occurs spontaneously with pyridoxal phosphate and ornithine[10]. Ornithine decarboxylase does not accept this Schiff-base as a substrate[10], and preincubation of the assays is required to equilibrate enzyme, free substrate, free cofactor, and Schiff-base. The seeming inhibition of the enzyme by 2 mM pyridoxal phosphate was overcome by concomitant use of 2 mM ornithine (Table 1). The Schiff-base did not interfere with the ion-exchange procedure, as it was destroyed by semicarbazide in the stopping mixture.

Formation of a Schiff-base from ornithine and pyridoxal phosphate will disturb any evaluation of the kinetics of ornithine decarboxylase, as it decreases the concentrations of free substrate and cofactor, respectively, depending on the ratio of the respective concentrations. In the 1/V-1/[S]-diagram a substrate concentration which is in fact smaller than calculated from the weighings must result in a Michaelis constant which is greater than the true value. This effect may be responsible for differences im K_m-values reported for ornithine (e.g., 30–40 µM in the brains of young rats[7,8], 20–460 µM in neonatal mouse brain[11], 60 and 350 µM, respectively, in rat liver[8,9]) as well as for pyridoxal phosphate (e.g., 4.7 µM in rat liver[7], and 10 µM in mouse fibroblasts[10]).

The present method allows the routine performance of up to 100 complete enzyme assays per day. It is especially suitable for the detection of changes of ornithine decarboxylase activity with developmental age or following experimental treatment. As an example, table 2 shows activities of ornithine decarboxylase in the brains of young Wistar rats at different postnatal days. These values are in good accordance with values obtained with the CO_2-trapping method[12].

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* Present address: Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, 39th and Rainbow Blvd, Kansas City, Kansas 66103, USA.

1 Morris, D.R., and Pardee, A.B., Biochem. biophys. Res. Comm. *20* (1965) 697.
2 Clark, J.L., Analyt. Biochem. *74* (1976) 329.
3 Djurhuus, R., Analyt. Biochem. *113* (1981) 352.
4 Maderdrut, J.L., and Oppenheim, R.W., Neuroscience *3* (1978) 587.
5 Lineweaver, H., and Burk, D., J. Am. chem. Soc. *56* (1934) 658.
6 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. biol. Chem. *193* (1951) 265.
7 Morris, G., Lau, C., and Slotkin, T., Eur. J. Pharmac. *88* (1983) 177.
8 Butler, S.R., and Schanberg, S.M., Life Sci. *18* (1976) 759.
9 Kitani, T., and Fujisawa, H., J. biol. Chem. *258* (1983) 235.
10 Clark, J.L., and Fuller, J.L., Eur. J. Biochem. *67* (1976) 303.
11 Laitinen, S.I., Laitinen, P.H., Hietala, O.A., Pajunen, A.E.I., and Piha, R.S., Neurochem. Res. *7* (1982) 1477.
12 Anderson, T.R., and Schanberg, S.M., J. Neurochem. *19* (1972) 1471.

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The enhanced induction of metallothionein by zinc, its half-life in the marine fish *Pleuronectes platessa*, and the influence of stress factors on metallothionein levels

J. Overnell, R. McIntosh and T.C. Fletcher

NERC Institute of Marine Biochemistry, St. Fittick's Road, Aberdeen AB1 3RA (Scotland), 20 March 1986

Summary. Intraperitoneal injection of zinc raised levels of a hepatic metallothionein-like species. Assuming that this species was metallothionein (MT) then levels were raised from approximately 20 µg/g to 300 µg/g in 7 days, and levels thereafter remained high for the next 4 weeks. The half-lives of the protein in liver and kidney from starved fish, measured using in vivo incorporation of ^{35}S cysteine at 11 °C, were approximately 27 days and 32 days respectively. The following agents failed to stimulate synthesis of MT in plaice: stress (due to catching), endotoxin, dexamethasone, cortisol and turpentine.
Key words. Metallothionein induction; zinc metallothionein half-life; plaice; stress; fish.

Metallothionein (MT) is a low molecular weight, heat stable, metal binding protein that has been isolated from a variety of vertebrate and invertebrate species[1]. It appears to be ubiquitous in vertebrate tissues and in mammals it is readily inducible by a variety of agents, including the metals copper, cadmium and zinc to which it binds[1,2]. Intraperitoneal (i.p.) injection of cadmium induces synthesis of MT in plaice liver. This MT has been isolated and characterized[3] and its partial amino acid sequence determined[4]: it is similar to mammalian MT. Metallothionein-like proteins have been demonstrated in a number of fish species as a result of metal exposure, and measurement of MT levels in fish has been suggested as a method of monitoring the degree of aquatic metal pollution[5]. Roch et al.[6] have found good correlation between hepatic MT levels in rainbow trout, caught downstream from a copper mine, and the copper level in the water. Seawater-adapted eels have been shown to respond to very high levels of mercury in the water (0.4 µg/g) by synthesizing MT[7]. Mercury was not, however, associated with MT in the estuarine fish, killifish, exposed for 4 weeks to 0.02 µg/g mercury[8]. In the case of freshwater trout, while water-borne zinc does induce synthesis of hepatic MT, cadmium alone does not[9,10]. In other freshwater species cadmium does induce synthesis of MT[11].

In mammals, a variety of non-metal agents, mostly associated with stress (including environmental stress, injury, bacterial infection, liver damage and glucocorticoid hormones) are also known to induce synthesis of MT. In order to investigate the possibility that stress could stimulate MT synthesis in fish we have investigated the effect of stress due to catching and the action of the glucocorticoid hormone cortisol and its synthetic analogue dexamethasone. In order to mimic bacterial infection and injury we have examined the effect of endotoxin and turpentine injection. For MT levels to be a useful parameter to study in fish, we need to know not only which factors are capable of stimulating synthesis of MT but also the half-life of the MT so formed. This paper reports the results of such studies.

Materials and methods. Animals. Mature plaice (200–400 g), seine-netted off the Aberdeenshire coast, were maintained unfed in a circulating seawater aquarium at 11 °C.

Induction experiments and sampling of tissue. Zinc (as sulphate) in 1.1% saline was injected i.p. as 5 successive, daily doses of 2

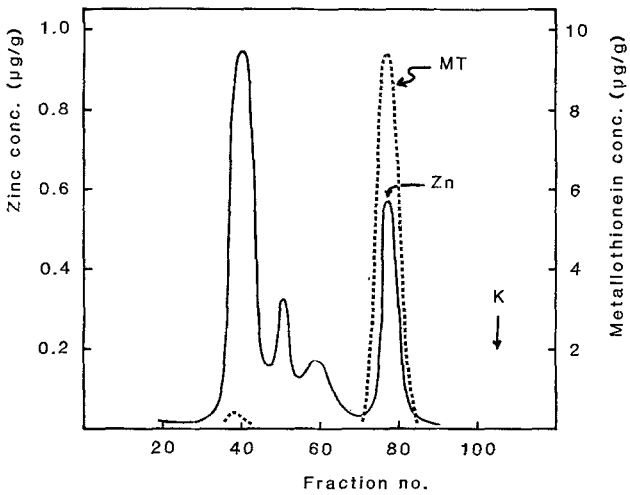

Figure 1. Sephadex G-75 chromatography of cytosol from wild plaice.

mg/kg. Animals were killed at intervals up to 7 days after initial injection and the following measured: liver MT, liver zinc, serum ceruloplasmin (using a *p*-phenylenediamine method[12]), and serum zinc. Blood was collected from the caudal vein and serum removed after clotting. Endotoxin (lipopolysaccharide B *E. coli* 0.111:B4; Difco) dissolved in 1.1% saline, was injected i.p. either as a single dose of 6 mg/kg or as 5 successive, daily doses of 2 mg/kg. Cortisol (21-phosphate disodium salt, Sigma Chemical Co.) dissolved in 1.1% saline was injected i.p. as 5 successive, daily doses of 2 mg/kg. Dexamethasone (Sigma Chemical Co.) dissolved in ethanol was injected as 5 successive daily doses of 2 mg/kg. Turpentine was injected s.c. as a single dose of 1.3 ml/kg.

Determination of metallothionein half-lives. Zinc MT was induced by i.p. injection of zinc (2 mg/kg fish) on days 1, 3 and 5. Radioactive (^{35}S) L-cysteine (New England Nuclear or Amersham International) was prepared for injection by dilution of the stock solution as received from the manufacturer (1000 Ci/m mole) to 30 µCi/ml with 1.1% saline. The injection solution was prepared immediately prior to use and was injected i.p., 2 ml/kg, on day 8. Non-radioactive L-cysteine (500 µmoles) was injected on day 12. Fish were killed at varying intervals after this and liver and kidney removed for analysis. Samples of ^{35}S MT were prepared for counting by a modification of the method of Held and Hoekstra[13]: each gram of tissue was homogenized with 4 ml of 20 mM Tris HCl pH 8.6 using a Potter-Elvehjem homogenizer and the homogenate either used immediately or stored at 70 °C. A sample of homogenate was heated at 90 °C for 4 min using an Eppendorf heating block, cooled and centrifuged for 2 min in an Eppendorf centrifuge 3200. The heat treated supernatant was desalted immediately on a column of Sephadex G-25 (0.9 × 12 cm) equilibrated in 20 mM Tris HCl pH 8.6. Fractions corresponding to the high molecular weight peak (the MT containing peak) and a low molecular weight peak were collected. The MT peak was evaporated to dryness at 60 °C, cooled and treated with 0.2 ml of performic acid (1 part of 100 vols hydrogen peroxide plus 9 parts of 100% formic acid) overnight in the refrigerator. The oxidized sample was applied to a 1 ml column of Dowex 50X8-400 equilibrated in 1 M Na phosphate buffer pH 1.8. The sample was eluted in 1.5 ml of the pH 1.8 buffer and counted with 2.5 ml of water and 10 ml of Instagel.

Counting. ^{35}S radioactive samples were counted on a Packard Tricarb model 3385 scintillation counter as for ^{14}C. Counting efficiency was approximately 75%.

Zinc analysis. Samples of homogenate (1 ml) were digested with 70% (w/v) nitric acid (5 ml), made up to 10 ml with water and analyzed for zinc by atomic absorption (Varian AA5) using standards made up in 35% (w/v) nitric acid.

Metallothionein analysis. Metallothionein was analyzed by a cadmium saturation assay[14], modified only by heat treating the homogenate instead of the cytosol and by carrying out the reactions at pH 8.6 instead of 7.4. The supernatant from the heat treatment was used immediately. ^{109}Cd was counted using a Packard Autogamma model 500C. This assay measures the number of cadmium equivalents bound strongly to a heat stable species. The sample is equilibrated with an excess of ^{109}Cd of known specific activity. The weakly bound cadmium is removed by addition of a large excess of hemoglobin which is sedimented after heat denaturation. The remaining activity is due to MT bound cadmium.

Results and discussion. The cadmium saturation assay was originally developed for rat MT and so this was evaluated for use with fish MT. It was found preferable to heat treat the homogenate instead of the cytosol because plaice livers were frequently fatty and this procedure usually eliminated problems of pipetting through a layer of fat since the fat sedimented with the heat denatured protein. The supernatant from heat treatment was found to be unstable and measurable MT activity was lost with a half-life of about 2 h at room temperature. Freezing the supernatant from heat treatment did not give a useful increase in its stability and the addition of 5 mM 2-mercaptoethanol to the homogenizing medium (in an attempt to stop oxidation of MT) caused a very large increase in the number of counts in the blank, rendering this procedure valueless. In contrast, MT concentration in the homogenate was largely unaffected by prior freezing of the homogenate at X70 °C. Increasing sample size of the heat-treated supernatant yielded calculated values for the MT content per gram of tissue, which rose to a plateau value. The cause of this has not yet been identified although this effect was not observed for partially purified MT fractions. For each determination several sample volumes were therefore taken and the highest value per gram of wet tissue recorded. The effect was so pronounced for kidney tissue that in most cases reliable values could not be determined.

Figure 1 is a chromatogram of cytosol from a plaice naturally high in MT. It can be seen that the peak of MT as measured by the cadmium saturation assay exactly coincides with a peak of zinc. The total MT in this peak corresponds to 273 µg. The zinc content of this peak corresponds to 286 µg of MT if we assume that zinc MT contains 5% by weight of zinc. The peak also contained a small amount of copper, and if present as MT would correspond to an additional 30 µg: cadmium was absent. It is considered unlikely that copper bound to MT would be determined by the assay. The amount of MT applied to the column on

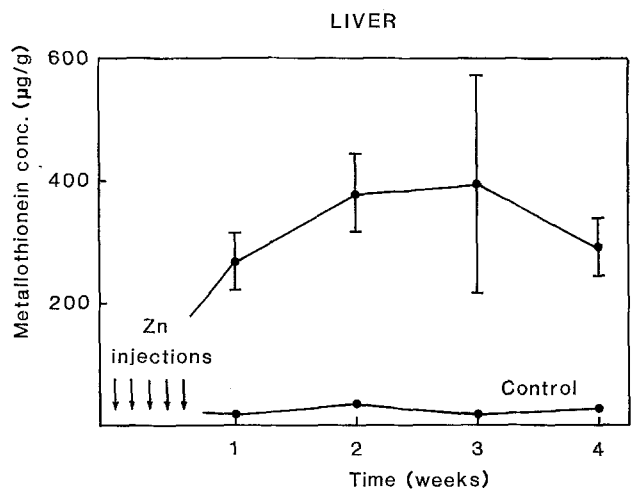

Figure 2. Effect of zinc injection on hepatic metallothionein concentrations. The bars represent standard errors from the means (n = 4,5) and the arrows the times of the daily injections of zinc.

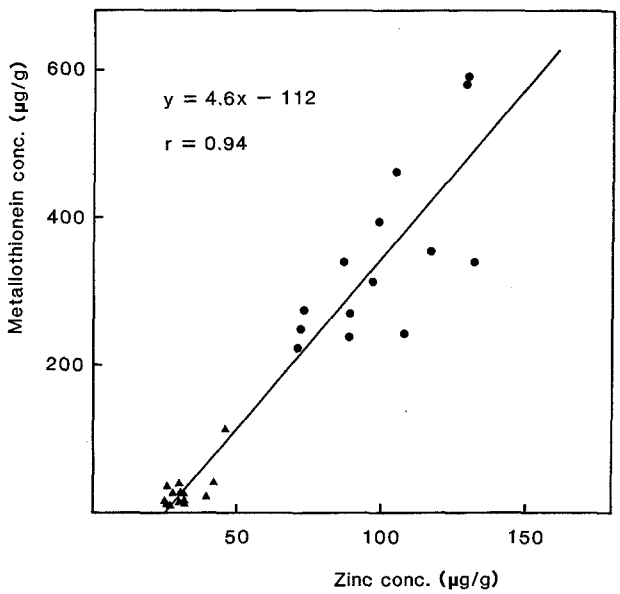

Figure 3. Relationship between zinc and metallothionein concentrations in the livers of control (▲) and zinc injected fish (●). Points represent individual fish from figure 2.

the basis of the assay for MT in the homogenate was 290 µg. These results are consistent with a satisfactory performance of the assay. The MT assay measures the number of equivalents of high affinity, heat stable, cadmium binding sites. The zinc peak and the maximum measured by the MT assay, which coincide in figure 1, are at the elution position of plaice cadmium metallothionein[3]. We are not aware of any reports of shortcomings of the assay due to lack of specificity and with the added information that in our hands the activity corresponds to the molecular weight of MT, we consider that the species being measured is plaice MT. However, to establish this point completely the species corresponding to the assay would need to be isolated and characterized by at least amino acid composition.

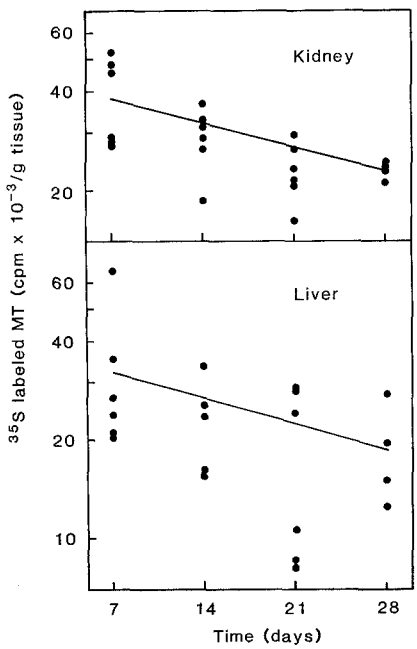

Figure 4. Disappearance of ^{35}S labeled MT from liver and kidney as a function of time after addition of labeled cysteine. The straight line is drawn between the mean values of the first and last time intervals studied.

During the time span studied there was no significant reproducible influence on hepatic MT levels caused by stress due to catching (over 14 days), cortisol (7 days), dexamethasone (7 days), endotoxin (7 days) and turpentine (5 days). Although serum zinc was unaffected, there was a modest increase in serum ceruloplasmin caused by endotoxin, cortisol and dexamethasone.

The effect of i.p. zinc injection on hepatic MT levels is illustrated in figure 2. Although there is considerable individual variation, it can be seen that high levels of MT are induced within a week of the start of injections and that these high levels are maintained for at least four weeks. Concomitant analyses of total zinc in the liver were made and the results are plotted as a graph of total MT *v* total zinc (fig. 3). This graph contains individual data points for both the control and zinc injected fish and may be compared with that reported by Scheuhammer et al.[15]. It can be seen that in plaice more of the zinc is present as non-MT component(s) than is the case for rats; from the slope of the regression we can say that in the case of plaice approximately one quarter of the additional zinc above the threshold value is sequestered as MT whereas in the rat approximately three quarters is so sequestered. All wild plaice livers contained MT, and the levels illustrated in figure 3 were typical, although occasionally very high values were found (unpublished observations).

Although it was felt that the assays for MT in plaice kidney were unreliable, it was found that the assay system for ^{35}S cysteine labeled MT worked satisfactorily for kidney samples. Thus we were able to investigate half-lives for both liver and kidney MT. In early experiments to determine half-lives of rat MT, it was deemed necessary to use a chase of non-radioactive L-cysteine to avoid possible re-utilization of label[16,17], however, more recent measurements have not used such chases and Oh et al.[18] found that a chase of 1000 µmoles/kg had only a small effect on the half-life. In this work we used a chase of 500 µmoles of L-cysteine/kg because we did not know whether it was needed or not. This value was based on the work of J.G. Bell (personal communication) who found a liver concentration of free cysteine of approximately 50 µmoles/kg in rainbow trout.

Figure 4 illustrates the reduction in the hepatic and renal labeled metallothioneins as a function of time. The lines were drawn through the means of the values of the first and last time intervals studied. The illustrated results give values of 26 and 29 days for the half-lives of the hepatic and renal MT. A repeat experiment gave values of 28 and 35 days. The lower molecular weight ^{35}S containing species (presumably cysteine or perhaps glutathione) had half-lives of approximately 9 and 13 days in liver and kidney respectively. The half-lives of the heat denatured, non-MT ^{35}S containing species were too long to measure. Individual variation was similar to that illustrated for ^{35}S MT. Total liver zinc and liver MT assays were performed on the same samples. The MT values showed a greater individual variation, but there was no evidence for significant loss of zinc or total MT during the time period.

It is curious, however, that total MT levels did not apparently fall during the time that the ^{35}S label was falling. This might have been due to the large individual variation of the MT determinations masking any decrease, but it seems more probable that the effect is real and indicates that in contrast to mammals, continuing MT synthesis makes good the MT degradation. This may be related to the much greater pool of non-MT zinc (fig. 3) in fish, compared with mammals, which could serve to act on the gene promoter and continue the induction of the gene.

There have been a number of studies of the half-life of hepatic and renal MT in mammals, although these seem to have been confined to rats. The three metals principally studied have been cadmium, copper, and zinc. In the case of cadmium it has been shown that the protein component turns over with a half-life of approximately 3–3.5 days[13,19]. However, the cadmium is not lost from the cell, but is re-incorporated into freshly synthesized MT so that the level of cadmium declines with a very long half-life.

With copper- and zinc-induced MT the half-lives of the protein are shorter and the metal and protein components of MT decline at the same rate[20,21]. For induction with zinc and the non-metallic inducers, the metal bound to MT is zinc. Reported values for the half-life of the protein component of zinc MT range from 9 h to 3 days[21-23,16-18] and a value of 1 day could probably be considered normal. When investigated, it has been found that the zinc status of the animal affects the half-life of zinc MT. Oh et al.[18] found little difference between the half-lives of hepatic and renal MTs in rats fed basic and zinc supplemented diets (3 days for hepatic and 2 days for renal), but the half-lives were considerably shorter in animals fed the supplemented diet and then returned to the basal diet (1.3 days for hepatic and 1.4 days for renal). Cain and Griffiths[23] reported values of 9.3 h and 14.7 h for the half-lives of rat hepatic MT I and MT II in the case of zinc injected rats and 16.4 and 26.1 h for the half-lives of the same MT iso forms from regenerating liver in non-injected rats. There is little published information on the activation energies for lysosomal protein degradation but if one assumed that the rate of protein degradation doubled for each 10 °C rise in temperature ($Q_{10} = 2$), then a half-life of rat MT of 1 day should correspond approximately to 7 days for the half-life of fish MT at 11 °C. The half-lives found in this study for fish MT are approximately 4 times this figure.

The results reported here indicate that zinc can produce enhanced levels of MT within a week, presumably by stimulating synthesis of fresh MT, and that this level remains high for at least 4 weeks at 11 °C. Fish MT levels could therefore represent a record of zinc exposure during the preceding weeks since we have not been able to show any effects due to stress. However, before conclusions can be drawn from MT levels in wild fish livers we need to know of any seasonal factors and of the influence on MT levels of dietary and water-borne metals.

1 Kägi, J.H.R., and Nordberg, M. (Eds), Metallothionein. Birkhäuser Verlag, Basel 1979.
2 Karin, M., Cell *41* (1985) 9.
3 Overnell, J., and Coombs, T.L., Biochem. J. 183 (1979) 277.
4 Overnell, J., Berger, C., and Wilson, K.J., Biochem. Soc. Trans. *9* (1981) 217.
5 Klaverkamp, J.F., Macdonald, W.A., Duncan, D.A., and Wagemann, R., in: Contaminant Effects on Fisheries, p.99. Eds V.W. Cairns, P.V. Hodson and J.O. Nriagu. John Wiley and Sons, Chichester 1984.
6 Roch, M., McCarter, J.A., Matheson, A.T., Clark, M.J.R., and Olafson, R.W., Can. J. Fish. aquat. Sci. *39* (1982) 1596.
7 Bouquegneau, J.M., Gerday, Ch., and Disteche, A., FEBS lett. *55* (1975) 173.
8 Weis, P., Mar. Envir. Res. *14* (1984) 153.
9 Thomas, D.G., Brown, M.W., Shurben, D., Solbé, J.F. de L.G., Cryer, A., and Kay, J., Comp. Biochem. Physiol. *82C* (1985) 55.
10 Thomas, D.G., Solbé, J.F. de L.G., Kay, J., and Cryer, A., Biochem. biophys. Res. Commun. *110* (1983) 584.
11 Kay, J., Brown, M.W., Cryer, A., Solbé, J.F. de L.G., Shurben, D., Garvey, J.S., and Thomas, D.G., in: Metallothionein and Other Low Molecular Weight Metal Binding Proteins. Ed. J.H.R. Kägi. Birkhäuser Verlag, Basel (1986) in press.
12 Houchin, O.B., Clin. Chem. *4* (1958) 519.
13 Held, D.D., and Hoekstra, W.G., J. Nutr. *114* (1984) 2274.
14 Eaton, D.L., and Toal, B.F., Sci. tot. Envir. *28* (1983) 375.
15 Scheuhammer, A.M., Onosaka, S., Rodgers, K., and Cherian, M.G., Toxicology *36* (1985) 101.
16 Cain, K., and Holt, D.E., Chem.-Biol. Interact. *28* (1979) 91.
17 Shaikh, Z.A., and Smith, J.C., Chem.-Biol. Interact. *15* (1976) 327.
18 Oh, S.H., Deagen, J.T., Whanger, P.D., and Weswig, P.H., Bioinorg. Chem. *8* (1978) 245.
19 Feldman, S.L., Squibb, K.S., and Cousins, R.J., J. Toxic. envir. Hlth *4* (1978) 805.
20 Bremner, I., Hoekstra, W.G., Davies, N.T., and Young, B.W., Biochem. J. *174* (1978) 883.
21 Feldman, S.L., and Cousins, R.J., Biochem. J. *160* (1976) 583.
22 Andersen, R.D., Winter, W.P., Maher, J.J., and Bernstein, I.A., Biochem. J. *174* (1978) 327.
23 Cain, K., and Griffiths, B.L., Biochem. J. *217* (1984) 85.

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Ginkgo biloba extract inhibits oxygen species production generated by phorbol myristate acetate stimulated human leukocytes

J. Pincemail[a], A. Thirion[b], M. Dupuis[a], P. Braquet[c], K. Drieu[c] and C. Deby[a]

[a]Laboratoire de Biochimie et de Radiobiologie, Institut de Chimie, B6, Université de Liège, Sart-Tilman, B-4000 Liège 1 (Belgique), [b]Centre de Transfusion Sanguine de Liège, rue Dos Fanchon, B-4000 Liège (Belgique), and [c]IHB, IPSEN Institute for Therapeutic Research, F-92350 Le Plessis-Robinson (France), 2 December 1985

Summary. A Ginkgo biloba extract (Gbe) containing flavonoids, among other compounds, was tested for the release of activated oxygen species (O_2^-, H_2O_2, $OH^\cdot$) during the stimulation of human neutrophils (PMNs) by a soluble agonist. The extract slows down O_2 consumption (respiratory burst) of stimulated cells by its inhibitory action on NADPH-oxidase, the enzyme responsible for the reduction of O_2 to O_2^-. Consequently, superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) production is significantly decreased when the PMNs stimulation is done in the presence of the extract at concentrations of 500, 250 and 125 µg/ml. Moreover, the hydroxyl radical generation ($OH^\cdot$) is very much decreased at concentrations as low as 15.6 µg Gbe/ml, which indicates that the extract also has free radical scavenging activity. Gbe is able at least to reduce very severely the activity of myeloperoxidase contained in neutrophils. This enzyme, secreted into the intra and extracellular medium, catalyzes the oxidation of chloride (Cl^-) by H_2O_2 to yield strong oxidants (HOCl, chloramines) which are implicated in inflammatory processes.
Key words. Ginkgo; activated leukocytes; oxygen; radical scavenger.

The action of various stimuli (particulate or soluble agonists) on human neutrophils (PMNs) produces a marked increase of enzymatic oxygen consumption (respiratory burst) which results in reduction of O_2 to superoxide (O_2^-) via the NADPH-oxidase system[1,2]. O_2^- rapidly dismutates to yield hydrogen peroxide (H_2O_2); myeloperoxidase contained in neutrophils catalyzes the oxidation of chloride (Cl^-) by H_2O_2 to yield hypochlorous acid (HOCl)[3] and derivatives called chloramines characterized by nitrogen-chlorine (N-Cl) bond[4,5]. H_2O_2 can also generate the hydroxyl radical ($OH^\cdot$) in the presence of iron (Fe^{2+}). Lactoferrin secreted during activation of neutrophils seems to be a natural catalyzer of this reaction[6]. All these substances are toxic