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Measurement of the Binding Capacity of Casein for Copper by Potentiometric Stripping Analysis

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ABSTRACT

The copper binding properties of casein were investigated; the conditional stability constant and the number of binding sites were evaluated by Potentiometric Stripping Analysis (PSA) and Gel Permeation Chromatography experiments. PSA, being insensitive to the presence of organic matter, allows measurements of the free form of the metal in normal conditions without disturbing the chemical equilibria in solution.

INTRODUCTION

It is well known that total metal determination is insufficient, and often misleading, in assessing the potential toxicity or the bioavailability of a metal (Schwarz, 1978; Underwood, 1977). More significant is the determination of the individual physico-chemical forms of the element (speciation) that contribute to its total concentration. In fact, the biochemical toxicity, or bioavailability, of a metal is strictly dependent on its oxidation state, its entrance route into the body and the nature of any ligands bound to it.

In the past decade many speciation methods (reviewed by Schwedt, 1983), have been developed for detecting the chemical forms of various metals present in food. These methods are based on a separation step followed by the measurement of the concentration of the metal in the different fractions. The stability constants of the complexes formed can be determined by several methods based on gel chromatography (Mantoura & Riley, 1975), equilibrium dialysis (Truitt & Weber, 1981; Cox *et al.*, 1984), ultrafiltration (Buffle & Staub, 1984), ion selective electrodes (Buffle *et al.*, 1977; Bresnahan *et al.*, 1978), and polarography (Florence, 1983).

Many studies of metal speciation have been carried out in different types of milk in order to assess the different bioavailability of various metals of nutritional interest (Lonnerdal, 1985). These studies principally concerned the distribution of metals among the different milk fractions (Evans & Johnson, 1980; Fransson & Lonnerdal, 1982, 1983, 1984; Lonnerdal *et al.*, 1982; Martin *et al.*, 1984; Saltman & Hegenauer, 1983). Methods used to estimate the metal binding capacity of casein include equilibrium dialysis (Harzer & Kauer, 1982) and measurement of the free metal ion concentration by ion selective electrodes (Dalgleish & Parker, 1980; Parker & Dalgleish, 1981).

In this paper, measurements of the apparent complexation capacity of milk casein, the conditional stability constant and the number of binding sites are examined by addition of Cu(II) ions followed by equilibration. The experimental technique used was Potentiometric Stripping Analysis (PSA) with a thin-film mercury glassy carbon electrode. The main advantage of this technique, developed by Jagner (1976), is that the electrodeposed metals are not stripped by a potential scan, as in Anodic Stripping Voltammetry (ASV), but are chemically oxidised by a suitable oxidising agent (the oxygen naturally present in the sample solution in our experiments) and potential difference is monitored as a function of time. The distinct advantage of this technique is the insensitivity to organic matter and therefore measurements can be carried out without disturbing the equilibria existing in solution.

EXPERIMENTAL

Instrumentation

All measurements were performed using a Radiometer ISS820 Ion Scanning System. The electrochemical cell consisted of a 40 ml thermostatted glass vessel in which a mechanical stirrer and three electrodes were inserted. A glassy carbon electrode (Radiometer F3500), a

platinum foil (Radiometer P1312) and a saturated calomel electrode (Radiometer K4040) were used as the working, counter and reference electrodes, respectively. The working electrode was coated with a mercury film before the starting of daily experiments as described previously by Mannino (1982).

Samples containing copper from gel filtration experiments were analysed in a SP90 Pye Unicam Atomic Absorption Spectrophotometer.

Analytical procedure

All experiments were performed in a 200 mM Tris/acetate buffer solution of pH = 6·8.

Increasing amounts of Cu(II) nitrate were added to 10 ml of casein (Merck, West Germany, N = 14·2% and P = 0·8%) suspended in buffer and then diluted to a final volume of 20 ml. Standard additions were performed with ABU80 Radiometer autoburette. Solutions were prepared at least 12 h before PSA measurements in order to attain equilibrium. Free copper concentrations were calculated from a calibration curve of copper dissolved in the same buffer. The electrolysis potential was −0·6 V vs SCE (Saturated Calomel Electrode); electrolysis time, 2 min or 1 min (depending on the copper concentration); chart speed, 1 cm/s.

Gel filtrations were performed by the method described by Mantoura & Riley (1975). Samples of casein (2 ml, 2 mg/ml), were dissolved in the eluant and applied to a column of Sephadex G25 (length 25 cm, bore 2·5 cm); they were eluted with Tris/acetate buffer containing concentrations of copper ranging from 8×10^{-6} M to $4{\cdot}7 \times 10^{-5}$ M; fractions of 2·5 ml were collected and their copper content determined by atomic absorption spectroscopy. K and n were calculated as reported by Mantoura & Riley (1975).

RESULTS AND DISCUSSION

All the experimental results were evaluated by the Ružić (1982) and Scatchard (in Segel, 1976) data treatments. By the Ružić data treatment the following relation is valid:

$$\frac{[M]_f}{[M]_t - [M]_f} = \frac{1}{K \times C_L} + \frac{[M]_f}{C_L}$$

where $[M]_t$ is the total concentration of the metal present in solution, C_L is the complexation capacity of the solution (in the case of large multifunctional molecules, such as proteins, C_L is interpreted as the concentration of individual functional groups acting independently as

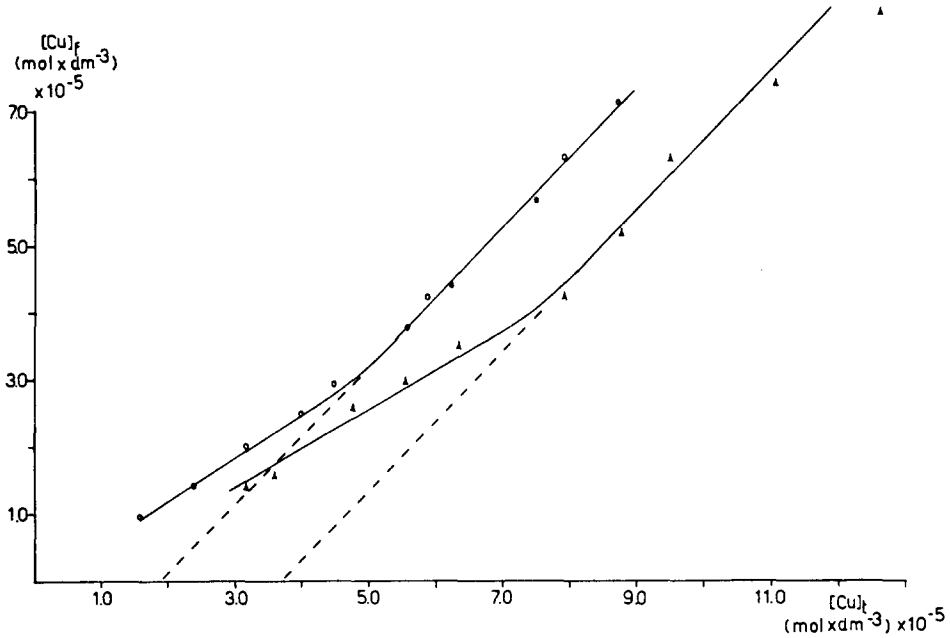

Fig. 1. Direct titration curves obtained plotting $[Cu]_{free}$ versus $[Cu]_{total}$ in experiments conducted with 0·05% (○) and 0·1% (△) casein and increasing amount of copper. From the intercept of the dotted lines on the x-axis the complexation capacity (C_L) can be evaluated.

ligands), $[M]_f$ is the concentration of the free metal and K is the conditional stability constant. By plotting the ratio of free to bound metal against the free metal concentration, a plot is obtained where the slope is the inverse value of the complexation capacity and the intercept the inverse of the product of the conditional stability constant and the complexation capacity. By this method C_L and K are determined by making use of the whole titration curve leading to more accurate results than those obtained by the method proposed by Shuman & Woodward (1973) that use only a part of the titration curve.

In the Scatchard data treatment the conditional stability constant and the number of sites are calculated graphically from the following relationship:

$$\frac{\bar{v}}{[M]_f} = Kn - K\bar{v}$$

where $\bar{v}$ is the average number of moles of metal bound per mole of protein and n is the number of sites per mole of protein.

In the case of similar and non-co-operative binding sites, a straight line is obtained, from which it is possible to extrapolate to find values for K and n.

The conditional stability constant for the complexes formed between

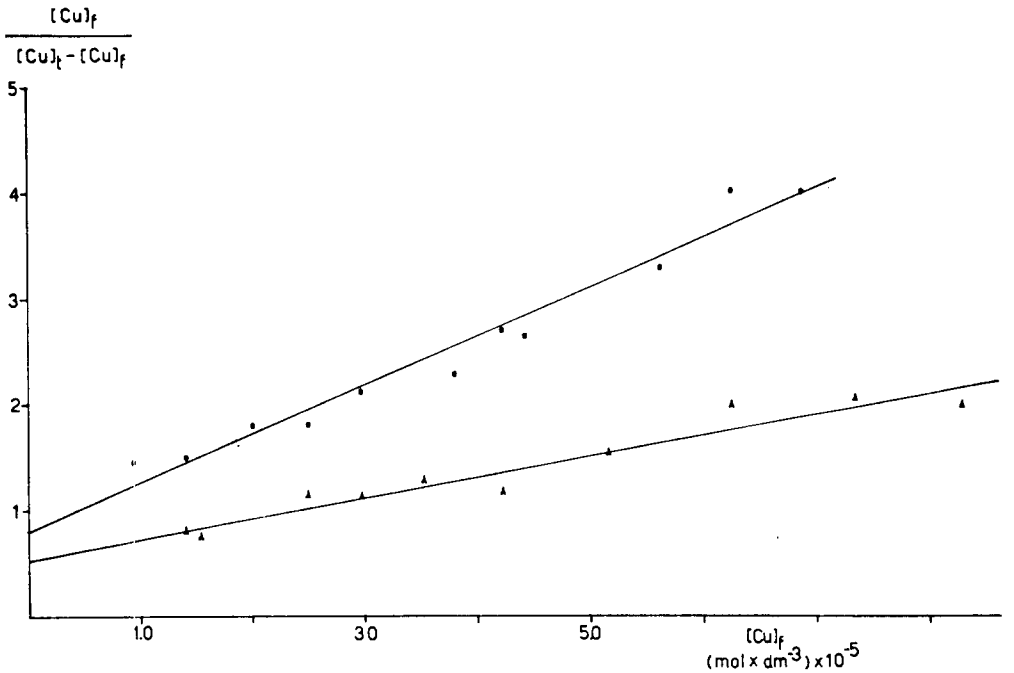

Fig. 2. Plot of the ratio of free to bound versus free metal, obtained from the experimental results shown in Fig. 1. The straight lines were obtained by least squares regression.

casein and copper, together with the average number of binding sites (n) are listed in Table 1. These values are comparable with those found by gel filtration ($n = 1{\cdot}2$, $\log K = 4{\cdot}5$).

In Fig. 1 the direct titration curves obtained by PSA measurements for two different casein concentrations are plotted. The data reported are the average of five determinations. The difference between independent measurements of the complexation capacity was not greater than 5%. Typical sets of data obtained for 0·05% and 0·1% of casein, plotted as required by the Ružić method, are shown in Fig. 2. The relative standard deviation of the slope of these, and similar, plots was found to be in the range from 6% to 10%. The linear relationship shows that copper and casein form a 1:1 complex.

It is important to stress that in this work all copper complexes that undergo reduction at potentials more positive than $-0{\cdot}6$ V versus SCE are considered as labile. By working at this deposition potential we found a well defined signal for copper, whereas other authors noticed, in anodic stripping experiments performed at our working pH, splitting of the copper signal and the appearance of two definite anodic peaks.

Scatchard plots of our results suggested that only one type of binding site was available. It should also be noted that the number of binding sites for

TABLE 1
Complexation Capacity (C_L), Conditional Stability Constant (K) and Number of Binding Sites (n), Evaluated by the Ružić Method with Experiments Performed at Different Casein Concentrations

Casein (%)	C_L[a]	*log K*	*n*
0·05	44×10^{-4}	4·8	1·0
0·10	52×10^{-4}	4·5	1·2
0·20	57×10^{-4}	4·5	1·3

[a] Expressed as moles of Cu/100 g of casein.

copper in the casein molecule is low and does not change when the ionic strength of the buffer is changed. Also, the conditional stability constant of the casein/copper complex does not change when the ionic strength of the medium has been increased from 0·2 to 0·4 by the addition of sodium chloride to the buffer. These data support the existence of quite specific binding sites for copper, and seem to rule out the possibility that the complexing capacity of casein simply lies on the electrostatic interaction among copper and the negatively charged groups of the casein molecule.

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