Haemolytic anaemia in ruminants fed forage brassicas: a review

Sophie Prache

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Review article

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S Prache

INRA-Theix, Laboratoire Adaptation des Herbivores aux Milieux, 63122 Saint-Genès-Champanelle, France

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Summary — This paper describes the present state of knowledge on metabolic disorders caused by the intake of forage brassicas, particularly the cellular and molecular changes leading to haemoglobin oxidation and precipitation as Heinz-Ehrlich bodies and to haemolysis. The factors responsible for variations in the haemolysis severity are then discussed as well as the measures for preventing pathological risks. Emphasis is placed on the identification of topics which need increased research activity.

forage brassica / haemolysis / anaemia / *S*-methylcysteine sulphoxide (SMCO) / ruminant

Résumé — L'anémie hémolytique chez les ruminants consommant des crucifères fourragères : revue bibliographique. *Ce texte présente d'abord les connaissances actuelles concernant les perturbations métaboliques provoquées par la consommation de crucifères fourragères, en particulier les perturbations cellulaires et moléculaires qui conduisent à l'oxydation de l'hémoglobine, à sa précipitation sous forme de corps de Heinz-Ehrlich et à l'hémolyse. Il examine ensuite les facteurs de variation de l'intensité de l'hémolyse, et présente les mesures de prévention actuellement connues qui permettent de limiter les risques pathologiques. Les points qui demeurent obscurs et nécessiteraient un effort de recherche sont soulignés.*

crucifère fourragère / hémolyse / anémie / S-méthylcystéine sulphoxide (SMCO) / ruminant

INTRODUCTION

Forage brassicas can play an important role in ruminant feeding. They can be grown between 2 main crops (during the summer or autumn/winter), they constitute a feed with a high nutritional value and they can be grazed *in situ* at periods when more traditional forage species are less abundant or of lower quality, which permits a reduction in the purchase of concentrates.

However, it is not recommended to use them without restriction, because of their typical antinutritional sulfur compounds: glucosinolates, which are goitrogenic, and *S*-methylcysteine sulfoxide (SMCO), which is

responsible for anaemia. The clinical signs associated with the ingestion of forage brassicas are: loss of appetite and digestion dysfunction; anaemia-linked disorders; a fall in the performance (milk yield, growth rate in the young, reproductive disorders); lesions in the liver and kidneys; reduction of wool growth with occurrence of bald areas; a photosensitization with an eventual pruritus; and sometimes a partial loss of the ears (particularly in sheep). In very susceptible species, such as the roe deer, cachexia has also been observed as well as lesions of the central nervous system which may lead to changes in visual and acoustic perceptions and to locomotion disorders inducing changes in the behaviour (Fehlberg *et al*, 1989). The disorders induced by these toxins (glucosinolates and SMCO) may develop separately, but some of them, such as icterus, fall in performance and reproductive disorders, seem to be due to the simultaneous action of both toxins (Giovanni, 1978; Smith, 1980). The severity of the disease is variable, but the condition may be fatal.

The aetiology of haemolytic anaemia and the different factors affecting its severity is the subject of this review.

The nature and severity of clinical signs associated with haemolysis can be varied: weakness, anaemia, icterus, elevation in heart and respiration rates partly for compensating the decreasing number of red blood cells, and a fall in performance. Haemoglobinuria can be observed in the most severe cases (Onderscheka *et al*, 1987a) and a massive prehepatic icterus, which can be fatal (Onderscheka *et al*, 1987a; Fehlberg *et al*, 1989). In the roe deer, hypoxia is suspected to be responsible for lesions of the central nervous system (Fehlberg *et al*, 1989). A subclinical icterus can also lead to carcass withdrawal or grading down because of a fat discolouration, particularly in sheep where cut pieces are not dressed and the fat tissue is normally white (Prache *et al*, 1990).

The severity of haemolytic anaemia is variable, but it can be fatal in the roe deer in which the hematocrit may drop by 75% from the normal values (Fehlberg *et al*, 1989). The most characteristic early symptom of the disease is the appearance of Heinz-Ehrlich bodies within the red blood cells a few days to 3 weeks after the beginning of feeding. These stainable granules located at the periphery of the cell are deposits of denatured methaemoglobin. They result from the irreversible oxidation of haemoglobin by dimethyl disulfide (CH_3-S-S-CH_3; DMDS), the secondary haemolysin produced in the rumen by fermentation of SMCO, the primary haemolysin present in forage brassicas.

GENERAL PRINCIPLES OF OXIDATIVE HAEMOLYSIS WITH FORMATION OF HEINZ-EHRLICH BODIES

Red blood cells are almost totally filled with haemoglobin (95%). The other components (5%) seem to be involved in the protection of haemoglobin from oxidation (Varet, 1990). The haemoglobin molecule consists of 4 polypeptide chains, 2 α-chains and 2 β-chains. These chains are folded in tridimensional structures. Each chain contains a heme, a small molecule including an atom of iron (Cohen-Solal and Dreyfus, 1990). Normally, the only bond between the heme and the surrounding chain is the bond between the heme iron and proximal histidine (Perutz, 1990). Two other amino acids, distal histidine and distal valine, are close to the heme but do not bind to it; distal histidine binds the oxygen fixed to the heme by a hydrogen bond (Perutz, 1990).

Hemichromes are the main constituents of Heinz bodies (Rachmilewitz *et al*, 1971; Winterbourn and Carrel, 1974; Winterbourn, 1990). The latter are composed of ferric haemoglobin, when the tertiary structure of the molecule becomes unstable and the dis-

tal histidine (reversible hemichrome) or an external ligand (irreversible hemichrome) occupies the sixth coordination position of the ferric heme (Winterbourn and Carrell, 1974; Winterbourn, 1990). Hemichromes are unstable and tend to precipitate to form Heinz bodies (Rachmilewitz *et al*, 1971; Perutz, 1990).

The amount of hemichromes present in red blood cells depends on 3 factors: 1) the rate of formation of ferric haemoglobin; 2) the rate of reduction of this protein by the reducing systems of red blood cells; 3) the rate of conversion of ferric haemoglobin to hemichromes. The quantity of hemichromes may increase when the reducing capacity of red blood cells decreases or when either of the other 2 rates increases.

Oxidation, denaturation and precipitation of haemoglobin

Oxidation, denaturation and precipitation of haemoglobin to form Heinz bodies are now well described at the molecular level for unstable human haemoglobins, which present defects in the protein chains (Rachmilewitz *et al*, 1971; Winterboum and Carrell, 1974; Reinhart *et al*, 1986; Winterbourn, 1990; Cohen-Solal and Dreyfus, 1990). However, the sequence of events leading to the formation of Heinz bodies within oxidized red cells (particularly those of ruminants fed on forage brassicas) has never been described at the molecular level. Several hypotheses have been put forward which take into account the similarities between unstable haemoglobins and oxidized haemoglobins in the formation of Heinz bodies (Winterbourn and Carrell, 1974).

Haemoglobin oxidation

This matter is documented particularly well in the publications of Winterbourn *et al* (Winterbourn and Carrell, 1974; Winterbourn *et al*, 1976; Winterbourn, 1990). Haemoglobin undergoes one-electron oxidations, which may take place in the heme (oxidation of heme iron) or in the protein chains (oxidation of thiol groups SH).

Oxidation of heme iron: formation of ferric haemoglobin

The atom of iron is oxidized according to the reaction $Fe^{2+} \longrightarrow Fe^{3+} + e^-$. Haemoglobin is then in the form of ferric haemoglobin or methaemoglobin (MetHb). This oxidation can occur by one of 3 routes. Firstly, even if the metabolism of the red blood cell is normal, oxyhaemoglobin (OxyHb) may be partly dissociated into methaemoglobin + O_2^- (superoxide anion). This natural autooxidation also produces indirectly peroxide H_2O_2 (Barry *et al*, 1981b). Secondly, reactions with the superoxide anion O_2^- or the peroxide H_2O_2 may occur directly or indirectly *via* an inactivation of the reducing systems of the red blood cell. Finally oxidation may occur by reaction with xenobiotics, oxidant drugs such as phenylhydrazine in humans.

Sulfhydryl oxidation: formation of disulfide bonds

In this case, the thiol groups (SH) situated at position 93 of the haemoglobin β chains are oxidized (the other 4 thiol groups of the haemoglobin tetramer are poorly accessible to oxidants). This sulfhydryl oxidation is explained by the role of scavengers of this groups towards the free oxidants, for instance O_2^- or H_2O_2, present or formed during oxidation or precipitation of haemoglobin. Haemoglobin can act as a source of free radicals (O_2^- during the formation of ferric haemoglobin) and as a sink of these radicals (during the sulfhydryl oxidation), and so sulfhydryl oxidation often accompanies the formation of ferric haemoglobin.

Oxidation gives either mixed disulfides (disulfide bonds between a chain of haemoglobin and another sulfur compound),

or interchain disulfide bonds (intra- or intermolecular).

Antioxidant systems of the red blood cell

Normal red blood cells maintain haemoglobin in its functional status (iron in the ferrous status) by: (i) restoring ferric haemoglobin to its ferrous form by reduction, according to the reaction $Fe^{3+} + e^{-} \rightarrow Fe^{2+}$ (Rapoport *et al*, 1968 cited by Rachmilewitz *et al*, 1971); (ii) metabolizing the superoxide anion by reaction with reduced glutathione, and by the superoxide dismutase, gluthatione peroxidase and catalase systems (Winterbourn and Carrell, 1974; Barry *et al*, 1981b); and (iii) maintaining the reducing environment necessary to the haemoglobin stability, by the glucose-6-phosphate dehydrogenase and glutathione reductase system (Barry *et al*, 1981b).

Role of reduced glutathione

Reduced glutathione (GSH) is a potent reductor whose role is to inactivate oxidants by reduction.

Reduction of ferrihaemoglobin

The plasma pH is slightly alkaline (7.35 – 7.45), which facilitates oxidation of ferrous ions. Protection of the heme iron from oxidation into Fe^{3+} requires the presence of a stronger reductor than Fe^{2+}, such as reduced glutathione. When redox couples Fe^{3+}/Fe^{2+} and oxidized glutathione/reduced glutathione are in the presence of each other, the following unilateral reaction is observed: reduced glutathione + Fe^{3+} —> oxidized glutathione + Fe^{2+}

Reduction of superoxide anion O_2^- *(Barry* et al, *1981b)*

Reduced glutathione reacts with superoxide anion and thus plays a role of detoxification of free oxidants (Smith, 1974).

GSH is oxidized and inactivated by these reactions. To maintain its intracellular concentration to a sufficient level, red blood cells generate GSH by stimulating the glucose-6-phosphate dehydrogenase (G-6-PD) and glutathione reductase systems (Smith, 1974; 1980). Heinz bodies may develop if for any reason the G-6-PD activity is impaired or if GSH level in red blood cells is insufficient (Smith, 1974, 1980; Winterbourn, 1990). In Finnish Landrace sheep, for example, an important proportion of animals present considerably less erythrocyte GSH concentration than that normally found in sheep, and the red blood cells of this GSH-low type sheep are more prone to Heinz body formation than those of GSH-high type sheep, even in classic feeding conditions (Tucker and Kilgour, 1973).

Erythrocyte GSH concentration varies with cell age, with the highest concentration in the reticulocytes and the lowest in aging cells (Tucker and Kilgour, 1973).

Role of superoxide dismutase

The cupro-enzyme superoxide dismutase, (EC 1.15.11, SOD) prevents GSH inactivation and haemoglobin oxidation by metabolizing rapidly the superoxide anion (Barry *et al*, 1982).

In newborn humans with low activities of the SOD, Rotilio *et al* (1977) observed Heinz-Ehrlich-body anaemias. Suttle *et al* (1987) first demonstrated that Cu deficiency in lambs grazing grass pasture predisposed the erythrocytes to Heinz-Ehrlich-body haemolysis. They produced lambs of different copper status through breeding or Cu supplementation, and observed that Heinz-body count was highest in the most Cu-deficient lambs and negatively related to SOD activity in the erythrocytes. Cu supplementation increased SOD activity, plasma Cu and blood haemoglobin concentrations, and reduced the proportion of erythrocytes containing Heinz bodies.

The animal body has the ability to store substantial quantities of copper in the liver (Barry *et al*, 1981b). If the copper supply is

lower than the requirements, the liver copper reserves are first affected, then the serum copper levels, and finally the concentrations in the critical sites of biochemical action (Suttle, 1976). According to Barry *et al* (1981b), a severe copper deficiency might cause a reduced SOD blood activity leading to reduced levels of GSH in red blood cells because of the subsequent accumulation of the superoxide anion. This accumulation would greatly accelerate conversion of haemoglobin to methaemoglobin leading to precipitation of the latter as Heinz bodies.

The activity of SOD also depends on the age of the erythrocyte, decreasing as the red blood cell aged (Strange *et al* 1992, in humans).

Role of glutathione peroxidase

The enzyme glutathione peroxidase (EC 1.11.1.9, GSH - Px) completes the detoxification of superoxide initiated by SOD, by catalysing the reduction of hydrogen peroxide, H_2O_2, to water. The unique hydrogen donor is GSH, which is regenerated by reduced nicotinamide adenine dinucleotide phosphate and glutathione reductase.

GSH-Px is the only known mammalian selenium-containing enzyme and its activity depends on Se concentration in blood. Barry *et al* (1981b) observed that blood GSH-Px activity in cattle fed grass pasture or kale was positively related to blood selenium concentration. More precisely, Bauersachs and Kirchgessner (1992) observed that Se deficiency in rats significantly reduced Se concentration and GSH-Px activity in serum, and that an excessive Se supply led to an increase in serum Se concentration, but had no more effect on serum GSH-Px activity.

In ruminants grazing grass pasture, Morris *et al* (1984) and Suttle *et al* (1987) demonstrated that Se deficiency predisposed animals to Heinz-Ehrlich-body anaemias, because of a probable deficiency in GSH-Px activity. Suttle *et al* (1987) produced lambs of different Se status through Se supplementation, and observed that lambs not treated with Se had a higher percentage of erythrocyte-containing Heinz bodies and a lower GSH-Px activity in the erythrocytes, although these 2 constituents were not correlated, probably because of the very uniform high and low Se status achieved by the 2 treatments.

The additive nature of the effects of Cu and Se deficiencies on Heinz-body formation is explained by the couple formed by the enzymes SOD and GSH-Px in the detoxification of superoxide (Suttle *et al*, 1987). However, the lesser effect of selenium than copper deficiency on Heinz-body formation might be attributed to the presence of an alternative enzyme, catalase (EC .1.11.1.6), which would compensate in part for the effects of reduced GSH-Px activity (Suttle *et al*, 1987). These authors have shown that Cu and Se supplementation of lambs grazing grass pasture markedly increased SOD and GSH-Px activities in the erythrocytes and had additive effects on the reduction of Heinz-body counts.

There seems to be an equilibrium within the red blood cells between the quantity of ferric hamoglobin spontaneously formed and the restoration of this oxidized material into functional haemoglobin. This equilibrium depends on the rate of conversion of oxyhaemoglobin to ferrihaemoglobin and on the efficiency of the antioxidating systems, which both depend on the superoxide anion O_2^-. If the antioxidating systems are insufficient relative to the oxidative stress undergone by the red blood cell, irreversible oxidative changes may be incurred by the haemoglobin which may be partly precipitated to form Heinz-Ehrlich bodies (Smith, 1974; 1980; Winterbourn, 1990)

Denaturation and precipitation of haemoglobin

The interactions between the α and β subunits of haemoglobin play a very important

role in the stability and function of the molecule. Any alteration in the conformation provokes an instability of the molecule (Rachmilewitz *et al*, 1971; Winterbourn and Carrell, 1974; Cohen-Solal and Dreyfus, 1990). Methaemoglobin is then much less stable than oxyhaemoglobin, with a higher tendency to be converted to hemichrome and to precipitate (Winterbourn and Carrell, 1974; Winterbourn, 1990).

Moreover, the structural modification of the molecule is accompanied by the formation of disulfide bonds within the molecule (Winterbourn and Carrell, 1974) and abundantly between the haemoglobin molecules (Winterbourn and Carrell, 1974; Reinhart *et al*, 1986). Some authors have demonstrated that simple α and β chains and polymers of 5 or 6 chains are bound by disulfide bonds (Winterbourn and Carrell, 1974). Disulfide bonds may also form between the haemoglobin molecule and the cell membrane, but in small quantities (Jacob *et al*, 1968b; Winterbourn, 1990), or between precipitated haemoglobin and reduced or oxidized glutathione, but sometimes in negligible quantities, even in the presence of excess glutathione (Winterbourn and Carrell, 1974).

Formation of disulfide bonds results from the oxidation of thiol groups of haemoglobin. When there is a distorsion of the molecule to form a hemichrome, part of the oxidative agents formed during precipitation may react with the precipitating molecule, instead of being released, and cause the regularly observed oxidation of 2 sulfhydryl groups (Winterbourn and Carrell, 1974). The oxidants formed would also react more with the sulfhydryl groups of haemoglobin than with those of GSH (Winterbourn and Carrell, 1974). According to these authors, the formation of mixed disulfides might also take place without oxidation by exchange with oxidized glutathione GSSG.

Haemoglobin precipitation results from the formation of irreversible hemichromes (Rachmilewitz *et al*, 1971; Winterbourn and Carrell, 1974) which are unstable and precipitate rapidly (Winterbourn, 1990) to form Heinz bodies. The contribution of the sulfhydryl oxidation of haemoglobin to precipitation is discussed (Jacob *et al*, 1986b; Winterbourn and Carrell, 1974). Winterbourn and Carrell (1974) suggest that this oxidation would be concomitant to precipitation, rather than being one of its causes. Heinz-body formation would result from the aggregation of precipitated haemoglobin.

With few exceptions, all Heinz bodies are located on the inner surface of the cell membrane. Several hypotheses have been put forward to explain this phenomenon.

1) The outermost layer of haemoglobin is first exposed to the oxidative agent when it diffuses into the red blood cell (Jacob *et al*, 1968a; Reinhart *et al*, 1986).

2) Denatured haemoglobin might be bound to the membrane: by disulfide bonds (Jacob *et al*, 1968a), although Reinhart *et al* (1986) suggest that these bonds are of minor importance; by hydrophobic bonds (Rifkind and Danon, 1965; Winterbourn and Carrell, 1973; Chan and Desforges, 1976); by spectrin, a membrane component located under the lipid layer; or the other membrane proteins (Reinhart *et al*, 1986).

3) It is also claimed that Heinz bodies form in the cytoplasm and migrate to the cell surface with time (Rifkind and Danon, 1965) where they become attached by hydrophobic interactions (Winterbourn and Carrell, 1973).

Removal of red blood cells containing Heinz-Ehrlich bodies

The ultimate cause of destruction of red blood cells after oxidative damage with Heinz-body formation is not yet well understood. Several hypotheses have been put forward.

Vulnerability of the membrane

Membrane vulnerability may be provoked by the peroxidation of membrane phospholipids because of the presence of free oxidants (peroxide and superoxide) (Winterbourn and Carrell, 1974; Suttle *et al*, 1987).

Alteration in the red blood cell membrane permeability

According to studies performed *in vitro* (Jocelyn, 1972), the blockage of the outer -SH groups of the red cell membrane resulting from the formation of disulfide bonds between membrane proteins and precipitated haemoglobin may lead to alterations in the membrane permeability and haemolysis.

Splenic entrapment due to an alteration in red blood cell deformability

As red blood cells have a larger diameter than some capillaries, they possess a high deformability, which facilitates their circulation along those capillaries. However, it has been suggested that the presence of Heinz bodies within the cell could reduce their deformability (Lubin and Desforges, 1972; Weinstein *et al*, 1975) because of the focal membrane rigidification conferred by their attachment to the membrane (Reinhart *et al*, 1986). This alteration of red blood cell deformability could cause biophysical disturbances in the microcirculation and lead to the sequestration of the red blood cells in the spleen where they would undergo a phagocytosis (Rifkind and Danon, 1965; Weed, 1970).

The physical presence of Heinz bodies is not the only factor responsible for the alteration in cell deformability. Other oxidative damage include peroxidation of the membrane phospholipids (Jain and Hochstein, 1979; Johnson *et al*, 1980), changes in the organization of the phospholipids within the membrane (Jain, 1985), loss of potassium and water (Orringer and Parker, 1977) and intracellular accumulation of calcium due to the inhibition of calcium-ATPase (Shalev *et al*, 1981).

Recognition by macrophages of the membrane areas with attached Heinz bodies

The above hypothesis has been refuted by some authors (Reinhart *et al*, 1986) who have shown that *in vitro* cell deformability may be affected only when the degree of coating of the membrane endoface by Heinz bodies is very high, which is never observed *in vivo*. According to these authors, red blood cells including Heinz bodies are thus most likely not sequestered in the spleen because of their filterability characteristics. It seems more likely to these authors that macrophages recognize the areas of attachment of Heinz bodies to the membrane, and depending on the size of the involvement, this results in either a focal pitting or an extended engulfment with subsequent destruction of the whole red blood cell.

Oxidation, denaturation and precipitation of haemoglobin and subsequent haemolysis thus constitute very complex phenomena and have not yet been totally elucidated. The next part of the paper will deal with the occurrence of these phenomena in connection with the intake of forage brassicas.

HEINZ-EHRLICH BODY ANAEMIAS ASSOCIATED WITH THE INTAKE OF FORAGE BRASSICAS IN RUMINANTS

The development of Heinz-Ehrlich-body anaemias in ruminants fed on forage brassicas largely results from the depletion of the red blood cells in protective mechanisms against naturally formed oxidants (superoxide anion O_2^-), particularly in erythrocyte GSH concentration. Several factors are involved in this depletion.

Factors involved in the formation of Heinz-Ehrlich bodies

Effects of dimethyl disulfide

Dimethyl disulfide (DMDS) is the secondary toxin produced by ruminal fermentation of SMCO, the primary toxin contained in these plants.

Earl and Smith (1982) suggest that the fate of SMCO ingested by the animal is probably essentially 2-fold: it is degraded either oxidatively after absorption, or by dethiolmethylation, the latter being the only pathway leading to the formation of DMDS and to haemolysis. Dethiolmethylation occurs mainly in the rumen where only 4 microorganisms possess a SMCO-lyase activity (*Lactobacillus, Veillonella alcalescens, Peptostreptococcus elsdenii* and *Anaerovibrio lipolytica*) (Smith, 1974). The affinity of DMDS for the red blood cell is very high (Earl and Smith, 1982). Within the red blood cell, it may rapidly participate in disulfide exchange reactions with essential sulfydryl groups.

GSH oxidation by DMDS

Smith *et al* (1974) suggested that a possible mode of action of DMDS in causing kale anaemia may be to convert reduced glutathione to oxidized glutathione, itself becoming reduced to methanethiol, according to the following route:

$$2GSH + CH_3\text{-}S\text{-}S\text{-}CH_3 \longrightarrow GSSG + 2CH_3SH$$

GSH = reduced glutathione
CH_3-S-S-CH_3 = DMDS
GSSG = oxidated glutathione
CH_3SH = methanethiol

Blockage of the outer-SH groups of the red cell membrane by DMDS

Another hypothesis put forward by Smith *et al* (1974) was that DMDS might react with the outer -SH groups of the membrane to give a mixed disulfide. According to *in vitro* observations (Jocelyn, 1972), it has been suggested that blockage of these groups may alter membrane permeability and lead to loss of capacity of the red blood cells to retain haemoglobin.

Reduction in GSH regeneration

The glutathione reductase is a thiol enzyme which transfers the hydrogen from NADPH to oxidated glutathione, leading to the regeneration of reduced glutathione. *In vitro* studies with the thiol enzyme papain have suggested that disulfur exchanges resulting from the reaction of DMDS with thiol groups might strongly modify or even inhibit the activity of thiol enzymes (Steven *et al*, 1981).

The potential reactions of DMDS with sulfydryl groups are however speculations, without sufficient experimental evidence to support them. The extent to which reactions observed *in vitro* operate *in vivo* has, as yet, not been studied; they also need to be critically tested *in vivo* before the mode of toxicity of DMDS is more fully understood.

According to these different hypotheses, the effect of DMDS might be 2-fold. It may oxidize GSH and reduce its regeneration, leading to a decrease in GSH concentration, which lowers the reducing power of the red cell.

Effects of antithyroid compounds

Forage brassicas contain glucosinolates, which are hydrolysed during chewing to give goitrogens. These compounds reduce iodine uptake by the thyroid, cause thyroid enlargement, and reduce its output of thyroxine (T4). Moreover, forage brassicas may be I-deficient (Barry *et al*, 1983a).

Iodine supplementation has been shown to alleviate the goitrogenic effects induced by the consumption of these plants, and also to enable the animals to partially counteract

haemolytic anaemia (Barry *et al*, 1983a, in lambs). These authors observed that I-supplementation of lambs grazing kale, though having no effect on Heinz body counts, increased erythrocyte GSH concentration and packed cell volume, and reduced blood methaemoglobin content. Moreover, compared to their counterparts (pasture-fed or control kale-fed), lambs grazing kale and supplemented with I showed increased heart muscle weight and cytochrome oxidase activity, both being compensatory mechanisms for the reduced blood oxygen-carrying capacity caused by the anaemia, whereas control kale-fed lambs showed a reduction in cytochrome oxidase activity in both heart and hind limb muscle.

Barry *et al* (1983a) suggested 2 possible mechanisms to explain effects of I supplementation on resistance to haemolytic anaemia: the first deals with the biochemical properties of erythrocytes and the second concerns muscle cytochrome oxidase.

1) Erythrocyte GSH concentration can be altered either through utilization by glutathione peroxidase/superoxide dismutase system, or by regeneration from glucose-6-P by glucose-6-P dehydrogenase/glutathione reductase system. According to Barry *et al* (1983a), the 2 former enzymes system being unaffected by I supplementation, it might be speculated that either the availability of glucose-6-P or the activity of the latter enzyme system might to some extent be dependent on thyroid hormones.

2) Cytochrome oxidase functions in the respiratory chain, linking O_2 uptake to the production of $NADH_2$ from the tricarboxylic acid cycle. Variations in the enzyme activity are therefore probably indicative of variations in O_2 uptake; increases in cytochrome oxidase activity may also reflect an increase in the amount of work done by heart muscle, and may indicate that the animal probably counteracts the reduced blood oxygen-carrying capacity by increased cardiac output. Differences in evolution of cytochrome oxidase activity in control or I supplemented kale-fed lambs suggest that cytochrome oxidase may also to some extent be dependent on thyroid hormones.

This, however, remains a speculative hypothesis without proof in the study reported by Barry *et al* (1983a), and clearly more research is required in this area.

It has been speculated, but not proven, that the protection afforded against haemolytic anaemia by I supplementation in kale-fed animals may be mediated more by T4 than by T3 (Barry *et al*, 1983a; 1985). In fact, in the presence of supplementary iodine, kale feeding is associated with an increase in plasma T4 to much higher values than those observed in pasture-fed counterparts (Barry *et al*, 1981b; 1983a; 1985, in lambs). T4 stimulates erythroetin-induced erythropoesis in human and mouse bone marrow cells with a potency slightly greater than T3 (Chopra and Solomon, 1980 cited by Barry *et al*, 1983a), and the increased T4 concentrations in kale-fed sheep may serve to stimulate synthesis of replacement erythrocyte.

Goitrogenicity of kale and beneficial effects of I supplementation on resistance to anaemia have not been observed in other studies (Barry *et al*, 1981b). These conflicting results may be due to differences: 1) in the iodine content of the experimental forages, 20 µg/kgDM (dry matter) in Barry *et al* (1983a) *vs* 70 µg/kgDM in Barry *et al* (1981b), *ie* the former is I deficient; 2) in animal species used (growing sheep for the former study and growing cattle for the latter), since, taking into account wool growth, the requirements of young sheep for iodine may be greater than for cattle (Barry *et al*, 1981b).

Effects of low copper content

Ruminants fed forage brassicas for long periods generally show Cu deficiency (Barry *et al*, 1981a, b, 1983b). This is due to 1) the low copper concentration of these plants

(4–5 mg/kg DM for kale); 2) the high sulfur content of these plants which reduces the proportion of truly available copper (Suttle and McLauchlan, 1976); and 3) the higher requirements of animals grazing these forages compared to pasture-fed counterparts (Barry *et al*, 1981b, in growing cattle; Barry *et al*, 1983b, in lambs).

Copper deficiency may be of variable intensity – mild to severe – with 3 stages: first, depletion from storage sites (liver reserves); second, depression in transport forms (plasma or serum Cu); and finally reduction in Cu concentration at critical sites (heart, erythrocytes, and Cu-containing enzymes) (Suttle and McLauchlan, 1976).

Depletion of liver and serum copper concentrations (Barry *et al*, 1981a, b, 1983b) may be more or less rapid and severe. In the experiment of Barry *et al* (1981b), serum copper concentration of cattle fed kale declined rapidly, from 0.8 mg/l at the beginning of kale feeding to 0.3 mg/l by week 12, and then remained in the range 0.2–0.3 mg/l. Barry *et al* (1983b) observed less severe depletion, in growing lambs, serum Cu concentration being reduced from 1.0 to 0.7 mg/l from weeks 18 to 24. Suttle *et al* (1987) observed little change in plasma Cu content of growing lambs, but it was very low (in the range 0.1–0.3 mg/l) at the beginning of rape grazing, which also lasted only 2 weeks. Cu supplementation of brassica forage-fed ruminants allows an increase in copper liver reserves (Barry *et al*, 1981b, 1983b) and plasma Cu concentration (Barry *et al*, 1981b; Suttle *et al*, 1987).

Heart muscle copper concentration has been shown to be slightly lowered in kale-fed cattle, which is counteracted by heart hypertrophy (Barry *et al*, 1981b). Copper supplementation increases both the concentration and total amount of copper in heart muscle, and avoids its enlargement (Barry *et al*, 1981b).

Some confusion exists in the literature regarding the effects of brassica forages on the activity of SOD in the erythrocytes. Barry *et al* (1983b) reported a decrease followed by a progressive slight recovery, whereas Suttle *et al* (1987) observed an increase. These apparently conflicting results may reflect differences in the previous grazing history of animals, which induced differences in their Cu-status at the beginning of brassica feeding. In Suttle *et al* (1987), animals grazed grass pasture of low Cu content for 24 weeks before transfer to rape. At that time, they were very Cu-deficient (plasma Cu and blood SOD activity were very low, in the range 0.11–0.30 mg/l and 417–661 U/g Hb respectively), and animals showed symptoms of haemolytic anaemia, Heinz body counts being in the range 6–9%. After grazing rape for 2 weeks, Heinz-body counts had risen to 25%, and blood SOD activity had increased to 1 057–1 069 U/g Hb, which however remains at a low level. Lambs used by Barry *et al* (1983b) were not Cu-deficient at the beginning of kale feeding, and symptoms of haemolytic anaemia began after introduction on kale. Blood SOD activity of animals grazing kale dropped from 5 177 U/g Hb to lower levels than for their pasture-fed counterparts by weeks 6 and 12 (2 977 and 2 677 U/g Hb *vs* 3 877 and 3 527 U/g Hb), and then slightly recovered to attain similar values by week 24 (2 727 vs 2 977 U g/Hb).

The biochemical basis of the effects of brassica feeding on blood SOD activity still await elucidation. It has been demonstrated that DMDS does not inactivate this enzyme (Barry *et al*, 1983b). Cu deficiency of brassica forages might depress Cu-containing enzyme activities, this hypothesis explaining the first stage of blood SOD activity depletion observed in the study of Barry *et al* (1983b). Injection of Cu to Cu-deficient rats has been shown to increase SOD activity to normal values after 36 h (Bohenkamp and Wesser, 1976), but in Cu-deficient sheep, 90 d of feeding on a Cu-adequate diet were required (Andrewartha and Caple, 1980; Barry *et al*, 1983b). It has been suggested that Cu may

attach to SOD during erythropoiesis, as this delay of 90 d corresponds to the life-span of erythrocytes in lambs (Barry *et al*, 1983b). In the experiment of Suttle *et al* (1987), the increase in blood SOD activity together with the rapid fall in blood haemoglobin content after only 2 weeks' grazing rape have been attributed to the fact that rape haemolysins probably induced a rapid and massive removal of red blood cells showing low enzyme activity. It may in fact have concerned a non-negligible proportion of red blood cells, since average blood SOD activity was very low at the beginning of rape feeding. As Cu supplementation have increased blood SOD activity and blood haemoglobin content, it may be suggested that blood SOD activity was below the minimum level necessary to ensure protection of RBC from O_2^-. In the experiment of Barry *et al* (1983b), blood SOD activity never dropped to such low levels, and the slight recovery observed after 12 weeks grazing kale was not due to the removal of predisposed erythrocytes, since it was not accompanied by a reduction in Hb content. It seems that blood SOD activity must have been above the minimum level necessary to protect erythrocytes from superoxide anion, since Cu supplementation, though increasing blood SOD, did not increase blood haemoglobin content or reduce Heinz body counts.

Less attention has been paid to muscle cytochrome oxidase activity, another Cu-containing enzyme, whose evolution in brassica-fed ruminants has been examined in only one study and in relatively mild conditions of Cu depletion (Barry *et al*, 1983b). Kale feeding for 24 weeks did not affect its activity in heart and hind-limb, but Cu supplementation considerably increased its total activity in the heart (Barry *et al*, 1983b), which is suggested to represent a defense mechanism against haemolytic anaemia.

It has also been suggested that the activity of the Cu-dependent plasma enzyme ceruloplasmin might be impaired in brassica-fed ruminants (Barry *et al*, 1981b). This enzyme functions by transporting iron between the liver, its site of storage, and the bone marrow, where haemoglobin is synthesized and red blood cells are formed. When its blood concentration is too low, iron mobilization from the liver may be impaired, and, as a consequence, haemoglobin synthesis in bone marrow may be reduced (Underwood, 1977 cited by Barry *et al*, 1981b). This phenomenon has not been measured directly, but is rather a hypothesis put forward by Barry *et al* (1981b). In cattle, 15 weeks after the beginning of kale feeding, they observed that: 1) the serum Cu concentration reached a minimum value; 2) iron started to accumulate in the liver; and 3) the haemoglobin concentration entered a second phase of decline. After 24 weeks of kale feeding, iron had massively accumulated in the liver of animals (5 g *vs* 0.2 g in pasture fed controls and 0.6 g in copper supplemented kale-fed cattle), while the haemoglobin concentration was extremely low. This however remains a speculative hypothesis without proof in this study and clearly further studies are required upon this area.

It is still uncertain whether low copper levels in brassica forages exacerbate anaemia. Some authors have speculated that this might be the case but others have not confirmed this hypothesis.

According to Barry *et al* (1981b), low copper levels in brassica forages may be partly responsible for the observed reduced concentration in erythrocyte GSH, probably because of insufficient activity of SOD, since Cu supplementation increases erythrocyte GSH and copper concentrations.

Some confusion exists regarding the effect of Cu supplementation of brassica forages fed ruminants on hematological parameters; 1) some authors report decreases in Heinz body counts (Barry *et al*, 1981b, in young cattle), and others no

change (Barry *et al*, 1983b; Suttle *et al*, 1987, in growing lambs); and 2) some authors report increases in blood Hb concentration (Barry *et al*, 1981b, who observed a rapid and complete recovery in blood Hb concentration, Suttle *et al*, 1987), while others no change (Barry *et al*, 1983b).

Different authors therefore disagree whether susceptibility of animals to brassica-induced anaemia is exacerbated or not by a concommitant Cu deficiency. Two hypotheses have been put forward to explain these conflicting results.

1) When blood SOD activity exceeds a minimum level, the protection of red blood cells from superoxide anion might be ensured, and Cu supplementation, though still increasing blood SOD activity, might have no more effect on hematological parameters. Blood SOD activity of brassica-fed control groups might have been below this level in the experiments of Barry *et al* (1981b) and Suttle *et al* (1987), and above it in the experiment of Barry *et al* (1983b).

2) Brassica forage haemolysins might sometimes overwhelm the influence of SOD, giving rise to excessive Heinz body formation unaffected by the Cu status of animals. This might have been the case in the study of Suttle *et al* (1987), where blood SOD activity was low even in Cu-supplemented rape-fed lambs, because of the grazing history of animals.

These hypotheses need to be tested critically before the role of low copper levels in brassica-forage-induced anaemia is clarified and the mechanisms involved are more fully understood. It must be pointed out that studies based on Heinz-body counts should have early blood sampling and a high blood sampling frequency, since Earl and Smith (1982) observed that Heinz-body counts were at maximum 10 d after the beginning of brassica feeding (70%), whereas at the 14th day, most of the red cells containing Heinz bodies had disappeared (Heinz body count about 15%).

Particularities in Se metabolism of ruminants fed forage brassicas

Se metabolism appears to differ between kale-fed and pasture-fed cattle (Barry *et al*, 1981b). Although blood GSH-Px is dependent upon blood Se concentration in both diets, in the kale-fed animals, GSH-Px and blood Se content are also positively related to erythrocyte GSH concentration, the substrate of GSH-Px (Barry *et al*, 1981b). Reductions in erythrocyte GSH concentration in cattle grazing kale for long periods, because of the several factors exposed earlier in this review, seem therefore to be associated with reduced erythrocyte Se status. Cu supplementation of kale-fed cattle, which leads to an increase in erythrocyte GSH concentration, therefore allows an increase in the activity of GSH-Px and in the blood selenium concentration (Barry *et al*, 1981b).

However, it does not seem that these particularities in Se metabolism exacerbate brassica-induced anaemia. In the study of Barry *et al* (1981b), for example, GSH-Px activity and blood Se concentration of animals fed on kale for 24 weeks never dropped to significantly lower values compared to their pasture-fed counterparts, most of them being indeed higher. Suttle *et al* (1987) observed that Se supplementation of rape-fed lambs, though increasing strongly GSH-Px activities, did not increase resistance to brassica-induced anaemia.

The hypotheses put forward to explain these results are the same as for effects of Cu supplementation.

1) When GSH-Px activity exceeds a minimum level necessary to ensure protection of red blood cells from oxidants, Se supplementation, though still increasing GSH-Px activity, might have no more effect on hematological parameters.

2) Brassica forage haemolysins might overwhelm the influence of GSH-Px, triggering Heinz-Ehrlich-body formation unaffected by

the Se status of the animals (Suttle *et al*, 1987).

These 4 sections have attempted to give an overview of our current state of knowledge on the mechanisms involved in the brassica-induced Heinz-Ehrlich body anaemia. It may be concluded that the intake of forage brassicas considerably disturbs the reducing environment of the red blood cell, which is then unable to generate the extra reducing power needed to reduce the oxidation derivatives and to preserve the integrity of cell membrane. A number of areas remain however speculative hypotheses and further studies are clearly required before the mode of toxicity of brassica forages is more fully understood.

Haemolysis-induced anaemia and prevention of the disorder

Description of the phenomenon

Haemolysis

The proportion of red blood cells containing Heinz bodies may reach 80%, the maximum being reached 10 d (Earl and Smith, 1982) to more than 4 weeks (Barry *et al*, 1981a) after the beginning of feeding. They are subjected to lysis so that their number decreases (Young *et al*, 1982; Fehlberg *et al*, 1989); it may be reduced by 60% (Greenhalgh *et al*, 1969) or even 75% (Fehlberg *et al*, 1989), the minimum being reached 3 to 5 weeks after the beginning of feeding (Greenhalgh *et al*, 1969; Fehlberg *et al*, 1989). This haemolysis can even cause a significant modification in plasma color (Onderscheka *et al*, 1987b). The first red blood cells concerned by haemolysis are those exhibiting the lowest reducing capacity, for instance, the most aged (Rigas and Koler, 1961 cited by Rachmilewitz *et al*, 1971).

Erythrocyte losses lead to a decline in haematocrit from 1 to 4 weeks after the beginning of feeding (Barry *et al*, 1981b, 1982; Fehlberg *et al*, 1989; Grongnet, 1982) the minimum value is generally reached between weeks 3 and 5 (Greenhalgh *et al*, 1969; Barry *et al*, 1981b, 1982; Fehlberg *et al*, 1989). In the most severe cases, haematocrit values may fall down to 30 to 50% of normal values (Greenhalgh *et al*, 1969; Fehlberg *et al*, 1989), which range around 35% in sheep and cattle and 60% in roe deer (Fehlberg *et al*, 1989). According to Fehlberg *et al* (1989), when the haematocrit level is 50% lower than normal in roe deer, the animal is condemned.

The blood haemoglobin concentration enters a decline when the proportion of red cells containing Heinz bodies reaches its maximum (Smith *et al*, 1974), *ie* between 1 and 4 weeks after the beginning of feeding (Smith *et al*, 1974; Barry *et al*, 1982; Fehlberg *et al*, 1989). In sheep and cattle, it may fall from 110–130 g/l to 60, or even 40 g/l in the most severe cases (Greenhalgh *et al*, 1969). In roe deer, Fehlberg *et al* (1989) recorded a decrease from 215 to 115 g/l. This minimum is reached by weeks 2–5 (Smith, 1974; Barry *et al*, 1981b, 1982; Fehlberg *et al*, 1989). In acute haemolysis, the rate of haemoglobin degradation may be so high that red pigments are observed in urine (Barry *et al*, 1982). In sheep and cattle, haemoglobinuria is observed when blood haemoglobin concentration is below 60 g/l (Smith, 1974). Haemoglobinuria has also been observed in roe deer (Onderscheka *et al*, 1987a). It may be fatal if the feeding of the animals is not modified.

Destruction of haemoglobin in the organism can lead to hemosiderosis in mononuclears (Fehlberg *et al*, 1989), as well as in the liver and kidney tissues (Onderscheka *et al*, 1987a). This is due to the overload of hemosiderin, a pigment composed of a protein substance containing up to 40% iron. It may also cause an icterus, as reported in dairy cows, in sheep and in roe deer (Greenhalgh *et al*, 1969; Smith, 1980; Onderscheka

et al, 1987a; Barnouin and Paccard, 1988; Fehlberg *et al*, 1989). Icterus can be slight; it can also be massive, in which case the condition may be fatal (Onderscheka *et al*, 1987a; Barnouin and Paccard, 1988). Icterus is both pre- and intra-hepatic (Fehlberg *et al*, 1989), as far as it results from 2 types of dysfunction: a haemolysis, which provokes an excessive production of bilirubin, and a liver dysfunction which disturbs the bilirubin metabolism. This results in an accumulation of bilirubin in the tissues. However, the increase in blood bilirubin concentration has never been quantified, nor has the severity of icterus. These measurements would nevertheless be of interest taking into account the commercial grading down resulting from a discoloration of the subcutaneous adipose tissue (Prache *et al*, 1990).

Phase of recovery

After the first stage of the disease, if brassica feeding is continued, animals are able to make a spontaneous but incomplete recovery within 2 to 5 weeks. The Heinz body count is reduced, slightly (Barry *et al*, 1981a) to strongly (Barry *et al*, 1981b, 1982; Earl and Smith, 1982), after 10 to 14 d according to Earl and Smith (1982), 21 to 28 d according to Barry *et al* (1982), and 4 to 6 weeks according to Barry *et al* (1981a). The red cell count, blood haemoglobin concentration and haematocrit increase after 3 to 5 weeks for the former, 3 to 7 weeks for the latter (Greenhalgh *et al*, 1969; Barry *et al*, 1981a, b, 1982; Grongnet, 1982). A return to normal is observed by week 9 to 10.

This period of recovery is due to an increase in hematopoiesis in response to haemolysis, and is associated with an increase in the number of young erythrocytes or reticulocytes (up to 4% of the red blood cells instead of 0.5 to 1.5% in healthy ruminants) (Greenhalgh, 1969; Tucker and Kilgour, 1973); and a greater than normal mean corpuscular volume of erythrocytes (macrocytosis) (Greenhalgh *et al*, 1969) resulting from an accelerated haemoglobin synthesis, which leads to a reduced number of mitoses of erythroblasts (Varet, 1990).

Recovery is generally incomplete and is followed by further cycles of fall and partial restoration of haemoglobin content if feeding is not modified. The arrival in blood of young erythrocytes and their subsequent poisoning explains these successive cycles. Earl and Smith (1982) noticed that blood DMDS level reaches a maximum value during the first haemolytic crisis, and then decreases rapidly with the arrival of young erythrocytes, and increases again as the new cell matures. Tucker and Kilgour (1973) reported a massive increase in the erythrocyte GSH concentration after the first haemolytic crisis, mainly associated with the reticulocyte population. This increase could explain the partial recovery of the animals and the further haemolytic crisis due to the aging of erythrocytes.

Stopping forage brassica feeding is the only means to obtain the recovery of the animals. Haemoglobin levels return to normal in 3 to 4 weeks (Smith, 1974), but the complete recovery of the animals requires 2 or 3 months (Tucker, 1969). The lifespan of red blood cells being 4 months on an average, numerous Heinz bodies and poorly functional red blood cells may persist during the time required for their renewal.

Factors affecting the intensity of haemolysis

The authors generally agree that consequences of anaemia on both health and performance of the animals may be severe. When the proportion of forage brassicas in the diet is limited, the risks of death due to an acute anaemia are limited. However, sometimes they constitute most or all of the diet, for instance, in grazing lambs fed on forage rape during fattening or for wild animals such as roe deer in winter (Onderscheka *et al*, 1987a, b; Fehlberg *et al*, 1989).

Different authors do not agree on the consequences of subclinical anaemia. Some consider that a slight anaemia does not lead to a fall in performance (Young *et al*, 1982), or to a detectable degradation in the health status (Barnouin and Paccard, 1988), the animal body also being able to adapt and recover (Barry *et al*, 1985). Others estimate that even a mild haemolytic anaemia is incompatible with a good health and maximum productivity (Penny *et al*, 1964). Smith (1974) reports the case of experimental goats exhibiting extremely low haemoglobin blood concentration of 30 to 40 g/l but which did not appear overtly ill. Barry *et al* (1981a) noted an inverse relationship between the degree of subclinical haemolytic anaemia and growth rate of lambs grazing kale.

The severity of anaemia may be extremely variable and is affected by many factors depending both on the animal and on the diet.

Factors depending on the animal

Susceptibility to anaemia varies among species. It is generally admitted that among domestic ruminants, cattle and goats are more susceptible than sheep to forage brassica anaemia. This might be due to differences in the enzyme potential (Giovanni, 1978) and copper requirements (Barry *et al*, 1981b). Roe deer seem to be particularly susceptible, much more than domestic ruminants (Fehlberg *et al*, 1989).

An effect of the physiological state has also been reported: heavily pregnant and lactating cows are at greater risk and show more severe clinical signs, probably because they eat more per unit body weight than do non-lactating or non-pregnant animals (Smith, 1974; Barnouin, 1990).

High yielding animals are often more susceptible to nutritional or physiological disorders. High yielding dairy cows, in particular, may be highly susceptible to anaemia, because the latter may occur at the same time as a post-partum haemoglobinuria.

The younger the animals and the greater the dietary incorporation of kale, the more affected they are. In fact, growing animals are more susceptible than adults to the combined action of antithyroid and haemolytic factors. Tataruch *et al* (1990), in roe deer, have also observed that the highest percentage of affected animals were fawns.

The haemolytic response and its severity can be accelerated by parasitism or an energy restriction (Giovanni, 1978). It is thus recommended to feed forage brassicas to healthy animals only.

The breeds may differ in haemoglobin types, and this may affect their susceptibility to oxidative stress. Sheep can be classified into haemoglobin genotypes AA, AB or BB. Types AA and AB can synthesize haemoglobin C whereas those of genotype BB are not (Tucker, 1969), and this might increase their capacity to recover from anaemia. However, the results of Barry *et al* (1982) indicate that the synthesis of haemoglobin C does not reach significant levels before 5 weeks of kale feeding. In Finnish Landrace sheep, an large proportion of animals show erythrocyte GSH deficiency (GSH concentration being one third or less of that normally found in sheep), which seems to be controlled by an autosomal recessive gene. When fed on kale, the GSH-low and GSH-high types become anaemic, but the GSH-low types are more severely affected (Tucker and Kilgour, 1973). As little is known on the genetic control of GSH concentration in the red blood cells, some authors have pointed out the interest of studying the selection of animals with high GSH concentration (Tucker and Kilgour, 1973; Smith, 1980), in order to offer a better resistance to haemolytic action of DMDS.

Finally, the severity of anaemia and the susceptibility of the animals seem to be extremely variable from one individual to another (Smith, 1974; Clegg and Evans, 1962; Eisfeld, 1990).

Factors depending on the diet

These factors concern the amount of ingested SMCO, the duration of feeding and the rate of conversion of SMCO to DMDS.

Effect of SMCO intake

Smith (1980) and his team were the first to study the relationship between the SMCO intake and the severity of anaemia. They demonstrated that a daily intake of 150 to 200 mg SMCO/kg LW (live-weight) is required to provoke an acute haemolytic response, that a subacute low grade anaemia is elicited by an intake of 100 to 150 mg/kg LW/d, and that an intake of 50 to 100 mg/kg LW/d only produces a slight increase in Heinz-body counts. According to Giovanni *et al* (1989), the consequences of an acute, or even subacute but prolonged anaemia can be important if the daily intake of SMCO exceeds 100 mg/kg LW. Barry *et al* (1982) observed that both the severity of anaemia and the rapidity to recover depended on the level of ingested SMCO in lambs fed kale and supplemented with various levels of synthetic SMCO.

The thresholds indicated above have been established by experiments made in goats, cattle and sheep. However, to our knowledge, there is no work on wild ruminants such as roe deer which are very susceptible to SMCO.

• *Effect of the SMCO content of forage.* During germination, SMCO, which occurs at a very low level in the grain, is rapidly synthesized. Its concentration in leaves increases strikingly at the time of most vigorous plant growth about 50 d after germination. The SMCO content is high (5 to 12 g/kg DM) relative to that of goitrogenic compounds (1–3 g/kg DM) (Giovanni *et al*, 1989).

Few studies have been made on the relationship between the SMCO content of forage and the proportion of red blood cells containing Heinz bodies. In lambs fed exclusively on stubble turnips, Smith (1980) observed the development of a Heinz body anaemia and a fall in the performance when the SMCO content of the plants was around 0.5% DM. By contrast, with contents of 0.2 to 0.3% DM, no significant decline in the blood haemoglobin concentration and no effect on the performance were observed.

There are numerous factors of variation in the SMCO content of forage bassicas. Some of them cannot be controlled or predicted. Heterogeneity in SMCO content has been observed for the same variety, from one year to another (Smith, 1980; Giovanni *et al*, 1989; MacFarlane Smith *et al*, 1990), from one date to another (Bradshaw and Borzucki, 1982, 1983; Griffiths *et al*, 1989), from one region to another and from one farm to another (Smith, 1974); this explains the difficulty in predicting pathological risks.

Variations in type and variety are documented in the publications of Smith (1974), Whittle *et al* (1976), and Giovanni *et al* (1989). They show the variation in SMCO contents of several forage brassicas throughout the year and, in particular, of several varieties of kale and rape. Their results can be summarized as follows. The SMCO content varies with the type of forage brassica: rape has on the average a 2-fold lower SMCO content than kale, *ie* 2–3 g/kg DM in October and 6 g/kg DM in January. The SMCO content varies with the variety. Selection for varieties with a low SMCO content (maximum value: 4–6 g/kg DM) seems to be the most promising means of reducing risks (Smith, 1974, 1980; Fales *et al*, 1987; Giovanni *et al*, 1989). Studies performed in England and cited by Fales *et al* (1987) have already led to a determination of the heritability of SMCO content. However, the factors of variation in SMCO synthesis seem to depend as much on the genetic potential of the plants as on the environmental conditions (Giovanni *et al*, 1989). It is therefore still difficult to classify the types of brassicas (MacFarlane Smith *et al*, 1990).

The effects of the structure and growth of the plant on the SMCO are documented in

the publications of Giovanni *et al* (1989) and of Griffiths and MacFarlane Smith (1989). The concentration of SMCO in the different parts of a single kale plant can vary about 5-fold (Smith, 1980). According to Smith (1980) and Giovanni *et al* (1989), in kale, the highest concentrations (12–20 g/kg DM) are found in young leaves, growing shoots and flowering parts. Giovanni *et al* (1989) compared the concentrations in leaves (preferred by animals) and stems of fodder kale during the period October to January for several years. They were similar in October, but increased in leaves between October and January, and were systematically higher than those of stems. This disagrees with earlier results of Smith (1974) in kale and of Griffiths and MacFarlane Smith (1989) in rape, who found no consistent differences between stems and leaves, during the period 9 to 22 weeks after sowing.

There are also some conflicting results regarding the evolution of the SMCO content of forage brassicas with the age of the plant. Smith (1980) and Giovanni *et al* (1989) report an increase, which they estimate as probably being due to the proportion of leaves, young or able to remain healthy and functional. In kale harvested at monthly intervals, for example, Giovanni *et al* (1989) observe 6 g/kg DM on average in October and 9–12 g/kg DM during winter for plants sown in early June. For later sowings (early July), the contents may be very high from October and till the winter (11.5 and 17 g/kg DM, respectively). Griffiths and MacFarlane Smith (1989), on the contrary, from weekly intervals harvesting, report considerable intra-seasonal variations in the evolution of 3 forage rape with age.

The growth rate of the plant may also be involved. Giovanni *et al* (1989) have observed that a rapid growth in autumn occurring after a period of slow growth in summer provoked a rise in SMCO contents compared with years of more regular growth.

The effect of the environment on SMCO content is extremely important and is one of the causes of the frequently observed and not well-controlled intravarietal variability.

A rise in SMCO content is observed between October and January. It is difficult to isolate the effect of the climate from that of the plant structure and age. However, climatic conditions during autumn and winter have a proper effect on SMCO synthesis or migration in the plant. During the short-day and falling-temperature periods, the SMCO content rises, particularly in leaves, although their growth is slower or even stopped (Giovanni *et al*, 1989). Moreover, frosting is traditionally thought to enhance the toxicity of kale, but this has never been demonstrated.

Culture conditions, in particular the sulfur content of the soil and the nitrogen fertilization can also affect the SMCO content of the plant.

Smith (1980) observed that when nitrogen fertilizer application was increased from 0 to 220 kg per hectare, the SMCO content of kale, rape and stubble turnips rose. More precisely, it was shown (MacDonald *et al*, 1981) that the effect of N application depended on the soil SO_4-S concentration. When it is high, SMCO synthesis is stimulated, but this effect is progressively reduced when the soil SO_4-S concentration declines below 9–10 mg/kg. According to these authors, SMCO represents a form of storage of S, which would be uptaken by the plant in excess relative to its requirements for synthesis of protein. By contrast, for a given level of N application, the level of potassium and phosphorus fertilization has little effect on SMCO content.

The possible effect of the previous culture on the SMCO content of the plant has also been mentioned (MacDearmid *et al*, 1982) without details on the factors involved.

According to Giovanni *et al* (1989), silage has little effect on SMCO content. However, they noticed that ensiled kale stored with

preservatives had higher SMCO contents (9–13 g/kg DM) than those ensiled without preservatives (7–10 g/kg DM). In contrast, Fales *et al* (1987) observed that ensiling significantly reduced the SMCO content of rape. However, this effect was extremely small (0.46 mg SMCO/kg DM on average). It is noteworthy that the forage analyzed in this study exhibited relatively small contents of SMCO. The wilting also studied by these authors had no effect on SMCO content.

The influence of the environment is thus extremely important, since its effect on the expression of the genetic potential seems to be at least as large as the genetic potential itself (Giovanni *et al*, 1989; MacFarlane Smith *et al*, 1990). Therefore, it remains difficult to predict the risk for the animal health status because most of the environmental factors cannot be controlled or predicted and their effects and interactions are not well known. The search for varieties with lower SMCO contents should permit to reduce this risk (Smith, 1974, 1980; Giovanni *et al*; 1989).

• *Importance of the dietary proportion of forage brassicas.* The severity and rapid development of anaemia is proportional to the contribution of forage brassicas to the diet.

In cattle, several authors have tested diets including 100%, 80%, 67% and 33% DM as kale or cabbage (Greenhalgh *et al*, 1970; Smith, 1980). Results show that the severity and rapid occurrence of anaemia increased with the dietary proportion of brassicas. With the diet including 33% DM, the number of red blood cells with Heinz bodies was only slightly above normal (Smith, 1980). In practice, a maximum level of about 30% of the total DM of the diet is recommended for kale (Greenhalgh *et al*, 1972; Giovanni *et al*, 1989). This level maintains the health status and leads to normal performance (Greenhalgh *et al*, 1972; Gordon, 1976). To our knowledge, no such an experiment has been performed on forage rape. Because of its low SMCO content, its level of dietary incorporation can be higher, but at an earlier stage of growth, the risk of intoxication by nitrates and nitrites is high. Thus it is recommended not to exceed 40% of the total DM (Giovanni, 1978).

In sheep, severe anaemia develops when 3 kg fresh weight or more kale are given to animals with a live-weight larger than 30 kg (Grant *et al*, 1968; Pelletier and Martin, 1973). In goats with a live-weight ranging between 50–70 kg, the daily intake of 5–6 kg fresh weight kale can induce anaemia after 5–6 weeks. In both species, the maximum level of kale intake ranges around 8% of the live-weight whereas in cattle it is around 4–5% (Giovanni, 1978).

Little information is available on the effect of the dietary level of forage rape in these species. An experiment comparing 3 levels of barley supplementation to lambs grazing rape has shown similar growth performance (Fitzgerald, 1984).

In roe deer zero-grazed with forage rape, Fehlberg *et al* (1989) have studied the effect of feeding other forages on health. They compared 2 groups of animals: group A was fed only forage rape *ad libitum*; while group B was fed the following forage *ad libitum*: forage rape, hay, fresh grass, corn, yoke-elm, and fir tree. The following results were obtained: rape intake was significantly lower in group B than in group A; the haematocrit decrease was conversely proportional to rape intake. Haematocrit of animals in group A was 20–30% lower than that of animals in group B; the number of red blood cells decreased and reached 23% of the normal value in group A and 50–70% in group B; the level of haemoglobin decreased. It was 30% lower in group A than in group B; the weight loss was more pronounced in group A and the first clinical signs (locomotion and digestive disorders) occurred earlier in group A (after 10 d) than in group B. In spite of differences in the development and severity between the

2 groups, the clinical signs were observed in all the animals and none survived. Death occurred on an average more rapidly in group A than in group B.

According to Frylestam *et al* (1990), the degree of use of rape fields by roe deer is influenced by their proximity to forests or tree stands where the animals usually have their refuges.

The feeding levels compatible with health and performance of farm species have thus been rather well studied for forage kale, probably because its SMCO content is the highest. By contrast, these levels have to be determined for other forage brassicas, particularly forage rape and its different varieties and for other animal species (particularly wild animals). Although the role of SMCO in brassica-induced anaemia was evidenced in 1974, it is not yet possible to relate the variations in blood parameters and animal performance to SMCO intake, because very few studies have been devoted to these variables.

• *Effect of the duration of SMCO intake.* When forage brassicas are grazed *ad libitum*, the only means to limit SMCO intake is to limit the duration of brassica feeding. To our knowledge, no experiment has been performed to compare the effect of different durations of forage brassica feeding on the variation of blood parameters and performance. In lambs, it has only been recommended to restrict forage rape feeding to the last 4–5 weeks of fattening.

When SMCO intake is stopped (total replacement of brassicas by other diets), the blood DMDS level falls sharply. A residual amount of DMDS, however, persists and is still detectable 80 d after the withdrawal of the source of SMCO (Earl and Smith, 1982). It is likely that this residual amount stems from DMDS chemically bound as mixed disulfides, but physical adsorption may also contribute to this retention (Earl and Smith, 1982).

Effect of the conversion rate of SMCO to DMDS

The severity of anaemia cannot be predicted from the SMCO intake alone, because the toxic action is mediated by the conversion of SMCO to DMDS (Earl and Smith, 1982). The conversion rate of SMCO to DMDS is influenced by several factors including the composition and metabolic activity of the rumen flora. Dietary composition, aside from SMCO content, is probably an important determinant of these factors (Smith, 1974). Some authors suggest the possibility to reduce or even suppress the ruminal conversion of SMCO, by chemical or dietary manipulation, or to speed the oxidative disposal of SMCO, in order to reduce DMDS production (Smith, 1974, 1980; Earl and Smith, 1982).

Barry *et al* (1982) have shown that for an identical dietary SMCO content, the basal diet supplemented with SMCO had a marked effect on the severity of anaemia. Synthetic SMCO was added to basal diets of either fresh kale (8 g SMCO/kg DM) or lucerne at the concentrations of 0, 0.2, 0.4, 0.8 and 1.6% DM and each diet was offered *ad libitum* to lambs kept indoors. For the same dietary SMCO content, the toxic effects were much more marked for kale than for lucerne. These authors suggested that the greater ratio of soluble to structural carbohydrate in kale compared with lucerne (2.3 *vs* 0.9) probably resulting in a microbial population developing in the rumen of kale-fed sheep that could metabolize SMCO to DMDS at a faster rate than in the rumen of lucerne-fed sheep.

Effect of various supplements

• *Copper supplement.* Cu supplementation has been shown to increase erythrocyte GSH concentration and GSH-Px activity (Barry *et al*, 1981b), and to increase heart cytochrome oxidase activity, the latter being considered as a defense mechanism against haemolytic anaemia (Barry *et al*, 1983b). However, its effect on hematological parameters still remain a matter of debate (Barry *et al*, 1981b,

1983b; Suttle *et al*, 1987; for more details, see above, *Effects of low copper content*).

• *Iodine supplement.* I-supplementation of growing sheep fed kale has been shown to enable the animals to resist the anaemia better, by increasing erythrocyte GSH content, heart muscle weight and heart cytochrome oxidase activity, the latter being compensatory mechanism for the reduced blood oxygen-carrying capacity. These effects were not observed in cattle (Barry *et al*, 1981b), perhaps because of differences in the iodine content of the experimental forages and in the animal species used, the requirements of young sheep for iodine being greater than for cattle because of wool growth (for more details, see above, *Effects of antithyroid compounds*).

• *Amino-acid supplement.* Barry *et al* (1981a) have shown that lambs grazing kale showed a temporary amino-acid deficiency corresponding to the time when anaemia was most severe and body growth most depressed. Injection of L-methionine and L-threonine to the animals 3 times a week increased the ratio of carcass weight gain (CWG) to empty-body weight gain (EBWG) and slightly stimulated the wool growth.

CONCLUSION

Forage brassicas feeding highly weaken or even exhaust the mechanisms of protection of red blood cells against oxidants. The haemolytic compound, SMCO, is consumed in benign form and becomes toxic by virtue of its digestive degradation to secondary haemolysin, DMDS. Mechanisms involved in haemolytic toxicity probably include first, disulfide exchange reactions of DMDS with various thiol groups of the red blood cells, and secondly, detrimental effects of antithyroids coupounds on factors involved in resistance to anaemia.

Haemoglobin is then oxidized; its molecular structure becomes more unstable and the outcome is the haemoglobin precipitation as Heinz-Ehrlich bodies and haemolysis. Little is known on the sequence of events undergone by the haemoglobin molecule between oxidation and the formation of Heinz-Ehrlich bodies. Hypotheses have been put forward on the basis of studies performed on human types of haemoglobins which are unstable because of structural defects, but the extent to which these transformations are similar to those undergone by normal haemoglobin of ruminants when fed on forage brassicas is, as yet, unknown. Furthermore, the ultimate cause of haemolysis is still discussed and different explanations have been proposed.

Haemolysis severity depends on numerous factors. It is still extremely difficult to predict the pathological risk because of the large number of factors involved, the lack of data about their effects and interactions, and the unpredictable and uncontrollable character of some of them. For example, the SMCO synthesis of the plant is a much or even more affected by the environmental conditions than by the genetic potential of the plant, which makes the selection for low SMCO varieties more difficult. Nevertheless the search for varieties with a low SMCO content in the forage (maximum: 4-6 g/kg DM) seems to be the most promising way of reducing pathological risks. Animal selection has also been proposed to improve the animal resistance to anaemia: selection for animals with a high level of GSH and/or glutathione reductase; study of the genetic determinism of GSH concentration in the red blood cell; influence of the type of haemoglobin on the capacity of resistance to anaemia. Up to now, very few studies have been performed on these topics.

The toxin responsible for haemolysis was evidenced almost 20 years ago. However, the relationship between the variation in blood parameters and the SMCO intake by the animal cannot be established, because there is still very little available information. This is due to the fact that the plant SMCO

content can vary in very large proportions as affected by numerous factors, and to the cost of SMCO determination. Further studies should be made to determine the relationships between the level of SMCO intake and the severity of the disease.

The haemolytic action of forage brassicas is mediated by the secondary toxin DMDS produced in the rumen from dethiolmethylation of SMCO, the primary toxin. The conversion rate of SMCO to DMDS and its factors of variation are still unknown, but it has been demonstrated that it depends on the dietary composition, and probably on the composition of the rumen flora. Some authors have mentioned the possibility of reducing or even supressing the degradation by dethiolmethylation of SMCO or to speed its oxidative degradation by modifying the composition of the diet or by chemical manipulation. However, very few studies have been devoted to this matter.

In practice, there is still no simple way of counteracting the toxic action of DMDS, and measures to prevent anaemia are limited to those aiming to restrict the SMCO intake, for instance, by limiting the dietary level of incorporation of brassicas. The recommended levels have been well studied in cattle grazing kale, but they have still to be determined for other forage brassicas (such as rape) and their varieties and for other animal species. The effect of feeding duration when these plants are grazed *ad libitum*, for example, by lambs during the fattening period, has not been studied much either. This lack of data can sometimes explain the underutilization of these forages.

Haemolysis can provoke an icterus (accumulation of bilirubin in the tissues). When it is severe, the condition may be fatal to the animal. A subclinal icterus can also be observed, without death, but it can lead to carcass seizures at slaughter by veterinary services or to a grading down because of fat discoloration. Blood bilirubin content has rarely been measured. Nothing has been published to date on the increase in the bilirubin content of tissues or on the quantification of fat discoloration and the means to prevent them.

These latter 2 themes are under study at present in our laboratory.

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