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Stimulated decay of superoxide caused by ferritin-bound copper

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The redox interaction between $O_2^{\bullet-}$ and ferritin cannot solely be regarded as a Fe(II) release reaction. We demonstrate that native copper bound to horse spleen ferritin and apoferritin, stimulated the decay of $O_2^{\bullet-}$ in a catalytic reaction. Copper was determined by atomic absorption spectrophotometry. Decay of $O_2^{\bullet-}$ was monitored spectrophotometrically as the decrease in $(A_{250}-A_{360})$ at pH 9.5. The catalytic effect was linearly related to the copper content of the protein. Ferritin copper was less efficient than equimolar $CuCl_2$, and iron-poor ferritin was more efficient than iron-rich ferritin. The results support a direct antioxidant function of ferritin.

Ferritin; Superoxide; Copper; Catalysis

1. INTRODUCTION

The major function of ferritin is sequestration and storage of iron in the cell. Iron is temporarily deposited as hydrous Fe(III) oxide phosphate microcrystals within the central cavity of the protein. The iron core may contain up to 4500 Fe(III) atoms [1,2].

There is substantial evidence from in vitro studies that low-M_r redox active substances can interact with the iron core and cause release of Fe(II) from ferritin [3]. Some studies have shown that $O_2^{\bullet-}$ can do this [4–6], and therefore ferritin has been suggested as a source of catalytic iron for the Haber-Weiss reaction resulting in enhanced production of free radicals and oxidative damage of the cells [7–9]. In this respect, ferritin can be regarded to have pro-oxidant properties.

On the other hand, reductive release of iron from ferritin by $O_2^{\bullet-}$ has been shown to be very limited [10]. Also, in the studies of iron release the use of a powerful, non-physiological chelator might disturb the thermodynamic equilibrium of the system and give erroneous results [11,12]. By omitting the chelator and instead measure the rate of decay of $O_2^{\bullet-}$ in the absence and presence of ferritin, we were able to show that ferritin stimulated the decay of $O_2^{\bullet-}$, and that the number of $O_2^{\bullet-}$ molecules which were degraded by ferritin, was in great incongruity with the low number of Fe(II) atoms which were released [13]. Together with the kinetic characteristics of the reaction [14], this indicates that the stimulated decay of $O_2^{\bullet-}$ was caused by a catalytic effect of ferritin. Thus, the antioxidant properties of ferritin exerted by its competency to prevent accumulation of toxic amounts of free iron [15,16], seems to be reinforced by its ability also to perform SOD activity. One way to explain the catalytic mechanism, is to postulate a cycling redox reaction of iron in situ initiated and maintained by $O_2^{\bullet-}$ [13]. On the other hand, a few studies have demonstrated that ferritin contains tiny amounts of copper [10,17,18]. Copper both in its ionic form [19] and bound to ligands such as Cu,Zn-SOD or chemicals [20,21] is capable of stimulating the dismutation of $O_2^{\bullet-}$. It was therefore of interest to clarify the role of copper in the catalytic breakdown of $O_2^{\bullet-}$ by ferritin. In the present paper we show that ferritin contains native copper, and demonstrate the importance of ferritin- bound copper for the stimulated decay of $O_2^{\bullet-}$.

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2. EXPERIMENTAL

2.1. *Materials*

Catalase (EC 1.11.1.6) (from bovine liver, thymol-free), bovine albumin, 2-amino-2-methyl-1-propanol and ceruloplasmin (bovine) were from Sigma Chemical Co (St. Louis, MO, USA). Ferritin (equine spleen, highly purified, 2 × crystallized with less than 0.001% Cd(II)) was obtained from Calbiochem Corp., La Jolla, CA, USA. It had an average iron content of 800 Fe/molecule. Horse spleen apoferritin, which was purchased both from Sigma and from Calbiochem, contained no detectable iron. Ferritin with a definite iron/protein ratio was obtained by fractionating native ferritin on a linear sucrose gradient [22].

Chelex-100 (200–400 mesh) was from Biorad, Richmond, CA, USA, and KO_2 was from Fluka AG, Buchs, Switzerland. KO_2 is a reactive reagent which should be handled very carefully. Accidental explosive reactions were avoided by keeping small volumes of KO_2 solutions in polystyrene test-tubes. Spilled KO_2 material was immediately removed and left-over material was diluted in abundant water.

Other chemicals were of the highest purity commercially available. Water was purified in the Milli-Q Water Purification System from Millipore Corp., Bedford, MA, USA, involving reversed osmosis, ion exchange and filtration. It was free of iron and copper. Buffers were filtered by a Millipore 0.22 μm (pore-size) filter prior to use. Catalase was passed through a Sephadex G-100 column to remove superoxide

dismutase activity, and catalase activity was defined according to [23]. All glassware was washed with acid to remove contaminating iron and other transition metals.

2.2 *Assays*

To study the affinity of ferritin for copper, ferritin dissolved in 10 mM HEPES buffer at pH 7.0 was passed several times through columns of Chelex-100. Between each passage, the content of iron, copper and protein in the sample was determined. New columns were used each time. Copper and iron were determined by atomic absorption spectrophotometry. Protein was determined as described in [24] with bovine serum albumin as a standard. The molar copper/ferritin ratio had a coefficient of variation of 5–8% when metal and protein was repeatedly determined in the same solution.

Isoelectric focusing of ferritin was performed using 2117 Multiphor II and 2209 Multitemp., LKB, Sweden. Approximately 0.1 nmol of protein was run on an Ampholite PAG plate manufactured by Pharmacia LKB Biotechnology, pI range 3.5–9.5. There was no significant difference in the isoelectric pattern of ferritin before and after 3 passages on Chelex-100 columns.

Decay of $O_2^{\bullet-}$ was studied in a medium which contained in a volume of 3 ml: 50 mM 2-amino-2-methyl-1-propanol, pH 9.5, 0.35 U/ml of catalase and 0.08 μM of ferritin (as protein). The temperature was 23°C. The decay of $O_2^{\bullet-}$ was monitored in a thermostatically controlled Hewlett-Packard HP 8450A diode array spectrophotometer as described previously [14,25]. Briefly, 100 mg of KO_2 was dissolved in 12.5 ml of 50 mM NaOH. 15 μl was transferred to the reaction medium and the declining concentration of $O_2^{\bullet-}$ was monitored as the decrease in absorbance (A_{250}–A_{360}). The rate of $O_2^{\bullet-}$ decay was expressed as the apparent pseudo-1st-order rate constant, calculated from the decay of $O_2^{\bullet-}$ from 16 to 4 μM, as described in [25]. The SOD activity of ferritin was defined as the rate of decay of $O_2^{\bullet-}$ which exceeded that of the spontaneous dismutation [13,25].

3. RESULTS

3.1. *Binding of copper to ferritin and apoferritin*

As shown in Table I(A), about 3/4 of the copper present in the original solution of native, unfractionated ferritin, was removed by one passage on Chelex-100. In the following two passages approximately 5 Cu atoms were removed per ferritin molecule, while subsequent passages of ferritin on Chelex-100 columns had no significant effect on its copper content. Thus, 9 10 Cu/ferritin molecules were resistant to removal by Chelex-100. There was no significant fall in the content of ferritin iron.

To control the effectiveness of Chelex-100 to remove copper and to get an idea of the affinity of ferritin for exogenously added copper, aliquots of a ferritin sample which had been treated 3 times on Chelex-100 and thus contained about 10 Cu/ferritin, were incubated on ice for one hour with $CuCl_2$ up to 52.5 mol Cu/mol ferritin. Then each sample was passed through a Chelex-100 column and the copper content was determined before and after this passage. As shown in Table I(B), one passage on Chelex-100 was sufficient to remove all the extra copper. Further, this experiment demonstrated that the chelating capacity of Chelex-100 was not a limiting step in the attempt to remove all copper from native ferritin.

While no copper could be detected in apoferritin from Sigma (Table II(A)), apoferritin from Calbiochem contained about 6 Cu/molecule. After one passage on Chelex-100, 1–2 Cu/molecule remained in the Calbiochem apoferritin, and this copper was resistant to 5 subsequent passages (Table II(C)). When the copper-free apoferritin was incubated with $CuCl_2$ as described above for ferritin, about 2 Cu/molecule were still bound after 3 passages on Chelex 100 (Table II(B)). This was in contrast to the results obtained with ferritin (Table I), where all the extra added copper could be removed by one passage on Chelex-100. After having been subjected to the same procedure, Calbiochem apoferritin also contained a final amount of 2 Cu/molecule (Table II(D)).

In control experiments, $CuCl_2$ added to copper-free albumin, was removed by one passage on Chelex-100. In comparison, ceruloplasmin contained an average of about 2 Cu/molecule which could not be removed by several passages on Chelex-100. However, extra added copper was completely removed by one subsequent passage on Chelex-100 (data not shown).

Table I

Content of copper in native ferritin

The table shows the mean of duplicate measurements in one out of 2–3 parallel experiments (A) Ferritin was passed through columns of Chelex-100 the number of times shown. (B) Ferritin which had been treated 3 times on Chelix-100 (as indicated by the arrow), was incubated with $CuCl_2$. The ferritin was then passed once again through Chelex-100.

Number of treatments on Chelex-100: 0	1	2	3	4	5	6
			(mol Cu/mol ferritin)			
(A) Native ferritin 56.7	14 7	12.7	9.9	9.8	8.8	8.9
(B) Native ferritin after incubation with $CuCl_2$ 14.4	10.0					
29.9	9 7					
41.9	10.1					
52.5	9.9					

To study if there was any correlation between the content of iron and copper in ferritin, we determined the Cu/protein ratio in ferritin fractions with a definite iron load in the range 20–2550 Fe/ferritin. After 3 passages on Chelex-100, all fractions with iron content above 500 Fe/molecule contained 14–16 Cu/molecule, while the fraction with the lowest iron load (20 Fe/molecule) contained about half of this amount (Table III).

3.2. *Influence of ferritin-bound copper on the rate of decay of $O_2^{\bullet-}$*

To reveal if the ability of ferritin to catalyze the decay of $O_2^{\bullet-}$ depended on its content of copper, we examined how apoferritin and ferritins with different copper content, obtained from the experiments described above, altered the rate of decay of $O_2^{\bullet-}$. There was a clear correlation between the amount of copper in native ferritin and the ferritin-induced $O_2^{\bullet-}$ decay (Fig. 1). However, the effect of ferritin was lower than that of equimolar concentrations of $CuCl_2$. In contrast, apoferritin with a certain copper content, had about the same effect as the corresponding concentration of $CuCl_2$.

Of the ferritin fractions shown in Table III, the SOD activity was about the same in all fractions. However, the ratio SOD activity/copper content was about twofold higher in iron-poor ferritin than in iron-rich ferritin fractions (Table III, Fig. 1).

Albumin or ceruloplasmin in the same concentrations as ferritin had no detectable SOD activity. Addition of $CuCl_2$ to albumin or ceruloplasmin had the same effect on the $O_2^{\bullet-}$ decay as that obtained with a pure, equimolar $CuCl_2$ solution (data not shown).

4. DISCUSSION

4.1. *Demonstration of specifically bound copper in ferritin*

The experimental design of this work diverged from that of previous studies of the binding of copper to ferritin. While others have studied the affinity by adding copper to the ferritin solution [26–28], we studied the resistance of native copper against removal by Chelex-100 which is a particularly strong copper chelator [29]. The graded resistance of copper to removal by Chelex-100 indicated the possibility of three orders of affinity with which copper could be bound to ferritin. The lowest affinity was represented by loosely bound copper which could be removed by the first treatment of ferritin with Chelex-100. This constituted all the exogenous copper which was added to ferritin and about 3/4 of the endogenous copper of native ferritin (Table I). We reckon that one treatment with Chelex was sufficient to chelate contaminating metal ions, and that the copper which then was left in ferritin was of biological origin and therefore can be designated as specific. However, also some of the specifically bound copper was probably removed at this stage. The molar ratio of specifically bound copper may therefore have been greater in vivo than that expressed by the Chelex-resistant fractions shown here, which we consider to be a minimum. Little is known about the number of specific copper binding sites in native ferritin, how copper is bound or released from ferritin, its submolecular localization and its function. In one of the few reports on this subject, Price and Joshi [28] added copper to ferritin and found 2–3 tightly

Table II

Content of copper in apoferritin

The table shows the mean of duplicate measurements in one out of 2–3 parallel experiments. (A and C) Apoferritin was passed through columns of Chelex-100 the number of times shown. (B and D) Apoferritin which had been treated once on Chelex-100 (as indicated by the arrow), was incubated with $CuCl_2$. The ferritin was then passed again through Chelex-100.

Number of treatments on Chelex-100:	0	1	3	6
	(mol Cu/mol ferritin)			
(A) Apoferritin (Sigma)	0.2	0.0		0.0
(B) Apoferritin (Sigma) after incubation with $CuCl_2$	9.1		2.2	
	45.0		2.1	
	90.0		2.0	
(C) Apoferritin (Calbiochem)	5.6	1.9		1.3
(D) Apoferritin (Calbiochem) after incubation with $CuCl_2$	11.4		2.1	
	40.6		2.2	
	87.4		2.2	

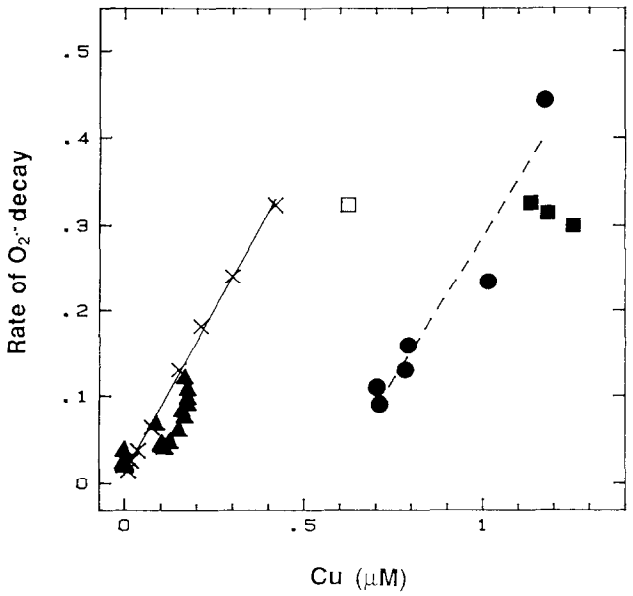

Fig. 1. Rate of $O_2^{\cdot-}$ decay in the presence of ferritin and $CuCl_2$. The rate of $O_2^{\cdot-}$ decay was calculated as described in Section 2 and compensated for the spontaneous dismutation ●, unfractionated native ferritin; ■, fractionated ferritin with > 500 Fe/ferritin; □, fractionated ferritin with ≤ 20 Fe/ferritin; ▲, apoferritin; ×, $CuCl_2$.

bound copper ions/subunit while 3–4 copper ions/subunit were loosely bound. In similar experiments, Macara et al. [27] found 2 copper ions/subunit. This gives an idea of how much copper that can probably be bound to the ferritin molecule. In contrast to these studies, our study was not designed to give the true estimate of the number of binding sites for copper in ferritin. Evidently, our method leads to underestimation of the number of copper ions bound to ferritin. However, while neither Price and Joshi [28] nor Macara et al. [27] found evidence for the presence of copper in the iron core, we came to an opposite conclusion. The difference is probably related to the open question of how copper is incorporated into the iron core. The negative results of the two other studies indicate that the experimental conditions did not promote fusion of exogenous copper ions with the iron core. On the contrary, the results of our experiment where copper was removed from ferritin step by step until we were left with a stable copper fraction with the highest order of affinity (Table I(A)), can reasonably be interpreted that there indeed was some copper present in the native iron core.

Between the loosely bound and the firmly bound copper, there was a smaller fraction with intermediate affinity which could be removed by three treatments of ferritin with Chelex-100 (Table I(A)). This might be copper which was less available to the functional ionic groups of the Chelex resin due to an interior localization for instance within the protein shell or at the surface of the iron core.

The apparent different orders of affinity could partly be explained by differences in the binding strength between the copper ions and the binding sites in the protein shell, and partly by the accessibility of the functional ionic groups of the Chelex resins to the copper ions. Thus, one would expect that for instance contaminating copper would be more loosely bound than native copper specifically bound to the surface of the ferritin molecule. And copper with the apparently highest affinity could be copper which was buried deeply in the iron core and therefore could not be touched by the chelator.

More copper was found in ferritin than in apoferritin (Tables I(A), II(A), II(C)), and there was more copper in iron-rich than in iron-poor ferritin fractions (Table III). This supports the suggestion above that there is some copper in the native iron core which apparently must reach a critical size to attain full copper binding capacity. Since unfractionated ferritin was a mixture of ferritin molecules which ranged from low to high iron content, it had an average copper content between the iron-rich and iron-poor fractions.

Copper-free apoferritin, but not copper-containing ferritin, could withhold some of the exogenously added copper despite subsequent treatments with Chelex-100 (Tables I, II). That could mean that the apoferritin had vacant high-affinity copper-binding sites. When those sites were occupied, extra copper could not be bound firmly enough to resist removal by Chelex-100. This interpretation was supported by the observation that the final copper content in apoferritin, after incubation with $CuCl_2$ and retreatment with Chelex-100, was the same whether or not the apoferritin contained some native copper beforehand (Table II(B), (D)).

We compared ferritin with ceruloplasmin which is a well-characterized copper binding protein [30]. As expected, copper could not be removed from ceruloplasmin by Chelex-100 [31,32]. It is therefore reasonable to

Table III

Copper and SOD activity in native, fractionated ferritin

Ferritin was fractionated according to its iron content. Ferritin iron, copper and the ratio SOD activity/copper content was determined The table shows the mean of duplicate measurements in one out of two parallel experiments.

Ferritin iron (mol/mol)	Ferritin copper (mol/mol)		SOD activity[a]	$10^3 \times$ SOD activity/copper content
	Before Chelex	After 3 × Chelex		
20	34.4	7.8	0.32	41
560	39.3	14.8	0.31	21
1440	39 6	14.2	0.33	23
2550	37 2	15.7	0.30	19

[a]After 3 × Chelex, calculated as described in section 2.

infer that ferritin is able to bind at least some copper with the same high affinity as ceruloplasmin.

At this point we conclude that horse spleen (apo)ferritin evidently contains specifically bound copper of native origin.

4.2. *Copper and the SOD activity of ferritin*

The ability of native, unfractionated ferritin to stimulate the decay of $O_2^{\bullet-}$ depended on its copper content (Fig. 1). Copper deficient apoferritin had no SOD activity. This demonstrated the importance of copper for the catalytic effect. On the other hand, since we did not succeed in removing all copper from ferritin, we could not test if copper was absolutely necessary for the SOD activity of ferritin, or if iron might have some catalytic effect of its own. On an equimolar basis, copper bound to ferritin was less effective than $CuCl_2$ to stimulate the decay of $O_2^{\bullet-}$, while copper bound to apoferritin was almost as effective as $CuCl_2$ (Fig. 1). Iron-poor ferritin had a higher ratio SOD activity/copper content than ferritin with more iron (Table III). This indicated that the size of the iron core had a negative influence on the capacity of copper to react with $O_2^{\bullet-}$. One possibility was that some of the copper was made kinetically inert as the iron core grew and passed a critical size. When we compared the activity of native, unfractionated ferritin with the activity of pure $CuCl_2$, it appeared that about 0.6 μM of the copper in ferritin was non-reactive with respect to stimulation of the $O_2^{\bullet-}$ decay (Fig. 1). We suggest that this was copper which was buried deeply in the iron core, protected from Chelex-100 and inaccessible to $O_2^{\bullet-}$. This explains the connection between the size of the iron core which influences the content of copper in the ferritin molecule as discussed above, and its apparent inhibiting effect on the catalytic activity of ferritin. The copper ions which evidently were responsible for the catalytic effect, had to be localized at sites which were accessible to $O_2^{\bullet-}$, i.e. in the protein shell or at the surface of the iron core.

We conclude that the ability of horse spleen ferritin to catalyze the decay of $O_2^{\bullet-}$ [13] depends on native copper situated in the ferritin molecule. This new insight may explain the diverging results reported in the literature for the bimolecular rate constant between ferritin and $O_2^{\bullet-}$, as this point was not considered in those studies [13,33]. Our results may extend the ideas of ferritin as an antioxidant. Of special interest is the well-known, but so far unexplained, rapid increase of iron-poor ferritin in plasma in inflammatory conditions ('the acute phase response'), when the tissues are exposed to increased oxidative stress [34,35].

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REFERENCES

[1] Theil, E.C. (1990) Adv. Enzymol. Relat. Aread. Mol. Biol. 63, 421–449.
[2] Crichton, R.R. (1990) Adv. Protein Chem. 40, 281–363.
[3] Funk, F., Lenders, J., Crichton, R.R. and Schneider, W. (1985) Eur. J Biochem. 152, 167–172.
[4] Biemond, P., van Eijk, H.G., Swaak, A.J.G. and Koster, J.F. (1984) J. Clin. Invest. 73, 1576–1579.
[5] Thomas, C.E., Morehouse, L.A. and Aust, S.D. (1985) J. Biol. Chem. 260, 3275–3280.
[6] Bolann, B.J. and Ulvik, R.J. (1987) Biochem. J. 243, 55–59.
[7] Biemond, P., Swaak, A.J.G., van Eijk, H. and Koster, J.F. (1988) Free Radical Biol. Med. 4, 185–198.
[8] Winterbourn, C.C., Monteiro, H.P. and Galilee, C.F. (1990) Biochim. Biophys. Acta 1055, 179–185.
[9] Reif, D.W. (1992) Free Radical Biol. Med. 12, 417–427.
[10] Bolann, B.J. and Ulvik, R.J. (1990) Eur. J. Biochem. 193, 899–904.
[11] Thorstensen, K. and Aisen, P. (1990) Biochim. Biophys. Acta 1052, 29–35.
[12] Thorstensen, K. and Romslo, I. (1990) Biochem. J. 271, 1–10.
[13] Bolann, B.J. and Ulvik, R.J. (1993) FEBS Lett. 318, 149–152.
[14] Bolann, B.J., Henriksen, H. and Ulvik, R.J. (1992) Biochim. Biophys. Acta 1156, 27–34.
[15] Halliwell, B. and Gutteridge, J.M.C (1986) Trends Biochem. Sci. 11, 372–375.
[16] Balla, G., Jacob, H.S., Balla, J., Rosenberg, M., Nath, K., Apple, F., Eaton, J.W. and Vercellotti, G.M. (1992) J. Biol. Chem. 267, 18148–18153.
[17] Theil, E.C. (1987) Annu. Rev. Biochem. 56, 289–315.
[18] Joshi, J.G. and Zimmermann, A. (1988) Toxicology 48, 21–29.
[19] Rabani, J., Klug-Roth, D. and Lilie, J. (1973) J. Phys. Chem. 77, 1169–1175.
[20] Klug-Roth, D. and Rabani, J. (1976) J. Phys. Chem. 80, 588–591
[21] Bielski, B.H.J., Cabelli, D.E., Arudi, R.L. and Ross, A B. (1985) J. Phys. Chem. Ref. Data 14, 1041–1100.
[22] Ulvik, R.J. and Romslo, I. (1979) Biochim. Biophys Acta 588, 256–271.
[23] Aebi, H. (1974) in: Methods of Enzymatic Analysis (Bergmeyer, H.U. ed.) vol. 2, pp. 673–684, Academic Press, New York/London.
[24] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[25] Bolann, B J. and Ulvik, R.J. (1991) Clin. Chem. 37, 1993–1999.
[26] Rüssel, H.A. (1970) Bull. Environ. Contam. Toxicol. 5, 115–124.
[27] Macara, I.G., Hoy, T.G. and Harrison, P.M. (1973) Biochem. J. 135, 785–789.
[28] Price, D.J. and Joshi, J.G. (1983) J Biol. Chem. 258, 10873–10880.
[29] (1986) Chelex-100 Chelating Ion Exchange Resin for Analysis, Removal or Recovery of Trace Metals, Bio-Rad Chemical Division, Richmond, CA.
[30] Cousins, R.J. (1985) Physiol. Rev. 65, 238–309.
[31] Morell, A.G. and Scheinberg, I.H. (1958) Science 127, 588–590
[32] Halliwell, B. and Gutteridge, J.M.C. (1990) Methods Enzymol. 186, 1–85.
[33] Buettner, G.R., Saran, M. and Bors, W. (1987) Free Radical Res. Commun. 2, 369–372.
[34] Worwood, M., Dawkins, S., Wagstaff, M. and Jacobs, A. (1976) Biochem. J. 157, 97–103.
[35] Birgegård, G. (1980) Clin. Sci. 59, 385–387.