The Thermal Decomposition of $(Cu^{I}_{8}Cu^{II}_{6}(D\text{-penicillamine})_{12}Cl)^{5-}$

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(Received May 6, 1986)

Abstract

$[Cu^{I}_{8}Cu^{II}_{6}(D\text{-penicillamine})_{12}Cl]^{5-}$ is metastable. Its thermal decomposition was investigated using electronic absorption at 518 nm. $[Cu^{I}_{8}Cu^{II}_{6}(D\text{-penicillamine})_{12}Cl]^{5-}$ disintegrated following sigmoidal characteristics at 85 °C. In the course of the decomposition, unspecifically bound Cu(II) was found which stimulated further decomposition. Trapping of this unspecific Cu(II) by EDTA or glycylglycyl-L-histidine (gly-gly-his) supported this conclusion. First order kinetics were observed in their presence. Likewise, additional $CuSO_4$ stimulated the decomposition. The concentration dependence of the reactivity of $CuSO_4$, EDTA, and gly-gly-his was examined. In contrast to $CuSO_4$, saturation of the disintegration rate was observed in the presence of more than 0.1 mM EDTA. While EDTA stimulated the initial rate compared to $[Cu^{I}_{8}Cu^{II}_{6}(D\text{-penicillamine})_{12}Cl]^{5-}$ alone, gly-gly-his was slightly inhibitory. Presumably, the intrinsic decay of $[Cu^{I}_{8}Cu^{II}_{6}(D\text{-penicillamine})_{12}Cl]^{5-}$ is seen in the presence of gly-gly-his. The activation parameters of the decomposition reaction in the presence of $CuSO_4$, EDTA, and gly-gly-his were derived from the temperature dependence. Gly-gly-his was used as a model for the binding site of copper in serum albumin. Both the model and the native protein affected the decomposition of $[Cu^{I}_{8}Cu^{II}_{6}(D\text{-penicillamine})_{12}Cl]^{5-}$ at 37 °C in a similar way. In bovine serum at 37 °C $[Cu^{I}_{8}Cu^{II}_{6}(D\text{-penicillamine})_{12}Cl]^{5-}$ disintegrated with a first order rate constant of 5.8×10^{-5} min^{-1}, or 8.3% per day. Furthermore, the effect of halides on $[Cu^{I}_{8}Cu^{II}_{6}(D\text{-penicillamine})_{12}Cl]^{5-}$ disintegration was investigated. Chloride stimulated the decomposition rate in a specific manner. Bromide and iodide were even more active. It was concluded that $[Cu^{I}_{8}Cu^{II}_{6}(D\text{-penicillamine})_{12}Cl]^{5-}$ is not stable in serum, but slowly decomposes. Attributable to this phenomenon, Cu(II) is liberated and is immediately sequestered by serum albumin and channelled into the usual way of copper metabolism**.

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**Abbreviations: CuPen: $[Cu^{I}_{8}Cu^{II}_{6}(D\text{-penicillamine})_{12}Cl]^{5-}$; gly-gly-his: glycyl-glycyl-L-histidine.

Introduction

D-Penicillamine (3,3-dimethyl-D-cysteine) is a very versatile drug. Besides being effective in curing heavy metal poisoning, it is used in the treatment of diseases as diverse as Morbus Wilson [1], cholestasis, primary biliary cirrhosis, neonatal jaundice [2, 3] and rheumatoid arthritis [4, 5]. At first sight, a common principle of action is not easily envisaged.

When looking for possible modes of action of D-penicillamine, the functional residues determining its reactivity have to be considered. First of all, there is a tertiary sulphhydryl group which supports three functions: (i) It is a reducing agent, e.g., capable of converting copper(II) to copper(I), itself being oxidized to penicillamine disulphide. (ii) It is a soft Lewis base, e.g., able to accommodate copper(II) and copper(I) at the same time. (iii) It can undergo exchange reactions with (mixed) disulphides. Secondly, the two methyl groups in the vicinity of the sulphhydryl group modify the reactivity of penicillamine and its metal complexes when compared with cysteine. Thus, the mixed valence complex $[Cu^{I}_{8}Cu^{II}_{6}(D\text{-penicillamine})_{12}Cl]^{5-}$ (CuPen) is stable against air oxidation in contrast to its cysteine analogue. Furthermore, the steric hindrance conferred by the methyl groups restricts the reactivity of penicillamine in reactions with large molecules (e.g., protein). Thirdly, the 2-amino and carboxyl groups characteristic of amino acids enable penicillamine to form chelate complexes. Furthermore, the vicinal position of sulphhydryl and amino groups enable penicillamine to react with aldehydes to form thiazolidine derivatives.

All these properties taken together render D-penicillamine especially suitable to interact with copper in the mammalian organism. Thus, 'reductive chelation' has been invoked as the process by which excess copper in Wilson's disease is sequestered and excreted renally [6]. In a similar way, accumulation of excess copper due to impaired biliary excretion might be reduced by D-penicillamine in liver diseases. In rheumatoid arthritis, copper has been shown to have a beneficial effect with animal experimental models and in man [7–9]. The basis for this action

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has not been revealed. No matter how, D-penicillamine might operate by mobilizing copper that can subsequently be disposed of in inflammatory regions.

It is not known in which form copper is excreted in the urine during therapy of Wilson's disease with D-penicillamine, and how it is mobilized. CuPen might be one possibility, being water-soluble and stable in the presence of serum albumin. However, it is not formed when physiological concentrations of copper bound to serum albumin are offered to penicillamine [10]. Therefore, CuPen does not arise in serum, but its formation in other compartments devoid of serum albumin cannot be excluded.

Instead of or in combination with D-penicillamine, CuPen might be administered in rheumatoid arthritis [8]. This would reduce side effects from D-penicillamine, counteract a renal loss of copper in long-term therapy with D-penicillamine alone, and provide additional copper that would propitiously influence the inflammatory process [9]. CuPen itself is only weakly toxic, partly due to its stability [8, 11].

Notwithstanding its stability, CuPen slowly decays dependent on several conditions [12]. Thus, it continuously releases copper which might support the intrinsic copper mobilization during inflammation. As this phenomenon may well be beneficial in the treatment of arthritis, the decomposition of CuPen was investigated in the presence of bovine serum, bovine serum albumin and glycylglycyl-L-histidine (gly-gly-his), a model of the copper binding site of serum albumin. The rate of decomposition was studied at eight different temperatures between 37 °C and 90 °C. The effect of excessive copper concentration was examined using $CuSO_4$. EDTA and gly-gly-his were employed to investigate the influence of copper chelators. Furthermore, the destabilizing effect of chloride and other halides was evaluated. The decomposition of the CuPen chromophore was monitored by electronic absorption spectrometry.

Experimental

Materials

Crystalline bovine serum albumin, fraction V bovine serum albumin and D-penicillamine were obtained from Sigma, München. Glygylglycyl-L-histidine and bovine calf serum were from Serva, Heidelberg. All other chemicals were of reagent grade quality except potassium iodide, which was of chemical grade.

CuPen was prepared as described by Birker and Freeman [13]. Additionally, the final solution was gel-filtrated using Superose 12 (crosslinked agarose) from Pharmacia, Uppsala. The purple aqueous solution was characterized by measuring electronic absorption at 518 nm; the extinction coefficient amounted to 1840 M^{-1} cm^{-1} expressed per Cu atom. Furthermore, the purple fraction migrated as one single band during gel permeation chromatography on Superose 12. The CuPen solution was stored at 4 °C in the dark, and low molecular weight fragments were removed by gel filtration prior to use.

Decomposition Measurement

Decomposition experiments at temperatures between 60 °C and 90 °C were performed in a thermostatted cuvette holder with stoppered 3 ml quartz cuvettes of 1 cm light path. Decay of the CuPen chromophore was monitored at 518 nm using a Unicam SP 1800 spectrophotometer and an SP 1805 programme control unit.

The total volume of the incubation mixture was 3 ml. It contained 19 mM tris, pH 6.3 (85 °C), and variable concentrations of EDTA, $CuSO_4$, gly-gly-his, NaCl, KCl, KBr, KI, or Na_2SO_4, respectively, given in the text. The reaction was started after a 10 min preincubation using 150 µl CuPen (1.7 mM Cu).

Decomposition experiments at 37 °C were performed in polystyrene tubes immersed in a water bath. The tubes contained 480 µl gel-filtrated CuPen and 300 µM EDTA, 100 µM $CuSO_4$, 600 µM gly-gly-his, or 600 µM serum albumin dissolved in 19 mM tris/H_2SO_4, pH 7.1 (37 °C) + 150 mM NaCl. Decomposition of CuPen in bovine serum was studied by adding 480 µl CuPen to 2.8 ml serum. At the indicated times a 500 µl aliquot was taken and absorbance at 518 nm was measured in a Beckman Model 25 spectrophotometer.

Evaluation

Rate constants were derived from absorbance *versus* time curves linearized assuming a process of first order kinetics. $\ln(A_0/A_t)$ (A_0 absorbance at zero time, A_t absorbance at time t) was plotted against time. Slope and intercept were calculated by linear regression analysis. The slope corresponded to the first order rate constant k_1. The straight line ideally passes through the origin.

Activation enthalpy and entropy were determined from the temperature dependence of the rate constants between 60 °C and 90 °C. From the thermodynamic transition state theory the equation follows:

$$\ln(k_1/T) = \ln(k/h) + \Delta S^{\ddagger}/R + \Delta H^{\ddagger}/(RT)$$

(T absolute temperature, k_1 experimental first order rate constant, k Boltzmann's constant, h Planck's constant, $\Delta S^{\ddagger}$ activation entropy, R gas constant, $\Delta H^{\ddagger}$ activation enthalpy). $\ln(k_1/T)$ was plotted against $1/T$, $\Delta H^{\ddagger}$ was derived from the slope and $\Delta S^{\ddagger}$ from the intercept after linear regression analysis. Temperature-dependent variation of pH was compensated by titration of the samples to pH 6.3 with H_2SO_4 or NaOH before the start of the reaction.

Results and Discussion

CuPen in aqueous solution is stable for weeks at room temperature. However, hints for its decomposition have been presented in earlier work [11–13]. CuPen releases Cu(II) at room temperature in the dark even in the absence of oxygen, which can be detected by inhibition of nitroblue tetrazolium reduction by xanthine oxidase and xanthine. This process is accelerated by visible light [12]. Boiling of aqueous CuPen solution leads to its bleaching over intervals of ten minutes [11]. Furthermore, CuPen is not stable in the presence of 0.15 M NaCl at ambient temperatures, disintegrating with a half-life of approximately 16 days. Decomposition in urine at pH 6 is much faster (complete within 50 h) [13].

Decomposition of CuPen

It was attempted to study the rate of the thermal decomposition of CuPen in more detail. 20 mM tris, pH 6.3 (85 °C) containing 0.15 M NaCl was heated to 85 °C. Aqueous CuPen (85 μM Cu) was added and the decrease of the red–violet chromophore was followed by measuring the electronic absorption at 518 nm. A sigmoidal A_{518} *vs.* time curve was noticed (Fig. 1), indicating an acceleration of the decomposition reaction by one or more reaction products. Unspecifically bound Cu(II) was assumed to be formed during decomposition and was expected to stimulate disintegration. This is consistent with the earlier observation that CuPen proved to be unstable in the presence of Cu(II) [11]. In order to support this conclusion, the same experiment was repeated in the presence of an excess of the copper chelator EDTA (300 μM) over penicillamine-bound copper. The chromophore decreased exponentially (Fig. 1); a straight line was obtained upon linearization of the absorption *vs.* time plot, assuming a first order process. Furthermore, the initial velocity was higher than in the absence of EDTA. When EDTA was replaced by $CuSO_4$ (100 μM) (Fig. 1), straight lines were again obtained after the same linearization procedure (Fig. 2). However, the decomposition was greatly stimulated. The addition of EDTA prevented acceleration by $CuSO_4$. Clearly, Cu(II) plays a marked role in the destabilization of CuPen.

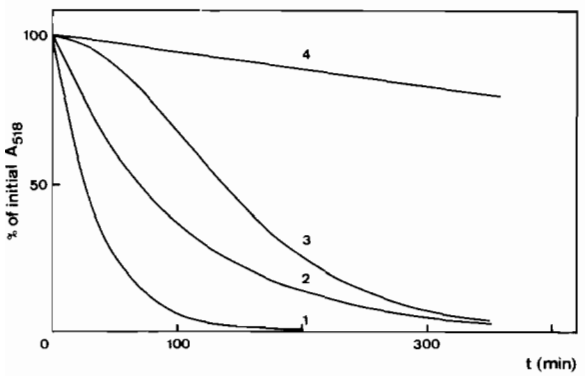

Fig. 1. Decrease of the CuPen chromophore at 518 nm in the presence of 185 μM $CuSO_4$ (1), 300 μM EDTA (2), 600 μM gly-gly-his (4), and in the absence of any additive (3) at 85 °C. Samples (1), (2) and (4) included 19 mM tris/H_2SO_4, pH 6.3 (85 °C) and 140 mM NaCl. (3) Shows the decomposition of a pure aqueous solution of CuPen.

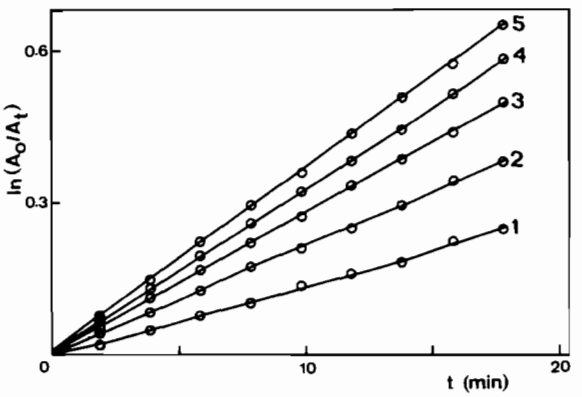

Fig. 2. Decomposition of CuPen at variable concentrations of added $CuSO_4$. To 2.8 ml of 20 mM tris/H_2SO_4, pH 6.3 (85 °C) containing 150 mM NaCl 50 μl of aqueous $CuSO_4$ were added. After a 5 min preincubation at 85 °C the reaction was started with 150 μl gel-filtrated aqueous CuPen (1.7 mM Cu). The final concentration of $CuSO_4$ was (1) 33 μM, (2) 67 μM, (3) 100 μM, (4) 133 μM, and (5) 167 μM, respectively. After another 5 min the decomposition was followed at 518 nm. The first order rate constants were obtained from the slopes.

Reactivity of Additional Copper

The reactivity of cupric copper on the thermal disintegration of CuPen was further examined by determining the concentration (Figs. 2, 3) and temperature dependency (Fig. 4). Between 0 and approximately 350 μM $CuSO_4$ a non-linear relationship between Cu(II) concentration and decomposition rate was observed with a kinetic order of 0.6 in Cu(II). Between 0.35 μM and 1.7 mM the concentration dependence was almost linear (with an experimental kinetic order of 0.9). This might be interpreted in terms of a change of reaction mechanism at higher Cu(II) concentrations. Up to 1.7 mM $CuSO_4$, no saturation was seen. The temperature dependence between 60 and 90 °C yielded the activation parameters of the decomposition in the presence of additional 185 μM Cu(II): activation enthalpy $\Delta H^{\ddagger}$ amounted to 127 ± 2 kJ mol^{-1}, activation entropy $\Delta S^{\ddagger}$ 44 ± 5 J K^{-1} mol^{-1}. The positive reaction entropy suggests an activated complex with reduced net order compared with its reactants. This might be due to the binding of Cu(II) to carboxyl groups at the periphery of the cluster.

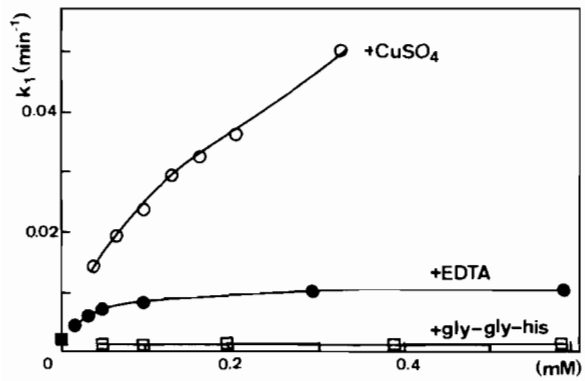

Fig. 3. Concentration dependence of the CuPen decomposition at 85 °C in the presence of $CuSO_4$ (○), EDTA (●), and gly-gly-his (□). Rate constants k_1 (min^{-1}) were derived according to the procedure described in the legend to Fig. 2 and in the presence of variable concentrations of $CuSO_4$, EDTA, and gly-gly-his. ■: experimental rate constant in the absence of any additive; the first 10 min of the reaction course were used to calculate the rate constant. The standard deviation of the determination of the rate constants was less than 2% in the presence of $CuSO_4$ or EDTA and less than 5% in the presence of gly-gly-his.

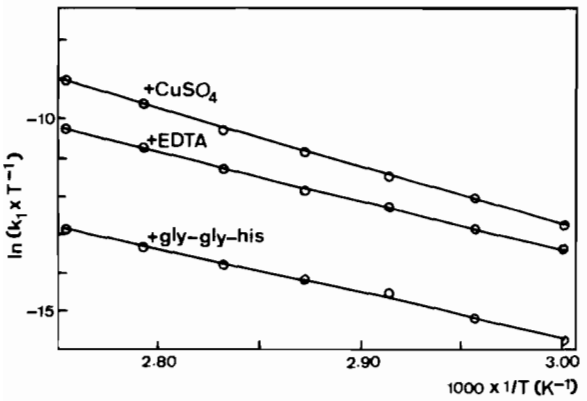

Fig. 4. Temperature dependence of the CuPen decomposition in the presence of 185 μM $CuSO_4$, 300 μM EDTA, and 600 μM gly-gly-his. The rate constants were obtained as described in the legend to Fig. 2, except that temperatures between 60 °C and 90 °C were used.

Reactivity of EDTA

Similar experiments were performed in the presence of EDTA (Figs. 3, 4). Linearization assuming first order kinetics yielded straight lines. In the presence of 300 μM EDTA, activation enthalpy was determined to 99 ± 1 kJ mol^{-1} and activation entropy to −43 ± 3 J K^{-1} mol^{-1}. However, at this concentration the decomposition was independent of EDTA. The reaction order relative to EDTA was zero. The concentration dependency of the reaction rate showed hyperbolic characteristics. Saturation was reached at approximately 100 μM EDTA (a 14-fold excess over CuPen cluster). Obviously, in close analogy to the enzyme-substrate complex in enzyme kinetics, a complex involving CuPen and EDTA is formed in a fast equilibrium process preceding the disintegration. This complex is considered to be the starting point for the decomposition reaction, which presumably is a monomolecular process. However, unlike enzyme-catalyzed reactions, both CuPen and EDTA are consumed during the decomposition of CuPen. This is true because only the EDTA which is not chelated to Cu(II) is effective in stimulating the disintegration of CuPen. When EDTA is added in less than stoichiometric amounts relative to copper, the decrease of the chromophore is diminished until an amount of Cu(II) has been released that is equivalent to the EDTA concentration. Thereafter, the decomposition proceeds sigmoidally as if no EDTA were present. The saturation observed at high EDTA concentrations is in contrast to the decomposition of CuPen in the presence of $CuSO_4$. In this case non-zero order kinetics in $CuSO_4$ and no detectable saturation suggest a reaction mechanism involving more than one molecule that is initiated by collisions between aqueous Cu(II) and CuPen.

Decomposition in the Presence of Biological Chelators

In order to learn more about the reactivity of CuPen under physiological conditions, the thermal decomposition of this copper complex was investigated in the presence of bovine serum, bovine serum albumin or glycyl-glycyl-L-histidine (gly-gly-his), a thermoresistant model of the specific amino-terminal copper binding site of both bovine and human serum albumin. Thus, the copper-chelating activity of serum albumin could be simulated at temperatures where albumin would normally denature.

In the case of gly-gly-his at 85 °C, no significant concentration-dependent rise of disintegration rate was observed. The decomposition was much slower than in the presence of EDTA. The rate was even less than in the absence of any additive. (In this case, however, traces of Cu(II) might catalyze the decomposition of the cluster.) From the temperature dependency of the disintegration reaction, the following activation parameters were obtained: activation enthalpy $\Delta H^{\ddagger}$ 97 ± 3 kJ mol^{-1}, activation entropy $\Delta S^{\ddagger}$ −71 ± 9 J K^{-1} mol^{-1}.

The reactivity of gly-gly-his and bovine serum albumin on CuPen disintegration was measured at 37 °C to determine whether or not this tripeptide would be a suitable model for serum albumin. The decay of the CuPen chromophore proceeded with the same characteristics, although insignificantly faster, when crystalline bovine serum albumin was added (Fig. 5). From the data of CuPen decomposition employing 600 μM bovine serum albumin (essentially globulin-free), the first order rate constant was estimated to be (1.4 ± 0.1) × 10^{-5} min^{-1}, or

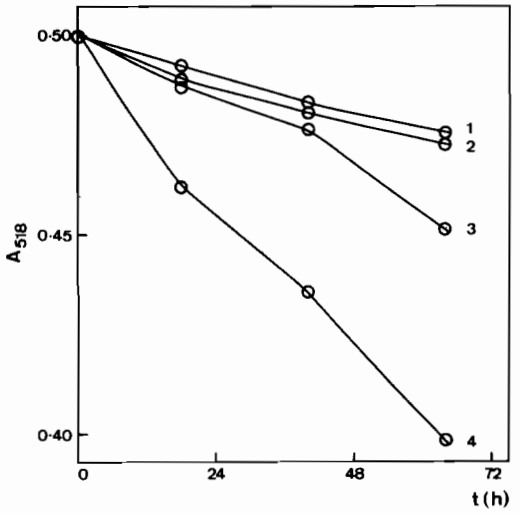

Fig. 5. CuPen decomposition at 37 °C in serum or in the presence of serum albumin or gly-gly-his. 480 µl of gel-filtrated CuPen was added to 2.8 ml of 20 mM tris/H_2SO_4, pH 7.1 (37 °C) containing 600 µM gly-gly-his (1), 600 µM crystalline bovine serum albumin (essentially globulin-free) (2), or 600 µM fraction V bovine serum albumin (98–99%, remainder mostly globulins) (3). Another 480 µl of CuPen was given to 2.8 ml of bovine calf serum (4). Incubation at 37 °C started at zero time. At the times indicated A_{518} was recorded in the supernatants of 500 µl aliquots. The remaining turbidity in the serum samples was corrected for by measuring A_{800} and subtracting $1.69 \times A_{800} + 0.202$ from A_{518}. This equation was empirically derived taking samples of different turbidity.

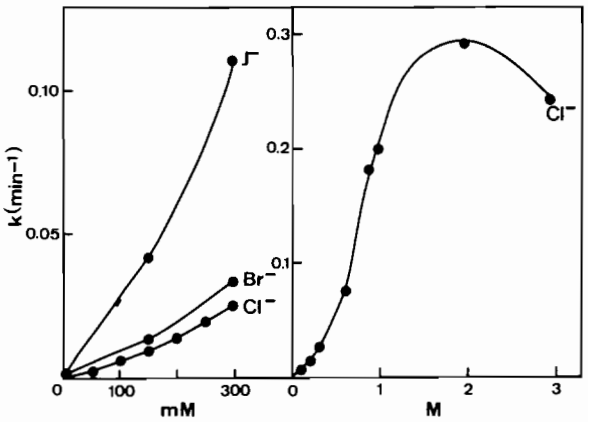

Fig. 6. CuPen decomposition in the presence of different halides. The rate constants were derived as described in the legend to Fig. 2, except that variable concentrations of chloride, bromide, or iodide were employed, respectively. $CuSO_4$ was replaced by 300 µM EDTA.

2.0% per day. When, however, fraction V of bovine serum albumin (containing 1–2% globulins) was used, the decomposition was slightly faster. The rate of decrease of the chromophore was significantly faster in bovine serum. The rate constant k_1 in this case was $(5.8 \pm 0.3) \times 10^{-5}$ min^{-1}, or 8.3% per day. Apparently some globulin-like serum component destabilizes CuPen and induces its decomposition.

Reactivity of Chloride, Bromide and Iodide

Furthermore, the reactivity of chloride, bromide, and iodide on thermal CuPen decomposition was examined. The dependency of the reaction rate on the concentration of the halides in the presence of 300 µM EDTA is shown in Fig. 6. Disintegration of CuPen is strongly dependent on chloride concentration; bromide and iodide are even more active. The reaction order in chloride (0–600 µM) was calculated to be 1.4. Blood plasma contains approximately 100 mM chloride and, therefore, supports CuPen decomposition due to this fact alone. The observed phenomenon is not a simple ionic strength or chaotropic effect. When sulphate was substituted for chloride at constant ionic strength, the decomposition rate decreased to one hundredth. The same result was obtained when iodide was replaced by urea. Halides of potassium instead of sodium did not change the rate significantly. According to this evidence, this effect is assumed to be specific for halides. This halide effect suggests a corresponding reactivity in the case of the preparation of CuPen. However, the concentration of a particular halide anion for optimal CuPen formation and maximal yield of CuPen is obtained at 0.3 M, while decomposition is most rapid at 2 M. Perhaps at higher than optimal halide concentrations decomposition processes predominate over formation during CuPen synthesis, thus suppressing the yield.

This halide effect is most pronounced in the presence of EDTA; 150 mM chloride stimulate the decomposition by a factor of 30. In the presence of gly-gly-his and $CuSO_4$, 150 mM chloride increase the rate by factors of 3 and 2.5, respectively.

The mechanism of the halide effect during disintegration of CuPen is not at all clear. In the case of iodide, the reductive activity might be important. Certainly, the role of halides during formation and disintegration is different. This is concluded from the fact that halide anions are absolutely necessary for formation of CuPen, being incorporated in the CuPen cluster as a symmetry center; on the contrary, CuPen even disintegrates in the absence of halides, albeit more slowly than in their presence. However, it cannot be decided whether traces of chloride, liberated during decomposition, might support the halide effect.

Conclusion

CuPen in aqueous solution is metastable. Even at room temperature it slowly decomposes liberating Cu(II). As a consequence, only freshly gel-filtrated solutions of CuPen should be used for experimental work.

The thermal decomposition of CuPen is susceptible to several conditions. Thus, chloride, bromide, and iodide accelerate the process by an unknown mechanism of complex kinetics. Moreover, Cu(II) stimulates the disintegration of CuPen, and Cu(II) formed during decomposition supports further decomposition of CuPen by way of autocatalysis. Cu(II) chelators, such as EDTA, gly-gly-his, or serum albumin, abolish this phenomenon. However, EDTA alone promotes CuPen disintegration. In contrast, gly-gly-his (and serum albumin) does not seem to affect the decomposition of CuPen. By chelating the unspecifically bound Cu(II) generated during the disintegration, it protects CuPen from autocatalytic acceleration of the reaction. Thus, in the presence of gly-gly-his the intrinsic decomposition of CuPen is presumably observed.

Our results lend support to the conclusion that CuPen is not stable in serum at physiological temperatures, but disintegrates only slowly. Extraneously bound Cu(II) generated during this process is chelated by serum albumin, thus being ineffective in accelerating the decomposition reaction. The albumin-bound copper is taken up by the liver and, eventually, by peripheral tissues.

2.0% of the copper of CuPen is removed per day in the presence of 600 μM serum albumin at 37 °C, 8.3% per day in serum. The recommended daily copper intake of 2 mg [14] would be furnished by the partial decomposition of 75 mg CuPen (corresponding to 24 mg of CuPen-bound copper), *i.e.*, approximately 1 mg CuPen/kg/day, renal elimination not considered. Thus, CuPen might help to replace copper that is released from the liver copper stores and used for the biosynthesis of ceruloplasmin, a major acute phase protein. (The concentration of ceruloplasmin in the blood is raised during systemic inflammation [9].) This might be beneficial as the present nutritional copper supply is considered to be low [14].

If penicillamine is effective by modulating copper metabolism, perhaps by mobilizing copper from inert storage sites, penicillamine might be replaced, at least in part, by CuPen. Usual doses of D-penicillamine of up to 1.5 g/day might be reduced, relieving its side effects. Administration of Cu^{2+} is not equivalent to CuPen, because Cu(II) (bound to serum albumin) is rapidly cleared from the blood by the liver [15]. CuPen, on the other hand, remains, at least partially, in the blood stream, a fact that is indicated by the renal excretion of CuPen after its administration [11]. Apart from the known thermal disintegration it is conceivable that CuPen is decomposed by oxidative metabolic processes. This might be particularly true at inflammatory sites where many reactive species including oxygen free radicals are released. Copper release from CuPen might be of particular benefit in this context, due to the anti-inflammatory properties of copper [9].

Acknowledgements

This study was supported by the Deutsche Forschungsgemeinschaft (We 401-17) and in part by the Fonds der Chemischen Industrie. U.D. was a recipient of a pre-doctoral fellowship of the Studienstiftung des Deutschen Volkes. The technical assistance of Helga Heinemann is greatly appreciated.

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