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Copper–phenanthroline-induced site-specific oxygen-radical damage to DNA

Detection of loosely bound trace copper in biological fluids

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Copper(II) ions, in the presence of 1,10-phenanthroline, O_2 and a reducing agent, degrade DNA with the release of thiobarbituric acid-reactive material. This reaction, dependent on the formation of oxygen radicals, was made the basis of a sensitive and specific assay for loosely bound copper in body fluids. When applied to certain extracellular fluids, trace amounts of copper could be detected in the lower micromolar range.

Previous studies have shown that the chelating agent 1,10-phenanthroline binds copper(II) ions and in the presence of a reducing agent and molecular O_2 degrades DNA (Sigman *et al.*, 1979; Downey *et al.*, 1980; Graham *et al.*, 1980; Gutteridge & Halliwell, 1982). This damage is inhibited by catalase and to a lesser extent by superoxide dismutase, implicating oxygen radicals in the destructive process (Que *et al.*, 1980; Gutteridge & Halliwell, 1982). It is likely that phenanthroline binds to the DNA molecule, causing a site-specific copper-dependent reaction close to the base and sugar moieties similar to that which occurs with the anti-tumour antibiotic bleomycin. Degradation of DNA by bleomycin shows a unique specificity for iron ions. Similarly, 1,10-phenanthroline shows a specificity for copper ions. Both reactions release thiobarbituric acid-reactive material from DNA (Gutteridge *et al.*, 1981; Gutteridge & Halliwell, 1982).

A sensitive technique has been developed that uses the bleomycin reaction to detect and measure iron salts in biological fluids (Gutteridge *et al.*, 1981). These iron salts are known to be involved in reactions leading to the formation of the highly reactive and damaging hydroxyl radical as well as lipid oxidation (Gutteridge *et al.*, 1982). Copper salts can catalyse these same reactions, often with greater efficiency. However, copper ions in biological material are invariably associated with ubiquitous amino groups, to which they readily ligate. Such binding does not necessarily prevent copper from participating in oxygen-radical reactions, but limits radical formation to the site of copper binding (Samuni *et al.*, 1981; Gutteridge & Wilkins, 1983). The copper available to 1,10-phenanthroline in biological fluids can be made the basis of a specific and sensitive assay of loosely bound copper in the lower micromolar range. This is measured as thiobarbituric acid-reactivity after site-specific oxygen-radical damage to DNA.

Materials and methods

Materials

Calf thymus DNA (type 1) and 1,10-phenanthroline were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Chelex 100 was from Bio-Rad Laboratories (St. Albans, Herts., U.K.). All other chemicals were of the highest grades available from BDH Chemicals (Poole, Dorset, U.K.).

Assay of copper

All reagents were prepared in Chelex-resin-treated distilled water in new plastic containers.

Copper–phenanthroline-dependent degradation of DNA

1,10-Phenanthroline was first dissolved in 0.2ml of ethanol and then made to 10ml with Chelex-resin-treated distilled water to give a final concentration of 1mM. DNA was prepared by dissolving 1mg/ml in Chelex-resin-treated water and leaving the solution to stand for 12h at 4°C before use. Phosphate buffer, pH6.5, contained 0.25M-phosphate (Na_2HPO_4/NaH_2PO_4). The 2-mercaptoethanol solution was prepared by adding 0.4ml to 100ml of Chelex-resin-treated water. All reagents were sufficiently stable when stored at 4°C. Before use reagents were further treated in new plastic Universal bottles with Chelex resin by adding 0.4g

per 10ml of reagent. Bottles were centrifuged at 2000 *g* for 10min to deposit all the resin before use.

The reaction mixture contained the following reagents added in the order stated: 0.4ml of calf thymus DNA (1mg/ml), 0.1ml of 1,10-phenanthroline (1mM), 0.05ml of NaN_3 (100mM), 0.05ml of copper salt or sample, 0.2ml of phosphate buffer, pH6.4 (0.25M), and 0.1ml of mercaptoethanol (0.4%, v/v). New plastic disposable tubes and pipette tips were used throughout. Each sample had its own blank prepared as described above, with omission of the 1,10-phenanthroline but with addition of 0.1ml of Chelex-treated water. A standard curve for copper concentrations ranging from 1 to 10μM was established by using $CuCl_2$. Tubes were mixed after the addition of each reagent, then incubated at 37°C for 1h in a shaking water bath. Then 0.1ml of 0.1M-EDTA was added to stop the reaction. Next 0.5ml of 1% (w/v) thiobarbituric acid in 50mM-NaOH and 0.5ml of 28% (w/v) trichloroacetic acid were added, and the contents were transferred to glass tubes for heating at 100°C for 10min. The tubes were left to cool, and the colour in each tube was extracted into 3.0ml of butan-1-ol by vortex-mixing. Phases were separated by centrifugation at 2000*g* for 5min, and the fluorescence of the butanol phase was read at 553nm after excitation at 532nm. Fluorescence units are expressed relative to a standard of Rhodamine B (3μM) as previously described (Gutteridge & Halliwell, 1982).

Results and discussion

Copper–phenanthroline-dependent degradation of DNA

Thiobarbituric acid-reactive material released from DNA by copper–phenanthroline has fluorescent properties indistinguishable from those of the material formed between malondialdehyde and thiobarbituric acid (Fig. 1). The reaction can be made the basis of a highly sensitive and specific assay for loosely bound copper in biological fluids that is accurate in the lower micromolar range (Fig. 2).

Mercaptoethanol is added to reduce cupric ions (Cu^{2+}) to the cuprous state (Cu^{+}) essential for activation of O_2 to a reactive species. Previous studies have shown that H_2O_2 is an important intermediate in the degradation of DNA by 1,10-phenanthroline and copper, since the reaction is substantially inhibited by catalase (Gutteridge & Halliwell, 1982). Addition of NaN_3 to the reaction mixture effectively inhibits any catalase that might arise in biological samples from cell lysis. The pH optimum for the reaction is 6.5, and the phosphate buffer must be carefully checked, and adjusted if necessary, after treatment with Chelex resin.

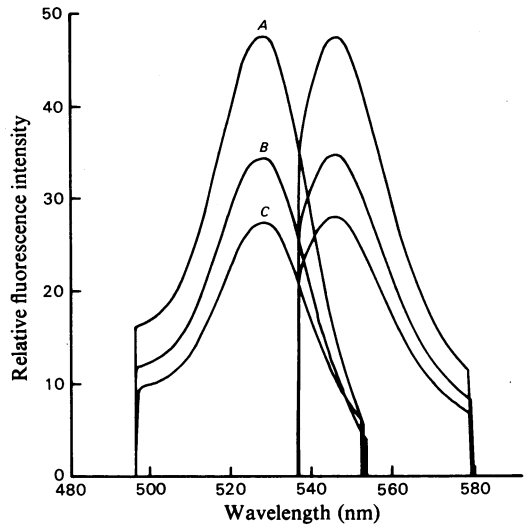

Fig. 1. *Fluorescence scans of the thiobarbituric acid-reactive chromogen extracted into butan-1-ol formed from (curve A) malondialdehyde (prepared from acid-hydrolysed 1,1,3,3-tetramethoxypropane) (5μM), (curve B) copper standard (8μM) and (curve C) sample of synovial fluid*
Fluorerscence intensity is given relative to a standard of Rhodamine B.

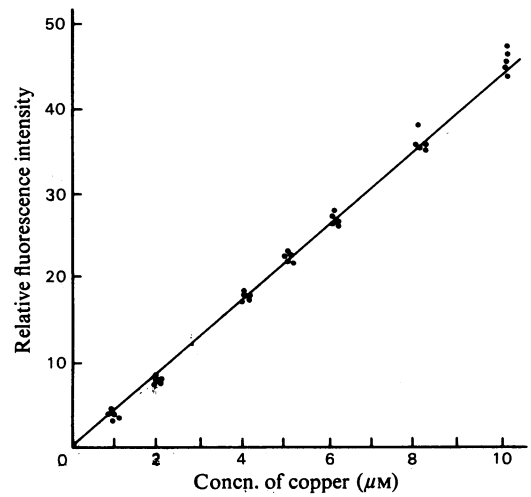

Fig. 2. *Standard curve prepared from $CuCl_2$ on five separate assays*
Fluorescence intensity is given relative to a standard of Rhodamine B.

Addition of EDTA stops the copper-dependent reaction with DNA and also prevents the breakdown of any lipid peroxides, that may be present, to form thiobarbituric acid-reactive material (Gutteridge & Quinlan, 1983). However, blanks

should be included for each sample to detect other thiobarbituric acid-reactive materials. Bile pigments are known to be thiobarbituric acid-reactive (Gutteridge & Tickner, 1978), but these have no fluorescent properties at 553nm and will not interfere in the assay. High concentrations of iron salts, if present, can be reduced by mercaptoethanol to the ferrous oxidation state, which reacts with 1,10-phenanthroline to yield a coloured complex absorbing at 520nm. However, this too has no fluorescent property at 553nm, and furthermore iron salts are only present in biological fluids at extremely low concentrations (Gutteridge *et al.*, 1981). The malondialdehyde–thiobarbituric acid chromogen is highly fluorescent and displays inner-filter effects. Fluorescence arising from copper concentrations greater than 10μM should be diluted with butan-1-ol to avoid self-quenching.

Presence of loosely bound copper in biological fluids measured by copper–phenanthroline-dependent degradation of DNA

Normal human serum has a mean total copper concentration of about 18μM (Scudder *et al.*, 1978), some 96% of which is associated with the protein caeruloplasmin. Normal human serum stored at −20°C contained no loosely bound copper available to 1,10-phenanthroline (Table 1), showing that 1,10-phenanthroline under these conditions does not remove copper from caeruloplasmin. However, serum freeze–thawed several times and then left at room temperature showed a detectable and increasing concentration of loosely bound copper, which after 6 days reached 8μM (Table 1). Samples of synovial fluid taken from patients with rheumatoid arthritis had loosely bound copper concentrations ranging from 0 to 2.3μM (Table 1). Cerebrospinal fluids could not be obtained from normal subjects, but samples taken from patients with suspected neurological disorders and found to be normal for cell counts and protein content and to be sterile on culture all contained micromolar concentrations of loosely bound copper ranging from 0.13 to 0.75μM. Two samples of sweat obtained from athletes after severe exercise contained substantial concentrations of loosely bound copper (Table 1). The samples of synovial fluid and sweat were stored frozen at −20°C for 2 months, and samples of cerebrospinal fluid were stored at 4°C for 2 weeks.

Cupric salts added to a solution of albumin or histidine could be fully recovered in the assay procedure (results not shown), suggesting a possible source of non-caeruloplasmin copper in biological fluids.

The method described measures a fraction of copper that, when complexed to 1,10-phenanthroline, can take part in oxygen-radical reactions leading to substantial molecular damage.

Table 1. *1,10-Phenanthroline-available copper in biological fluids*

Normal serum was obtained from healthy volunteers. Samples of synovial fluids were a gift from Dr. D. R. Blake, Department of Rheumatology, University of Birmingham, Birmingham, U.K. Samples of cerebrospinal fluid were a gift from Mr. P. Lamport, Department of Microbiology, Whittington Hospital, London N.19, U.K. Samples of sweat were a gift from Mr. D. M. Heeley, Alfred Chester Beatty Body Dynamics Laboratory, Cranbrook, Kent, U.K. Synovial fluids and cerebrospinal fluids were taken for authentic medical purposes. Values are given as means ± S.D. for the numbers of samples shown in parentheses.

	Concn. of copper (μM)
Normal serum (15)	0
Normal serum freeze–thawed and left at room temperature for 6 days*	8.0
Synovial fluid from rheumatoid patients (13)	0.54 ± 0.76
Cerebrospinal fluids (8)	0.46 ± 0.21
Sweat fluid (2)	16.60 ± 1.4

* One sample of pooled serum from five normal subjects.

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