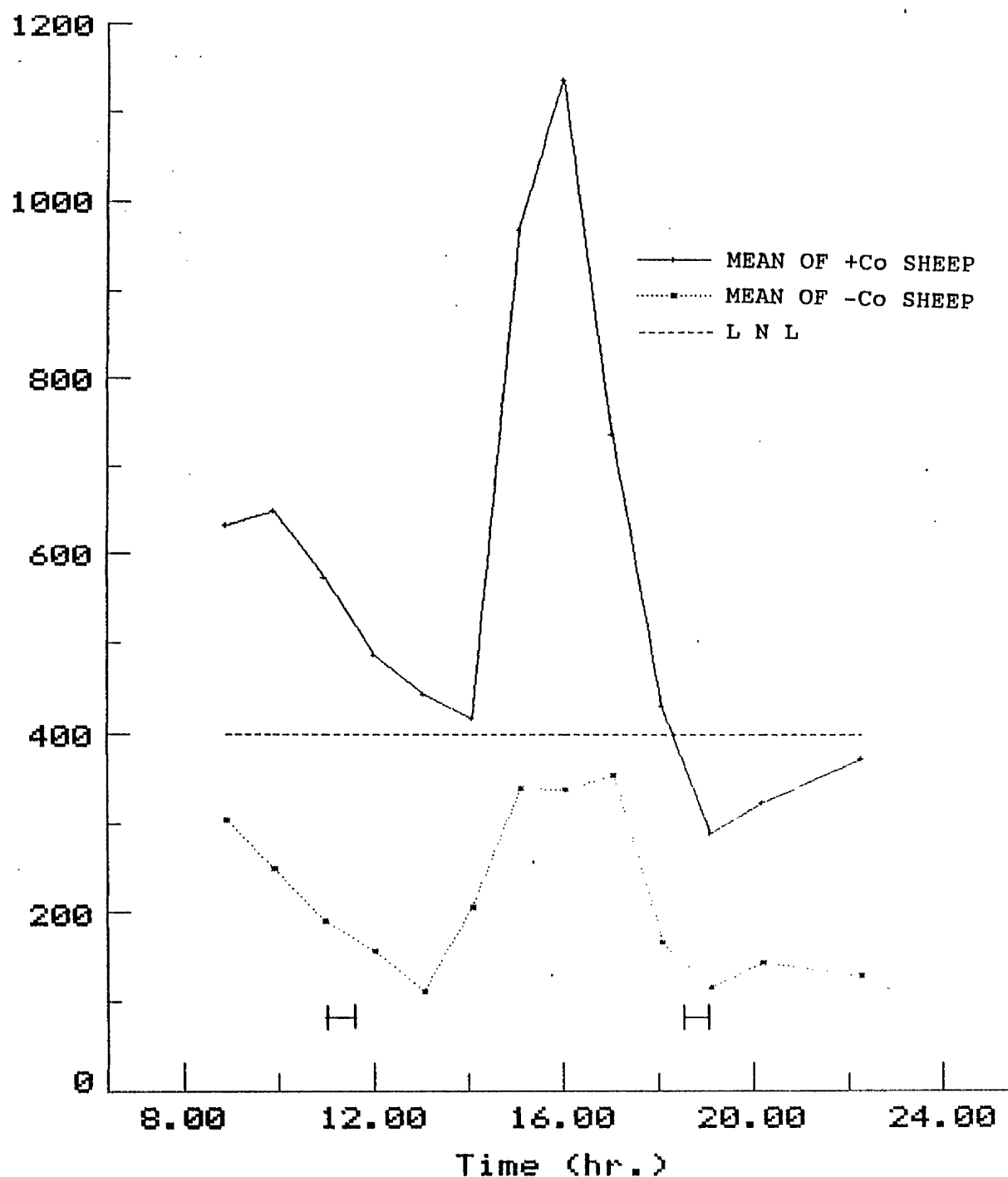
Figure 9. Mean serum vitamin B12 concentrations in Co-supplemented (+Co) and non-supplemented (-Co) wethers, between 08:50 and 22:15 on day 99. (⊢⊣ = feeding).

feed, on the evening of day 50. A comparison between B12 concentrations on days 98 and 99 is presented in Figure 10.

4.3.8 Day 99 - Serum MMA.

The mean serum MMA results for day 99 are presented in Figure 11 and Appendix 11. Levels in the -Co wethers were above the UNL throughout the day, while those in the +Co group were below this threshold. Statistically significant differences between treatments were evident at all samplings ($P<0.05$), except those collected at. 08:55, 11:00, 18:05, 20:10 and 22:30.

In contrast to day 98, there was no evidence of a response in the serum MMA concentrations of +Co animals to either the 11:00, or the 18:30 feeds. MMA levels in these sheep tended to rise throughout the day, with an intermediate fall at approximately 16:00.

Serum MMA concentrations in the -Co treatment showed a similar response to feeding, as on day 98. However, these changes were not as marked as those of the previous day. The response to the 11:00 feeding by the -Co animals, represented a rise of 37 per cent (non-significant), between 11:00 and 12:00.

Similar to day 98, serum MMA concentrations in all the sheep tended to fall at the end of the sampling period, between 20:10 and 22:30. A comparison between serum MMA concentrations on days 98 and 99 is presented in Figure 12.

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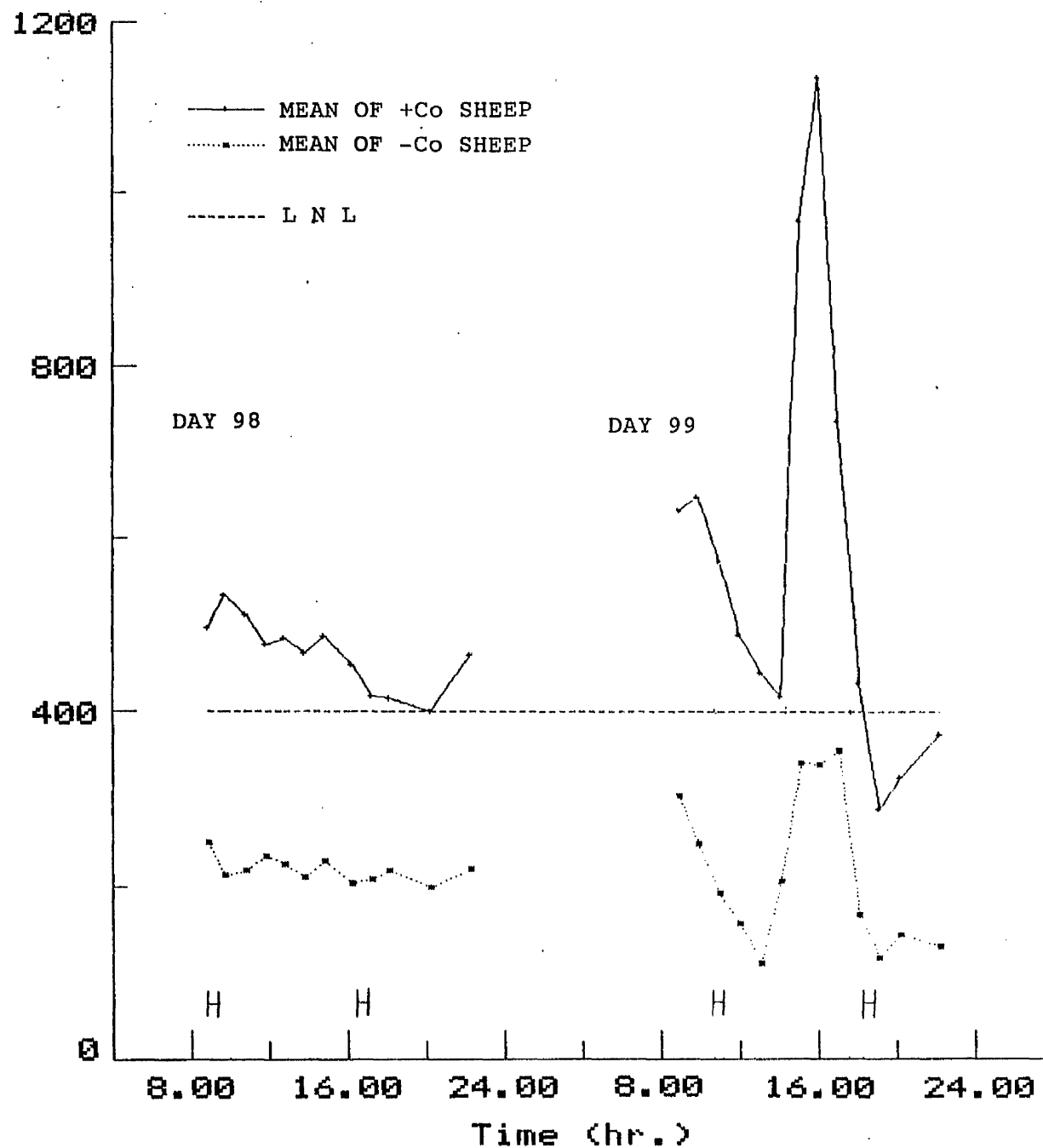
Figure 10. Mean serum vitamin B12 concentrations in Co-supplemented (+Co) and non-supplemented (-Co) wethers, on days 98 and 99. (⊢⊣ = feeding).
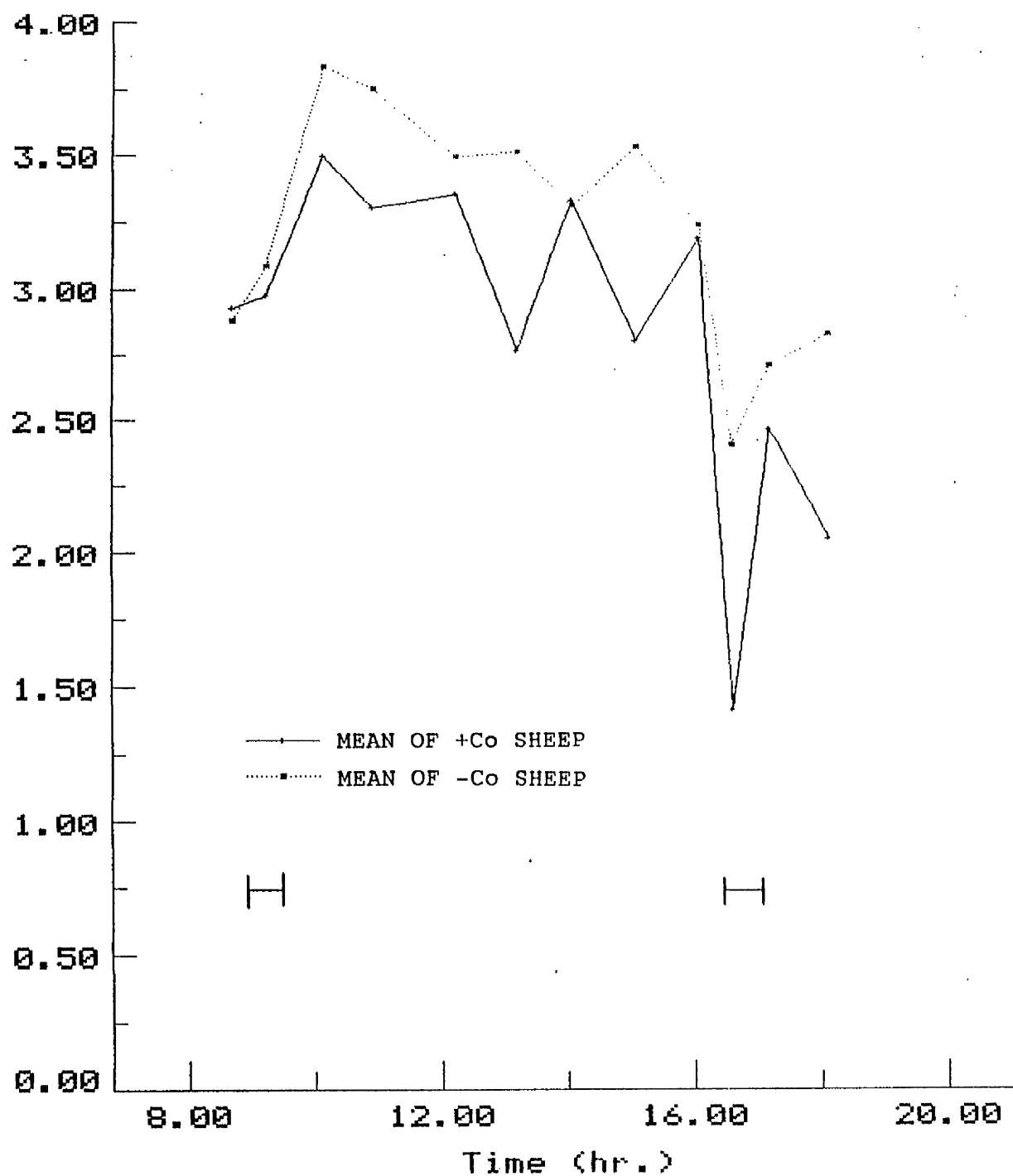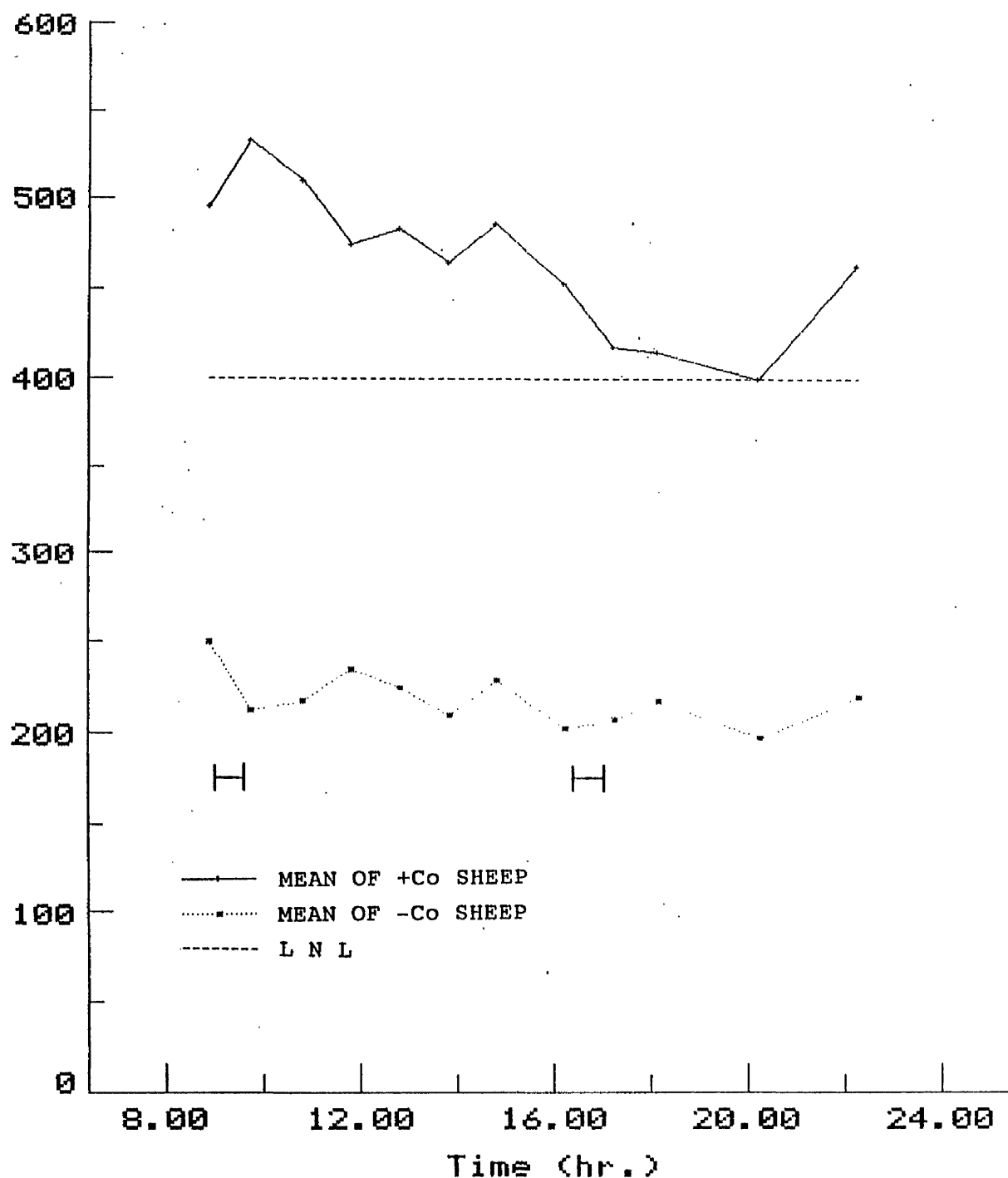
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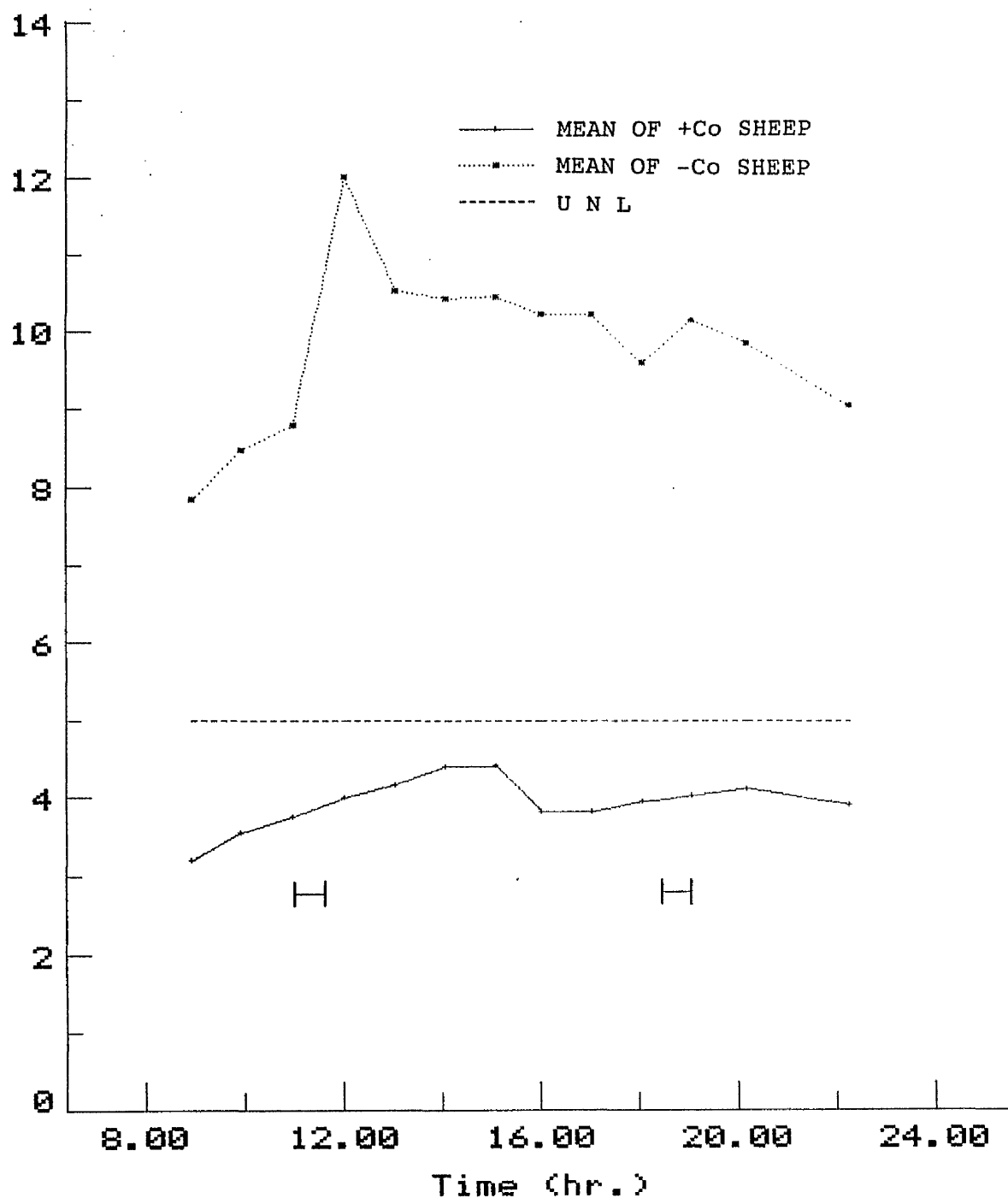
Figure 11. Mean serum MMA concentrations in Co-supplemented (+Co) and non-supplemented (-Co) wethers, between 08:50 and 22:15 on day 99. (⊢⊣ = feeding).

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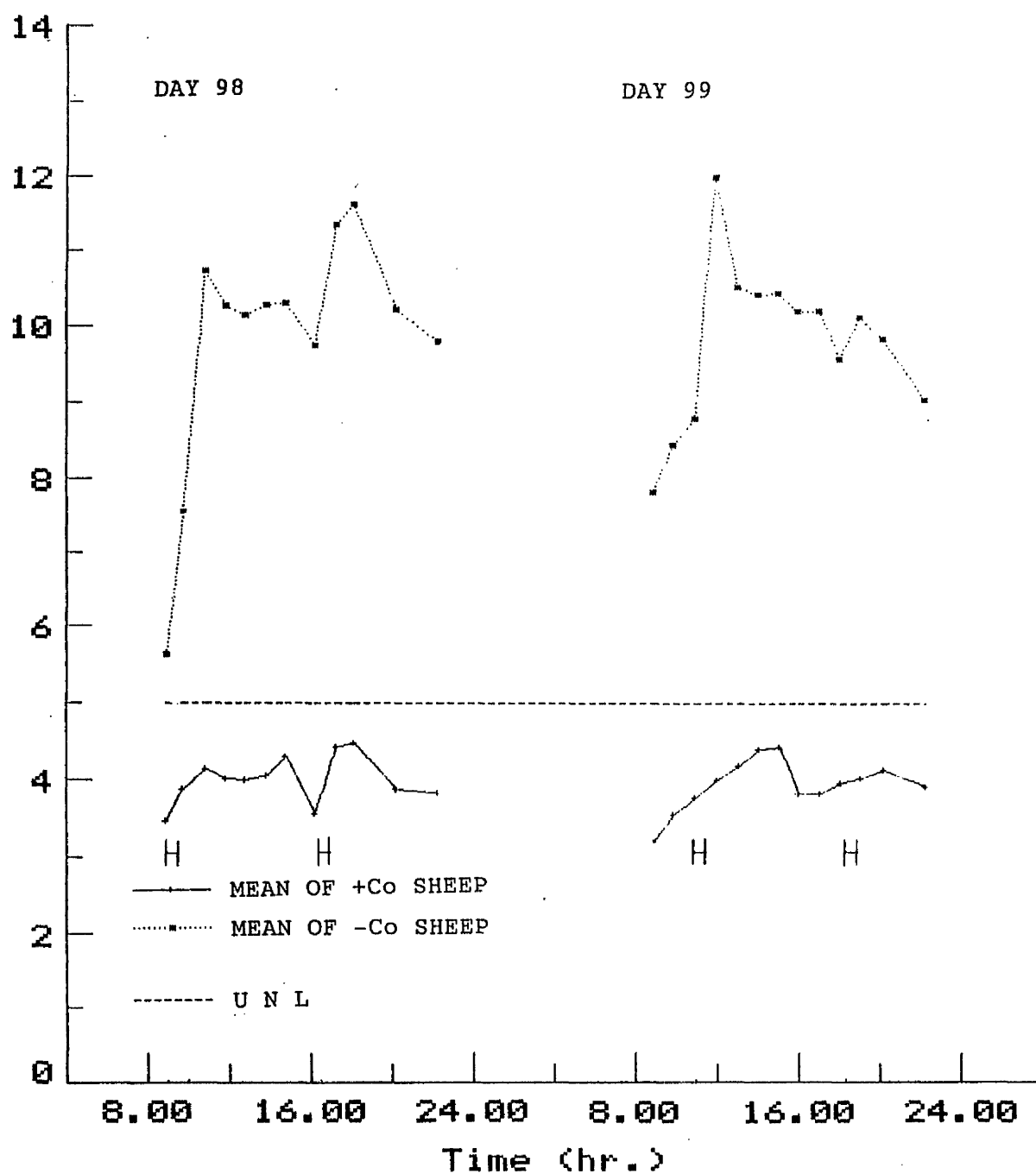
Figure 12. Mean serum MMA concentrations in Co-supplemented (+Co) and non-supplemented (-Co) wethers, on days 98 and 99. (⊢⊣ = feeding).

4.3.9 Days 100 and 101 - Serum Vitamin B12.

The data for mean serum B12 concentrations in both treatments on days 100 and 101 are presented in Table 2.

Table 2. The response of mean serum vitamin B12 concentrations (ng/l $\pm$ SED) in +Co and -Co wethers to starvation on days 100 and 101.

DAY	SAMPLING TIME	HOURS STARVATION	SERUM B12 +Co	SERUM B12 -Co	Sig. Dif.
100	09:50	14.75	606 $\pm$ 219	238 $\pm$ 64	*
100	17:15	22.25	579 $\pm$ 225	229 $\pm$ 45	*
101	11:00	40.00	861 $\pm$ 302	330 $\pm$ 120	*
101	17:00	46.00	933 $\pm$ 249	428 $\pm$ 165	*

Although differences within treatments, between samplings over these two days were not significant, there was a trend for serum B12 levels to increase with prolonged feed deprivation. This was evident in both treatments and was most marked on day 101.

4.3.10 Day 100 and 101 - Serum MMA.

Due to an accidental loss of sera, MMA concentrations in the blood of sheep on days 100 and 101 could not be determined.

4.4 DISCUSSION.

4.4.1 Variability in Serum Vitamin B12 and MMA Levels.

The first notable aspect of the results was the variability of serum B12 data in comparison with serum MMA results. This was evident in the weekly samplings (Figures 1 and 2) and also in the intensive sampling periods on individual days. Although weekly samples were taken at the same time of day on each occasion, Figure 1 demonstrated that great variability existed in serum B12 concentrations between samplings in the same group of wethers.

Variation of this kind leads to difficulties in the use of serum B12 data in the diagnosis of Co deficiency. Mean serum B12 concentrations in the -Co sheep dropped below the LNL of 400 ng/l, on day 44 (343 ng/l, Figure 1). By day 50, the corresponding level in these animals was 550 ng/l and did not drop below the LNL again until day 73 (373 ng/l). Findlay (1972) observed that it might be difficult to obtain an accurate and precise diagnosis from such variable data.

In contrast, the concentrations of MMA in serum were less variable (Figure 2). When levels in the -Co wethers rose above the UNL of 5.0 umol/l, this rise was marked and consistent with a sharp increase between day 73 (3.66 umol/l) and day 79 (6.26 umol/l).

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4.4.2 <u>Diurnal Fluctuations in Serum Vitamin B12 and MMA Levels.</u>

In response to feeding, serum vitamin B12 concentrations in both +Co and -Co sheep increased. However, this pattern was not consistent between days, or animals. For example, while the response to the evening feed on day 50 (Figure 3) and both feeds on day 99 (Figure 9) were evident, there was no such change in serum B12 levels on day 98 (Figure 7). Further, only three animals showed a marked rise in serum B12 concentrations after the evening feed on day 50 (Figures 4 and 5), although this had a discernable effect on the mean values. An opposite effect was also noted on day 50, when mean serum B12 levels in both treatments showed a slight drop immediately after the morning feed.

The results collected from this experiment did not reveal why serum B12 concentrations tended to rise after feeding. However, reasons can be speculated from evidence in the literature. That feeding can result in an increase of B12 levels in blood was reported by Somers and Gawthorne (1969). Similar to the results of this experiment, these workers found that this response varied between individual sheep. The rise in blood B12 levels after the evening feed on day 50, could be detected within an hour of the feed being offered. Somers and Gawthorne (1969) discovered a response only after 4 hours post-feeding, which was similar to the delay in response to the morning feed evident on day 99. However, the

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sampling regime employed by these workers did not allow for earlier detection of fluctuations in serum B12 concentrations.

That an input of Co to the rumen, in this case from feeding, results in increased ruminal production of vitamin B12 and subsequent absorption across the mucosa of the small intestine into the circulation, was established by Hedrich et al (1973). Thus the response shown to feeding in this experiment, although inconsistent, was probably due to increased ruminal microbial production of B12 and absorption into circulation.

The results presented suggested that the response of serum MMA concentrations to twice daily feeding, was a fall just before, followed by a restoration of levels after feed intake. This effect was detected on days 50, 98 and 99 (Figures 6, 8 and 11, respectively). On some occasions (for example, in the -Co and +Co wethers after the evening feed on day 98) the post-feeding increase raised serum MMA concentrations above the level evident pre-feeding. As with serum B12, the response varied between animals and sampling days, but was far more consistent than the rise in serum B12 levels due to feeding.

The results of this experiment did not reveal why serum MMA concentrations responded to feed intake in such a manner. As with the serum B12 data, the responses may

have been linked with ruminal effects. The fall in serum MMA concentrations just before the evening feeds on days 50, 98 and 99 may have been associated with the physiological effects of the 'anticipation' of feeding in the sheep.

It is interesting to note that the response of serum MMA post-feeding was actually a rise and not a fall in concentrations. As the response of serum B12 was to rise, it might have been expected that the response of serum MMA levels would have been to fall. The anticipated pattern would be that as serum B12 rose post-feeding, liver B12 concentrations might also have been augmented resulting in increased activity of the methylmalonyl CoA mutase enzyme. This would have enhanced the conversion of methylmalonyl CoA to succinyl CoA, with a subsequent drop in serum MMA levels. This did not happen.

The rise in serum MMA concentrations after feeding, may have been associated with an increased propionate load on the liver at such times. That feed intake leads to a surge in propionate production and absorption, is well documented (McDonald et al, 1981). In a Co-sufficient situation, this input of propionate to the liver would result in an increase in methylmalonyl CoA production (Williams et al, 1969). Some of this might be degraded and excreted, accounting for a short increase in serum MMA levels. As the propionate is catabolised liver concentrations of methylmalonyl CoA would fall, along with serum MMA. Such a case was demonstrated by all the

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wethers on day 50 (Figure 6) and by the +Co sheep on day 98 (Figure 8). In these two instances, a short rise in serum MMA levels post-feeding was followed by a decline, before a second increase after the evening feed.

In a Co-deficient situation, the post-feeding rise in serum MMA due to increased propionate load on the liver, is again evident. However, there is insufficient vitamin B12 present to act as co-factor for the enzymic conversion of methylmalonyl CoA to succinyl CoA. Thus, methylmalonyl CoA levels in the liver rise post-feeding, but are not dissipated and this is reflected by serum MMA concentrations. Such a case was evident in the -Co animals on days 98 and 99 (Figures 8 and 10), where a plateau in serum MMA levels was established after the post-morning feed rise, which was not dissipated before the post-evening feed response.

The increases in serum concentrations of B12 and MMA post-feeding, resulted in the presence of exogenously stimulated fluctuations in the circulating levels of these diagnostic parameters. Such variations were found by Somers and Gawthorne (1969) for B12 and were proposed for MMA by Andrews and Hogan (1972).

The question arises as to whether there are other endogenously stimulated fluctuations in serum B12 or MMA concentrations, which are not associated with the exogenous stimulation of feeding pattern? In a trial with grazing sheep, which were given no daily supplementary

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feed, Millar et al (1984) found no evidence of diurnal fluctuations in serum B12 levels. This could have been due to a more even pattern of feed intake during the day. In this situation, there would be periods of feeding and other periods of ruminating, resting and sleeping. However, surges in B12 and propionate production after isolated feedings would not occur.

While this reasoning might explain the absence of diurnal fluctations in the data of Millar et al (1984), these workers also found no evidence of other, endogenous, daily fluctuations in serum B12 concentrations. The results from the smaller housed experiment undertaken here, would substantiate these findings. No variations in serum B12 or MMA levels, other than those due to feeding pattern, were apparent on any of the three intensive sampling days. Small rises in the serum B12 concentrations in sheep at the end of days 98 and 99, associated with falls in serum MMA levels, were evident. These were most likely to be related to the absence of feed at these times, rather than to any other factor. However, sampling over a full 24 hr. period was not undertaken. Thus, endogenous fluctuations which may have occurred during the night, could not be detected.

4.4.3 The Effect of Delayed Feeding.

Delaying both the morning and evening feeds by 2 hours on day 99 compared to day 98, had a profound effect on the response to feed intake of serum B12 concentrations,

but not on serum MMA levels. This was best illustrated by the plots of serum B12/MMA against time for both days, presented in Figures 10 and 12.

Compared to the results from day 98, delaying feeding for 2 hours on day 99 had the effect of steadily reducing serum B12 concentrations in all sheep, until after the 11:00 feed. This may have been a reflection of the absence of substrate and energy input for the production of B12 by rumen microorganisms. Thus, less vitamin was absorbed and detected in circulation. However, the rise in serum B12 levels following the 11:00 feed was dramatic and much more marked than it had been to the evening feed on day 50. This was even more surprising when there had only been a small response to the morning feed in the +Co wethers, in the whole analysis of day 98.

The explanation for such a dramatic rise in serum B12 levels after witholding feed, was probably associated with the rumen microbes which produce the vitamin. The period of activity and growth by microbial populations in the rumen following feed intake (McDonald et al, 1981), may have been enhanced by simple extension of the period of relative quiescence, prior to feeding. Thus, the rise in microbial B12 production after the morning feed (11:00) on this occasion, may have occurred in a burst, with a resultant surge in serum B12 concentrations.

The evening (18:30) feed on day 99, although 2 hours later than the evening feed of day 98, was given after

the usual time interval between feeds. In this way, the populations of rumen microorganisms may have returned to a customary state by the time the evening feed was given. Thus, no dramatic surge in serum B12 response to the evening feeding was apparent after 18:30 on day 99.

The effect of delaying feeding time on serum MMA concentrations was not marked (Figure 12). The rise in MMA levels in serum after feeding on day 99 was consistent with the responses evident on days 50 and 98. However, serum MMA concentrations did show an increase over the three samplings taken prior to the 11:00 feed on day 99 (Figure 11). These coincided with concurrent falls in serum B12 levels.

It may be postulated that in this extended fast, serum MMA concentrations showed small rises prior to delayed feeding, due to continuation of basal propionate catabolism in the liver, which would produce methylmalonyl CoA, while decreased concentrations of B12 were available to act as a co-factor for the conversion and dissipation of methylmalonyl CoA to succinyl CoA. MMA levels in serum would thus rise during such time.

It must be stressed that these explanations for the effect of feeding on serum B12 and MMA concentrations are speculative. They can only be refuted or substantiated if a similar experiment is undertaken, in which rumen microbial B12 production and influx rates of B12 and propionate to the liver were measured, with more frequent collection of blood samples. This could be achieved by

using rumen fistulated sheep with duodenal and hepatic portal vein re-entrant canulae. This would enable the response of B12 and propionate production to measured Co and feed intakes, along with effects on serum levels of B12 and MMA, to be determined.

4.4.4 The Effect of Starvation.

The effect of starvation on days 100 and 101, was initially to depress and then to increase vitamin B12 concentrations in serum. The fall in circulating B12 between the 09:50 and 17:15 samplings on day 100, was perhaps due to declining levels of microbial vitamin production and thus animal absorption. With some of the vitamin in blood being used or stored in the liver, serum B12 concentrations fell. However, starvation would reduce the propionate load and therefore the necessity for B12, in the liver. A basal level of B12 input from the rumen to the host animal would continue in these 46 hours of absent feed intake. Thus, the balance between liver demand for B12 from serum and the input of vitamin to circulation from the rumen might change, resulting in the net increase in serum B12 levels as starvation continued.

Although the increases in serum vitamin B12 between 14.75 and 46.00 hours of starvation were not statisically significant within treatments, they represented rises of 54 and 79 per cent in serum B12 concentrations for the +Co and -Co treatments, respectively. This increased mean

serum B12 levels in the -Co group from diagnostically deficient to sufficient concentrations, without the addition of Co supplementation.

The effect of starvation on serum MMA levels could not be determined. However, with rising concentrations of serum B12 and a lack of propionate load on the liver, it may be postulated that the result would have been a marked depression in serum MMA status. This scenario would agree with that proposed by Rice et al (1987).

4.4.5 Consequences for the Diagnosis of Cobalt Deficiency.

The results of this experiment suggested that the -Co wethers were sub-clinically Co-deficient from day 70 onward. That is, they showed mean serum vitamin B12 levels consistently between 200 and 400 ng/l, with serum MMA concentrations between 5.0 and 15.0 umol/l.

The investigation produced strong evidence for the presence of diurnal fluctuations in serum B12 and MMA concentrations, due to the exogenous stimulation of feeding pattern. As such, the time of sampling in relation to feeding may have a profound effect on the use of these two markers in the diagnosis of Co deficiency in sheep.

The feeding response was most prevelant in the serum B12 data. Increases of over 100 per cent in B12 concentrations due to a feed intake response may render the employment of such data, useless for diagnosis. This

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would be the case if sampling for diagnostic purposes was performed shortly after feeding.

However, Millar et al (1984) have shown that when sheep are at pasture and no supplementary feed is given, diurnal fluctuations in serum B12 concentrations do not exist. In this situation, the points stated here would have no relevance to the use of serum B12 levels in the diagnosis of Co deficiency.

The data presented suggested that serum B12 was a sensitive indicator of Co intake and ruminal vitamin production in the ovine. This was in agreement with MacPherson et al (1976) and Sutherland (1980). However, there was considerable variation both within and between animals in serum B12 response to Co intake, over the long (weeks) and short (hours) term. Such variation was generally more marked in the Co-sufficient sheep, compared to deficient controls. These findings reiterated those of Somers and Gawthorne (1969), Findlay (1972), Millar and Albyt (1984) and Millar et al, (1984)

Despite the evident variation, serum B12 levels reflected declining Co intake in both groups of wethers (Figure 1). It should also be noted that no false diagnoses would have been made of the +Co sheep, using serum B12 concentrations and that the risks of a false diagnosis in the -Co group only existed for approximately three weeks.

Serum MMA concentrations tended to fall just before and rise after feed intake. Such effects might lead to

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confusion in the use of this marker for the diagnosis of Co deficiency. If blood samples are taken just before or after feeding, the data obtained may imply a false diagnosis of Co status. This would be particularly relevant in the detection of sub-clinical Co deficiency, where levels of MMA in serum might be slightly above or at the UNL of 5.0 umol/l.

The data collected during starvation of the sheep would suggest a need for caution, if serum B12 or MMA levels are to be used in the diagnosis of clinical and severe ovine Co deficiency. In such cases, the effects of Co deficiency induced inappetence on liver function, via rumen microbial influences, may result in apparent low serum MMA concentrations and vitamin B12 levels which would imply only marginal deficiency, or even adequacy.

4.4.6 Recommendations for the Diagnosis of Cobalt Deficiency in Pregnant Ewes.

The results of Chapters 3 and 4 can be drawn together to produce recommendations for the use of serum B12 and MMA concentrations, in the diagnosis of Co deficiency in pregnant ewes. The most accurate and reliable technique for the prognosis and diagnosis of Co deficiency, particularly in the sub-clinical form, is to use the criteria presented in Chapter 3, Table 2. Blood should be collected several hours before the provision of supplementary feed and several hours after the last supplementary feed intake. Extraordinarily high

concentrations of serum MMA should be excluded from diagnostic analysis. When serum B12 concentrations are used, consideration must be given to the analytical technique employed and the criteria applied to the results. Where clinical Co deficiency is suspected, the determination of low serum MMA concentrations should be viewed with caution, as these may in some circumstances lead to a false negative diagnosis.

CHAPTER 5.

THE EFFECT OF COBALT DEFICIENCY IN THE PREGNANT EWE ON REPRODUCTIVE PERFORMANCE AND LAMB VIABILITY.

5.1 INTRODUCTION.

In a review of Co deficiency in ruminant animals, Mills (1981) suggested that there was a lack of research evidence on the effects of the disease on reproductive performance. Few reports can be cited which relate to this aspect of the syndrome. Thus, there is a need to study the influences of this deficiency disease on the reproducing ewe and subsequent lamb performance, particularly in the sub-clinical region.

Dunlop (1946b) reported that sub-clinical Co deficiency on farms in the south west of Scotland, had a detrimental effect on sheep production. More recently, Duncan et al (1981) reported that clinically Co-deficient ewes produced fewer lambs with incidence of stillbirths and neonatal mortalities, compared to Co-sufficient animals. Garton et al (1981) related these findings to vitamin B12 and MMA status of the dams, and adverse neuropathology evident in both ewes and lambs. However their investigations employed very small numbers of animals and were therefore not conclusive.

The objectives of this work were to investigate the effects of Co deficiency in pregnant hill sheep, on reproductive performance and neonatal lamb viability. Two housed experiments with hill ewes were undertaken and tupping and lambing performances were recorded in sub-clinically Co-deficient dams and sufficient controls. The viability of lambs from ewes of sufficient and deficient Co status was measured up to four weeks of age.

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Observations were related to ewe and lamb Co/vitamin B12 status, by measurement of serum B12 and MMA concentrations.

5.2 MATERIALS AND METHODS.

5.2.1 Animals and Treatments.

The animals used in the two investigations were the same as those already detailed in Chapter 3, for the study of the diagnosis of Co deficiency in pregnant ewes. Thus, the Experiments 1 and 2 discussed in this chapter, refer to the same sheep and experiments as Chapter 3.

The treatments imposed on the ewes in both investigations, were described in Chapter 3. The NS (non-supplemented) group in both experiments received a Co-deficient intake for the whole of pregnancy. However, whereas in Experiment 1 the NS sheep were rendered sub-clinically Co-deficient only after tupping, in Experiment 2 these animals experienced this degree of disease during mating.

The HS treatment in Experiment 1 were placed on a Co-deficient intake for the second half of gestation only whilst in Experiment 2 this group were sub-clinically Co-deficient for the first half of pregnancy and replenished from mid-term. In both experiments, the FS ewes received a Co-sufficient intake for the whole of pregnancy.

Thus, in Experiment 1 the emphasis of the investigation was on the effects of Co deficiency in the second half of

gestation. · Experiment 2 concentrated more on the consequences of disease imposed at tupping and during the first half of pregnancy. These differences were reflected by the HS treatment, while the NS and FS groups of both experiments acted as negative and positive controls, respectively.

5.2.2 Experiment 1 (1985/86).

5.2.2.1 Cobalt deficient ration.

All sheep were introduced to a Co-deficient diet on 30/9/85. The Co contents of the feeds were detailed in Chapter 3, Table 1. Day zero of the experiment was at the first blood sampling on 10/10/85 and the ration fed thereafter is presented in Table 1.

This diet was designed to meet all the nutrient requirements, excluding Co, of pregnancy and lactation for ewes with multiple lambs. Small amounts of sodium chloride, dicalcium phosphate and magnesium were added to the ration, but these supplements contained insignificant levels of Co. All the sheep were on a rising plain of nutrition up to tupping, in an attempt to obtain a flushing effect.

Table 1. Basal Co-deficient ration fed to all ewes in Experiment 1.

DAYS	FEED (Kg/hd/d)				Co INTAKE (mg/hd/d)
	HAY	MAIZE 1	MAIZE 2	MILK	
PREGNANCY					
0 - 11	0.40	0.30	0.075	—	0.034
12 - 76	0.66	0.25	0.050	—	0.048
77 - 127	0.90*	0.40	0.100	—	0.067
128 - 140	0.90*	0.67	0.150	—	0.076
LACTATION					
141 -	1.00*	0.64	0.250	0.10	0.086

KEY:

HAY = Timothy hay
MAIZE 1 = Micronised maize
MAIZE 2 = 60 % Maize gluten
MILK = Skimmed milk powder
* = Fed _ad libitum_

5.2.2.2 Tupping, gestation and lambing management.

The 60 ewes in this experiment were cycle synchronised using 'Veramix' progesterone vaginal implant sponges (Upjohn Ltd., Crawley, Sussex). For mating the sheep were split into four groups of 15, each separately penned. They were mated to four experienced, Co-sufficient Suffolk rams. These tups were rotated around the pens for a four day period, encompassing the peak of each tupping activity. The rams were thus moved from pen to pen, twice daily.

The sequence of management is presented in Table 2.

Table 2. Tupping, gestation and lambing in the ewes of Experiment 1.

DATE	DAY	ACTION
30/09/85	-10	Introduction of Co-deficient ration.
01/10/85	- 9	Progesterone sponges into all ewes and dosed with albendazole general wormer.
14/10/85	4	Sponges taken out of half of the ewes.
15/10/85	5	Sponges taken out of remaining ewes.
16/10/85	6	Ewes put to tup. Peak of 1st tupping period.
30/10/85	20	1st of weekly oral Co supplements to HS and FS ewes.
01/11/85	22	Peak of 2nd tupping period.
20/12/85	71	Ultrasonic scanning of ewes for lambs.
30/12/85	81	Co supplement removed from HS ewes.
15/01/86	97	All ewes dosed with a flukacide.
10/02/86	123	All ewes vaccinated against clostridial diseases.
05/03/86- 15/03/86	146- 156	1st lambing period.
24/03/86- 31/03/86	165- 172	2nd lambing period.

The ewes were weighed and condition scored fortnightly. Condition scores were determined by the author on each occasion, to eliminate scorer bias and the procedure was carried out in accordance with the recommendations of ADAS Leaflet 787 (1984).

5.2.2.3 Measurements of fertility and gestational performance.

A. Conception rate.

When running the tups with the ewes, keel paint was employed to enable observation of mating progress. A conception rate for each treatment at each of the two tupping periods (TP) was calculated as follows:

TP1

$$\frac{\text{No: marked in TP1 -- No: returns in TP2}}{\text{No: marked in TP1}} \times 100$$

TP2

$$\frac{\text{No: marked in TP2 -- No: of these scanned empty}}{\text{No: marked in TP2}} \times 100$$

B. Reabsorption rate.

Implantation of the developing fertilised oocyte (blastocyst), occurs approximately one cycle's length after ovulation and fertilisation. In the ovine, the cycle is about 16.5 days long and is equivalent to the time between the peak activity of two tupping periods. If conception occurred, but implantation failed and the blastocyst was reabsorbed, this could be recorded for each treatment at the first tupping period as follows:

TP1

$$\frac{\text{No: marked in TP1, not at TP2, and scanned empty}}{\text{No: marked at TP1}} \times 100$$

C. Lambing rate.

A prospective lambing rate for each treatment was calculated from the scanning data as follows:

Lambing rate at scan =

$$\frac{\text{No: of lambs from the scan}}{\text{No: of ewes put to tup}} \times 100$$

Two actual lambing rates were obtained for each treatment group, using the following equations:

Lambing rate at birth =

$$\frac{\text{No: of lambs born}}{\text{No: of ewes put to tup}} \times 100$$

Lambing rate at 4 weeks =

$$\frac{\text{No: of lambs alive at 4 weeks of age}}{\text{No: of ewes put to tup}} \times 100$$

The length of gestation in each ewe was calculated as the period from the peak day of the tupping period in which conception occurred, to the day of parturition. A spread of gestation length and the mean for each treatment could thus be obtained.

5.2.2.4 Biochemical profile of the ewes.

The ewes were bled fortnightly throughout the experiment. Samples were analysed for whole blood haemoglobin (Hb) and glutathione peroxidase (GSH+Px) concentrations. Plasma copper (Cu), calcium (Ca) and

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magnesium (Mg) levels were also determined. Serum vitamin B12 and MMA concentrations were measured by microbiological assay and capillary GC, respectively. Plasma glucose was determined spectrophotometrically.

5.2.2.5 Lambing arrangements.

Before lambing, the main pens were mucked out, but not disinfected.

Wherever possible ewes were moved to small lambing pens before parturition. If the ewe and lamb(s) were healthy, they were removed from the lambing pen after 24 hours and placed in a mothering up pen. After a further 48 hours, half the ewes and lambs from each treatment were turned out to grass and taken off the experiment. The other half were returned to the main pens for further investigation.

The lambs were sexed, weighed and their umbilical cords dipped in an iodine solution, within 6 hours of birth. Prophylactic dosing of new born lambs with antibiotics was not undertaken, but where necessary such treatment was given for ill-health and the incident noted.

The numbers of stillbirths and neonatal mortalities occurring in each treatment group were recorded. For the purposes of these investigations, a neonatal mortality was defined as the death of a lamb which was born alive, any time up to four weeks of age. Post mortem examinations were carried out on all dead lambs and the livers analysed for Co content.

The lambs kept indoors after parturition and mothering

up were blood sampled at two and four weeks of age. Serum B12 and MMA concentrations were determined in these samples.

5.2.3 Experiment 2 (1986/87).

5.2.3.1 Cobalt deficient ration.

All sheep were introduced to a Co-deficient diet on 10/10/86. The feeds and general design of the diet were the same as those used in Experiment 1. Day zero of the experiment was at the first blood sampling on 10/9/86, and the ration fed is detailed in Table 3. Due to low blood GSH+Px concentrations, all ewes received an oral dose of 4 mg Se/head on day 63.

Table 3. Basal Co-deficient diet fed to all ewes in Experiment 2.

DAYS	HAY	FEED (Kg/hd/d) MAIZE 1	MAIZE 2	MILK	Co INTAKE (mg/hd/d)
PREGNANCY					
0 - 72	0.58	0.25	0.10	—	0.044
73 - 161	0.83*	0.30	0.10	—	0.061
162 - 208	0.70*	0.41	0.12	—	0.058
209 - 228	0.59*	0.64	0.15	—	0.058
LACTATION					
229 -	0.66*	0.65	0.25	—	0.066

KEY:

HAY = Timothy hay
MAIZE 1 = Micronised maize   MAIZE 2 = 60 % Maize gluten
MILK = Skimmed milk powder   * = Fed ad libitum

The same mineral supplements added to the ration in Experiment 1, were also used for the diet in Experiment 2. The ewes in Experiment 2 were 'flushed' in the same manner as those in Experiment 1.

5.2.3.2 Tupping, gestation and lambing management.

Cycle synchronisation and management of tupping was similar to that employed in Experiment 1. However, four Co-sufficient Blue-faced Leicester ram lambs were used for mating in this second experiment. After the first cycle, the number of ewes marked with keel paint was unexpectedly low. Therefore, these tups were supplemented with four more experienced Blue-faced Leicester rams, before the second tupping period began. A third tupping period was also employed, to compensate for the poor tup performance at the first cycle.

The sequence of management is presented in Table 4.

All ewes were weighed and condition scored as for Experiment 1.

Table 4. Tupping, gestation and lambing in the ewes of Experiment 2.

DATE	DAY	ACTION
24/09/86	15	All ewes dosed with albendazole, general wormer.
10/10/86	31	Introduction of Co-deficient ration.
22/10/86	43	1st of weekly oral Co supplements to FS ewes.
12/11/86	63	All ewes dosed with 4 mg selenium/hd.
21/11/86	73	Progesterone sponges into all ewes.
04/12/86	86	Sponges taken out of half of the ewes.
05/12/86	87	Sponges taken out of remaining ewes.
06/12/86	88	Ewes put to tup. Peak of 1st tupping period.
22/12/86	104	Peak of 2nd tupping period.
07/01/87	120	Peak of 3rd tupping period.
14/01/87	127	All ewes dosed with a flukacide.
13/02/87	157	Ultrasonic scanning of ewes for lambs.
17/02/87	161	1st of weekly oral Co repletion supplements to HS ewes.
10/04/87	212	All ewes vaccinated against clostridial diseases.
27/04/87– 02/05/87	229– 234	1st lambing period.
10/05/87– 20/05/87–	242– 252	2nd lambing period.
27/05/87– 04/06/87	259– 267	3rd lambing period.

5.2.3.3 Measurements of fertility and gestational performance.

A. Conception rate.

Conception rates were calculated for each treatment at each tupping period, in the same manner as those determined in Experiment 1. The following equations were employed:

TP1

$$\frac{\text{No: marked in TP1 -- No: returning in TP2}}{\text{No: marked in TP1}} \times 100$$

TP2

$$\frac{\text{No: marked in TP2 -- No: returning in TP3}}{\text{No: marked in TP2}} \times 100$$

TP3

$$\frac{\text{No: marked in TP3 -- No: of these scanned empty}}{\text{No: marked in TP3}} \times 100$$

B. Reabsorption rates.

Reabsorption rates in each treatment at the first two tupping periods, were calculated as follows:

TP1

$$\frac{\text{No: marked in TP1, not in TP2, but returning for TP3 or scanned empty}}{\text{No: marked in TP1}} \times 100$$

TP2

$$\frac{\text{No: marked in TP2, not in TP3, but scanned empty}}{\text{No: marked in TP2}} \times 100$$

For each treatment, these reabsorption rates were combined, to give an overall percentage value.

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C. Lambing rate.

Lambing rates were calculated for the ewes in each treatment at scanning, birth and four weeks of age, in exactly the same manner as for Experiment 1.

The mean and spread of gestation length for each treatment was determined by the method used in Experiment 1.

5.2.3.4 Biochemical profile of the ewes in Experiment 2.

In Experiment 2, blood samples were collected and analysed by the same procedures and techniques employed in Experiment 1.

5.2.3.5 Lambing arrangements.

Before lambing, the main pens were mucked out and thoroughly disinfected. This was in contrast to Experiment 1, where the pens were mucked out, but not disinfected before lambing.

Lambing arrangements, record taking and blood sampling in Experiment 2, were kept as close as possible to those employed in Experiment 1. However, in this second investigation it was considered desirable to monitor how vigorous the lambs appeared post partum, as a guide to any later effects on lamb viability. For this purpose, most births were observed first hand (Plate 3). In each case, the time taken for each lamb to perform three functions, namely standing, finding the udder and suckling, were recorded.

The point of first 'standing' was taken when a lamb achieved balance with all four feet on the ground and the legs straight. When a lamb first touched the udder with any part of the head, was viewed as 'finding the udder.' When a lamb took a teat into the mouth and sucked, was recorded as the first 'suckle.'

Where lambings were attended and no assistance required, births were allowed to take place in the main pens. Ewes in labour were not placed in the small lambing pens so that handling did not interfere with parturition, or observation of the lambs' first movements. After observations were complete the ewe and lamb(s) were moved to a lambing pen for 24 hours and thence to a mothering up pen. In Experiment 2, all the ewes and lambs were eventually returned to the main pens for further investigation (Plate 4).

If a ewe required assistance, it was removed to a lambing pen immediately. When a dystocia was considered to have markedly weakened a ewe and its lamb(s), lamb vigour data from the birth were not included in statistical analysis. If a weak lamb did not suckle within 2 hours of parturition, it was encouraged and put on the teat. Where necessary in such situations 150 ml of colostrum from the ewe was given to the lamb at 4 hour intervals, by stomach tube. Prophylactic dosing of new born lambs with antibiotics was not undertaken, but where necessary such treatment was given for ill-health and the incident recorded. The lambs were bled at 2 and 4 weeks

Plate 3. A HS treatment ewe lambing in one of the main pens. Experiment 2.

Plate 4. Four week old lambs with their ewes. Experiment 2.

of age and sera were analysed for B12 and MMA concentrations.

5.2.4 Statistical Analysis - Experiments 1 and 2.

Data for ewe liveweight, condition score, whole blood Hb and GSH+Px, plasma Ca, Cu, Mg and glucose, were tested for significant difference among treatments at each sampling, using one way analysis of variance. The analysis of serum B12 and MMA results was detailed in Chapter 3.

Treatment differences in the numbers of barren ewes, conception, reabsorption and lambing rates, number of lambs born, stillbirths, neonatal mortalities and incidence of lamb ill-health, were tested using Chi-squared analysis.

Differences in lamb mean serum B12 and MMA concentrations and lamb vigour data among treatments, were tested using one way analysis of variance. Where a significant difference existed between treatments, the degree of significance was ascertained using the least significant difference analysis.

Lamb birthweight data were tested by two way analysis of variance, using singles and twins as blocks. Variation due to differences in the numbers of singles and twins born in each treatment, could then be eliminated from the test. Mean lamb birthweight in each treatment was also adjusted to account for the different numbers of singles and twins in each group.

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5.3 RESULTS.

5.3.1 Experiment 1.

5.3.1.1 Ewe liveweight and condition score.

The ewe liveweight and condition score data from Experiment 1 are presented in Figures 1 and 2, respectively and Appendix 12. There were no significant differences among treatments for either mean liveweight or condition score values, at any sampling date. However, a trend was evident in which the NS ewes lost and the FS sheep gained ground in terms of liveweight, relative to all the animals as a whole. This was evident in the data obtained during the weight loss incurred by lambing. For example, the respective mean liveweights of the NS and FS treatments just before lambing on day 133, were 62.9 and 62.8 Kg. After lambing on day 189, the corresponding weights were 52.9 and 56.4 Kg. This trend was not reflected in the condition score data.

5.3.1.2 Biochemical profile of the ewes.

Whole blood Hb and GSH+Px data for the ewes in Experiment 1 are presented in Figures 3 and 4, respectively. There were no significant differences among treatments for mean whole blood Hb or GSH+Px concentrations, at any sampling date. Levels of these two parameters in all treatment groups were within normal ranges throughout the experiment, implying that the sheep were selenium sufficient with no signs of anaemia.

There were no significant differences in mean

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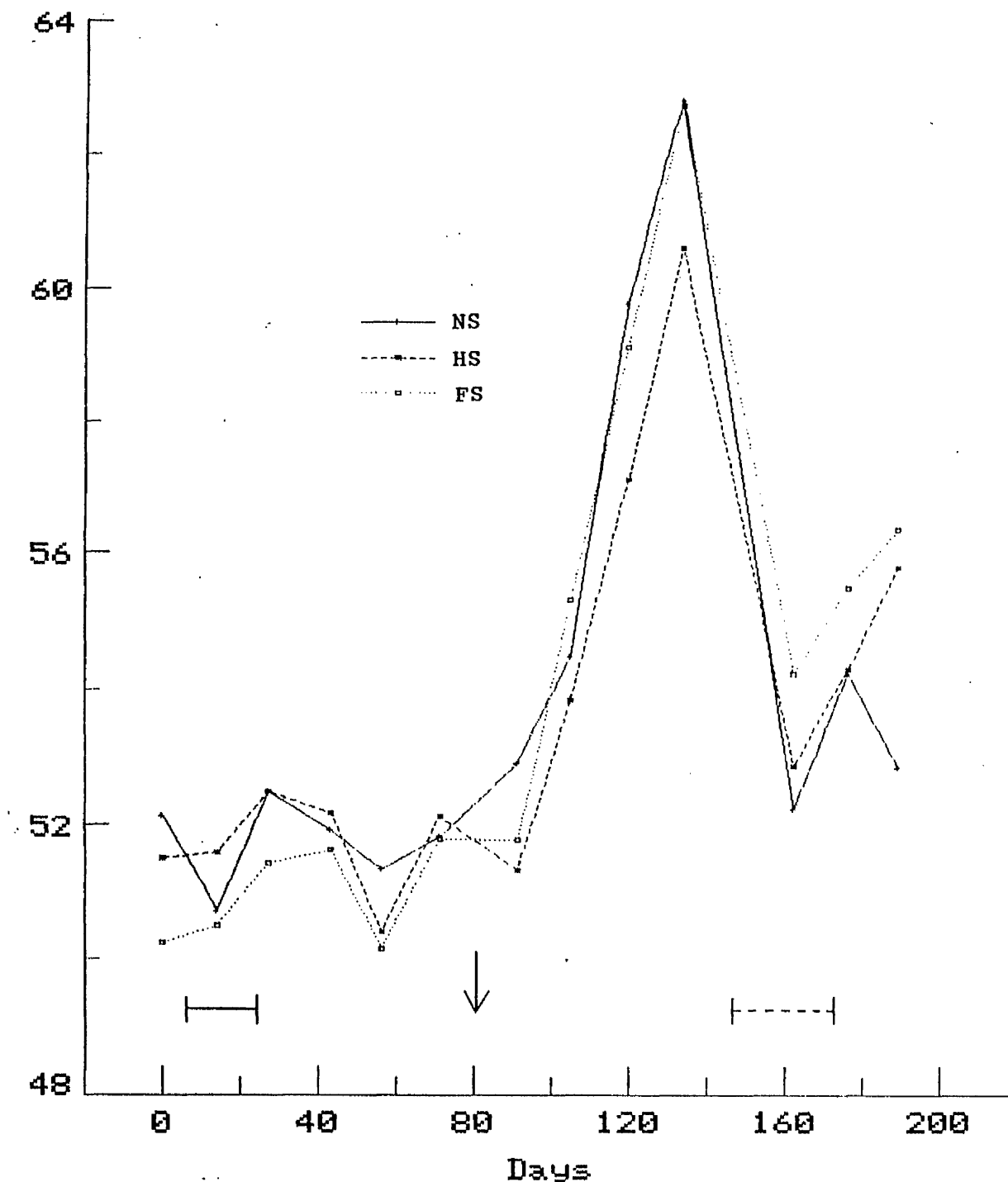
Figure 1. Mean liveweight of the ewes in Experiment 1. (⊢————⊣ = Tupping period; ⊢----⊣ = Lambing period; ↓ = Point at which the weekly oral Co supplement was removed from the HS sheep).
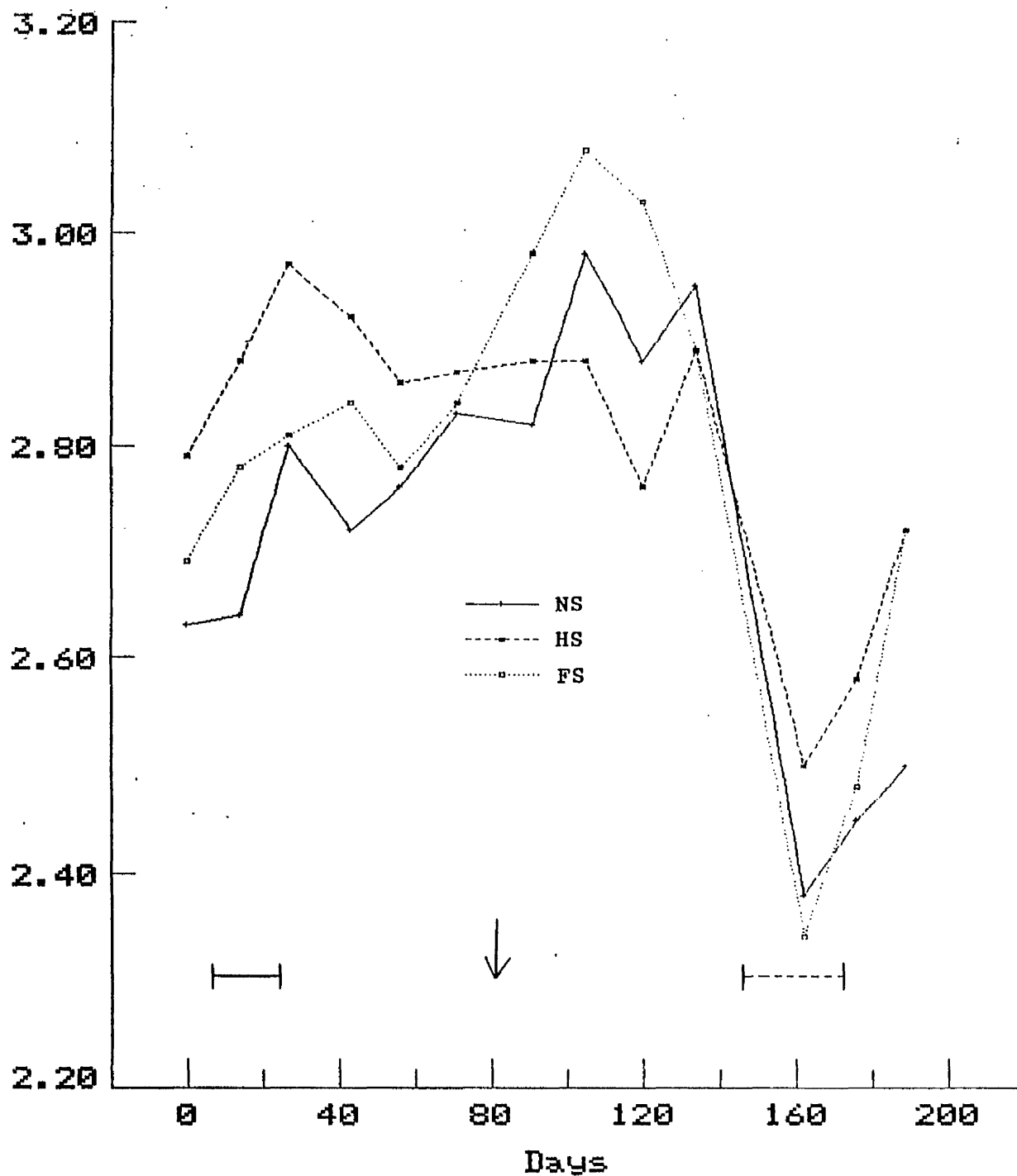
Figure 2. Mean condition score of the ewes in Experiment 1.
(⊢———⊣ = Tupping period; ⊢-- ⊣= Lambing period; ↓ = Point at which
the weekly oral Co supplement was removed from the HS sheep).
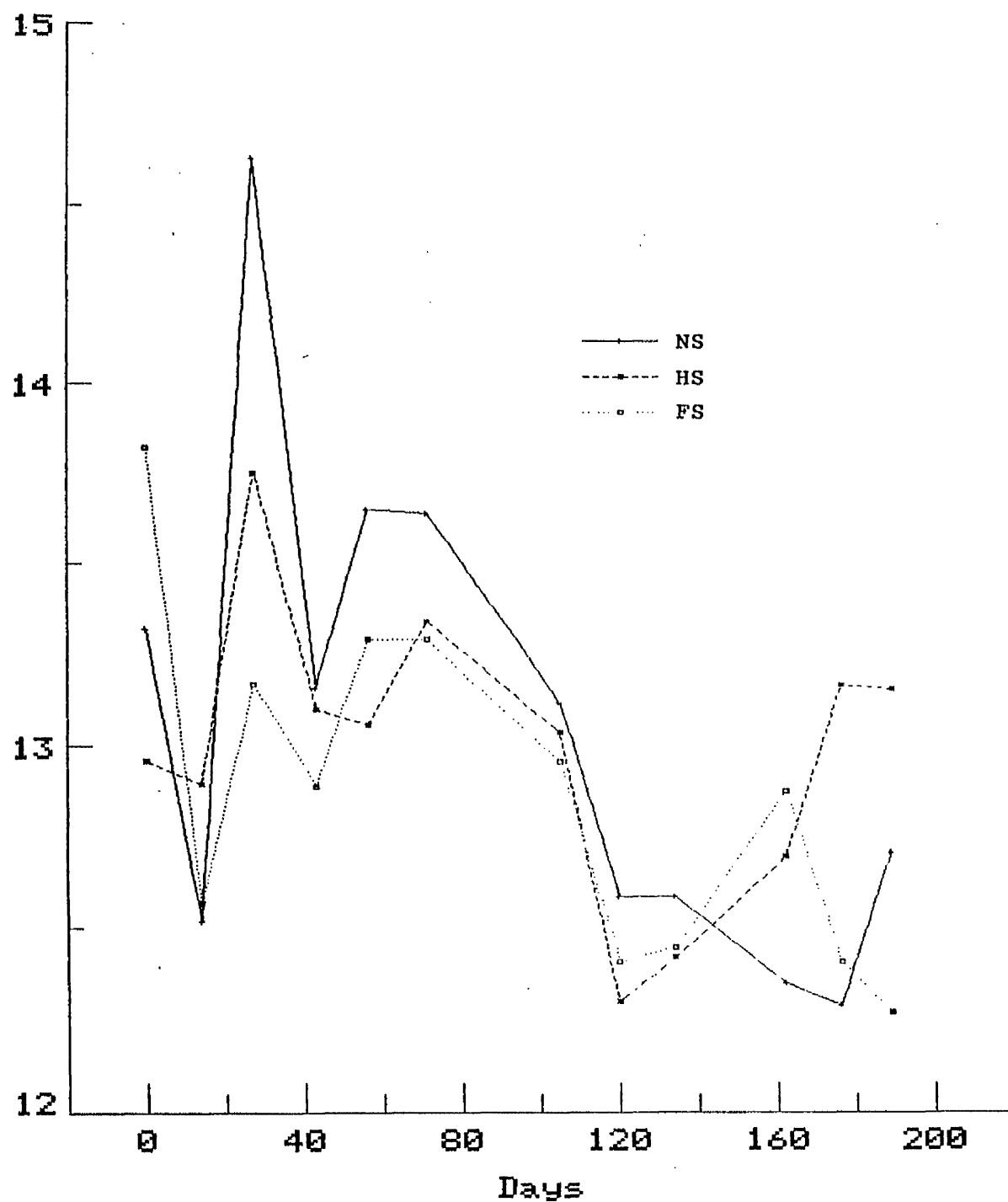
Figure 3. Mean whole blood haemoglobin concentrations in the ewes of Experiment 1.

GSH+Px (Units/ml cells in whole blood)
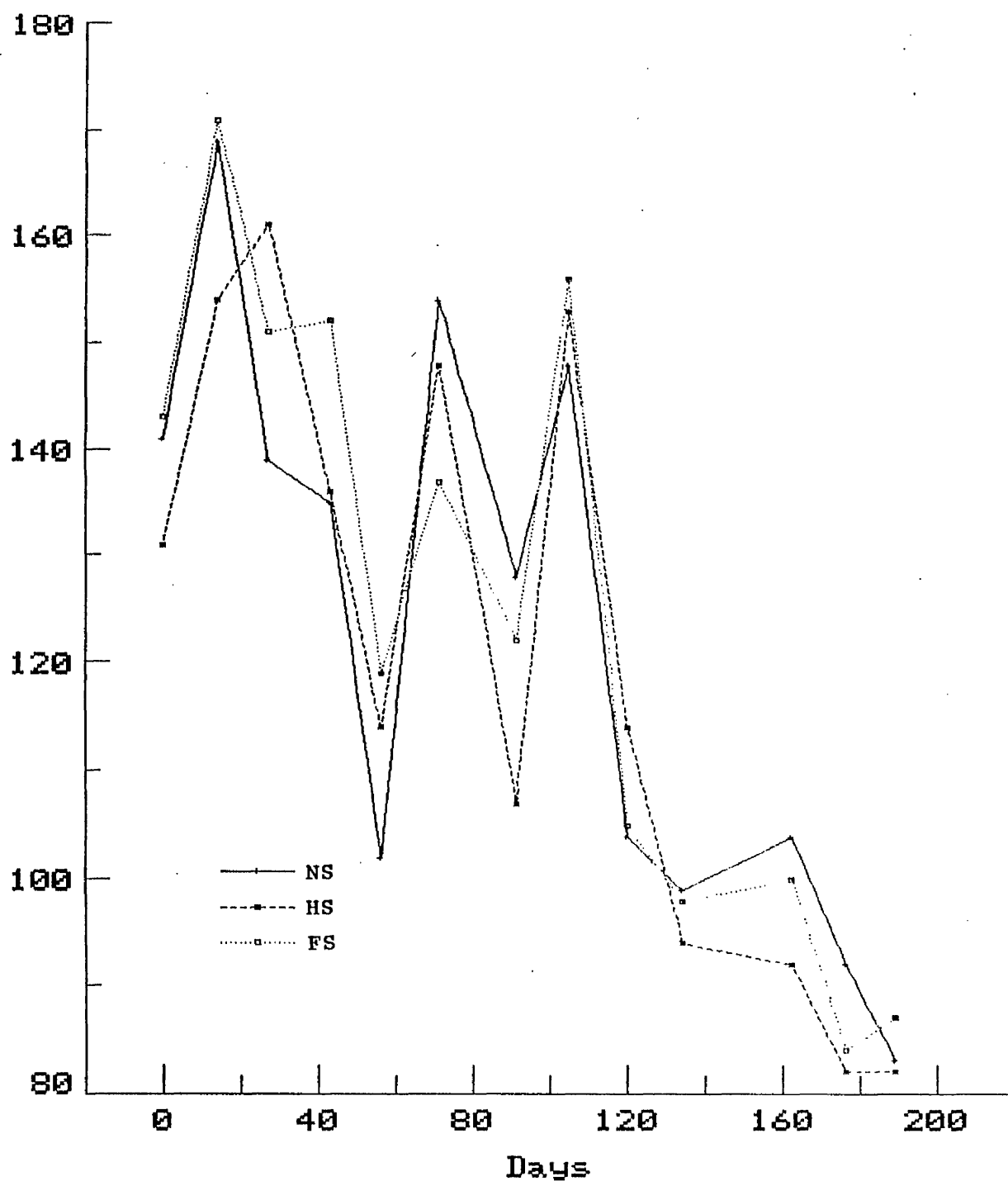
Figure 4. Mean whole blood glutathione peroxidase concentrations
in the ewes of Experiment 1.

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concentrations of plasma Ca, Cu or Mg among groups, at any sampling date. Treatment mean values for plasma Cu and Mg were within the normal ranges of 9.4 to 19.0 umol/l and 0.7 to 1.2 mmol/l, respectively, throughout the experiment. Low plasma Ca levels (less than 2.0 mmol/l) were recorded in all groups as lambing approached. These were immediately corrected in the short term by injections of calcium borogluconate (Crown Chemicals Co. Ltd., Kent) and in the long term by extra additions of dicalcium phosphate to the diet.

Levels of plasma glucose within treatments at different sampling dates, were very varied. However, there were no significant differences in mean plasma glucose levels among groups at any sampling date. Further, concentrations were always within the normal range between 60 and 75 mg/100 ml (MacPherson et al, 1976). This implied adequate energy supply to all the ewes and normal energy metabolism throughout gestation.

5.3.1.3 Ewe serum vitamin B12 and MMA concentrations.

Mean serum B12 and MMA concentrations in the treatments of Experiment 1 were presented in Chapter 3. The data from these two diagnostic markers suggested that the FS ewes were Co-sufficient throughout Experiment 1. The HS sheep were sub-clinically Co-deficient at lambing and remained so until the end of the experiment. The NS ewes became sub-clinically Co-deficient before lambing and suffered from this degree of the disease until the end of

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the experiment.

5.3.1.4 Ewe fertility and gestational performance.

The fertility and gestational performance of the ewes in Experiment 1 are presented in Table 5. There were no significant differences among treatments for the parameters presented in Table 5, except for lambing rate to 4 weeks (see Appendix 13). The lambing rate to 4 weeks of age in the NS group (40 per cent), was significantly lower than those shown by the HS (80 per cent) and FS (95 per cent) ewes ($P<0.05$).

Table 5. Ewe fertility and gestational performance in Experiment 1.

PARAMETER	TREATMENT		
	NS	HS	FS
No: of ewes to the tup	20	20	20
Conception rate, TP1 (%)	71	78	74
Conception rate, TP2 (%)	80	80	80
Reabsorption rate (%)	6	6	11
No: ewes barren at scan	4	2	4
Lambing rate at scanning (%)	125	100	90
Lambing rate at birth (%)	95	85	105
Lambing rate at 4 weeks (%)	40	80	95

The mean ($\pm$ SED) length of gestation in the NS, HS and FS groups, were 147.4 $\pm$ 1.1, 147.9 $\pm$ 0.8 and 148.3 $\pm$ 0.7 days, respectively, with no significant differences among treatments.

The frequency distribution of lambings in each treatment is presented in Figure 5. The percentage of lambing ewes in each group that gave birth in the two lambing periods, is presented in Figure 6. There were no significant differences among treatments for any of these measurements.
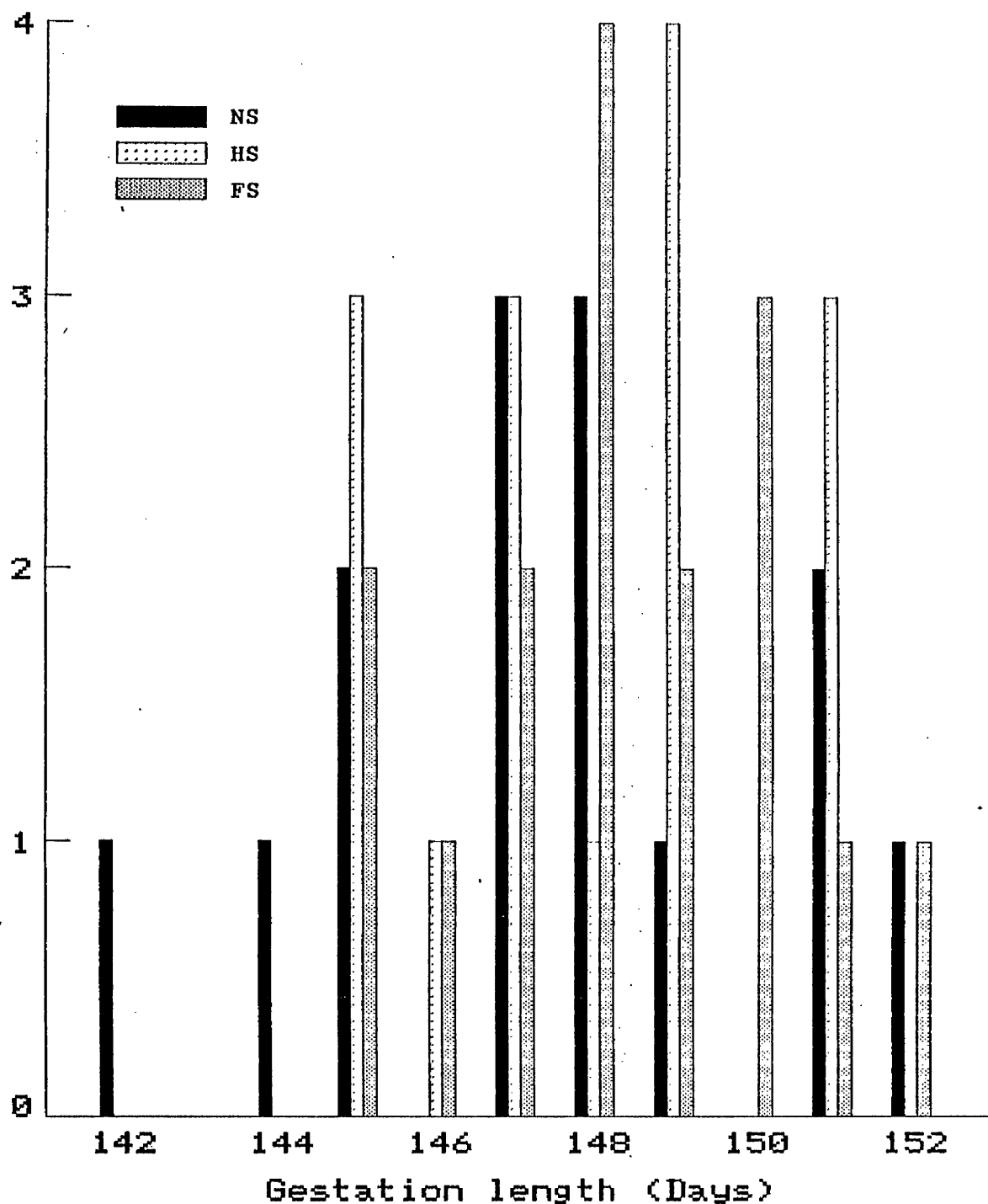
Figure 5. Histogram showing lambing frequency relative to gestation length in the ewes of Experiment 1.
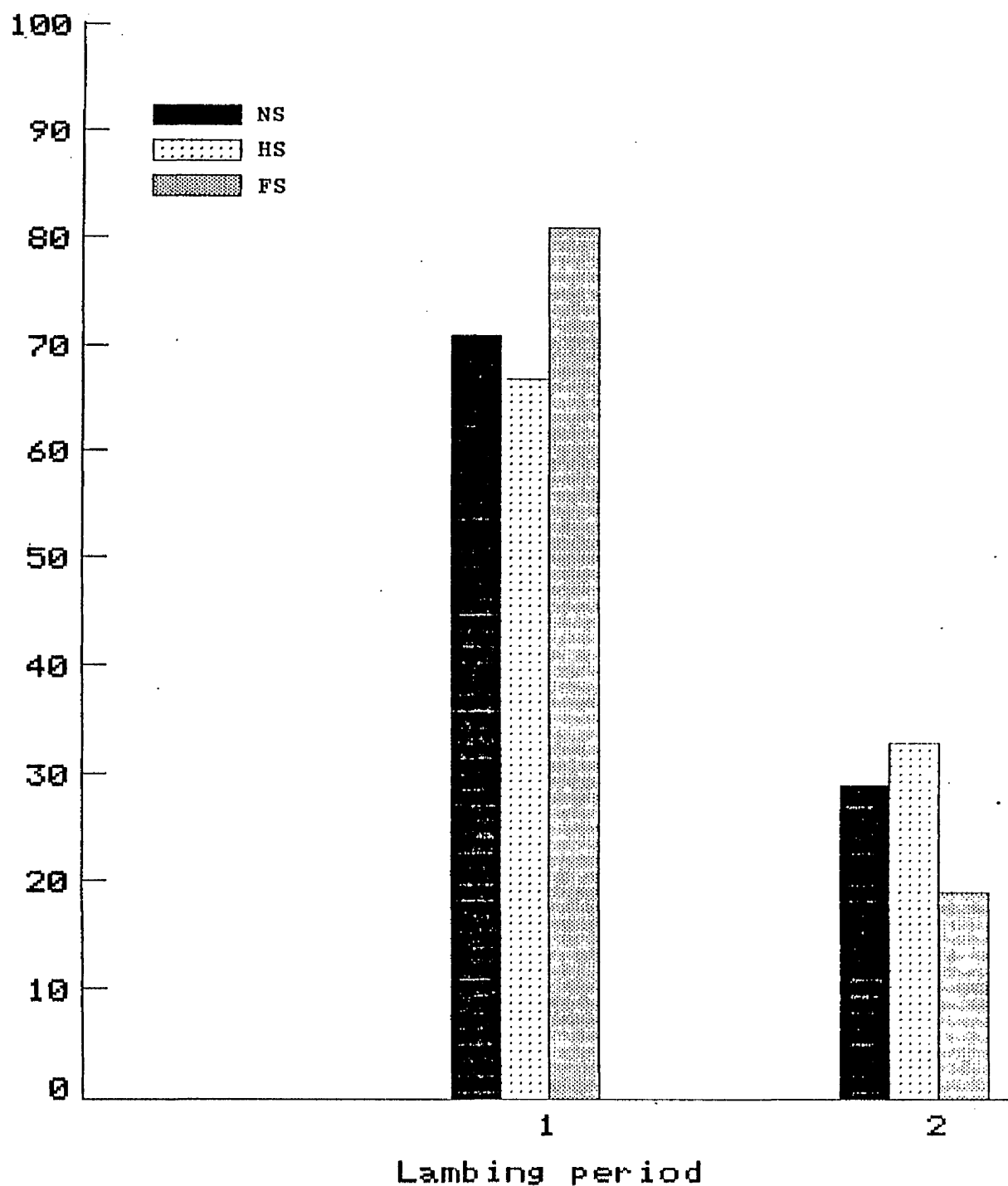
Figure 6. Histogram showing the percentage of ewes in each treatment, which gave birth in the two lambing periods of Experiment 1.

5.3.1.5 Lambing records and post mortem results.

The lambing data for each treatment group in Experiment 1 are presented in Table 6. There were more neonatal mortalities in the NS lambs, compared to the HS and FS neonates ($P<0.001$, see Appendix 14). Significantly more lambs from the HS ewes were given antibiotic treatment for ill-health, than those from the NS and FS sheep ($P<0.05$). An example of the statistical analysis of lamb birthweights is presented in Appendix 15.

The liver Co contents of stillborn lambs, or those dying in the first four weeks post partum, are presented in Table 7.

Details of the post mortem examinations of dead lambs from this experiment, are presented in Table 8.

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Table 6. Lambing record from Experiment 1.

PARAMETER	TREATMENT		
	NS	HS	FS
Lambs born	19^a	17^a	21^a
Mean birthweight (Kg) Av. SED = 0.26	5.1^a	4.6^a	5.1^a
Stillbirths	2^a	0^a	1^a
Neonatal mortalities	9^a	1^b	1^b
Treated for ill-health	1^a	4^b	0^a

Values in rows with different superscripts, differed at least at $P<0.05$.

Table 7. Liver Co contents of dead lambs from Experiment 1.

	TREATMENT		
	NS	HS	FS
No: of samples	6	1	1
Mean Co content (mg/Kg fresh wght)	0.017	0.04	0.16

Table 8. Details of post mortem examinations of dead lambs from Experiment 1.

Tag	AGE (Days)	CAUSE OF DEATH
TREATMENT - NS		
--	Stillbirth	Dystocia. Haemorrhage of liver during birth.
--	Stillbirth	Dystocia.
51	9	Slight infectious prenatal brain lesion, cause unknown. Significant E. coli infection of kidney, lung and intestinal lesions.
52	10	No significant findings.
53	27	Bacterial bronchopneumonia and nephritis with tubular degeneration. Degenerative changes in liver.
75	10	No significant findings.
-- TWINS	1	No significant findings.
--	2	Gastritis and enteritis. E. coli and Clostridium perfringens isolated from small intestine.
94	6	No significant findings.
99	22	Lesions suggested crushing.
100	2	Diffuse myositis in skeletal muscle. Tubular nephritis in kidney.
TREATMENT HS		
--	2	Small, premature lamb (2.7 Kg).
TREATMENT FS		
--	Stillbirth	Dystocia.

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5.3.1.6 Lamb serum vitamin B12 and MMA concentrations.

Treatment mean serum B12 concentrations at 2 and 4 weeks post partum in the lambs from Experiment 1, are presented in Table 9. Mean serum MMA levels in all lambs at 2 and 4 weeks post partum were 3.40 $\pm$ 0.24 and 3.73 $\pm$ 0.26 umol/l, respectively, with no trends or significant differences amongst treatments being evident.

Table 9. Lamb serum vitamin B12 concentrations from Experiment 1.

MEAN VIT. B12 (ng/l) AT:	NS	TREATMENT HS	FS	SIG. DIF.	SED
2 weeks pp	147[a]	153[a]	271[b]	***	25
4 weeks pp	150[a]	164[a]	259[b]	*	42

Means in rows with sifferent superscripts were significantly different.

5.3.2 Experiment 2.

5.3.2.1 Ewe liveweight and condition score.

The ewe liveweight and condition score data from Experiment 2 are presented in Figures 7 and 8, respectively and Appendix 16. There were no significant differences among treatments for either mean liveweight or condition score values, at any sampling date. However, a general trend of the FS group gaining and the NS sheep losing ground for both parameters, relative to all the animals as a whole, continued through gestation and lambing. This was evident in the liveweight data, where

Liveweight (Kg)
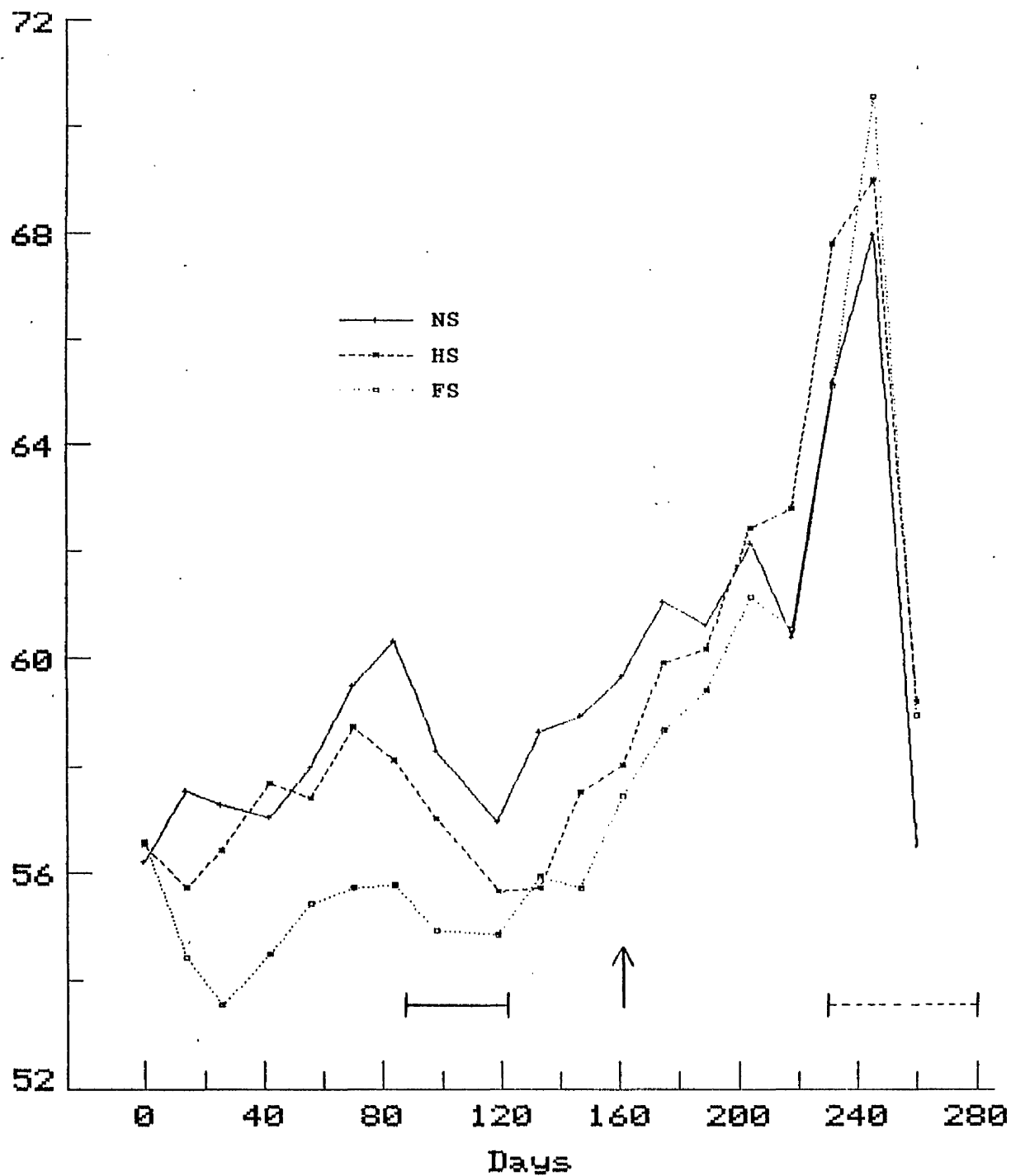
Figure 7. Mean liveweight of the ewes in Experiment 2. (├────┤ = Tupping period; ├────┤ = Lambing period; ↑ = Start of weekly oral Co supplementation of the HS sheep).
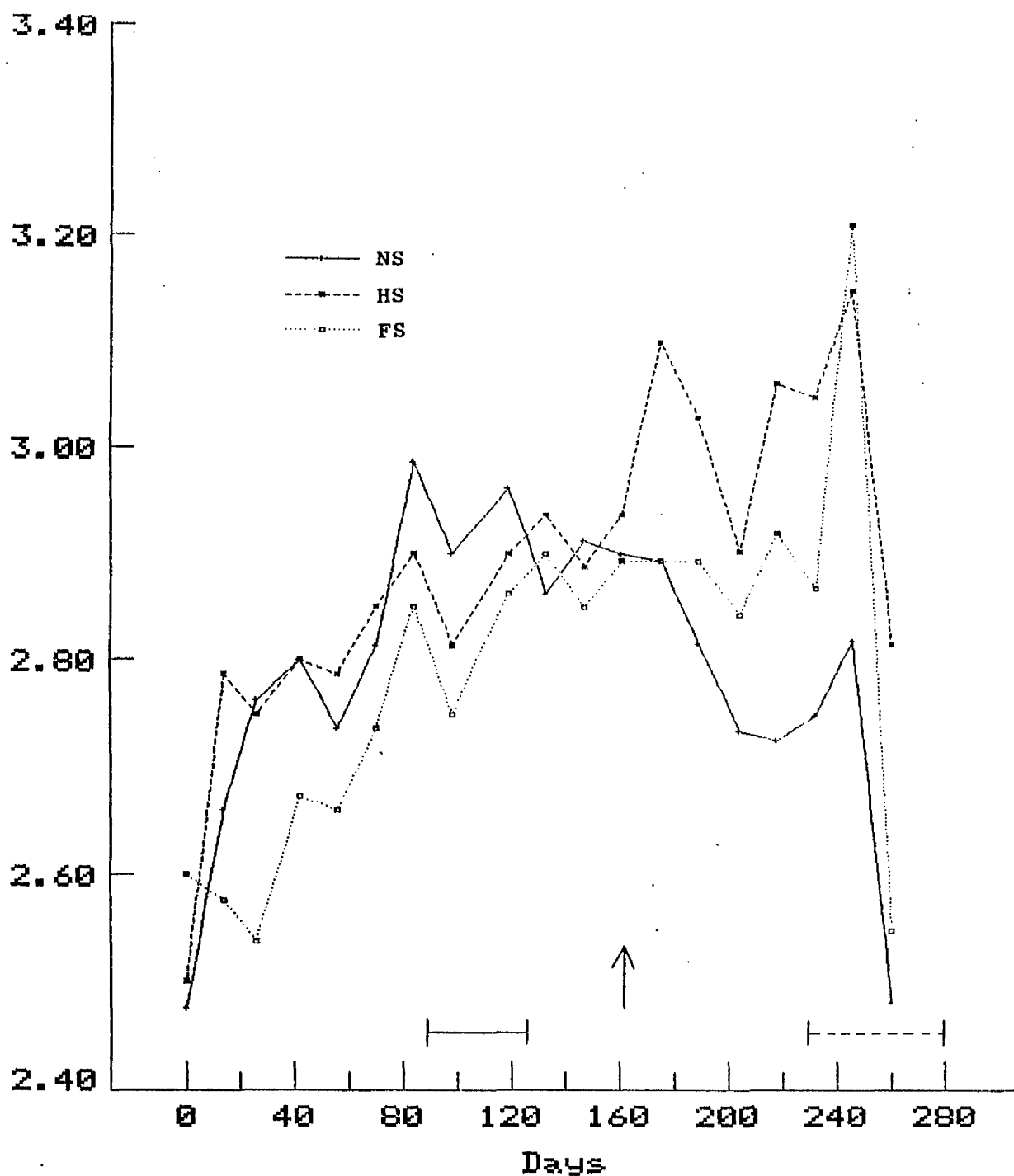
Figure 8. Mean condition score of the ewes in Experiment 2. (├────┤ = Tupping period; ├── ─┤ = Lambing period; ↑ = Start of weekly oral Co supplementation of the HS sheep).

the mean values for the NS and FS ewes at the start of tupping (day 87) were 60.3 and 55.8 Kg, respectively. At the last weighing after lambing (day 260), this 4.5 Kg advantage of the NS group had been lost and the equivalent values were 56.5 and 59.0 Kg. A similar trend between the NS and FS treatments was revealed by the condition score results, where mean values were 2.99 and 2.85 (day 87) and 2.48 and 2.55 (day 260), respectively.

5.3.2.2 Biochemical profile of the ewes.

Whole blood Hb and GSH+Px data for the ewes of Experiment 2 are presented in Figures 9 and 10, respectively. There were no significant differences among treatments for mean whole blood Hb or GSH+Px concentrations, at any sampling date. Levels of Hb in all treatment groups were within the normal range (above 9.0 g/100 ml whole blood) throughout the experiment, implying that the sheep at no time suffered from anaemia.

Mean GSH+Px concentrations were in the deficient range (less than 27 units/ml), implying selenium deficiency, on day 26. All ewes were given an oral dose of 4 mg Se/hd as sodium selenate ($Na_2SeO_4 . 7H_2O$) before tupping, on day 63. Mean GSH+Px levels in all groups then increased to normal values and remained so for the whole of pregnancy and lactation.

No confidence could be placed in the plasma glucose concentrations determined in ewes from Experiment 2. This was due to continual problems with the analytical
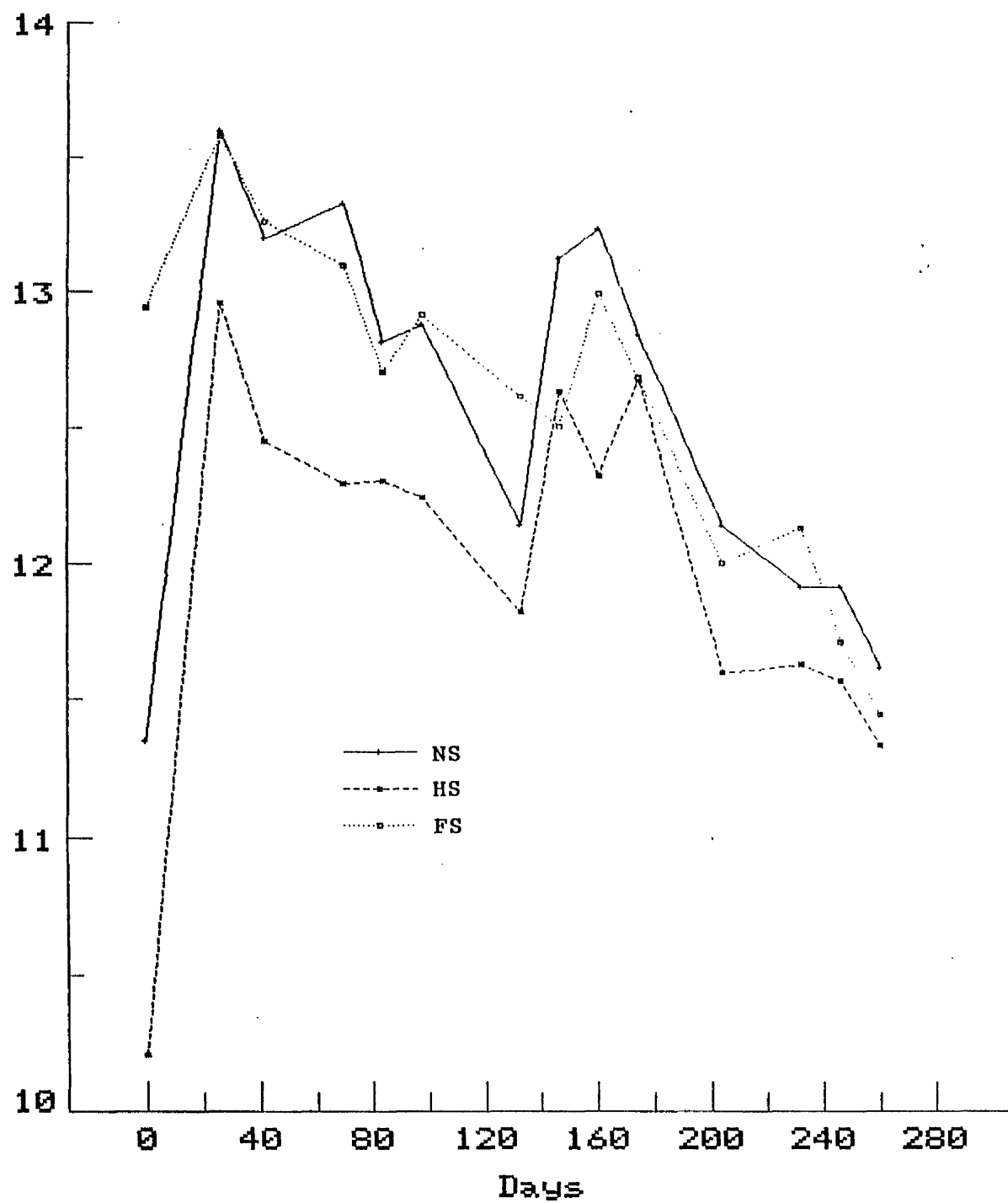
Figure 9. Mean whole blood haemoglobin concentrations in the ewes of Experiment 2.

GSH+Px (Units/ml cells in whole blood)
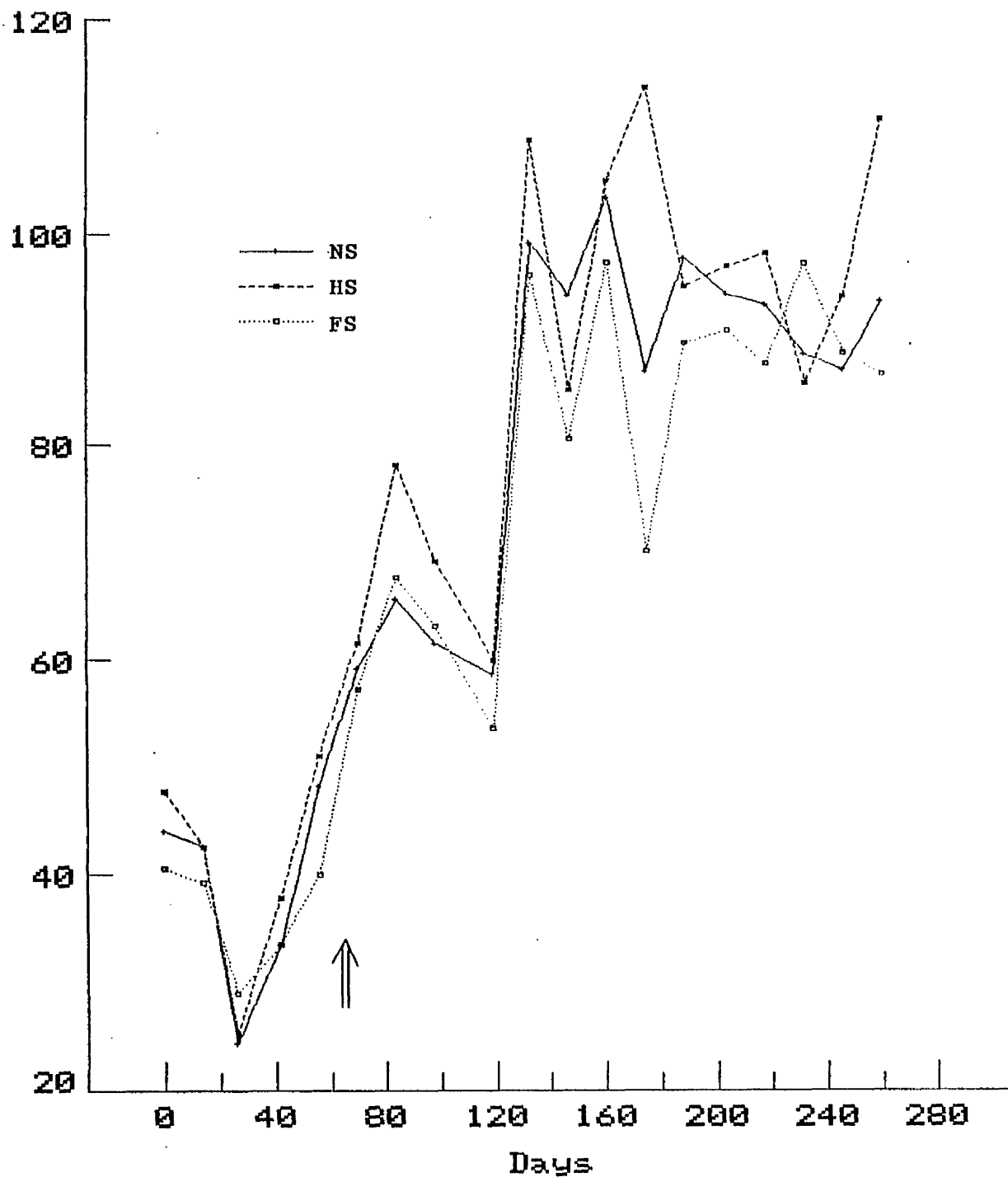
Figure 10. Mean whole blood haemoglobin concentrations in the ewes of Experiment 2. (⇑ = Timing of single oral dose of 4 mg/hd selenium, to all sheep).

technique of large variations in the results. These variations appeared to be due to the method, rather than any effect of treatment on the sheep. However, energy status in a sample of twelve ewes (four from each treatment) was tested on day 218 by measurement of serum beta-hydroxybutyrate levels. These were all normal (less than 0.80 mmol/l) and ranged from 0.10 to 0.42 mmol/l. These indicated normal energy metabolism in the sheep just before lambing.

There were no significant differences in the mean concentrations of plasma Ca, Cu or Mg amongst treatments, at any sampling date. Levels of these three minerals in all groups were within the normal ranges throughout the investigation.

5.3.2.3 Ewe serum vitamin B12 and MMA concentrations.

Mean serum B12 and MMA concentrations in the ewes from Experiment 2 were presented in Chapter 3. The FS ewes were Co-sufficient throughout Experiment 2. The HS sheep were sub-clinically Co-deficient for the first half of pregnancy, but were sufficient for the second half and the remainder of the experiment. The NS ewes were rendered sub-clinically Co-deficient at tupping and the disease progressed to the clinical form at lambing.

5.3.2.4 Ewe fertility and gestational performance.

The fertility and gestational performance of the ewes in Experiment 2 are presented in Table 10. There were no

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significant differences among treatments for any of the parameters shown. However, there was a tendency for the NS and HS groups to have lower lambing rates at birth and 4 weeks, compared to the FS sheep.

Table 10. Ewe fertility and gestational performance in Experiment 2.

PARAMETER	TREATMENT		
	NS	HS	FS
No: of ewes to the tup	20	20	20
Conception rate, TP1 (%)	46	25	33
Conception rate, TP2 (%)	67	67	57
Conception rate, TP3 (%)	100	100	100
Reabsorption rate (%)	16	25	20
No: of ewes barren at scan	1	3	1
Lambing rate at scan (%)	130	120	120
Lambing rate at birth (%)	105	105	115
Lambing rate at 4 weeks (%)	85	100	115

The frequency of lambings in each treatment are presented in Figure 11. The percentage of lambing ewes in each treatment that gave birth in each of the three lambing periods, are presented in Figure 12. The mean length of gestation in the NS, HS and FS sheep, was 146.1 ± 1.0, 145.7 ± 0.6 and 146.8 ± 0.7, respectively. There were no significant differences among treatments, for any of these measurements.
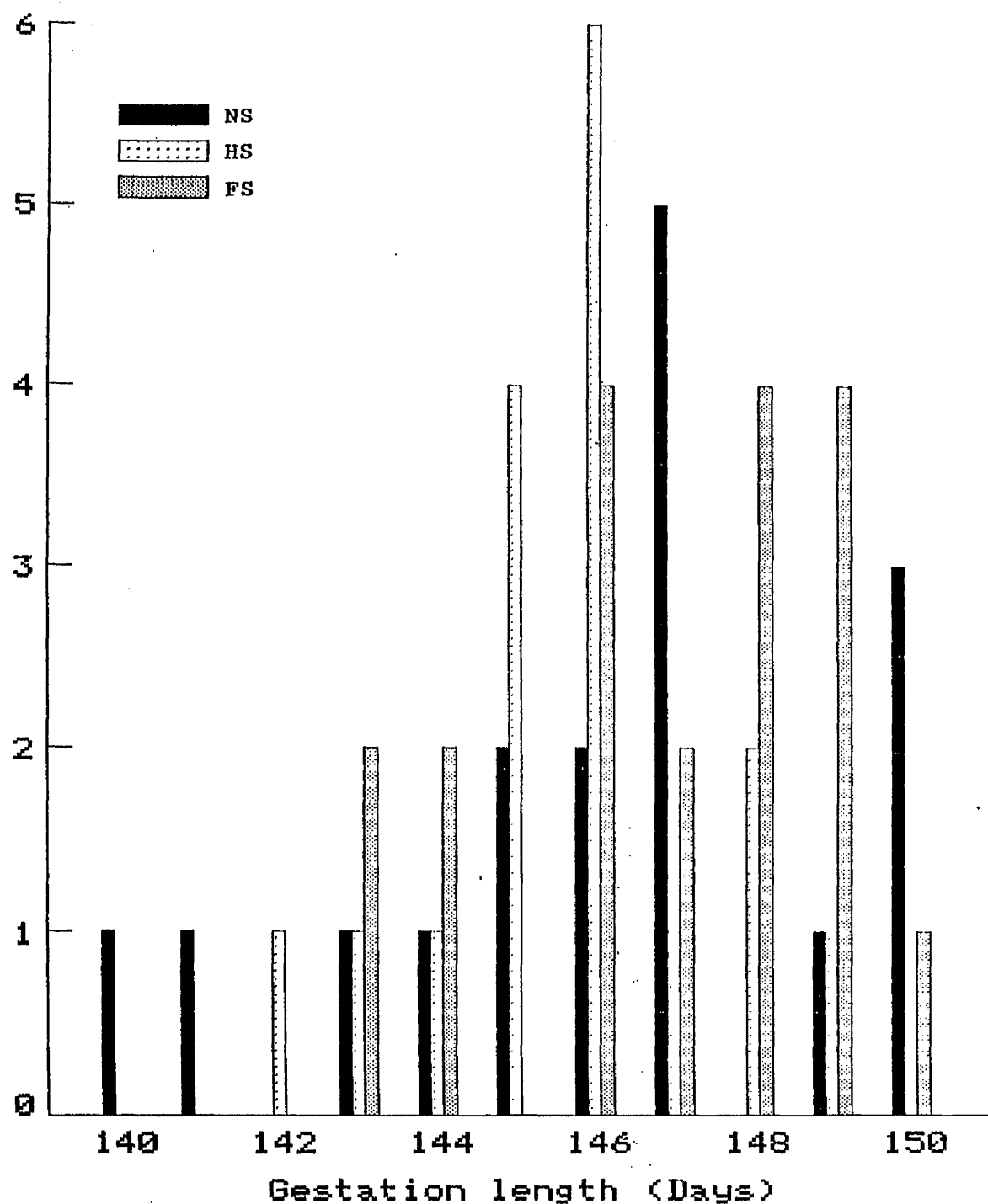
Figure 11. Histogram showing lambing frequency relative to gestation length in the ewes of Experiment 2.
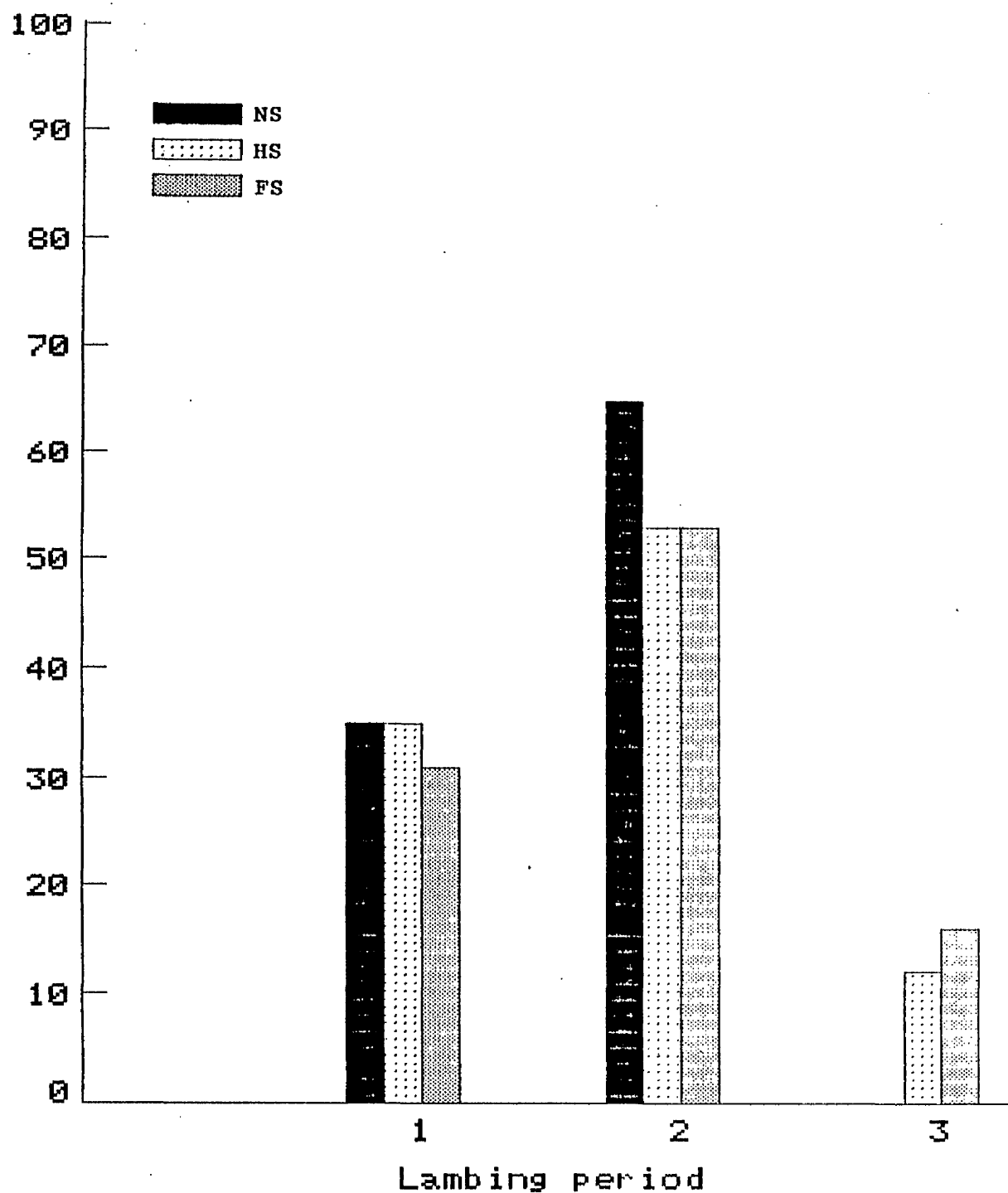
Figure 12. Histogram showing the percentage of ewes in each treatment, which gave birth in the three lambing periods of Experiment 2.

5.3.2.5 Lambing records, post mortem results and lamb vigour.

The lambing data for each treatment in Experiment 2 are presented in Table 11.

Table 11. Lambing record from Experiment 2.

| PARAMETER | TREATMENT | | |
	NS	HS	FS
No: of lambs born	21	21	23
Mean birthweight (Kg) SED = 0.27	4.3	4.8	4.7
Stillbirths	4	1	0
Neonatal mortalities	0	0	0
Treated for ill-health	1	2	1

There were no significant differences among treatments for any of the parameters in the lambing record, except the number of stillbirths. The NS group had a significantly higher incidence of stillbirths, compared to the HS and FS sheep (P<0.05). Two ewes in the NS group were scanned as carrying twins, but were found to be empty at lambing time. One other sheep in this treatment aborted twin lambs.

The post mortem results for the lamb deaths in Experiment 2 are presented in Table 12. Liver samples taken from three of the stillborn lambs in the NS treatment and samples from the twins aborted by a NS ewe,

showed a deficient mean Co content of 0.014 ± 0.002 mg/Kg fresh weight. A similar sample from the stillborn lamb in the HS group revealed an adequate Co level of 0.11 mg/Kg fresh weight.

Lamb vigour data are presented in Table 13.

Table 12. Results of <u>post</u> <u>mortem</u> examinations of dead lambs from Experiment 2.

TAG	AGE (Days)	CAUSE OF DEATH

TREATMENT NS

-- Stillbirth   No significant findings.

-- Stillbirth   Mummified lamb (2.2 Kg).

-- Stillbirth   Dystocia.

-- Stillbirth   Dorsal and lateral curvature of the thoracic spine, of unknown aetiology. Some colonies of <u>Pasteurella</u> <u>haemolytica</u> (type 15) isolated from lungs.

TREATMENT HS

-- Stillbirth   Staphylococcus aureus isolated from liver tissue. Neuropathology revealed lesions suggestive of toxoplasmosis.

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Table 13. Measurements of lamb vigour.

TIME FROM BIRTH TO:- (Min.)	TREATMENT			SIG. DIF.	SED
	NS	HS	FS		
Standing	22.4^{ab}	28.7^{a}	15.3^{b}	*	4.9
Finding udder	40.7^{a}	43.5^{a}	23.7^{b}	**	5.8
Suckling	76.2^{a}	60.5^{b}	30.7^{c}	***	6.9

Means in rows with different superscripts differed significantly.

5.3.2.6 Lamb serum vitamin B12 and MMA concentrations.

Treatment mean serum B12 and MMA concentrations in the lambs of Experiment, 2 at 2 and 4 weeks post partum, are presented in Table 14.

Table 14. Lamb serum vitamin B12 and MMA concentrations in Experiment 2.

WEEKS post partum		TREATMENT			SIG. DIF.	SED
		NS	HS	FS		
Vit. B12 (ng/l)	2	53^{a}	153^{b}	300^{c}	***	40
	4	94^{a}	248^{b}	330^{b}	***	64
MMA (umol/l)	2	15.0^{a}	1.0^{b}	1.1^{b}	***	3.1
	4	20.3^{a}	2.4^{b}	2.3^{b}	***	4.4

Means in rows with different superscripts, differed at $P < 0.001$.

5.4 DISCUSSION.

The effects of Co deficiency in pregnant ewes on reproduction evident in Experiments 1 and 2, will be discussed in terms of the fertility and gestational performance of the sheep and lamb viability. These results will be related to the degree of Co deficiency disease experienced by the ewes in the two experiments and the serum vitamin B12 and MMA status of the neonatal lambs.

5.4.1 General Effects on the Ewes.

The extent of the deficiencies imposed on the ewes in both Experiments 1 and 2 were reflected by the liveweight and condition score data (Figures 1, 2, 6 and 7). While there was a tendency for the NS sheep to lose ground relative to the other animals in terms of these two parameters, no significant differences among treatments were evident. This indicated that the degree of Co deficiency imposed on the NS and HS groups in both experiments was sub-clinical, although the NS sheep were experiencing the clinical disease at lambing in Experiment 2, due to the extended period on a low Co diet in this second experiment.

The analysis of plasma glucose levels in Experiment 1 and beta-hydroxybutyrate concentrations in Experiment 2 supported these conclusions. The data from these determinations implied that all the animals in Experiment 1 had a normal energy metabolism. The energy status of

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the sheep in Experiment 2 could also be presumed to be normal, up to the start of lambing, when the beta-hydroxybutyrate determinations were made.

The sub-clinical Co deficiencies imposed on the ewes in Experiments 1 and 2 did not affect whole blood Hb concentrations (Figures 3 and 7). Thus, the animals were at no time suffering from anaemia, which can be associated with severe clinical Co deficiency (MacPherson et al, 1976).

In both experiments the whole blood GSH+Px (Figures 4 and 8) and plasma Ca, Cu and Mg concentrations in the sheep were usually in the normal range. Where levels of GSH+Px and Ca were low, relevant supplementation was provided immediately. For this reason, selenium or other mineral deficiencies did not complicate the investigation of the effect of Co deficiency in these experiments.

5.4.2 Fertility and Gestational Performance.

In both experiments a Co-deficient intake did not affect the tupping performance of the ewes in terms of conception rates, reabsorption rates, or the number of ewes barren at scanning (Tables 5 and 9). This was even the case in Experiment 2, where the NS and HS sheep were rendered sub-clinically Co-deficient during the mating period. In this second experiment, 10 per cent (4 out of 40) of the animals on a Co-deficient intake (NS and HS treatments) were barren at scanning compared to only 5

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per cent (1 out of 20) of those given a sufficient Co supply (FS group). However, this difference was not statistically significant.

The poor conception rates evident in all treatments at tupping periods 1 and 2 in Experiment 2, were due to the use of inexperienced ram lambs for mating. The high reabsorption rates recorded for all groups in this experiment were also caused by these tups, who appeared to mark ewes without actually joining. Thus, a large number of sheep were apparently returning to service, when in fact many of these had not been tupped in a previous cycle.

These results were in contrast with those of Dunlop (1946b), who reported greater infertility in "sub-minimally" Co-deficient ewes, compared to Co-dosed controls. However, if the animals in these two experiments had been rendered sub-clinically Co-deficient earlier before mating and tupped by deficient rams, then the data might have been more in agreement with that of Dunlop (1946b).

Despite these negative results, the imposed deficiencies did adversely affect the gestation and lambing performance of the sheep in both experiments. These effects could be summarised by the observation that lambs from sub-clinially Co-deficient ewes had a decreased pre- and post-natal viability, compared to those from sufficient controls.

This adverse effect was evident in the lambing rate

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data from both experiments. The prospective lambing rates calculated at scanning were not actually achieved by the NS and HS ewes, but were accomplished by the FS animals. This drop in lambing rate between scanning and birth in the NS and HS treatments was due to an increased number of abortions and probable reabsorptions in the deficient sheep, compared to FS controls. For example, in Experiment 2, two NS ewes were scanned as carrying twins, but were found to be empty at lambing. These animals either aborted or reabsorbed their lambs in the second half of gestation.

The depression in lambing rate between scanning and birth in both experiments, was more marked in the NS than in the HS treatment. However, it was still a problem in the HS ewes. This fact implied that Co supplementation of sub-clinically Co-deficient sheep in either the first (Experiment 1) or second (Experiment 2) half of pregnancy, was not adequate to protect these ewes fully from pre-natal lamb losses due to the deficiency.

A further decrease in lambing rate between birth and 4 weeks of age was evident in both investigations. In Experiment 1 this was most marked in the NS group, where lambing rate between these two times dropped from 95 to 40 per cent. This was due to the increased number of neonatal mortalities occurring in this treatment, compared to the FS controls, (Table 6). In Experiment 2, this depression in lambing rate was again evident in the

HS and NS treatments, but this time was caused by increased numbers of stillbirths, rather than neonatal mortalities, relative to the FS group.

These results are in agreement with those of Quirk and Norton (1987) who reported increased numbers of reabsorptions, abortions and stillbirths in Co-deficient ewes compared to sufficient controls. These workers tried to characterise the effects of clinical Co deficiency on reproductive performance and subsequent lamb growth. They established four treatments: 'C' - 8 non-supplemented controls; 'LC' - 6 given 0.03 mg Co/head/day in a weekly oral dose; 'HC' - 6 given 0.06 mg Co/head/day in a weekly oral dose; and 'CB' - 6 given a Co bullet and grinder. All treatments were administered from before tupping. The effects of these treatments on ewe reproductive performance are shown in Table 15.

Table 15. The reproductive performance of ewes recieving various levels of Co supplementation.

PARAMETER	TREATMENT GROUP			
	CB	HC	LC	C
Ewes cycling/ewes joined (%)	100	100	100	88
Returns to service/ewes cycling (%)	0	0	0	29
Ewes lambing/ewes joined (%)	83	83	83	50
Lambs born/ ewes joined (%)	116	83	83	50

(After Quirk and Norton, 1987).

Further to these results, Co-supplemented sheep were heavier than non-supplemented controls throughout pregnancy and lactation (P<0.05). Lambs from the HC and LC treatments had greater birthweights than those from C dams (P<0.05), and CB, HC and LC lambs grew faster in the 14 weeks of lactation before weaning, than the C lambs (P<0.05).

The detailed study of Quirk and Norton (1987) also attempted to relate serum B12 and urinary MMA and FIGLU concentrations to these findings. Serum B12 levels in C group ewes were in the clinical range and less than a mean of 160 ng/l throughout the trial, while those in the other treatments indicated Co sufficiency.

Urinary MMA and FIGLU were not detected in the CB, HC and LC ewes during pregnancy and lactation, but consistently high levels (up to means of 4847 umol MMA/l and 232 umol FIGLU/l urine) were evident in the C group sheep throughout the experiment. These concentrations were indicative of clinical Co deficiency in the non-supplemented animals.

It is important to note that the results of Quirk and Norton (1987) were obtained from clinically Co-deficient and sufficient ewes. The investigations discussed here implied that such effects can occur in sub-clinically deficient animals.

In Experiments 1 and 2. trends were also discernable in the data relating to differences in gestation length among treatments. In both experiments there was a

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tendency for the NS ewes to lamb over a longer period than either the HS or FS groups. This was evident from Figures 5 and 11, which showed that the NS animals started lambing three days before the FS treatment, in both experiments. The NS and HS groups of both experiments also had slightly shorter mean gestation lengths than the FS sheep.

These observations of greater inconsistency in gestation length in sub-clinically Co-deficient sheep, compared to sufficient controls, may be linked to the cyclical hormonal changes which Mgongo et al (1985) reported in clinically deficient goats. These workers found evidence of inconsistent oestrus cycle length in Co-deficient animals, compared to sufficient counterparts. However, the trend reported here for Co-deficient sheep to have shorter gestation lengths, can not be supported by any direct evidence in the literature.

5.4.3 Lambing Records, post mortem Examinations and Lamb Vigour.

Sub-clinical Co deficiency in the NS and HS ewes of Experiments 1 and 2 did not have any marked effect on the numbers of lambs born, or the mean lamb birthweights among treatments. However, a profound adverse affect on post-natal lamb viability was evident. These results were indicated by the lambing records, presented in Tables 6 and 10.

Although there was a tendency in both experiments for the NS and HS treatments to yield fewer lambs than the FS group, these differences were not statistically significant. In addition, there was a trend for the HS ewes in Experiment 1 and the NS sheep in Experiment 2 to produce lighter lambs than the FS animals, but again these differences were not statistically significant.

Decreased post-natal viability of lambs from Co-deficient ewes, compared to those from sufficient dams, was evident in both experiments. In Experiment 1, this was manifest by a significantly higher number of neonatal mortalities in the NS, compared to the HS and FS treatments (9 vs. 1 vs. 1, respectively, $P < 0.05$). In Experiment 2, the effect was found in a significantly higher number of stillbirths in the NS relative to the HS and FS groups (4 vs. 1 vs. 0, respectively, $P < 0.05$). These effects led to the depression of lambing rate in the NS ewes, discussed in the previous section. These observations are supported by the work of Duncan et al (1981), who reported an increased incidence of stillbirths and perinatal mortalities in lambs from a small group of clinically Co-deficient sheep, compared to sufficient controls.

This impairment of post-natal viablility in lambs requires explanation. In the two experiments described the effect seemed to be related in part to the ability of the sheep to resist infection before lambing, and the

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ability of the lambs to resist infection post partum.

This conclusion can be drawn from the results of the post mortem examinations of dead lambs in both experiments, presented in Tables 7 and 11. In two of the stillbirths from Experiment 2 (one in each of the NS and HS treatments), bacterial infections were implicated in the cause of death. Further, in 4 of the 9 neonatal mortalities in the NS group of Experiment 1, bacterial infections were again implicated in the cause of death.

However, there may be a flaw in this argument as neonatal mortalities were recorded in Experiment 1, but not in Experiment 2. This discrepancy must be explained if the theory is to be substantiated. In the first experiment, the pens were mucked out and re-bedded before lambing, but not disinfected. In the second investigation, the pens were again mucked out, but were also thoroughly washed and disinfected. The level of pathogenic challenge to new born lambs in Experiment 1, was therefore probably greater than that experienced in Experiment 2. This could account for the discrepancy in the incidence of neonatal mortalities between experiments.

A major cause of the decreased ability of lambs from Co-deficient ewes to resist infection, compared to sufficient controls, was revealed by the lamb vigour data of Experiment 2 (Table 13). These results showed that lambs from both the NS and HS treatments took, on average, at least twice as long to start suckling,

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relative to lambs from the FS group. Such an effect was also noted subjectively in Experiment 1. In general, lambs from Co-deficient ewes did not appear to be weaker or 'less willing' to stand and suckle than sufficient controls. Rather, lambs from deficient sheep simply took longer to perform these tasks.

It may therefore be postulated that lambs with depressed vigour from Co-deficient ewes would suckle less frequently in the first hours and days of life. Suckling frequency was not measured in these experiments, but it may be that a lamb from a NS or HS dam which took more than 60 minutes to start suckling, fed from the teat less frequently than a lamb from a FS sheep, which started to suckle after only 30 minutes.

If this were the case, then the levels of energy and passive immunity acquired by NS and HS lambs from their mothers' colostrum and milk would be reduced, relative to that achieved by the FS lambs. Thus, lambs from Co-deficient ewes would have a decreased energy supply and ability to resist infection compared to those from sufficient controls. Such an effect would account for the increased incidence of neonatal mortalities in the NS group and numbers requiring anti-biotic treatment for ill-health in lambs from HS group in Experiment 1, and the overall depression in post-natal lamb viability evident in both experiments.

Interestingly, Garton et al (1981) reported cases of

neuropathological abnormalities in lambs born to clinically Co-deficient ewes. These workers related their findings to an observed depression in viability in lambs from these deficient dams, compared to sufficient controls. However, in the post mortem examinations carried out in both of the present experiments no significant neuropathological effects could be found in any of the treatment groups.

There are no similar experiments reported in the literature which would support or refute the evidence from Experiments 1 and 2, concerning lamb vigour. However, it is likely that depressed lamb vigour due to sub-clinical Co deficiency in dams would result in the offspring of such sheep having a decreased post-natal viability. This may be related to a reduced energy intake in early life and to an impaired mothering ability in deficient ewes. These parameters were not measured in the experiments reported here. To assess their influence on lamb vigour and subsequent viability, determinations of lamb energy status would need to be made. This could be achieved by continued weighing of the new born lambs at regular intervals and by analysis of lamb plasma samples for glucose concentrations. Behavioural studies of dams post partum, would enable an assessment of mothering ability to be made.

The effect of sub-clinical Co-deficiency in the ewe on the transfer of passive immunity from dam to lamb and consequent lamb viability, will be discussed fully in

Chapter 6. It should be noted here however, that Co repletion of the HS sheep in the second half of pregnancy in Experiment 2, did not significantly improve the vigour of the lambs from this treatment above that evident in the NS group. Further, Co supplementation of the HS ewes in the first half of gestation in Experiment 1 did not ensure post-natal viability in these lambs equivalent to that found in the FS group.

5.4.4 Lamb Serum Vitamin B12 and MMA Status.

The effects of sub-clinical Co deficiency in pregnant ewes on lamb viability, could be related in some degree to the serum vitamin B12 and MMA concentrations of the lambs. The exact relationship was not clear, but this section will attempt to discuss the relevance of the data obtained.

In Experiment 1, lambs from Co-deficient ewes had significantly lower serum B12 levels at 2 and 4 weeks post partum than those from the sufficient sheep (Table 9). In Experiment 2, lambs from the NS and HS ewes at 2 weeks, and those from the NS dams at 4 weeks post partum, had significantly lower concentrations of B12 in serum compared to lambs from the FS treatment (Table 14). Vitamin B12 in all of the lambs would be derived from pre-natal placental transfer and post-natal absorption from colostrum and milk (Quirk and Norton, 1987). That Co supplementation of deficient ewes raises the B12 content

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of their milk, above non-supplemented controls, was established by Hart and Andrews (1959) and O'Halloran and Skerman (1961).

These latter workers also reported that lambs from deficient sheep have lower liver B12 concentrations than those born to sufficient animals. These results were confirmed by the analysis of liver Co content in dead lambs from Experiment 1 (Table 7). These showed that lambs from the NS and HS ewes, which were sub-clinically Co-deficient at lambing, had lower levels of Co in liver than those from the sufficient FS treatment.

In Experiment 2 there was a significant graded treatment effect in lamb serum B12 concentrations in the first month post partum (Table 14). FS group lambs had the highest levels and the NS lambs the lowest, with those in the HS lambs intermediate (P<0.001). These results implied that even though the HS ewes received Co supplementation from mid-pregnancy, stores had not built up to the extent that B12 status of the HS group lambs was equivalent to that of the FS controls. Whether this was caused by less placental transfer of the vitamin, or decreased levels of B12 in the colostrum and milk of the HS group compared to the FS treatment, could not be ascertained from the data collected.

The lamb mean serum vitamin B12 concentrations in all treatments of both Experiments 1 and 2, at 2 and 4 weeks post partum, were below the lower normal limit of 400 ng/l for sheep. Levels in lambs from the NS group at both

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samplings in both experiments, and those from the HS treatment at 2 and 4 weeks of age in Experiment 1 and at 2 weeks *post* *partum* in Experiment 2, were below 200 ng/l. These concentrations would suggest clinical Co deficiency in mature sheep.

Quirk and Norton (1987) also reported low serum B12 levels in lambs from clinically Co-deficient and sufficient ewes. These workers found that serum B12 concentrations in pre-weaned lambs were variable, but were generally below 160 ng/l, regardless of the Co status of the dams.

The significance of these 'low' concentrations of serum B12 in neonatal lambs is not clear. This is basically because the B12 requirements of such 'pre-ruminant' lambs, are not known. It may be that the B12 status evident in the FS lambs was adequate at 2 and 4 weeks after lambing. At this time the vitamin is not needed as an enzyme co-factor in the catabolism of propionate, as this metabolic pathway will not be fully developed until the lamb converts from a milk to a roughage/concentrate ration. During and after the development of a reticulorumen at around weaning time and subsequent establishment of a rumen microflora and production of propionate, the requirement for B12 would increase and become high. However, these lambs had not reached this stage and the serum B12 concentration evident in lambs from FS ewes, although apparently low, may have been

adequate for optimal viability and growth at this stage.

In comparison, the neonatal lamb requirement for B12 as a co-factor to the methyltetrahydrofolate methyltransferase enzyme (Chapter 1, Figure 3.), in the metabolism of homocysteine and folate, may be significant. For this reason, the possibility that the evident differences in lamb serum B12 levels between treatments had an effect on lamb viability, can not be ignored.

Some discrepancy was evident between Experiments 1 and 2 in the lamb serum MMA data at 2 and 4 weeks post partum. In Experiment 1 there were no significant differences in mean serum MMA levels among treatments, while in Experiment 2 lambs from NS ewes had higher concentrations of this metabolite in serum, compared to the HS and FS groups (P<0.001). Further, the mean levels of serum MMA found in these NS treatment lambs at 2 and 4 weeks of age, were 15.0 and 20.3 umol/l, respectively. Such values would be indicative of clinical Co deficiency in mature sheep.

These discrepancies require explanation. It may be postulated that serum MMA in lambs arose solely from absorption of the metabolite from ewe colostrum and milk. It would not be caused by abnormal propionate metabolism (as it is in mature sheep) as this volatile fatty acid would not be produced in the immature rumens of these lambs. In Experiment 1 none of the ewes were showing very high levels of MMA in serum at lambing time.

Consequently, little was present in colostrum and milk and available for absorption by the lambs. However, in Experiment 2 the NS dams had very high concentrations of serum MMA at lambing (up to a mean of 145 umol/l on day 260). Thus, enough was probably present in the colostrum and milk of these ewes to be absorbed by their offspring and appear in lamb serum in significant amounts. In Experiment 2 the NS ewes were the only sheep to show low serum B12 and high MMA concentrations at lambing time. Consequently, the NS lambs were the only ones in this experiment to have significant levels of the metabolite in serum at 2 and 4 weeks post partum.

There is no known role for MMA in such young lambs and the possible consequences of the differences shown in Experiment 2 are therefore not clear. Garton et al (1981) stated that high serum MMA concentrations may be related to failure of Co-deficient ewes to produce viable lambs. However, a greater number of lambs survived to 4 weeks of age in the NS treatment of Experiment 2 (17), than survived in the NS group of Experiment 1 (8). This was despite the NS ewes and lambs of Experiment 2 having much higher serum MMA concentrations than the NS dams and lambs of Experiment 1. These results are not consistent with the statement of Garton et al (1981).

5.4.5 Consequences for Farming Practice.

The results of these two experiments indicated that the reproductive performance of Co-deficient ewes was impaired, relative to Co-sufficient controls. This was evident in reduced gestational and lambing performance in the sheep and depressed post-natal vigour and viability in their lambs. These findings were in agreement with those already reported by Dunlop (1946b), Duncan et al (1981), Garton et al (1981) and Quirk and Norton (1987).

These workers however, with the possible exception of Dunlop (1946b), gained such evidence from ewes of sufficient and clinically Co-deficient status. In contrast, the two experiments described here suggested that similar detrimental effects can occur where only sub-clinical Co deficiency exists. Further, this work also suggested that some depression in reproductive performance would be evident if ewes were sub-clinically deficient for only the first or second half of pregnancy. Thus, in terms of prevention, full protection from these adverse effects would only be afforded by Co supplementation of deficient ewes before tupping and maintenance of an adequate Co intake throughout pregnancy and lactation.

The increased mortality and morbidity of the lambs from Co-deficient ewes demonstrated in these two experiments, may have been associated with the housing of these animals. The pathogenic challenge which the sheep of both investigations experienced, would not have been the same

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as those present in the field situation. Therefore, it may be difficult to relate the findings of these investigations and those of Duncan et al (1981) and Garton et al (1981), directly to practical farming circumstances. However, that Co-deficient ewes have reduced fertility and gestational performance, with depressed lamb viability, compared to sufficient controls, has been established in the field situation by Quirk and Norton (1987).

On this basis, the findings reported here have implications for lamb viability on the farm. If the lambing of sub-clinically Co-deficient ewes takes place on the open hill or upland pastures, it may be expected that lambs which took much longer to suckle and thus gain energy, have less chance of survival than those from sufficient dams which get to the teat more quickly. This would be particularly relevant during inclement weather conditions where lambs from deficient ewes which are slow to rise and suckle, would be more susceptible to loss by hypothermia. This is especially relevant in countries such as Scotland, where lambing conditions can be harsh and Co deficiency is a particular problem of upland and hill type farms.

How apparent the effects of sub-clinical Co deficiency might be in the field situation, would depend on the lambing regime of individual farms. If lambing takes place on the open hill or upland, or is difficult to

monitor closely, the effect may be recorded as large numbers of stillbirths and low lambing rates, with a number of barren ewes. The data from these experiments suggests that in reality some of these lambs would have been born alive, but were not sufficiently vigorous to survive. If this is normal for a farm, suspicions that improvememts could be made would not be aroused. Where lambing takes place on more closely supervised in-by pastures, the effect would be manifest by a number of barren ewes and stillbirths with a great need for assisting lambs to the teat.

5.4.6 Future Work.

The detrimental effects of sub-clinical Co deficiency in pregnant ewes on reproductive performance are evident from the results presented. However, many of the exact causes are still not known. For example, further work is required to ascertain why Co-deficient ewes fail to carry lambs to full-term and produce non-viable offspring. Increased rates of reabsorption, abortion and stillbirths may be due to abnormal hormonal balance during gestation and parturition (Mgongo et al, 1985). The observed depression in lamb vigour may be a consequence of subtle neurological and hormonal changes, poor mothering ability, or low serum B12 concentrations. These aspects require further investigation.

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CHAPTER 6.

THE EFFECT OF COBALT DEFICIENCY IN THE PREGNANT EWE ON NEUTROPHIL FUNCTION AND THE TRANSFER OF PASSIVE IMMUNITY.

6.1 INTRODUCTION.

Evidence of the detrimental effects of Se and Cu trace element deficiencies on immunocompetent cell function in steers, was reported by Boyne and Arthur (1979 and 1981). MacPherson et al (1987) have also shown that Co deficiency in steers results in a reduction in the ability of isolated neutrophils to kill the yeast organism Candida albicans in vitro. Kaplan and Basford (1976) established that hexose monophosphate shunt (HMPS) pathway activity was depressed in leucocytes isolated from vitamin B12 deficient patients. However, there is no evidence in the literature to suggest that Co/B12 deficiency in sheep might have similar effects.

The transfer of passive immunity from ewe to lamb in the first 12 to 24 hours after parturition is a complex process, which is influenced by many factors (Halliday, 1965). It involves the absorption of immunoglobulin (Ig) by the neonate lamb, from ingested colostrum. The neonatal lamb is incapable of synthesising Ig's de novo and the acquisition of maternal Ig's in this passive manner is essential to the ability to resist infection and survive in early life (Campbell, 1974). However, there is no evidence in the literature to suggest that Co deficiency in pregnant ewes might have any adverse effect on the transfer of passive immunity from dam to lamb.

The ability of the pregnant ewe to resist pathogenic infection and effectively to transfer passive immunity in the first hours post partum, are essential to the

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viability of pre- and post-natal lambs. The results presented in Chapter 5 suggested that lambs from sub-clinically Co-deficient ewes had depressed viability before and after lambing. In some cases this was shown at post mortem to be associated with pathogenic bacterial infections.

The objectives of this study were to investigate the effects of Co deficiency in pregnant ewes, on cellular immune function and the transfer of passive immunity to newborn lambs. One aspect of immune function in pregnant sheep was determined, by measurement of the ability of isolated neutrophils to phagocytose and kill C. albicans cells in vitro. The transfer of passive immunity was monitored by the estimation of IgG concentrations in ewe colostrum and lamb serum samples and by the zinc sulphate turbidity test.

6.2 MATERIALS AND METHODS.

6.2.1 Animals and Treatments.

The ewes and lambs employed in the two investigations to be described, were the same as those already detailed in Chapter 3 and Chapter 5. Thus, Experiments 1 and 2 discussed in this chapter, refer to the same sheep and experiments of these previous chapters. Observations on immune competence could be directly related to Co/B12 status in the animals and other effects of the deficiency disease.

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The treatments and diets imposed on the animals in both investigations were described in Chapter 3 and Chapter 5. The blood sampling regimes for the ewes and lambs and lambing routines of both Experiments 1 and 2, were also detailed in these chapters.

6.2.2 Neutrophil Function.

In both experiments, 24 ewes comprising 8 from each treatment were monitored for neutrophil function by the neutrophil function test (NFT, Chapter 2, section 2.9). These 8 sheep from each group were randomly selected and split into two groups of 4. Thus, at each fortnightly sampling blood from 12 ewes (4 from each treatment) was employed for the NFT. The two groups of 12 were tested alternately at samplings throughout the experiments.

6.2.3 The Transfer of Passive Immunity.

The lambs of both experiments were bled at 2 and 4 weeks of age. In the first experiment, only those lambs whose mothers were kept inside after parturition were used in this investigation. In the second experiment, all ewes and lambs were kept on trial post partum and thus all available lambs were employed in this study.

To determine the level of passively acquired immunity obtained by the lambs, serum samples were analysed for IgG concentration (Chapter 2, section 2.10) and by the zinc sulphate turbidity test test (ZST, Chapter 2, section 2.11).

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Where lambings were attended in both experiments, samples of colostrum were taken from the ewes before suckling commenced. Approximately 10 ml of colostrum from each teat were taken and mixed together, to form the sample from a particular ewe. Whey was extracted from these pre-suckling colostrum samples and analysed for IgG content.

In both experiments, prophylactic dosing of new born lambs with anti-biotics was not undertaken. However, where ill-health was apparent the lamb(s) was treated with intramuscular injections of terramycin (Pfizer, Pfizer Central Research, Kent) and the incident recorded. The occurrence of ill-health and neonatal mortalities in the lambs of Experiments 1 and 2, could be compared and related to IgG and ZST values measured in these animals.

6.2.4 Statistical Analysis.

In both experiments, one way analysis of variance was used to test for significant differences in mean per cent (%) kill of C. albicans among treatments at each fortnightly sampling. This statistical test was also employed to analyse differences among treatments for mean colostrum IgG content, and lamb serum ZST values and IgG concentrations at 2 and 4 weeks post partum.

A regression analysis of treatment mean lamb serum ZST values on IgG levels was also performed.

6.3 RESULTS AND DESCRIPTION OF TRENDS IN THE DATA.

The results will be presented along with a description of the data. This will be derived directly from, and will contain no interpretation of, the results.

6.3.1 Neutrophil Function.

The mean % kill of C. albicans by isolated neutrophils from the ewes of each treatment, for Experiments 1 and 2, are presented in Figures 1 and 2 and Appendices 17 and 18, respectively.

6.3.1.1 Experiment 1.

The mean candidacidal activity of neutrophils in all three treatments was within the range 35 to 54 % kill, with no significant differences among groups, until day 70 (Figure 1). At this point, activity in the NS ewes (28.75 % kill) was significantly below that of the two Co supplemented treatments (FS = 36.75 %, HS = 36.25 %, P<0.01).

This first significant difference in the NFT occurred 43 days after mean serum vitamin B12 concentrations in the NS sheep (Chapter 3, Figure 1) had fallen to the region of the lower normal limit (LNL). On day 70, serum B12 levels in the NS treatment were lower than those in the HS and FS groups (P<0.001). At this stage, the mean serum MMA level in the NS animals was still below the upper normal limit (UNL) and not significantly different from those in the HS and FS ewes (Chapter 3, Figure 2).

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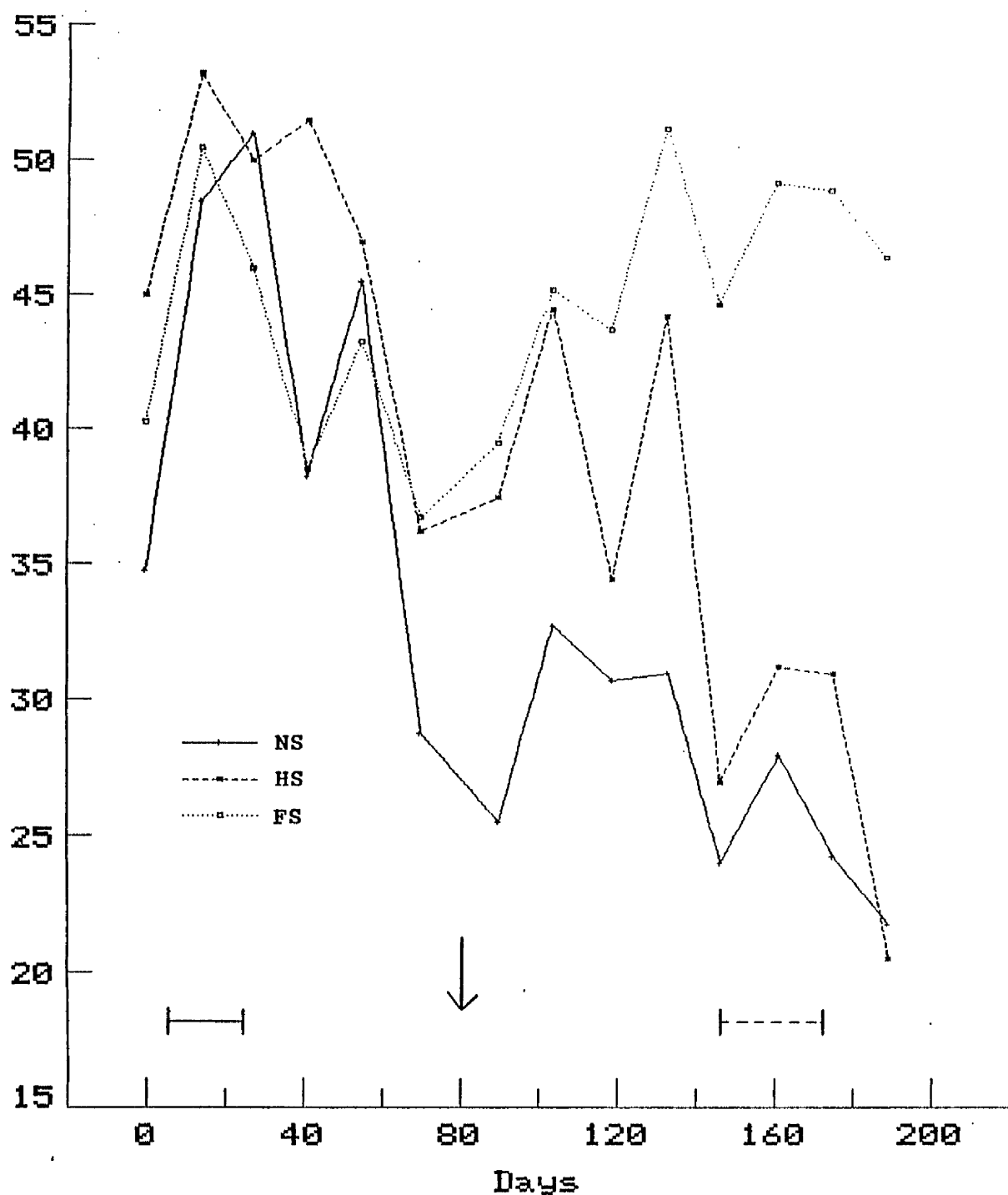
Figure 1. Candidacidal activity of neutrophils isolated from the ewes of Experiment 1, measured by the NFT. (├────┤ = Tupping period, ├───┤ = Lambing period, ↓ = Removal of the weekly oral Co supplement from the HS sheep).

% Kill (of C. albicans)
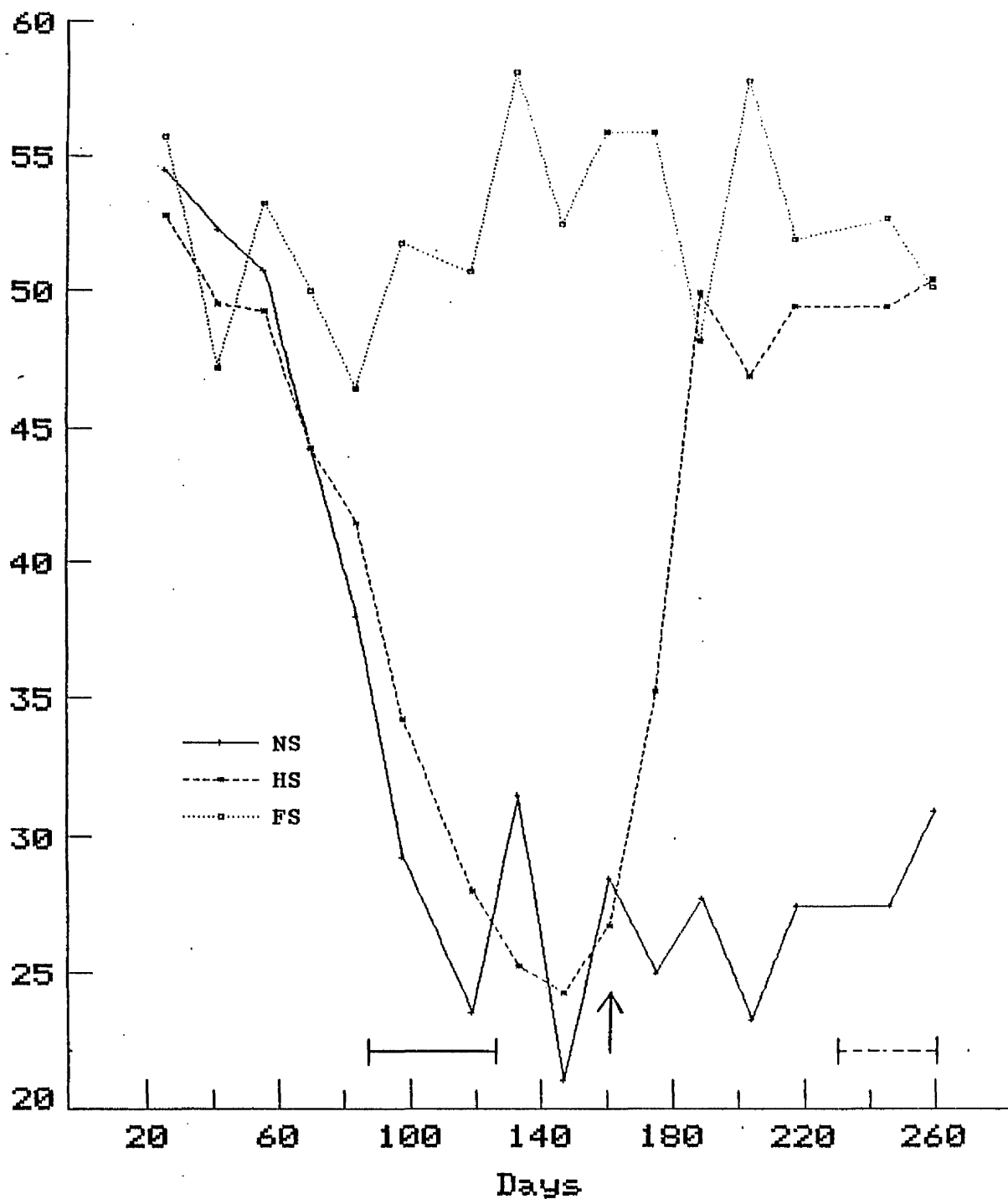
Figure 2. Candidacidal activity of neutrophils from the ewes of Experiment 2, measured by the NFT. (├────┤ = Tupping period, ├----┤ = Lambing period, ↑ = Start of weekly oral Co supplementation of the HS sheep).

The HS ewes were placed on a Co-deficient intake from day 82 onward. The % kill of C. albicans by neutrophils from these animals fell to be significantly lower than the Co-sufficient FS sheep on day 133 (P<0.001), but were still above the NS group (P<0.001). On day 146, candidacidal activity in the HS treatment had fallen to be equivalent to and not significanlty different from that shown by the NS sheep (27 vs 24 % kill, respectively).

This drop in candiacidal activity in the HS ewes to the level shown by the NS sheep, occurred 42 days after the mean serum vitamin B12 concentrations in the HS group had fallen to the region of the LNL (Chapter 3, Figure 1). On day 146, serum B12 levels in the HS treatment were lower than those in the FS group (P<0.001), but were not significantly different from the NS sheep. At this stage, the mean serum MMA concentration in the HS animals was below the UNL of 5.0 umol/l and not significantly different from the FS sheep (Chapter 3, Figure 2).

Once candidacidal activity of neutrophils from either the NS or HS treatments had fallen significantly below that shown by the Co-sufficient animals, the % kills remained low and showed a tendency to fall even further. By the end of the experiment on day 189, the values for these two treatments were 21.75 and 20.5 % kill, respectively. This was less than half that of the FS ewes (46.5 %). Mean % kill by neutrophils from the FS animals remained high throughout the experiment, within

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the range 36.75 to 51.25 %.

6.3.1.2 Experiment 2.

From the start of this experiment, the NS and HS treatments were both on a Co-deficient intake. Neutrophil dysfunction was initially evident in these two groups on day 70, with candidacidal activity falling below that of the Co-sufficient FS ewes (Figure 2). These differences first became significant at day 98 ($P < 0.01$), when the mean % kill shown by neutrophils in the NS, HS and FS treatments was 29.25, 34.25 and 51.75 %, respectively. This first significant difference in neutrophil activity, occurred 28 days after the mean serum vitamin B12 concentrations in the two deficient groups had fallen to the region of the LNL (Chapter 3, Figure 3). At day 98, serum B12 levels in the NS and HS ewes were significantly lower than those of the FS animals ($P < 0.001$). At this stage, mean serum MMA concentrations in the NS and HS treatments were below the UNL, but were significantly higher than those in FS group ($P < 0.01$).

Candidacidal activity in neutrophils from the NS ewes continued to fall to a basal level, within the range 21 to 31 % kill. As in Experiment 1, this was half that shown by the FS ewes ($P < 0.001$), whose mean % kill remained high and between 46.5 and 58.25 % throughout Experiment 2.

The % kill of C. albicans by neutrophils from the HS

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treatment fell to a low of 24.25 % at day 146. These ewes were repleted with Co from day 161. In consequence, candidacidal activity in this group rose significantly above the NS ewes (P<0.05), but still below the FS animals (P<0.01) on day 175. When sampled on day 189, % kill by HS sheep neutrophils had risen further to the 'normal' range of the FS ewes and still significantly above that of the NS treatment (P<0.001).

This return to normal neutrophil function in the HS treatment occurred 28 days after the start of repletion. However, the mean serum vitamin B12 concentrations in these ewes did not rise above the LNL until day 204; 43 days after the start of repletion.

6.3.2 The Transfer of Passive Immunity.

6.3.2.1 Colostrum IgG concentrations.

In Experiments 1 and 2, the mean concentrations of IgG in pre-suckled colostrum samples from all the ewes were 12,310 ± 562 (n = 21) and 11,381 ± 489 (n = 32), respectively. There were no significant differences among treatments in either experiment.

6.3.2.2 Lamb serum IgG and ZST data.

The results for mean lamb serum IgG and ZST values at 2 and 4 weeks post partum in Experiments 1 and 2, are presented in Tables 1 and 2, respectively.

In Experiment 1, lambs from HS ewes had significantly less IgG in serum at 2 and 4 weeks post partum, compared

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to those from the NS and FS groups (P<0.05).

Table 1.　　Lamb　mean　serum　IgG　and　ZST　levels –
Experiment 1.

| IgG (mg/100ml) | TREATMENT | | | SIG. | |
	NS	HS	FS	DIF.	SED
2 wks pp	1220^a	757^b	1484^a	*	162
4 wks pp	925^a	657^b	938^a	*	68

ZST (Arbitrary units)

	NS	HS	FS		
2 wks pp	11.8^a	9.1^a	12.7^a	NS	2.5
4 wks pp	8.7^a	9.0^a	12.1^a	NS	2.2

Table 2.　　Lamb　mean　serum　IgG　and　ZST　levels –
Experiment 2.

| IgG (mg/100ml) | TREATMENT | | | SIG. | |
	NS	HS	FS	DIF.	SED
2 wks pp	2470^a	2204^a	3601^b	*	543
4 wks pp	1799^a	1523^a	2908^b	**	401

ZST (Arbitrary units)

	NS	HS	FS		
2 wks pp	15.2^a	14.9^a	20.4^b	*	3.1
4 wks pp	13.7^{ab}	11.4^b	17.1^a	*	2.8

pp = post partum

For data in both Tables 1 and 2, means in rows with
different superscripts differed significantly.

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No significant differences among treatments for lamb ZST test values were evident in this experiment. However, at 2 weeks after lambing the HS group showed mean ZST levels 3.6 units below the FS animals and at 4 weeks, both the NS and HS group lambs had values over 3 units less than the FS treatment.

In Experiment 2, mean lamb serum IgG concentrations and ZST test values for all treatments were generally higher than those found in Experiment 1. Lambs from the NS and HS groups had significantly less IgG in serum, compared to the FS controls, at 2 ($P<0.05$) and 4 ($P<0.01$) weeks post partum.

Mean ZST values in NS and HS lambs were significantly below the FS treatment 2 weeks after lambing ($P<0.05$). At 4 weeks post partum, the mean ZST values of the HS lambs were again significantly less than those of the FS group ($P<0.05$). However, levels in the NS animals at this time, while not significantly above those in the HS lambs, were not significantly different from the FS treatment.

A statistically significant regression was found between ZST values and IgG concentrations in lamb serum at 2 and 4 weeks post partum (Figure 3). The data for this regression were drawn from the treatment group means of both experiments (Tables 1 and 2). The regression equation is presented below:
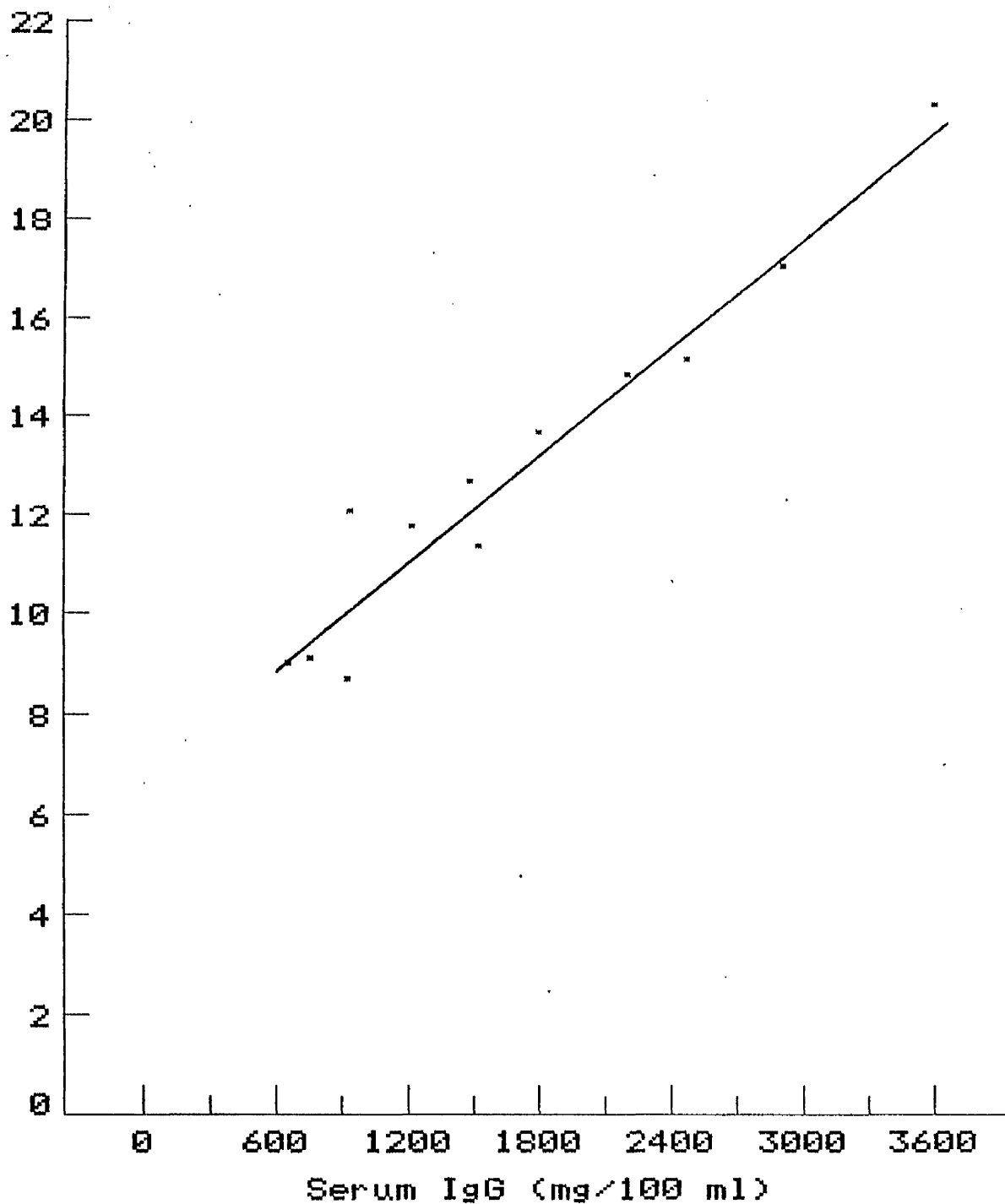
Figure 3. Scatter diagram showing the relationship between values of ZST and concentrations of IgG in lamb serum at 2 and 4 weeks post partum, in Experiments 1 and 2.
$y = 0.0037x + 6.66$ ($r = 0.94$,   RSD = 0.89, $n = 12$, $P < 0.001$).

$$y = 0.0037x + 6.66$$

RSD for variation about y = 0.89

r = 0.94   n = 12   (P<0.001)

Where, y = serum ZST value (arbitrary units) and x = serum IgG concentration (mg/100 ml).

6.3.2.3 ZST values and incidence of lamb mortality and morbidity.

6.3.2.3.1 Experiment 1.

In this experiment, there were 9 neonatal mortalities in the NS treatment and one each in the HS and FS groups. The details of these were presented in Chapter 5, Table 8. Five of the 9 lambs from NS ewes which died within 4 weeks of birth, had post mortem ZST values below the normal threshold of 14 units. One other had an adequate ZST value (>14) and the test could not be performed on the other 3 lambs, due to haemolytic changes.

The ZST values of lambs requiring treatment for ill-health in Experiment 1, are presented in Table 3.

6.3.2.3.2 Experiment 2.

In this experiment, there were no neonatal mortalities in any treatment. One lamb in each group required treatment for ill-health and the ZST values in these animals are presented in Table 4.

Table 3. Incidence of ill-health in lambs from Experiment 1.

TAG	AGE (Days)	REASON FOR TREATMENT AND ZST VALUES
NS GROUP		
89	12 + 16	Scouring. Inadequate ZST (9 and 13).
HS GROUP		
93	8	Scouring. Inadequate ZST (5 and 5).
86	5, 6 + 7	Joint ill. Inadequate ZST (8 and 8).
88	5	Scouring. Inadequate ZST (8 and 11).
90	4	Scouring. Inadequate ZST (11 and 14).

Table 4. Incidence of ill-health in lambs from Experiment 2.

TAG	AGE (Days)	REASON FOR TREATMENT AND ZST VALUES
NS GROUP		
44	40 + 41	Scouring. Inadequate ZST (8 and 11).
HS GROUP		
76	5	Joint ill. Inadequate ZST (13 and 13).
FS GROUP		
70	5	Joint ill. Adequate ZST (15 and 15).

In Tables 3 and 4, the numbers in brackets refer to the ZST value for that lamb at 2 and 4 weeks post partum, respectively.

6.4 DISCUSSION.

The results of this investigation will be discussed in two sections. The first will deal with the depression of neutrophil function in Co-deficient ewes, compared to sufficient controls, which was evident in Experiments 1 and 2. The second will relate the general decrease in passive immunity acquired by lambs from Co-deficient ewes, relative to those from sufficient dams, to post partum lamb viability in both experiments.

6.4.1 Neutrophil Function in Pregnant Ewes.

Before the data on neutrophil candidacidal activity can be related to the ability of pregnant ewes to resist pathogenic infection, the limitations of this determination must be considered. The NFT is a relatively subjective test. Results are dependent on operator observation of isolated neutrophils containing either dead or viable candida cells. Although in both experiments all tests were performed by the same operator, the author has observed that exact percentage results can be operator dependent.

It was also clear from the data presented in Figures 1 and 2 that the candidacidal activity of a particular treatment, receiving a constant Co intake, varies between samplings. For example, the FS group of both investigations showed various levels of % kill over the experimental periods. This was presumably due to subtle changes in test conditions between sampling dates. These

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may have involved slight variations in the numbers of neutrophils and yeast cells used and in the methylene blue stain employed. However, where differences in neutrophil function between Co-sufficient and deficient treatments existed, the statistical significance obtained was large (up to P<0.001).

Additional limitations of the NFT were described by Stinnett (1983), who stated that the technique is a gross determination. PMN's possess a normal 'overkill' of pathogens. Therefore, this worker suggested that differences in microbicidal killing between 'treated' and 'control' groups of less than one half a log, are probably not relevant in terms of host immune resistance. However, the method was stated to be a useful primary screen of cellular immune function.

With these points noted, the results of Experiments 1 and 2 suggested that Co deficiency in pregnant ewes had a depressive effect on the candidacidal activity of circulating neutrophils. However, this could not be related to incidence of disease. The differences obtained between Co treated and untreated groups in these two experiments, did not reach the large proportions discussed by Stinnett (1983). Further, there were no treatment trends in the amount or degree of recorded incidences of pathogenic disease in the ewes during pregnancy. However, MacPherson et al (1976) reported that Co deficiency in sheep can lead to increased

susceptibility to infective disease. In addition, it is likely that a 50 per cent reduction in microbicidal activity due to sub-clinical Co deficiency, which was observed in both Experiments 1 and 2, would have some detrimental effect on the ability of the deficient animals to resist infection.

It is important to note that neutrophils are an essential and integral part of a hosts immune system and ability to resist pathogens. As circulating PMN's they are employed as a major part of the general phagocytic and killing function of the system, along with macrophages and leucocytes, both non-specifically and in conjunction with antigen specific humoral immune factors. Therefore, a reduction in neutrophil microbicidal activity of 50 per cent, would presumably have an adverse affect on host animal immune resistance.

The results presented do not provide evidence of where dysfunction(s) in the neutrophil cell occurs in a Co/vitamin B12 deficient situation. Gershwin, et al (1985) described the NFT as being relatively non-specific, in that a measured abnormality in killing does not indicate where the defect may actually arise. However, Kaplan and Basford (1976) reported a depressive effect of vitamin B12 deficiency on HMPS pathway activity in human leucocytes and this may be a universal effect on higher animal PMN's. The HMPS pathway produces NADPH in the cell, which is oxidised by a flavoprotein oxidase to yield the killing agents superoxide (O_2^-) and hydogen

peroxide (H_2O_2). Thus a reduction in HMPS activity would account for a depression in the ability of PMN cells to kill engulphed microbes. Such a mechanism would also explain the reduction in candidacidal activity of neutrophils from sub-clinically Co-deficient sheep, demonstrated in this study.

In the two experiments described here and in the investigation of Paterson and MacPherson (1987) with calves, the level of Co deficiency necessary to precipitate a detrimental effect on neutrophil function was sub-clinical. Indeed, in both experiments neutrophil function in a treatment group was significantly affected when mean serum B12 concentrations in the animals were in the range 200 to 400 ng/l, and before serum MMA levels had risen above the UNL of 5.0 umol/l. It was argued in Chapter 3 that 'sub-clinical' Co deficiency in pregnant ewes was indicated only when concurrent concentrations of B12 and MMA in serum, were in the ranges 200 to 400 ng/l and 5.0 to 15.0 umol/l, respectively. Therefore, the evidence suggested that neutrophil dysfunction due to Co deficiency could occur early in the development of the condition, even before sub-clinical disease was detectable.

The adverse effect of Co deficiency on neutrophil function was probably mediated directly by low Co/B12 levels. Animals in all treatments were feeding normally with equal energy intakes and normal energy metabolism

when depression in neutrophil candidacidal activity first became evident. As the deficiency was sub-clinical and no differences in liveweight among treatments were found, it can be contended that the effect was direct through a deficit in Co/vitamin B12 supply to the neutrophils, rather than due to depression of energy input or balance which may result from the effect of Co deficiency on propionate metabolism and clinical inappetence.

The effect of Co repletion of the HS ewes in Experiment 2 on neutrophil function, supported this theory. Co supplementation of these sheep resulted in a rapid return to normal candidacidal activity in neutrophils isolated from this group. This evidence was consistent with similar repletion studies in cattle by Paterson and MacPherson (1987) and also with vitamin B12 therapy of pernicious anaemic patients in the work of Kaplan and Basford (1976). Mean % kill by HS sheep neutrophils was equivalent to that in the FS group, only 28 days after the start of Co supplementation. This was the same time period that mean serum MMA concentrations took to return below the UNL in this treatment. Restoration of neutrophil function also occurred before serum B12 levels rose above the LNL in response to repletion. These observations are in accord with the theory that the effect of Co/B12 deficiency on neutrophil function, was direct on the PMN cells. If the effect had been mediated via a lack of energy, caused by vitamin B12 deficiency, then neutrophil function would have been restored only

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after serum B12 concentrations had returned to sufficient levels and after normal energy balance had been attained.

6.4.2 Future Research.

It is evident from this discussion that more work is needed on the intracellular effects of Co/vitamin B12 deficiency on neutrophils and other PMN's. This is necessary if exact mechanisms for the detrimental effects of deficiency are to be obtained. It would be pertinent to concentrate on the HMPS pathway, as this seems a likely site of action for B12. Close investigation of reduced neutrophil function as a result of Co deficiency and its effects on the ability to resist specific pathogenic infections, is also necessary to determine the practical relevance of the data presented.

6.4.3 The Transfer of Passive Immunity.

6.4.3.1 Colostrum IgG concentrations.

In comparison with the literature, the levels of IgG in colostrum in these two experiments appeared to be adequate for the normal transfer of passive immunity from ewe to lamb. The concentrations of IgG in pre-suckled colostrum were higher than those quoted as normal for Scottish Blackface ewes by Halliday (1978a). This worker reported a range of 9000 to 9600 mg/100 ml for this breed. However, the levels found in Experiments 1 and 2 did approximate to the mean of 11,500 mg/100 ml, reported

by Hunter et al (1977) for the Columbia breed of sheep.

Many factors influence the level of Ig's present in ewe colostrum (Halliday, 1978a). In Experiment 1, the ewes were of crossbred origin from Swaledale rams and in Experiment 2, half of the sheep were of similar breeding. These differences may partly account for the range of concentrations in Halliday's (1978a) animals, not coinciding with those evident in these two experiments. Differences in nutrition between investigations also have a profound effect on the Ig levels of pre-suckled ovine colostrum (Halliday, 1978b). Shubber et al (1979b) stated that errors could be made in the interpretation of colostral Ig estimations, if the proportion of whey in the samples was not taken into account. These workers therefore recommended that the results be expressed on a whey content basis. Like the majority of investigations pertaining to the transfer of passive immunity, the proportion of whey in samples of colostrum in Experiments 1 and 2 was not determined.

6.4.3.2 Lamb serum IgG and ZST levels.

To determine the biological and practical significance of the IgG and ZST results in terms of the ability of lambs to resist infection and survive, it is important to compare them with normal values for serum concentrations reported in the literature. Unfortunately, there is no published evidence to suggest what normal levels in 2 and 4 week old lambs might be. However, Halliday (1971a and

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1978a) stated that normal concentrations of IgG in Scottish Blackface lambs at 24 hr. post partum were in the range 1560 to 2500 mg/100ml, depending on litter size. The results from these two experiments did not distinguish between single and twin lambs, as there was no apparent effect of litter size on serum IgG levels at the sampling times employed. This was also reported to be the case by Hunter et al (1977) and Halliday (1971b).

For these reasons it is difficult to make direct comparisons between the study detailed here and 'normal' values quoted by previous workers. It is perhaps logical that differences in passive immune status due to litter size, whilst evident at 24 or 48 hr. after parturition, become non-existent at 2 and 4 weeks of age. Several weeks after birth the absorbed Ig's are well distributed within the body interstitium (Mayhofer, 1984). The situation is further complicated when consideration is given to the fact that the lambs' own active immune system can begin to acquire functional competence at approximately 4 weeks of age.

With these points noted, the levels of serum IgG evident in Experiments 1 and 2 were high compared to those quoted in the literature. Normally, Ig concentrations in lamb serum reach a peak between 12 and 24 hr. post partum when absorption begins to close down, and fall consistently thereafter (Shubber et al, 1979a). The apparent high levels in these investigations at 2 and

4 weeks after parturition could have been due to differences in the analytical techniques employed between these experiments and those of other workers. Differences in experimental diets may also have caused this discrepancy. The fact that the animals were housed in Experiments 1 and 2, and therefore not at pasture, may also have had an influence. More contact between ewe and lamb may occur in the indoor situation with more frequent suckling activity and greater acquisition of Ig's, compared to outdoor field experiments such as that conducted by Halliday (1978a).

The onset of competence in the lambs' own immune system did not appear to influence the 'high' serum ZST and IgG levels evident in the lambs of Experiments 1 and 2. Serum IgG concentrations and ZST levels in these neonates fell between 2 and 4 weeks of age. This was presumably due to interstitium distribution and utilisation of the passively acquired IgG. If the lambs' own immune function had been significant at 4 weeks post partum, then an increase rather than a decrease in concentrations between 2 and 4 weeks might have been expected.

The normal range for lamb serum ZST test levels from 2 days of age, is generally accepted to be any value above 14 units. By this criterion, all mean levels for serum ZST in all treatments were low in Experiment 1. The reasons for this are not clear, especially as the concentrations of IgG in pre-suckled colostrum samples in this experiment were adequate. In Experiment 2, the mean

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serum ZST levels for all treatments at 2 weeks after lambing and for lambs from FS ewes at 4 weeks post partum, were above 14 units. Mean values for the NS and HS group lambs 4 weeks after parturition, were less than 14 units. These increased values in the second investigation coincided with higher IgG concentrations in Experiment 2, compared with Experiment 1.

. In order to try and ascertain a normal concentration for lamb serum IgG, a regression analysis of mean lamb serum ZST levels on mean IgG values was calculated for all treatment groups in both experiments (Figure 3). If the 'threshold' ZST value of 14 is imposed on the regression equation, the corresponding IgG concentration was 1973 mg/100ml serum. Mean serum IgG concentrations in all the groups of Experiment 1 were below this level. In Experiment 2, all treatment mean IgG values were above 1973 mg/100 ml serum at two weeks post partum. Those in the NS and HS groups were below this value, 4 weeks after lambing.

The question arises as to whether these two values can be used as indicators of the adequate transfer of passive immunity, under the experimental conditions employed? If this were the case, then lambs with serum levels below 14 units for the ZST test and 1973 mg/100 ml serum for IgG concentration, would be more susceptible to infection than those with levels greater than these.

The answer to this question lies in comparing evidence

in the literature with the lambing records and post mortem findings from these two experiments. Campbell (1974) reported that lambs receiving little or no colostrum and thus presumably with low levels of passively acquired Ig's in serum, were more likely to die after a measured E. coli infection than neonates receiving a normal colostrum intake. In a field investigation, Harker (1974) reported that neonatal mortalities occurred in lambs with a mean ZST value of 5.7. Survivors had mean ZST levels far above 14 units. A cause and effect relationship between low neonatal lamb serum Ig concentrations and ZST values and increased susceptibility to infection and death, is therefore established in the literature.

There were significantly more neonatal mortalities in lambs from the Co-deficient NS ewes in Experiment 1 (P<0.05), compared to those from either the HS or FS sheep (9 vs 1 vs 1, respectively). In 4 of these 9 cases, there were no significant findings at post mortem. One lamb which possibly died by accidental crushing had an inadequate ZST value. In the 4 other cases, isolated pathogens were implicated in the cause of death and in all 3 serum ZST levels were inadequate (<14 units). The one neonatal mortality in the HS group in Experiment 1, was of a small premature lamb. The death in the FS treatment was due to infection as a result of inadequate iodine dipping of the umbilical cord. This latter lamb was thus weak and died accidentally with a serum ZST

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value of just 1 unit.

In the 5 lambs requiring treatment for ill-health in Experiment 1 (1 from the NS and 4 from the HS groups), all appeared to be due to pathogenic attack. All these lambs had inadequate ZST levels at 2 and 4 weeks post partum. None of the lambs from FS ewes were observed in ill-health.

There were no neonatal mortalities in Experiment 2. This was possibly due to changes in management practice in the preparation of pens for lambing, which were discussed in Chapter 5. Of the 3 lambs needing treatment for ill-health (one in each group), all were for pathogenic infection. Two of these neonates (from NS and HS ewes) had inadequate ZST values. The lamb from the FS group had adequate ZST levels.

The results from both experiments thus indicated a detrimental effect of sub-clinical Co deficiency in pregnant sheep, on the transfer of passive immunity from ewes to lambs. The resultant depression in serum Ig concentrations and ZST levels in lambs from deficient dams, compared to sufficient controls, was evident in both experiments. That these differences resulted in lambs from Co-deficient ewes being more susceptible to pathogenic infection was demonstrated by the increased numbers of neonatal mortalities and incidences of ill-health in these lambs, compared to sufficient controls.

A scenario can thus be composed from the results, which

would explain the influence of Co deficiency on the transfer of passive immunity. In these housed investigations, the lambs from deficient ewes obtained colostral Ig's immediately after parturition, as all lambs were seen to suckle within the first hours of life. However, the levels acquired were not generally sufficient to provide effective and sustained resistance to infection, until such time as the lambs' own immune system gained competence and produced an endogenous Ig supply. The peak in lamb serum Ig concentrations occurs between 12 and 24 hr. post partum. The acquisition of colostral Ig's to this peak must be adequate to sustain resistance over this period, considering that the passively absorbed Ig's will be continually used and degraded. Lambs from Co-sufficient ewes managed to obtain adequate levels of passive resistance to survive until they could produce their own Ig's. Some of those from Co-deficient sheep did not obtain such levels and the pathogenic infections which resulted could have caused the observed increases in morbidity and mortality, possibly complicated by other effects of Co deficiency.

The reasons for a depression in the levels of passively acquired immunity in lambs from Co-deficient ewes, compared to sufficient controls, may be complex. It did not appear to involve treatment differences in the concentration of Ig's in pre-suckled colostrum, as these were not evident. The main cause seemed to result from depressed vigour in lambs from deficient ewes, compared

to sufficient animals (Chapter 5, Table 13). This was the case whether the dams were sub-clinically Co-deficient for just the first half (HS group), or for the whole (NS group) of gestation. This was demonstrated by the observation in Experiment 2 that lambs from NS and HS ewes took significantly longer to stand, find the udder and start suckling, than those from the FS group.

The direct cause of these differences in lamb vigour were not clear. The effect in terms of the acquisition of passive immunity, was probably a general decreased frequency in suckling in lambs from the NS and HS treatments, relative to FS counterparts. Although suckling frequency was not measured, it was clear that lambs from deficient ewes (which took on average twice as long to commence suckling) showed markedly reduced activity in the crucial first hours of life, including suckling behaviour, relative to controls from sufficient dams. Thus, in the vital first 12 to 24 hr. after parturition, lambs from Co-deficient groups probably did not suckle enough to obtain sufficient levels of colostral Ig's, for effective resistance to infection. Consequently, these lambs showed depressed post-natal viability, relative to the FS treatment controls.

6.4.3.3 Future research.

There may be other factors affecting this scenario, such as a possible depression of mothering activity and

maternal encouragement of the lamb to suckle in sub-clinically Co-deficient ewes. These further influences need investigation, along with study of the specific effects of Co/vitamin B12 deficiency on neonatal lambs, if the detailed causes of the depressed lamb viability and acquisition of passive immunity, which were observed in both experiments, are to be understood.

CHAPTER 7.

A SURVEY OF COBALT STATUS IN PREGNANT EWES ON FARMS IN SCOTLAND IN 1988.

7.1 <u>INTRODUCTION.</u>

The results and discussions of the work already reported in this project suggested that Co deficiency could have a profound detrimental effect on the pregnant ewe. The evidence established that sub-clinical Co deficiency could lead to depressed pre- and post-natal lamb viability, relative to sheep that were sufficient in the trace element. These effects were recorded in ewes which were deficient for either the first or the second half of gestation, but were most marked when a Co-deficient status was maintained for the whole of pregnancy.

The problems associated with the prognosis and diagnosis of Co deficiency in pregnant sheep were discussed in Chapter 3 and Chapter 4. The data presented suggested that the most accurate and precise means of diagnosing the syndrome, particularly the sub-clinical form of the disease, was by the determination of serum MMA and B12 concentrations. However, it was found that close consideration should be given to the analytical techniques and diagnostic criteria employed, and to the time of sampling in relation to supplementary feeding.

Although this diagnostic approach may provide the most accurate and earliest indication of Co deficiency in pregnant ewes, the main method of diagnosis used in practice is still the analysis of serum for vitamin B12 levels only (Millar and Penrose, 1980, MacPherson, 1982 and Taylor and Greer, 1982).

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The adverse effects of Co deficiency on sheep farming have long been recognised in Scotland (Hogg, 1831). Previous surveys and reports of Co deficiency, particularly on hill and upland farms, have shown that the disease can occur throughout Scotland (Corner, 1939, Stewart et al, 1941 and 1942, Dunlop, 1946a and Boddie, 1947). The occurrence and severity of the syndrome have been observed to vary from season to season, according to climatic conditions and grass growth (Stewart et al, 1946 and Andrews, Stephenson, Anderson and Faithful, 1958, MacPherson, Voss and Dixon, 1978, MacPherson, 1982 and McDonald and Suttle, 1983).

Co deficiency in pregnant ewes can be alleviated by the administration of intraruminal slow release Co bullets (Hart and Andrews, 1959 and O'Halloran and Skerman, 1961). This form of treatment has also proved successful for other classes of sheep (Connolly and Poole, 1967, Poole and Connolly, 1967 and Whitelaw and Russel, 1979).

In view of the harmful effects of sub-clinical Co deficiency, the problems with diagnosis and the history of the disease in Scotland, the objectives of this survey were to assess the distribution and severity of the syndrome on a sample of farms throughout Scotland. The results were compared and discussed in terms of the conclusions already made from the data of this project. The use of an isolated sampling and analysis of one parameter, namely serum vitamin B12, to diagnose Co

deficiency in field situations, was also assessed.

On one farm, a study was made of the effect of supplementation by Co bullets, given to ewes before tupping and at mid-pregnancy, on the serum B12 concentrations of ewes and lambs.

7.2 MATERIALS AND METHODS.

7.2.1 General Survey.

Fifteen farms throughout Scotland (designated the letters A to O) were chosen for the survey. Each had a proportion of hill and/or upland pastures within their land. All the farms were in areas notorious for the syndrome, or had a previous history of Co deficiency, or low serum B12 concentrations had been evident in sheep from the farm in the past.

All the farms were visited between 25/01/88 and 17/02/88. This time period corresponded approximately to mid-pregnancy in the ewes, as tupping had begun on most of the farms on the traditional date of 12th November in the previous year. Between 10 and 20 randomly picked pregnant sheep were sampled on each farm. The recent grazing and supplementary feeding history of the sheep was obtained from the farmers. Serum vitamin B12 concentrations were determined in all samples by the microbiological assay, using Lactobacillus leichmannii as the test organism.

7.2.2 Experiment at Farm O.

One farm (Calla, Carnwarth, Lanarkshire - Farm'O') was visited on various occasions during the 1987/88 breeding season. On 17/10/87 (day zero) 60 ewes were randomly selected from the breeding flock on the hill. On this date, before the start of tupping, 20 of the sheep ('CB') were tagged and administered with an intraruminal cobalt oxide bullet ('S Permaco', Tasman Vaccine Laboratories [UK] Ltd., Bury St, Edmonds, Suffolk, England - active ingredient 30 per cent). 20 others ('MPCB') were tagged, but did not receive a Co bullet until mid-pregnancy. A further 20 ('NCB') were also tagged, but did not receive Co supplementation at any time during gestation or lactation. Blood samples were taken from all 60 ewes on this date and on 17/12/87 (day 41).

At approximately mid-pregnancy (13/02/88 - day 109) all 60 sheep were again brought down off the hill and blood sampled. The MPCB ewes each received a Co bullet on this occasion.

Approximately 8 weeks before lambing, the ewes were taken off the hill and confined on in-bye pastures. All were fed daily on a supplementary commercial concentrate mix. On 15/06/88 (day 230) a random selection of the ewes and 12 lambs from each treatment were blood sampled and the farmer was asked for his opinion of the lambing performance in each group. At this final sampling date, the lambs were approximately 6 weeks old.

At each sampling, blood was analysed for serum vitamin

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B12 concentration, by the microbiological assay.

7.3 <u>RESULTS.</u>

7.3.1 <u>General Survey.</u>

The results of the survey are presented in Table 1. On farms A, D, E, H, J, K, N and O (* in Table 1) no supplementary feed had been given to the ewes at grass before they were sampled. On farms L and M (# in Table 1) the history of supplementary feeding was unknown. On farm B the ewes had grazed arable stubble for three weeks before the sampling. On farm C the ewes were fed molasses for two days prior to the sampling. On farm F the ewes had been fed molasses and supplementary hay. On farm G the ewes were housed and fed silage for 10 days prior to the sampling. On farm I the ewes were given an oral dose of cobalt sulphate two weeks before and had free access to silage in the field at the time of sampling. The farms where some form of supplementary feed was provided to the ewes before sampling, are marked in Table 1 by '&.'

Table 1. Serum vitamin B12 concentrations in pregnant ewes on 15 farms in Scotland in 1988.

FARM	NUMBER OF EWES	BREED	MEAN SERUM VIT. B12 (ng/l)	RANGE
*A Grainston, Stirlingshire	16	Mules	>1250	>1250
&B Souchin, Stirlingshire	14	Mules	>1250	>1250
&C Auchinlay, Stirlingshire	15	Scottish Blackface	1185	750 - >1250
*D Langbank, Stirlingshire	15	Scottish Blackface	1203	960 - >1250
*E U.Auchinlay, Stirlingshire	15	Scottish Blackface	795	370 - >1250
&F Braco Castle, Perthshire	15	Mules	1179	770 - >1250
&G Drum, Dumfries and Galloway	15	Mules	1220	800 - >1250
*H Clerkhill, Dumfries and Galloway	15	South Cheviots	1101	500 - >1250
&I Douganhill, Kirkcudbright	15	Scottish Blackface	1237	1050 - >1250
*J Drumbuie, kirkudbright	15	Scottish Blackface	1168	690 - >1250
*K Houdston, Ayrshired	12	Scottish Blackface	>1250	>1250
#L Rougag,	10	Unknown	916	520 - >1250
#M Achnahanaid, Skye	12	Scottish Blackface	>1250	>1250
*N Scorrybreck, Skye	7	Scottish Blackface	>1250	>1250
*O Calla, Lanarkshire	20	Scottish	>1250	>1250

7.3.2 <u>Experiment at Farm O.</u>

The results of the additional analysis and treatments at Farm O, are presented in Table 2 and Figure 1.

Table 2. Mean serum vitamin B12 concentrations (ng/l) in the ewes of Farm O.

DAY OF EXP.	NCB	TREATMENT MPCB	CB	SIG. DIF.	SED
0	845^a	786^a	837^a	NS	76
41	1026^a	1389^b	1767^c	***	170
109	2190^a	2332^a	2117^a	NS	135
230	480^a	696^a	2150^b	***	322

In Table 2, means in rows with different superscripts differed significantly.

On day 230 the range of serum B12 concentrations in the NCB, MPCB and CB group ewes were 260 to 820, 150 to 2500 and 380 to 1840 ng/l, respectively. Mean serum B12 concentrations in lambs from NCB, MPCB, and CB ewes on day 230 were 123, 200 and 392 ng/l, respectively, with no significant differences among treatments.

According to the farmer, there were no differences among treatments in gestational or lambing performance.
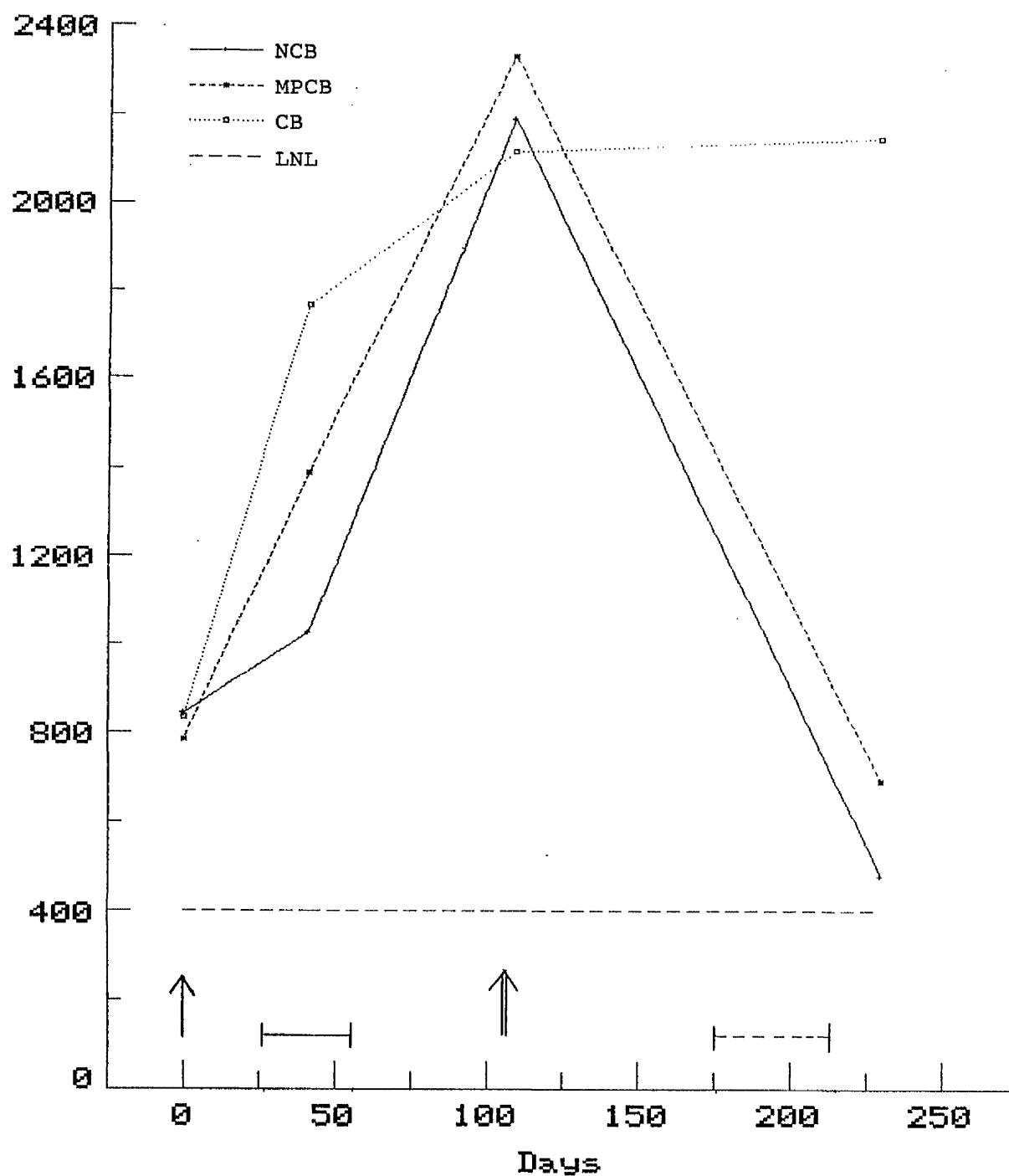
Figure 1. Mean serum vitamin B12 concentrations in the experimental ewes at Farm O. (├────┤ = Tupping period;├────┤ = Lambing period; ↑ = Administration of Co bullets to the CB sheep; ⇑ = Administration of Co bullets to the MPCB sheep).

7.4 <u>DISCUSSION.</u>

The results will be discussed in two sections. Firstly, the data and implications of the survey will be considered. Secondly, the results from the experiment at Farm O will be discussed.

7.4.1 <u>General Survey.</u>

The results of the Co survey were generally negative in terms of the detection of Co deficiency. On all 15 farms the mean serum vitamin B12 concentrations in the ewes sampled, were above the lower normal limit (LNL) of 400 ng/l. A serum B12 level below this threshold was only evident on one farm (Farm E) and this was found in only one sheep (370 ng/l). Thus, the data indicated that the mid-pregnant ewes on these farms were Co-sufficient at the time of sampling.

The negative results of the survey may have been associated with seasonal variations in Co status. All the farms sampled were either in known Co-deficient areas, or had a history of low serum B12 concentrations in sheep. On this occasion the ewes were sampled at the beginning of the year, between grass growing seasons. At this time sward length is low and Co status may rise in sheep due to increased soil ingestion (Andrews <u>et</u> <u>al</u>, 1958 and MacPherson, 1982). During the grass growing season when sward length is higher, soil ingestion tends to diminish and Co status declines in deficient areas. The effect of soil intake on increasing Co status may be

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more marked if the autumn and winter are particularly wet. In this situation the increased rainfall leads to soil contamination of the existing herbage and thus a greater intake of soil by grazing sheep. That soil ingestion can boost the Co status of sheep was reported by MacPherson et al, (1978) and McDonald and Suttle, (1983).

Particularly high rainfalls were experienced in Scotland in the Autumn of 1987, just before this survey was undertaken. Many of the farmers in the survey complained of the extraordinarily wet conditions at this time. Thus, the high Co status of ewes in mid-pregnancy in this survey of farms with histories of Co deficiency, was probably due to seasonal variations and climatic conditions.

An additional reason for the high Co status of ewes on these farms was the provision of supplementary feeding, and on Farm I the sheep were orally dosed with Co shortly before the sampling. It was evident from Table 1 that the ewes on farms where supplementary feed was offered before the sampling, had generally higher serum B12 concentrations than those on farms where such additional feeding had not been provided. Farmers may give hill and upland breeding ewes supplementary feed from approximately mid-pregnancy onward. In areas where Co deficiency can be a problem in some seasons, a dose of Co may also be administered at this time and supplementary

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feed introduced earlier to alleviate any effects which might occur.

The results of the survey only indicated the Co status of the ewes at mid-pregnancy and it has been argued here that the occurrence of Co deficiency can vary according to season and climatic conditions. Further, the data reported in Chapter 3 and Chapter 4 suggested that Co status as reflected by serum B12 and MMA concentrations can change within a matter of hours to weeks under the influence of Co intake. The results of Chapter 3 also provided evidence that post-natal viability of lambs was depressed when ewes had been sub-clinically Co-deficient in either the first or second half of gestation. For these reasons, the data in Table 1 only relate to the ewes at the time of sampling and provide no indication of Co status at other times during pregnancy and lactation. Some ewes may have been Co-deficient earlier in gestation (although this is unlikely) and Co status may have fallen to deficient levels by the end of lambing and/or lactation.

A single sampling of pregnant ewes using only the determination of serum vitamin B12 as a diagnostic marker, does not therefore provide sufficient evidence on which to base advice to farmers. A farmer will wish to know the Co status of a flock and whether or not there is a need for Co supplementation. Where Co status is adequate, the sole analysis of serum for vitamin B12 suffices in indicating the situation. However, where Co

deficiency is evident, then the data of Chapter 3 suggested that the estimation of serum for MMA levels was also necessary to provide confirmatory evidence of the diagnosis. Further, the presence or absence of the syndrome during pregnancy can only be ascertained if Co status is monitored regularly. This may be achieved by sampling a proportion of ewes at tupping, mid-pregnancy, lambing and possibly also during lactation. However, such a sampling regime would be costly and impractical.

A decision must be made on how best to diagnose Co deficiency in pregnant ewes on the farm. The ewes on the farms in this survey could have been Co-deficient prior to tupping and/or in the first half of pregnancy. Such an occurrence would have had detrimental effects on gestational performance and lamb viability, despite Co status being adequate in mid-pregnancy. Such a possiblity could not be determined by a single sampling in mid-pregnancy. However, because of the impracticalities of frequent sampling, diagnoses will continue to be made on the results of blood analysis from single sampling occasions. For these reasons, it may be pertinent for farmers in Co-deficient areas to dose breeding ewes prophylactically with Co bullets before tupping and dispense with a diagnostic sampling during pregnancy. Where sampling before dosing is preferred however, this is best performed before tupping.

7.4.2 Experiment at Farm O.

The Co status of all 60 experimental ewes on Farm O, indicated by serum B12 concentrations, was adequate on day zero when Co bullets were administered to the CB sheep (Table 2). This treatment raised the serum B12 levels of the CB ewes significantly above those in the NCB and MPCB sheep on day 41 (P<0.001).

The serum B12 concentrations in all three groups were raised at mid-pregnancy on day 109 (Table 2 and Figure 1), when the MPCB ewes received Co bullets. During lactation on day 230, the mean serum B12 level of the CB sheep was not different from that shown on day 109. However, mean serum B12 concentrations in the NCB and MPCB ewes had fallen markedly between days 109 and 230 and were not significantly different, although both were significantly lower than the mean level in the CB animals (P<0.001). This fall in serum B12 concentrations in the MPCB ewes occurred despite the administration of a Co bullet to each sheep in the group on day 109. Further, serum B12 levels as low as 260 and 150 ng/l were recorded on day 230 in the NCB and MPCB groups, respectively. These were well below the lower normal limit of 400 ng/l, although mean concentrations for both groups were above this threshold.

Serum vitamin B12 concentrations in all the ewes rose between day zero and day 109 (Figure 1). This result was consistent with the discussion of seasonal and climatic influences on Co status. During this time (mid-October to

mid-Febuary) sward length would have been low and diminishing, with increasing soil ingestion by the sheep. Consequently, the Co status of the ewes improved.

The fall in serum B12 levels in the MPCB group between days 109 and 230 was unexpected. The Co bullet treatment of these sheep on day 109 should have provided adequate Co to give a high serum B12 status over this time period. Indeed, the Co bullets administered to the CB ewes on day zero provided an adequate Co supply thoughout the experiment and this was reflected by the high serum B12 concentrations in these animals through to day 230.

That Co bullet treatment is effective in raising the Co status of pregnant ewes was reported by Hart and Andrews (1959), O'Halloran and Skerman (1961) and Quirk and Norton (1987).

The unsuccessful use of Co bullets in the MPCB ewes on this occasion, could have been caused by two reasons. Firstly, the treatment may have been successful but was administered too late in pregnancy to provide adequate Co and B12 stores for the increased requirements of gestation and lactation. That pregnancy and lactation markedly increase the requirement of ewes for B12 and thus Co, was reported by Quirk and Norton (1987). However, this reason is unlikely as the Co bullets administered to the CB ewes 109 days before those given to the MPCB sheep, provided enough Co for pregnancy and lactation, as mean serum B12 concentration in the CB

group remained high through to day 230.

The second explanation may have involved regurgitation of the bullets by the MPCB ewes, or the coating of the bullet surface with calcium phosphate. The bullets given to the MPCB sheep were the same as those provided to the CB group and they were administered by the same operator and balling-gun. As treatment of the CB ewes was successful, it is unlikely that regurgitation by the MPCB sheep was the cause of low serum B12 concentrations on day 230.

Dewey, Lee and Marston (1969) reported that Co bullets in the rumen could be rendered useless, due to surface coating with calcium phosphate. This effect reduces the release of Co from the bullet. This was more likely to occur when sheep were fed concentrates in the diet, but the effect was variable between animals. The MPCB ewes were given Co bullets just before the start of supplementary concentrate feeding of all the sheep on Farm O. Further, there was a wide range of serum B12 levels in this group on day 230 (150 to 2500 ng/l). Thus, it is likely that the overall failure of the Co bullet treatment of the MPCB ewes was due to inconsistent but significant coating of the bullet surface in the rumen.

The fall in serum B12 concentrations in the NCB and MPCB ewes between days 109 and 230, indicated the increased Co requirement and B12 utilisation of these animals during late pregnancy and lactation. Seasonal variations probably also influenced this fall in Co

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status. As the spring grass growing season had commenced by day 230, the increased sward height and decreased soil contamination of the herbage would have contributed to the fall in Co intake and serum B12 levels in these groups.

The mean serum B12 concentration in the NCB ewes on day 230 (480 ng/l) was not below the LNL. However, a number of sheep in this group had serum B12 levels below this threshold. Whether or not these individual ewes were Co-deficient could not be determined, as serum MMA concentrations were not measured. Despite this observation, these low values and the fluctuations in serum B12 levels overall during the experiment (Figure 1), demonstrated the need for monitoring Co status throughout pregnancy and lactation, rather than drawing conclusions from a single sampling. This was also concluded from the results of the general survey.

The effect of these low serum B12 concentrations in the NCB group on lamb growth could not be ascertained, as lamb growth rates were not measured. However, it was evident that the serum B12 levels before tupping and at mid-pregnancy gave no indication of the fall to low concentrations which were observed during lactation. Thus, constructive and accurate advice could only be given to the farmer if Co status was monitored throughout the breeding season.

The serum B12 levels in the lambs from all three groups

were analogous to those found in the housed experiments described in Chapter 5, and those reported by Quirk and Norton (1987). These investigations reported concentrations of between 50 to 400 ng/l in lambs from Co-sufficient and deficient ewes. On Farm O, serum B12 concentrations in lambs from Co treated (CB) ewes were higher than those in lambs from non-supplemented (NCB) dams and the MPCB group. However, all mean values were below the LNL of 400 ng/l.

It is important to note the 'low' lamb serum B12 concentrations recorded in this field situation. Most of the ewes in all three treatments were Co-sufficient throughout pregnancy and up to the point of sampling of the lambs at approximately 6 weeks of age. In Experiments 1 and 2 of Chapter 5, similar serum B12 levels were found in 2 and 4 week old lambs from Co-deficient and sufficient ewes. It thus seems that the occurrence of serum B12 concentrations below 400 ng/l is normal in pre-weaned lambs from Co-sufficient mothers.

From the results of this field experiment and the experiments described in Chapter 5, the normal range of serum B12 concentrations in neonate lambs would appear to be in the region of 200 to 400 ng/l. However, as evident in the investigations of Chapter 5 and the work of Quirk and Norton (1987), the direct effects of serum B12 levels below 200 ng/l in pre-weaned lambs from Co-deficient ewes are unclear and this aspect requires further study.

CHAPTER 8.

CONCLUSIONS.

8.1 THE DIAGNOSIS OF COBALT DEFICIENCY IN THE PREGNANT EWE.

The results detailed in Chapters 3 and 4 suggested that serum MMA is a more accurate and precise diagnostic marker of Co deficiency, in the pregnant ewe, than serum B12. This is particularly the case for the sub-clinical form of the disease.

Serum MMA concentrations are less variable and relate more directly to Co/vitamin B12 deficiency in the liver than serum B12 levels. Analysis of serum for B12 may yield false positive diagnoses. In contrast, there is no problem from diagnosing false negatives by the analysis of serum for MMA. However, because of the possibility of very high serum MMA concentrations due to liver damage, this marker can not be recommended for use in the diagnosis of Co deficiency in a flock, on the basis of a small number of samples. For diagnosis in this situation, 10 per cent or at least 30 sheep in a flock should be sampled.

Although serum MMA concentrations provide the most accurate marker of Co deficiency, serum B12 levels are a sensitive indicator of Co intake. The analysis of serum B12 is also cheaper than the determination of serum MMA. Thus, if Co deficiency is to be indicated prognostically as well as diagnostically, then the dual analysis of sera for B12 and MMA content is necessary.

If serum B12 concentrations are used as a diagnostic marker, determination by either the microbiological assay

using L. leichmannii as the test organism, or the RIA (Becton and Dickinson) is adequate. However, care must be taken in the choice of diagnostic criteria which may be applied to data obtained from these techniques. The criteria must be relevant to the method employed, the diet fed and the physiological state of the sheep. For pregnant ewes fed a ration high in concentrates, the range 160 ng B12/l serum to 340 ng/l should be used for the diagnosis of sub-clinical Co deficiency with the RIA. For the same sheep fed a roughouge type diet, the range 200 to 400 ng/l can be employed to indicate sub-clinical disease, regardless of the analytical technique used.

The results of Chapter 4 suggested that the analysis of serum B12 or MMA concentrations in blood samples collected around the time of supplementary feeding, could yield false diagnoses of Co deficiency. Although the small housed experiment with wethers did not indicate the presence of endogenously stimulated diurnal fluctations in serum B12 or MMA levels, marked changes in the concentrations of these two markers were caused by the exogenous stimulation of twice daily feed intake. Thus, where supplementary feed is offered to pregnant ewes, sampling for diagnostic purposes should only be undertaken outwith the time period of one hour before and two hours after the provision of such feeds.

The results of Chapter 7 suggest that the use of serum B12 concentrations alone, determined in blood samples taken at one point during gestation, is inadequate for

diagnosing the Co status of a flock of ewes over the whole of tupping, pregnancy and lactation. For the provision of accurate advice to farmers, the Co status of a flock should be monitored throughout the breeding season, by analysis of serum B12 and MMA levels. However, such an approach is impractical and will not prove cost effective. Thus, diagnostic sampling should be undertaken during the grass growing season previous to tupping. This will ascertain the Co status of a flock when soil contamination of the herbage is low, and when Co status is likely to be at its lowest. Sampling at such a time will determine the likelihood of Co deficiency occurring in the flock during the year and will enable the most accurate and cost effective advice to be given to the farmer.

8.2 THE EFFECTS OF COBALT DEFICIENCY IN THE PREGNANT EWE.

The results of Chapter 5 suggested that sub-clinical Co deficiency in pregnant ewes can lead to a depression in pre- and post-natal lamb viability. Co-deficient ewes have a higher incidence of reabsorptions, abortions and stillbirths, compared to sufficient controls. Lambs from Co-deficient ewes are less vigorous and have a higher incidence of morbidity and mortality, than those from sufficient dams.

The results of Chapter 6 suggested that Sub-clinical Co deficiency also has a detrimental effect on the immune

status of ewes and neonate lambs. Deficient ewes show a 50 per cent reduction in neutrophil microbicidal activity, compared to sufficient controls. Less vigorous lambs from sub-clinically Co-deficient ewes obtain a lower level of passively acquired immunity and vitamin B12, than those from sufficient dams. The effects of low serum B12 concentrations in neonate lambs are not clear. However, the increased morbidity and mortality in lambs from Co-deficient ewes, is associated with increased susceptibility to infection as a result of the depression in the level of passively acquired immunity.

These detrimental effects were evident irrespective of whether the ewes had been sub-clinically Co-deficient for either the first or second halves of pregnancy only. However, they were most marked if the sheep had been deficient for the whole of gestation. Thus, full protection from the adverse effects of Co deficiency would only be afforded if ewes in Co-deficient areas were supplemented during the whole of tupping, pregnancy and lactation.

In the tables of this thesis, '*' denotes a significant difference at P<0.05, '**' at P<0.01 and '***' at P<0.001.

APPENDIX 1.

Mean Serum Vitamin B12 Concentrations (ng/l) in the Ewes of Experiment 1, Analysed by the Microbiological Assay.

DAY OF EXP.	TREATMENT NS	HS	FS	SIG. DIF.	SED
0	613^a	797^a	1001^a	NS	146
14	457^a	609^a	589^a	NS	116
27	369^a	594^a	681^a	NS	102
41	442^a	1158^b	1208^b	***	115
55	375^a	1358^b	1399^b	***	176
70	354^a	843^b	867^b	***	93
90	396^a	765^b	1109^c	***	113
104	227^a	414^b	943^c	***	87
119	275^a	419^b	554^c	***	47
133	145^a	197^a	532^b	***	35
146	258^a	237^a	791^b	***	67
161	348^a	251^a	772^b	**	184
175	212^a	239^a	553^b	**	124
189	149^a	199^a	651^b	***	149

Means in rows with different superscripts differed significantly.

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APPENDIX 2.

Mean Serum MMA Concentrations (umol/l) in the Ewes of Experiment 1.

DAY OF EXP.	TREATMENT NS	HS	FS	SIG. DIF.	SED
0	2.45^a	2.38^a	2.40^a	NS	0.13
14	2.50^a	2.48^a	2.45^a	NS	0.10
27	2.59^a	2.61^a	2.66^a	NS	0.05
41	2.79^a	2.90^a	2.80^a	NS	0.19
55	3.07^a	2.92^a	3.14^a	NS	0.18
70	3.15^a	3.11^a	3.04^a	NS	0.09
90	3.22^a	3.19^a	3.16^a	NS	0.03
104	3.57^a	3.31^b	3.20^c	*	0.01
119	4.77^a	3.79^b	3.03^c	***	0.05
133	5.14^a	3.89^b	3.40^b	***	0.39
146	6.02^a	4.41^b	3.49^b	**	0.59
161	6.24^a	7.60^a	3.54^b	**	0.99
175	7.44^a	9.85^a	3.62^b	*	2.10
189	8.78^a	4.83^a	3.43^b	**	1.71

Means in rows with different superscripts differed significantly.

APPENDIX 3.

Mean Serum Vitamin B12 Concentrations (ng/l) in the Ewes of Experiment 2, Analysed by the Microbiological Assay.

DAY OF EXP.	NS	TREATMENT HS	FS	SIG. DIF.	SED
0	2152^a	2383^a	2329^a	NS	181
14	1545^a	1559^a	1955^a	NS	220
26	1398^a	1449^a	1671^a	NS	208
42	406^a	444^a	517^a	NS	77
56	509^a	436^a	711^b	***	71
70	425^a	401^a	700^b	***	64
84	235^a	228^a	348^b	***	22
98	231^a	234^a	567^b	***	50
119	199^a	210^a	395^b	***	27
133	152^a	220^a	681^b	***	63
147	187^a	206^a	513^b	***	59
161	161^a	165^a	625^b	***	36
175	108^a	308^b	496^c	***	73
189	98^a	329^b	598^c	***	92
204	91^a	498^b	560^b	***	102
218	171^a	481^b	743^c	***	122
232	173^a	512^b	610^b	***	100
246	162^a	718^b	816^b	***	147
260	184^a	730^b	975^b	***	215

Means in rows with different superscripts differed significantly.

Mean Serum Vitamin B12 Concentrations (ng/l) in the Ewes of Experiment 2, Analysed by the RIA.

DAY OF EXP.	NS	TREATMENT HS	FS	SIG. DIF.	SED
0	1550[a]	1773[a]	1860[a]	NS	205
14	1241[a]	1303[a]	1433[a]	NS	145
26	887[a]	710[a]	884[a]	NS	109
42	392[a]	344[a]	397[a]	NS	59
56	389[a]	363[a]	466[a]	NS	48
70	340[a]	344[a]	536[b]	**	57
84	235[a]	178[b]	305[c]	***	26
98	176[a]	192[a]	428[b]	***	35
119	159[a]	193[a]	411[b]	***	29
133	158[a]	186[a]	495[a]	***	41
147	198[a]	172[a]	600[b]	***	51
161	158[a]	169[a]	558[b]	***	34
175	109[a]	261[b]	403[c]	***	55
189	101[a]	294[b]	470[c]	***	65
204	53[a]	437[b]	525[c]	***	76
218	193[a]	408[b]	613[c]	***	89
232	163[a]	465[b]	571[b]	***	104
246	137[a]	675[b]	794[b]	***	150
260	143[a]	600[b]	782[b]	***	161

Means in rows with different superscripts differed significantly.

APPENDIX 5.

Mean Serum MMA Concentrations (umol/l) in the Ewes of Experiment 2.

DAY OF EXP.	NS	TREATMENT HS	FS	SIG. DIF.	SED
0	1.28^a	1.28^a	1.14^a	NS	0.28
14	1.80^a	1.49^a	1.76^a	NS	0.33
26	1.83^a	1.43^a	2.11^a	NS	0.38
42	1.29^a	1.49^a	1.54^a	NS	0.33
55	1.93^a	1.41^a	1.70^a	NS	0.32
70	2.44^a	1.85^a	1.22^a	NS	0.43
84	3.31^a	3.43^a	1.85^a	NS	0.69
98	4.19^a	3.94^a	1.18^b	***	0.85
119	6.28^a	4.80^a	1.24^b	***	1.25
133	5.41^a	5.08^a	1.22^b	***	1.33
146	8.04^a	6.40^a	1.50^b	**	2.00
161	6.56^a	6.15^a	1.54^b	***	1.16
175	16.59^a	5.76^b	2.77^b	***	2.85
189	13.17^a	2.02^b	1.36^b	***	3.34
204	18.68^a	1.99^b	2.34^b	**	6.35
218	11.17^a	2.07^b	1.55^b	***	2.10
232	33.99^a	2.43^b	1.68^b	***	8.00
246	46.06^a	2.63^b	1.83^b	***	9.34
260	145.24^a	3.05^b	2.89^b	***	38.26

Means in rows with different superscripts differed significantly.

APPENDIX 6.

Mean Serum Vitamin B12 Concentrations (ng/l) in the Wethers Throughout the Experimental Period, Sampled Before the Morning Feed.

DAY OF EXP.	TREATMENT +Co	TREATMENT -Co	SIG. DIF.	SED
0	>1250	>1250	NS	--
10	1136	713	*	117
16	>1250	1193	NS	57
23	955	595	NS	199
29	>1250	1190	NS	37
37	1213	1028	NS	142
44	663	343	**	86
50	793	550	NS	138
58	>1250	538	***	100
73	1025	373	**	150
79	530	240	**	63
85	601	320	**	75
94	683	229	*	156
98	495	259	**	69
99	633	304	*	123
100	606	238	*	114
101	861	330	*	163

APPENDIX 7.

Mean Serm MMA Concentrations (umol/l) in the Wethers
Throughout the Experimental Period, Sampled before the
Morning Feed.

DAY OF EXP.	TREATMENT +Co	-Co	SIG. DIF.	SED
0	1.78	2.26	NS	1.50
10	1.03	1.46	NS	0.64
16	2.46	2.82	NS	0.61
23	2.46	2.25	NS	0.67
29	2.61	3.21	NS	0.60
44	2.33	3.65	NS	1.05
50	2.93	2.88	NS	0.71
58	2.14	3.56	NS	0.62
73	1.66	3.66	NS	0.74
79	2.76	6.26	NS	1.93
85	3.18	7.29	NS	2.45
94	2.78	8.70	*	2.24
98	3.47	5.62	NS	1.49
99	3.20	7.84	NS	2.37

APPENDIX 8.

Serum Vitamin B12 Concentrations (ng/l) in the 8 Wethers at each Sampling Time on Day 50.

SAMPLING TIME	1	2	3	4	5	6	7	8
		+Co		ANIMAL NUMBER		−Co		
08:43	770	1050	490	860	400	740	480	580

Feeding between 08:52 and 09:30

SAMPLING TIME	1	2	3	4	5	6	7	8
09:15	780	1050	490	860	440	820	440	600
10:08	670	950	480	830	400	740	400	510
10:56	730	990	530	900	410	750	440	590
12:15	760	1090	530	780	420	750	235	600
13:13	800	940	400	650	480	670	330	430
14:06	620	910	440	690	320	560	360	420
15:06	740	960	500	660	300	610	400	400
16:06	840	1020	560	660	310	620	400	400
16:37	840	1140	600	780	300	610	450	430

Feeding between 16:50 and 17:20

SAMPLING TIME	1	2	3	4	5	6	7	8
17:13	840	1080	600	2000	320	650	1320	430
18:08	760	1070	640	600	430	660	430	380

APPENDIX 9.

Serum MMA Concentrations (umol/l) in the 8 Wethers at each Sampling Time on Day 50.

SAMPLING TIME	1	2 +Co	3	4	ANIMAL NUMBER 5	6 -Co	7	8
08:43	4.02	2.10	3.98	1.62	3.77	2.45	2.29	3.01

Feeding between 08:52 and 09:30

SAMPLING TIME	1	2	3	4	5	6	7	8
09:15	4.08	2.36	3.63	1.84	3.33	2.65	2.82	3.54
10:08	4.45	2.40	4.22	2.92	3.63	3.08	3.09	5.55
10:56	4.49	1.76	4.64	2.34	3.72	3.21	3.54	4.57
12:15	4.46	2.72	3.99	2.28	3.86	2.92	2.86	4.35
13:13	1.74	2.78	4.10	2.47	3.80	3.60	2.91	3.78
14:06	3.52	3.63	3.93	2.29	3.48	3.13	2.81	3.85
15:06	1.90	2.69	4.24	2.40	3.99	3.53	3.31	2.42
16:06	3.59	2.44	4.25	2.52	2.33	3.32	3.22	4.12
16:37	1.52	1.63	1.51	1.02	3.25	2.97	1.39	2.02

Feeding between 16:50 and 17:20

SAMPLING TIME	1	2	3	4	5	6	7	8
17:13	3.84	1.09	1.86	3.08	1.72	1.44	3.10	4.63
18:08	1.47	1.17	4.52	1.08	3.82	1.79	1.32	4.41

APPENDIX 10.

Mean Serum Vitamin B12 (ng/l) and MMA (umol/l) concentrations in the Wethers at each Sampling Time on Day 98.

SAMPLING TIME	VITAMIN B12 +Co	-Co	SIG.	SED	MMA +Co	-Co	SIG.	SED
08:54	495	250	*	69	3.47	5.62	NS	1.49
Feeding between 09:00 and 09:30								
09:45	533	213	*	107	3.88	7.57	NS	3.11
10:50	510	218	**	69	4.14	10.73	NS	2.84
11:50	475	235	*	83	4.01	10.27	NS	2.75
12:50	483	225	*	78	4.00	10.15	NS	3.45
13:50	465	210	**	66	4.05	10.28	NS	2.91
14:50	486	229	*	82	4.30	10.31	NS	2.81
16:15	453	203	**	42	3.56	9.75	*	2.02
Feeding between 16:30 and 16:55								
17:16	418	208	**	52	4.42	11.36	NS	3.00
18:10	415	218	*	62	4.48	11.64	NS	3.61
20:14	400	198	*	69	3.88	10.23	NS	3.14
22:17	463	220	**	63	3.83	9.81	NS	2.85

APPENDIX 11.

Mean Serum Vitamin B12 (ng/l) and MMA (umol/l) Concentrations in the Wethers at each Sampling Time on Day 99.

SAMPLING TIME	VITAMIN B12 +Co	-Co	SIG.	SED	MMA +Co	-Co	SIG.	SED
08:56	633	304	*	123	3.20	7.84	NS	1.94
09:56	649	249	**	83	3.55	8.47	*	1.99
10:59	574	191	**	89	3.76	8.80	NS	2.08
Feeding between 11:03 and 11:40								
12:02	488	156	*	127	4.00	12.02	*	2.44
13:04	445	111	**	55	4.17	10.54	*	2.27
14:05	418	206	NS	87	4.40	10.44	*	2.37
15:07	970	341	*	177	4.42	10.46	*	2.28
16:03	1138	340	**	124	3.82	10.23	*	2.23
17:04	738	355	NS	213	3.82	10.23	*	2.50
18:05	433	166	*	64	3.95	9.60	NS	2.41
Feeding between 18:30 and 18:35								
19:06	289	116	*	58	4.03	10.15	*	2.37
20:11	325	143	*	70	4.12	9.86	NS	2.49
22:16	374	129	**	51	3.91	9.06	NS	2.39

APPENDIX 12.

Mean Liveweight (Kg) and Condition Score of the Ewes in Experiment 1.

DAY OF EXP.	LIVEWEIGHT			CONDITION SCORE		
	NS	HS	FS	NS	HS	FS
0	52.1	51.5	50.2	2.63	2.79	2.69
14	50.7	51.6	50.5	2.64	2.88	2.78
26	52.5	52.5	51.4	2.80	2.97	2.81
41	51.9	52.2	51.6	2.72	2.92	2.84
55	51.3	50.4	50.2	2.76	2.86	2.78
70	51.8	52.1	51.8	2.83	2.87	2.84
90	52.9	51.3	51.8	2.82	2.88	2.98
104	54.5	53.9	55.3	2.98	2.88	3.08
119	59.8	57.1	59.2	2.88	2.76	3.03
133	62.9	60.6	62.8	2.95	2.89	2.89
161	52.3	52.9	54.3	2.38	2.50	2.34
175	54.3	54.3	55.5	2.45	2.58	2.48
189	52.9	55.8	56.4	2.50	2.72	2.72

APPENDIX 13.

The Use of Chi-Squared in the Statistical Analysis of Ewe Fertility and Gestational Performance Data.

Example from Experiment 1 - Lambing per cent to 4 Weeks.

Contingency Table.

| | TREATMENT | | |
	NS	HS	FS
1. No: of ewes with 0 lambs at 4 weeks pp	14	5	5
2. No: of ewes with 1 lamb at 4 weeks pp	5	13	11
3. No: of ewes with 2 lambs at 4 weeks pp	1	2	4

pp = post partum

	NS	HS	FS	ROW TOTAL
1.	14 (8.0)	5 (8.0)	5 (8.0)	24
2.	5 (9.7)	13 (9.7)	11 (9.7)	29
3.	1 (2.3)	2 (2.3)	4 (2.3)	7
COLUMN TOTAL	20	20	20	60

'Expected' values (in brackets) =

$$\frac{\text{Row Total X Column Total}}{\text{Overall Total}}$$

Chi-squared =

$$(\text{The sum of}) \quad \frac{(\text{Observed} - \text{Expected})^2}{\text{Expected}}$$

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Total Chi-squared =    4.50 + 1.12 + 1.12 +
                       2.25 + 1.15 + 0.18 +
                       0.76 + 0.05 + 1.19 +

                    = 12.34   (P<0.05)
```

Degrees of freedom = (3-1) X (3-1) = 4

Critical values of Chi-squared with 4 degrees of freedom at P<0.05, P<0.01 and P<0.001 are 9.49, 13.28 and 18.46, respectively.

APPENDIX 14.

The Use of Chi-Squared in the Statistical Analysis of the Lambing Records.

Example from Experiment 1 - Neonatal Mortalities.

Contingency Table.

| | TREATMENT | | |
	NS	HS	FS
1. No: of lambs living at 4 weeks pp	10	16	20
2. No: of lambs dying before 4 weeks pp	9	1	1

pp = post partum

	NS	HS	FS	ROW TOTAL
1.	10 (15.3)	16 (13.7)	20 (16.9)	46
2.	9 (3.7)	1 (3.3)	1 (4.1)	11
COLUMN TOTAL	19	17	21	57

Total Chi-squared = 1.86 + 0.38 + 0.55 +
7.76 + 1.59 + 2.30

$$= 14.43 \quad (P<0.001)$$

Degrees of freedom = (2-1) X (3-1) = 2

Critical values of Chi-squared with 2 degrees of freedom at $P<0.05$, $P<0.01$ and $P<0.001$ are 5.99, 9.21 and 13.82, respectively.

The Statistical Analysis of Lamb Birthweight Data,
Accounting for different Numbers of Single and Twin
Births in each Treatment.

Example from Experiment 1.

Raw birthweight (Kg) data.

| | TREATMENT | | |
	NS	HS	FS
	3.6,3.8	4.1,4.2	3.3,3.0
	4.1,4.7	3.8,3.4	5.6,4.6
	4.8,3.5	5.5	3.0,5.2
	5.5,4.0	5.5	4.4,3.9
	5.4	5.4	4.5,5.5
	5.8	5.4	5.4
	6.2	5.7	5.6
	5.5	5.3	5.6
	5.3	6.3	5.2
	5.3	6.0	5.4
	3.8	6.0	5.4
	6.8	5.0	5.2
	6.1	2.7	7.0
	5.1	3.8	5.7
	5.9	3.6	5.5
			5.8
No: of recordings	19	17	21
Crude means	5.0	4.8	5.0

Birthweights shown together relate to twins. Those
shown alone relate to single births. The data were
analysed using the EDEX statistical computing package,
devised for animal experimentation by the Animal Research
Council, Edinburgh. Birthweights were analysed by a two
way analysis of variance, with the number of singles and
twins separated as blocks. Variation due to single and
twin births could thus be eliminated from analysis of
variance due to differences in birthweight. The means
were adjusted by the computing package, according to the
number of single and twin births occurring in each
treatment.

Analysis of variance.

VARIABLE	DF	SS	MS	F	PROBABILITY
Blocks	1	21.9882	21.9882	36.038	***
Treatmants	2	3.1118	1.5559	2.550	NS
Blocks/ Treatments	2	0.2219	0.1110	0.182	NS
Error	51	31.1171	0.6101		
Total	56	56.4389			

Coefficient of Variation = 15.81 per cent

Adjusted means

NS = 5.1   HS = 4.6   FS = 5.1   Average SED = 0.26

There was a significant difference in birthweights
between singles and twins.

There were no significant differences among treatments
for birthweight.

There were no significant interactions between single/
twin births and treatments.

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APPENDIX 16.

Mean Liveweight (kg) and Condition Score of the Ewes in Experiment 2.

DAY OF EXP.	LIVEWEIGHT			CONDITION SCORE		
	NS	HS	FS	NS	HS	FS
0	56.2	56.5	56.6	2.48	2.50	2.60
14	57.6	55.7	54.4	2.66	2.79	2.58
26	57.3	56.4	53.3	2.76	2.75	2.54
42	57.1	57.7	54.5	2.80	2.80	2.68
55	58.0	57.4	55.4	2.74	2.79	2.66
70	59.5	58.7	55.7	2.81	2.85	2.74
84	60.3	58.2	55.8	2.99	2.90	2.85
98	58.3	57.0	54.9	2.90	2.81	2.75
119	57.0	55.7	54.9	2.96	2.90	2.86
133	58.7	55.7	56.0	2.86	2.94	2.90
146	59.0	57.6	55.7	2.89	2.91	2.85
161	59.7	58.1	57.5	2.90	2.94	2.94
175	61.1	60.0	58.7	2.89	3.10	2.89
189	60.7	60.2	59.4	2.82	3.03	2.89
204	62.2	62.5	61.2	2.74	2.90	2.84
218	60.4	62.8	60.6	2.73	3.06	2.92
232	65.3	67.9	65.2	3.05	2.75	2.87
246	68.1	69.1	70.7	2.82	3.15	3.21
260	56.5	59.2	59.0	2.48	2.82	2.55

APPENDIX 17.

Mean Per Cent Kill of <u>C. albicans</u> by Neutrophils Isolated
from the Ewes of Experiment 1 at each Sampling Date.

DAY OF EXP.	NS	TREATMENT HS	FS	SIG. DIF.	SED
0	34.75^a	45.00^a	40.25^a	NS	4.91
14	48.50^a	53.25^a	50.50^a	NS	4.06
27	51.00^a	50.00^a	46.00^a	NS	3.86
41	38.25^a	41.50^a	38.50^a	NS	2.14
55	45.50^a	47.00^a	43.25^a	NS	2.63
70	28.75^a	36.25^b	36.75^b	**	3.08
90	25.50^a	37.50^b	39.50^b	***	5.00
104	32.75^a	44.50^b	45.25^b	**	4.91
119	30.75^a	34.50^a	43.75^a	NS	6.38
133	31.00^a	44.25^b	51.25^c	***	6.29
146	24.00^a	27.00^a	44.75^b	***	7.12
161	28.00^a	31.25^b	49.25^c	***	7.03
175	24.25^a	31.00^b	49.00^c	***	7.85
189	21.75^a	20.50^a	46.50^b	***	9.53

Means in rows with different superscripts differed
significantly.

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APPENDIX 18.

Mean Per Cent Kill of <u>C. albicans</u> by Neutrophils Isolated from the Ewes of Experiment 2.

DAY OF EXP.	TREATMENT NS	TREATMENT HS	FS	SIG. DIF.	SED
26	54.50^a	52.75^a	55.75^a	NS	1.83
42	52.25^a	49.50^a	47.25^a	NS	3.37
55	50.75^a	49.25^a	53.25^a	NS	3.87
70	44.25^a	44.25^a	50.00^a	NS	2.39
84	38.00^a	41.50^a	46.50^a	NS	4.17
98	29.25^a	34.25^a	51.75^b	**	4.22
119	23.50^a	28.00^a	50.75^b	***	2.67
133	31.50^a	25.25^b	58.25^c	***	2.59
146	21.00^a	24.25^a	52.50^b	***	3.57
161	28.50^a	26.75^a	56.00^a	***	4.08
175	25.00^a	35.25^b	56.00^c	***	3.91
189	27.75^a	50.00^b	48.25^b	***	3.66
204	23.25^a	47.00^b	58.00^c	***	4.36
218	27.50^a	49.50^b	52.00^b	***	4.41
246	27.50^a	49.50^b	52.75^b	***	2.93
260	31.00^a	50.50^b	50.25^b	***	2.38

Means in rows with different superscripts differed significantly.

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