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# Conditioned hypocuprosis: some effects of diet on copper storage in ruminants



*1968* *Centre for Agricultural Publishing and Documentation*
*Wageningen*

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# Contents

# 1 Introduction

The importance of copper in nutrition was discovered some forty years ago, and much data have since accumulated on its metabolism and mode of action in the organism. Yet much remains unknown about the factors governing its absorption and utilization. That these factors exist, became evident soon after the original discovery of its importance with the demonstration that in certain areas of the world, cattle and sheep grazing on pastures of apparently normal copper content did not thrive and exhibited symptoms of copper deficiency which could be alleviated either by oral or parenteral administration of copper or by removing the animals to other pastures very often of no better copper level.

This led to the conclusion that under natural conditions two types of copper deficiency occur: the simple type induced by deficiency of the element in the fodder and the conditioned or complex type in which copper is present in normal concentrations but factors make it unavailable. Research was undertaken with the aim of defining the nature of these factors.

The Australian finding that molybdenum limits copper storage and that this limitation was greatly dependant on inorganic sulfate in the diet was a great step in this direction, and the Australian workers advanced the hypothesis that molybdenum and sulfate may be the factors behind natural conditioned copper deficiency. While this hypothesis was found to be valid in some areas, it was not in others. For example, a recent report from Australia indicated that molybdenum and sulfate were not always responsible for conditioned copper deficiency. Swayback disease in lambs remains, to the best of our knowledge, a conditioned copper deficiency not due to molybdenum or sulfate. The literature reports similar hypocuprosis affecting grazing livestock in many different parts of the world which could not be caused by deficiency of copper, or by its unavailability through excess of molybdenum and inorganic sulfate. The possibility of other factors which complicate copper metabolism must be considered. The search for these factors limiting copper storage and producing the deficiency syndrome represents one of the most interesting fields of work on copper. Three different approaches are being used to define them:

1. Classical pasture analysis in affected and healthy areas to detect any abnormality or difference in composition.
2. The search for any difference in the compounds in which copper occurs in the plant and the animal after pasturing in normal and in affected pastures.
3. The experimental production of hypocuprosis or the inhibition of its occurrence by manipulating dietary factors other than copper.

My study is intended to provide more information on the factor(s) precipitating conditioned copper deficiency in the Netherlands and reports the effect of urea, ammonium sulfate and iron on the copper storage in ruminants.

# 2 Review of the literature

Shortly after the original discovery of HART *et al.* (1928) that copper in addition to iron is essential for the hemoglobin regeneration in milk-anemic rats, several investigators were able to relate some longknown naturally occurring debilitating diseases in grazing livestock to the deficiency of copper in the herbage. SJOLLEMA (1933a) was the first to show that in areas where the disease 'likzucht' is known, the copper content of the herbage is usually low and sick animals can be cured by giving them copper sulfate. This was followed by the demonstration of a number of diseases in different parts of the world with low copper in blood and liver; animals responded to copper therapy and the copper content of the affected pastures was in most cases subnormal. Outstanding among these diseases are 'enzootic ataxia' or 'swayback' of sheep in Australia and England, respectively (BENNETTS and CHAPMAN, 1937; BENNETTS and BECK, 1942; DUNLOP *et al.*, 1939), falling disease of bovines (BENNETTS *et al.*, 1941, 1942a, b) and the loss of pigment and abnormal keratinization of wool in sheep in Australia (MARSTON, 1946, 1949; MARSTON and LEE, 1948), calf pine in Scotland (JAMIESON and ALLCROFT, 1950), and scouring of cattle in the Netherlands (SJOLLEMA, 1938; BROUWER *et al.*, 1938), New Zealand (CUNNINGHAM, 1944, 1946) and England (ALLCROFT and PARKER, 1949). Similar disorders were reported from many other localities (MCELROY and GLASS, 1950; RUSSELL and DUNCAN, 1956) and it became apparent that copper was involved in a wide range of functions in the body (see ADELSTEIN and VALLEE, 1962; UNDERWOOD, 1962; GALLAGHER, 1964).

Investigations on natural hypocuprosis soon showed that no consistent relation existed between the copper intake and the occurrence of copper deficiency. Thus while all cattle and sheep on copper deficient pastures develop hypocuprosis, also on some pastures with adequate copper the disorder develops. To explain the hypocuprosis which develops with diets apparently normal in copper, it has been often suggested that some factor, or factors, interfere with copper storage either by limiting its absorption, increasing its excretion, or probably by a combination of both.

## 2.1 Symptoms of copper deficiency

Copper is concerned with a variety of metabolic processes and deficiency of this element will, therefore, ultimately lead to failure of these processes. A multitude of symptoms has been related to a dietary deficiency of copper in different species.

They include loss of appetite and condition, slow growth, roughness and discoloration of the coat, shedding of the hair, skeletal changes, anemia, scouring, abnormal keratinization of wool and ataxia through faulty myelination of the central nervous system in sheep, decrease in milk yield and, in severe deficiency, sudden death from heart failure due to myocardial atrophy and replacement fibrosis in cattle (UNDERWOOD, 1962). Reproductive disturbances have been also associated with copper deficiency in cattle (ALLCROFT and PARKER, 1949; SEEKLES, 1960) and in rats (DUTTS and MILLS, 1960).

Of these symptoms, some are specific and some not confined to copper deficiency. They differ not only from one species to another but also within the same species under varying conditions. For instance, the nervous lesions are quite typical for sheep and especially lambs where they frequently appear before birth; loss of pigment and abnormal keratinization of sheep's wool will occur at copper intakes not low enough to cause the appearance of other symptoms. Diarrhea in cattle is more severe when excess molybdenum aggravates copper deficiency.

On deficient diet, liver copper falls until reserves are exhausted. After a period whose length depends on certain factors, the liver becomes unable to maintain copper in blood and the level there falls. The various functions dependent on copper start to compete for the limited supply available and some functions, which compete less, will be impaired before others which continue to obtain the needed copper. This is responsible for the characteristic sequence with which the symptoms appear in the different species when the animal is put on a copper deficient diet. According to VAN DER GRIFT (1955), blood copper in milking cows cannot keep its physiologic level and deficiency symptoms start to appear when liver copper has dropped below 20-25 ppm of dry matter. The level found by MARSTON (1952) for sheep is 20 ppm.

## 2.2 Conditioned copper deficiency and factors interfering with copper metabolism

Practically no other trace element exceeds copper in the diversity of the factors which govern its absorption, excretion and utilization within the organism. Indeed, the story of conditioned copper deficiency in livestock is that of the factors which antagonize its metabolism. During the last three decades much knowledge has been gained about the nature of some of these factors and it is quite certain that other new factors will be found. The mechanism of interference with copper metabolism is not clear but sufficient data from both laboratory and field experience have accumulated to warrant the conclusion that species, copper status and the relative amounts of copper and interacting factors determine the nature and the extent of interaction.

### 2.2.1 Copper, molybdenum and inorganic sulfate

Stimulated by the similarity of the symptoms of a local molybdenosis in cattle in England to those described by BROUWER *et al.* (1938) for copper deficient cattle in the Wieringermeer Polder, FERGUSON *et al.* (1938, 1943) successfully treated scouring cattle with copper. Since then sufficent evidence has accumulated to indicate that molybdenum and copper are biological antagonists. Thus DICK and BULL (1945) demonstrated that excess molybdenum depleted liver copper in sheep. CUNNINGHAM (1950), DAVIS (1950) and COMAR *et al.* (1949) confirmed and extended this finding to other species . BULL (1951) showed that copper retention in sheep liver was actually favoured by diets low in molybdenum. Copper largely prevented the growth-inhibiting effect of molybdenum in a variety of species (COMAR *et al.*, 1949; GRAY and ELLIS, 1950; AARRINGTON and DAVIS, 1953; GRAY and DANIEL, 1954).

Convincing as this may be, some workers were unable to demonstrate copper antagonism for molybdenum or to associate molybdenum with natural conditioned hypocuprosis in livestock (STEWART *et al.*, 1946; GREEN, 1950, 1951; ALLCROFT, 1952; LEWIS and ALLCROFT, 1953). MARSTON (1950, 1952) found that, when copper intake was sufficient, molybdenum supplements to sheep diminished copper reserves, however not to the extent of clinical hypocuprosis even when this diet was continued for two years; while on copper deficient pastures the same treatment significantly decreased the rate of copper depletion from the liver and maintained a significantly higher level of blood copper than in the controls. Despite high blood copper, sheep developed deficiency symptoms, according to MARSTON, through the deficiency of physiologically active copper. He concluded that molybdenum limits copper metabolism in sheep only when it is superimposed on a deficient copper intake. KULWICH *et al.* (1953) confirmed the hypothesis that excess copper not physiologically active may be accumulated on diets with excess molybdenum. They found that molybdenum at the very high level of 1000 ppm increased copper retention especially in the liver and kidney of rat and swine and they were unable to detect any benefit of copper in overcoming the growth depression caused by molybdenum in rats.

All these findings indicated that other factors can modify the effect of molybdenum on copper metabolism and that molybdenum is only one cause among probably many in complicated deficiency. None of the elements examined by CUNNINGHAM (1950), zinc, manganese, tungsten, vanadium, chromium, rhenium, uranium and tantalum, lowered tissue copper. Iron, nickel and, at lower intakes, calcium and zinc were similarly without effect (DICK, 1954b).

The discovery of the influence of inorganic sulfate on the molybdenum-copper interaction is credited to DICK (1953; 1954a; 1954b; 1956a; 1956b; 1956c). His main findings were as follows:

a. Molybdenum alone or sulfate alone do not limit copper storage.

b. At very low sulfate intakes, molybdenum within the range 0.3-100 mg per sheep

per day, has no effect on blood copper.
c. Within the range 30-90 mg molybdenum per day, and at higher sulfate intakes, blood copper rises immediately in proportion to molybdenum and sulfate intakes and slowly after a delayed period at lower intakes.
d. At constant normal molybdenum and sulfate intakes, molybdenum within a certain range will depress copper storage. No further depression will occur with more molybdenum.
e. The amount of molybdenum needed for a certain depression in liver copper will diminish as sulfate intake increases.
f. At very high molybdenum intakes (60-90 mg per day), liver copper is maintained if the diet contains sufficient inorganic sulfate. Wool changes will develop quickly despite normal copper reserves in liver and the higher level in blood.

These apparently conflicting results were explained (DICK, 1956a) by the hypothesis that sulfate interferes with, or at high concentration prevents the passage of molybdenum across membranes. Transport of copper across such membranes will be impeded or prevented, according to molybdenum concentration. DICK (1954b) suggested that high molybdenum and sulfate intakes in sheep decrease the absorption and increase the excretion of copper. Yet no increase in the excretion rate of radioactive copper could be detected in sheep under the same dietary conditions (MILLS, 1961).

The influence of molybdenum and inorganic sulfate on copper metabolism was amply confirmed and extended to other species (WYNNE and MCCLYMONT, 1955, 1956; MYLREA, 1958; VAN DER VEEN and KEENER, 1964). WYNNE and MCCLYMONT (1956) surmised that it is the proportion and not the absolute intake of sulfate which is important. In sheep a diet with only 0.8 ppm molybdenum and 0.4 % sulfate in dry matter significantly reduced copper in blood and liver. MILLS and FELL (1960) and FELL, WILLIAMS and MILLS (1961) induced demyelination in lambs by giving pregnant ewes molybdenum and sulfate.

Despite this, conditioned copper deficiency in many parts of the world could not be explained by pasture values for molybdenum, copper and sulfate. For example in 1949, GREEN (1951) stated that "no relationship has been observed between copper and molybdenum in England where hypocupremia occurs or coincides with molybdenosis, neither is the molybdenum content of the pasture the operative factor".

ALLCROFT and LEWIS (1956a, 1956b, 1957; LEWIS and ALLCROFT, 1960) re-evaluated this statement in the light of the role of inorganic sulfate and arrived at the same conclusion. These workers partially confirmed the results of DICK, but they could not repeat such a decrease in liver copper of pregnant ewes on diets normal in copper and normal to high in molybdenum whatever the sulfate intake. There was even an increase in copper storage. They also produced pasture analysis figures for farms with swayback and normal farms. These were of the order of 14.5 ppm copper, 1.35 ppm molybdenum and 0.65 % sulfate in dry matter of affected pastures. For innocuous pastures the figures were 16.6, 1.44 and 0.52, respectively.

On the basis of similar analysis in places where conditioned copper deprivation occurs in cattle they concluded "...There should therefore be ample sulfate present to allow even small amounts of molybdenum to exert its full limiting effect on copper storage in pastures of normal copper and molybdenum content on both affected and healthy farms", and they suggested that still other factors are involved.

Similarly, sufficient sulfate in pastures has been reported where copper deficiency occurs with or without excess molybdenum in New Zealand (CUNNINGHAM, 1955). HARVEY *et al.* (1961) found that the liver copper in grazing cattle in Queensland decreased throughout the year and that the rate of decrease was greater in these cows than in cattle stall-fed on freshly cut pasture. From copper, molybdenum and sulfate intakes, they concluded that the pasture contains factors other than molybdenum and inorganic sulfate which interfere with copper metabolism in cattle. UNDERWOOD (1962) also pointed to the possibility that with some of the manifestations generally associated with copper deficiency, copper may be only secondarily involved.

Still more puzzling is the difference in response to molybdenum and sulfate in ruminants and in rats. In rats, molybdenum promotes copper storage in liver (MILLER *et al.*, 1956) and the addition of sulfate prevents the increase in liver copper and enhances the growth depression observed in rats on high molybdenum intakes (VAN REEN and WILLIAMS, 1956; MILLER *et al.*, 1956; GRAY and DANIEL, 1964). MILLS (1960) suggested that this species difference could be explained by the more limited ability of rats to reduce sulfate and then only in the cecum and colon. GRAY and DANIEL (1964) report that the copper status of the animal and the proportions of copper, molybdenum and sulfate in diet determine the nature of the interaction. Sulfate enhanced the decrease in liver copper in rats receiving molybdenum only when copper was depleted and the diet was copper deficient. In undepleted rats receiving adequate dietary copper, no hypocuprosis was noticed and sulfate completely prevented all other metabolic disorders resulting from excess molybdenum.

Since rats given much molybdenum have low liver sulfide oxidase activity (MILLS *et al.*, 1958), some workers contended that disturbance in the rat's copper metabolism in due to the fixation of copper in tissues as copper sulfide (HALVERSON *et al.*, 1960; SIEGEL and MONTY, 1961). Yet in ruminants where there is a decrease in tissue copper without any increase in the rate of copper excretion (MILLS, 1961), it was claimed that copper, molybdenum and sulfate interact in the digestive tract (MILLS, 1960, 1964; MILLS and QUARTERMAN, 1963).

### 2.2.2 Copper and zinc

An antagonistic relationship exists in the rat and probably also in sheep and pig between copper and zinc, in which excess zinc reduces copper in tissues and fluids. In the rat, zinc toxicity in characterized by anemia, severe retardation of growth (SMITH and LARSON, 1946), a decrease in the activity of some copper activated

enzymes (DUNCAN *et al.*, 1953; VAN REEN, 1953; MAGEE and MATRONE, 1960), blood and tissue hypocuprosis (GRANT-FROST and UNDERWOOD, 1958).

Dwarfism is apparently at least partly a separate effect of zinc toxicity which unlike the other manifestations is not cured by copper or copper and iron (DUNCAN *et al.*, 1953; COX and HARRIS, 1960; may own work, unpublished). The anemia of zinc toxicity is most probably caused by lack of both copper and iron in the animal and also by a zinc-copper antagonism in the cell. Liver iron decreases in zinc toxicity (COX and HARRIS, 1960) but an impaired absorption of either copper or iron could not be demonstrated (MAGEE and MATRONE, 1960). Diets rich in zinc increased the excretion of copper in urine. These results are not in agreement with those of VAN CAMPEN (1966) and VAN CAMPEN and SCAIFE (1967) who were able to demonstrate a significant depression in copper $^{64}$ uptake when the isotope and zinc were directly introduced into ligated segments of the rat's stomach or the duodenum in vivo.

The copper-zinc interaction is unlikely to be involved in natural conditioned hypocuprosis in livestock. Experimental evidence on this point, however, is scanty. DICK (1954b) demonstrated a marked decrease in the liver copper in sheep eating as much as 100 mg zinc and 30 mg copper daily but when the daily zinc intake was only 20 mg liver copper was constant. The high tolerance of monogastrics to zinc suggests that the amount needed to impede copper metabolism in grazing livestock might be much higher than is likely to be present naturally.

### 2.2.3 Copper and protein

In addition to molybdenum, sulfate and zinc, the protein level of the diet may be an important determinant of copper storage in liver. As early as 1953, SEEKLES observed that copper deficiency in the Netherlands is especially encountered in cattle on lush growing pastures normal in copper content and suggested that the high protein level may be the cause of the observed hypocuprosis. Since that time, sufficient evidence has accumulated to indicate the importance of this factor in determining the copper status of laboratory animals and livestock. Thus DICK (1956 b, c) showed that sheep on the same molybdenum intake will store less copper in their livers when either sulfate or gluten is added to the ration. He considered that this effect of protein resulted from oxidation of protein sulfur to sulfate. WALLACE *et al.* (1960) found that the toxicity of 750 ppm copper (as sulfate) fed to growing pigs decreases as the protein level increased from 15 to 25 %. MCCALL and DAVIS (1961) showed that rats on a protein-rich diet (25 %) store considerably less copper in their livers than rats on a protein-low diet (10 %). A similar effect of high protein ration in lowering liver copper storage has been also reported for sheep (MCPHERSON and HEMMINGWAY, 1965).

Similarly the studies of AMMERMAN *et al.* (1963), GOODRICH and TILLMAN (1965, 1966a) and COMBS *et al.* (1966) clearly indicated the interaction between level and source of protein and the concentration of copper in liver of both sheep and swine.

Quite recently BOSMAN (1966) succeeded in demonstrating a correlation between the ratio starch equivalent : digestible crude protein and conditioned copper deficiency in the Netherlands, in which the blood copper concentration in cattle was found to decrease as the proportion of starch equivalent to crude protein decreases. The relationship between protein intake and the occurrence of conditioned hypocuprosis in grazing livestock is a most promising aspect of work on the problem. However, with the diversity of the protein sources and the numerous metabolic pathways of protein, it will be difficult to find the mechanism of the antogonism with copper metabolism.

### 2.2.4 Copper and iron

The importance of copper in nutrition emerged in a study of iron deficiency and the effect of diet on anemia. In addition to iron, copper was needed for the regeneration of hemoglobin in rats with milk anemia. There has been ample confirmation in many species of the importance of copper in iron metabolism. Copper-deficient rats store more iron in their bodies; supply of copper caused these stores to disappear and the hemoglobin level to rise. Since copper is not a constituent of the hemoglobin molecule, it was concluded that copper was not needed for iron absorption but for the use of iron stores for hemoglobin synthesis. Evidence has not been conclusive and some workers could not detect an increase in iron stores of copper-deficient rats. However it was confirmed that the portion of iron stored as hemoglobin increased when copper was added and the specificity of the two elements for that purpose was recognized.

DICK (1954b) found that both ferrous sulfide and ferrous sulfate significantly reduced liver copper storage in sheep. He concluded, without any direct evidence, that ferrous sulfide forms hydrogen sulfide in the rumen and precipitates copper as copper sulfide whereas with ferrous sulfate the effect observed must have been due to a combined effect of sulfate and molybdenum "which was certainly present in the ration". While he demonstrated a negative influence of other sulfate sources with molybdenum on copper storage in sheep, he could not decrease liver copper with elemental sulfur or sodium thiosulfate, which would both liberate hydrogen sulfide in the rumen. Earlier, BENNETTS and CHAPMAN (1937), MARSTON (1950, 1952) and SCHULZ *et al.* (1951) observed a large increase in tissue iron in copper-deficient sheep. Recently HENNEUX *et al.* (1963) described conditioned hypocuprosis in cattle and sheep grazing pastures near an iron factory and heavily contaminated with iron. But they could not detect any difference from controls in excretion of labelled copper given once by mouth to sheep which had received excess iron for five months. SUTTLE and MILLS (1964) found that iron supplements to pigs lessened the increase in serum copper and transaminase value, and protected against jaundice associated with toxic doses of copper. ANTHONY and NIX (1965) demonstrated a significant decrease in liver copper in calves receiving iron supplements.

### 2.2.5 Copper and other elements

HILL *et al.* (1963) found that cadmium toxicity in rats involved a copper factor; copper corrects the mortality caused by cadmium. VAN CAMPEN (1966) showed that copper uptake from the intestine was severely limited by cadmium. Mercury seemed to have a slight effect but it was insignificant. Silver was without effect.

Earlier DICK (1954b) showed that high dietary intakes of calcium "as carbonate" diminish copper accumulation in sheep's liver.

## 3 Conditioned hypocuprosis in the Netherlands

The discovery of conditioned copper deprivation in this country dates back to the discovery that copper deficiency occurs in grazing stock under natural conditions. SJOLLEMA (1933a), noticed that 'likzucht' could be cured by giving the affected cattle copper sulfate, although pastures where the disorder occurred were not necessarily low in this element. Later on it appeared that 'likzucht' was a cobalt deficiency, sometimes complicated by copper deficiency. Nevertheless the work of SJOLLEMA stimulated further research on similar natural disorders, during which conditioned copper deprivation was found in different places (SJOLLEMA, 1933b, 1938; BROUWER *et al.*, 1938; FRENS, 1941; KAPPELLE, 1951; HOFSTRA, 1952; VAN DER GRIFT, 1955).

HOFSTRA and VAN DER GRIFT confirmed SJOLLEMA'S observation that the disorder is confined to pasture and they further demonstrated that copper reserves of most Dutch cattle are depleted during pasturing and replenished in the stall. The rate of depletion was independent of the copper content of the pasture and could indeed be rapid in liver of cattle on pastures with as much as 10 ppm copper. Replenishment with copper in the stall is closely dependent on copper intake. The onset of clinical hypocuprosis thus seems to depend on three factors: the rate of depletion on the particular pasture, the duration of pasturing and the initial size of the copper reserve. Unless otherwise stated, copper depletion, hypocuprosis, copper deprivation, or whatever term may be used describe the condition in which copper reserves are diminished with or without clinical symptoms.

The disorder is by no means confined to a particular type of soil. It occurs most on these soils which favour water stagnation (HARTMANS, 1962). The general opinion, as yet unproven, is that the disease is more prevalent on improved pastures, especially where lush after a wet season. Calves appear to be more sensitive. According to SEEKLES (1956) some 60 % or more of Dutch dairy cows are low in copper in liver, very often also in blood during the pasturing period.

The disorder is characterized by loss of condition and appetite, discoloration and symmetrical thinning of the hair, a decrease in milk yield and milk fat, and scouring. Anemia is not invariably present, and a decrease in fertility has rarely been reported (SEEKLES, 1960).

These symptoms, or some of them, develop early in autumn or late in summer after cattle have been sufficiently long on pasture and copper stores in liver have fallen below 20-25 ppm dry matter (VAN DER GRIFT, 1955).

Biochemically, there is an increase in the iron stores in the liver (HOFSTRA, 1952)

and an increase in inorganic phosphate in blood (SEEKLES, 1956).

Dutch workers have been actively trying to find the nature of contributary factors. According to HOFSTRA (1952), improvement in condition of grazing cows when stalled or moved to another pasture could not be related to an increase in copper intake. That copper content of pasture is not directly responsible for hypocuprosis is also shown by the recovery on hay from nocuous pastures, even if low in copper.

BROUWER *et al.* (1938) reported an improvement of the condition of scouring cows given clover instead of grass of similar copper content. Neither could they find any difference in the P, K, Ca, Na, Mg, Cl, S, Mn, Fe and proximate constituents between normal and nocuous pastures.

HARTMANS and VAN DER GRIFT (1964) detected a highly significant difference in total sulfur in herbage from two groups of farms with different rates of copper depletion. Their pasture composition is reproduced in Table 1.

The liver biopsy technique introduced by VAN DER GRIFT in 1953 yielded many interesting results and allowed checking of the different hypotheses put forward over the years. However, since only some of the hypotheses have been worked out,

*Table 1. Chemical composition*[1] *of the herbage in two groups of farms with different rates of change in liver copper content of the grazing cattle.*

| | | Average liver Cu in autumn expressed as % of spring value | | | | Significance of difference (Wilcoxon's test) |
|---|---|---|---|---|---|---|
| | | 7 (rapid fall) | | 28 (slow fall) | | |
| | | mean | range | mean | range | |
| Crude protein | % | 20.5 | 12.4–26.6 | 19.2 | 16.7–20.6 | |
| K | % | 2.85 | 1.97–3,69 | 2.75 | 2.36–3.00 | |
| Na | % | 0.16 | 0.08–0.29 | 0.14 | 0.07–0.27 | |
| Mg | % | 0.24 | 0.21–0.27 | 0.22 | 0.18–0.25 | |
| Ca | % | 0.55 | 0.42–0.64 | 0.55 | 0.51–0.60 | |
| Cl | % | 1.49 | 1.04–1.78 | 1.42 | 1.30–1.54 | |
| S | % | 0.37 | 0.35–0.41 | 0.28 | 0.23–0.35 | $p < 0.01$ |
| P | % | 0.44 | 0.33–0.54 | 0.43 | 0.40–0.48 | |
| Cu | ppm | 10.1 | 8.0–11.3 | 10.2 | 8.0–12.5 | |
| Mo | ppm | 3.34 | 2.15–5.93 | 2.57 | 1.87–3.85 | |
| Mn | ppm | 305 | 60–430 | 172 | 110–210 | |
| Fe | ppm | 165 | 110–271 | 139 | 80–231 | |
| mequiv (K+Na+Ca+Mg)/ mequiv (Cl+S+P) | | 1.26 | 1.09–1.30 | 1.30 | 1.06–1.46 | |
| Ca — S — P | mequiv | — 399 | — 452— 352 | — 319 | — 410— 242 | $p < 0.10$ |
| Inorganic S | % | 0.155 | 0.094–0.205 | 0.091 | 0.024–0.119 | $p < 0.10$ |
| Dry matter | % | 18.7 | 11.5–24.6 | 15.9 | 12.7–17.8 | $p < 0.10$ |

[1] Values, except dry matter, on dry matter basis.

Source: Hartmans and Van der Grift (1964).

I enumerate these hypothese here and will discuss their validity in view of new literature.

HOFSTRA (1952), stimulated by the resemblance of the symptoms of hypocuprosis to those of deficiency of vitamin B, put forward the hypothesis that the disorder is actually a vitamin B deficiency, through disturbance of microbial synthesis of the vitamin complex by eating pasture. He attributed the beneficial effects of extra copper to removal of inhibition on vitamin synthesis in the rumen. But this hypothesis does not explain the decrease in tissue and blood copper after cows have been sufficiently long on pasture or show why copper is equally curative in rations or injections.

WIND and DEIJS (1952) postulated that the acid base relation, expressed as the ratio (K + Na + Ca + Mg)/(Cl + S + P) is the primary determinant of copper utilization, more copper being needed as the base excess decreases. However, the range of situations where hypocuprosis was not related to base excess in later experiments, led them to conclude that it is the balance between Ca — (S + P) which is important, more copper being needed to offset a low value (DEIJS *et al.*, 1956). Subsequent investigations invalidated this hypothesis (HARTMANS, 1960) and established that only the sulfur is active in this relation (HARTMANS and VAN DER GRIFT, 1964). However a hypocuprosis comparable to that occurring in pasture could not be obtained even with excessive amounts of calcium and sodium sulfate given to two groups of cows for 2½ months.

An earlier suggestion, by VAN KOETSVELD (1955), was that excess ingestion of sulfate will tend to convert soluble copper in the rumen into insoluble copper sulfide, biologically unavailable (DICK, 1954b; SCHULZE *et al.*, 1936; BOWLAND *et al.*, 1961). MILLS (1960) in Scotland detected a high sulfide level in the rumen, yet not in the abomasum, of sheep fed on much molybdenum and much sulfate which was inversely correlated with the soluble copper in the rumen.

SEEKLES (1948, 1955 and personal communication 1967) believes that the imbalance of the ration during pasturing (high protein in grass and the release of active peptitdes in the gastrointestinal tract) increases the intestinal movement thereby decreasing copper absorption.

The interesting and thorough work of BOSMAN and DEIJS on the forms in which copper occurs in pasture, rument contents and feces of cows indoors and on pasture deserves to be mentioned. It was found that grass possesses a stronger copper-binding capacity than hay and that chlorophyll and various amino acids containing the S—S and the S—H groups can bind copper in vitro (DEIJS and BOSMAN, 1959). However, only very small amounts of copper porphyrins (copper pheophytin) could be detected in grass, rumen contents and feces from cattle on pasture, and no hypocuprosis occurred when cystein was administered to a stalled cow (DEIJS and BOSMAN, 1961; BOSMAN, 1964). BOSMAN (1964) was able to fractionate total copper in grass, rumen contents and feces from cattle on hay and on pasture according to the solubility in different solvents. She observed that a considerable portion of copper in grass could be extracted with 0.1 N acetic acid and that the size of this

fraction decreased considerably in solid rumen contents and feces of grazing cows. With cows fed on a winter ration, the size of this fraction in the solid rumen contents and feces was slightly higher. It is unfortunate that such a partition of copper has not been reported for hay and it is clear that work on this principle should be extended before general conclusions can be drawn. At any rate, she doubted whether much copper remained available to grazing cows in the rumen through the formation of copper sulfide. Further work to verify this hypothesis is in progress and preliminary reports has recently appeared (BOSMAN, 1965, 1966).

Final reference should be made to the correlation between the incidence of hypocuprosis and water stagnation in surface soil (HARTMANS, 1962). The significance of this finding cannot yet be fully understood.

Most Dutch workers think that molybdenum and sulfate cannot be held responsible for conditioned hypocuprosis. But direct experimental evidence to support this are lacking. According to WYNNE and MCCLYMONT (1956), molybdenum levels in dry matter as low as 1 ppm can strongly inhibit copper storage if sulfate in diet is sufficiently high. Molybdenum is frequently more in Dutch pastures. The general value for molybdenum content, given by WIND (1954), is 3-5 ppm in grass grown on peat soil and 1-2 ppm on sandy soils. WIND and DEIJS (1952) report 4 ppm in grass grown on peat soil and WIND (1951) mentions 20-30 ppm in clover and 8 ppm in grass in the Wieringermeer Polder. KAPPELLE (1951) found 2.4-4.2 ppm molybdenum in grass from nocuous pastures and 5.4 ppm molybdenum in 'one sample' of normal grass. HARTMANS and VAN DER GRIFT (1964) report a mean of 3.34 and a range of 2.15 to 5.93 ppm in farms where hypocuprosis developed very quickly. The sulfate content of the grass in relation to time during pasturing and the soil type has been studied bij 'T HART (1945), GRASHUIS *et al.* (1953) and VAN KOETSVELD (1955). It was found that sulfate was highest in grass grown in peat soils and rises during pasturing on all soil types to reach the height of 1.5 % of dry matter towards the end of pasturing, when hypocuprosis in cattle is most frequent (VAN KOETSVELD, 1955). The figures for sulfate content of grasses on different soil types are reproduced in Table 2.

These figures enable a daily intake of sulfate of more than 100 g per cow so that perhaps hydrogen sulfide is formed in the rumen and precipitates copper as copper sulfide (VAN KOETSVELD, 1955). This hypothesis appears to enjoy now the most support among Dutch workers, and a lot of work has been and is still being done to verify it. Yet no clear proof has emerged to indicate the formation of copper sulfide inside the rumen of cattle with conditioned hypocuprosis.

Table 3 shows the relation between sulfate and nitrogen in grass and the copper content of the grass at different sulfate levels. The figures for copper, molybdenum and sulfate in Dutch pastures are within the range where hypocuprosis induced by molybdenum or molybdenum and sulfate has been either naturally reported or experimentally produced. A survey of copper, molybdenum and sulfate in Dutch pastures in relation to the occurrence of hypocuprosis, and a controlled experiment

on the proportions of molybdenum and sulfate needed to upset the metabolism of copper in cattle in the Netherlands is badly needed. Probably, as in Britain, other factors await discovery. The possibility, however, that at least some outbreaks of hypocuprosis in this country can be accounted for by the proportions of these three elements cannot yet be eliminated.

*Table 2. Influence of season and kind of soil on sulfate percentage in DM of grass according to different workers.*

| | 'T HART (1945) | | | | GRASHUIS *et al.* (1953) | VAN KOETSVELD (1955) |
|---|---|---|---|---|---|---|
| | clay | sand | peat | average | mixed peat and clay | sand |
| May | 0.89 | 0.94 | 1.04 | 0.96 | 0.86 | 1.22 |
| June | 0.79 | 0.92 | 1.10 | 0.94 | 1.18 | 1.07 |
| July | 0.89 | 0.87 | 1.57 | 1.11 | 1.67 | 0.79 |
| August | — | — | — | — | 1.53 | 0.98 |
| September | 1.35 | 1.08 | 1.48 | 1.30 | 2.18 | 1.07 |
| October | 1.51 | 1.51 | 1.58 | 1.53 | — | — |

*Table 3. Percentages dry matter and their chemical constituents in grass*[1].

| Date | Dry matter | Crude protein | True protein | Total S as sulphate | Inorganic S | Organic S | N/S | Cu ppm |
|---|---|---|---|---|---|---|---|---|
| 17.5 | 18.7 | 30.8 | 26.2 | 1.22 | 0.63 | 0.59 | 12 | — |
| 1.6 | 16.0 | 29.4 | 24.8 | 1.04 | 0.46 | 0.58 | 13 | 29.6 |
| 21.6 | 15.5 | 25.0 | 21.4 | 1.09 | 0.59 | 0.50 | 11 | 25.1 |
| 5.7 | 16.4 | 19.5 | 16.2 | 0.78 | 0.53 | 0.25 | 8 | 20.0 |
| 19.7 | 12.6 | 21.5 | 18.3 | 0.80 | 0.54 | 0.26 | 21 | 18.6 |
| 2.8 | 17.7 | 19.6 | 16.2 | 0.88 | 0.53 | 0.35 | 10 | 18.4 |
| 16.8 | 17.4 | 23.7 | 21.0 | 1.08 | 0.66 | 0.42 | 10 | 19.0 |
| 2.9 | 18.7 | 19.8 | 18.2 | 1.02 | 0.68 | 0.34 | 9 | 25.0 |
| 13.9 | 16.0 | 19.3 | 17.9 | 1.03 | 0.71 | 0.32 | 9 | 15.0 |
| 27.9 | 15.6 | 19.0 | 17.3 | 1.17 | 1.02 | 0.15 | 7 | 17.2 |

[1] Van Koetsveld (1955).

# 4 Experiments with cows

When faced with a problem of the kind described in the preceding chapter, the investigator would expect some difference(s) in composition between grass and hay as the reason for copper depletion. If this is so, and if copper depletion could be obtained in cows on a hay ration by giving one or more of the suspected substances, or if copper storage instead of depletion could be initiated in cows on pasture by giving them one or more constituents of hay, there would be a strong indication of the nature of conditioning factor(s). This idea formed the basis of all subsequent experiments. This kind of approach has the advantage over research with hay and grass as such that one or, if desired, more factors can be separately studied. An indoor experiment was preferred because of the better control of conditions than in a pasture experiment.

The composition of hay and grass differs in so many respects (WATSON and NASH, 1960) that it would be impossible to investigate all aspects in one series of experiments. We decided to start by investigating the influence of rumen ammonia on copper storage in the liver. Ammonia is formed easily and rapidly from the nitrogenous fractions of pasture grass. This fraction comprises both non-proteins as well as proteins (MCDONALD, 1948; EL SHAZLEY, 1952) and is more abundant in grass, especially lush grass (on which copper is most severely depleted) than in hay.

Because of grazing habit, the ammonia concentration in rumen fluid from cattle on pasture is high and fluctuates during the day between narrow limits so that in these cattle the ammonia curve during daylight is practically a straight line. But the same curve for indoor-fed cows is rather V-shaped (Fig. 1) and the concentration of ammonia can drop as low as 2 mg per 100 ml or even lower some eight hours after feeding. Maximum values (the arms of the V), similar to those of cattle on pasture, are only reached shortly after feeding.

TILLMAN (personal contact with Dr. J. VAN DER GRIFT) could not induce copper intoxication in calves when toxic doses of copper were administered with a synthetic ration in which urea supplied the nitrogen. These facts encourage the opinion that an experiment should be carried out to investigate the effect on the copper storage in the liver of flattening the V-curve of stallfed cows at the maximum ammonia level so that it will become comparable to the curve for grazing cows. As urea is known to be broken down very quickly in the rumen to ammonia and carbon dioxide, this substance would meet the requirements, if a suitable method could be found of administering it in such a way that the curve is flattened. Therefore some preliminary work had to preceed the experiments:

1. to estimate the amount of urea needed daily per cow and
2. to find an easy way of administering this amount so that the experimental cows attain a diurnal ammonia curve similar to that of cows on pasture.

## 4.1 Preliminary work

First of all the ammonia curve in the rumen fluid was estimated in cows on a winter ration and on pasture. The results indicated that, whereas for stallfed cows the daylight curve is V-shaped, that for grazing cows is almost horizontal and is always above the value of 20 mg per 100 ml. Furthermore it was noticed that maximum values were recorded for cows in the stall immediately after feeding, the curve then declines to reach its lowest value immediately before the next feed.

To achieve a curve similar to that of pasture, the following trials were carried out on stallfed cows:

a. Administering urea through a rumen fistula. The urea was very quickly broken down, causing a sharp increase in ammonia in rumen fluid, then an equally sharp decrease so that the ammonia concentration had returned completely to normal in less than two hours after giving the urea.

b. Mixing sugar beet pulp with urea and letting cows eat the mixture at more frequent intervals. Good results were obtained when 100 g urea were mixed with sugar beet pulp and cows were given enough mixture to provide 25 g urea four times per day. The extra urea must be provided in the periods when ammonia tends to decrease. The disadvantage of this method was that, while the curve was kept well above the value of 25 mg per 1000 ml during daylight, the ammonia concentration in the rumen fluid was, as normal, very low during the night. Complete success was only achieved when urea was provided three times during the night.

c. To slow down the liberation of ammonia, sugar beet pulp was soaked in a urea solution untill saturation and subsequently dried. All urea was absorbed by the pulp and there were no losses during drying. But when cows ate this urea-pulp, ammonia was as rapidly liberated in the rumen as in a.

d. With a special designed infusion apparatus, 385 g urea, dissolved in 5 liters water, was infused continuously into the rumen of a stallfed cow. This resulted in highest and lowest values in the curve of 58 and 35 mg ammonia per 100 ml, respectively. On the same day the curve for another cow, without infused urea, fluctuated between 23 and 6 mg per 100 ml. The results of this trial are given in Fig. 2. The upper curve in Fig. 2 for the stallfed cow receiving urea by intraruminal infusion is similar to that for grazing cows (Fig. 1). The curve of ammonia concentration in the rumen fluid of stallfed cows without urea (lower curve in Fig. 2) was always lower. Although the curve for the cow receiving urea was higher than for grazing cows, it still represented a level which may occur in cows grazing on normal Dutch pastures. However this was not considered to be an obstacle since the height of the curve can be regulated by changing the amount of urea infused.

*Fig. 1. Diurnal ammonia curve in rumen fluid from a stallfed cow, and daytime curve in grazing cows.*

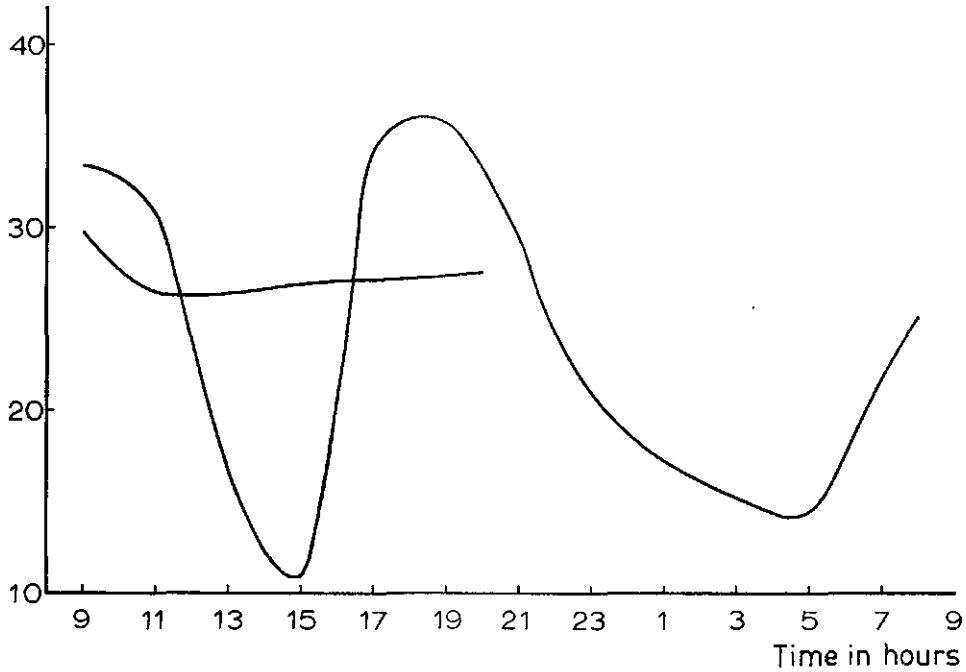



*Fig. 2. Diurnal ammonia concentration in rumen fluid with and without intraruminal infusion of urea.*

*The technique of infusion* After slight modifications, some made during the experiment, the infusion apparatus (Fig. 3 and 4) consisted simply of an inverted bottle hanging about two meters above the lying cow. The bottle was fitted with a cork through which two tubes passed, a long one to admit air protruding above the solution and a short one extending only a few millimeters into the bottle. A rubber hose was fixed to the short tube and led to a small plastic tap which was fitted in a 5 ml calibrated cylinder of a plastic syringe. The tap regulated the flow of the liquid, which could be observed by the drops falling into the syringe tube. This in turn was fixed above the cow to a wooden or iron bar which was loosely attached by its upper end only to a horizontal bar in the stall so that it could only swing. This prevented any tendency to pull the bottlecork downwards and automatically minimized the distance between the syringe and the fistula when the cow lay down. The lower end of the cylinder was connected by a piece of rubber tube to a 50 ml plastic bottle which acted as a reservoir if flow was temporary blocked, though this seldom occurred. A rubber hose was attached to the plastic bottle at one end and screwed to the fistula with a screw plastic joint at the other end; it conducted the fluid to the rumen. The fistula and the screw joint were like those used bij VAN ADRICHEM (1962). To avoid blockage, the hose connecting the plastic bottle to the fistula must meet certain requirements. This hose should be very elastic but not too soft-walled. Elasticity was necessary to avoid undue discomfort to the cow through pulling on the fistula and a degree of firmness was needed to keep the hose open if it bends. Latex hose was found adequate for the purpose. The length of the tube should be such that it would not hang down with a deep bend when the cow stands nor be too stretched if the cow lies. A long hanging hose invariably led to troubles with infusion since the infused fluid accumulated in the U-shaped bend and did not always flow into the rumen. It was found convenient to attach the hose to the fistula by a screw junction to facilitate disconnection. The urea solution entered the rumen by a plastic tube inserted 15 cm through the fistula opening and fixed to the inner side of the screw of the fistula. There it could diffuse through perforations in the plastic tube guarded by nylon gauze. Without these precautions there was a great chance that flow of the liquid would be blocked by rumen contents which invariably accumulate in the fistula opening or in the plastic tube inside the rumen. To allow free escape of the rumen gases with each rumen contraction the small plastic bottle was perforated at its upper side. This perforation was connected to a tube that was attached with its other end to the top of the bottle containing the solution to be infused.

The infusion rate was at first controlled by calculating the time taken to fill the calibrated cylinder or a part of it, but soon it proved to be more accurate and less tedious to count the number of drops passing the tap in half a minute. A little experience was needed to control such an apparatus as the number of drops varied and frequent adjustment to correct the rate of flow proved necessary. Despite this limitation, the apparatus suited the purpose of the experiment well and experiments with this apparatus lasted over 6 months without major troubles.

## 4.2 Experiments with with urea at Hoorn

*Materials and methods* The Hoorn experiments were on 6 Dutch Friesian cows, 3 with permanent rumen fistulae as the experimental group (cow 8, 30 and 84), and three unfistulated cows as controls (cow 24, 31 and 99). Cows 31 and 99 both appeared to be in calf when the experiment began in January 1964 and calved in the same October.

The cows were stalled next to each other and a preliminary period was kept before the experiment began. The copper status of the cows was assessed at intervals during the preliminary and experimental periods by copper determination in blood and liver samples. Blood was sampled by jugular puncture and liver by aspiration biopsy from the 12th intercostal space as described by VAN DER GRIFT (1955) (see also page 43). Rumen fluid was withdrawn by suction from the fistula and by the stomach tube described by VAN ADRICHEM (1962) for unfistulated cows. The rumen fluid was collected into a 300-ml Erlenmeyer flask containing 2 ml concentrated sulfuric acid. Ammonia was estimated in this sample by distillation into sulfuric acid after neutralization and by titration of the remaining sulfuric acid against sodium hydroxide.

The daily ammonia curve was determined once a week for the experimental group and as a check once only for the controle during the whole experiment. The rumen fluid was withdrawn at the times when the ammonia concentration is normally lowest and highest without infusion, at 09.00, 13.30, 19.30 and 23.30 h. Only during the first urea experiment was ammonia estimated also at 11.00 h. Copper was estimated by wet ashing and estimating the colour intensity of copper-dithiocarbamate in a Beckman spectrophotometer type B at 430 nm.

The ration of the cows during this and all subsequent experiments consisted of hay and concentrates meeting the Dutch standards for the requirement of starch equivalent. Samples of the components of the ration were analysed for copper at intervals during the experiment. Table 4 shows the mean values for the analyses of the components used during all the Hoorn experiments. Calculated from these figures and the ration intake, the daily intakes of copper, molybdenum and total sulfur amounted to 145 mg, 26 mg and 31 g per cow, respectively.

The amount of urea infused was sufficient to maintain ammonia in rumen fluid above 20 mg per 100 ml. As already mentioned, the day ammonia curve in rumen fluid was estimated once a week in the experimental group and from this estimate the provision of urea was calculated for the next week. Generally the infusion gave no practical difficulties or disorders in the cows, provided that infusion was slow enough.

*The first urea experiment* After a brief preliminary period during which the copper status of the cows was assessed twice, the experiment started on 21 January 1964 and ended on 20 March 1964. The daily infusion of urea in the experimental group averaged 280 g per cow. The ammonia concentrations in the rumen fluid

*Table 4. Composition of the ration [1] and estimated daily intake of copper, molybdenum and sulfate during the experiment with cows at Hoorn.*

| | | Hay | Dried pulp | Linseed oilmeal | Total |
|---|---|---|---|---|---|
| Composition | | | | | |
| Crude protein [2] | % | 12.48 | 9.73 | 28.68 | |
| Ether extract | % | — | — | 5.69 | |
| Nitrogen-free extract | % | 45.56 | 64.77 | 52.13 | |
| Crude fiber | % | 34.19 | 22.67 | 8.35 | |
| Ash | % | 9.28 | 4.26 | 6.57 | |
| Dry matter | % | 82.53 | 86.69 | 90.78 | |
| Copper | ppm | 9 | 8 | 27 | |
| Molybdenum | ppm | 2.45 | — | 0.91 | |
| Total sulfur | % | 0.23 | 0.32 | 0.29 | |
| Daily intake | | | | | |
| Copper | mg | 89 | 7 | 49 | 145 |
| Molybdenum | mg | 24.3 | — | 1.6 | 25.9 |
| Total sulfur | g | 22.8 | 2.8 | 5.3 | 30.9 |

[1] Values, except dry matter, on dry matter basis.
[2] Kjeldahl, N x 6.25.

during the experiment are shown in Table 5. Fig. 5 and Table 6 show the changes in copper content of blood and liver.

The infusion apparatus was highly successful for the experiment, the ammonia level in rumen fluid of the experimental group remained higher than 20 mg per 100 ml except rarely. A drop in ammonia concentration in rumen fluid at 13.30 h, as in the control group, did not occur in the experimental cows. Since the infusion was continuous day and night, the ammonia concentration in rumen fluid in experimental cows must have also remained high during the night.

During the preliminary period copper stores in liver increased in both groups, as expected. Afterwards liver copper in the control group remained almost constant throughout the experiment, while in the experimental group no consistent changes could be observed: at first the mean liver copper increased slightly (17 January-7 February 1964) but no significance was attached to this rise since it reflected a fluctuation in one cow only (cow 8). At the next sampling (28 February 1964) liver copper decreased uniformly in all three experimental cows as might be expected if urea was depressing copper utilization. At this date liver copper in the experimental group was significantly lower than at the beginning ($P < 0.025$) but the difference in change from the controls was still not significant ($0.1 < P < 0.2$).

But on the next date (20 March 1964) the mean liver copper in the experimental group increased, so that neither change from the beginning nor difference from the change in the controls were significant, and a careful study was needed of factors which might have led to this unexpected rise. It was discovered that the urea used

*Table 5. Ammonia concentration in rumen fluid (mg/100 ml) during the first urea experiment with cows at Hoorn (in round values).*

| | Date of sampling | Sampling time | | | | | Mean |
|---|---|---|---|---|---|---|---|
| | | 09.00 | 11.00 | 13.30 | 19.30 | 23.30 | |
| Cow 8 | 28.1.64 | 23 | 22 | 20 | 30 | 24 | 23.8 |
| | 5.2.64 | 32 | 42 | 32 | 28 | — | 33.5 |
| | 12.2.64 | 36 | 36 | 37 | 36 | 25 | 34.3 |
| | 19.2.64 | 36 | 32 | 29 | 32 | 34 | 32.6 |
| | 4.3.64 | 24 | 21 | 26 | 27 | 21 | 23.9 |
| | 12.3.64 | 39 | 35 | 40 | 27 | 29 | 33.9 |
| | 19.3.64 | 31 | 25 | 16 | 24 | 23 | 23.7 |
| Cow 30 | 28.1.64 | 32 | 28 | 25 | 31 | 22 | 27.6 |
| | 5.2.64 | 37 | 36 | 39 | 41 | — | 38.3 |
| | 12.2.64 | 40 | 37 | 36 | 42 | 40 | 38.9 |
| | 19.2.64 | 32 | 31 | 35 | 40 | 37 | 35.0 |
| | 4.3.64 | 30 | 31 | 30 | 36 | 29 | 31.3 |
| | 12.3.64 | 33 | 30 | 24 | 19 | 26 | 26.3 |
| | 19.3.64 | 31 | 22 | 25 | 24 | 15 | 23.4 |
| Cow 84 | 28.1.64 | 27 | 24 | 19 | 25 | 18 | 22.5 |
| | 5.2.64 | 41 | 37 | 31 | 30 | — | 34.9 |
| | 12.2.64 | 35 | 30 | 23 | 31 | 22 | 28.1 |
| | 19.2.64 | 36 | 33 | 30 | 35 | 27 | 32.5 |
| | 4.3.64 | 32 | 29 | 22 | 25 | 14 | 24.6 |
| | 12.3.64 | 37 | 33 | 36 | 28 | 25 | 31.8 |
| | 19.3.64 | 31 | 27 | 18 | 23 | 17 | 23.3 |
| Cow 24 | 12.2.64 | 23 | — | 9 | 19 | — | 17.0 |
| Cow 31 | 12.2.64 | 19 | — | 9 | 17 | — | 14.9 |
| Cow 99 | 12.2.64 | 18 | — | 6 | 13 | — | 12.7 |

for infusion immediately after the previous biopsy date (28 February 1964) was severely contaminated with copper so that each cow in the experimental group would have received 42 mg extra copper from urea. This was important enough to justify a repetition of the experiment.

The changes in blood copper remained insignificant in both groups, although a tendency towards a decrease was more marked in the experimental than the control group. No correlation was found between copper changes in liver and blood in either group.

*The second urea experiment* The second urea experiment used copper-free urea. The cows, infusion technique, rations and management were the same as previously, except that ammonia in rumen fluid was not estimated at 11.00 h. The first urea

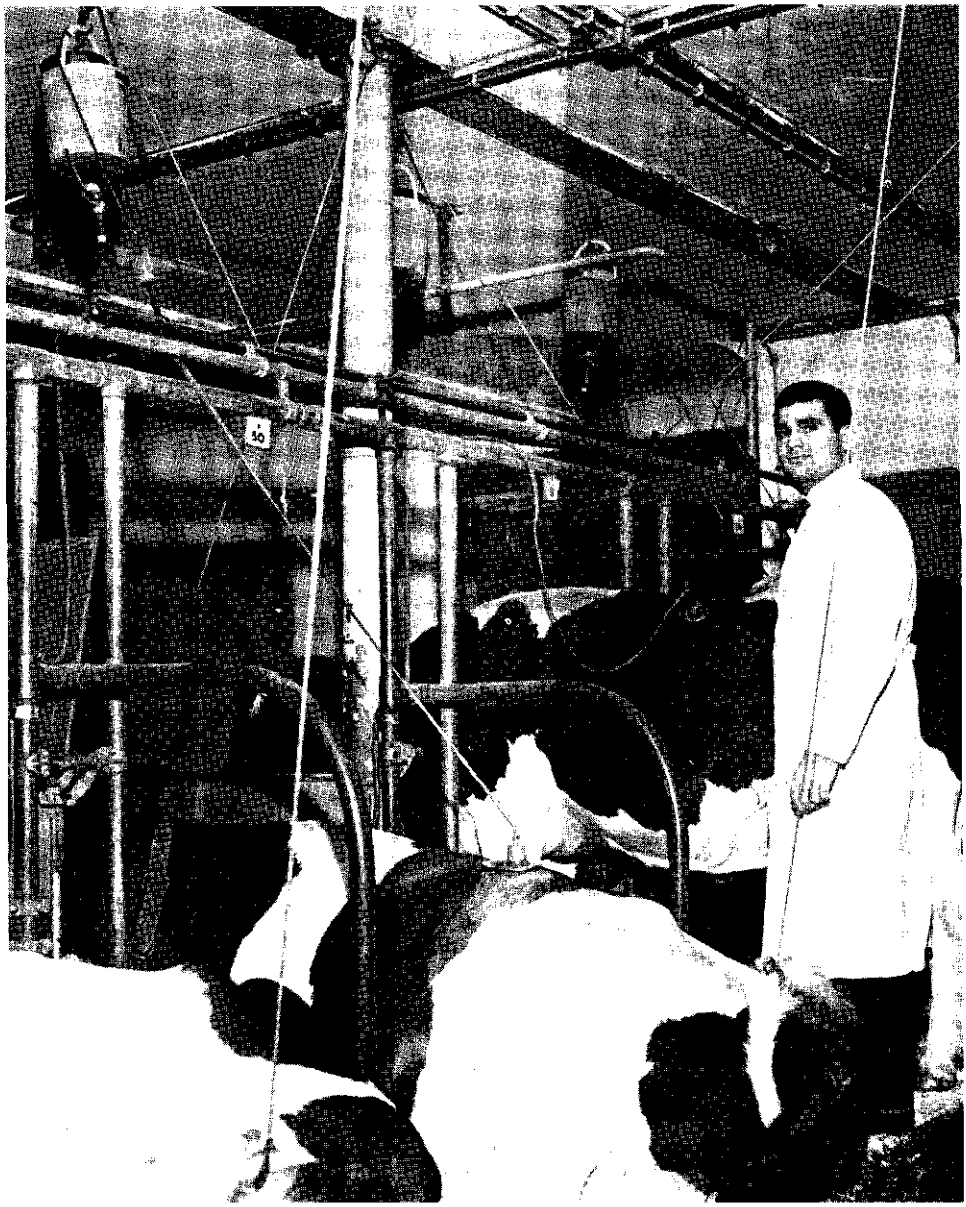


*Fig. 3. The apparatus for continuous intraruminal infusion connected to the fistulated cows of the experimental groups. For a detailed description of the infusion apparatus see text.*

*Table 6. Copper content of blood and liver during the first urea experiment with cows at Hoorn (1964).*

| Cow number | Blood (mg/l) | | | | | Liver (ppm in DM) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2.1 | 17.1 | 7.2 | 28.2 | 20.3 | 2.1 | 17.1 | 7.2 | 28.2 | 20.3 | 20.3/17.1 × 100 |
| 8 | 1.22 | 1.19 | 1.02 | 0.92 | 0.82 | 422 | 386 | 440 | 318 | 310 | 80 |
| 30 | 1.05 | 1.53 | 1.02 | 1.02 | 0.98 | 236 | 362 | 366 | 304 | 317 | 88 |
| 84 | 1.02 | 0.96 | 1.04 | 1.08 | 0.95 | 191 | 215 | 200 | 177 | 225 | 105 |
| Mean | 1.10 | 1.23 | 1.03 | 1.01 | 0.92 | 283 | 321 | 335 | 266 | 284 | 88 |
| 24 | 1.04 | 0.93 | 0.97 | 0.91 | 0.94 | 240 | 314 | 290 | 290 | 297 | 95 |
| 31 | 1.11 | 0.96 | 0.92 | 0.88 | 1.00 | 203 | 237 | 271 | 290 | 278 | 117 |
| 99 | 1.31 | 1.02 | 1.05 | 0.94 | 0.92 | 187 | 271 | 270 | 245 | 243 | 90 |
| Mean | 1.15 | 0.97 | 0.98 | 0.91 | 0.95 | 210 | 274 | 277 | 275 | 273 | 100 |

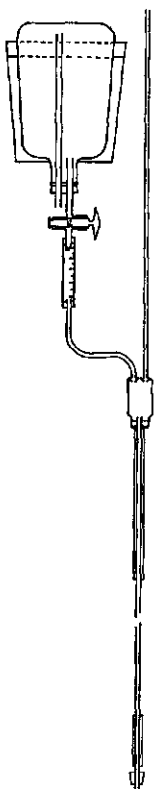


*Fig. 4. Schematic drawing of the apparatus used for intraruminal infusion during the experiment with cows at Hoorn.*

*Table 7. Ammonia concentration in the rumen fluid (mg/100 ml) during the second urea experiment with cows at Hoorn (in round values).*

| | Date of sampling | Sampling time | | | | Mean |
|---|---|---|---|---|---|---|
| | | 09.00 | 13.30 | 19.30 | 23.30 | |
| Cow 8 | 13.4.64 | 43 | 28 | 36 | 23 | 32.4 |
| | 20.4.64 | 24 | 27 | 23 | 24 | 24.4 |
| | 27.4.64 | 35 | 34 | 33 | 20 | 30.5 |
| | 5.5.64 | 27 | 25 | 48 | 45 | 36.3 |
| | 12.5.64 | 47 | 33 | 35 | 25 | 35.2 |
| Cow 30 | 13.4.64 | 39 | 35 | 36 | 22 | 33.1 |
| | 20.4.64 | 38 | 32 | 33 | 25 | 31.9 |
| | 27.4.64 | 26 | 40 | 36 | 38 | 34.8 |
| | 5.5.64 | 21 | 23 | 42 | 39 | 31.5 |
| | 12.5.64 | 58 | 42 | 45 | 34 | 44.7 |
| Cow 84 | 13.4.64 | 32 | 26 | 34 | 24 | 29.1 |
| | 20.4.64 | 53 | 30 | 30 | 22 | 33.8 |
| | 27.4.64 | 26 | 40 | 39 | 30 | 33.7 |
| | 5.5.64 | 23 | — | 33 | 16 | 23.9 |
| | 12.5.64 | 40 | 27 | 32 | 21 | 30.0 |
| Cow 24 | 19.5.64 | 25 | 8 | 22 | — | 18.2 |
| Cow 31 | 19.5.64 | 23 | 10 | 25 | — | 19.2 |
| Cow 99 | 19.5.64 | 20 | 5 | 14 | — | 13.2 |

experiment ended on 20 March 1964 and on 10 April 1964 copper status was again assessed and the experiment started. The mean rate of urea infusion was 306 g per cow per day this time as the weekly estimates of ammonia showed that 280 g urea was not enough to maintain constantly high ammonia in rumen fluid. The experiment ended on 22 May 1964.

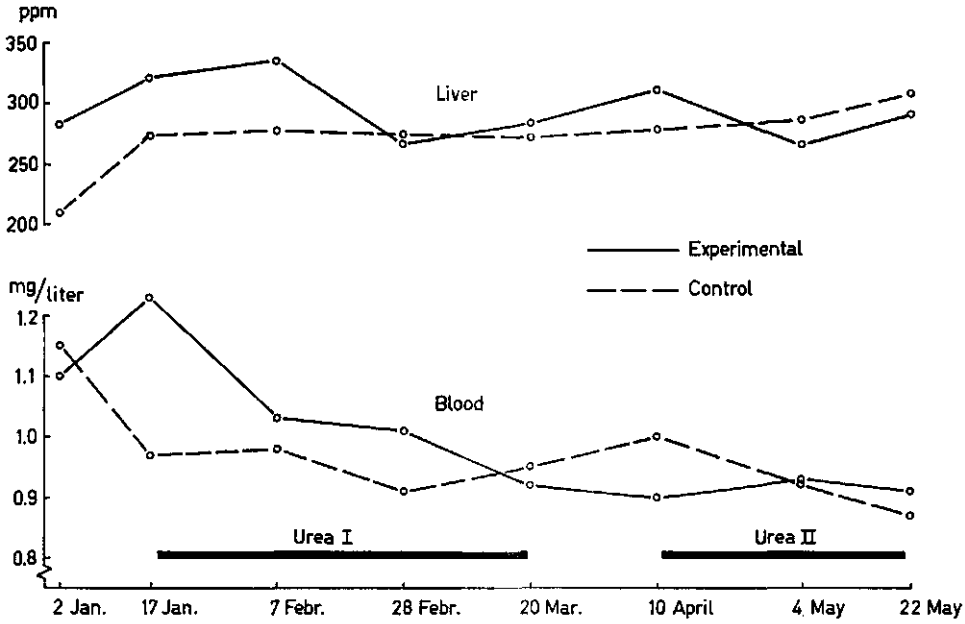
Table 7 shows the estimates of ammonia and Table 8 and Fig. 5 show those of blood and liver copper. This experiment did not show any significant depression of copper utilization by rumen ammonia. As during the first experiment, the mean liver copper in the experimental group decreased at first and then increased so that the value was lower at the end than that at the beginning. In the controls, the liver copper slowly but steadily rose till the end of the experiment. Both the changes within or between groups were never significant. Blood copper similarly showed no significant changes or differences.

On average each cow received 131 and 142 g nitrogen as urea per day during the first and the second urea experiments, respectively. The greater of these figures is still less than half the difference in nitrogen intake between cows on pasture and in

*Table 8. Copper content of blood and liver during the second urea experiment with cows at Hoorn (1964).*

| | Blood (mg/l) | | | | Liver (ppm in DM) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 20.3 | 10.4 | 4.5 | 22.5 | 20.3 | 10.4 | 4.5 | 22.5 | 22.5/10.4 × 100 |
| Cow 8 | 0.82 | 0.84 | 1.02 | 0.88 | 310 | 456 | 326 | 356 | 78 |
| 30 | 0.98 | 0.93 | 0.92 | 0.94 | 317 | 251 | 238 | 298 | 119 |
| 84 | 0.95 | 0.94 | 0.86 | 0.92 | 225 | 226 | 230 | 216 | 96 |
| Mean | 0.92 | 0.90 | 0.93 | 0.91 | 284 | 311 | 265 | 290 | 93 |
| Cow 24 | 0.94 | 0.94 | 0.94 | 0.86 | 297 | 269 | 270 | 331 | 123 |
| 31 | 1.00 | 0.81 | 0.83 | 0.88 | 278 | 294 | 296 | 316 | 107 |
| 99 | 0.92 | 1.24 | 1.00 | 0.86 | 243 | 270 | 291 | 274 | 101 |
| Mean | 0.95 | 1.00 | 0.92 | 0.87 | 273 | 278 | 286 | 307 | 110 |

*Fig. 5. Changes in blood and liver copper concentration in cows during both experiments with urea at Hoorn.*




stall. With urea liver copper decreased by 12 % during the first experiment and by 7 % during the second. The percentage change in the controls were nil and 10 % increase, respectively. For the two experiments mean liver copper in the experimental group decreased by 10 % and in controls it increased by 12 % of initial. Although the decrease observed in the experimental group was not like that in

cattle on pasture (where after a period of equal length a value of 30 % of the initial value is often reached) the fact remains that urea appears to have slightly depressed copper storage in the liver. It was decided therefore, not to omit the possible effect of this variable from the next experiments.

## 4.3 Experiments with urea and ammonium sulfate at Hoorn

Copper in ionic form readily forms complexes. MILLS (1955a, 1955b, 1956a, 1956b) showed that copper in herbage was largely present in the form of water-soluble organic complexes which are more easily utilized by copper-deficient rats than inorganic copper and he found evidence that it was absorbed in the form of complexes. He could not find any difference in the availability of copper from herbage causing swayback and from normal herbage to rats.

In the presence of sulfate, known to be reduced inside the rumen to sulfide (LEWIS, 1954; ANDERSON, 1956), copper may be liberated from the complexes and precipitated as copper sulfide. VAN KOETSVELD (1955) claimed that the effect of molybdenum and sulfate on copper metabolism, which DICK found, is directly due to sulfate on copper and not an interaction through molybdenum. Quite recently HARTMANS and VAN DER GRIFT (1964) reported a significant decrease in storage of copper in liver in two groups of cows after giving an excess of calcium sulfate and sodium sulfate. The next experiment tested whether results were similar when in addition to the ration a source of sulfate was given in the concentration normally present in pasture. Owing to the results obtained already with urea, I decided to keep it in the experiments.

At first thiourea was the sulfur source but as the infused cows lost considerable weight after a few days and showed a multitude of symptoms suggestive of interference by thiosulfate with basal metabolism, it was replaced by ammonium sulfate instead.

*The first experiment with urea and ammonium sulfate* This experiment was with the same cows. The last experiment with urea ended on 22 May 1964 and on 27 May 1964 the cows in the experimental group were infused with a mixture of urea and thiourea. After four days this was stopped, allowing the animals two weeks to recover and on 9 June 1964 the copper status of all cows was assessed and an experiment with urea and ammonium sulfate was started. The desired infusion rate was 42 g sulfur and 162 g nitrogen per cow per day. The amount of nitrogen was the same as in 350 g urea infused during the last days of the second urea experiment and approaches that infused during earlier urea experiments. Theoretically each cow should receive 270 g urea and 173 ammonium sulfate daily. The actual mean rates were 185 and 164 g respectively. As before, the day ammonia curve was estimated in rumen fluid once a week for the experimental group. When the level was found too high on 17 June 1964, the rate of urea infussion was slowed to 150 g per cow per day. The urea and sulfate apportioned for four days were

dissolved in tap water and 5 liters of this solution, containing the calculated amount of urea and sulfate, were put in the apparatus for infusion each day. Proximate composition and analytical data of the ration were as during earlier experiments (Table 4). Because of the farm program, the experiment had to be ended on 1 July 1964. Tables 9 and 10 and Fig. 6 give the results. Ammonia was not estimated in the control group; the level of ammonia in this group would be like that in earlier experiments.

Liver copper decreased in all cows in the experimental group so that the mean decreased from 343 ppm to 266 ppm, bij 22 %. Unfortunately, because of the wide individual variation this decrease did not reach significance ($0.1 < P < 0.2$). But in the controls the decrease was only 4 %: from 309 ppm to 296 ppm. The difference in change between the group was not significant either ($0.1 < P < 0.2$), probably because of the brevity of the experiment and the fewness of cows each group. Blood copper increased in both groups but especially in the controls. Again, changes within or between groups were not significant.

*The second experiment with urea and ammonium sulfate* After the first urea and sulfate experiment (on 1 July 1964) all cows were on pasture till 18 August 1964, when they returned to stalls and their copper status was assessed.

After three more weeks, copper status was again assessed and the previous experiment was repeated until 16 October 1964. Ammonia was not estimated during the grazing period. The daily rates of infusion of urea and ammonium sulfate were

*Fig. 6. Changes in blood and liver copper concentration in cows during both experiments with urea and ammonium sulfate during grazing and during the experiment with ammonium sulfate at Hoorn.*

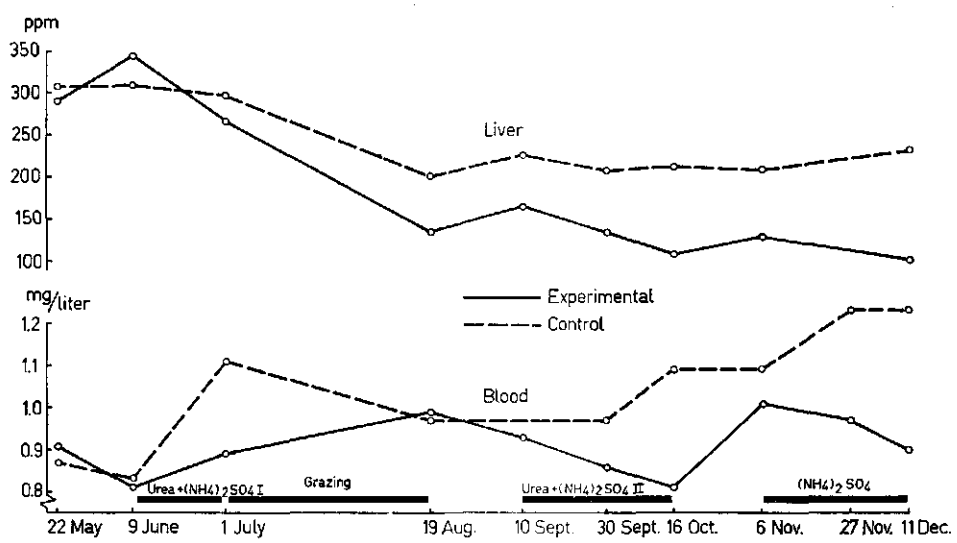


*Table 9. Ammonia concentration in rumen fluid (mg/100 ml) during the first experiment with urea and sulfate with cows at Hoorn (in round values).*

| | Date of sampling | Sampling time | | | | Mean |
|---|---|---|---|---|---|---|
| | | 09.00 | 13.30 | 19.30 | 23.30 | |
| Cow 8 | 12.6.64 | 40 | 42 | 50 | — | 44.2 |
| | 17.6.64 | 36 | 58 | 36 | 25 | 38.8 |
| | 24.6.64 | 22 | 20 | 39 | 33 | 28.4 |
| Cow 30 | 12.6.64 | 46 | 30 | 44 | — | 39.8 |
| | 17.6.64 | 38 | — | — | — | — |
| | 24.6.64 | 52 | 63 | 86 | 89 | 72.5 |
| Cow 84 | 12.6.64 | 49 | 44 | 59 | — | 50.8 |
| | 17.6.64 | 66 | 60 | 72 | 80 | 69.6 |
| | 24.6.64 | 26 | 24 | 37 | 31 | 29.4 |

*Table 10. Copper content of blood and liver during in first experiment with urea and sulfate with cows at Hoorn (1964).*

| | Blood (mg/l) | | | Liver (ppm in DM) | | | |
|---|---|---|---|---|---|---|---|
| | 22.5 | 9.6 | 1.7 | 22.5 | 9.6 | 1.7 | 1.7/9.6 x 100 |
| Cow 8 | 0.88 | 0.74 | 0.91 | 356 | 472 | 329 | 70 |
| 30 | 0.94 | 0.90 | 1.00 | 298 | 338 | 306 | 90 |
| 84 | 0.92 | 0.79 | 0.76 | 216 | 218 | 162 | 74 |
| Mean | 0.91 | 0.81 | 0.89 | 290 | 343 | 266 | 78 |
| Cow 24 | 0.86 | 0.80 | 1.31 | 331 | 323 | 284 | 88 |
| 31 | 0.88 | 0.81 | 0.97 | 316 | 312 | 306 | 98 |
| 99 | 0.86 | 0.88 | 1.05 | 274 | 293 | 298 | 102 |
| Mean | 0.87 | 0.83 | 1.11 | 307 | 309 | 296 | 96 |

264 and 184 g per cow, respectively; the corresponding ammonia level in rumen fluid during the experiment is shown in Table 11.

Liver copper decreased greatly in both groups during grazing (Table 12 and Fig. 6) despite the normal copper content of the pasture (8 ppm in dry matter). In the experimental group the depletion in 48 days was 50 % and in the controls 32 %, average 41 %. The decrease during grazing was significant in the control group ($P < 0.025$) and nearly so in the experimental group ($0.05 < P < 0.1$). The result is more significant when it is remembered than in stalls the control cows had previously increased their liver copper by 41 % between 2 January 1964 and 1 July

*Table 11. Ammonia concentration in rumen fluid (mg/100 ml) during the second experiment with urea and sulfate with cows at Hoorn.*

| | Date of sampling | Sampling time | | | | Mean |
|---|---|---|---|---|---|---|
| | | 09.00 | 13.30 | 19.30 | 23.30 | |
| Cow 8 | 26.08 64 [1] | 24 | 17 | 19 | 7 | 16.8 |
| | 14.09.64 | 68 | 40 | 38 | 35 | 45.3 |
| | 23.09.64 | 38 | 26 | 43 | 37 | 36.0 |
| | 29.09.64 | 27 | 20 | — | 13 | 19.7 |
| | 6.10.64 | 51 | 30 | 46 | 33 | 40.2 |
| | 12.10.64 | 35 | 39 | 47 | 38 | 40.0 |
| Cow. 30 | 26.08.64 [1] | 23 | 12 | 21 | 6 | 15.7 |
| | 14.09.64 | 58 | 39 | 42 | 30 | 42.2 |
| | 23.09.64 | 38 | 39 | 45 | 35 | 39.4 |
| | 29.09.64 | 48 | 62 | — | 42 | 50.3 |
| | 6.10.64 | 73 | 49 | 51 | 36 | 52.1 |
| | 12.10.64 | 53 | 38 | 39 | 40 | 42.4 |
| Cow 84 | 26.08.64 [1] | 24 | 10 | 16 | 11 | 15.0 |
| | 14.09.64 | 61 | 48 | 38 | 19 | 41.5 |
| | 23.09.64 | 50 | 21 | 35 | 25 | 32.7 |
| | 29.09.64 | 37 | 23 | — | 21 | 27.1 |
| | 6.10.64 | 46 | 32 | 50 | 33 | 40.3 |
| | 12.10.64 | 44 | 44 | 47 | 29 | 41.1 |
| Cow 24 | 7.10.64 | 21 | 12 | 21 | — | 17.8 |
| 31 | 7.10.64 | 24 | 17 | 23 | — | 21.6 |
| 99 | 7.10.64 | 23 | 21 | 28 | — | 24.1 |

[1] Samples taken before the start of the intraruminal infusion.

*Table 12. Copper content of blood and liver during grazing and during the second experiment with urea and sulfate with cows at Hoorn (1964).*

| | Blood (mg/l) | | | | | Liver (ppm in DM) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | grazing | | second exp. | | | grazing | | | second exp. | | | |
| | 1.7 | 18.8 | 10.9 | 30.9 | 16.10 | 1.7 | 18.8 | % | 10.9 | 30.9 | 16.10 | % |
| Cow 8 | 0.91 | 0.90 | 0.95 | 0.92 | 0.78 | 329 | 216 | 66 | 253 | 232 | 182 | 72 |
| 30 | 1.00 | 0.93 | 0.90 | 0.83 | 0.78 | 306 | 113 | 37 | 154 | 107 | 100 | 65 |
| 84 | 0.76 | 1.13 | 0.95 | 0.82 | 0.87 | 162 | 73 | 45 | 84 | 64 | 39 | 46 |
| Mean | 0.89 | 0.99 | 0.93 | 0.86 | 0.81 | 266 | 134 | 50 | 164 | 134 | 107 | 65 |
| Cow 24 | 1.31 | 0.98 | 0.97 | 0.92 | 1.04 | 284 | 216 | 76 | 280 | 246 | 210 | 75 |
| 31 | 0.97 | 0.95 | 1.01 | 0.89 | 0.92 | 306 | 199 | 65 | 179 | 190 | 222 | 124 |
| 99 | 1.05 | 0.99 | 0.93 | 1.11 | 1.31 | 298 | 184 | 62 | 215 | 182 | 200 | 93 |
| Mean | 1.11 | 0.97 | 0.97 | 0.97 | 1.09 | 296 | 200 | 68 | 225 | 206 | 211 | 94 |

1964. The decrease for all six cows during grazing and the difference in change from the controls during the previous period in stall (2 January-1 July 1964) were both highly significant ($P < 0.001$). After the cows had returned to stall, liver copper again increased in both groups: in the experimental group only till the next experiment began but in the controls until the experiment ended.

Urea and ammonium sulfate significantly decreased liver copper in the experimental group ($P < 0.025$) so that copper in liver decreased to 65 % of its initial (10 September 1964) value. In the control group it decreased by only 6 % and again the difference between the change in the two groups was not significant. However, comparison of this experiment with the first urea and ammonium sulfate experiment shows that the duration of the experiment greatly influenced the amount of copper depleted from the liver. While liver copper in the experimental group decreased in the first experiment by only 22 % in 21 days, the corresponding decrease in the second experiment was 35 % in 35 days. In both experiments with urea and ammonium sulfate the difference between groups in the decrease in liver copper did not reach significance. But in each case the difference between the mean values certainly suggests that the treatment inhibited copper storage in liver. As well as in liver, copper in blood also decreased in the experimental group, so that at the end of the experiment, on 16 October 1964, the change in concentration from the beginning (10 September) was significant ($P < 0.05$). In the controls blood copper rose and the final difference in change between the two groups was significant ($P < 0.05$). Differences between the control and the experimental groups were rather similar in both experiments with urea and sulfate; during the first the treatment gave a smaller increase in copper concentration than in controls but in the second the copper concentration actually decreased.

Since copper stores slightly decreased with urea and more markedly with urea and ammonium sulfate, studies were continued on the effect of the same amount of ammonium sulfate alone on copper storage.

## 4.4 Experiment with ammonium sulfate at Hoorn

Three weeks after the end of the previous experiment, another was commenced on 6 November 1964 lasting 35 days with ammonium sulfate alone. The technique and ration were the same as earlier. It was decided to infuse the experimental cows with the same amount of ammonium sulfate as during the previous experiments, 173 g per cow per day. Urea was not used. The day ammonia curve was estimated in rumen fluid from the experimental group once during the preliminary period of three weeks and each week during the experiment. Ammonia was estimated once during the experiment for the control cows. The actual daily infusion of ammonium sulfate was 184 g per cow, supplying the same amount of sulfur as in the previous experiment. The ammonia curve in the rumen fluid was distinctly lower in the experimental group than in previous experiments when the infusion contained also

urea but was still slightly higher than in earlier controls (Table 13).

Changes in blood and liver copper are shown in Table 14 and in Fig. 6. Mean liver copper decreased in the experimental group by 21 % while that of the control cows actually increased by 12 %. Both the difference in change within and between groups were not significant. Again blood copper in the experimental group decreased, while that of the controle increased. The change in each group was not significant ($0.05 < P < 0.1$), but the change in blood copper was significantly lower in the experimental group than in the controls ($P < 0.05$).

The rate of decrease in liver copper in the experimental group (21 % in 35 days), was less than in the previous experiments with sulfate and urea (22 % in 21 days and 35 % in 35 days). However, when the mean change in the control group during the different experiments is considered, the decrease in liver copper could be completely accounted for by sulfate alone.

*Table 13. Ammonia in rumen fluid (mg/100 ml) during the experiment with ammonium sulfate with cows at Hoorn.*

| | Date of sampling | Sampling time | | | | Mean |
|---|---|---|---|---|---|---|
| | | 09.00 | 13.30 | 19.30 | 23.30 | |
| Cow 8 | 4.11.64 [1] | 19 | 9 | 22 | — | 16.6 |
| | 10.11.64 | 32 | 16 | 15 | 6 | 17.4 |
| | 18.11.64 | 24 | 15 | 31 | — | 23.2 |
| | 30.11.64 | 24 | 12 | 24 | — | 20.4 |
| | 8.12.64 | 32 | 19 | 15 | 11 | 19.2 |
| Cow 30 | 4.11.64 [1] | 17 | 16 | 21 | — | 18.0 |
| | 10.11.64 | 36 | 19 | 21 | 9 | 21.3 |
| | 18.11.64 | 23 | 12 | 32 | — | 22.2 |
| | 30.11.64 | 30 | 16 | 22 | — | 22.4 |
| | 8.12.64 | 34 | 18 | 22 | 9 | 21.1 |
| Cow 84 | 4.11.64 [1] | 24 | 9 | 20 | — | 17.5 |
| | 10.11.64 | 32 | 23 | 17 | 7 | 19.8 |
| | 18.11.64 | 26 | 15 | 25 | — | 22.0 |
| | 30.11.64 | 32 | 7 | 26 | — | 21.8 |
| | 8.12.64 | 35 | 7 | 19 | 6 | 16.7 |
| Cow 24 | 10.12.64 | 24 | 10 | 19 | — | 17.8 |
| Cow 31 | 10.12.64 | 29 | 14 | 18 | — | 20.0 |
| Cow 99 | 10.12.64 | 26 | 12 | 20 | — | 19.5 |

[1] Samples taken before the start of the intraruminal infusion.

*Table 14. Copper content of blood and liver during the experiment with ammonium sulfate with cows at Hoorn (1964).*

| | Blood (mg/l) | | | | Liver (ppm in DM) | | | |
|---|---|---|---|---|---|---|---|---|
| | 16.10 | 6.11 | 27.11 | 11.12 | 16.10 | 6.11 | 11.12 | 11.12/6.11 x 100 |
| Cow 8 | 0.78 | 0.91 | 0.90 | 0.82 | 182 | 180 | 116 | 64 |
| 30 | 0.78 | 0.99 | 1.05 | 0.96 | 100 | 134 | 138 | 103 |
| 84 | 0.87 | 1.12 | 0.97 | 0.92 | 39 | 68 | 46 | 68 |
| Mean | 0.81 | 1.01 | 0.97 | 0.90 | 107 | 127 | 100 | 79 |
| Cow 24 | 1.04 | 1.06 | 1.22 | 1.13 | 210 | 182 | 170 | 93 |
| 31 | 0.92 | 0.98 | 1.10 | 1.07 | 222 | 208 | 235 | 113 |
| 99 | 1.31 | 1.22 | 1.38 | 1.48 | 200 | 229 | 284 | 124 |
| Mean | 1.09 | 1.09 | 1.23 | 1.23 | 211 | 206 | 230 | 112 |

## 4.5 Experiment with ammonium sulfate with or without urea at Wageningen

The experiments described so far were carried out on cows at the experimental farm of the Hoorn Institute for Research in Animal Nutrition and because of the farm program the experiments had to be ended before the total research program was completed. As cows became available at the experimental farm of the Laboratory for Animal Physiology of the Agricultural University at Wageningen I was able to set up another experiment in the hope of confirming earlier results on the function of sulfate in copper metabolism and the possible relation of ammonia to that effect.

Seven Dutch Friesian cows were available, born in 1962 except one, born in 1959. They were arranged in two groups similar in liver copper concentration: a group of three (cow 14, 17 and 19) given urea and ammonium sulfate and a group of four (cow 15, 16, 18 and 20) given ammonium sulfate. As none of the cows had a fistula, the compounds were mixed with the concentrates.

The daily amount of the compounds was weighed, divided into four equal portions given with the concentrates at 06.00, 11.00, 16.00 and 21.30 h. The daily ration consisted of 6 kg concentrate mixture of sugar beet pulp and concentrate (Rundveemeel A), 8 kg wilted silage and 5 kg hay per cow. The hay and the silage were both given twice a day at 06.30-07.00 h and at 16.30-17.30 h. The daily ration supplied approximately 201 mg copper and 5.1 mg molybdenum per cow.

Ammonia was not estimated in rumen fluid so that it is not possible to make sure whether urea in the diet caused the same diurnal curve for ammonia rumen fluid as with infusion. However the frequent feeding and provision of supplements should maintain a high ammonia level for most of the time. The copper status of

the cows was assessed by periodic estimation of copper in liver and blood.

The experiment started on 3 February 1965 and ended on 26 March 1965. No difficulties were encountered with the sulfate group but in the group given urea and sulfate cows refused to eat all the concentrates. After some unsuccessful attempts to induce the cows to eat all the supplemented concentrates, half the urea was withdrawn. The daily supply was then 172 g ammonium sulfate and 136 g urea. Thus the amount of ammonium sulfate given to both groups was almost the same as in the preceding experiments, while the amount of urea given to one group was almost half as much as in similar previous experiments.

Table 16 shows the changes in copper status of the cows.

The mean liver copper of the sulfate group first increased and then decreased very slowly; the overall change was a decrease of 12 ppm. The mean liver copper in the group given urea and sulfate decreased more rapidly at first but increased at the last observation; the overall change was a decrease of 24 ppm. Neither the difference in change between groups nor the changes within each group were significant and indeed they hardly exceeded analytical error.

It is to be noted that the cows received another hay, early spring hay, during the last three weeks of the experiment. But this would hardly explain the increase of the last observation in liver copper in the group given urea and sulfate. No major relevant difference could be detected by analysing the two hays (Table 15) and the change of hay did not affect the rate of decrease in liver copper in the sulfate group. It seems very probable that the more rapid decrease in liver copper at the beginning of the experiment in the group given urea and sulfate was caused by the refusal of the concentrate mixture which supplied most of the dietary copper. The decrease in liver copper in both groups during the experimental period (81 days) was much less than that recorded in earlier briefer experiments. Examination of the group mean values shows that the changes in each group can be largely accounted for by fluctuations in one cow.

The failure to confirm the decrease in liver copper with sulfate, with or without urea could have been caused by infusing the supplement at Hoorn and giving it by mouth at Wageningen. Various of the possible mechanisms of interference with copper metabolism would demand that the supplement and the copper be present simultaneously in the rumen or in the gut. Such possible mechanisms include the

*Table 15. Dry matter composition of the two hays supplied in the cow's experiment at Wageningen with sulfate with or without urea.*

| | Crude protein % | True protein % | Crude fiber % | Fe ppm | Mo ppm | Cu ppm | S % |
|---|---|---|---|---|---|---|---|
| Hay I | 12.4 | 8.7 | 33.7 | 163 | 0.2 | 7.7 | 0.27 |
| Hay II (last 3 weeks) | 16.0 | 11.0 | 28.1 | 123 | 0.3 | 9.6 | 0.31 |

*Table 16. Change in copper content of blood and liver during the cows' experiment with sulfate with or without urea at Wageningen (1965).*

| | Blood (mg/l) | | | | Liver (ppm in DM) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1.2 | 9.3 | 5.4 | 26.4 | 1.2 | 9.3 | 5.4 | 26.4 | 26.4/1.2 × 100 |
| Cow 14 | 0.92 | 0.80 | 0.80 | 0.76 | 240 | 194 | 155 | 152 | 63 |
| 17 | 1.15 | 0.94 | 0.89 | 0.90 | 258 | 242 | 237 | 270 | 105 |
| 19 | 1.04 | 0.62 | 0.66 | 0.66 | 164 | 181 | 154 | 169 | 103 |
| Mean | 1.04 | 0.79 | 0.78 | 0.77 | 221 | 206 | 182 | 197 | 89 |
| Cow 15 | 0.80 | 0.70 | 0.72 | 0.61 | 292 | 332 | 305 | 316 | 108 |
| 16 | 0.66 | 0.65 | 0.57 | 0.59 | 116 | 150 | 150 | 112 | 96 |
| 18 | 0.78 | 0.84 | 0.94 | 0.83 | 208 | 197 | 171 | 152 | 73 |
| 20 | 0.90 | 0.84 | 0.77 | 0.66 | 277 | 266 | 252 | 264 | 95 |
| Mean | 0.78 | 0.76 | 0.75 | 0.67 | 223 | 236 | 220 | 211 | 95 |

direct or indirect binding of the copper, the supplement making the copper unavailable by a change in the gut contents, interference by the supplement with the primary or enzymatic reactions of copper absorption. Such interference would have been possible in the Hoorn trials where the supplements were infused directly and continuously into the rumen but in the Wageningen trial the supplements would be released quickly and would disappear before the copper was released from the feeding stuffs.

Probably also the lower intake of molybdenum and the higher intake of copper in this trial (5.1 mg molybdenum and 201 mg copper against 26 mg and 145 mg, respectively in Hoorn trials) have accounted wholy or partly for the negative results.

The difference in supply of urea (only 136 g per cow per day in the Wageningen experiment) is hardly relevant as the decrease in the sulfate group was also less than in earlier experiments. It is also quite possible that still more factors are involved.

The blood copper in both groups decreased, insignificantly in the sulfate group ($0.10 < P < 0.20$) and nearly significantly in the sulfate group ($0.05 < P < 0.1$). The decrease in all seven cows was significant ($P < 0.025$).

## 4.6 General discussion of the experiments with cows

The results, so far can be summarized in the following six points:

1. Slow infusion of 280-350 g urea per day into the rumen of stalled cows raised the diurnal ammonia curve in rumen fluid to levels similar to those in grazing cows. The least ammonia in rumen fluid at any time of the day was similar to the higher levels recorded for cows on normal Dutch pastures.

2. Urea 280-350 g per day infused slowly into the rumen had no clear effect on copper storage in liver or on copper concentration in blood.
3. When 172 g ammonium sulfate and 270 g urea were daily infused into the rumen, liver and blood copper decreased both significantly.
4. Infusion of the same amount of sulfate without urea decreased blood and liver copper.
5. When in another experiment, the same amount of sulfate with or without 136 g urea was provided in four portions per day, blood but not liver copper decreased.
6. In liver, but not in blood, copper decreased considerably and rapidly when cows were turned out to pasture normal in copper content.

These results have been reached after performing a series of experiments on a group of cows in the experimental farm of the Hoorn Institute for Research in Animal Nutrition and an experiment on another group of cows in the experimental farm of the Laboratory for Animal Physiology at Wageningen. At Hoorn the first experiment in the series was started on 2 January 1964 and the last was ended on 11 December 1964. Cows 31 and 99 of the control group in the Hoorn experiments were pregnant and both calved in October 1964. Before the general discussion of results, the question must be answered whether pregnancy affected the results.

In serial biopsies on five pregnant cows, VAN DER GRIFT (1955) found that liver copper decreased four months before calving and practically recovered two months after calving. The extensive treatise of UNDERWOOD (1962) makes no mention of the effect of parturition or gestation on copper in cow's liver. The data of VAN DER GRIFT were probably too limited to justify a general statement, they do not agree with our results. Thus in June, no change in the concentration of copper in the liver was noticed in the two pregnant cows. During grazing, the decrease in liver copper in the two cows was similar to that in nonpregnant cows. After grazing the liver copper had increased in cow 24 but remained almost constant in the other two pregnant cows till calving. After calving there was no consistent change in either cow. These data indicate that the estimated concentration of copper in the biopsy sample represents the true figure and that pregnancy did not affect the concentration of the element in the liver. If it was correct that pregnancy decreases copper in liver as found by VAN DER GRIFT, my conclusion is still better supported as the concentration of the element in the liver during the second urea-sulfate experiment in the control group would have been even higher and the difference between the groups greater.

Table 4 shows the contents of copper molybdenum and total sulfur in the ration used at Hoorn. The calculated daily intake of these constituents was 145 mg copper, 26 mg molybdenum and 31 g sulfur. Such amounts of molybdenum and sulfur are unlikely to have hindered copper storage in the experiments since cows in the control group actually stored copper. When ammonium sulfate was infused in the experimental group, it is not improbable that excess sulfate together with molybdenum have hindered copper storage. At similar intakes of copper, molybdenum and sulfate, there will be a steady decrease in liver copper, a delayed rise in

blood copper and finally a sharp decrease when liver copper in depleted. (DICK, 1956a). In my experiment a steady decrease of both blood and liver copper was noticed. UNDERWOOD (1962) mentions that sulfate and molybdenum either decrease or increase blood copper depending on their relative concentration and probably also on other factors.

My experiment cannot tell how far molybdenum could be incriminated as the cause of the ensuing hypocuprosis in Hoorn experiments with sulfate, with or without urea. MILLS (1960) showed that sulfate reduction in the rumen of sheep is faster with than without molybdenum. In the publication of BOSMAN (1966), the highest values of sulfide in the rumen fluid from grass fed cows corresponded to the highest molybdenum content in the grass.

HARTMANS and VAN DER GRIFT (1964) obtained a decrease in liver copper with sulfate. However, in their experiment the ratio of copper to molybdenum intake per cow was 71/4.4 as opposed to 145/26 and 201/5.1 in Hoorn and Wageningen trials, respectively.

Thus it appears probable that molybdenum has contributed, at least in part to the hypocuprosis in Hoorn trials. Disregarding the possible importance of this factor, on the grounds that Dutch pastures contain only a few parts per million molybdenum, is certainly unjustified. The failure to confirm any decrease in liver copper in the Wageningen trial further supports this argue.

Despite lower copper and sulfate intakes and a higher molybdenum intake by the control group in Hoorn than by cows in the Wageningen experiment, copper storage did occur in the former group indicating that sulfate also depresses copper storage.

TILLMAN (personal contact with Dr. J. VAN DER GRIFT, 1961) could not poison calves with toxic doses of copper in diets with urea. I could not detect any significant effect of urea on copper metabolism. Probably copper-ammonia complex is equally well absorbed as diet copper. The decrease in blood copper in the experimental group during the first urea experiment only resulted from extreme deviation in one cow and is probably of no significance. It could not be repeated. The difference with TILLMAN'S results must be sought in something other than urea. Colleagues of TILLMAN proved a significant influence of protein source and level on copper storage in lambs and swine (AMMERMAN *et al.*, 1963; COMBS *et al.*, 1966). Copper storage in liver was severely depreased with soybean meal but not with casein. There was no difference in copper storage in lamb tissues with either soybean meal or urea served as the major source of dietary nitrogen (AMMERMAN, 1965). Such differences were demonstrated when elemental sulfur was present and less markedly when sulfate served as the source of dietary sulfur (GOODRICH and TILLMAN, 1965, 1966a). Generally copper retention was highest when sheep ate elemental sulfur and urea and least with soybean protein and sulfate. They concluded that both sulfate and protein act to prevent the absorption of copper. Their results, and those of other studies on the function of inorganic sulfate in copper metabolism (WYNNE and MCCLYMONT, 1956; EVANS and DAVIS, 1963; HARTMANS and VAN DER GRIFT, 1964; GOODRICH and TILLMAN, 1966b) are in harmony with

mine. In one series of experiments ammonium sulfate with or without urea, infused slowly into the rumen at the daily rate of 163 g N and 42 g S, decreased liver copper. There was no significant difference from the controls and this largely resulted from the high standard error associated with the small groups used and the brevity of the experimental period. Despite the lack of statistical evidence, it seems reasonable that sulfate interfere with copper storage in liver. A more severe copper depletion was demonstrated also by HARTMANS and VAN DER GRIFT (1964) with more sulfate. As in our experiments, blood copper decreased significantly with sulfate, liver copper in both experiments was far from depleted. A decrease in blood copper seems doubtful while the stores in liver are still adequate and it would be interesting to demonstrate whether this decrease in blood copper continued till symptoms of copper deficiency develop. In my experiments the lowest blood value was 0.57 mg copper per liter and in the experiment of HARTMANS and VAN DER GRIFT the value was 0.40 mg per liter. In neither experiment was there any clinical symptom of copper deficiency.

Sulfate, with or without urea, depressed liver copper far more at high than at low molybdenum intake, however the sulfate was given. Blood copper decreased, independent of molybdenum intake or route of intake. In all cases, blood copper decreased while liver stores were still adequate. These two facts suggest that sulfate has two independant effects on copper in the body: in the gut, it may be the formation of copper sulfide which hinders absorption and consequently diminishes liver stores; the second effect is inside the body and diminishes blood copper. My limited evidences suggests that only the first effect is accentuated by molybdenum.

A similar suggestion has recently been made by DEIJS (1966) [1]. Hydrogen sulfide formed in the rumen would prevent copper absorption by converting it to copper sulfide, thus diminishing liver storage. Through absorption of hydrogen sulfide any copper absorbed would still be converted to copper sulfide, which would be removed from the blood, further decreasing its concentration.

The effect of sulfate on blood copper is also proven by the results of an experiment with radioactive copper [2], Table 17. A dose of $^{64}$copper was injected into the gluteal muscles in each of the cows comprising the Wageningen experiment and in each of four control cows. More radioactive copper was retained, after 19 of 38 hours at the site of injection, in the groups given either sulfate or sulfate and urea than in the controls. However, the percentage of the dose mobilized to the liver was similar in each case. It appeared that copper was less mobile in the groups given sulfate with or without urea, but that as a compensation more copper from the mobilized dose in these groups find its way to the liver than in the controls.

The mechanism of action of sulfate in depressing liver copper storage remains now to be discussed. As stated previously, the hypothesis of copper sulfide forma-

[1] Lecture at Utrecht, 26 May 1966.

[2] I am grateful to Dr. W. Binnerts who kindly performed this experiment.

*Table 17. Percentages of the dose of radioactive copper retained at the site of injection or transferred to the liver after intramuscular injection of $^{64}Cu$ (experiment with cows at Wageningen).*

| | Treatment | Percentages of initial dose | | |
|---|---|---|---|---|
| | | injection site | | liver after 38 h |
| | | after 19 h | after 38 h | |
| Cow 14 | urea + ammonium sulfate | 67.5 | 54.1 | 23.1 |
| 17 | | 53.6 | 40.1 | 22.6 |
| 19 | | 56.5 | 45.7 | 27.4 |
| Cow 15 | ammonium sulfate | 62.5 | 49.5 | 23.3 |
| 16 | | 69.3 | 50.6 | 18.7 |
| 18 | | 46.1 | 33.9 | 30.9 |
| 20 | | 79.0 | 63.0 | 16.1 |
| Cow 1 | control | 43.6 | 34.2 | 17.2 |
| 2 | | 43.2 | 36.5 | 28.5 |
| 3 | | 26.6 | 25.5 | 29.4 |
| 4 | | 19.2 | 11.7 | 24.9 |
| Mean | urea + ammonium sulfate | 59.2 | 46.6 | 24.4 |
| Mean | ammonium sulfate | 64.2 | 49.2 | 22.1 |
| Mean | control | 33.2 | 27.0 | 24.9 |

tion in the rumen is the one gaining ground among workers in this country to explain conditioned hypocuprosis in grazing cattle. Several points favour this hypothesis. Grass is richer in crude protein and total sulfur than hay (VAN KOETSVELD, 1955). My results and those of others (HARTMANS and VAN DER GRIFT, 1964) indicate a significant decrease in liver copper storage when sulfate is given to stalled cows.

Together with BOSMAN, some studies were made to verify the formation of copper sulfide in our experimental cows and also in some other cows (BOSMAN, 1965). The formation of sulfide in the rumen increased markedly with infused sulfate and urea. Sulfide level in the rumen fluid from cows on pasture was higher than in stalled cows but similar to values in the experimental cows receiving urea and sulfate. Incubation with rumen fluid of certain amino acids, especially those containing a sulfhydril group, increased sulfide formation in vitro. These results recall those of MILLS (1960) who found that sulfate alone or with molybdenum raised the sulfide level in the sheep's rumen; more with than without molybdenum. Molybdenum and sulfate also diminished soluble copper in both the rumen and abomasum. Soluble copper was inversly correlated with sulfide level in the rumen but not in the abomasum. In my experimental cows, no such inverse relation between sulfide and soluble copper in the rumen could be demonstrated. On the contrary, soluble

copper was higher in rumen fluid from cows given sulfate and urea than in the controls.

The significance, therefore, of the sulfide level in rumen and its relation to copper nutrition cannot yet be explained. Urea does not seem to enhance sulfide formation inside the rumen: sulfide level was equally high with urea and sulfate as with sulfate alone. Present evidence on the hypothesis of copper sulfide formation in the gut are far from conclusive. The demonstration by DICK (1956b), that not all sources of hydrogen sulfide are effective in diminishing liver copper in sheep, further weakens this hypothesis.

Reference has been made also to the correlation between the quotient starch equivalent/digestible crude protein and the utilization of copper (BOSMAN, 1966). She postulated that the balance between protein breakdown and synthesis in the rumen will be shifted at a low quotient so that ammonia and sulfide increase in the rumen. The level of ammonia in the rumen determines the concentration of urea in the blood. She demonstrated a correlation between the quotient starch equivalent/digestible crude protein in grass and the sulfide level in the rumen: in steers low blood copper occured only when the quotient was low (3.0-4.1) and the urea concentration in the blood was high. My negative results during both urea experiments indicate that the quotient starch equivalent/digestible crude protein in the ration does not necessarily always determine the copper status of cattle.

Despite the similarity of the fall of liver copper with sulfate with or without urea to that on pasture (however I unfortunately did not estimate sulfate), the difference in the behaviour of blood copper suggests other factors. VAN DER GRIFT (1955) and HARTMANS (1960) found that blood copper fell on pasture only after liver was depleted. The fall in copper status is therefore not fully explained by an influence of sulfate on liver copper (HARTMANS and VAN DER GRIFT, 1964).

Other factors which may or may not interact with sulfate are indicated by the difference between the Hoorn experiments with a decrease in liver copper and the Wageningen experiment without a decrease despite diets similar in sulfate.

ALLCROFT and LEWIS (1956a, 1956b) compiled figures for copper, molybdenum and inorganic sulfate in pastures causing swayback and in normal pastures. No significant difference could be detected and they concluded that other factors complicating copper metabolism await discovery. The situation in the Netherlands is probably similar to that in England. In our experiment, compositional differences between grass and hay were tested if they might have affected copper storage in cows. Ammonia was ineffectual while ammonium sulfate in the amounts used decreased both liver and blood copper. Assuming an intake of 15 kg dry matter per cow daily, equal amounts of sulfate will be supplied by pastures containing 0.8 % sulfate. However, I do not claim that sulfate explains the observed hypocuprosis in grazing cattle under normal Dutch conditions, but it probably contributes to it. Further work is required to distinguish the importance of this and other factors.

There are many field and laboratory observations indicating an effect of protein source and level and many theories which would explain such an action. Amino

acids form strong complexes with copper. High levels of copper decrease nitrogen retention, probably by chelating with amino acids. Protein may give rise to sulfate in the body. Molybdenum also influences the effect of sulfate. Further studies are therefore needed on the effect of protein and molybdenum on copper status.

# 5 Experiments with sheep

In view of the increase in body iron known to occur in copper deficient sheep and cattle and of the repeated indications in the literature that iron may complicate copper storage and metabolism in grazing livestock (see Chapter 2), it is strange that the effect of iron on copper absorption has received little attention.

My next experiments were intended to narrow this gap in the literature and they report the effect of excess of two iron salts on copper storage in sheep's liver. Copper was given in diet to enhance any difference and was estimated periodically in blood and liver.

It was first necessary to master a technique for obtaining biopsy samples from sheep's liver [1].

## 5.1 The technique of liver biopsy in sheep

In the Netherlands liver biopsy in healthy sheep had never before been done in feeding experiments. Therefore some lambs were first made available to try different techniques and to gain experience in obtaining biopsy samples. I first tried the same technique as used for cows by VAN DER GRIFT (1955). He inserted a cannula in the 12th intercostal space, a hand's breadth ventral to the dorsal boundary of the depression of the right flank with a boring movement ventrally and forwards to pierce the visceral surface of the liver. He aspirated the sample cut with a syringe.

For sheep the same technique was tried but the site of the operation was somewhat changed. The cannula was introduced a few centimeters below the dorsal boundary of the triangular depression of the right flank into the same intercostal space. Liver samples could always be obtained but the lambs died of internal hemorrhage. The smaller visceral surface of the liver and the more anterior position of the whole organ than in cows would make the chance of boring, in search of the liver, in the portal fissure instead of the liver proper, very high.

DICK (1944, 1952) describes a technique for liver biopsy in the lying sheep, which seems now to be universally used. The sheep is fixed in the lying position with its back on the operation table and a cannula is introduced through the 9th intercostal

[1] I would like to mention with gratitude the help received from Dr. J. van der Grift and Drs. J. M. van Leeuwen in this respect.

space into the chest cavity, boring through the diaphragm and into the liver which lies immediately posterior to it. This method is certainly safe, but the samples obtained were small and sometimes no sample could be obtained.

SISSON (1965) shows that the parietal surface of the liver almost entirely adjoins the diaphragm and that the whole organ is further to the right and anterior than in cows. Studies at the Hoorn slaughterhouse showed that a safe method could also be developed for obtaining large biopsy samples in the standing sheep from the 9th or 10th intercostal space. This method can be described as follows.

The operation was carried out in the 9th intercostal space a few centimeters dorsal to the mid-lateral line (Fig. 7). The instruments used were described by VAN DER GRIFT (1955) for liver biopsy in calves.

The sheep stood on a suitable table and was shorn at the site of biopsy. The position was marked with tincture of iodine, disinfected and locally anesthetized. A cut, 1 to 1.5 cm long was made in the skin and underlying muscle. The cannula with the trocar inside was inserted into the incision in a medial and slightly ventral direction until the diaphragm was felt. This was then pierced and the trocar withdrawn. The cannula was immediately inserted further ventral parallel to the rib cage. After piercing the diaphragm, the cannula usually met the parietal surface of the liver. If not, the cannula could be further inserted ventral and slightly medial or slightly posterior. When the cannula was felt cutting the liver, it was further inserted in the same direction and as vertical as possible and the sample of the liver bored was aspirated with a syringe.

The advantages of this method were its satefy and speed. In more than a hundred cases only one debilitated sheep died; it had been rejected from the experiment much earlier. Only two assistants were needed to hold the sheep, one standing in front and the other behind. If the sheep suddenly lay down, it was not harmed since the cannula was pulled out of the body. The sample obtained was large and if in doubt the cannula was half withdrawn from the liver and re-inserted. If the cannula was inserted vertical, no harm resulted because there are no major blood vessels in this area. The 9th intercostal space should be used for lambs less than 3 months old and the 10th intercostal space for older ones.

## 5.2 The first experiment

The trial was run with 25 ram lambs born in March or early April 1965 and arranged in 5 groups as equal as possible in age, liveweight, gains, parentage and copper content of liver. Treatments were:

Group 1. basal diet
Group 2. basal diet and copper
Group 3. basal diet, copper and ferrous sulfate
Group 4. basal diet, copper and ferrous chloride
Group 5. basal diet, copper and ammonium sulfate

The trial began on 21 June 1965 after a preliminary assessment of copper status

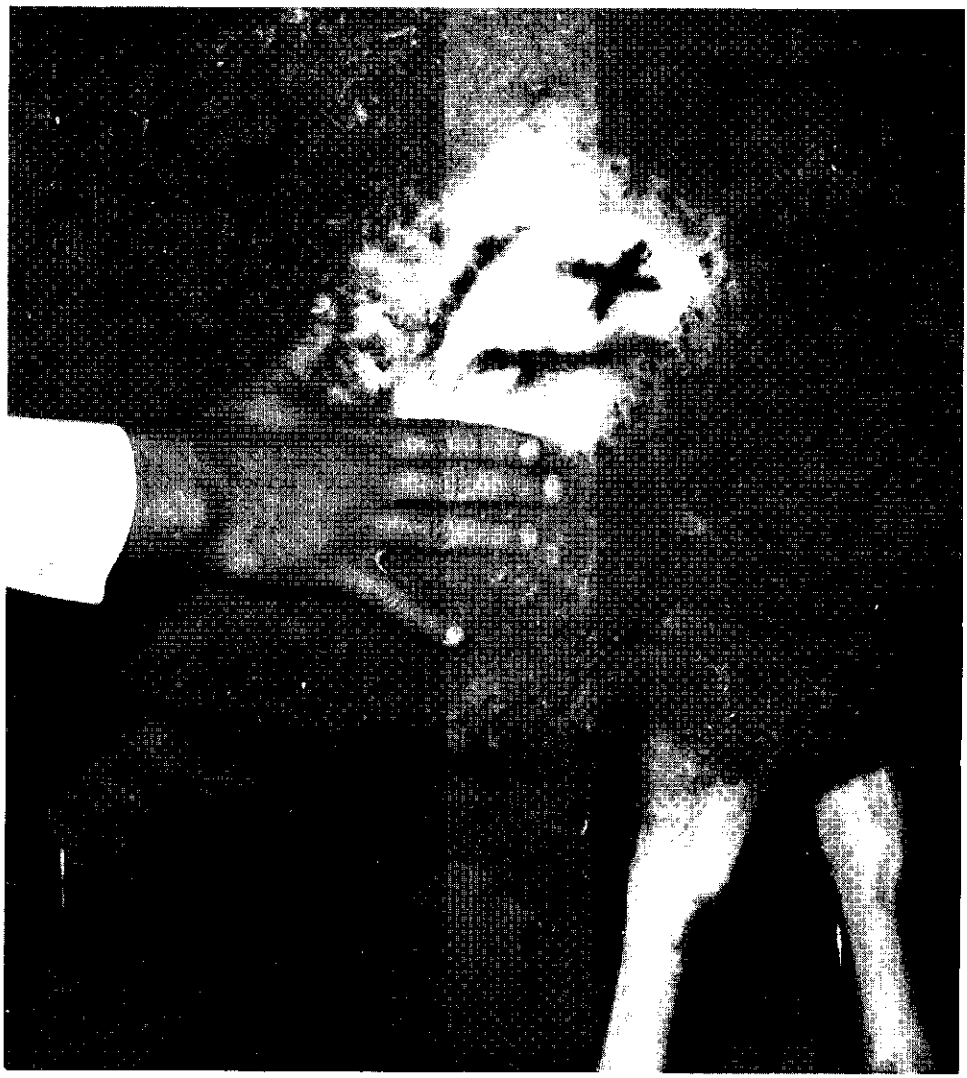


*Fig. 7. The site of biopsy (at the cross) and its relation to the midlateral line and the last rib.*

(15 June 1965). Status was again assessed on 5 August, 45 days later and the trial ended on 16 September 1965.

*Supplements* Copper sulfate ($CuSO_4.5H_20$, analytical reagent) was mixed with concentrates: 20 mg copper in 1 kg concentrates. At first, each lamb was allowed 0.6 kg concentrates per day. The allowance was gradually raised to 1.3 kg. Danger of copper poisoning could be predicted by periodic estimates of glutamic oxalacetic transaminase (SGOT) in serum. If there was danger of poisoning SGOT rose suddenly (VAN ADRICHEM, 1965; TODD and THOMPSON, 1965). This happened on 31 August 1965 and groups 2 and 5 were removed from the trial. When there was such a danger, the amount of copper-supplemented concentrates was limited to 1 kg and the rest of the allowance (0.3 kg) was supplied as unsupplemented concentrates. The important point was to keep the intake of copper supplemented concentrates equal for sheep in groups 2, 3, 4 and 5. But difference in the actual intake of copper did occur between the iron groups (3 and 4) and the iron-free groups (2 and 5) through differences in intake of concentrates.

Iron supplements ($FeSO_4.7H_20$ and $FeCl_2.3H_2O$) were at first mixed with the concentrates: 5.2 g Fe in 1 kg concentrates. But two difficulties soon arose. The first was the rancidity of the concentrates mixed with ferrous chloride and the second was that sheep receiving iron (groups 3 and 4) refused to eat all concentrates. Thus the actual intake of copper was less in these groups than in groups 2 and 5. Any residues of concentrates were weighed to allow calculation of copper intake and of other supplements. Flavoring agents, sucrose and the gradual feeding of iron were all tried without success. The iron supplements could be successfully administered in gelatin capsules twice daily but it was a lot of trouble. Finally drenching with the iron supplements was tried and found adequate. The amount of iron equivalent to the amount provided in concentrates was weighed daily, dissolved in water with a little linseed mucilage to protect the sheep's gut and the sheep was drenched twice daily at or just before providing concentrates. After a few days, however, some sheep were still leaving much of their concentrates and the drench was limited to 2.6 g iron per sheep per day for the rest of the experiment. After this (22 August 1965) much less concentrates were rejected in groups 3 and 4.

Ammonium sulfate was mixed with the concentrates to supply 8.9 g sulfate per kg concentrates. To allow comparisons between groups 3 and 5 intakes of sulfate were kept as equal as possible.

*Experimental arrangements* The sheep were housed with arrangements for individual feeding. The basal ration was concentrates and hay to provide the requirement of starch equivalent (WEIDE, personal contact). Hay was up to 0.5 kg per sheep per day. Any occasional remnants were not weighed. Samples of the ration were analysed for copper. The composition of the concentrate mixture is given in Table 18.

Copper status was followed by periodic analysis of blood and liver copper. Blood

samples were obtained from the jugular vein and liver samples by aspiration biopsy.

Sheep in the groups 2, 3, 4 and 5 should have received equal amounts of copper. But this could not be realized because of the low intake of concentrates by the iron groups (3 and 4), during the first month, before drenching was introduced. In addition the experiment ended 16 days earlier for groups 2 and 5. Therefore, any interpretation of the results should consider the total intake of copper by the different groups, as given in Table 19. During the first period, between the start of the experiment and the first biopsy, the total intake of copper was lower in the iron groups (3 and 4) than in groups 2 or 5. This disadvantage was counteracted by the prolongation of the experimental period for the iron groups. During the second period, between the second biopsy and the end of the experiment, the intake of copper was higher in groups 3 and 4 than in groups 2 and 5. Considering the experimental period as a whole, this pattern was maintained so that the ultimate results are reasonably comparable still with the existence of a certain margin of safety. Similarly the sulfate intake was higher in group 3 than group 5 during the second period and the whole experimental period, but not during the first period.

*Results* The results of this experiment are shown in Table 20 and Fig. 8. In Fig. 9 the change in liver copper concentration in the different groups as a percentage of copper intake is given.

The results for each two groups were compared with the Student t-test. In addition, the results for alle five groups were submitted to a Student-Newman-Keuls

*Table 18. Composition of the concentrate mixture used during both sheep experiments (values, except dry matter, on dry matter basis).*

| Crude protein % | True protein % | Ether extract % | Crude fiber % | Ash % | Dry matter % | Mo ppm |
|---|---|---|---|---|---|---|
| 17.65 | 15.86 | 4.87 | 11.62 | 5.27 | 88.48 | 1.23 |

*Table 19. Calculated mean total copper intake in mg by the different groups of sheep during the first experiment (1965).*

| | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 |
|---|---|---|---|---|---|
| From 15.6 to 5.8 | 695 | 1398 | 1096 | 989 | 1402 |
| From 6.8 to 31.8 | — | 1076 | — | — | 1055 |
| From 6.8 to 16.9 | 837 | — | 1630 | 1476 | — |
| Total | 1532 | 2474 | 2726 | 2465 | 2457 |

*Table 20. Copper content of blood and liver during the first experiment with sheep (1965).*

| Group | Blood (mg/l) | | | | Liver (ppm in DM) | | | |
|---|---|---|---|---|---|---|---|---|
| | 15.6 | 5.8 | 31.8 | 16.9 | 15.6 | 5.8 | 31.8 | 16.9 |
| 1 Negative control | | | | | | | | |
| sheep 507 | 0.84 | 0.86 | — | 1.54 | 322 | 401 | — | 530 |
| 514 | 1.00 | 0.75 | — | 1.14 | 485 | 626 | — | 948 |
| 520 | 0.89 | 0.68 | — | 1.84 | 442 | 521 | — | 883 |
| 537 | 0.94 | 0.82 | — | 1.05 | 268 | 440 | — | 647 |
| 547 | 0.79 | 0.66 | — | 1.26 | 216 | 316 | — | 496 |
| 2 Copper | | | | | | | | |
| sheep 501 | 1.20 | 1.99 | 1.18 | — | 432 | 875 | 1215 | — |
| 528 | 1.02 | 0.76 | 0.86 | — | 360 | 800 | 991 | — |
| 534 | 1.11 | 0.76 | 0.90 | — | 437 | 817 | 1214 | — |
| 545 | 0.84 | 0.82 | 1.23 | — | 367 | 950 | 1230 | — |
| 562 | 0.88 | 0.73 | 1.10 | — | 215 | 717 | 1034 | — |
| 3 Copper + ferrous sulfate | | | | | | | | |
| sheep 502 | 0.94 | 0.66 | — | 1.18 | 710 | 732 | — | 1272 |
| 517 | 1.41 | 0.92 | — | 1.00 | 441 | 651 | — | 1117 |
| 541 | 0.73 | 0.70 | — | 0.88 | 176 | 262 | — | 458 |
| 544 | 0.89 | 0.80 | — | 0.84 | 296 | 304 | — | 524 |
| 558 | 0.78 | 0.66 | — | 0.96 | 224 | 306 | — | 852 |
| 4 Copper + ferrous chloride | | | | | | | | |
| sheep 508 | 1.29 | 0.97 | — | 1.30 | 456 | 572 | — | 1110 |
| 510 | 0.92 | 1.05 | — | 0.85 | 287 | 546 | — | 855 |
| 524 | 0.82 | 1.08 | — | 1.10 | 238 | 411 | — | 804 |
| 540 | 0.75 | 1.08 | — | 1.10 | 274 | 444 | — | 736 |
| 560 | 0.98 | 1.33 | — | 1.24 | 392 | 569 | — | 838 |
| 5 Copper + ammonium sulfate | | | | | | | | |
| sheep 506 | 0.69 | 0.76 | 1.04 | — | 411 | 608 | 816 | — |
| 509 | 0.80 | 0.79 | 0.99 | — | 260 | 622 | 745 | — |
| 527 | 0.69 | 0.68 | 0.83 | — | 416 | 944 | 1148 | — |
| 538 | 0.88 | 0.78 | 0.96 | — | 399 | 944 | 1268 | — |
| 559 | 1.22 | 0.77 | 0.96 | — | 230 | 704 | 874 | — |
| Means | | | | | | | | |
| Negative control | 0.89 | 0.75 | — | 1.37 | 347 | 461 | — | 701 |
| Copper | 1.01 | 1.01 | 1.05 | — | 362 | 832 | 1137 | — |
| Copper + ferrous sulfate | 0.95 | 0.75 | — | 0.97 | 369 | 451 | — | 845 |
| Copper + ferrous chloride | 0.95 | 1.10 | — | 1.12 | 329 | 508 | — | 869 |
| Copper + ammonium sulfate | 0.86 | 0.76 | 0.96 | — | 343 | 764 | 970 | — |

test (KEULS, 1952). The conclusions were similar in each case. These conclusions are:

a. Under normal conditions, copper content of liver was a function of total intake. At higher intakes, either as copper sulfate or as natural copper in the ration, liver

*Fig. 8. Course of blood and liver copper concentration in the different groups of sheep during the first experiment.*

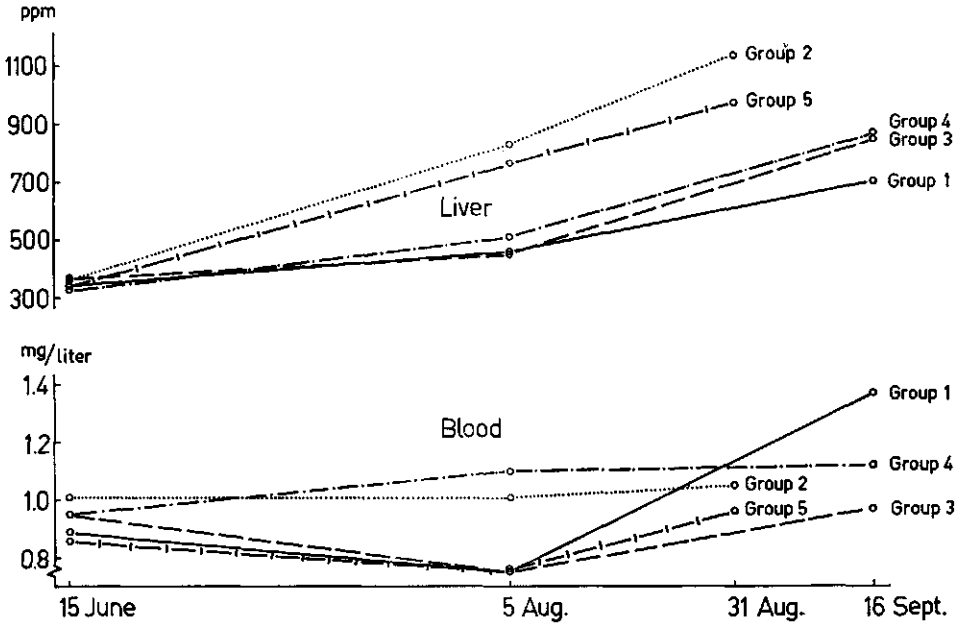
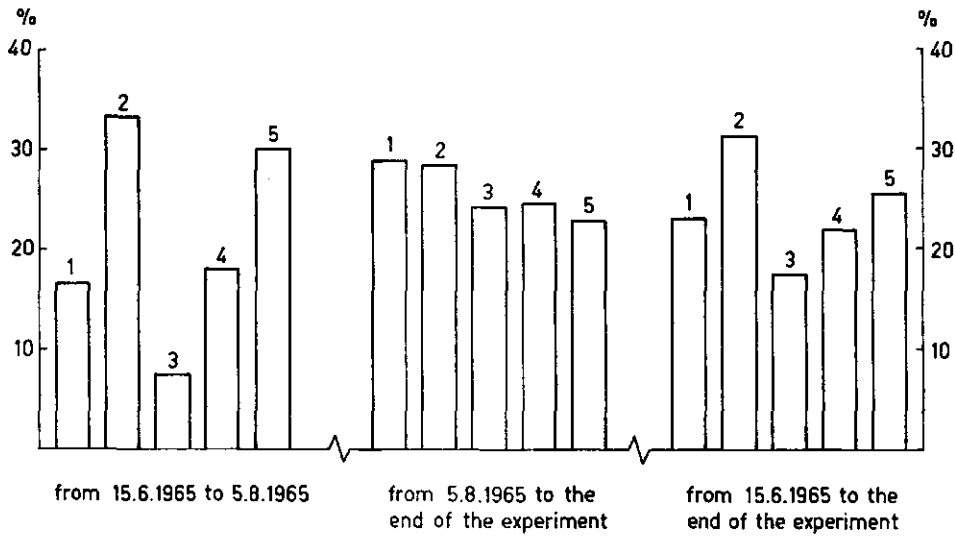



*Fig. 9. Change in liver copper concentration (in ppm) as a percentage of total copper intake (in mg) during the first experiment with sheep.*



copper increased and the size of the increase depended on copper intake. Copper concentration in the liver increased in groups 1 and 2 and the difference in increase between the two groups was highly significant ($P < 0.001$).

b. At high intakes of iron, the storage of copper in liver was limited. Despite the similar or higher copper intake by the iron groups (3 and 4), the increase in the liver copper was significantly less than in group 2. Iron limited copper storage more markedly during the first period of the experiment. The mean rises in liver copper in groups 1, 2, 3 and 4 during this period were 114, 470, 82 and 179 ppm, respectively, and the corresponding total copper intakes were 695, 1398, 1096 and 989 mg. During the second period of the experiment, iron limited liver storage much less and the higher copper intakes with iron would probably explain the larger increase in mean liver copper than in group 2. During the whole experiment the mean increase in liver copper in groups 1, 2, 3 and 4 were 354, 775, 476 and 540 ppm, respectively, and the corresponding total copper intakes were 1532, 2474, 2726 and 2465 mg, respectively. Groups 3 and 4 each show a statistically significant difference in the rate of copper storage from group 2 ($P < 0.025$ and $< 0.005$, respectively). When the iron groups were combined, this difference was highly significant ($P < 0.005$). So powerful was the effect of iron, especially in group 3, that no significant difference could be detected between the change in this group and group 1 which received no extra copper.

c. The difference between the increase in liver copper in groups 3 and 4 was not significant. But, when considered in relation to the results in group 5 and in relation to the difference in copper intake between the groups, the mean values suggest that ferrous sulfate was more potent than ferrous chloride in diminishing copper storage in liver.

d. Ammonium sulfate slightly diminished copper storage in liver. In this experiment the mean increase in group 5 was 627 ppm, against 775 in group 2 on equal copper intake, but the difference was not significant.

e. In groups 1, 3 and 5 blood copper decreased during the first period and increased during the second. Both the decrease and the subsequent increase were significant in group 1 ($P < 0.05$ and $P < 0.025$, respectively), and nearly significant in group 3 ($0.05 < P < 0.10$), while in group 5, the initial slight insignificant decrease was followed by a highly significant increase ($P < 0.001$). The overall effect was that there was no significant change in blood copper in either group 3 or 5, but in group 1 there was a significant rise ($P < 0.05$). In groups 2 and 4 there was a steady slight increase in blood copper during both periods of the experiment, but the level in both groups at the end of the experiment was not significantly higher than at the beginning. Neither there was any significant difference in change in blood copper between the different groups.

Thus iron significantly diminished copper storage in liver without a similar effect on blood.

It is difficult to resolve how far the depression of copper storage in the liver in sheep in group 3 was caused by iron and how far by sulfate because of the differ-

ence in copper intakes between groups 3 and 5 and because of the difference in duration. Nevertheless some conclusions on this point may be drawn.

Iron limits copper storage more than the sulfate since in group 4 the rise in liver copper was still lower than in group 5, and since the difference in the increase in liver copper between groups 2 and 5 was not significant. Thus the significant difference between groups 2 and 3 must be largely due to iron. During the second period (from 5 August 1965 till the end), when the sheep in group 3 ate considerably more sulfate (also more copper) than in group 5, the rise in mean liver copper concentration in group 3 was greater than in group 5 while during the first period (from the start till 5 August 1965), when group 3 ate less sulfate than group 5, the effect of sulfate in group 5 was negligible in comparison with group 2. During this period the mean rise in group 3 was 82 ppm, even less than in group 1 (114 ppm) on a far smaller intake of copper.

The difference in the response of blood copper to the iron treatment in groups 3 and 4 suggests that the significant decrease in blood copper in group 3 and the subsequent significant increase was due to sulfate and not to iron. This is partly in harmony with my results with cows. The general conclusion from this experiment is that iron independent of sulfate, diminishes copper storage in liver and that it probably does not interfere with the physiologic balance of copper inside the body between the tissue and the fluid.

Reference has just been made to the less marked effect of iron in limiting liver copper storage in groups 3 and 4 during the second period. In relation to mean change in liver copper and the copper intakes, the inhibiting effect of sulfate in group 5 seemed to exceed that of iron. During the first period precisely the opposite was seen. Several reasons can be proposed to explain this contradiction. When the experiment began, copper and iron supplements were both mixed with the concentrates. Later on, from 20 July 1965, iron was given daily in a drench, but not the copper. Four days later the drench was limited to 2.6 g iron per sheep per day. Sheep given iron drenches did not eat all their concentrates until 1 to 3 hours from drenching. This might prevent any interaction between iron and copper within the sheep. DICK (1954 b) mentions that without supplemental copper, 4 g iron daily from ferrous sulfide did not significantly affect liver copper, whereas, when 30 mg supplementary copper was simultaneously added, such treatment significantly reduced liver copper concentration. In the present experiment the copper supplement was mixed with the concentrate mixture in the amount which supplied 20 mg extra copper per kg concentrates. From 27 August 1965 only 1 kg copper-supplemented concentrates was allowed daily to the sheep in the groups 2, 3, 4 and 5 and the rest of the allowance (0.3 kg) was supplied as unsupplemented concentrates used for group 1. This was done when, according to SGOT activity, the danger of copper intoxication was predicted in sheep in group 2 and 5. This meant that less supplemental copper and more copper naturally present in the ration was administered during the second period than in the first.

It is not certain whether it was the change in copper source, the lowering of the iron dose, or the time period which diminished the antagonism of iron to copper storage during the second period. Therefore a second experiment was necessary.

## 5.3 The second experiment

Some anomalous results obtained during the first experiment showed the need for a second experiment to confirm the antagonism of iron to copper storage in liver and to investigate further aspects of the problem. Reference has already been made to the fact that, despite of the practice of drenching, the intake of concentrates and so of copper were lower with iron than in other groups. To counteract this difficulty, the drench supplied both supplements. Iron dose was increased in some groups to 5.2 g per sheep per day and iron was given either with or without copper.

When the first experiment ended, liver copper was high in most groups. In fact the first experiment ended for groups 2 and 5 when SGOT estimation predicted that deaths from copper poisoning were likely. Sheep in groups 2 and 5 were then turned out to pasture to diminish copper in liver before further experiments. The sheep remained on pasture for about 10 days. They then returned to stalls and on 16 September 1965 copper status was assessed for all the groups. The second experiment started on 20 September after the results for liver copper were known for all sheep. Copper in liver did not decrease during this short grazing period, but, in fact, slightly increased.

In order to harmonize the existing facts with the designed working plan, the following groups and treatments were used during this experiment:

Group 1. the same sheep as during the first experiment; each received 10 mg copper daily.

Group 2. sheep no. 506, 527, 528, 545 and 559, received no supplement.

Group 3. the same sheep as during the first experiment; each received 10 mg copper and 5.2 g iron as ferrous sulfate daily.

Group 4. the same sheep as during the first experiment; each received 2.6 g iron as ferrous chloride daily, no copper was given.

Group 5. sheep 501, 509, 534, 538 and 562; each received 5.2 g iron as ferrous sulfate daily; no copper was given.

In other words: the groups 1, 3 and 4 consisted during this experiment of the same sheep as in the first experiment. The sheep in group 2 and group 5 during the first experiment were redistributed into two new groups: group 2 and group 5 for the second experiment. The reason for this was to avoid any effect from the different origin of their high values for liver copper. In this way group 1 acted as a control for group 3 and group 2 as a control for groups 4 and 5.

The copper supplements were again supplied by copper sulfate ($CuSO_4.5H_20$, analytical reagent). Supplements were dissolved in water and given as a drench twice daily at the same time as concentrates. As during the first experiment, iron supplements were prepared immediately before administration. Ration, housing and

management of the sheep were as during the earlier experiment.

The experiment started on 20 September 1965 and ended on 29 October 1965. In groups 1, 3, and 5 copper status was also determined once between these two dates. Sheep 545 from group 2 and sheep 501 from group 5 had to be eliminated early in the experiment because of SGOT value. Sheep 534 was excluded from group 5 and sheep 560 from group 4 because of ill thrift probably caused by the administration of iron. The results from these sheep were not included in the statistical treatment at the end of the experiment. In group 3, sheep 502 and sheep 541 had to be eliminated after 6 October 1965, the first through incipient copper poisoning and the second because of ill thrift.The actual result from these two sheep were excluded from the statistical treatment of group 3 only after 6 October 1965. This, unfortunately, introduced the need to divide the experiment into two periods, and the differences observed between the groups thus decreased. Sheep in which the SGOT activity indicated incipient copper poisoning (usually accompanied by a large increase in liver copper) were also excluded. In the iron groups these were the sheep with the least response to iron administration. This was so for sheep 502, but not certainly so for sheep 534 which was excluded early in the experiment.

*Results* The results of this experiment are shown in Tables 21 and 22 and in Fig. 10. The practice of drenching with copper supplements and the discarding of sheep with ill thrift has narrowed the differences in copper intake between the experimental and control groups so that the groups are reasonably comparable.

The results for all five groups ware submitted to an analysis of variance. When there was a statistically significant difference between the means showing the greatest difference, one of these means was discarded and the Snedecor's F test was applied again on the other means . . . etc. In addition, the results of each two groups were also compared with the Student 't' test. In each case the conclusions were similar.

As during the first experiment, liver copper in groups 1 and 2 increased and the size of the increase depended on copper intake. In the iron groups (groups 3 and 4), on the other hand, liver copper increased less. Thus during the first period (from

*Table 21. Calculated mean total copper intake in milligrams of the different groups of sheep during the second experiment.*

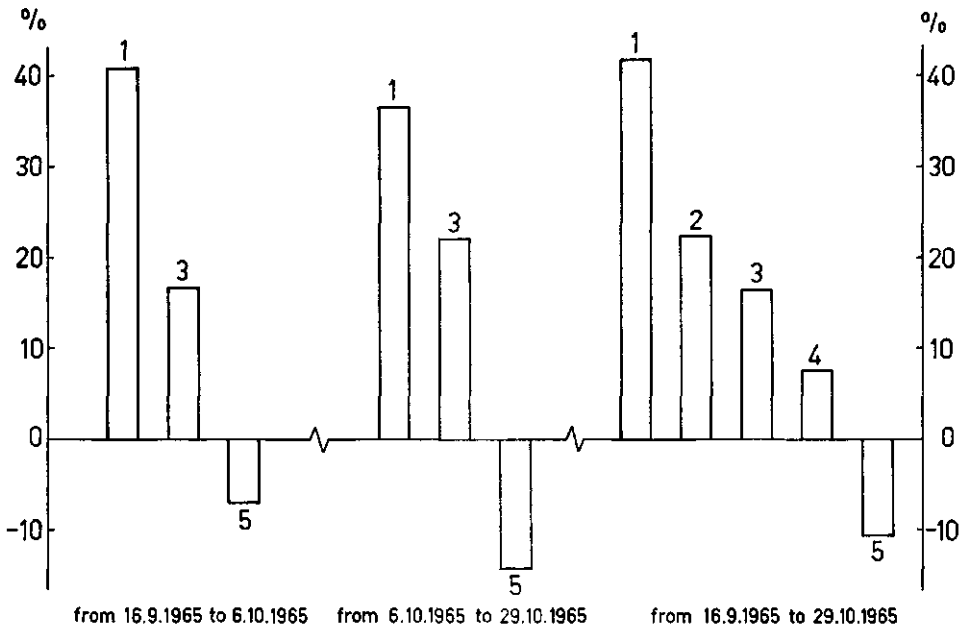
| | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 |
|---|---|---|---|---|---|
| From 16.9 to 6.10 | 515 | | 528 | | 327 |
| From 7.10 to 29.10 | 643 | | 600 | | 331 |
| Total | 1158 | 785 | 1128 | 763 | 658 |

*Table 22. Copper content of blood and liver during the second experiment with sheep (1965).*

| Group | Blood (mg/l) | | | Liver (ppm in DM) | | |
|---|---|---|---|---|---|---|
| | 16.9 | 6.10 | 29.10 | 16.9 | 6.10 | 29.10 |
| 1 Copper | | | | | | |
| sheep 507 | 1.54 | 1.28 | 1.18 | 530 | 673 | 942 |
| 514 | 1.14 | 1.37 | 1.68 | 948 | 1159 | 1648 |
| 520 | 1.84 | 1.27 | 1.14 | 883 | 1083 | 1185 |
| 537 | 1.05 | 1.31 | 1.28 | 647 | 876 | 973 |
| 547 | 1.26 | 1.32 | 1.20 | 496 | 762 | 980 |
| 2 Negative control | | | | | | |
| sheep 506 | 0.96 | — | 0.82 | 834 | — | 928 |
| 527 | 0.88 | — | 0.98 | 1255 | — | 1469 |
| 528 | 1.21 | — | 1.04 | 927 | — | 1219 |
| 559 | 1.02 | — | 0.94 | 1099 | — | 1204 |
| 3 Copper + ferrous sulfate 2.6 g/day | | | | | | |
| sheep 502 | 1.18 | 1.26 | — | 1272 | 1402 | — |
| 517 | 1.00 | 0.98 | 0.90 | 1117 | 1198 | 1296 |
| 541 | 0.88 | 0.82 | — | 458 | 592 | — |
| 544 | 0.84 | 1.50 | 0.86 | 524 | 575 | 694 |
| 558 | 0.96 | 0.92 | 0.89 | 852 | 898 | 1080 |
| 4 Ferrous chloride 2.6 g/day | | | | | | |
| sheep 508 | 1.30 | — | 1.16 | 1110 | — | 1177 |
| 510 | 0.85 | — | 0.90 | 855 | — | 970 |
| 524 | 1.10 | — | 0.78 | 804 | — | 828 |
| 540 | 0.90 | — | 0.97 | 736 | — | 758 |
| 5 Ferrous sulfate 5.2 g/day | | | | | | |
| sheep 509 | 0.98 | 1.47 | 1.20 | 724 | 788 | 700 |
| 538 | 1.26 | 0.94 | 0.79 | 1500 | 1405 | 1293 |
| 562 | 1.09 | 1.30 | 0.92 | 1080 | 1041 | 1101 |
| Means | | | | | | |
| Copper | 1.37 | 1.31 | 1.30 | 701 | 911 | 1146 |
| Negative control | 1.02 | — | 0.94 | 1029 | — | 1205 |
| Copper + ferrous sulfate 2.6 g/day | 0.97 | 1.10 | 0.88 | 845 | 933 | 1023 |
| Ferrous chloride 2.6 g/day | 1.04 | — | 0.95 | 876 | — | 933 |
| Ferrous sulfate 5.2 g/day | 1.11 | 1.24 | 0.97 | 1101 | 1078 | 1031 |

16 September 1965 to 6 October 1965), the increase in liver copper was significantly less than in group 1 ($P < 0.005$). After 6 October 1965, the increase in mean liver copper in the three remaining sheep of group 3 was 133 ppm against an increase of 235 ppm in group 1, but the difference between the two groups was not significant. However, a value of copper concentration in the liver at the end of the experiment could be estimated for each of sheep 501 and 541 from the increase in liver copper concentration in each sheep during the first period and the duration of that period. When this was done (estimated values 1545 ppm and 746 ppm for sheep 501 and

*Fig. 10. Change in liver copper concentration (in ppm) as a percentage of total copper intake (in mg) during the second experiment with sheep.*




541, respectively), group 3 showed a statistically significant difference in the rate of liver storage of copper during the whole experiment in comparison with group 1 ($P < 0.025$). This was also the case when both sheep were excluded ($P < 0.05$).

The increase in mean liver copper in group 4 during the whole experiment was lower than that in group 2 and the difference between the two groups was nearly significant ($0.05 < P < 0.10$). There was no significant difference in the rate of liver storage of copper between group 3 with iron and group 2 without, despite the higher copper intake by the former group.

In group 5, liver copper gradually decreased during both periods of this experiment and the difference from group 2 at the end of the experiment was significant ($P < 0.05$). Depletion of the copper never actually occured in any other group or sheep in either experiment whether receiving copper supplements or not. This was also clear after 6 October 1965 in sheep 502 from group 3 which after that date and till the end of the experiment received only 5.2 g iron daily from ferrous sulfate and no supplemental copper.

No conclusions could be drawn from the changes in blood copper in the different groups.

The results are not completely in agreement with those of DICK (1954b) who found that iron (4 g per sheep per day as ferrous sulfide) limited copper storage only when administered with copper (30 mg per sheep per day). My experiment included two groups, one receiving 5.2 g iron as ferrous sulfate, and the other 2.6 g

iron as ferrous chloride. No supplemental copper was given to either group. At the end of the experiment, there was a less liver storage of copper in both groups than in the control group. Indeed in the group given most iron, copper stores already present in the liver were depleted.

## 5.4 General discussion of the experiments with sheep

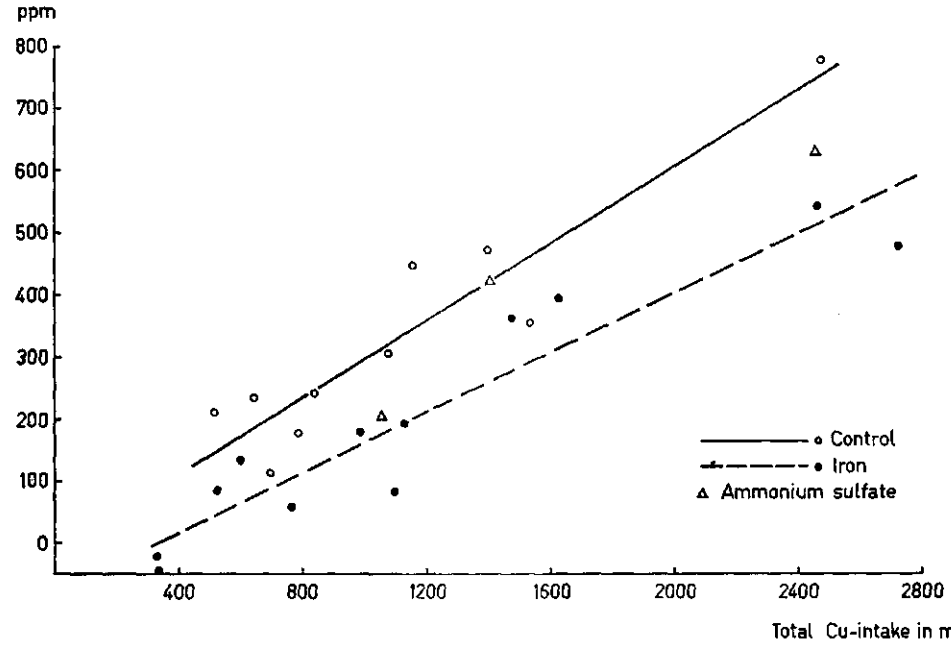
The first experiment has clearly shown that iron, either as ferrous sulfate or as ferrous chloride, providing 2.6 g iron per sheep per day significantly hindered utilization of a copper supplement as indicated by copper storage in liver. The results for group 5 during the first experiment indicated that the slower storage in liver in group 3 was largely due to iron and not to sulfate. But the mean values obtained, and my results with cows given ammonium sulfate, and those of others (HARTMANS and VAN DER GRIFT, 1964) with other sulfate sources, would suggest that sulfate might also have promoted the effect of iron.

The second experiment confirmed the results obtained during the first and showed that the proportional intake of copper and iron determines the extent of interference by iron in copper storage in liver. This is apparent from differences in copper intake and change in the mean liver copper in group 3, 4 and 5.

Despite all efforts, intakes of concentrates were less in groups given iron because the sheep refused to eat them completely, especially in the first experiment. DICK (pers. comm., 1966) encountered similar difficulty with ferrous sulfate but not with saccharated ferrous carbonate. The difference between groups in copper intake hindered comparison of the figures for liver copper. To alleviate this difficulty, the actual copper intakes were calculated from records of food intake for each sheep. Most workers have expressed chemical values as contents in the whole liver. The weight of liver was assumed to be a certain proportion of bodyweight or to be correlated to bodyweight by a regression. However I found that liver weight was not a constant proportion of live weight or dead weight and its rate of growth varied within and between groups of sheep. I have therefore compared total copper intakes with copper concentration, per unit dry weight of liver. I believe this procedure has given more accurate results and avoids the errors likely to have occurred if the total copper content of whole liver was calculated.

When total intake of copper during each period was plotted against change in liver copper in groups without iron, a direct relation was found with the highly significant linear regression $\bar{y} = 0.3095\ \bar{x} - 11.54$. The same applied to the iron groups where the regression equation was $\bar{y} = 0.2412\ \bar{x} - 79.95$. These two regressions are shown in Fig. 11. Data from both experiments, and from the groups with both intakes of iron were used to calculate the regression. The triangles in Fig. 11 are those for group 5 during the first experiment. They are too few to calculate a regression but their pattern in the figure is suggestive. These curves completely confirm the conclusion that iron has hindered liver storage of copper: as can be seen, the mean rise in liver copper concentration at a given copper intake

*Fig. 11. Regression of copper intake on change in mean liver copper concentration in the iron and control groups. Hollow triangles represent observations in the ammonium sulfate group during the first experiment. For regression formulae see text.*




is much higher without iron than with it.

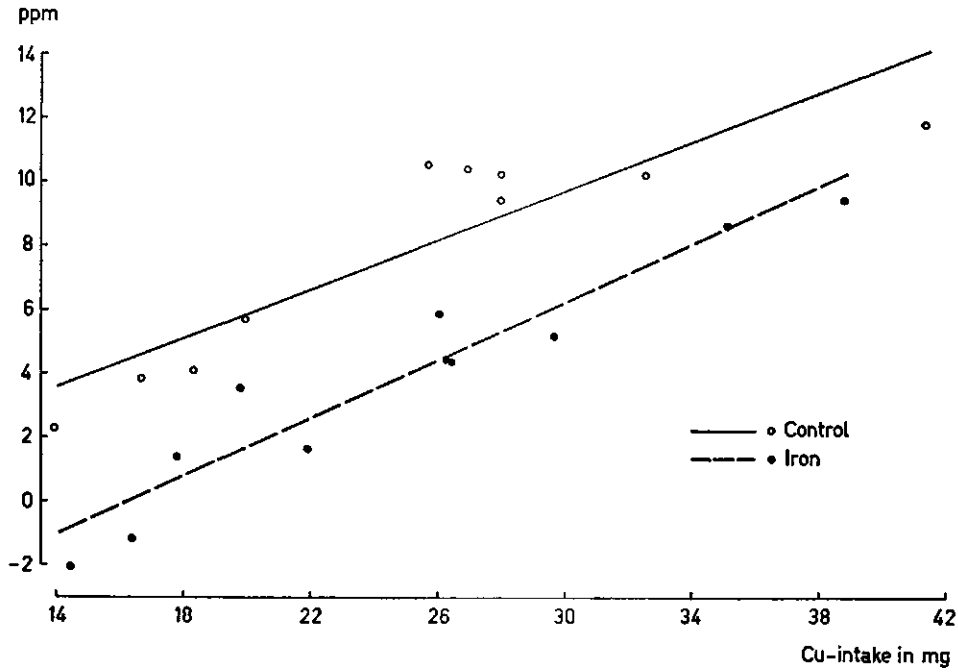
A quantitative relationship between interference by iron and the extra copper needed to counteract this interference (expressed in daily intake) could be found if the length of the period of intake was considered. The two resulting regressions are shown in Fig. 16. The formulae for these regressions are $\bar{y} = 0.3783\ \bar{x} - 1.673$ and $\bar{y} = 0.4506\ \bar{x} - 7.320$ for the control and iron groups, respectively. These regressions again confirm the depression by iron of liver copper storage, and show that this effect decreased considerably at higher intakes of copper. The high copper intake during the second period of the first experiment (38 mg per sheep per day) may account for the smaller effect of iron than in the first period of the same experiment (mean copper intake 20 mg per sheep per day).

These regressions will provide a mean for estimating the physiological requirement of copper in sheep. From the regression formula for control sheep an intake of 4.4 mg copper per sheep per day is sufficient for maintenance. For the iron groups maintenance requires 16.2 mg copper per sheep per day, indicating that, if the ration supplies excess iron, significantly more copper must be present for a positive copper balance.

The requirement of 4.4 mg copper per sheep per day, calculated for control sheep, agrees well with estimates by other workers.

The nature of the diet affects the copper requirement; this explains the high

*Fig. 12. Regression of daily change in mean liver copper concentration on daily copper intake in the iron and control groups. For regression formulae see text.*




figures reported by some authors and the lower figures reported by others. According to BECK (1941, 1951), pastures containing 4-6 ppm copper in dry matter and low in molybdenum supply enough copper to meet the requirements of English purebred and crossbred sheep. This corresponds to a daily intake of approximately 4-6 mg copper. DICK (1954b) estimated the requirement of crossbred sheep at 1 mg copper daily, or less. My experiment cannot indicate whether antagonists were present in the basal ration in sufficient amount to affect copper requirement. Our figure of 4.4 mg conforms with that (4-6 mg) generally accepted for sheep under natural conditions (UNDERWOOD, 1962). It is also in accordance with that reported by MURTY (1957) who obtained a regression equation relating copper retention to copper intake. According to his equation, the maintenance requirements, taken as the intake corresponding to zero retention, equals 3.85 mg per sheep per day.

In the first experiment I only once tried to estimate copper in rumen fluid. Samples of rumen fluid from sheep in groups 1, 2, 3 and 4 were withdrawn by stomach tube. Equal amounts from the samples were pooled for each group and copper was estimated in the fluid phase of the composite sample. For groups 1, 2, 3 and 4 this was 612, 938, 538 and 775 $\mu$g copper per liter, respectively. The mean copper intakes during that day were 12 mg for the first group and 36 mg for the other three groups. These data harmonize with the results of the biopsy but are too limited to permit any conclusion on how iron works. The possibility of iron af-

fecting the rumen pH is doubtful in view of the absence of such a change in cows given excess iron (COUP and CAMPBELL, 1964). Perhaps iron diminished the solubility of copper within the digestive tract.

DICK (1954b) found that both ferrous sulfate and ferrous sulfide hindered copper storage in liver. He regarded the results with sulfate as having arisen from the combined effect of sulfate and molybdenum which was 'certainly present in the ration'. The positive outcome obtained with ferrous sulfide was ascribed to the liberation of hydrogen sulfide which precipitated the physiologically inactive copper sulfide. But his failure to secure a similar result with other sources of sulfur and the results of my experiments implies that iron must be another element complicating copper storage in the liver of sheep. In the experiment of DICK, part of the observed effect may have been due to interaction of molybdenum and sulfate but, in our experiment, this seems unlikely because of the changes observed in blood and copper level in the groups given sulfate, and the minute molybdenum content of the ration. Neither is sulfate likely to be the major cause of the decrease in copper storage in the liver in group 3 during the first experiment and in group 3 and 5 during the second experiment. Copper storage in liver was also hindered with ferrous chloride.

The results for group 4 during the second experiment indicate that a considerable amount of copper was stored in liver when sheep ate 13-16 mg copper and 2.6 g iron per day. Storage was, however, less than in controls. At the same copper intake, stores already present in liver were depleted only when iron intake was increased to 5.2 g per sheep daily. Such intakes of iron are exceedingly high and would probably never occur except in industrially contaminated pastures. Recently, COUP and CAMPBELL (1964) described a condition characterized by scouring, decrease in milk production and bodyweight and coat changes in cows grazing pastures irrigated with bore water rich in iron. By comparing cows receiving much iron, they concluded that iron caused the symptoms. Perhaps less iron still complicates copper storage, especially if copper intake is marginal. More study of copper status of sheep and cattle under different dietary conditions and with different intakes of iron is needed before definite conclusions can be drawn.

## Summary

In the Netherlands, the copper status of cattle decreases during summer, when cows are turned out to pasture and increases during winter when they are stall-fed, in spite of the very often equal copper intakes in both seasons. Similar disorders in grazing livestock, in which copper deficiency occurs while the animal is ingesting amounts of copper generally accepted to exceed its requirement, have been reported from different parts of the world. In all these cases it was suggested that pastures contain some factors which interfere with copper utilization and storage in the animal. In some areas molybdenum, either alone or together with inorganic sulfate, was indentified as the factor interfering with copper storage. However, judging by the occurrence of conditioned hypocuprosis on pasture of normal molybdenum and sulfate content, it is apparent that still other factors must be involved.

In Chapter 2 the literature relative to the factors which affect copper storage has been reviewed.

In the Netherlands, conditioned hypocuprosis occurs in cattle grazing on pastures low in molybdenum content. This would tend to suggest that molybdenum can not be incriminated as the cause of the observed hypocuprosis in this country. Further, hay from affected pastures is curative in spite of the similar copper content. This indicates that the factor or factors involved are only present in grass and disappear during drying.

Chapter 3 describes conditioned hypocuprosis in the Netherlands, hypotheses advanced by Dutch workers to explain its occurrence and such work as has been done to verify these hypotheses.

The experimental part of this work reports the effect on the copper status of stallfed cattle from adding some of the components which are known to be more abundant in grass than in hay. Firstly that fraction was investigated which is broken down in the rumen with the liberation of ammonia and, secondly, the sulfate. In addition an experiment was carried out with sheep to study the effect of excessive iron intakes on copper storage.

*Experiments with cattle* The investigations with cattle consisted of a series of experiments carried out on the same cows (Hoorn experiments) and one on other cows (Wageningen experiment). In the Hoorn experiments the effect of urea, urea and ammonium sulfate and ammonium sulfate alone on copper storage was examined; in the experiment in Wageningen the effect of ammonium sulfate alone or in combination with urea were compared. In both experiments the copper status

of the cattle was assessed by periodic estimation of this element in blood and liver biopsies. In the Hoorn experiments the substances under investigation were administered by slow intraruminal infusion. An infusion apparatus intended for the purpose is described in Section 4.1.

*Hoorn experiments* Six Friesian cows were divided into a control group and an experimental group of three cows each and were fed on a ration supplying daily 145 mg copper, 26 mg molybdenum and 31 gram sulfur per cow.

*Hoorn experiments with urea* It was shown that the ammonia concentration in the rumen liquor from cows on pasture is constantly high during the day, whereas in cows fed indoors similar high values of ammonia are only reached shortly after feeding. Two experiments were carried out, during which urea was administered to one group of cows in such amounts that the concentration of ammonia in the rumen liquor from these cows at any given time corresponded to that observed in pasturing cows. In neither of these experiments could a consistent significant change in liver and blood copper be observed.

*Hoorn experiments with urea and ammonium sulfate* Approximately 42 g sulfur as ammonium sulfate per cow daily, together with urea in amounts sufficient to maintain a constant high concentration of ammonia in the rumen liquor were given by slow intraruminal infusion to one group of cows. After 21 days, copper in the liver of these group had decreased by 22 % but in the controls by only 4 %. The decrease in the experimental group did not reach significance ($0.1 < P < 0.2$). The difference in decrease between the two groups was also insignificant ($0.1 < P < 0.2$).

Cows were then turned to pasture for 48 days. In spite of adequate copper in the pasture, copper in liver decreased drastically in all cows. The mean decrease in liver copper in all cows was 41 % and this decrease was highly significant ($P < 0.001$).

Afterwards the cows returned to the stall for a second experiment with urea and ammonium sulfate lasting 35 days. At the end of this experiment, a significant decrease in liver and blood copper was observed in the cows receiving urea and ammonium sulfate ($P < 0.025$). However, in comparison with the control cows, the differences observed were not significant with the liver, but significant with the blood ($P < 0.05$).

It was suggested that urea and ammonium sulfate together hindered copper storage in the liver.

*Hoorn experiment with ammonium sulfate* Approximately 42 g sulfur as ammonium sulfate was given daily by slow intraruminal infusion. After 35 days mean liver copper in the experimental group had decreased by 21 % but in the controls it had increased by 12 %. Neither of these differences within or between the groups were significant. Blood copper decreased in the experimental cows and the difference from the controls was significant ($P < 0.05$).

But, by comparison with the mean change in the control group in this and earlier experiments, it would appear that sulfate alone hindered liver storage of copper.

*Wageningen experiment* Seven Friesian cows were available and these were divided into a group of three receiving urea and ammonium sulfate and another group of four receiving only ammonium sulfate. There was no control group. The supplements were mixed with the concentrates: 172 g ammonium sulfate supplying 42 g sulfur were administered daily per cow in both groups. Urea was at first 270 g daily per cow in the group receiving urea and ammonium sulfate but when it was noticed that the cows did not eat all their concentrates, this was reduced to half. The daily amount of copper and molybdenum intake by each cow from the ration was calculated at 201 mg and 5.1 mg, respectively.

No consistent changes were observed in the course of liver copper in either group during the experimental period (81 days). There was a trend towards a decrease in the mean value for both groups. A clear decrease in the blood copper was observed in both groups and the decrease was nearly significant in the urea-sulfate group ($0.05 < P < 0.1$). In all seven cows, the decrease in blood copper was significant ($P < 0.025$).

In cows given sulfate, with or without urea, injected $^{64}$copper was less mobile than in control cows. However, the percentage of the dose mobilized to the liver was similar in each case.

It was concluded that the difference in copper and molybdenum intakes or in the method of giving the supplements in Hoorn and Wageningen trials might explain the much lower decrease in liver copper in the last experiment. The importance of molybdenum was emphasized. However, molybdenum alone would not fully explain this difference. It was also maintained that other factors may probably still be involved.

In view of the decrease in blood copper which occurred when ammonium sulfate was given either orally or by infusion, irrespective of the change in liver copper and molybdenum intake, it was concluded that sulfate has two independant effects on copper metabolism, one in the digestive tract which results in the decrease of copper absorption and consequently the gradual depletion of liver copper and the other in the intermediate metabolism decreasing blood copper. It appeared that only the first effect is augmented by molybdenum.

Despite the similar size of decrease in liver copper with sulfate as with cattle turned to pastures, it was concluded that this factor may only partly contribute to the decrease in liver copper in pasture. The early decrease in blood copper when sulfate with of without urea, was given, which is not paralleled in grazing cows, and the failure to confirm a decrease in liver copper in the Wageningen experiment suggest that still other factors are involved.

*Experiments with sheep* Two experiments served to investigate the effect of two sources of iron, namely ferrous sulfate and ferrous chloride on copper storage in sheep. The copper status of sheep was assessed periodically in blood and liver biopsies. Section 5.1 describes a method for carrying out liver biopsy in the standing sheep.

In the *first experiment* lambs were distributed into five groups of five as negative or positive controls (1 and 2), or with ferrous sulfate (3), ferrous chloride (4) or ammonium sulfate (5). Supplementary copper as copper sulfate was mixed with the concentrates of the last four groups. Iron was given for the most part as a drench supplying 2.6 g iron per lamb per day. Total copper intakes of individual sheep were calculated. The experiment was concluded two and a half weeks earlier in group 2 and 5 and this, together with the differences in intake of concentrates, led to a difference in the total copper intake between the different groups. Any resulting differences in copper status would not interfere with the conclusions drawn, since the iron groups ingested totally more copper than their controls.

At the end of the experiment, there was highly significantly lower rate or storage of liver copper in the groups given copper and iron than in the group given only copper ($P < 0.005$). No significant difference could be detected in rate of accumulation of liver copper between groups 2 and 5. It was concluded that iron limits copper storage in the liver.

The *second experiment* was a continuation to the first one. Again five groups were available, treated as follows: group 1 receiving copper, group 2 negative control, group 3 receiving copper and ferrous sulfate (2.6 g Fe per sheep per day), group 4 receiving ferrous chloride in the same iron dose and no copper and group 5 receiving ferrous sulfate (5.2 g Fe per sheep per day) and no copper. All supplements including the copper were given as a drench.

The results of this experiment confirmed the earlier conclusion that excess iron interferes with copper storage in the liver. Thus it was found that either 2.6 g Fe or 5.2 g Fe limited copper storage in the liver especially at lower copper intakes. In the group given the more iron and no copper supplement, actual depletion of the liver copper stores was observed.

No consistent changes in blood copper which could possibly be related to iron could be observed in either experiment.

When the estimated mean intakes of copper during the different periods of both experiments and the corresponding change in liver copper during the same periods were plotted for the groups which had received no iron, a relation was evident, which followed the regression equation $\bar{y} = 0.3095\ \bar{x} - 11.54$. For the groups which had received iron the relation could be expressed by the regression equation $\bar{y} = 0.2412\ \bar{x} - 79.95$. These two regressions are given in Fig. 15. In daily terms, the two regressions shown in Fig. 16 were obtained (regression equations $\bar{y} = 0.3783\ \bar{x} - 1.673$ and $\bar{y} = 0.4506\ \bar{x} - 7.320$ for the control and iron groups, respectively). Is was calculated that a daily intake of 4.4 mg copper per sheep is sufficient to maintain liver copper under normal dietary conditions. The groups which received iron needed 16.2 mg copper per sheep per day for maintenance.

It was concluded that excess iron interferes with copper storage in the liver but it was maintained that similarly high iron intakes as those given during this experiment will be only rarely encountered under ordinary grazing conditions. Less iron may hinder copper storage when copper intake is marginal.

## Samenvatting

Het is bekend dat de koperstatus van rundvee in Nederland gedurende de zomer als de dieren in de weide grazen, afneemt en gedurende de wintermaanden wanneer zij op stal staan, toeneemt.

Dit ondanks het feit dat de koperopname in beide jaargetijden (d.w.z. op gras, resp. stalrantsoen) vaak vergelijkbaar is. Soortgelijke stoornissen bij grazend vee worden vermeld uit verschillende delen van de wereld: koperdeficiëntie treedt op terwijl het dier koperhoeveelheden opneemt, waarvan men algemeen aanneemt dat zij de behoefte overtreffen. In al deze gevallen werd verondersteld dat gras factoren bevat die de koperbenutting en -opslag in het dier beïnvloeden. In sommige streken werd bewezen dat molybdeen, hetzij alleen, hetzij samen met anorganisch sulfaat, de koperbenutting belemmerde. Maar door het voorkomen van voorwaardelijk kopergebrek in weiden met een normaal molybdeen en sulfaatgehalte moet worden aangenomen, dat nog andere factoren een rol spelen.

In hoofdstuk 2 wordt de literatuur betreffende de factoren die invloed hebben op de koperopslag besproken.

In Nederland komt voorwaardelijke hypocuprose voor bij rundvee dat graast op weiden met een laag molybdeengehalte. Dit lijkt er op te wijzen dat molybdeen niet de oorzaak kan zijn van de waargenomen hypocuprose in dit land. Verder blijkt hooi van weiden waarop voorwaardelijk kopergebrek voorkomt, genezend te werken ondanks het vergelijkbare kopergehalte. Dit wijst er op dat de betrokken factor of factoren alleen aanwezig zijn in het gras en dat hij (zij) verdwijnen tijdens het hooien.

In hoofdstuk 3 worden de voorwaardelijke hypocuprose in Nederland, hypotheses die naar voren zijn gebracht door Nederlandse onderzoekers over de mogelijke oorzaak en het werk dat gedaan is om die hypotheses te toetsen, beschreven.

In het experimenteel gedeelte van deze dissertatie wordt de invloed van het toedienen van sommige componenten, die meer voorkomen in gras dan in hooi op de koperstatus van koeien op stalrantsoen, onderzocht. Allereerst werd die fractie onderzocht waaruit ammoniak vrij komt bij de afbraak in de pens. De tweede onderzochte stof was het sulfaat. Bovendien werd nog een proef gedaan met schapen om het effect van een overmatige ijzeropname op de koperopslag te bestuderen.

*Experimenten met rundvee* De onderzoekingen bij koeien bestonden uit een serie proeven met dezelfde koeien (Hoornse proeven) en één experiment met andere

koeien (Wageningse proef). In de Hoornse proeven werd de invloed van ureum, ureum en ammoniumsulfaat en ammoniumsulfaat alleen op de koperopslag nagegaan; in het Wageningse experiment werd het effect van ammoniumsulfaat alleen of samen met ureum vergeleken. Bij beide proeven werd de koperstatus van de dieren vastgesteld door periodiek dit element te bepalen in het bloed en in leverbiopsieën. Bij de Hoornse experimenten werden de te gebruiken stoffen toegediend door langzame infusie in de pens. Het infusieapparaat dat voor dit doel werd gebruikt, is beschreven in 4.1.

*Proeven te Hoorn* Zes zwartbonte koeien werden verdeeld in een controlegroep en een experimentele groep van 3 koeien elk. De koper-, molybdeen- en zwavelopname per dier per dag op het proefrantsoen bedroeg resp. 145 mg, 26 mg, 31 g.
*Proeven met ureum te Hoorn* De ammoniakconcentratie in pensvocht van koeien in de weide is overdag constant hoog, terwijl bij op stal staande dieren vergelijkbare hoge ammoniakgehalten alleen bereikt worden vlak na het voeren. Twee proeven werden gedaan, waarbij aan één groep koeien ureum werd gegeven in hoeveelheden zodanig, dat de ammoniakconcentraties in het pensvocht van deze koeien op elk moment overéénkwamen met de gehalte in grazende dieren. In beide proeven werden willekeurige, insignificante veranderingen gevonden in het koper in lever en bloed. Toch was er een neiging tot daling van het leverkopergehalte bij de dieren die ureum kregen.

Hierom werd besloten het geven van ureum in de volgende proeven te continueren.

*Proeven met ureum en ammoniumsulfaat te Hoorn* Aan één groep koeien werd dagelijks ongeveer 42 g zwavel per koe in de vorm van ammoniumsulfaat, samen met ureum, door middel van langzame infusie in de pens gegeven. De hoeveelheden ureum was zo groot dat een constant hoge ammoniumconcentratie in het pensvocht werd gehandhaafd. Na 21 dagen was het koper in de lever van deze groep gedaald met 22 %, in de controlegroep slechts met 4 %. De daling bij de proefgroep was echter niet significant ($0{,}1 < P < 0{,}2$). Het verschil in daling tussen de twee groepen was ook insignificant ($0{,}1 < P < 0{,}2$). De koeien kwamen daarna 48 dagen in de weide. Ondanks een adequaat kopergehalte van het gras nam het kopergehalte in de lever bij alle koeien drastisch af. De gemiddelde daling van het koper in de lever berekend over alle koeien was 41 % en deze daling was zeer significant ($P < 0{,}001$).

Daarna kwamen de koeien weer op stal voor een tweede proef met ureum en ammoniumsulfaat die 35 dagen duurde. Aan het eind van dit experiment werd een significante afname van het koper in de lever en het bloed waargenomen van die koeien die ureum en ammoniumsulfaat kregen ($P < 0{,}025$). In vergelijking met de controlegroep waren de waargenomen verschillen echter niet significant in de lever, doch wel wat betreft het bloed ($P < 0.05$).

Het lijkt dus waarschijnlijk dat ureum en ammoniumsulfaat samen de koperopslag in de lever hebben geremd.

*Proef met ammoniumsulfaat te Hoorn* Aan elke koe werd per dag ongeveer 42 g

zwavel in de vorm van ammoniumsulfaat gegeven door middel van pensinfusie. Na 35 dagen was het gemiddelde kopergehalte in de lever gedaald met 21 %, maar bij de controlegroep was het toegenomen met 12 %. Geen van deze verschillen in of tussen de groepen was significant. Het kopergehalte in het bloed van de proefkoeien nam af en het verschil met de controledieren bleek significant te zijn ($P < 0,05$).

Uit een vergelijking van de gemiddelde verandering bij de controlegroep in deze proef en in eerder genomen experimenten, blijkt dat sulfaat het opslaan van koper in de lever remde.

*Proef te Wageningen* Zeven zwartbonte koeien waren beschikbaar en deze werden verdeeld in een groep van 3 dieren die ureum en ammoniumsulfaat kregen en een tweede groep van 4 dieren die alle ammoniumsulfaat kregen. Er was geen controlegroep. De supplementen werden gemengd met het krachtvoer; per koe per dag werd 172 g ammoniumsulfaat (d.w.z. 42 g zwavel) verstrekt aan beide groepen. Er werd in het begin dagelijks 270 g ureum per koe gegeven in de groep die ureum en ammoniumsulfaat kreeg, maar toen bleek dat de koeien niet al hun krachtvoer opnamen werd dit tot de helft teruggebracht. De dagelijkse hoeveelheid koper en molybdeen per koe in het rantsoen werd berekend op respectievelijk 201 mg en 5,1 mg. In geen van beide groepen werden vaste veranderingen waargenomen in het verloop van het leverkopergehalte tijdens de proefperiode (81 dagen). Bij beide groepen vertoonde de gemiddelde waarde een neiging tot dalen. Een duidelijke daling van het bloedkopergehalte trad op bij beide groepen en die afname was bijna significant in de ureumsulfaatgroep ($0,05 < P < 0,1$). In alle zeven koeien was de daling van de koperspiegel van het bloed significant ($P < 0,025$).

In koeien die sulfaat kregen, met of zonder ammoniak, was geïnjecteerd $^{64}$koper minder mobiel dan in controlekoeien. Het percentage van de dosis dat naar de lever werd getransporteerd, was echter in beide gevallen vergelijkbaar.

Het verschil in de opname van koper en molybdeen of in de toedieningsmethoden van de supplementen in de Hoornse en Wageningse proeven zou de veel kleinere daling van het leverkoper in de laatste proef kunnen verklaren. Het belang van molybdeen werd onderstreept, molybdeen alleen echter kon niet volledig verantwoordelijk worden gesteld voor het gevonden verschil. Het blijft tevens waarschijnlijk dat andere factoren hierbij ook een rol spelen. Gezien de verlaging van de bloedkoperspiegel die optrad als ammoniumsulfaat, hetzij oraal, hetzij door infusie, werd gegeven, ongeacht de veranderingen in het leverkopergehalte en de molybdeenopname, werd de conclusie getrokken, dat sulfaat twee onafhankelijke effecten heeft op het kopermetabolisme. Ten eerste doet het de koperresorptie in het spijsverteringskanaal afnemen met als gevolg een geleidelijke uitputting van het leverkoper. Ten tweede wordt het intermediair metabolisme beïnvloed, waardoor het bloedkoperniveau wordt verlaagd. Het bleek, dat alleen het eerste effect door molybdeen wordt versterkt.

Ondanks het feit, dat de verlaging van het gehalte aan koper in de lever even groot was met sulfaat dan wanneer het vee in de weide liep, werd geconcludeerd

dat deze factor slechts ten dele bij zou dragen tot de leverkoperafneming in de weide. Het al zeer gauw afnemen van het bloedkoper wanneer sulfaat, met of zonder ureum, wordt verstrekt (wat zich niet voordoet bij koeien in de weide) en het feit, dat de afneming van het koper in de lever niet werd bevestigd in het Wageningse experiment, wijzen er op dat er nog andere factoren bij betrokken zijn.

*Proeven met schapen* Twee proeven werden gedaan om het effect van twee ijzerbronnen, nl. ferrosulfaat en ferrochloride, op de koperopslag in schapen na te gaan. De koperstatus van schapen werd periodiek vastgesteld aan de hand van het gehalte in het bloed en in leverbiopsieën. In 5.3 wordt een methode beschreven om leverbiopsie uit te voeren bij staande schapen. In de eerste proef werden de lammeren onderverdeeld in 5 groepen van vijf, waarbij een negatieve en een positieve controlegroep (1 en 2), een groep die ferrosulfaat kreeg (3), een die ferrochloride kreeg (4) en tenslotte een groep waaraan ammoniumsulfaat verstrekt werd (5). Een kopersupplement in de vorm van kopersulfaat werd voor de laatste 4 groepen gemengd met het krachtvoer.

Gedurende het grootste gedeelte van de proef werd de dieren ijzer in vloeibare vorm ingegeven. Ieder lam kreeg 2,6 g ijzer per dag. De totaal opgenomen hoeveelheden koper in de verschillende groepen werden berekend uit de individuele koperconcentratie van de rantsoenen.

Het experiment werd met de groepen 2 en 5 twee en een halve week eerder beëindigd. Dit samen met de opnameverschillen voor krachtvoer leidde tot een verschil in de totaal opgenomen koperhoeveelheden tussen de verschillende groepen. Wat voor verschil in koperstatus dan ook hieruit resulteert zal echter geen invloed hebben op de conclusies, daar de groepen die ijzer kregen in totaal meer koper opnamen dan hun controledieren.

Aan het eind van de proef was er een zeer significant lagere koperopslag in de lever in de groepen die koper en ijzer kregen t.o.v. de groep die alleen koper kreeg ($P < 0{,}005$). Er werd geen significant verschil gevonden in de ophoping van leverkoper tussen de groepen 2 en 5. Dus moet de conclusie luiden dat ijzer de koperopslag in de lever belemmerde.

Na afloop van de eerste proef bleek het kopergehalte van de lever bij de dieren uit de groepen 2 en 5 enorm te zijn verhoogd. Er bestond gevaar voor kopervergiftiging wat werd bevestigd door de bepaling van de serum-glutamine-oxaalazijnzuur-transaminase activiteit. Daarom werd besloten, ten einde het leverkopergehalte te verlagen, de dieren in de weide te brengen tot het begin van de tweede proef.

De tweede proef was een vervolg op de eerste. Dezelfde vijf groepen werden gebruikt. De dieren uit de groepen 2 en 5 werden echter opnieuw ingedeeld. Groep 1 kreeg koper, groep 2 was de negatieve controle, groep 3 kreeg koper en ferrosulfaat (2,6 g Fe per schaap per dag), groep 4 ferrochloride, dat dezelfde hoeveelheid ijzer bevatte, en geen koper en groep 5 ferrosulfaat (5,2 g Fe per schaap per dag) en geen koper. Alle supplementen, het koper inbegrepen, werden in vloeibare vorm ingegeven. De resultaten van dit experiment bevestigden de eerder getrokken conclusie

dat een overmaat ijzer de koperopslag in de lever belemmert. Op deze manier werd gevonden dat zowel 2,6 g als 5,2 g ijzer de koperopslag in de lever remt, speciaal als de koperopname laag is. In de groep waaraan meer ijzer en geen koper werd verstrekt, werd zelfs een vermindering van de leverkopervoorraad waargenomen.

Er werden in geen van beide proeven vaste veranderingen in het bloedkopergehalte gevonden die verband zouden kunnen houden met het verstrekte ijzer.

Wanneer de gemiddelde opnamen van koper gedurende de verschillende perioden van beide proeven werden uitgezet tegen de daarmee verband houdende veranderingen in het leverkopergehalte gedurende diezelfde perioden voor die groepen die geen ijzer kregen, was er een duidelijke relatie volgens de regressievergelijking $\bar{y} = 0{,}310\ \bar{x} - 11{,}54$. Wat betreft de groepen waaraan wel ijzer werd gegeven, kon de relatie worden uitgedrukt in de regressievergelijking $\bar{y} = 0{,}241\ \bar{x} - 79{,}95$. Deze twee regressielijnen worden gegeven in fig. 15. Indien men de resultaten per dag berekent, worden de twee regressielijnen uit fig. 16 verkregen (regressievergelijkingen $\bar{y} = 0{,}378\ \bar{x} - 1{,}673$ en $\bar{y} = 0{,}451\ \bar{x} - 7{,}320$ voor de controle- en de ijzergroepen, respectievelijk).

Bij extrapolatie wordt gevonden dat een dagelijkse opname van 4,4 mg koper per schaap voldoende is bij normale rantsoenen om de leverkopervoorraad op hetzelfde niveau te handhaven. De groepen waaraan ijzer werd gegeven, hadden daarvoor 16,2 mg koper per schaap per dag nodig. Hieruit volgt, dat een overmaat ijzer de koperopslag in de lever belemmert, maar we kunnen vaststellen dat een ijzeropname van vergelijkbare grootte als gedurende deze proef werd gegeven bij normaal grazen zelden voorkomt.

Minder ijzer zou echter de koperopslag kunnen belemmeren als de koperopname marginaal is.

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