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Antioxidative Constituents in *Heterotheca inuloides*

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Abstract—Sesquiterpenoids, 7-hydroxy-3,4-dihydrocadalin and 7-hydroxycadalin, and flavonoids, quercetin, kaempferol and their glycosides, isolated from *Heterotheca inuloides* (Asteraceae), a Mexican medicinal plant known as "arnica", were evaluated as antioxidants. These compounds showed potent scavenging activity on diphenyl-*p*-picrylhydrazyl (DPPH) radical. Microsomal lipid peroxidation induced by Fe(III)–ADP/NADPH was inhibited by both terpenoids and flavonoids, though only flavonoids possessed superoxide anion scavenging activity in microsome. Flavonoids also scavenged enzymatically and non-enzymatically generated superoxide anion. On the other hand, mitochondrial lipid peroxidation induced by Fe(III)–ADP/NADH was inhibited only by sesquiterpenoids. Furthermore, these terpenes protected mitochondrial enzyme activity against oxidative stress. These results showed that two types of antioxidants existed in the dried flower of *H. inuloides* and would contribute to protection of tissues against various oxidative stresses. © 1997 Elsevier Science Ltd.

Introduction

It is becoming increasingly apparent that the inadvertent overproduction of reactive oxygen species may overwhelm the protective oxidant defenses resulting in oxidative tissue injury.[1] Reactive oxygen species have been implicated in several different diseases including cardiovascular disease, chronic gut inflammation, cancer and AIDS.[2,3] The generated active oxygen is catalyzed by transition metals, such as Fe^{2+} and Cu^{+}, and subsequently leads to lipid peroxidation, resulting in cell death and neoplasia.[4] Lipid peroxidation is a typical free radical oxidation and proceeds via a cyclic chain reaction.[5] The propagation cycle is broken by enzymatic inactivation of oxygen species or by non-enzymatic reactions due to the intervention of free radical scavengers and antioxidants.[6–8] Some antioxidants have been reported to prevent cancer and coronary heart disease,[9] to protect myocardium from experimental myocardial infraction,[10] and to be a prophylactic agent against some neuronal symptoms of aging.[11] A number of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been developed but their use has begun to be restricted because of their toxicity.[12,13] Vitamin E (α-tocopherol) is an effective natural antioxidant but has limited usage.[14] As a result, there is considerable interest in the food industry and in preventative medicine in the development of natural antioxidants from botanical sources.[15,16]

The dried flowers of *H. inuloides* have been used for the treatment of postoperative thrombophlebitis and pathicuse, and externally for acne, bruises and muscle aches in Mexico.[17] Previously, sesquiterpenoids, 7-hydroxy-3,4-dihydrocadalin (**1**) and 7-hydroxycadalin (**2**), were characterized as antibacterial agents from the dried flower of *H. inuloides*.[18] The flavonoids, quercetin (**7**), kaempferol (**10**) and their glycosides (**8**, **9**, **11**) were also isolated from the same source and showed tyrosinase inhibitory activity.[19] In our continuous search for

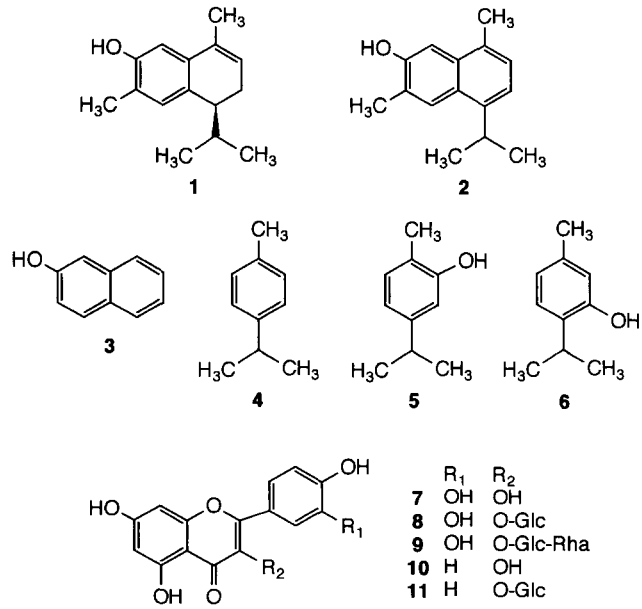

Figure 1. Antioxidative constituents in *H. inuloides* and related compounds.

Table 1. Antioxidative activities of sesquiterpenoids in *H. inuloides* and related compounds

	IC_{50} (μg/mL) [a]			
			Membrane lipid peroxidation	
Compound	Autoxidation	DPPH radical	Mitochondoria	Microsome
1	5.0	4.1	0.65	2.0
2	8.6	1.5	1.55	2.0
3	>30	1.2	>30	>30
4	>30	>30	>30	>30
5	17.1	>30	6.5	4.0
6	16.7	>30	6.9	3.8

[a]Inhibitory activity was expressed as the mean of 50% inhibitory concentration of triplicate determinations, obtained by interpolation of concentration–inhibition curves.

bioactive substances from *H. inuloides*, 7-hydroxy-3,4-dihydrocadalin (**1**) was found to exhibit potent inhibitory activity against animal tissue lipid peroxidation.[20] This report characterizes the antioxidative and superoxide scavenging activity of the constituents of *H. inuloides*.

Results and discussion

Membrane lipids are abundant in unsaturated fatty acids. These unsaturated molecules are most susceptible to oxidative processes,[21] particularly linoleic acid.[22] Thus, this study examined the effect of terpenoids from *H. inuloides* and related compounds on autoxidation of linoleic acid. Sesquiterpenoids **1** and **2** showed potent antioxidative activity against linoleic acid autoxidation; almost 80% inhibition was observed at 10 μg/mL of **1** or **2**. Structurally these sesquiterpenes, especially compound **2**, contain β-napthol (**3**) and *p*-cymene (**4**) moieties. The antioxidative activites of these related compounds were compared. β-Napthol (**3**) and *p*-cymene (**4**) showed almost no effect on linoleic acid autoxidation up to a concentration of 30 μg/mL. Carvacrol (**5**) and thymol (**6**) exhibited antioxidative activity. Their 50 % inhibitory concentrations are shown in Table 1. Lipid peroxidation is a typical free radical oxidation and proceeds via a cyclic chain reaction.[5] The radical scavenging activity of these compounds, which can be measured as decolorizing activity following the trapping of the unpaired electron of diphenyl-*p*-picrylhydrazyl (DPPH), was examined. As shown in Table 1, sesquiterpenoids from *H. inuloides* (**1**, **2**) exhibited potent DPPH radical scavenging activity. β-Napthol (**3**) was also a potent free radical scavenger. On the other hand, *p*-cymene (**4**), carvacrol (**5**) and thymol (**6**) showed no effect on DPPH radical scavenging up to a concentration of 30 μg/mL.

Flavonoids are known to be natural antioxidants and radical scavengers.[23] The radical scavenging activity of flavonoids isolated from *H. inuloides* is shown in Table 2. Quercetin (**7**), kaempferol (**10**) and their glycosides (**8**, **9**, **11**) exhibited potent DPPH radical scavenging activity. It is well established that lipid peroxidation is one of the reactions set into motion as a consequence of the formation of free radicals in cells and tissues. The one-electron reduction products of O_2, superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxy radical (OH·) actively participate in the initiation of lipid peroxidation.[24] Several oxidative enzymes, such as xanthine oxidase, produce the O_2^- radical as a normal product of the one-electron reduction of oxygen, resulting in tissue injury.[24] The scavenging activity of flavonoids from *H. inuloides* against enzymatic-generated superoxide anion by xanthine oxidase system is shown in Table 2. Some of them were found to be potent O_2^- scavengers. The O_2^- scavenging activity of

Table 2. Antioxidative activities of flavonoids isolated from *H. inuloides* and related compounds

	IC_{50} (μg/mL)[a]			
		O_2^- generation		Microsomal lipid peroxidation
Compound	DPPH radical	XOD	Non-enzymatic	
7	0.9	>30	6.6	7.0
8	1.9	5.3	4.1	23.5
9	2.4	7.8	8.1	>30
10	4.2	7.8	7.3	2.6
11	17.2	>30	9.6	>30

[a]Inhibitory activity was expressed as the mean of 50% inhibitory concentration of triplicate determinations, obtained by interpolation of concentration–inhibition curves.

these flavonoids was further confirmed by the non-enzymatic O_2^- generating reaction. All of flavonoids inhibited non-enzymatic O_2^- generation (Table 2). Conversely, sesquiterpenoids from *H. inuloides* showed no inhibition against both enzymatic or non-enzymatic O_2^- generation (data not shown).

Membrane lipids are particularly susceptible to oxidation not only because of their high polyunsaturated fatty acid content but also because of their association in the cell membrane with enzymatic and non-enzymatic systems capable of generating free radical species.[25] Microsomes, especially smooth surfaced endoplasmic reticulum, easily produce lipid peroxides and are thought to supply the peroxidation products to other tissues.[26] NADPH-cytochrome P-450 reductase is involved in NADPH-induced microsomal lipid peroxidation. Lipid peroxidation, which can be measured by the thiobarbituric acid (TBA) method, occurs when rat liver microsomes are incubated with Fe(III)–ADP/NADPH.[27] A common synthetic antioxidant BHT completely inhibited this peroxidation at 10 μg/mL (data not shown). As shown in Table 1, sesquiterpenoids from *H. inuloides* (**1**, **2**) strongly inhibited the production of lipid peroxides induced by microsomal NADPH oxidation; complete inhibition was observed at 10 μg/mL of **1** or **2**. Structure–activity relationship studies showed a similar pattern to linoleic acid autoxidation (Table 1). β-Napthol (**3**) and *p*-cymene (**4**) had no effect; carvacrol (**5**) and thymol (**6**) showed antioxidative activity against microsomal peroxidation. Some flavonoids isolated from *H. inuloides* also showed inhibition of microsomal lipid peroxidation (Table 2). In particular, the flavonols aglycon, quercerin (**7**) and kaempferol (**10**) were potent antioxidants in microsomes. Superoxide anion and hydrogen peroxide can be converted into hydroxy radicals,[4] which result in lipid peroxidation of biological membrane.[28] The cellular sources of superoxide anion in mammalian cells include the microsomal electron transfer chain, entailing a slow electron transfer to O_2 via NADPH-cytochrome P-450 and NADPH-cytochrome b_5 reductase.[29] Succinoylated cytochrome *c* was used for the detection of superoxide radicals produced by liver microsomes incubated with NADPH.[30] A time dependent reduction of modified ferricytochrome *c*, which was superoxide dismutase (SOD)-inhibitable, was observed soon after the addition of NADPH (Figure 2). Flavonoids isolated from *H. inuloides* inhibited this reduction. Figure 2 shows the effect of quercetin (**7**) on the generation of superoxide anion in rat liver microsome. In comparison, sesquiterpenoids **1** and **2**, which are potent antioxidants in microsomes, had no effect.

Redox reactions frequently occur in mitochondria, which are constantly susceptible to oxidative stress.[31] At two sites of the mitochondrial electron transport system, electrons leak and react with oxygen to generate superoxide anions and, subsequently, hydrogen peroxide.[32] Lipid peroxides produced by hydroxy radical (OH·), derived from H_2O_2 and O_2^-, affect mitochondrial function.[33] Lipid peroxidation by submitochondrial particles is supported by NADH or NADPH in the presence of ADP and Fe(III).[34] In the case of BHT, complete inhibition was obtained at 10 μg/mL (data not shown). Flavonoids isolated from *H. inuloides* had no effect on mitochondrial lipid peroxidation up to 30 μg/mL; which differs in the case of microsomes. Sesquiterpenoids from *H. inuloides* (**1**, **2**) showed more potent antioxidative activity against mitochondrial lipid peroxidation than against the peroxidation of microsomes. Complete inhibition was observed at 1 and 3 μg/mL of **1** and **2**, respectively. The 50% inhibitory concentrations of related compounds are also shown in Table 1. Various oxidative stresses affect the mitochondrial enzyme activities.[35] NADH-cytochrome *c* reductase

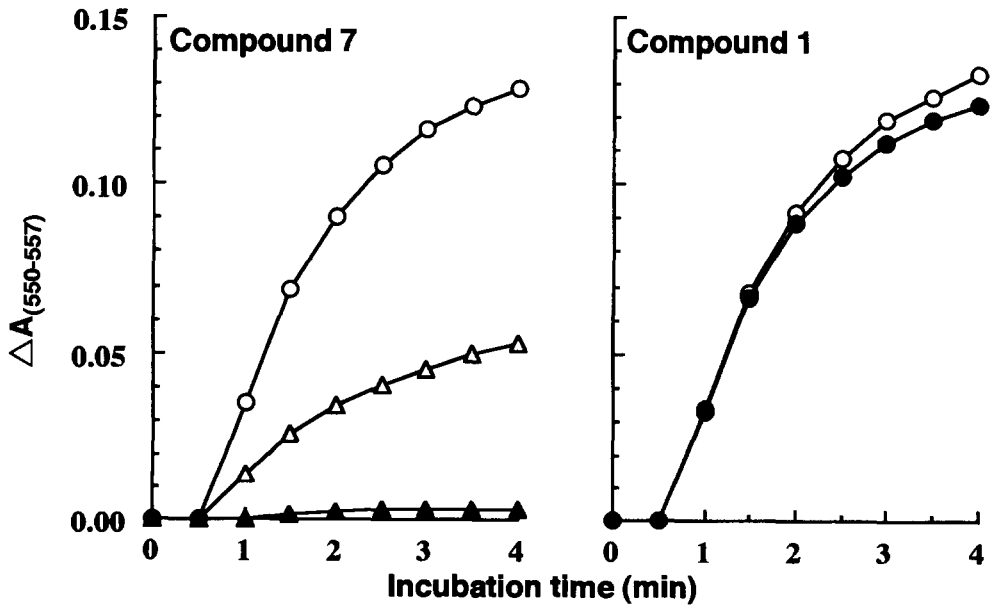

Figure 2. Effects of antioxidative constituents in *H. inuloides* on superoxide anion generation in rat liver microsome. (●) 30 μg/mL, (▲) 10 μg/mL, (△) 3 μg/mL, (○) control.

and succinate cytochrome *c* reductase are the most sensitive sites to mitochondrial peroxidative injury. NADPH-dependent lipid peroxidation in submitochondrial particles results in a remarkable loss of these enzyme activities.[36] When rat liver mitochondria were incubated with Fe(III)–ADP/NADPH, membrane lipid was peroxidized and NADH- and succinate-cytochrome *c* reductase activities were decreased; almost complete loss of activity was observed for 2 and 1.5 hr incubation, respectively. An antioxidative sesquiterpene **1** from *H. inuloides* at 1 μg/mL protected both enzyme activities against NADPH-induced peroxidation.

Many plant secondary metabolites exhibit antioxidative activity,[37] and mechanisms of antioxidant action have been proposed.[38,39] In particular, flavonoids have been well studied for their structure–activity relationships.[40–42] Among the antioxidative compounds in *H. inuloides*, sesquiterpenoids (**1**, **2**) have a hydroxyl group adjoined to a methyl group. This hydroxyl function would contribute to radical scavenging activity because β-napthol (**3**) was a potent DPPH-radical scavenger. Sesquiterpenoids in *H. inuloides* also possess a *p*-cymene moiety, though this particular structure would not directly contribute to their antioxidative activity. Structurally similar compounds, carvacrol (**5**) and thymol (**6**), however, possessed antioxidant activity, and have an electron donating methyl or isopropyl group in the *ortho* position of a phenolic hydroxy function. Phenoxy radicals which would be generated in the process of antiperoxidation may be stabilized not only by their aromatic character, but also by the presence of the electron donating group.[43] The antioxidative materials acting in biological systems are classified as preventive antioxidants and chain-breaking ones.[44] The flavonoids in *H. inuloides* (**7**–**11**) scavenged superoxide anion generated enzymatically or non-enzymatically and in microsome, which would be included in preventive antioxidants. On the other hand, sesquiterpenoids isolated from *H. inuloides* (**1**, **2**) strongly inhibited lipid peroxidation without affecting superoxide radical generation, which would be included in chain-breaking antioxidant.

Present results showed that two types of antioxidants exist in the dried flower of *H. inuloides*. These different effects would contribute to the various pharmaceutical activities of this remedy. The combination antioxidative effect of terpenoids and flavonoids in *H. inuloides* in whole cell system and in vivo are under investigation.

Experimental

7-Hydroxy-3,4-dihydrocadalin (**1**), 7-hydroxycadalin (**2**), quercetin (**7**), quercetin-3-β-glucoside (**8**), quercetin-3-β-rutinoside (**9**), kaempferol (**10**) and kaempferol-3-β-glucoside (**11**) were previously isolated from the dried flower of *H. inuloides*.[18,19] Carvacrol, thymol, *p*-cymene, β-naphthol, xanthine oxidase, cytochrome *c*, BHT, TBA, ADP and α-tocopherol were purchased from Sigma Chemical Co. (St. Louis, MO). NADH and NADPH were obtaind from Oriental Yeast Co., Ltd (Tokyo, Japan). Succinoylated ferricytochrome *c* was prepared according to the method described by Kuthan and Ullrich.[30] Other chemical reagents were of commercial grade.

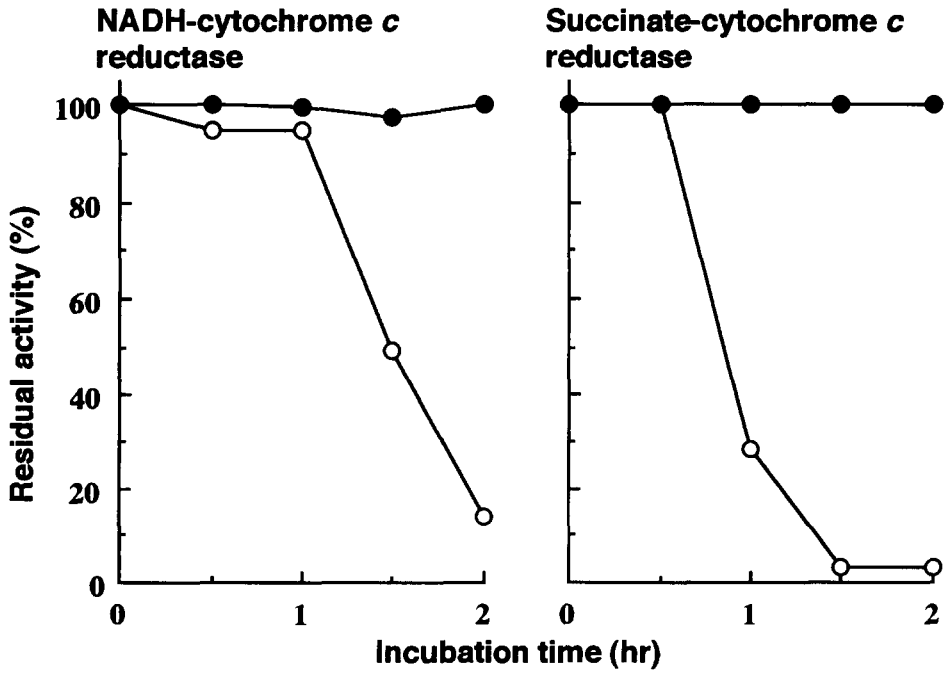

Figure 3. Effects of compound **1** on NADPH-dependent lipid peroxidation of mitochondrial respiratory chain. Each plot is the mean of triplicate determinations. (●) 1 μg/mL, (○) control.

Assay of autoxidation

Oxidation of linoleic acid was measured by the modified method described previously.[45] Different amounts of samples dissolved in 30 μL EtOH were added to a reaction mixture in a screw cap vial. Each reaction mixture consisted of 0.57 mL of 2.51% linoleic acid in EtOH and 2.25 mL of 40 mM phosphate buffer (pH 7.0). The vial was placed in an oven at 40 °C. After 5 days incubation, 0.1 mL aliquot of the mixture was diluted with 9.7 ml of 75% EtOH, which was followed by adding 0.1 mL of 30% ammonium thiocyanate. Precisely 3 min after the addition of 0.1 mL of 20 mM ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance at 500 nm was measured.

Radical scavenging activity on DPPH

The reaction mixture consisted of 1 mL of 100 mM acetate buffer (pH 5.5), 1 ml of ethanol and 0.5 mL of 2.5 mM ethanolic solution of diphenyl-*p*-picrylhydrazyl (DPPH). After allowing the mixture to stand at room temperature for 20 min, the absorbance of the remaining DPPH was determined colorimetrically at 517 nm. The scavenging activity was measured as the decrease in absorbance of the DPPH expressed as a percentage of the absorbance of a control DPPH solution.[46]

Assay of superoxide anion

Superoxide anion was generated enzymatically by the xanthine oxidase system. The reaction mixture consisted of 40 mM sodium carbonate buffer (pH 10.2) containing 0.1 mM xanthine, 0.1 mM EDTA, 50 μg/mL bovine serum albumin, 25 mM nitroblue tetrazolium, and 3.3×10^{-3} units xanthine oxidase (XOD, EC 1.2.3.2) in a final volume of 3 mL. After incubation at 25 °C for 20 min, the reaction was terminated by the addition of 0.1 mL of 6 mM $CuCl_2$. The absorbance of formazan produced was determined at 560 nm.[47]

The non-enzymatic generation of superoxide anion was measured in the mixture composed of 10 μM phenazine methosulfate, 78 μM NADH, 25 μM NBT and 0.1 M phosphate buffer (pH 7.4). After 2 min of incubation at room temperature, the absorbance at 560 nm was measured.[48]

Preparation of mitochondria and microsomes

Livers of Wistar male rats weighing 100–150 g were removed quickly and dropped into ice-cold 3 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 0.1 mM EDTA. Mitochondria were obtained as a pellet after centrifuge at 15,000 *g* according to the method of Johnson and Lardy,[49] and then resuspended in 100 mM HEPES buffer (pH 7.2). Submitochondrial particles were prepared by sonication[50] of mitochondrial suspension for 1 min at 4 °C using a Model 450 Sonifier (Branson Ultrasonics Corporation, U.S.A). Microsomes were obtained as a pellet after centrifuge at 105,000 *g* for 60 min,[51] and then the pellet was resuspended in the buffer containing 70 mM sucrose, 0.21 M mannitol, 0.1 mM EDTA and 3 mM HEPES (pH 7.4).

Protein concentrations of the suspensions were determined by the method of Lowry et al.[52]

Measurement of lipid peroxidation

The NADPH-dependent peroxidation of microsomal lipid was assayed by the modified method described by Pederson et al.[27] Rat liver microsomes (equivalent 0.2 mg protein) were incubated at 37 °C in 1 mL of reaction mixture containing 0.05 M Tris-HCl (pH 7.5), 2 mM ADP, 0.12 mM $Fe(NO_3)_3$, and 0.1 mM NADPH. The reaction was initiated by the addition of NADPH. After 5 min, 2 mL of TCA–TBA–HCl reagent (15 % w/v trichloroacetic acid; 0.375 % thiobarbituric acid; 0.25 N HCl) and 90 μL of 2 % BHT were added to the reaction mixture. The solution was heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 *g* for 10 min. The absorbance of thiobarbituric acid (TBA) reactive substances in the supernatant was determined at 535 nm.[53]

Mitochondrial lipid peroxidation was assayed by the modified method described by Takayanagi et al.[34] Rat liver submitochondrial particles (equivalent 0.3 mg protein) were incubated at 37 °C in 1 mL of reaction mixture containing 50 mM HEPES–NaOH (pH 7.0), 2 mM ADP, 0.1 mM $FeCl_3$, 10 μM rotenone and 0.1 mM NADH. The reaction was initiated by the addition of NADH. After 5 min, the reaction was terminated and lipid peroxidation was determined by the same TBA method as for the microsomal peroxidation.

Detection of superoxide anion in microsome

Rat liver microsomes were diluted to a final concentration of 0.4 mg/mL in 0.1 M Tris buffer (pH 7.7) containing 0.1 mM EDTA and 30 μM succinoylated ferricytochrome *c*.[30] The reaction mixtures were incubated at 37 °C, and after 30 s, NADPH at 0.2 mM was added. The reduction of succinoylated cytochrome *c* was monitored using the wavelength-pair 550–557 nm[30] in a MPS-2000 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) equipped with a TCC temperature controller. The addition of an excess of SOD allowed to differentiate between O_2^- mediated and direct enzymic reduction of cytochrome *c*.

Mitochondrial peroxidation and assay for enzyme activity

NADPH-dependent peroxidation of rat liver submitochondrial particles were achieved in a medium contain-

ing 0.1 M mannitol, 5 mM potassium phosphate (pH 7.4), 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM ADP and 0.3 mM $FeCl_3$ at 25 °C.[54] The reaction was started by the addition of 0.5 mM NADPH. At intervals during incubation, mitochondrial suspensions were taken out from the mixture and NADH–cytochrome *c* reductase and succinate–cytochrome *c* reductase activities were measured.

The reductase activity was assayed by measuring the increase in the absorbance at 550 nm resulting from the reduction of cytochrome *c*. The reaction mixtures contained 50 mM potassium phosphate buffer (pH 7.4), 5 mM NaN_3, 2.1 mg of oxidized cytochrome *c* and 200 μM NADH or 20 mM sodium succinate in a total volume of 3 mL.[55]

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