Food Chemistry **29** (1988) 143–155

The Chemistry and Stability of the Haem–Protein Complex in Relation to Meat

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(Received 23 December 1986; revised version received and accepted 27 October 1987)

ABSTRACT

An account of the current understanding of the structure of haemoglobin and myoglobin is presented. The chemistry of these haemoproteins is reviewed in relation to reactions in meat and denaturation processes. The effects of solvents on the precipitation and dissociation of haemoproteins are considered in terms of their role in methods of analysis of nitric oxide haemoproteins, free haem and total haemoprotein in meat and model systems.

INTRODUCTION

The chemistry of the colour of meat is the chemistry of the haemoproteins haemoglobin and myoglobin. Extensive information is available on the chemical structure of myoglobins and haemoglobins from several species. All vertebrate haemoproteins studied have an iron content of 0·30–0·35% which yields values for the minimum molecular weight of 16 000–18 000 daltons. All of them contain one prosthetic group (haem) per polypeptide chain (globin), which consists of 140 to 160 amino acid residues (Antonini & Brunori, 1971). Haemoglobins are composed of two different types of polypeptide chains, which have been called α- and β-chains (Perutz, 1965). Myoglobins contain only one type of polypeptide chain in the species so far examined. Most of the amino acid residues in haemoglobin and myoglobin are part of helical regions (Antonini & Brunori, 1971). Figure 1 shows a schematic drawing of the three-dimensional model of sperm whale

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Food Chemistry 0308-8146/88/$03·50

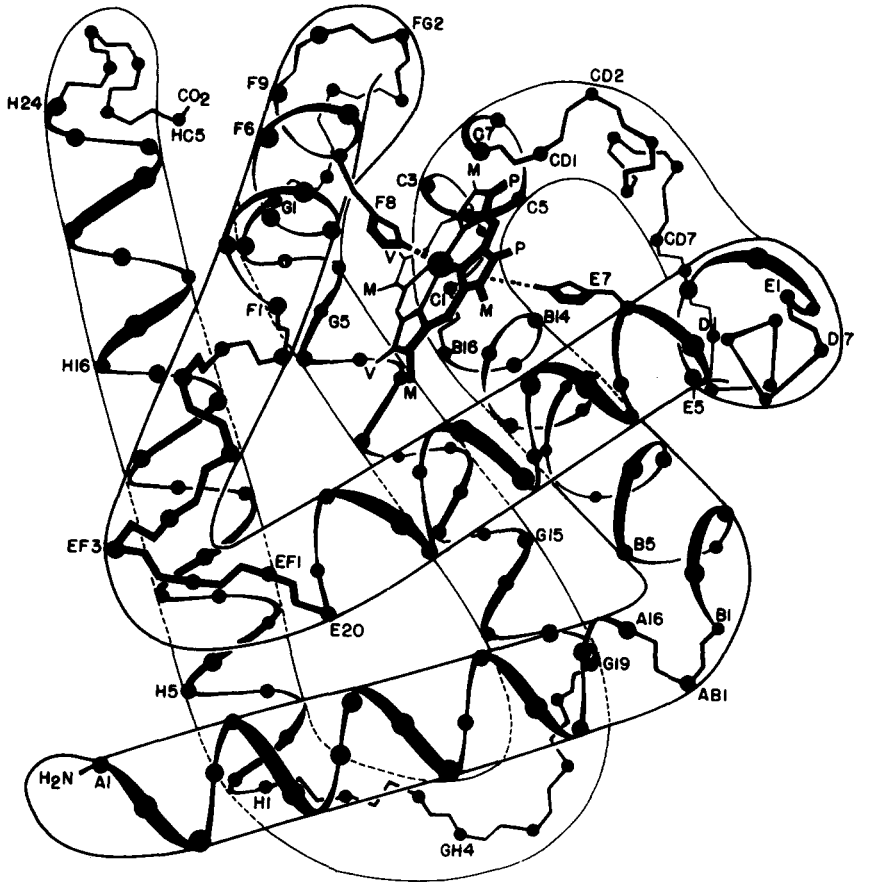

Fig. 1. Three-dimensional structure of myoglobin showing location of haem with iron atom coordinated to histidine groups labelled F8 and E7. Amino acid residues in helical regions are labelled with a single letter and number whilst residues in non-helical regions are labelled with two letters and a number. Side groups on the haem are identified by M = methyl, V = vinyl, P = propionic acid. Reproduced from Dickerson (1964) with permission from Academic Press.

myoglobin, illustrating the arrangement of the residues in the helical and non-helical regions. Nearly all polar side chains, located on the surface of the molecule, are in contact with water, whereas non-polar residues lie in the interior of the molecule where the contact with water is minimised (Kauzmann, 1959).

THE HAEM MOIETY

The haem consists of an iron atom and a large planar ring, the porphyrin (protoporphyrin IX), whose structure is that of four pyrrole rings linked

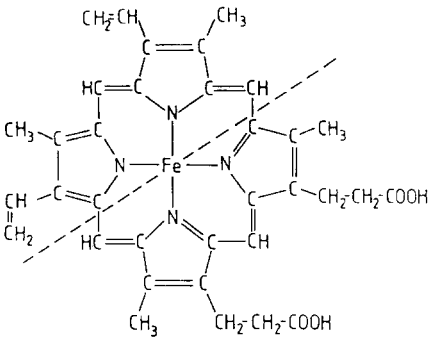

Fig. 2. Structure of haem.

together by methene bridges as shown in Fig. 2. There are a large number of naturally occurring porphyrins with different substituents on the pyrrole nuclei. However, only the one shown in Fig. 2 has been found to occur naturally in haemoglobin and myoglobin.

The iron may be found in oxidation states II or III. Ferrous protoporphyrin is very easily autoxidised to ferric protoporphyrin. In the ferric complex there is one residual positive charge and the complex is usually isolated as a halide, most commonly the chloride, which may be represented by the structure shown in Fig. 3a. This is often called haematin chloride but the chloride ion is clearly coordinated to the metal to form a pentacoordinate square pyramidal complex (Falk & Perrin, 1961) and the name chlorohaemin, extensively used in the German literature, is preferable (Falk, 1964). Other nitrogenous substances, such as pyridine, ammonia, globin or any other protein, may be bound to the iron of the haem; these (hexacoordinate) complexes of ferrous porphyrins are known as haemochromes and those of ferric porphyrins as haemichromes (Lemberg & Legge, 1949). The octahedral stereochemistry of a pyridine haemochrome is shown in Fig. 3b. For systematic purposes, the essentially square planar compounds are designated as porphyrin chelates and the square pyramidal

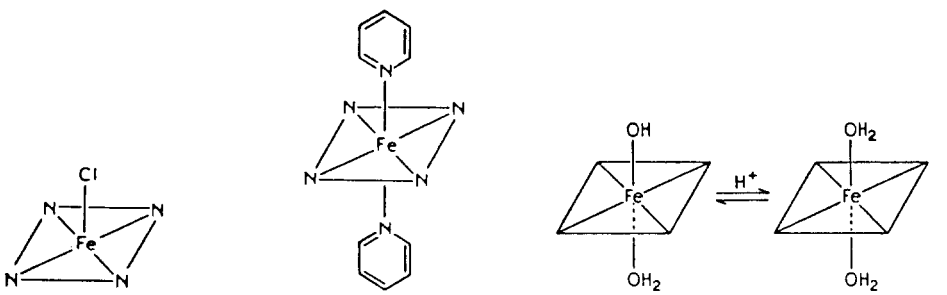

Fig. 3. Structures of derivatives of haem. (a) Chlorohaemin. (b) Pyridine haemochrome. (c) Hydroxyhaemin (haematin).

and octahedral derivatives as metalloporphyrin complexes (Falk, 1964). In alkaline solution halide ions are replaced by the hydroxide ion forming hydroxyhaemin or haematin whose structure is shown in Fig. 3c.

Most natural porphyrins have carboxylic acid side chains and all have basic nitrogen atoms. They are thus ampholytes and have some solubility in aqueous acids and alkalis. If the carboxyl groups are masked, e.g. by esterification, solubility in alkaline solution is lost. On the other hand, if the nitrogen atoms are coordinated to a metal ion, solubility in acid media is lost. The esters of porphyrins and their metal chelates are, of course, much more soluble in organic solvents (Falk, 1964); haems and porphyrins are readily dissolved in acidified organic solvents such as acetone–HCl, ethyl acetate–acetic acid, diethyl ether–acetic acid and methanol–oxalic acid. These solvents are also protein precipitants and are used widely for the extraction of the tetrapyrroles from biological materials. Haem associates loosely with solvents containing carbonyl or hydroxyl groups, such as acetone and ethanol (Hamsik, 1930).

DERIVATIVES OF HAEMOPROTEINS

The iron atom of the haem has four of its coordination sites involved with bonds to porphyrin. In the protein complex, the fifth site is linked to the imidazole nitrogen of a histidine residue as shown in Fig. 4, whilst the sixth is available for the binding of other ligands. The oxidation state of the iron and the type of the ligand bound to the iron centre determine the colour and reactivity of haemoproteins. Deoxyhaemoglobin and deoxymyoglobin lack a ligand in the sixth position and are the Fe(II) derivatives (high spin) found under low oxygen tensions. Oxymyoglobin and oxyhaemoglobin, the diamagnetic ferrous forms, are stable derivatives under high oxygen tension. Nitric oxide haemoglobin and nitric oxide myoglobin are the products of the reaction of the haemoproteins with nitrite ion in the presence of a reducing agent. They are similar low spin Fe(II) complexes, as are carboxyhaemoglobin and carboxymyoglobin.

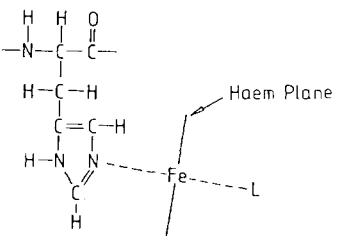

Fig. 4. Coordination of a nitrogen atom of histidine to the iron in haem.

Methaemoglobin and metmyoglobin are ferric haemoproteins (high spin). The sixth ligand is water (or the hydroxyl group at alkaline pH). Nitric oxide methaemoglobin and nitric oxide metmyoglobin are, respectively, the products of the reaction between nitrite ion and methaemoglobin and metmyoglobin and have iron in the Fe(III) state (high spin).

THE HAEM–GLOBIN BOND

In addition to enabling the haem group to perform its physiological function as an oxygen-carrier, the globin converts the insoluble free haem into a soluble complex and protects the haem from oxidation; oxidation of the iron takes place if the protein is denatured.

Molecular interactions between the haem and the protein are very complex and involve about 90 Van der Waals contacts and the haem iron forms a coordinate bond with the imidazole of histidine F8 (see detail in Fig. 1 for myoglobin) and a weak interaction with histidine E7, the distal histidine. The iron atom is slightly out of the porphyrin plane (by about 0·03 nm) towards the side of the histidine F8 imidazole ring. Perutz *et al.* (1968) have reported similar interactions between the haem group and the α- and β-chains of haemoglobin.

The haem moiety in haemoproteins is not a fixed prosthetic group. Reversible dissociation into haem and apoproteins may be achieved (Rossi Fanelli *et al.*, 1958). However, in both haemoglobin and myoglobin the affinity of the haem for the protein at neutral pH is very high (Antonini & Brunori, 1971) and a finite dissociation can be demonstrated only in displacement or haem transfer experiments (Rossi Fanelli & Antonini, 1960) of the type:

$$\text{Haem}\text{–}X\text{–Protein} + \text{Haem}\text{–}Y \rightleftharpoons \text{Haem}\text{–}Y\text{–Protein} + \text{Haem}\text{–}X$$
$$\text{Haemoprotein}\text{–}X + \text{Globin}\text{–}Y \rightleftharpoons \text{Haemoprotein}\text{–}Y + \text{Globin}\text{–}X$$

At neutral pH the haem–globin equilibrium constant has been estimated to be of the order of 10^{12}–10^{15} M (Banerjee, 1962; Gibson & Antonini, 1963). Dissociation of the haem is increased considerably at acid pH (Lewis, 1954). Preparations of globin are, therefore, based on acid splitting of the corresponding haemoprotein and separation of the apoprotein from the haem by the use of organic solvents (Ascoli *et al.*, 1981).

If the pH of a haemoglobin or myoglobin solution is lowered to $<$ pH 3–4, the characteristic linkage of the prosthetic group with the protein is ruptured, while the protein is denatured. The resulting haem compound has a well defined spectrum with three absorbance bands in the visible region (512, 540 and 640 nm) and is called *acid haematin* (Lemberg & Legge, 1949).

THE BASIS OF COLOUR IN MUSCLE FOOD

The muscle haem pigment myoglobin is the principal, but not the whole, source of colour in meat. Even in a well bled piece of meat the blood pigment, haemoglobin, might comprise 20–30% of the total pigment present and sometimes more (Fox, 1966). Although most of the reactions of the two pigments are similar, several which are of importance in meat colour, such as autoxidation, reaction with nitrite ion and denaturation, have different rates. In fresh meat there is a dynamic cycle such that in the presence of oxygen the three pigments, oxymyoglobin, deoxymyoglobin and metmyoglobin and the corresponding haemoglobin forms are constantly interconverted. Uptake of oxygen by myoglobin results in conversion of the purple reduced pigment to the bright red oxygenated compound. However, the haem may also be oxidised to the brown or grey metmyoglobin, particularly when the oxygen tension is low (Walters, 1975). When the meat is fresh the production of reducing substances endogenous to the tissue will constantly reduce the pigment to the purple myoglobin and the cycle continues if oxygen is present. It is suggested that the rate of metmyoglobin formation on the surface of beef slices is dependent on at least two factors (Ledward, 1985): the oxygen consumption rate and the activity of an enzymic reducing system. In most practical situations the activity of the reducing system is the most important factor and this can be affected by the time, temperature and pH history of the muscle. Generally, exposure to high temperatures and low pH leads to increased rates of metmyoglobin formation.

In the case of cooked meat it was suggested that the complexes present are denatured haemoproteins, where the protein may be any of several denatured proteins present in cooked meat. Reflectance spectra of cooked meat and the precipitates obtained on heating aqueous muscle extracts, mixtures of bovine serum albumin (BSA) and horse heart myoglobin were similar (Ledward, 1971). The affinity of the haematin for denatured BSA was much greater than for the apomyoglobin aggregate. It has been proposed that in cooked meat the haemoproteins are mainly di-imidazole complexes, the imidazole residues being supplied by the histidine groups of the bound protein (Ledward, 1974). Heating of ground beef samples, haematin and meat extract solutions results in destruction of the iron–porphyrin complex (Schricker & Miller, 1983). The thermal denaturation of sperm whale myoglobin has been investigated by Awad & Deranleau (1968). A suggested mechanism involves a conformational disturbance in the region of the haem group in the first phase, unfolding of the helical regions in the molecule in the second phase, followed by a sequence of aggregation steps leading to precipitation in the third phase. Hägerdal & Martens (1976) studied the effect of heat treatment on sperm whale myoglobin at various water contents

but were not in favour of the two state transition suggestion for the mechanism of denaturation in this system, and pointed out that both the aggregates and the soluble fraction might consist of native as well as denatured molecules. Haemoglobin and myoglobin are also relatively easily denatured by stirring of their solutions and haemoglobin is particularly sensitive in this respect (Asakura *et al.*, 1974; Ohnishi & Asakura, 1976). Considerable care in handling solutions is necessary.

It is well known that nitrite ion added to meat reacts with myoglobin (and haemoglobin of trapped red blood cells), to form the cured meat colour. From practical experience with curing (Cassens *et al.*, 1979), the general scheme of events is that when nitrite ion is first added to meat, the colour is changed from the purple-red colour of myoglobin to the brown of metmyoglobin; with time and reducing conditions the colour is converted to the dark red of nitric oxide myoglobin. Heat denaturation (if used) converts the pigment to the stable nitric oxide haemochrome, which is pink. Nitric oxide complexes are photodissociable (Giddings, 1977) and, in the presence of oxygen, their stability is limited by the rate of loss of nitric oxide. Tarladgis (1962) proposed that the pigment of cooked cured meat was a haem compound (nitric oxide myochrome), containing nitric oxide groups at both axial coordination sites rather than the so far widely accepted denatured globin–nitric oxide haemochrome description. This is the currently accepted structure for the pigment of cooked cured meat.

There are three potential problems arising from the curing of meat: the possible formation of nitrosamines, the presence of residual nitrite ion (consumption of which increases the total body burden of nitrite ion) and concern over unknown reactions of nitrite ion (Cassens *et al.*, 1979). The concern over the use of nitrite ion as a meat curing agent arose in the 1970s in response to sporadic detection of nitrosamines in various cured meats. The question is one of risk/benefit, involving risk from preformed nitrosamines and the action of residual nitrite ion, and benefit from protection against botulism.

Nitrite ion also functions as an antioxidant in cured meat products in three possible ways (Igene *et al.*, 1985): by the formation of a stable complex with haem pigments thereby preventing the release of nonhaem iron for subsequent catalysis of lipid oxidation; by directly interacting with the nonhaem iron (Fe(II)) from denatured haem pigments and, to a lesser extent, by stabilisation of the unsaturated lipids within the membranes. Stabilisation of the porphyrin ring, preventing release of iron (Fe(II)) during the cooking process, appeared to be the most important mechanism (Igene *et al.*, 1985; Chen *et al.*, 1984; Kanner *et al.*, 1984). Efforts have been made to reduce the amount of nitrite and to develop alternative methods of meat curing. In such an attempt, Hannan (1981) proposed the use of a substance

with a proven ability to prevent the formation of nitrosamines and supplementing the action of nitrite ion with another preservative, e.g. sorbic acid, or physical methods of preservation such as freezing or irradiation so as to allow much lower levels of nitrite ion. As a last resort nitrite ion would have to be replaced by some other preservative. Shahidi *et al.* (1984) developed an alternative method for producing cooked meat colour by preparing dinitric oxide haemochrome. It is now required to identify an antioxidant to perform similar functions to nitrite ion and to devise some way of imparting antimicrobial properties to the system. It has become clear that a combination of sorbic acid and nitrite ion is inappropriate since these two preservatives react with one another to form a potentially mutagenic reaction product (Namiki *et al.*, 1981).

The possibility of developing an alternative curing system appeared very remote in the earlier stages; the main research was directed towards ways of reducing the amount of residual nitrite ion. Frouin (1977) suggested that there are numerous compounds in meat capable of fixing nitric oxide and thereby greatly decreasing the possible risk. Some of these compounds, e.g. nonhaem proteins, form complexes with nitrite ion which, however, are not stable and decompose, particularly at low pH and high temperatures (Ito *et al.*, 1983). When myoglobin was incubated with nitrosated protein, nitric oxide haemochrome could be extracted from the reaction mixture in the presence of ascorbic acid. Lougovois (1982) suggested that addition of haemoprotein (e.g. blood pigment) to cured meat products, would be expected to increase the amount of pigment converted to the nitric oxide form and thus to reduce the amount of nitrite ion available for reaction with nonhaem constituents, some of which may present a potential for formation of carcinogenic *N*-nitroso compounds. This follows the suggestion made by Keefer (1976) that certain biological compounds trap nitric oxide and suppress its nitrosamine-forming ability.

A dramatic reduction in the content of nitrite ion in meat products was suggested by Bucsko (1982); this proposal was that it was possible to reduce the nitrite content of heat processed Brühwürst products considerably whilst retaining the characteristic colour and colour stability of the finished product, by addition of ascorbic acid. Wenzel & Schaal (1977) revealed that addition of blood to cured meat products resulted in substantial reduction of residual nitrite ion available for nitrosation reactions. This approach does, however, lead to potential problems with microbial contamination in practice.

In meat products, partial substitution of meat by vegetable proteins results in colour fading. Addition of nitric oxide haemoglobin prevents this drawback and restores a meat-like colour to the product (Noel *et al.*, 1984). With haemoglobin concentrations in the range 3–10%, the colour of the

resulting product can be readily set anywhere on a Frankfurter-type sausage red meat colour scale but the level of residual nitrite ion is similar to that usually found in traditional cured meat products.

DETERMINATION OF NITRIC OXIDE HAEMOPROTEINS

Anderton & Locke (1955) first described a method whereby the highly coloured nitric oxide haem moiety was rendered soluble in some organic solvents by treatment of the cooked cured meat with acetone. Hornsey (1956) described a simple and rapid method for extracting the nitric oxide haem pigment from cooked cured meat. Selective extraction as a nitric oxide–haem–acetone complex was achieved by the use of an acetone–water solvent. The acetone:water ratio was shown to be critical, maximum extraction being obtained with a final ratio of 4:1, due allowance being made for the moisture present in meat. After filtration, the absorbance of the extract was measured at 540 nm for nitric oxide pigment and, using the extinction coefficient at this wavelength, the concentration of the pigment calculated. It was reported that other forms of meat pigments, e.g. oxidised, reduced or oxygenated forms, were not extracted. In order to determine the concentration of total pigments, concentrated hydrochloric acid was introduced in the acetone–water solvent used for the extraction, and acid haematin was formed from any free haem and nitric oxide pigments present. The absorbance of this extract at 640 nm and the extinction coefficient of acid haematin at this wavelength were then used to obtain the concentration of the total pigment present in the sample.

A slight modification of the Hornsey method for the determination of nitric oxide pigment was reported by Gantner (1960); instead of measuring the absorbance of the nitric oxide–haem–acetone complex, which was found to photodissociate, this derivative was converted to acid haematin by the addition of hydrochloric acid to the extract. The acid haematin was then measured in the same way as for total pigment.

Hägerdal (1978), studying the stability of myoglobin to acetone treatment, revealed that at acetone concentrations of 50–70%, myoglobin underwent complete irreversible transition, whereas at low ($<30\%$) and high ($>90\%$) acetone concentrations, only a fraction of the myoglobin sample underwent irreversible transition.

It is not clear whether the 80% acetone solvent used for the extraction of nitric oxide pigments cleaves the haem–denatured globin bond or the globin is replaced by another ligand. It is well established, however, that the resulting extracted haem compound has no globin attached to it (Möhler, 1973). Deatherage & Moffat (1979) have examined the structure of nitric

oxide haemoglobin using difference Fourier analysis of X-ray data. They suggested that the iron ions move to the distal histidine on both α- and β-chains; the porphyrin plane moves with the iron. It is not clear whether this motion of the haem could weaken the haem–globin bond and render it susceptible to rupture by the organic solvent. Ibers & Holm (1980), on the other hand, have suggested that the iron–imidazole nitrogen bond in nitric oxide haemoglobin is apparently ruptured.

The method reported by Hornsey (1956) has been used by many workers in meat system studies for the determination of nitric oxide pigment and total pigment (Walters *et al.*, 1968; Lougovois, 1982) and similarly in model (solution) systems of haemoproteins (Fujimaki *et al.*, 1975).

The modification of Hornsey's method by Gantner (1960) for the determination of nitric oxide pigment has also been applied to the determination of *free haem* in solutions of haemoproteins (Fronticelli & Bucci, 1963). In these experiments it was desired to measure the extent to which haem dissociated from haemoproteins as a function of pH and suitably buffered solutions were extracted by adding acetone until the final composition was acetone:water, 4:1 v/v. The protein was precipitated and any free haem appeared in the extract; this was measured as acid haematin. The same method was used by Trautman (1966) to measure free haem in an attempt to determine whether acetone-soluble haem increased concomitantly with loss of observed colour and decrease in pH of post-rigor pork muscles. It was found that the amount of free haem present in muscle extracts increased as their pH was lowered, but it was not possible to detect any free haem in the muscles themselves.

It has been recently demonstrated that phosphate species greatly enhance the liberation of haem when haemoglobin or myoglobin is precipitated from aqueous solution with acetone (Wedzicha & Ladikos, 1985). The reason for this effect has been identified as a lowering of pH when mono- and dihydrogen orthophosphate salts are precipitated from aqueous solution by the addition of an organic solvent (Wedzicha & Ladikos, 1986). Indeed, when haemoglobin or myoglobin are precipitated from solutions buffered with phosphate, the visible spectrum of the supernatant more closely resembles that of acid haematin than hydroxyhaemin or haematin. A model for the process of release of haem involves an initial change in pH which is fast on the timescale of protein precipitation and acid catalysed loss of haem from the haemoprotein. The extent of release of haem is then dependent on the relative rates of the two rate-determining processes (Wedzicha *et al.*, 1988). These findings indicate the need for caution when using acetone precipitation for the determination of nitric oxide haemoproteins or free haem in the presence of haemoglobin or myoglobin in phosphate buffer. The problems do not arise if other weak acids and their conjugate bases, which

do not precipitate when an appropriate volume of organic solvent is added, are also present and are capable of buffering the change in pH. Thus, haem is not released from solutions of haemoproteins in a mixture of acetate and phosphate buffers, or from slurries of meat in phosphate buffer.

ACKNOWLEDGEMENT

We are grateful to the University of Leeds for a Scholarship to one of us (DL).

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