New insights into the pathogenesis of copper toxicosis in Wilson's disease: evidence for copper incorporation and defective canalicular transport of caeruloplasmin

George F. E. CHOWRIMOOTOO*, Hafez A. AHMED† and Carol A. SEYMOUR*‡

Departments of *Clinical Biochemistry and Metabolism and †Biochemical Medicine, St George's Hospital Medical School, London SW17 0RE, U.K.

Previous studies have suggested that copper is incompletely incorporated into caeruloplasmin, the major plasma form of copper-transporting protein, in the genetic copper toxic condition, Wilson's disease. In this paper we have investigated the role of copper and caeruloplasmin in the abnormal biliary copper transport that characterizes Wilson's disease. Using SDS/PAGE and Western blotting, we have demonstrated the presence of holocaeruloplasmin in liver samples from patients with Wilson's disease (abnormal biliary copper excretion) and in control patients (normal biliary copper excretion). The presence of holocaeruloplasmin was also confirmed by measurement of caeruloplasmin oxidase activity using staining with o'-Dianisidine. In contrast with the findings in liver tissue, holocaeruloplasmin was absent from bile from patients with Wilson's disease, but as

expected it was present in the bile from control subjects. We have also identified and partially characterized a 189–200 kDa protein from purified human biliary canalicular membranes which binds copper and possesses caeruloplasmin-like activity when probed with a specific human anti-caeruloplasmin antibody. In conclusion, we have demonstrated that copper incorporation in caeruloplasmin is normal in patients with Wilson's disease contrary to previous reports. Secondly, we have shown that the defect in Wilson's disease appears to lie in the biliary canalicular excretion of holocaeruloplasmin resulting in its retention within the hepatocyte causing copper toxicosis. Finally we have identified and partially characterized a caeruloplasmin-binding protein from biliary canalicular membranes which may provide a link to the gene defect in Wilson's disease.

INTRODUCTION

The bodily homeostasis of copper depends solely on its excretion from the liver into bile [1]. After its absorption from the gut, copper is first bound to albumin and then transported to the liver [2]. Here it is either used for the synthesis of copper-containing enzymes such as cytochrome oxidase and superoxide dismutase or is incorporated into caeruloplasmin [3–5]. The gene for caeruloplasmin is located on chromosome 3q25 [6] and codes for a 1046-amino acid glycoprotein found in the γ -globulin fraction of plasma [7]. Caeruloplasmin contains six or seven copper atoms per molecule [8] and is secreted into the plasma. Its major role is to transport copper to extrahepatic tissues, but it also has several other functions [4,9].

Caeruloplasmin is variably absent from plasma in the autosomal recessive disorder of copper metabolism, Wilson's disease, which is also associated with copper toxicosis due to impairment of copper excretion. The recently cloned gene for Wilson's disease has been located to chromosome 13q14.3 [10–12]. It encodes an as yet uncharacterized copper-transporting ATPase similar to that observed in bacteria and Menkes disease [13].

We have suggested previously that caeruloplasmin plays an important role in biliary copper excretion [14]. In addition, the biochemical pathway for the incorporation of copper into caeruloplasmin is thought to be impaired in Wilson's disease [15,16]. The present study confirms the previous work of Sato and Gitlin [17] showing that the two forms of caeruloplasmin, namely holocaeruloplasmin (which contains bound copper) and apocaeruloplasmin (the protein after removal of bound copper by heat treatment), can be separated by SDS/PAGE by virtue of

their different electrophoretic mobilities. We have investigated further the incorporation of copper into caeruloplasmin and the involvement of caeruloplasmin in biliary copper excretion in normal subjects and in patients with Wilson's disease.

MATERIALS AND METHODS

Affinity-purified polyclonal sheep anti-human caeruloplasmin antibody (peroxidase conjugate) was purchased from Serotec, Oxford, U.K. Hybond N membrane and enhanced chemiluminescence Western-blotting detection reagents were all from Amersham International, Amersham, Bucks., U.K. All other chemicals were from either Sigma, Poole, Dorset, U.K. or BDH, Dagenham, Essex, U.K.

Liver homogenates

Liver tissue (non-Wilson's cirrhosis and Wilson's disease cirrhosis) was obtained either by percutaneous needle biopsy where clinically indicated or after explants from liver transplantation. At the time of collection, the tissue (about 2 g) was immediately snap-frozen as 1 cm cubes in liquid nitrogen and stored at -80°C . At the time of use, the tissue was powdered by crushing with a mortar and pestle under liquid nitrogen. Samples were taken into SVE buffer [0.25 M sucrose, 0.1 M EDTA, pH 7.4, and 0.1 % (v/v) ethanol] containing the proteinase inhibitors 1 % (w/v) antipain and 1 % (w/v) leupeptin and homogenized in a Dounce homogenizer (Kontes Glass Company) using approx. 50 strokes of a tight-fitting pestle. The homogenates were then centrifuged at 800 g for 10 min. The pellet was discarded and the supernatant stored in 500 μl aliquots at -20°C .

‡ To whom correspondence should be addressed.

Bile collection

Cholecystokinin-stimulated bile samples were collected from fasting subjects by cannulation of the bile duct through the ampulla of Vater, at the time of endoscopic retrograde cholangiopancreatography and stored at -20°C .

SDS/PAGE and Western blotting

Human caeruloplasmin was purified from out-dated plasma [18] for use as standard control and for preparation of apo-caeruloplasmin [19]. Samples of liver homogenates and bile samples (containing $150\text{ }\mu\text{g}$ of protein) from controls and patients with Wilson's disease were subjected to SDS/PAGE (8% gels) as described previously [20]. Proteins from the gel were transferred to a nylon membrane using a semidry blotter (Bio-Rad) for 90 min at a constant current of 1 mA/cm^2 . The blot was blocked in 10% milk solution containing 50 mM Tris/150 mM NaCl, pH 7.4, and 0.02% (w/v) NaN_3 for 2 h and incubated for a further 3 h with sheep anti-human caeruloplasmin conjugated to horseradish peroxidase. After the blot had been washed several times in 50 mM Tris/150 mM NaCl buffer, pH 7.4, caeruloplasmin was detected using an enhanced chemiluminescence method as described by Amersham.

Measurement of caeruloplasmin oxidase activity in liver samples

Liver homogenates (containing $150\text{ }\mu\text{g}$ of protein), without heating before loading, were subjected to SDS/PAGE (8% gel) to determine the presence of holocaeruloplasmin, which could be identified by its oxidase activity. The gel was briefly rinsed in 0.1 M sodium phosphate buffer, pH 5.7, and incubated with the same buffer, but containing 0.1% o'-Dianisidine and 30% ethanol at 37°C for 90 min. The gel was left at room temperature until staining for oxidase activity was clearly visible.

Measurement of caeruloplasmin oxidase activity in serum

Serum caeruloplasmin oxidase activity was measured as described by Henry et al. [21] with slight modification. Serum samples (2 mg of protein) from patients with Wilson's disease and controls were added to 0.1 M sodium acetate buffer, pH 6.0, containing 0.25% *p*-phenylenediamine dichloride. The reaction was performed at 37°C . For blank samples, 0.1% NaN_3 was added instead of the sample. Absorbance was read after 10 min at 520 nm and again after 40 min. Caeruloplasmin oxidase activity (units) was calculated as follows:

$$\text{Oxidase} = (A_{520} \text{ at } 40 \text{ min} - A_{520} \text{ at } 10 \text{ min}) \times 1000$$

Preparation of biliary canaliculi membranes

Biliary canaliculi membranes were prepared from normal liver tissue as previously described [22]. Wedges of histologically normal liver tissue (0.8–1.2 g) taken at the time of laparotomy for gall stones were homogenized in 15 ml of Tris buffer (0.25 M sucrose in 5 mM Tris/HCl, pH 8; 4°C) using a Potter/Elvehjem homogenizer. The homogenates were pelleted sequentially at 1000 *g* for 10 min using an SS-34 fixed-angle rotor in Sorval RC-5B. The 1000 *g* pellet was resuspended in 0.25 M sucrose and adjusted to 35% sucrose solution by adding 55% sucrose before gradient centrifugation. The refractive index was monitored using a refractometer. The gradients consisted of 55, 45, 35, 25 and 15% (w/w) sucrose in 5 mM Tris/HCl, pH 8, with the resuspended 1000 *g* membrane pellet replacing the 35% sucrose solution, and were centrifuged at 100000 *g* for 18 h using a 6×14 swing-out rotor in an MSE-65 superspeed ultracentrifuge. The

separated layers were visualized against light before being taken up with a syringe into centrifuge tubes. The separated samples were then washed and resuspended in the homogenization buffer. Membranes were identified by marker enzymes and electron microscopy as previously described [22,23].

Detection of caeruloplasmin-bound protein in biliary canaliculi membranes

Biliary canaliculi membranes were solubilized in SDS electrophoresis sample buffer and subjected to SDS/PAGE (8% gel), transferred to a nylon membrane and immunoblotted using the polyclonal antibody to caeruloplasmin, as described above, with the exception that, after the blot was blocked with milk, it was incubated in either the presence or absence of purified caeruloplasmin in 50 mM Tris/150 mM NaCl buffer, pH 7.4, before the washing steps.

Ethics

This study had approval from the South West Thames Local Research Ethics Committee.

RESULTS

Apocaeruloplasmin and holocaeruloplasmin in serum and liver tissues by Western immunoblotting

Figure 1 (top) shows the presence of holocaeruloplasmin (lane 2) with different mobility on SDS/PAGE from apocaeruloplasmin

Figure 1 SDS/PAGE of purified plasma apocaeruloplasmin and holocaeruloplasmin stained with Coomassie Blue (top) and Western blot of holocaeruloplasmin in liver homogenates (bottom)

Top, holocaeruloplasmin (apparent molecular mass 84–89 kDa) and apocaeruloplasmin (apparent molecular mass 132 kDa) have different mobilities on SDS/PAGE. Heated holocaeruloplasmin has the same mobility as apocaeruloplasmin produced by chelation dialysis. Lane 1, molecular-mass markers; lane 2, unheated purified holocaeruloplasmin; lane 3, purified apocaeruloplasmin prepared by chelation dialysis (unheated); lane 4, heated purified holocaeruloplasmin. Bottom, holocaeruloplasmin is shown by Western immunoblotting in unheated liver samples after SDS/PAGE (8% gel) as described in the text. Lane 1, purified plasma apocaeruloplasmin (not heated); lane 2, purified unheated holocaeruloplasmin; lane 3, purified heated holocaeruloplasmin; lanes 4–6, control liver tissues; lanes 7–9, liver tissues from patients with Wilson's disease. The holocaeruloplasmin form is clearly present in substantial amounts in liver tissues from both patients with Wilson's disease and controls. The sample and standard were run and blotted from the same gel, but with different X-ray exposure times (approx. 2 min for the standard and approx. 10 min for the samples).

Figure 2 Western blot of holocaeruloplasmin in bile

Detection of holocaeruloplasmin is shown in bile samples after SDS/PAGE (8% gel) and immunoblotting as described in the text. Lane 1, purified unheated plasma apocaeruloplasmin; lane 2, unheated holocaeruloplasmin; lane 3, heated holocaeruloplasmin; lanes 4 and 5, normal (control) bile; lanes 6–8, bile from patients with Wilson's disease. The 132 kDa form is seen in purified apocaeruloplasmin and holocaeruloplasmin heated before SDS/PAGE. Holocaeruloplasmin (molecular mass 89–90 kDa) is seen in unheated normal bile samples. Both apo- and holo-caeruloplasmin forms are absent from bile of patients with Wilson's disease. The sample and standard were run and blotted from the same gel, but with different X-ray exposure times (approx. 2 min for the standard and approx. 10 min for the samples).

Figure 3 Caeruloplasmin oxidase activity in liver samples

Top, liver homogenates, unheated before SDS/PAGE, incubated with 0.1% o'Dianisidine at 37 °C and examined for oxidase activity as described in the text. Lane 1, molecular-mass markers; lanes 2–4, liver tissue from patients with Wilson's disease; lanes 5 and 6, control liver tissue. Bottom, caeruloplasmin oxidase activity in purified caeruloplasmin samples. Lane 1, molecular-mass markers; lane 2, heated holocaeruloplasmin (apocaeruloplasmin); lane 3, unheated holocaeruloplasmin. As expected apocaeruloplasmin does not possess the caeruloplasmin oxidase activity.

produced by chelation dialysis (lane 3) and heated holo-caeruloplasmin (lane 4).

Holocaeruloplasmin (with apparent molecular mass 84–89 kDa) is detected by immunoblotting in all the liver homogenate samples from patients with Wilson's disease and control tissues (Figure 1, bottom). Thus caeruloplasmin is synthesized and present in the liver in Wilson's disease even when plasma caeruloplasmin is reduced or absent. The changes in plasma caeruloplasmin are a well-established but not clearly explicable feature of Wilson's disease.

Holocaeruloplasmin in bile samples

Figure 2 shows that holocaeruloplasmin is also present in bile from normal subjects confirming that caeruloplasmin is involved in biliary copper excretion. However, in contrast with the liver (Figure 1, bottom), neither the holo nor the apo forms of caeruloplasmin could be detected in the bile of patients with Wilson's disease. It should be noted that it is still possible for caeruloplasmin to be excreted in the bile in amounts that cannot be detected by Western blotting but these are likely to be negligible. Nevertheless even a significantly reduced level of caeruloplasmin in the bile clearly indicates its defective export.

Caeruloplasmin oxidase activity

Figure 3 (top) shows that caeruloplasmin oxidase activity is detectable in liver homogenates from both patients with Wilson's disease and control subjects. This is consistent with the results shown in Figure 1 (bottom) and suggests that the mechanism of copper incorporation remains intact in Wilson's disease. It should be noted that apocaeruloplasmin does not stain for caeruloplasmin oxidase activity (Figure 3, bottom, lane 2) in contrast with holocaeruloplasmin (Figure 3, bottom, lane 3).

Measurement of caeruloplasmin oxidase activity in serum

Caeruloplasmin oxidase activity [expressed as units/mg of protein; mean $\pm$ S.D. (no. of patients)] is absent or markedly reduced in samples from patients with Wilson's disease [7.63 ± 4.39 (4)] compared with control samples [150.67 ± 87.73 (3)]. This suggests that, although holocaeruloplasmin can be detected in substantial amounts in the liver in Wilson's disease (Figure 1, bottom), this

Figure 4 Electron micrograph (magnification $\times 35000$) of the human biliary canalicular membrane-rich fraction

Table 1 Enzyme characterization of isolated biliary canaliculi membranes

The specific activities of the enzymes were measured at 37 °C. Relative enrichment in the ratio of the specific activity in the biliary canaliculi membranes to that in the homogenate. The results are means $\pm$ S.D. obtained with six samples.

Marker enzyme	Specific activity in homogenate (μ mol of product/h per mg of protein)	Relative enrichment
Alkaline phosphatase	0.28 ± 0.16 (6)	58.1 ± 21
5'-Nucleotidase	1.09 ± 0.18 (6)	61.3 ± 17
Ouabain-sensitive Na^+/K^+ -ATPase	0.53 ± 0.21 (6)	2.2 ± 0.1
NADPH-cytochrome <i>c</i> reductase	1.85 ± 0.23 (6)	0.75 ± 0.3
Succinate-cytochrome <i>c</i> reductase	0.68 ± 0.19 (6)	0.25 ± 0.10
Acid phosphatase	0.47 ± 0.11 (6)	0.90 ± 0.37

Table 2 Recovery of protein and marker enzyme activity of isolated biliary canaliculi membranes

The results show recovery of protein and marker enzyme activity (5'-nucleotidase, alkaline phosphatase) of isolated biliary canaliculi membranes as described in the text, and are means $\pm$ S.D. obtained with six samples.

	Protein concentration (mg/g of liver)	Distribution (%)	
		5'-Nucleotidase	Alkaline phosphatase
Homogenate	139 ± 17	100	100
Bile canaliculi membrane	0.125 ± 0.13	29.9	27.5

Figure 5 Detection of caeruloplasmin-binding protein in biliary canaliculi membrane

Left, solubilized biliary canaliculi membranes subjected to electrophoresis on SDS/8% polyacrylamide gel followed by immunoblotting with polyclonal caeruloplasmin antibody. Lanes 1 and 2 show, after incubation of the blots with caeruloplasmin and blocking with milk solution, the presence of a high-molecular-mass protein (189–200 kDa) detected by the anti-human caeruloplasmin antibody suggesting a caeruloplasmin-like protein. Right, a similar gel but containing biliary canaliculi membranes in which the blots had not been incubated with caeruloplasmin after blocking with milk solution, and where the high-molecular-mass protein is still evident.

is not reflected in the serum. Caeruloplasmin must therefore be retained in the liver, suggesting variably defective secretion of caeruloplasmin from liver to plasma.

Detection of a caeruloplasmin-binding protein in biliary canaliculi membrane

Figure 4 shows the appearance of purified biliary canaliculi membranes and Table 1 indicates enzyme characterization of these membranes using alkaline phosphatase as the predominant

membrane marker. Recoveries of these biliary canaliculi membrane preparations showing the enriched preparation are indicated in Table 2 and are similar to previous results [22,23]. Preincubation of Western blots with caeruloplasmin before incubation with an anti-human caeruloplasmin antibody shows the presence of a high-molecular-mass (189–200 kDa) protein in the biliary canaliculi membranes with an activity identical with caeruloplasmin (Figure 5, left), as has been shown in a preliminary communication [14]. Control membranes (Figure 5, right), not preincubated with caeruloplasmin before incubation with the caeruloplasmin antibody, also demonstrate caeruloplasmin. This suggests that a caeruloplasmin-like or caeruloplasmin-binding protein is present in these purified biliary canaliculi membranes.

DISCUSSION

Previous studies based on radioactive copper uptake *in vitro* and *in vivo* suggested that copper incorporation into caeruloplasmin is defective in Wilson's disease [15]. In this paper we have clearly demonstrated that this is not the case. We find that holocaeruloplasmin, the copper-containing protein, is present in liver tissue from both controls and patients with Wilson's disease. This suggests that copper is incorporated normally into caeruloplasmin in Wilson's disease. It is therefore unlikely that the gene defect in Wilson's disease affects this step in the regulation of copper metabolism as has been previously suggested [11,15]. More importantly, whereas holocaeruloplasmin is present in normal bile samples, neither the holo nor the apo forms of the protein were detectable in bile from patients with Wilson's disease. Furthermore in Wilson's disease, serum caeruloplasmin oxidase activity, which is an indication of the presence of holocaeruloplasmin, is negligible. Thus in Wilson's disease holocaeruloplasmin is indeed synthesized, but is retained within the hepatocyte, not excreted into bile and incompletely secreted into plasma. This provides, for the first time, evidence for the specific involvement of caeruloplasmin in copper excretion and in the pathogenesis of copper toxicosis in Wilson's disease.

We find that apo and holo forms of caeruloplasmin can be readily separated by SDS/PAGE (Figure 1, top) by virtue of their different mobilities. This confirms previous studies [17]. This difference in mobility is not a consequence of incomplete SDS solubilization of the unheated samples, since the apoprotein form produced by chelation dialysis has been shown to migrate with a similar velocity to that of heated apocaeruloplasmin [17]. These observations could be explained by conformational changes in the tertiary structure of the protein [17]. It was noted that the readdition of copper to apocaeruloplasmin returned its electrophoretic mobility to a value close to that of the unheated holocaeruloplasmin (84–89 kDa) [17].

The defect in Wilson's disease may involve altered transport of copper bound to caeruloplasmin across the biliary canaliculi membranes in such a way that copper fails to be excreted. This is possible as we have identified a high-molecular-mass protein (189–200 kDa) in biliary canaliculi membranes which cross-reacts with specific purified human caeruloplasmin antibody. This protein could be a candidate for binding to caeruloplasmin before its export into bile. The presence of caeruloplasmin activity in the normal control biliary membranes not incubated with caeruloplasmin might be explained by endogenous caeruloplasmin binding tightly under non-physiological conditions. Alternatively dimerization of caeruloplasmin could occur which in Wilson's disease could result in its entrapment in the liver. This would in turn prevent its binding to biliary canaliculi membranes, a process that is necessary for its excretion into bile.

In a similar way α_1 -antitrypsin, when polymerized, has been shown to be entrapped within the liver [24].

The gene for Wilson's disease has been shown to encode a P-type copper-transporting ATPase [10,12]. A number of genetic mutations have been described but none have been found to correlate with the copper overload in Wilson's disease [25]. It is also not yet clear how the predicted copper transporter is involved in copper excretion. The putative copper transporter nevertheless does contain six to seven copper-binding motifs, as does caeruloplasmin [10–12] and could have a role in transport of caeruloplasmin across the biliary canalicular membrane into bile. The suggested location of this putative transporter is the endoplasmic reticulum, since the incorporation of copper into caeruloplasmin occurs at an early stage in the biosynthetic pathway soon after translation. However, earlier investigations using HepG2 cells have indicated that conformational changes are associated with copper incorporation into caeruloplasmin which do not affect the rate of excretion of caeruloplasmin from such cells [26]. The present study has shown that copper is incorporated normally into caeruloplasmin in Wilson's disease. Furthermore these observations support our hypothesis that copper toxicosis in Wilson's disease results from failure of caeruloplasmin excretion into the bile rather than a defect in the incorporation of copper into caeruloplasmin. The 200 kDa protein that we have identified may be a candidate for the Wilson's disease transporter protein or may bind to it. It is possible that a similar transporter might also exist for secretion of caeruloplasmin into plasma. The copper transporter in Wilson's disease may have different affinities for the 132 kDa (plasma) and 125 kDa (biliary) caeruloplasmin forms which we have identified previously [27].

The biosynthetic step involved in either caeruloplasmin or copper transport across the biliary canalicular membrane is unclear, although McArdle and Van Den Berg [28] have investigated vesicular copper transport, and previous chronic copper-loading studies performed in rats have indicated that copper is transported from plasma to bile in a saturable carrier-mediated process against a concentration gradient [29]. Similarly, kinetic data on copper uptake and efflux in isolated rat hepatocytes is consistent with transport across the membrane that involves a specific carrier protein [30].

It is well established that caeruloplasmin has a major role in copper transport to extrahepatic tissues [2]. Nevertheless, the mechanism by which it releases copper at these sites is unknown. There is some evidence that caeruloplasmin binds to other non-hepatocyte cell membranes and that the dissociation of copper from caeruloplasmin occurs after its binding at the cell surface. The copper is then released and taken up by cells [31,32]. In hepatocytes a different mechanism seems to exist; native caeruloplasmin is first desialylated at the endothelial cell [33] and is then taken up by hepatocyte membrane receptors that recognize the exposed galactosyl-recognition site [2,33]. This is believed to be a way of removing old or partially degraded caeruloplasmin molecules from circulation [2]. A similar mechanism may exist whereby caeruloplasmin is taken up by a receptor or transport protein in the biliary canalicular membrane before its export, and therefore that of copper, into bile. The presence of caeruloplasmin receptors has been noted in a number of different cell types including erythrocytes [34,35], liver endothelium [36], heart [37] and lymphocytes [38], but such receptors have yet to be identified in the biliary canalicular membrane.

In conclusion, we have shown that: (a) copper incorporation into caeruloplasmin is normal in liver tissue from patients with Wilson's disease; (b) in this disease the impairment of biliary copper excretion is likely to be due to defective caeruloplasmin export into bile; (c) biliary canalicular membranes possess a novel caeruloplasmin-binding protein. The relationship between caeruloplasmin and the putative Wilson's disease copper transport merits further study.

We are grateful to the South Thames Health Authority for a Research and Development grant for support of this work. We thank Dr. Bernard Portmann and Dr. C. Summerton for some bile samples, Dr. Robert Mahler and Professor Mone Zaidi for helpful discussions and Mrs Vivienne Marvell for typing the manuscript.

REFERENCES

- 1 Sass-Kortsak, A. and Bearn, A. G. (1965) *Adv. Clin. Chem.* **8**, 1–67
- 2 Harris, E. (1991) *Proc. Soc. Exp. Biol. Med.* **196**, 130–140
- 3 Sternlieb, I. (1980) *Gastroenterology* **78**, 1615–1628
- 4 Gutteridge, J. M. C. and Stock, J. (1981) *Crit. Rev. Clin. Lab. Sci.* **14**, 259–329
- 5 Frieden, E. (1981) in *Metal Ions in Biological Systems* (Siegel, H., ed.), pp. 117–142, Marcel Dekker, New York
- 6 Takahashi, N., Ortel, T. L. and Putnam, F. W. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 390–394
- 7 Yang, F., Naylor, S. L., Lum, J. B. et al. (1986) *Proc. Natl. Acad. Sci.* **83**, 3257–3261
- 8 Ryden, L. and Bjork, I. (1976) *Biochemistry* **15**, 3411–3417
- 9 Dameron, C. T. and Harris, E. D. (1987) *Biochem. J.* **248**, 669–675
- 10 Petrukhin, K., Fisher, S. G., Pirastu, M. et al. (1993) *Nature (London) Genetics* **5**, 338–343
- 11 Yamaguchi, Y., Heiny, M. E. and Gitlin, J. D. (1993) *Biochim. Biophys. Res. Commun.* **197**, 271–277
- 12 Tanzi, R. E., Petrukhin, K., Chernov, I. et al. (1993) *Nature (London) Genet.* **5**, 344–350
- 13 Vulpe, C., Levinson, B., Whitney, S., Packman, S. and Gitschier, J. (1993) *Nature (London) Genet.* **3**, 7–13
- 14 Chowrimootoo, G. F. E. and Seymour, C. A. (1994) *Biochem. Soc. Trans.* **22**, 190
- 15 Sternlieb, L., Morell, A. G., Tucker, W. D., Greene, M. W. and Scheinberg, I. H. (1961) *Clin. Invest.* **40**, 1834–1840
- 16 Gibbs, K. and Walshe, J. M. (1921) *Clin. Sci.* **41**, 189–202
- 17 Sato, M. and Gitlin, J. D. (1991) *J. Biol. Chem.* **266**, 5128–5134
- 18 Oosthuizen, M. M. J., Johannes, L. N., Myburgh, J. A. and Crookes, R. L. (1985) *Anal. Biochem.* **146**, 1–6
- 19 Holtzman, N. A. and Gaumnitz, B. M. (1970) *J. Biol. Chem.* **245**, 2350–2353
- 20 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 21 Henry, R. J., Chiamori, N., Jacobs, S. L. and Segalove, M. (1960) *Proc. Soc. Exp. Med. Biol.* **104**, 620–624
- 22 Wisher, M. H. and Evans, W. H. (1975) *Biochem. J.* **146**, 375–388
- 23 Song, C. A., Rubin, W., Rifkin, A. B. and Kappas, A. (1969) *J. Cell. Biol.* **141**, 124–132
- 24 Lomas, D.-A. and Carell, R. W. (1993) *Am. J. Physiol.* **265**, L211–L219
- 25 Schilsky, M. L. (1994) *Hepatology* **20**, 529–533
- 26 Parent, J. B., Bauer, H. C. and Olden, K. (1985) *Biochim. Biophys. Acta* **846**, 44–50
- 27 Davis, W. and Seymour, C. A. (1992) *Q. J. Med.* **85**, 77
- 28 McArdle, H. J. and Van Den Berg, G. J. (1992) *J. Nutr.* **122**, 1260–1265
- 29 Harada, M., Sakisaka, S., Yoshitake, M., Shakadohs, S., Gondoh, K., Sata, M. and Tanikawa, K. (1993) *Hepatology* **17**, 111–117
- 30 Schmith, R. C., Hisham, M. and Darwish, J. C. (1983) *Am. J. Physiol.* **243**, G183–G191
- 31 Owen, C. A., Jr. (1965) *Am. J. Physiol.* **209**, 900–904
- 32 Orena, S. J., Goode, C. A. and Linder, M. C. (1986) *Biochem. Biophys. Res. Commun.* **139**, 822–829
- 33 Tavasoli, M., Kishimoto, T. and Kataoka, M. (1986) *J. Cell Biol.* **102**, 1298–1303
- 34 Saenko, E. L. and Yarapolov, A. I. (1990) *Biochem. Int.* **20**, 215–225
- 35 Barnes, G. and Frieden, E. (1984) *Biochem. Biophys. Res. Commun.* **125**, 157–162
- 36 Kataoka, M. and Tavasoli, M. (1984) *Exp. Cell Res.* **155**, 232–240
- 37 Stevens, M. D., Disivetoo, R. A. and Harris, E. D. (1984) *Biochemistry* **23**, 261–266
- 38 Kakatao, M. and Tavasoli, M. (1985) *Exp. Hematol.* **13**, 806–810