BIOCHEMICAL JOURNAL LETTERS

Non-caeruloplasmin-bound copper ('phenanthroline copper') is not detectable in fresh serum or synovial fluid from patients with rheumatoid arthritis

It is reported that individual rheumatoid synovial fluids stored at -20°C for 2 months or at $+4^{\circ}\text{C}$ for up to 36 h contain micromolar concentrations of non-caeruloplasmin-bound copper, as measured by the phenanthroline assay (Gutteridge, 1984, 1986). Normal or rheumatoid sera do not contain phenanthroline copper under such conditions (Gutteridge, 1984, 1986). The phenanthroline assay measures a fraction of copper that, when complexed to 1,10-phenanthroline, can take part in oxygen radical reactions leading to the degradation of DNA (Gutteridge, 1984). In human synovial fluid, such copper is thought to be loosely bound to albumin or to amino acids such as histidine and able to catalyse the free-radical-mediated reactions which have been implicated as an important part of the destructive process that occurs within the rheumatoid joint (Gutteridge, 1984, 1986). Copper bound to caeruloplasmin is not detected by the phenanthroline assay.

In contrast with the results of Gutteridge (1984, 1986), we have previously demonstrated in collaboration with him (Gutteridge *et al.*, 1985; Winyard *et al.*, 1986) that no phenanthroline copper is measurable in fresh *pooled* human synovial fluid or serum. Storage of pooled synovial fluid at $+4^{\circ}\text{C}$ results in the formation of phenanthroline copper within 1 week, accompanied by a decrease in the oxidase activity of caeruloplasmin (Gutteridge *et al.*, 1985; Winyard *et al.*, 1986), suggesting that phenanthroline copper originates from the degradation of caeruloplasmin. This raised the question of whether phenanthroline copper is indeed present in individual rheumatoid synovial fluids if they are assayed immediately after aspiration.

To answer this question, we have established the phenanthroline copper assay in laboratories close to the rheumatology ward at Selly Oak Hospital, Birmingham. This has allowed us to measure the phenanthroline copper concentration in fresh sera and synovial fluids from rheumatoid patients within 4 h of collection. We have then assayed the same fluids again after storage for 2 months at -20°C . Paired sera and synovial fluids were obtained from patients with classical rheumatoid arthritis (American Rheumatism Association criteria); all patients had active synovitis. Samples were centrifuged at 1500 *g* for 10 min to remove cells, divided into portions and held at 4°C for assay the same day and at -20°C for assay after 2 months of storage. Phenanthroline copper was determined by rigorously following the method of Gutteridge (1984). In our hands, the inter-assay coefficient of variation was 6.5% and the detection limit was 0.1 $\mu\text{mol/l}$.

No phenanthroline copper was detectable in any of the six fresh rheumatoid synovial fluids which were tested within 4 h of aspiration. There was sufficient sample to store three of these fluids at -20°C for 2 months, at which time phenanthroline copper was detected in all three samples, the concentrations being 2.5, 2.3 and 1.9 $\mu\text{mol/l}$. None of the rheumatoid sera ($n = 7$) contained phenanthroline copper, neither when fresh nor following 2 months storage. In contrast, Gutteridge has reported mean phenanthroline copper concentrations of $0.54 \pm 0.76 \mu\text{mol/l}$ in 13 synovial fluid samples stored at -20°C for 2 months (Gutteridge, 1984) and $0.73 \pm 0.66 \mu\text{mol/l}$ in 12 synovial fluids stored for a maximum of 36 h at 4°C (Gutteridge, 1986). In the latter study phenanthroline copper was detectable in 11 out of 12 synovial fluids. Our data suggest that even these short periods of storage may result in an artifactual detection of phenanthroline copper in rheumatoid synovial fluid. In agreement with our results, Gutteridge (1984, 1986) did not detect phenanthroline copper in either fresh or stored normal or rheumatoid sera.

In rheumatoid arthritis, the mean total copper concentration rises to around 26 $\mu\text{mol/l}$ from about 18 $\mu\text{mol/l}$ in normal human serum (Scudder *et al.*, 1978a). About 95% of serum copper is thought to be associated with caeruloplasmin (Harrison & Hoare, 1980). By calculating the concentration of non-caeruloplasmin copper from these values, the phenanthroline assay should detect about 1.3 μmol of copper/l in rheumatoid serum, well within the detection limit of the assay (see above). Our inability to detect phenanthroline copper in rheumatoid serum suggests that the serum labile copper pool is smaller than reported in the literature or that the amount of phenanthroline copper detected is not a simple function of the concentration of non-caeruloplasmin-bound copper.

In contrast with our results, there are reports that an increased proportion of rheumatoid serum and/or synovial fluid copper is dissociated from caeruloplasmin (Niedermeier, 1965; Lorber *et al.*, 1968; Aiginger *et al.*, 1978), although other workers have not reproduced these findings (Scudder *et al.*, 1978a,b). All of these studies relied on an indirect calculation of the amount of non-caeruloplasmin-bound copper by comparing the total copper concentration with the caeruloplasmin concentration, assuming seven copper atoms per caeruloplasmin molecule. Furthermore, the conditions of sample storage are not clear and in some studies the caeruloplasmin concentration was determined from the serum oxidase activity, which falls on storage at -20°C (Gutteridge *et al.*, 1985; Winyard *et al.*, 1986). Although cupric salts added to a solution of albumin or histidine are fully recovered by the phenanthroline assay (Gutteridge *et al.*, 1985), it is possible that there is a fraction of non-caeruloplasmin copper in human extracellular fluids which is inactive. This might be the copper detected by

others (Niedermeier, 1965; Lorber *et al.*, 1968; Aiginger *et al.*, 1978). With regard to this, Weiss & Linder (1985) have recently reported that 10–15% of rat plasma copper is bound to a new copper transport protein, transcuprein.

We suggest that the detection of increased levels of non-caeruloplasmin copper in rheumatoid synovial fluids is an artifact, resulting from the release of copper from caeruloplasmin by proteolytic and/or oxidative attack during storage (see Ryden, 1984). Syed *et al.* (1982) and Evans *et al.* (1985) have proposed that storage of caeruloplasmin or its isolation leads to the production of type 2 ('normal') copper which remains bound to the protein. The production of type 2 copper during storage of extracellular fluids could give rise to copper that is active in the phenanthroline assay. However, type 2 copper is not itself active, since a commercial caeruloplasmin preparation, which presumably contains type 2 copper, does not contain phenanthroline copper (Gutteridge *et al.*, 1985).

The phenanthroline copper assay measures a fraction of copper that, when complexed to 1,10-phenanthroline, can take part in oxygen radical reactions (Gutteridge, 1984). Since we found that no copper was active in this manner in synovial fluid samples assayed immediately after aspiration, it appears unlikely that copper plays a significant role in the transition-metal-catalysed radical reactions postulated to be important in the destructive process within the rheumatoid joint.

P. G. W. and D. R. B. acknowledge financial support from the Arthritis and Rheumatism Council. H. P. is a Sheldon Clinical Research Fellow supported by the West Midlands Regional Health Authority. We thank Dr. J. M. C. Gutteridge of the National Institute for Biological Standards and Control,

London, for his help with establishing the phenanthroline copper assay in Birmingham.

Paul G. WINYARD,* Hardev PALL,† Joe LUNEC‡ and David R. BLAKE*

Departments of *Rheumatology and †Neurology, The Medical School, University of Birmingham, Birmingham B15 2TJ, and ‡Department of Biochemistry, Selly Oak Hospital, Birmingham B29 6JD, U.K.

Aiginger, P., Kolarz, G. & Willvonseder, R. (1978) *Scand. J. Rheumatol.* **7**, 75–78

Evans, R. W., Madden, A. D., Patel, K. J., Gibson, J. F. & Wrigley S. K. (1985) *Biochem. Soc. Trans.* **13**, 627–629

Gutteridge, J. M. C. (1984) *Biochem. J.* **218**, 983–985

Gutteridge, J. M. C. (1986) *Biochim. Biophys. Acta* **869**, 119–127

Gutteridge, J. M. C., Winyard, P. G., Blake, D. R., Lunec, J., Brailsford, S. & Halliwell, B. (1985) *Biochem. J.* **230**, 517–523

Harrison, P. M. & Hoare, R. J. (1980) *Metals in Biochemistry*, Chapman and Hall, London

Lorber, A., Cutler, L. S. & Chang, C. C. (1968) *Arthritis Rheum.* **11**, 65–71

Niedermeier, W. (1965) *Ann. Rheum. Dis.* **24**, 544–548

Ryden, L. (1984) in *Copper Proteins and Copper Enzymes* (Lontie, R., ed.), pp. 37–100, CRC Press, Boca Raton, FL

Scudder, P. R., Al-Timini, D., McMurray, W., White, A. G., Zoob, B. C. & Dormandy, T. L. (1978a) *Ann. Rheum. Dis.* **37**, 67–70

Scudder, P. R., McMurray, W., White, A. G. & Dormandy, T. L. (1978b) *Ann. Rheum. Dis.* **37**, 71–72

Syed, M. A., Coombs, T. L., Goodman, B. A. & McPhail, D. B. (1982) *Biochem. J.* **207**, 183–184

Weiss, K. C. & Linder, M. C. (1985) *Am. J. Physiol.* **249**, E77–E88

Winyard, P. G., Blake, D. R., Gutteridge, J. M. C., Brailsford, S. & Lunec, J. (1986) *Br. J. Rheumatol.* **25**, 108–109

Received 12 January 1987

Non-caeruloplasmin copper and the phenanthroline assay

The phenanthroline assay was introduced in 1984 [1] as a first attempt to detect copper ions, in human body fluids, that might participate in free radical reactions. The assay measures copper bound to albumin or to histidine, but not to caeruloplasmin. However, other work (reviewed in [2] and [3]) has since shown that copper ions bound to histidine or albumin do not generate reactive radicals in free solution, which suggests that phenanthroline-detectable copper cannot necessarily be regarded as a pro-oxidant form of copper [2–4].

In a collaborative study between my laboratory and Dr. Blake's group [6] no phenanthroline-detectable copper was found in fresh pooled serum. That paper discussed in detail the possibility that the 'low-molecular-mass' copper pool reportedly present in serum is, in fact, a consequence of mishandling or storage. Hence the statement of Winyard *et al.* [5] that the 'serum labile copper pool is smaller than has been reported in the literature' seems correct and confirms the conclusions of [6]. It was first established in 1984 [1] and confirmed in 1985 [6] that storage of pooled serum or synovial fluid, initially containing no phenanthroline-detectable copper,

caused such copper to appear. This raised the possibility that samples of synovial fluid positive in the phenanthroline assay had been stored too long, or mishandled [6]. Indeed, it has already been pointed out in the literature that a positive result must be regarded with suspicion [7]. Hence I encouraged Winyard *et al.* [5] to set up the phenanthroline assay in their laboratory and to perform immediate assays on fluids taken straight from the patient. They have now done this (although only within 4 h of aspiration) on six fluids and found nothing [5].

The results of Winyard *et al.* [5] support the earlier conclusions [6] that the phenanthroline-detectable copper reported in synovial fluid samples is an artifact of storage. However, six experiments is an insufficient number to prove this and fluids should preferably be analysed *immediately*. Altogether I have assayed more than 100 synovial fluids supplied by Dr. Blake's laboratory without a clear pattern for the appearance of copper emerging. For example, one batch of 17 synovial fluids stored for 6 months at -20°C had a mean phenanthroline-detectable copper of $0.52\ \mu\text{M}$ with some 50% showing no phenanthroline-detectable copper. On the other hand, a batch of 12 samples stored for 36 h or less at 4°C had a mean phenanthroline-detectable