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Mechanism of Biochemical Action of Substituted 4-Methylbenzopyran-2-ones. Part 3: A Novel Mechanism for the Inhibition of Biological Membrane Lipid Peroxidation by Dioxygenated 4-Methylcoumarins Mediated by the Formation of a Stable ADP-Fe-Inhibitor Mixed Ligand Complex

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Abstract—7,8-Dihydroxy-4-methylcoumarin (DHMC) and 7,8-diacetoxy-4-methylcoumarin (DAMC) have been reported to effectively inhibit in-vivo lipid peroxidation in rat tissues induced by CCl_4 and paraquat. DHMC was found to readily impart green colour to the lipid peroxidation incubation mixture containing ADP and Fe^{3+}, whereas DAMC formed green complex only upon incubation with liver microsomes, confirming our earlier observation that liver microsomal deacetylase hydrolyses DAMC to DHMC. Sensitive pH metric technique revealed the formation of ADP-Fe-DHMC ternary complex with highest stability, while Fe-DHMC and ADP-DHMC had negligible stabilities concluding that ADP-perferryl ion formation is prevented by DHMC resulting in the production of stable ternary mixed ligand complex (ADP-Fe-DHMC), thereby inhibiting the formation of $O_2^{-\cdot}$, and eventually other reactive oxygen species (ROS) responsible for membrane lipid peroxidation.

Introduction

Lipid peroxidation initiated by oxygen radicals eventually results in membrane degradation and cell death.[1] The role of oxidative processes involving free radicals in the pathogenesis of various diseases including cancer and vascular diseases are now well established.[2] Antioxidants are thought as remedies to overcome the lethal action of oxygen free radicals.[3] Intensive efforts are being made to discover newer antioxidants with greater efficiency to intercept the processes of oxidative stress. Our earlier work[4] described dioxygenated 4-methylcoumarins (4-methylbenzopyran-2-ones) as superb antioxidants and radical scavenging agents and also hypothesised the possibility of a chromophoric mixed ligand complex involving 4-methylcoumarins to mediate the inhibition of membrane lipid peroxidation. In this paper, experimental results are reported substantiating such a viewpoint, and demonstrating the formation of a stable coordination complex involving ADP, Fe and the dioxygenated 4-methylcoumarin (ligand) viz. 7,8-dihydroxy-4-methylcoumarin (DHMC).

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Materials and Methods

Chemicals

NADPH, ADP, trichloroacetic acid (TCA), xanthine, xanthine oxidase and cytochrome C were obtained from Sisco Research Laboratory (Mumbai, India). Tris, $FeCl_3$, thiobarbituric acid (TBA), dimethyl sulfoxide (DMSO), HCl, CCl_4, KCl, acetic acid, NaOH, n-butanol and pyridine of high purity were procured from local suppliers. Paraquat was procured from Sigma Chemical Co., St. Louis, MO (USA). 7,8-Diacetoxy-4-methylcoumarin (DAMC) and 7,8-dihydroxy-4-methylcoumarin (DHMC) were synthesised in our laboratory by the well-known Pechmann condensation.[5]

Animals

Male rats of Wistar strain weighing around 190–200 gm, fed on rat chow supplied by Hindustan Lever Ltd. (Mumbai, India) were used.

Assay for inhibition of lipid peroxidation

The details of the assay procedure are described in our earlier communication.[4] In short, the reaction mixture in a final volume of 2 mL consisted of 0.025 M tris-HCl (pH 7.5), liver microsomes (1 mg protein), 3 mM ADP and 0.15 mM $FeCl_3$. The tubes were preincubated for 10 min at 37 °C followed by the addition of the test compounds (where indicated) at a concentration of 100 μM in 0.2 mL of DMSO and then again incubated for 10 min at 37 °C. The reaction was started by the addition of 0.5 mM NADPH for initiation of enzymatic lipid peroxidation and incubated for 10 min at 37 °C. The reaction was terminated by the addition of 0.2 mL of 50% TCA, followed by the addition of 0.2 mL of 5 N HCl and 1.6 mL of 30% TCA and 2.0 mL of 0.8% TBA. The tubes were heated in an oil bath at 95 °C for 30 min, cooled and centrifuged at 3000 rpm. The intensity of colour of thiobarbituric acid reacting substance (TBARS) formed was measured at 535 nm and calculated using the extinction coefficient, $1.56\times10^5\,M^{-1}\,cm^{-1}$. The lipid peroxidation was found to be linear up to 15 min under the conditions described here.

IC_{50} value

The different concentrations of inhibitor ranging from 0.01 to 100 μM were incubated as described above to calculate the inhibitor concentration for 50% inhibition (IC_{50}).[6]

UV spectrum of green complex

DHMC (100 μM) was incubated at 37 °C with 0.025 M tris-HCl (pH 7.5), 3 mM ADP and 0.15 mM $FeCl_3$. The reaction mixture was transferred to spectrophotometer cuvette and scanned against reagent blank.

Superoxide scavenging activity

The reaction mixture consisted of 50 mM phosphate buffer (pH 7.4), 2.5 mM xanthine, 0.3 mM cytochrome C and 100 μM test compound (where indicated) in a final volume of 3.0 mL. The reaction was initiated by the addition of 0.3 units of xanthine oxidase and superoxide radical production was quantitated by measuring the reduction in the absorption of cytochrome C at 550 nm.

Carbon tetrachloride-induced hepatic lipid peroxidation

The rats were separately injected ip DAMC (300 mg/kg b.w.) or DHMC (200 mg/kg b.w.) in 0.2 mL DMSO, 30 min later animals received another dose of DAMC or DHMC with 0.25 mL CCl_4. The animals were sacrificed 1, 2 and 3 hr after the injection of carbon tetrachloride, liver excised and 10% tissue homogenate was prepared in 0.15% KCl. To a sample of 0.2 mL of tissue homogenate, 0.2 mL of 8.1% SDS, 1.5 mL of 20% acetic acid solution (adjusted to pH 3.5 with NaOH) and 1.5 mL of 0.8% aqueous solution of thiobarbituric acid were added. The mixture was made up to 4 mL with water and then heated at 95 °C in an oil bath for 1 h. After cooling the tubes, 5 mL of a mixture of n-butanol and pyridine (15:1, v/v) was added and the contents were shaken vigorously followed by centrifugation at 4000 rpm. The organic layer was taken and its absorbance due to TBARS was recorded at 532 nm against the proper reagent blank.

Paraquat-induced hepatic and pulmonary lipid peroxidation

The rats were separately injected ip DAMC (300 mg/kg b.w.) or DHMC (200 mg/kg b.w.) in 0.2 mL DMSO, 30 min later animals received another dose of DAMC or DHMC along with paraquat (100 mg/kg b.w.). The animals were sacrificed 2 h after the paraquat injection, liver and lung were excised, tissue homogenate was prepared and TBARS formed was measured as described for CCl_4 treatment described above.

Chemical speciation and formation constants studies on Fe(III), ADP and 4-methylcoumarin complexes

pH-metric technique is a robust and versatile way of measuring ionization and distribution of drugs in biological fluids,[7] and assessing their interaction with trace metal ions. pH-metric titrations have been carried out with the digital radiometer (Copenhagen) PHM83 with a combined glass electrode. Glass electrode was calibrated before the titrations as described by Martell and

Motekaitis.[8] The AUTOCAL pH-meter was calibrated over the pH range of 0 to 14 before performing the titrations to read the pH directly. To ensure constant ionic strength (0.1 M) during the titrations, an inert electrolyte, sodium perchlorate, $NaClO_4$ (Fluka) was added in requisite amounts. A solution of tetramethylammonium hydroxide (TMAH) (E. Merck) in DMF/water was used as the titrant. Metal ion solution was prepared from Analar (BDH) samples of the corresponding chloride and was standardised by the conventional methods as described by Vogel.[9]

The titrations were performed in a covered glass-jacketed titration cell under a stream of presaturated nitrogen. All measurements were made in 80% DMF/water medium at 25 °C (±0.5 °C) maintained constant by using Julabo VC type thermostat. Solution concentrations of ligand in the presence and absence of metal ions were in the order of 10^{-3} M. Stepwise dissociation constants within range of the potentiometric titrations (up to pH 12.0) were calculated by fitting the pH data with the help of the program PKAS.[8] Formation constants of the complexes were determined by direct potentiometric titration using the program BEST. Correction factors[10] were applied for the glass electrode in aqueous N,N-dimethylformamide solutions. A Pentium computer was used for computing the results.

Results

The present study has concentrated on the mechanism of antioxidant action of the two dioxygenated 4-methylcoumarins, namely DHMC and DAMC. The results tabulated in Table 1 highlight the remarkable capability of DHMC and DAMC to inhibit the initiation of microsomal lipid peroxidation, they inhibit to the extent of 87 and 89%, respectively. These compounds exhibit significantly low IC_{50} values as compared to vitamin E.

Table 1. Inhibition of NADPH-dependent lipid peroxidation in rat liver microsomes by dioxygenated 4-methylcoumarins

Inhibitor	Microsomal lipid peroxidation	IC_{50} (nM)
	nmol TBARS formed/mg protein	
—	8.81	—
DHMC	1.14	7,900
DAMC	0.97	250
4-Methylesculetin	4.61	32,000
Vitamin E	4.72	31,620

The values represent mean of 10 different experiments with variation < 5%. The details are described under Materials and Methods.

It is observed that the dioxygenated 4-methylcoumarins when added to the liver microsomal lipid peroxidation incubation mixture imparted green colour. The data shown in Table 2 clearly indicates that the dihydroxy compound (DHMC) readily imparts green colour to the incubation mixture, while the diacetoxy compound becomes chromophoric only after incubation with the rat liver microsomes. Chromophoric complex exhibited λ_{max} at 600 nm, typical of green coloured constituent (Fig. 1). The fact that DAMC imparted green colour only upon incubation with liver microsomes points out the role of microsomal enzyme in the hydrolysis of DAMC. It is thus evident that DAMC forms the chromophoric complex (Table 2) and also contributes to the inhibition of microsomal lipid peroxidation. It is worthwhile to note that Vitamin E imparts no green colour. We have employed the versatile pH metric technique to study the interactions of DAMC and DHMC with Fe(II) and Fe(III).

Table 2. Specificity for the formation of green coloured coordination complex

Principal compounds of reaction mixture			Formation of green complex when added separately		
ADP	Fe^{3+}	Rat liver microsomes (RLM)	DAMC	DHMC	Vitamin E
+	+	–	Nil	Intense	Nil
+	+	+	Intense[a]	Intense[b]	Nil

The reaction mixture includes phosphate buffer, ADP, Fe^{3+}, test compound (100 μM) and liver microsomes (where indicated).

[a] The intensity of colour deepened with time.

[b] Intense colour developed rapidly.

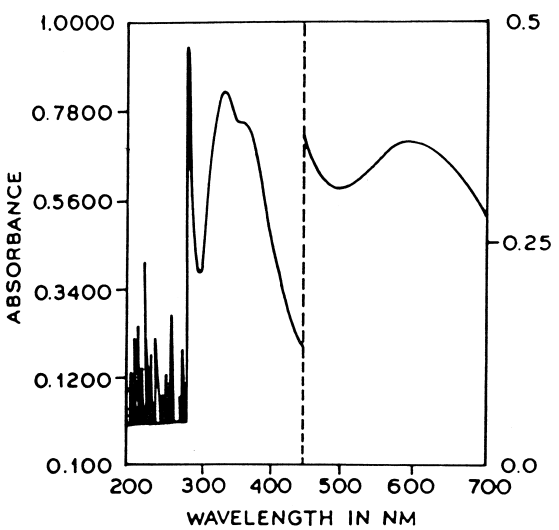

Figure 1. Chromophoric complex exhibited λ_{max} typical of green coloured complex.

The results in Table 3 include pH metric titration data for the calculation of protonation constants of DHMC which are found to be 9.50 and 5.87 using PKAS program.[8] Throughout this investigation, the function minimized is the weighted average of the sums of the squares of deviation between calculated and observed pH values and σ_{fit}. The pH metric titration data for the calculation of overall equilibrium constant for Fe(III)-7,8-dihydroxy-4-methylcoumarin complex is shown in Table 4. The overall equilibrium constant for Fe(III)-DHMC-ADP mixed ligand complex as determined by pH metric titration data is documented in Table 5. Potentiometric equilibrium measurements data of the ligand solution in the absence and presence of metal ion is tabulated in Table 6. All proton association constants of DHMC and formation constants of the 1:1 and 1:2 metal ligand complexes were obtained with the program BEST.[11]

The results described so far confirm the ability of DHMC and DAMC as superb inhibitors of lipid peroxidation in vitro. The data included in Table 7 highlights the superoxide scavenging capacity of DHMC and DAMC. Superoxide radical generated during the action of xanthine oxidase on xanthine reduces the cytochrome C yielding intense absorption at 550 nm. The superoxide scavenging activity was based on inhibition of the reduction of cytochrome C. Accordingly DAMC and DHMC scavenged the superoxide radical to the extent of 3.4- and 4.3- folds, respectively (Table 7).

The results documented in Tables 8 and 9 reveal the potential of DAMC and DHMC to inhibit lipid peroxidation in tissues of rat in vivo. The administration of DAMC/DHMC caused around 70% inhibition of hepatic lipid peroxidation 1–2 hrs after CCl_4 treatment (Table 8). Effect of pretreatment of rats with DAMC/DHMC on paraquat-induced tissue lipid preoxidation is shown in Table 9. Both DAMC and DHMC could

Table 3. Titration data for calculation of protonation constants of 7,8-dihydroxy-4-methylcoumarin

ml (TMAH)	pH	ml (TMAH)	pH
0.0	3.119	1.3	9.839
0.1	3.269	1.4	9.899
0.2	3.599	1.5	9.929
0.3	5.559	1.6	9.999
0.4	6.569	1.7	10.059
0.5	7.189	1.8	10.099
0.6	8.319	1.9	10.149
0.7	8.959	2.0	10.169
0.8	9.229	2.1	10.219
0.9	9.419	2.2	10.249
1.0	9.549	2.3	10.279
1.1	9.669	2.4	10.299
1.2	9.759	2.5	10.329

$pK_1 = 9.50$ $pK_2 = 5.87$ $\sigma_{fit} = 0.0000$.

Table includes pH-metric titration data for the calculation of protonation constants which are found to be 9.50 and 5.87.

Throughout this investigation the function minimized is the weighted average of the sums of the squares of deviation between calculated and observed pH values, σ_{fit}.

Table 4. Titration data for Fe(III)-7,8-dihydroxy-4-methylcoumarin complex

mL (TMAH)	pH	mL (TMAH)	pH
0.0	3.56	2.0	10.02
0.1	3.60	2.1	10.08
0.2	3.72	2.2	10.15
0.3	3.90	2.3	10.19
0.4	4.30	2.4	10.23
0.5	4.85	2.5	10.28
0.6	5.95	2.6	10.31
0.7	6.34	2.7	10.36
0.8	6.59	2.8	10.39
0.9	6.82	2.9	10.47
1.0	7.07		
1.1	7.29		
1.2	7.61		
1.3	8.22		
1.4	8.92		
1.5	9.34		
1.6	9.56		
1.7	9.73		
1.8	9.85		
1.9	9.95		

pH-metric titration data for the calculation of overall equilibrium constant for Fe(III)-7,8-dihydroxy-4-methylcoumarin complex.

Table 5. Titration data for Fe(III)-DHMC-ADP complex

mL (TMAH)	pH	mL (TMAH)	pH
0.0	3.58	1.6	9.76
0.1	3.66	1.7	9.93
0.2	3.81	1.8	10.00
0.3	4.14	1.9	10.08
0.4	4.80	2.0	10.14
0.5	5.35	2.1	10.21
0.6	5.72	2.2	10.30
0.7	6.06	2.3	10.35
0.8	6.37	2.4	10.39
0.9	6.75	2.5	10.43
1.0	7.36	2.6	10.45
1.1	8.07	2.7	10.48
1.2	8.75	2.8	10.51
1.3	9.26	2.9	10.53
1.4	9.40		
1.5	9.53		

The pH-metric titration data for the calculation of overall equilibrium constant for Fe(III)-DHMC-ADP mixed ligand complex.

reduce liver lipid peroxidation in vivo by 28 and 23%, respectively. The compounds were less effective in inhibiting lipid peroxidation in pulmonary tissue.

Discussion

The initiation of liver microsomal lipid peroxidation in vitro is accomplished by the reduction of ADP-chelated iron by NADPH-linked cytochrome P-450 reductase.[12] The oxenoid moiety of the ADP-perferryl ion is eventually responsible for the peroxidative process. Several classes of compounds known as 'antioxidants' are able to halt the membrane lipid peroxidation.[13] Among them plant phenolics demonstrate remarkable antioxidant activity.[14–16] In the present work, the attention is focussed on dioxygenated 4-methylcoumarins, DHMC and DAMC which exhibit superb antioxidant activity (Table 1). DAMC is nearly 126 times more potent than vitamin E in effecting the inhibition of NADPH dependant liver microsomal lipid peroxidation (Table 1). 6,7-Dihydroxy-4-methylcoumarin (commercially available as 4-methylesculetin) was found to be an inferior antioxidant compared to DHMC, the former having much higher IC_{50} value than the latter. For this reason, attention is focussed on 7,8-dioxygenated 4-methylcoumarins in this paper. The polyphenolic structure of DHMC facilitates the inhibition of lipid peroxidation by scavenging of free radicals or involving chelation of transition metals[4] in some manner. The formation of chromophoric complex (Table 2) provided some clues regarding the nature of the interaction between the three main constituents catalysing NADPH-dependent membrane lipid peroxidation, namely ADP, Fe(III) ion and the ligand (the inhibitor DHMC). The ternary combination of the three moieties mentioned above could only impart the green colour.

During the pH metric titration, the function minimized was the σ_{fit}. The data included in Figure 2 describes the percentage distribution of ligand (DHMC) among different forms (H_2L, HL and L) as a function of pH. Species distribution curve (Fig. 3) indicates very little (=5%) formation of Fe(III)-DHMC complex, 100% formation of ADP-DHMC-Fe(III) mixed ligand complex at biological pH values is clear from the species distribution curve (Fig. 4). The Fe^{2+} ion was also found to form the mixed ligand of maximum stability with ADP and DHMC in a manner similar to Fe^{3+} ion (data not shown). It is evident from these observations that

Table 6. Overall equilibrium constants for Fe(III)-DHMC and Fe(III)-DHMC-ADP complex

	Log β_{HL}	Log βH_2L	Log β_{ML}	Log βML_2	σ_{fit}
Fe(III)-DHMC	9.5000	15.3700	4.1752	7.2752	0.06514
Fe(III)-DHMC -ADP			7.7930		0.05474

$\beta_{HL}=[HL]/[H^+][L]$; $\beta_{H2L}=[H_2L]/[H^+]^2[L]$; $\beta_{ML}=[ML]/[M][L]$; $\beta ML_2=[ML_2]/[M][L]^2$

Potentiometric equilibrium measurement data of the ligand solutions in the absence and presence of metal ion.

All proton association constants of DHMC and formation constants of the 1:1 and 1:2 metal-ligand complexes were obtained with the program BEST with the aid of Pentium Computer. Throughout this investigation, the function minimized is the weighted average of the sums of the squares of deviations between calculated and observed pH values, σ_{fit}.

Table 7. Scavenging of superoxide radical by DAMC/DHMC

	Cytochrome C reduced (nmol/min)	Superoxide scavenging (no. of folds)
Control	8.19	0
DAMC	2.38	3.44
DHMC	1.90	4.31

Superoxide radical generated during the reaction catalysed by xanthine oxidase is utilised for the assay of scavenging action of the compounds. Details are given under Materials and Methods. The values expressed are mean of three different experiments with variation < 5%.

Table 8. Inhibition of CCl_4-induced rat liver lipid peroxidation in vivo by DAMC/DHMC

Compound administered	CCl_4 treatment (h)	Inhibition of lipid peroxidation (%)
DAMC	1	72
	2	68
	3	40
DHMC	1	71
	2	68
	3	47
α-Tocopherol	1	21
	2	18

The values are mean of three observations with variation < 5%.

Table 9. Inhibition of paraquat-induced lipid peroxidation in rat tissues by DAMC/DHMC

Tissue	Inhibition of tissue lipid peroxidation (%)	
	DAMC	DHMC
Liver	28	23
Lung	15	21

The values are mean of three observations with variation < 5%.

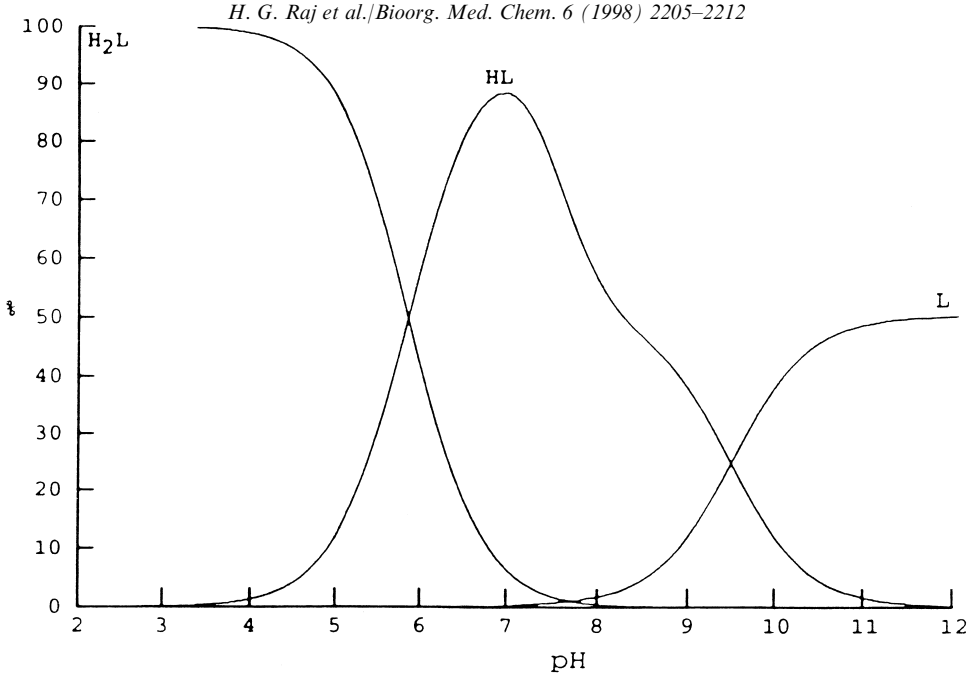

Figure 2. Species distribution curve of 7,8-diydroxy-4-methylcoumarin indicating ionization as a function of pH. The figure describes the percentage distribution of ligand (DHMC) among its different forms (H_2L, HL and L) versus pH.

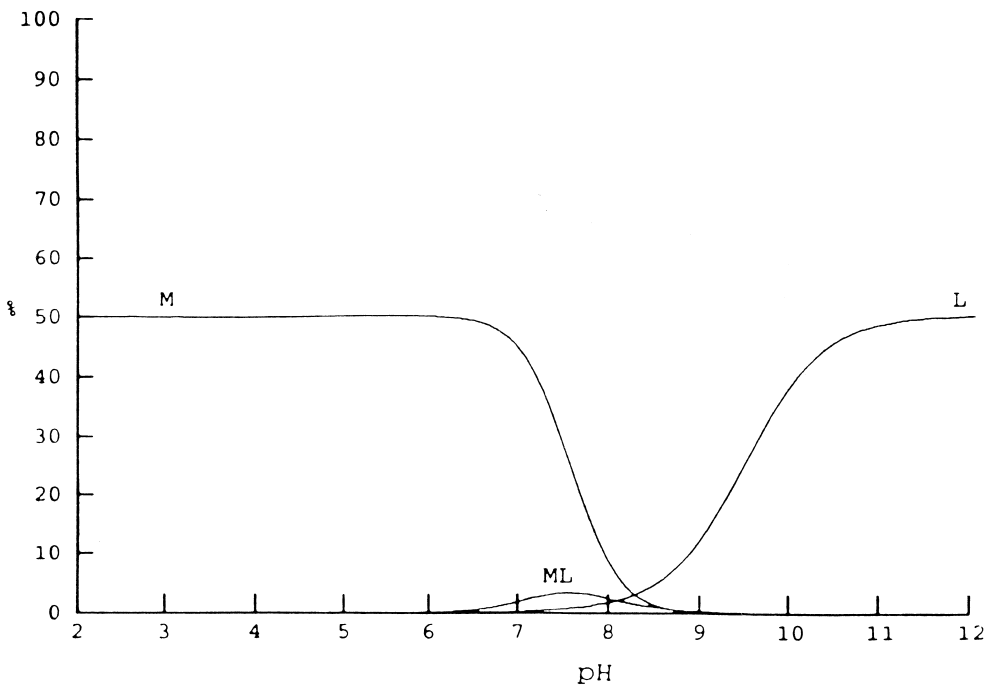

Figure 3. Species distribution of Fe(III) -DHMC as a function of pH. The figure describes the percentage distribution of Fe(III) among different complexes versus pH in the Fe(III)-DHMC system. This species distribution curve indicates very little (approx. 5%) formation of Fe(III) -DHMC complex.

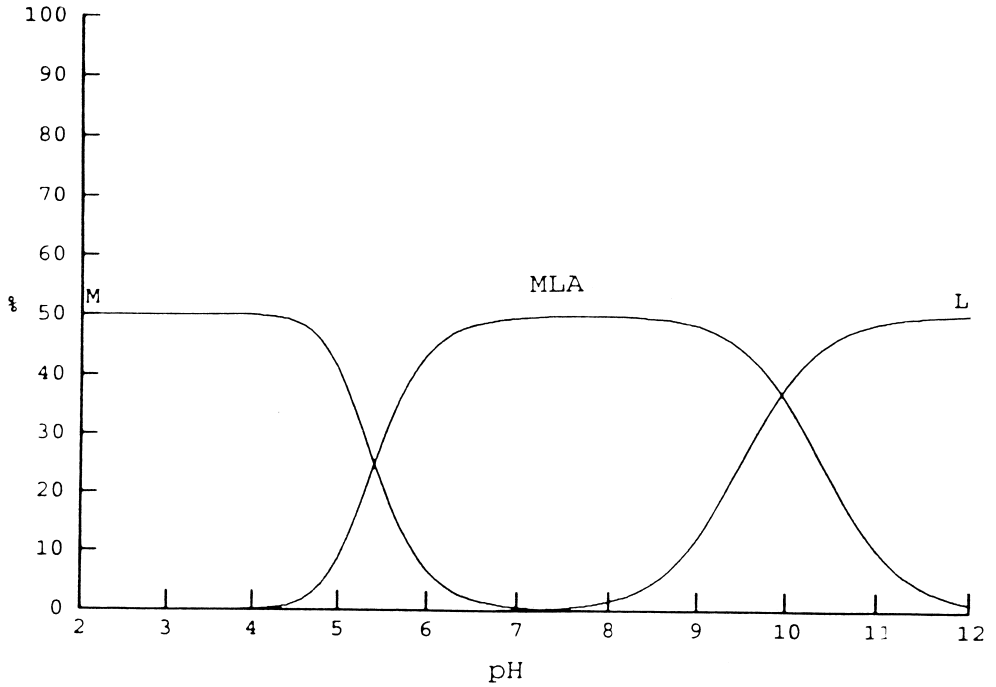

Figure 4. Species distribution of Fe(III) -DHMC -ADP as a function of pH. The figure describes the percentage distribution of Fe(III) among different complexes versus pH in the Fe(III)-DHMC-ADP system. This curve indicates 100% formation of Fe(III)-DHMC-ADP mixed ligand complex at biological pH value.

the ternary coordination complex ADP-Fe-DHMC has the maximum stability. It is conceivable that the formation of ADP-Fe-DHMC can dislodge the molecular oxygen from forming the ADP-perferryl and thereby prevents the formation of O^{-}_{2} (Fig. 5) and ultimately the other reactive oxygen species (ROS). Although chelation of transition metal by antioxidant ligands has been considered a possibility for inhibition of membrane lipid peroxidation,[15–17] no report has so far envisaged the formation of ADP-Fe-antioxidant (ligand). We have shown for the first time that Fe-antioxidant (ligand) has insignificant stability and hence of no consequence in the prevention of the formation of ADP-perferryl leading to the generation of ROS. Although ADP-Fe-DHMC formation is shown to be greatly inhibitory for the lipid peroxidation process (Table 1), one may still wonder whether ADP-Fe-DHMC can activate the molecular oxygen. The data included in Table 10 convincingly indicated that preformed ADP-Fe^{2+}-DHMC/ ADP-Fe^{3+}-DHMC when acted upon with rat liver microsomes and NADPH failed to initiate lipid peroxidation. It can thus be concluded that ADP-Fe-DHMC is a stable complex devoid of releasing free Fe and can hardly activate the molecular oxygen. It is also interesting to note that the unstable Fe^{2+}-DHMC/Fe^{3+}-DHMC (Fig. 4) at once forms the stable ADP-Fe-DHMC mixed ligand complex in the presence of added ADP and halts lipid peroxidation (Table 10). Formation

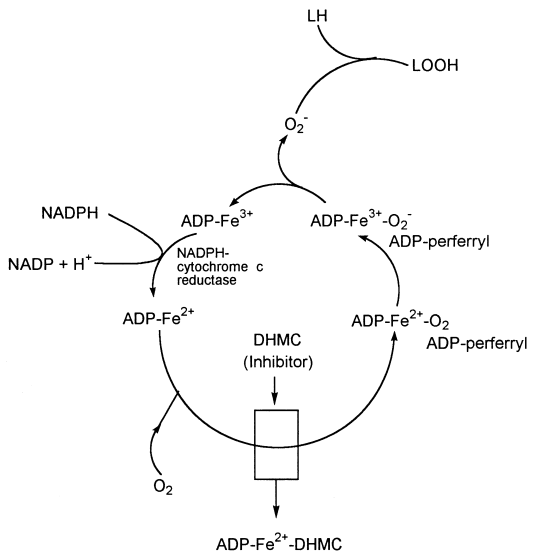

Figure 5. Inhibition of NADPH-dependent microsomal lipid peroxidation by DHMC.

Table 10. Inhibition of lipid peroxidation by ADP-Fe-DHMC mixed ligand complex

Tube no.	Reaction mixture	Lipid peroxidation (nmole TBARS formed/mg protein)
1	ADP-Fe^{3+} + LM + NADPH	6.85
2	ADP + Fe^{3+} + LM + NADPH	6.61
3	ADP-Fe^{3+}-DHMC + LM + NADPH	0.11
4	ADP-Fe^{3+}-DHMC + ADP + LM + NADPH	0.15
5	ADP + Fe^{2+} + LM + NADPH	3.27
6	ADP-Fe^{2+}-DHMC + LM + NADPH	0.0
7	ADP-Fe^{2+}-DHMC + ADP + LM + NADPH	0.0
8	Fe^{3+}-DHMC + ADP + LM + NADPH	0.15
9	Fe^{2+}-DHMC + ADP + LM + NADPH	0.0

LM: Rat liver microsomes.

The complexes ADP-Fe^{3+}-DHMC, ADP-Fe^{2+}-DHMC, ADP-Fe^{2+}, ADP-Fe^{3+}, Fe^{3+}-DHMC and Fe^{2+}-DHMC were preformed by mixing of particular components and kept aside at 37 °C for 10 mins. Later the other components were added to the respective tubes as indicated above and the contents incubated for 10 min at 37 °C. In the case of tube Nos. 2 and 5, the components were mixed and immediately incubated for 10 min at 37 °C.

The concentration of the components of the reaction mixture and assay of TBARS were as mentioned under Materials and Methods.

The values represent average of three separate experiments.

of ADP-Fe-antioxidant (ligand) could assume a crucial role in the prevention of ROS, while the other mechanism, viz. the radical scavenging (Table 7), repair of the damage of biomolecules caused by ROS, etc.[3] also play an important part. Earlier studies from our laboratory demonstrated the microsome mediated conversion of DAMC to DHMC (Fig. 6) possibly through the action of microsomal deacetylase (unpublished data). Hence the antioxidant action of DAMC in vivo is contingent upon the formation of DHMC which is primarily responsible for inhibition of lipid peroxidation. In addition, pulse radiolysis studies have indicated that an initiating free radical can convert DAMC and DHMC to the phenoxyl radical (unpublished data). These observations explain the ability of DAMC and DHMC to scavenge the superoxide radicals (Table 7). DAMC and DHMC, exhibiting low IC_{50} values (Table 1) for inhibition of initiation of liver microsomal lipid peroxidation have proved to be good inhibitors of in vivo lipid peroxidation of rat tissues under the intoxication of CCl_4 and paraquat (Tables 8 and 9). It is conceivable that the mechanisms of antioxidant action of dioxygenated 4-methylcoumarins through the formation of a

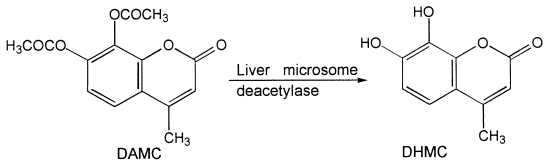

Figure 6. Liver microsome catalysed deacetylation of DAMC.

stable ADP-Fe-ligand can be largely applicable to the action of other inhibitors which prevent the formation of ROS.

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