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FEBS Letters 341 (1994) 263–267

FEBS 13796

FEBS LETTERS

# Ascorbic acid can either increase or decrease low density lipoprotein modification

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Received 11 February 1994

**Abstract**

In freshly prepared low density lipoprotein (LDL), ascorbate inhibited LDL oxidation by macrophages at the higher concentrations tested (60–100 μM). In contrast, with LDL that had been allowed to autoxidise in the refrigerator (3°C) for at least 10 weeks after isolation (mildly oxidised or minimally-modified LDL), ascorbate did not inhibit the modification of LDL in the presence of macrophages. Ascorbate actually modified autoxidised LDL itself in the absence of macrophages to greatly increase its uptake by macrophages. The modification of autoxidised LDL by ascorbate increased the levels of thiobarbituric acid-reactive substances in the medium and was completely inhibited by the antioxidant butylated hydroxytoluene. Thus the effects of ascorbate on unoxidized LDL can be very different to those on mildly oxidised LDL.

*Key words:* Antioxidant; Ascorbic acid; Atherosclerosis; Low density lipoprotein; Macrophage; Vitamin C

## 1. Introduction

It has been well documented that low density lipoprotein (LDL) plays an important role in atherosclerosis, the underlying cause of coronary heart disease and strokes. Oxidatively modified LDL is taken up by macrophages at a much greater rate than native LDL, via 'scavenger' receptors [1,2] leading to the formation of cholesterol-laden 'foam cells' [3]. Foam cells are characteristic of many early and late atherosclerotic lesions. The role of antioxidants as a defence against lipid peroxidation has thus been considered as potentially important in the protection against atherosclerosis.

Ascorbic acid (vitamin C) is a major water-soluble antioxidant in plasma. It has been shown to inhibit LDL oxidation by a water-soluble azoinitiator, activated neutrophils or cigarette smoke [4], and by metal ions and cells [5,6,7]. Physiological concentrations of ascorbate inhibit LDL oxidation by human monocyte-derived macrophages or copper ions and help to preserve its endogenous lipid-soluble antioxidants [5,6]. Ascorbate increases the lag phase before LDL is oxidised by copper ions [8].

Ascorbate has been reported to regenerate the antioxidants probucol [9] and α-tocopherol [10] from their radicals in oxidising LDL particles, and to decrease the in vivo oxidation of LDL in genetically scorbutic rats [11]. Retsky et al. [7] have suggested two mechanisms by which ascorbate may protect LDL against oxidation; ascorbic acid may scavenge free radicals in the aqueous phase or dehydro-L-ascorbic acid (the oxidation product of ascorbic acid), or its decomposition products, may modify LDL leading to decreased copper binding to the LDL particle and increased resistance to copper-dependent oxidation.

Ascorbate can inhibit atherosclerosis in cholesterol-fed rabbits even when the plasma cholesterol levels are not decreased by it [12,13]. Another study has demonstrated an inverse relationship between ascorbate levels in human plasma and the occurrence of coronary heart disease (although low levels of vitamin E were a stronger risk factor) [14,15] and stroke [14]. The risk of angina is inversely related to the plasma ascorbate level, but this is partly due to the low plasma ascorbate levels found in smokers [16]. The mortality from cardiovascular disease and all causes has been reported by Enstrom et al. to be strongly inversely related to ascorbate intake in males and more weakly in females [17]. Contrary to these results, two studies on antioxidant vitamin intake [18,19] showed no apparent effect of ascorbate on coronary heart disease.

Ascorbate can act as a prooxidant as well as an antioxidant [20,21]. We report here that ascorbate can either increase or decrease the modification of LDL by macrophages depending upon the age of the LDL preparation.

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*Abbreviations:* BHT, butylated hydroxytoluene; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; LDL, low density lipoprotein; MDA, malondialdehyde; TBARS, thiobarbituric acid-reactive substances.

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*SSDI* 0014-5793(94)00181-T

## 2. Materials and methods

### 2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), foetal calf serum (FCS), gentamicin (10 mg/ml), penicillin/streptomicin (5,000 i.u./5,000 μg per ml), amphotericin B (250 μg/ml) and glutamine (200 mM) were purchased from Gibco, Uxbridge, Middlx., UK. Ham's F-10 medium was from ICN Flow, High Wycombe, Bucks, UK. Ferrous sulphate, Phenol red solution (0.5% w/v), L-ascorbic acid (sodium salt) and tetramethoxypropane were from Sigma Chemical Co., Poole, Dorset, UK. Falcon multiwell tissue culture plates (18 mm and 22 mm diameter wells) were from Fahrenheit, Bradwell Abbey, Milton Keynes, Bucks., UK and female Swiss T.O. mice were purchased from Tuck and Son, Battlesbridge, Essex, UK.

### 2.2. Isolation and radioiodination of LDL

LDL (1.019–1.063 g/ml) was isolated from blood donated by healthy volunteers by sequential density ultracentrifugation as previously described [22], and radiolabelled with $Na^{125}I$ using iodine monochloride [23]. The $^{125}I$-labelled LDL was mixed with nonlabelled LDL to give a specific activity of 20–40 cpm/ng and diluted to 2 mg protein/ml with buffer containing 100 μM EDTA for storage at 3°C. When used at 100 μg protein/ml, this would give a final EDTA concentration of 5 μM. This standardisation of the EDTA concentration was considered important as EDTA can either stimulate or inhibit LDL oxidation by cells depending on its concentration [24].

### 2.3. Modification of LDL by cells

Resident peritoneal macrophages were isolated from female Swiss T.O. mice by peritoneal lavage [25]. The peritoneal cells were plated at $0.75 \times 10^6$ cells per 18 mm well, and used for the modification of LDL. $^{125}I$-labelled LDL (100 μg protein/ml) was incubated with macrophages (macrophage-modified LDL) or in cell-free wells (control LDL) at 37°C, under 5% $CO_2$ for 16 or 18 h in 0.4 ml of Ham's F-10 medium supplemented with $FeSO_4$ (6 μM final) [26], Phenol red (12 μg/ml) and gentamicin (50 μg/ml), with varying concentrations of ascorbate. For experiments in which a thiobarbituric acid-reactive substances (TBARS) assay was used, cells were plated at $1.5 \times 10^6$ cells per well in 22 mm diameter wells with 1 ml medium per well. After incubation, the modified medium was collected and centrifuged (MSE Microcentaur, 13,000 rpm for 2.5 min) to remove any detached cells.

### 2.4. Uptake of modified LDL by cells

For measurement of uptake of modified LDL, mouse peritoneal macrophages ($1.25 \times 10^6$ cells per 22 mm diameter well) or mouse J774 macrophage-like cells (plated at $0.2 \times 10^6$ cells per 22 mm diameter well and used the day after) were used ('target' macrophages). Macrophage-modified or control LDL was diluted to 10 μg protein/ml in DMEM containing 10% (v/v) FCS, penicillin/streptomicin (100 i.u./100 μg per ml) and gentamicin (50 μg/ml) for the peritoneal cells or in DMEM containing 20% (v/v) heat-inactivated FCS, amphotericin B (1 μg/ml), L-glutamine (2 mM) and penicillin/streptomicin (10 i.u./10 μg per ml) for the J774 cells (media in which cells cannot oxidise LDL [27]). The LDL was then incubated with the 'target' macrophages or cell-free wells (1 ml/well) for 22 h.

The degradation of modified LDL was assessed by measuring the radioactive noniodide trichloroacetic acid-soluble degradation products in the medium [22]. The degradation products in the cell-free wells were subtracted from the values for the macrophage wells. The cells were washed, lysed with 0.2 M NaOH, and the cell protein and cell-associated radioactivity measured [23]. Any radioactivity remaining in the cell-free wells was subtracted from the values for the macrophage wells. Since much radioactivity remains in the cells with oxidised LDL, the cell-associated values were added to the degradation values to obtain total LDL uptake [28].

### 2.5. Thiobarbituric acid-reactive substances assay

The method used was modified from that described by Bernheim et al. [29]. Butylated hydroxytoluene (BHT) and EDTA at final concentrations of 20 μM and 1 mM, respectively, were added to 250 μl samples of modified or control LDL (100 μg protein/ml) to prevent further oxidation. 2 ml of 0.335% (w/v) thiobarbituric acid in 10% (w/v) trichloroacetic acid was added to each sample and incubated at 95°C for 15 min. The absorbance was read at 535 nm. Standards were prepared using tetramethoxypropane.

## 3. Results

The effect of ascorbate on the macrophage-mediated oxidation of LDL was studied by incubating mouse peritoneal macrophages with $^{125}I$-labelled LDL in Ham's F-10 medium containing various concentrations of ascorbate. A second set of macrophages ('target' macrophages) was used to determine the rate of uptake of the LDL. In the absence of ascorbate, the macrophages oxidised the $^{125}I$-labelled LDL so that it was taken up much faster than the control LDL by the second set of macrophages (Fig. 1A). As reported before [28], the oxidised $^{125}I$-labelled LDL was resistant to degradation and much of its radioactivity accumulated within the cells. $^{125}I$-labelled LDL uptake was therefore calculated by adding the degraded noniodide trichloroacetic acid-soluble radioactivity released into the medium to the cell-associated radioactivity.

Ascorbate inhibited the modification of 'fresh' LDL by macrophages, with 80 μM giving a total or near total inhibition in the experiment shown in Fig. 1A. At lower concentrations, the ascorbate might have slightly increased the modification of LDL by macrophages, since greater modification of LDL was seen at 20 μM and 40 μM than occurred in the absence of ascorbate. This effect, although slight, was observed in all experiments on 'fresh' LDL (LDL used within 7 weeks of isolation). Control LDL (LDL incubated in cell-free wells) was taken up somewhat faster after incubation with about 40 μM ascorbate. Although the extent of modification was only low, this effect was observed in a number of experiments.

The effects of ascorbate on the same batch of LDL 10 weeks after the start of its isolation from blood are shown in Fig. 1B. With increasing concentrations of ascorbate there was an increase, rather than a decrease, in LDL modification by macrophages. There was also a great increase in the modification of the control LDL. About half of the ascorbate-modified LDL that was taken up by the macrophages over 22 h was not degraded by them (Fig. 1B) and so it appears that ascorbate-modified LDL, like oxidised LDL [28], is resistant to lysosomal degradation. The only difference between the two experiments (other than that they had, of course, to be carried out at different times) was the duration of storage of the isolated LDL.

Fig. 2 demonstrates the different effects of ascorbate on 'fresh' and 'aged' LDL within the same experiment. Ascorbate inhibited LDL modification by macrophages when the LDL was 'fresh', with the exception of the lower doses. It did not inhibit the modification of LDL in the presence of macrophages, and modified the con-

trol LDL itself, however, when the 'aged' LDL was used. The 'fresh' LDL, whose oxidation by macrophages was inhibited by ascorbate when it was 5 weeks old (Fig. 2A), was not effectively protected by ascorbate from modification in the presence of macrophages when it was 20 weeks old and indeed ascorbate itself modified the 'aged' LDL in a dose-dependent manner in the absence of macrophages (Fig. 2B).

The effects of ascorbate on 'aged' LDL were not due to any contaminating iron or copper ions in the ascorbate solution as the levels of iron and copper in 100 $\mu$M ascorbate solution would have been less than 1 nM each,

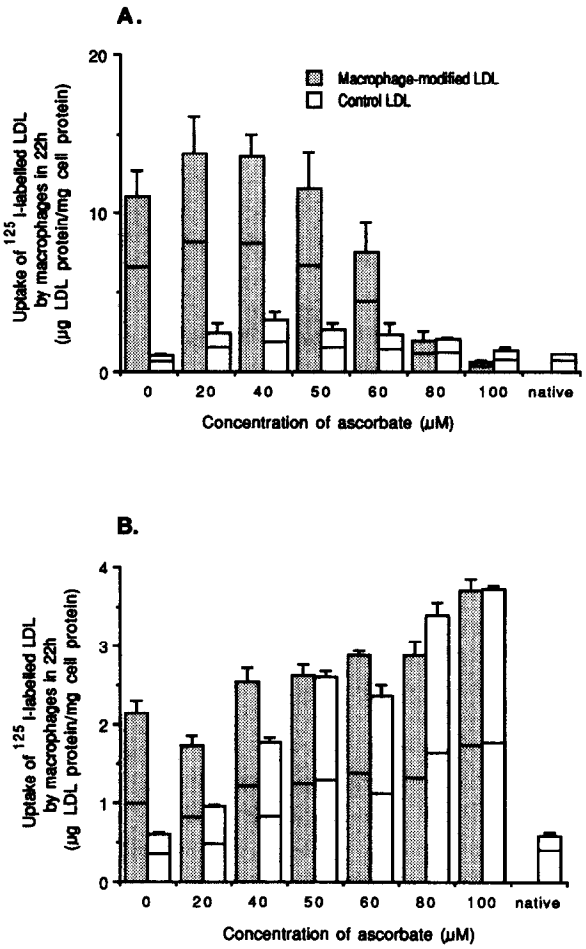


Fig. 1. $^{125}$I-labelled LDL (100 $\mu$g protein/ml) was incubated for 18 h in triplicate wells with mouse peritoneal macrophages (macrophage-modified LDL) or in cell-free wells (control LDL) in Ham's F-10 medium and the indicated amount of ascorbic acid. The LDL was then diluted to 10 $\mu$g protein/ml in serum-containing DMEM and incubated with 'target' macrophages (mouse peritoneal macrophages in Fig. 1A; J774 macrophages in Fig. 1B) or in cell-free wells for 22 h. The rates of degradation (lower area of bar) and cell association (upper area of bar) of the LDL were then measured. The total height of the histobar indicates the uptake (degradation plus cell-association) of the LDL. The uptake of native (non-incubated) LDL is also shown. (A) LDL batch A was used 7 weeks after starting its isolation from blood; (B) LDL batch A was used 10 weeks after starting the isolation. The mean ± S.E.M. of triplicates are shown.

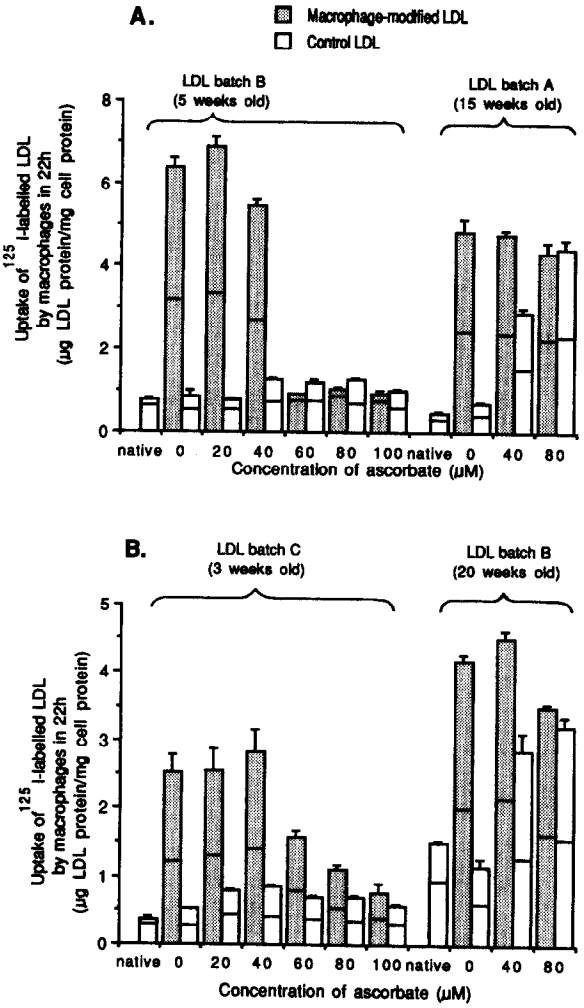


Fig. 2. 'Aged' or 'fresh' $^{125}$I-labelled LDL (100 $\mu$g protein/ml) was incubated for 18 h in triplicate with macrophages or in cell-free wells in Ham's F-10 medium and the indicated amounts of ascorbate. The LDL was then diluted to 10 $\mu$g protein/ml with serum-containing DMEM and incubated with 'target' J774 macrophages or in cell-free wells for 22 h. The rates of degradation (lower area of bar) and cell-association (upper area of bar) of the LDL were then measured. (A) LDL batch B (5 weeks old; 'fresh') and LDL batch A (15 weeks old; 'aged'), tested in the same experiment; (B) LDL batch C (3 weeks old; 'fresh') and LDL batch B (20 weeks old; 'aged'), tested in the same experiment. The mean ± S.E.M. of triplicates are shown. Note the different effects of ascorbate on 'fresh' and 'aged' batch B LDL.

as determined by atomic absorption spectroscopy (in a 100 mM solution of ascorbate).

Fig. 3 shows that adding 100 $\mu$M ascorbate increased the modification of 'aged' LDL incubated both in the presence or absence of macrophages, as measured by macrophage uptake or TBARS. (Adding ascorbate directly to the TBARS assay tubes did not increase the absorbance above the reagent blanks, either in the presence or absence of LDL.) Adding the antioxidant BHT together with the ascorbate during the modification stage totally prevented the enhanced uptake of the LDL by the

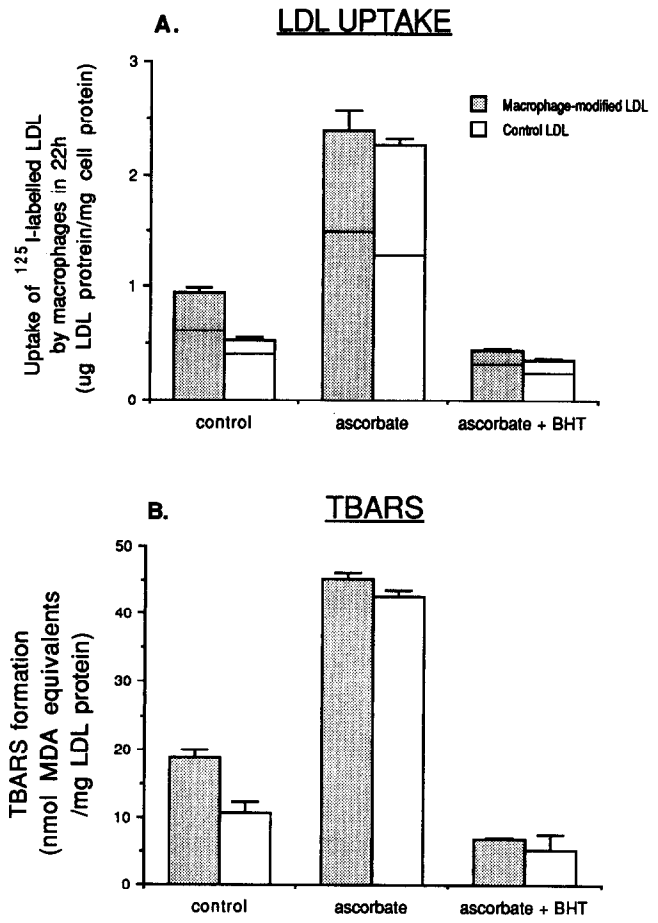


Fig. 3. 'Aged' $^{125}$I-labelled LDL (100 $\mu$g protein/ml; 29 weeks old) was incubated for 16 h in triplicate with macrophages or in cell-free wells in Ham's F-10 medium without ascorbate, with 100 $\mu$M ascorbate or with 100 $\mu$M ascorbate plus 20 $\mu$M BHT. Samples were then assayed for TBARS (B) or the LDL was diluted to 10 $\mu$g protein/ml in serum-containing DMEM and incubated with 'target' J774 macrophages or in cell-free wells for 22 h. The rates of degradation (lower area of bar) and cell-association (upper area of bar) were then measured (A). The mean ± S.E.M. of triplicates are shown. These results were confirmed by another experiment.

target macrophages and the increased formation of TBARS.

## 4. Discussion

Ascorbate is known to have antioxidant properties. It is able to scavenge aqueous free radicals [20,4] and to regenerate α-tocopherol from the α-tocopheryl radical [30,31]. It has been shown that ascorbate can protect plasma and LDL lipids from peroxidative damage [32,33,4], and it may inhibit the binding of copper ions to LDL [7].

In the presence of transition metal ions, however, ascorbate is well known to sometimes act as a prooxidant rather than as an antioxidant, since it can reduce transition metal ions. In the presence of hydrogen peroxide this will lead to hydroxyl radical production by the Fenton reaction [34]:

$$\text{e.g. } Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

Hydroxyl radicals have been shown to initiate lipid peroxidation [35]. Thus, in contrast to the above observations, one may expect ascorbate to act sometimes as a prooxidant [20,7]. Indeed, Hunt et al. [21] have shown that ascorbate can both inhibit and promote the formation of ceroid (polymerised oxidised lipids) in a model system, depending on its concentration. When trace levels of transition metals are present, low concentrations of ascorbate can increase ceroid formation in macrophages [21].

The study described here demonstrates that ascorbate can either inhibit or promote the modification of LDL. Ascorbate was observed to marginally increase the modification by macrophages of 'fresh' LDL at concentrations of approximately 40 $\mu$M or below, whilst above this concentration a strong inhibition of LDL modification by macrophages was seen, with total inhibition at between 60 $\mu$M and 100 $\mu$M ascorbate (Figs. 1A and 2). The mean plasma level of ascorbate in various European populations varies between approximately 20 and 55 $\mu$M [15].

In contrast, when the same LDL had 'aged' for a period of at least 10 weeks, ascorbate did not inhibit the modification of LDL in the presence of macrophages and actually caused a large and dose-dependent modification of LDL in the absence of macrophages (Figs. 1B and 2).

To demonstrate that it was not a change in experimental conditions that was causing the observed differences in the effects of ascorbate on 'fresh' and 'aged' LDL of the same batch, two batches of LDL of different ages were tested in the same experiment (Fig. 2A). As expected, ascorbate inhibited the oxidation of the 'fresh' LDL but modified the 'aged' LDL itself. The LDL whose oxidation by macrophages was prevented by ascorbate when 'fresh' (Fig. 2A), showed cell-free modification in the presence of ascorbate after 'ageing' (Fig. 2B).

It appears therefore that ascorbate has a modifying effect upon LDL that has already undergone some change, most probably autoxidation, upon storage. LDL is known to autoxidise slowly upon storage [36] and LDL stored for long periods has been used as a model for mildly oxidised (or minimally modified) LDL [37], which has been postulated to be involved in the very early stages of atherogenesis [37].

There was an increase in TBARS in 'aged' LDL incubated with ascorbate in the absence of cells suggesting that the oxidation of the LDL was involved in the modification process. In addition, adding the antioxidant BHT together with the ascorbate prevented the increase

in TBARS and the enhanced uptake of the LDL by the target macrophages. It may possibly be of interest that ascorbate has been shown to react with lysyl residues under autoxidising conditions [38].

Previous studies have demonstrated LDL accumulation in regions of arteries susceptible to the development of atherosclerotic lesions and have shown that LDL is retained in these regions for longer than in lesion-resistant sites and that the retention time is increased by cholesterol feeding [39]. If some limited degree of oxidation of LDL were to occur during this time, analogous to what happens during storage in a refrigerator over a period of months [37], this raises the possibility that ascorbate may promote further modification of LDL within the arterial wall.

In summary, ascorbate can either stimulate or inhibit LDL modification in vitro depending upon how long the LDL has been stored and hence presumably upon its 'background' level of autoxidation. Therefore if mildly oxidised LDL were to occur in plasma or in atherosclerotic lesions, it cannot be automatically assumed that ascorbate would act as an antioxidant against its further modification. Not all epidemiological studies have shown ascorbate to be a protective agent against cardiovascular disease [18,19], and the finding that ascorbate can sometimes augment LDL modification in vitro suggests that further studies should be carried out to ascertain whether or not ascorbate is truly anti-atherogenic in vivo.

*Acknowledgements*: We thank Drs. David T. Hart and Julia Eckersley for kindly providing the J774 cells, Justin Richards for his excellent technical assistance and the Wellcome Trust for a Research Leave Fellowship (D.S.L.). S.E.S. is a SERC CASE student in collaboration with SmithKline Beecham Pharmaceuticals, Welwyn. We thank Dr. Catherine A. Rice-Evans (United Medical and Dental Schools of Guy's and St. Thomas's Hospitals, London) for her comments on the work and Dr. Steve P. Hopkin (Zoology, University of Reading) for kindly showing us how to perform the atomic absorption spectroscopy for iron and copper.

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