The Synthesis of Binuclear Copper(II) Complexes and the Study of their Catecholase Activity

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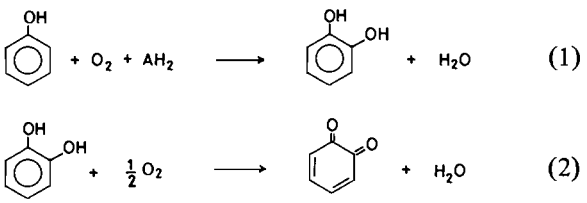
Three new binuclear copper(II) complexes have been prepared with shift bases derived from the condensation of 2,6-diformyl-4-methylphenol with the amino acids lysine, glutamic acid and arginine. The binuclearity of the complexes was characterized from optical, magnetic and electron spin resonance studies. Their ability to catalyze the dioxygen oxidation of several substituted catechols was measured using oxygen uptake and optical techniques.

The role of copper(II) ions in biological systems is the subject of much interest [1–9]. These copper metalloenzymes are involved in the metabolic processes of hydrozylation, oxygen transport, electron transfer and catalytic oxidation.

Tyrosinase or polyphenoloxidase is a copper enzyme which is found in many different plants and animals. Its primary function is to catalyze the air oxidation of phenols (cresolase activity) and *o*-diphenols (catecholase activity).



Tyrosinase obtained from mushrooms contains two pairs of copper(II) ions [10, 11]. The copper(II) ions in each pair are antiferromagnetically coupled (spin coupled Cu(II) pairs are often called type III copper). Information on the mechanism of oxidation catalyzed by tyrosinase is scant due to its molecular heterogeneity and difficulties suggest mechanistic elucidation using model compound. Ochiai proposed a mechanism which exploits the need for contiguous copper ions as coordination sites for the hydroxyl oxygens of *o*-diphenol and the binding of dioxygen (Figs. 1a and 1b).

1a 1b

Fig. 1. Ochiai's mechanism [1].

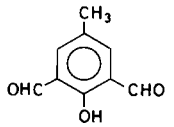


Fig 2. 2,6-Diformyl-4-methylphenol.

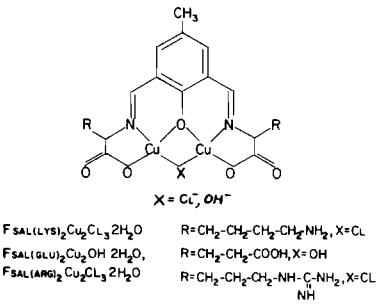


Fig. 3. Proposed structures for the binuclear copper(II) complexes.

Binuclear copper sites may also mediate the forbidden reaction between the triplet oxygen and singlet organic substrates.

The purposes of this work is to prepare water soluble binuclear copper complexes from 2,6-diformyl-4-methylphenol (Fig. 2), like those shown in Fig. 3. The complexes will be characterized and tested for catecholase activity.

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Experimental

Synthesis

2,6-Diformyl-4-methylphenol

Muto's method [12] was modified to prepare this ligand. 25.0 g of *p*-cresol and 25.0 g of hexamethylenetetramine were ground together into a fine powder. The pulverized mixture was added to a mixture of 114.0 g P_2O_5 and 136.0 g of 85% phosphoric acid at 160 °C while stirring vigorously. The complete mixture was then cooled in an ice bath to room temperature then hydrolyzed with 150 ml of deionized water. A pale yellow crystalline product was recovered by steam distillation and vacuum dried. The ligand ir and melting point (130–132 °C) were compared with literature values [12].

Fsal(Lys)$_2$Cu$_2$Cl·2HCl·2H$_2$O (Fsal(Lys))

0.608 g (0.0037 m) of lysine were dissolved in a minimal amount of water. 0.631 g (0.0037 m) of $CuCl_2 \cdot 2H_2O$ and 0.300 g (0.0018 m) of 2,6-diformyl-4-methylphenol were dissolved in 75.0 ml of ethanol, and the solution was heated to 70 °C. The lysine solution was then added and the mixture was stirred at 70 °C for ten minutes and then allowed to cool. The product precipitated as a dark green powder. It was filtered, washed with ethanol, and vacuum dried at 60 °C. The product gave a melting point of 187–9 °C with decomposition above 195 °C.

Because of the free amine groups on lysine, the complex precipitates as an amine hydrochloride with molecular weight 687.02 g/mol. Analysis from Galbraith Laboratory:

	%C	%H	%N	%Cu	%Cl
Found	37.50	5.11	7.35	18.86	15.25
Calc.	36.70	5.10	8.11	18.59	15.47

Fsal(Arg)$_2$Cu$_2$Cl·2HCl·2H$_2$O (Fsal(Arg))

1.0 g (0.0061 m) of 2,6-diformyl-4-methylphenol and 2.08 g (0.0122 m) of $CuCl_2 \cdot 2H_2O$ were dissolved in 200 ml of ethanol and the solution was heated to 70 °C. 2.125 g (0.0122 m) of arginine were dissolved in a minimal amount of water, and added to the ethanol solution. The mixture was stirred at 70 °C for 10 minutes and then allowed to cool. The dark green microcrystalline product was washed in cold ethanol and vacuum dried at 60 °C for one hour.

The complex precipitates as an amine hydrochloride with a molecular weight of 742.97 g/mol. It has a melting point of 183–85 °C. Analysis from Galbraith Laboratory:

	%C	%H	%N	%Cu	%Cl
Found	33.43	4.99	14.61	16.80	14.36
Calc:	33.91	4.71	15.07	17.07	14.33

Fsal(Glu)$_2$Cu$_2$(OH)·2H$_2$O (Fsal(Glu))

An aqueous solution of glutamic acid was prepared by dissolving 0.679 g (0.0037 m) of glutamic acid in a small amount of water. 0.631 g (0.0037 m) of $CuCl_2 \cdot 2H_2O$ and 0.300 g (0.00183 m) of 2,6-diformyl-4-methylphenol were dissolved in 75 ml of ethanol at 70 °C. The amino acid solution was added and the mixture was stirred for ten minutes at that temperature. The solution was allowed to cool. The solution was then carefully neutralized with ethanolic ammonium hydroxide. The product precipitated as fine pale green crystals when the solution reached the neutral point. The product was washed with cold ethanol and vacuum dried at 60 °C. Analysis showed that this complex contains a hydroxyl group as the second bridging group and has a molecular weight of 599.35 g/m. Analysis:

	%C	%H	%N	%Cu	%Cl
Found	37.53	4.30	3.85	21.36	0
Calc:	38.04	4.01	4.67	21.16	0

Physical Methods

IR studies

Infrared spectra of these complexes were measured in a KBr disk with a Beckman Aculab II Spectrophotometer in the region 4000–600 cm^{-1}. The spectra were used to determine that the Schiff base condensation was complete and to characterize the complexes.

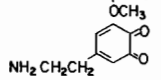
Optical studies

The visible spectra of Fsal(Lys), Fsal(Arg), and Fsal(Glu) were determined in the 400–800 nm region on a Cary Model 17 Spectrophotometer. The spectrum of each solid complex was recorded in a Nujol mull, and the solution spectra were determined in a 10^{-3} *M* aqueous solution using Coleman 1 cm quartz cells.

Magnetic studies

The magnetic susceptibilities of the solid complexes were determined at room temperature by the Gouy method using a system similar to the one developed by Eaton and Eaton [13]. The systems was calibrated with cobalt mercury tetrathiocyanate.

Table I Catechols Used in Oxidation Studies

Catechol	O-Quinone	λ(NM)	∈1
Pyrocatechol		390	1417
4-methylcatechol		495	2140
4-T-Butylcatechol		400	1140
3-methoxycatechol		420	1570
Dopamine		465	2455

[1] J H Waite, Anal Biochem, 75,211(1976)

The solution susceptibilities were determined in water at 34 °C by the Evans method [14]. The studies were done on a Varian EM 390-90 MHz Spectrometer. The concentration of each complex was 10^{-2} *M* in an aqueous solution containing 2% t-butyl alcohol as the reference. Diamagnetic corrections were calculated from a table of Pascal's constants.

ESR studies

The electron spin resonance spectra of these complexes were measured on a Varian 4502 Spectrometer. The spectra of the solid compounds were determined on undiluted powder samples, and the solution spectra were measured in n-butanol. The spectra were recorded at room temperature.

Kinetic studies

Initial rate studies. To determine the catalytic oxidase activity of these complexes, oxidation studies were performed with five different substituted catechols. The oxidation of each catechol to its corresponding *o*-quinone was followed spectrophotometrically by choosing the strongest absorption band of the quinone and monitoring the increase in absorbance at this wavelength as a function of time.

The substituted catechols used in this study are listed in Table I. A 10^{-1} *M* solution of each catechol was made in water. A fresh catechol solution was made for each study, and each solution was stored in a dark container during the study to minimize photo-oxidation. A potassium phosphate buffer solution of 0.125 ionic strength was made up and adjusted to pH 7.1 with K_2HPO_4. A 10^{-3} *M* solution of each complex was made in the phosphate buffer solution. The final pH of each copper complex solution was 7.05–7.10.

The oxidation studies were conducted as follows: 3.0 ml of Fsal(Lys) solution was added to a 1 cm quartz cell, and allowed to equilibrate in the spectrophotometer cavity which was at a constant temperature of 25 °C. A 0.3 ml sample of the desired catechol was quickly added, the solution stirred, and the cell closed. The absorbance of the solution was then continually monitored at the appropriate wavelength for the absorbance of the oxidation product (see Table I). This procedure was repeated three times with each catechol. The initial rate of formation of *o*-quinones was determined as the average slope of the tangent to the absorbance curve at time zero. The initial oxidation rate was determined for each copper complex with each catechol.

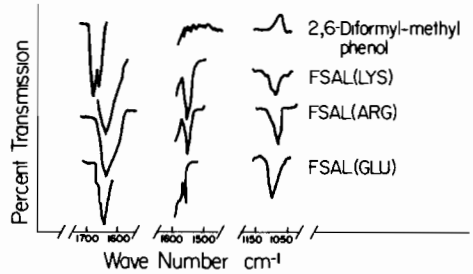


Fig. 4. Pertinent infrared spectra of 2,6-diformylmethylphenol, Fsal(Lys), Fsal(Arg) and Fsal(Glu).

Oxygen uptake studies

The rate of usage of molecular oxygen during the oxidation of 4-methyl-catechol by the Fsal complexes was determined by studying the decrease of molecular oxygen in the reaction system as a function of time. The oxygen concentration was determined by using an Orion model 97-08 oxygen electrode coupled to an Orion Model 701A digital pH meter and a recorder.

A 10^{-4} *M* solution of Fsal (Lys) was prepared in the buffer previously discussed. A 3×10^{-2} *M* solution of 4-methylcatechol was prepared in water. 15.0 ml of the Fsal(Lys) solution was added to the electrode cell, and allowed to equilibrate. 0.3 ml of the catechol solution was injected into the cell while the system was totally closed to the atmosphere. The final concentrations in the electrode cell were 10^{-3} *M* 4-methylcatechol and 10^{-4} *M* Fsal(Lys). The decrease in concentration of molecular oxygen in the system was monitored for 30 minutes. This study was repeated three times with each copper complex.

Results and Discussion

The structure proposed for these new complexes (Fig. 2) can be inferred from the following analytical data.

The elemental analysis of each compound, which is included in the experimental discussion shows excellent agreement with the calculated results.

The pertinent infrared spectra of the Fsal complexes are shown in Fig. 4. The spectrum of each

TABLE II. Optical Spectra.

Complex	λ(nm)	
	Solid[a]	Solution[b] (ε)
$Fsal(Lys)_2Cu_2Cl_3 \cdot 2H_2O$	680	680(173)
$Fsal(Arg)_2Cu_2Cl_3 \cdot 2H_2O$	700	675(120)
$Fsal(Glu)_2Cu_2OH \cdot 2H_2O$	680	700(120)

[a]Spectra were obtained in Nujol mull. [b]Spectra were obtained in H_2O.

TABLE III. Effective Magnetic Moments.

Complex	μ_{eff}[a]	
	Solid	Solution[b]
$Fsal(Lys)_2Cu_2Cl_3 \cdot 2H_2O$	1.64	1.42
$Fsal(Arg)_2Cu_2Cl_3 \cdot 2H_2O$	1.63	1.32
$Fsal(Glu)_2Cu_2OH \cdot 2H_2O$	1.54	1.41

[a]Magnetic moment per copper ion. [b]Solvent is H_2O.

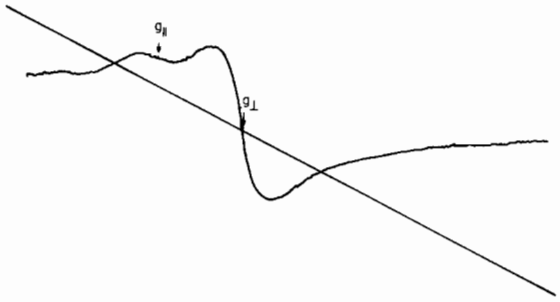


Fig. 5. The esr spectrum of Fsal(Lys) in solid state (powder).

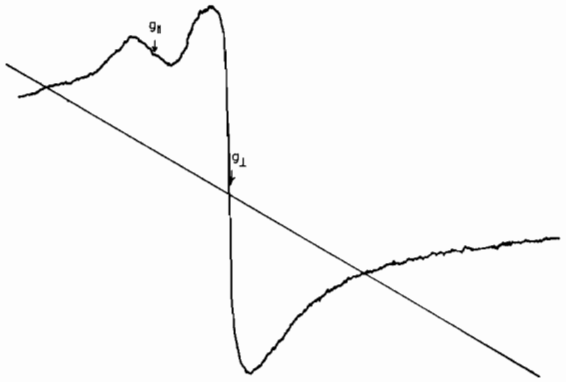


Fig. 6. The esr spectrum of Fsal(Lys) in solution of n-butanol.

complex is generally complicated but several characteristic bands can be used to establish the dimeric structure. Disappearance of the aldehyde stretching frequency at 1680 cm^{-1} indicates the complete Schiff base condensation. The C=N stretching frequency occurs in the range 1630–1650 cm^{-1} for these Schiff base complexes which is in agreement with the assignments of Okawa and Kida [15]. The band arising at 1545–50 cm^{-1} is the result of skeletal vibrations of the aromatic ring. This band is indicative of the binuclear structure in which the metal ions are bridged by a phenolic oxygen [15, 16]. The band in the 1080–90 cm^{-1} region can be assigned to the C–N stretch.

All the Fsal complexes prepared in this study exhibit broad d–d transitions with maxima at 680–700 nm (Table II). These broad absorptions are actually envelopes of the individual Cu(II) transition which cannot be resolved. The absorption maxima and molar absorptivities compare favorably with those reported for other binuclear copper(II) complexes with N_2O_2 environments [17]. These broad absorptions are indicative of a distorted octahedral sytem with solvent molecules weakly coordinated at the apical positions. The similarity of the transition maximum of each complex in solid and in solution indicates that the complexes maintain their binuclear structure in solution (see Table II).

The values of the magnetic moments measured for these complexes are given in Table III. They are

TABLE IV. ESR Data.

Complex	g⊥		g∥	
	Powder	Solution[a]	Powder	Solution[a]
$FSal(Lys)_2Cu_2Cl \cdot 2HCl \cdot 2H_2O$	1.99	1.98	2 29	2.29
$Fsal(Arg)_2Cu_2Cl \cdot 2HCl \cdot 2H_2O$	1.99	1.98	2.31	2.29
$Fsal(Glu)_2Cu_2OH \cdot 2H_2O$	1.98	1.98	2.29	2.29

[a]Solution is H_2O.

TABLE V. Initial Oxidation Rates.[a]

Catechol	R ($\times 10^4$ mol/l min)		
	$Fsal(Lys)_2Cu_2Cl\cdot 2HCl\cdot 2H_2O$	$Fsal(Arg)_2Cu_2Cl\cdot 2HCl\cdot 2H_2O$	$Fsal(Glu)_2Cu_2OH\cdot 2H_2O$
4-t-Butylcatechol	10.116	0.986	0.189
3-Methoxydatechol	7.909	0.416	0.497
4-Methylcatechol	0.541	0.133	0.087
Pyrocatechol	2.635	0.115	0.082
Dopamine	0.090	0.077	0.061

[a]Concentrations. $C_{Fsal\ complex} = 10^{-3}$ M/L; pH = 7.05, $C_{catechol} = 10^{-7}$ M/L. T = 25 °C.

all well below the calculated spin-only value for copper(II), and indicate that the copper ions are spin paired. These values are somewhat lower than the values of the chloride ion bridged copper complexes reported by Okawa *et al.* for binuclear complexes prepared from 2,6-diformyl-4-methylphenol and glycine and alanine, but are higher than the glycine complex bridged by the hydroxyl ion [15].

The X-band ESR spectra of powder and n-butanol samples of Fsal(Lys) are shown in Figs. 5 and 6 respectively. Data from the ESR studies are given in Table IV. The absorption signal for each complex consists of an extremely broad band centered at 3400 G with a width of 1000 G. The absorptions are asymmetric giving values of $g_\perp$ and $g_\parallel$ for each complex. These spectra are consistent with others reported for dimeric copper(II) complexes, and are indicative of spin-coupled copper centers [15, 20, 21]. Hyperfine splitting due to the spin of the copper nucleus was not detected because of the extremely broad signal. No $\Delta M = \pm 2$ absorption signal at around 1500 G was observed, again because of extreme signal broadening.

The ESR spectra in solutions of n-butanol are surprisingly similar to their solid spectra. The magnetic anisotropy found in the spectra of powdered solids is also found in solution spectra. Hydrogen bonding, viscosity, molecular size and the shape of the complexes in n-butanol restrict the molecular motion to prevent a motionally averaged system.

The results of the study of the catecholase activity of the dicopper complexes with a series of substituted catechols are given in Table V. Each of the Fsal complexes showed oxidase activity toward each catechol in the study. Under the reaction conditions the rates ranged from 6×10^{-6} mol/liter min to 1000×10^{-6} mol/liter min. Fsal(Lys) was found to be approximately one order of magnitude more active than Fsal(Arg) and two orders of magnitude more active than Fsal/(Glu). The difference in reactivity of the complexes toward the catechols is not entirely understood. Steric and inductive contributions from the amino acid residues surrounding the copper centers influence the oxidase activity of the complexes, but the combination of the two effects is not predictable. It is tempting to attribute the activity enhancement, at least in part, to the bridging chloride ion.

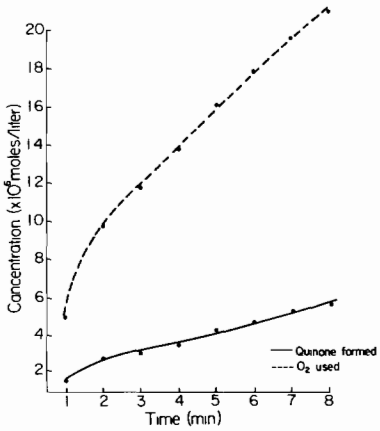


Fig. 7. The mol of oxygen uptake and the mol of 4-methyl-*o*-quinone produced in the Fsal(Lys) (10^{-4} *M*) catalyzed oxidation of 4-methylcatechol (10^{-3} *M*) with time.

The reactivity of each of the Fsal complexes toward the series of catechols studied is generally in the order expected based on the inductive effect of the substituents on the benzene ring. The shift of electron density to the phenolic oxygens facilitates a stronger phenolic oxygen–copper(II) bond which was previously shown to enhance the oxidation rate [22]. Oxidation rates are not affected by electronic shifts alone. Hammett plots which show no straight line correlation between the log of rates and σ values of the catechol substituents infer significant effects on the rates due to steric hindrance.

Correlation of the rate of oxygen uptake to mol of *o*-quinone formed during the oxidation is shown for Fsal(Lys) catalyzed oxidation of 4-methylcatechol in Fig. 7. For the first 8–10 minutes, these

studies show that four mol of oxygen are used for every mol of catechol oxidized. Similar results were found for the oxidation of 4-methylcatechol oxidized by Fsal(Arg) and Fsal(Glu). These results are inconsistent with reaction 1 which is usually reported. The extreme reactivity of the *o*-quinone may account for the large amount of oxygen used. *O*-quinones are known to oligomerize, add oxygen to form epoxides and undergo ring opening to produce several acidic compounds [23]. These processes utilize additional oxygen. Previous studies of oxidation by dicopper catalysts in this laboratory [24] show a decrease in the pH of the reaction media, precipitation of a dark polymer and complex spectra of products which cannot be identified either as catalyst, *o*-diphenol or *o*-quinone. Thus, the mechanism for the oxidation reaction seems to be a complex one which cannot be determined until all of the oxidation products have been identified.

In summary, three new water soluble binuclear copper(II) complexes were synthesized and characterized. They were shown to catalyze the air oxidation of several *o*-diphenols in aqueous solution. The catalytic activities for the oxidation reactions were measured as initial rates of *o*-quinone produced and by the oxygen uptake of the reaction with time. The reactivity of the series of *o*-diphenols is generally in the order expected based on the inductive effect of the substituents on the benzene ring. Steric effects are also believed to be important. The study shows the oxidation mechanism to be a complex one requiring more oxygen than previously thought.

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