tronic parameters listed in Tables II and III. Furthermore on inclusion of a π^2 term, its coefficient was found to be not significantly different from zero. It seems therefore, that, within its range in this study, the second-order contribution of π is minimal.

The significant partial correlation to π indicates an influence of the hydrophobic–hydrophilic character on the relative activities of the coumarins.

The partial correlation to the HOMO energy, an index of electron donor ability,[26] may be interpreted[6,13] as an indication that charge-transfer complex formation may occur at the site of the critical reaction.

The excitation energy of a molecule is a parameter of an analogous nature to a free energy of reaction or activation, thus substituent effects on the former may well parallel these effects on the latter.[14] Hence, it is not too surprising to find the EE to be in significant partial correlation with log BA. It is also interesting to note that in eq 11 and 19 the π term coefficient was no longer significant at the $p < 0.05$ level. This seems to indicate that the EE term incorporates part of the variance in π relevant to its effect on log BA.

Finally, the partial correlation to the sum of the net charges on the C-2/O-11 (NET 2 + 11) and C-2/O-1 (NET 1 + 2) groups seems to implicate these positions as possible sites of H bonding[26] or of charge transfer. Ease of electron donation in any case would increase sensibly as the net charge at these points becomes more negative, as reflected in eq 6–11 and 14–19.

The data presented here suggest that the induction of drug metabolism by coumarins may be associated with the carbonyl (C-2/O-11) and the α-pyran (O-1/C-2) moieties of the molecules. It should be clarified that in fact the absolute need for the carbonyl or the α-pyran moieties for this biological activity has not been established. This study has simply delineated a pattern of charge distribution that seems to favour it. Such activity might well be achieved by reproducing this pattern with the use of any other fortuitous combination of substituents on this or other molecules exhibiting a similar electronic geography. However, the electronic properties on the carbonyl and α-pyran moieties of the lactone ring seem to be salient to the capability of the various coumarins to induce drug-metabolizing enzymes in the liver of the rat to varying degrees. The stabilization of this structure by 4-Me substitution[28] may also play a role in the structure–activity relationship. The degree of resolution in the present study does not allow more specific conclusions to be drawn concerning the drug–receptor site interaction involved. The mechanism by which these compounds exert their action on the liver cell remains to be elucidated.

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Interaction of Ergothioneine with Metal Ions and Metalloenzymes[1]

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The interaction of ergothioneine (I) with a number of divalent metal ions was determined using a pH titration method. Formation constants for complexes containing 2 moles of ligand and 1 mole of metal ion were found to be $10^{18.2}$ (Cu^{2+}), $10^{11.6}$ (Zn^{2+}), $10^{8.2}$ (Ni^{2+}), and $10^{8.2}$ (Co^{2+}). A study of the inhibitory power of I on selected Zn and Cu metalloenzymes was made. None of the 4 Zn-containing enzymes, including 3 dehydrogenases and alkaline phosphatase, were inhibited even if preincubated for 6 hr with 10^{-2} M I. Four different Cu enzyme systems were investigated, including uricase, ascorbate oxidase, MAO, and a number of polyphenol oxidases. Of these, only the polyphenol oxidases were inhibited by I. Kinetic measurements using *Psalliota campestris* (mushroom) polyphenol oxidase showed that inhibition by I does not involve removal of Cu from the enzyme, but is reversible and displays characteristics of both competitive and noncompetitive inhibition. This type of inhibition may result from the presence of Cu at noncatalytic as well as catalytic sites of mushroom polyphenol oxidase.

Ergothioneine, the betaine of thiolhistidine, was first described by Tanret over 50 years ago.[2] This fungal alkaloid has often engaged the interest of investigators because of its obvious potential as a metabolically important compound; however, most studies in this regard have proven to be disappointing. Melville[3] has extensively reviewed this and other aspects of ergothioneine chemistry and biochemistry.

A previously unexplored approach to the elucidation of a possible metabolic role for ergothioneine lies in its metal ion binding potential. Qualitative information regarding metal binding is supplied by the fact that purification of ergothioneine from natural sources includes a precipitation step using Cu_2O.[4] Also, Mann and Leone[5] showed that an unusually high concn of ergothioneine in boar serum prevented Cu^{2+} inhibition of sperm motility and fructolysis.

This paper reports findings on the interaction of

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ergothioneine with Cu^{2+}, Zn^{2+}, Ni^{2+}, and Co^{2+}, transition metal ions known to play important roles in metabolism. In conjunction with these studies the inhibitory effect of ergothioneine on some Zn- and Cu-requiring enzymes has been determined.

Experimental Section

Chemicals.—L-Ergothioneine (Nutritional Biochemical Corp.) was recrystd before use. The recrystd material was pure according to spectrophotometric assay[6] and chromatog properties.[7] $CuCl_2 \cdot 2H_2O$, $NiCl_2 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$, and $ZnCl_2$ were Baker Analyzed Reagent Grade. All solns were prepd with glass distd, degassed H_2O.

Determination of Metal Complex Formation Constants.—The pH titration method of Bjerrum as employed by Calvin and Wilson[8] was used to determine the free ligand concn, [A], and the average number of ligands bound per mole of metal ion, $\bar{n}$. The pK_a value for the monoprotic form of ergothioneine was calcd from the pH titration data for ligand only using an equation derived by Ricci[9] which contains terms for H^+ and OH^- and therefore yields more reliable values for pK_a in low and high pH ranges.

Formation constants were computed from exp values of $\bar{n}$ and [A], and approximate values for K_1 and K_2 (estimated from values for [A] at $\bar{n}$ values of 0.5 and 1.5) using a least-squares curve-fitting technique applied to a modification of a general expression for metal ion binding.[10]

Titrations.—Titration of ergothioneine and the interaction of ergothioneine with Cu^{2+}, Zn^{2+}, Ni^{2+}, and Co^{2+}, were measured at 25°. The receiving vessel was water jacketed and placed under a constant surface stream of N_2. Titrations were made with a previously calibrated Gilmont microburet (1 ml total vol) contg a soln of approximately 1 *N* NaOH prepd from 50% NaOH (Fisher) and standardized against potassium hydrogen phthalate. pH was determined with a Radiometer pH meter, Model 22, using semimicro electrodes. The pH meter was standardized against buffers of known pH before exp readings were taken.

A measured vol of ligand soln was placed in the 25-ml titration chamber, and a measured vol of metal salt was added from a calibrated micropipet. Total concn of ligand was usually 10^{-2} *M* and the ligand to metal ion ratio was set at 2:1. The initial pH was recorded after temp equil occurred. Immediate mixing was ensured by the use of a magnetic stirrer. pH was again recorded. Standard base was added slowly with the tip of the microburet placed just below the surface of the liquid. pH readings were taken after each addn of base, permitting sufficient time for equil to be achieved. The same procedure was followed when titrating solns contg only ligand. Average values from at least 2 different titrations were used to calc pK_a values and formation constants.

Enzyme Studies.—The inhibitory effect of 5×10^{-4} *M* ergothioneine was measured on the following enzyme systems.

1. Zn Enzymes.—Yeast alcohol dehydrogenase (YADH) was obtd from Sigma Chemical Co. as a lyophilized prepn. Activity was measured at 25° using the method of Williams, *et al.*[11]

Glutamic acid dehydrogenase (GDH) from bovine liver was obtd as an $(NH_4)_2SO_4$ suspension from Sigma Chemical Co. Activity was measured spectrophotometrically according to the method of Strecker.[12]

Alkaline phosphatase from calf liver was obtd from Sigma Chemical Co. Enzyme activity was assayed spectrophotometrically.[13]

Uricase from porcine liver was obtd from Sigma Chemical Co. Activity was assayed spectrophotometrically using the method of Mahler.[14]

2. Cu Enzymes. Ascorbic Acid Oxidase (AAO) from *Cucurbita pipo condensa*, the Yellow Summer Squash.—Squash rind (62 g) was minced and mixed with 20 ml of 0.1 *M* sodium phosphate, pH 6.0. The resulting mash was squeezed through cheesecloth and the expressed juice served as enzyme. AAO activity was measured manometrically.[15]

Monoamine Oxidase (MAO) from Bovine Blood.—Whole blood (4 l.), obtd from a slaughterhouse, was allowed to coagulate for 2 hr at +4°. The coagulum was squeezed through cheesecloth, and 5 g of sodium citrate was added per l. of filtrate. The filtrate was centrifuged at 6500*g* at +4°. Reagent grade Me_2CO, previously cooled to −10°, was added with stirring to make a mixt 45% in Me_2CO. The ppt was removed by centrifugation at 2000*g*. Cold Me_2CO was added to the supernatant to bring its total concn to 60% (v/v). The mixt was again centrifuged at 2000*g*, and the residue was dissolved in a min vol of cold 0.2 *M* sodium phosphate, pH 7.2. Undissolved material was removed by centrifugation. The final step in purification was gel filtration using G-100 Sephadex. Elution was made with 0.05 *M* sodium phosphate, pH 7.2. Enzyme was assayed spectrophotometrically following Tabor, *et al.*[16]

Polyphenol Oxidase (PPO) from White Potatoes.—Peeled white potato (30 g) was finely diced and ground in a mortar and pestle. Distd H_2O (10 ml) was added and blended. The mixt was filtered through glass wool, and the filtrate was centrifuged to remove starch granules. The supernatant was dild with an equal vol of Me_2CO at −10°. The flocculent ppt formed was centrifuged down and the Me_2CO supernatant was discarded. The ppt was taken up in 5 ml of 0.1 *M* sodium phosphate, pH 7.0. A small amount of insol material was removed by centrifugation. The clear soln served as enzyme.

PPO from *Tenebrio molitor*.—Meal worm pupae (1.6-g lot) was homogenized in 5 vol of 0.1 *M* sodium phosphate, pH 7.0. The mixt was centrifuged, and the centrifugate was treated as in the prepn of potato PPO.

PPO from the Mushroom, *Coprinus atramentarius*.—Stalk and cap tissue (12 g) was minced with 6 ml of distd H_2O. The minced tissue was pressed through cheesecloth and the juice served as enzyme.

PPO from the mushroom, *Psalliota campestris*, was purchased from the Sigma Chemical Co. as the Grade 2 prepn. No contaminating laccase activity was found.

Routine assays on the 4 PPO's were made using an assay medium contg 8.0×10^{-3} *M* L-dihydroxyphenylalanine (L-dopa), purchased as the Grade A prepn from Calbiochem Corp., and 0.1 *M* sodium phosphate, pH 7.0. Temp was set at 25°. The increase in absorbance at 475 mμ due to the formation of 2-carboxy-2,3-dihydroindole-5,6-benzoquinone (dopachrome) was monitored with a Bausch and Lomb Spectronic 505. Initial velocities were taken from the linear portion of the absorbance increase.

The following kinetic experiments were performed using *Psalliota* PPO. (1) A soln was prepd which contd 0.267 mg of PPO/ml in 0.1 *M* sodium phosphate, pH 7.0, and 10^{-3} *M* ergothioneine. A control contd identical concns of buffer and enzyme, but no ergothioneine. Temp of the mixt was kept at 25° during the incubation. At 10-min intervals for a total of 1 hr, activity measurements were performed using 0.1-ml aliquots of enzyme. Assays were made using the standard assay system. (2) PPO activity was measured in the presence of 1×10^{-4}, 2×10^{-4}, and 4×10^{-4} *M* ergothioneine using several different rate-limiting concns of L-dopa (see Figure 1).

Results and Discussion

Metal Ion Interaction Studies.—Table I lists formation constants calcd from titration data. Note that complexes containing 2 moles of ligand per mole of metal ion are formed in every case. This ratio is a maximum value since preliminary studies using ligand to metal ion ratios of up to 10:1 showed that the completely formed complexes contained 2 moles of ligand per mole of metal ion.

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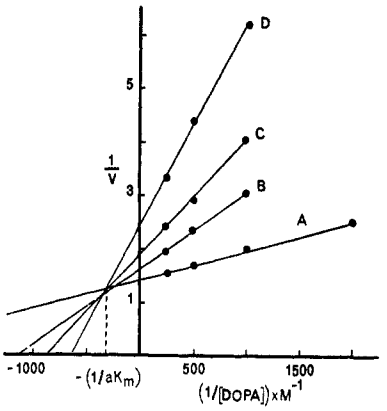

Figure 1.—The effect of varying substrate concn on ergothioneine inhibition of *Psalliota* polyphenoloxidase. Enzyme concn was 14 μg/ml. Velocities are expressed as increase in A_{475}/min. Each point represents the average of 6–12 measurements. Data are fitted with a regression line calcd by the method of least squares and weighted according to Wilkinson.[17] A, substrate only; B, substrate plus 10^{-4} *M* ergothioneine; C, substrate plus 2×10^{-4} *M* ergothioneine; D, substrate plus 4×10^{-4} *M* ergothioneine.

TABLE I

FORMATION CONSTANTS FOR ERGOTHIONEINE–METAL ION COMPLEXES[a]

Metal ion	log K_1	log K_2
Cu^{2+}	9.74	8.60
Zn^{2+}	5.72	5.72
Ni^{2+}	4.12	3.95
Co^{2+}	3.98	3.96

[a] Method of calculation and experimental conditions are given in the Experimental Section.

The relative stability of the ergothioneine complexes is $Cu^{2+} > Zn^{2+} > Ni^{2+} = Co^{2+}$. This order can be correlated with the second ionization potential of the component metals, an observation which is similar to that found for a wide variety of ligands investigated by Irving and Williams.[18] The degree to which ergothioneine interacts with divalent metal ions approximates that of a number of biologically important ligands, including glycine[19] and histamine,[20] a compound structurally similar to ergothioneine.

A pK_a of 10.39 was obtained for the deprotonation of the uncharged thiolimidazole group of ergothioneine. Although this value is in the appropriate range for deprotonation of aliphatic SH groups (pK_a of cysteine is 10.34), Lawson and Morley[21] suggest that thiolimidazoles are in the thione form in the uncharged state and the ionized species exists as a thiolate ion.

Two phenomena were observed during the titration experiments which seem worthy of mention. During the ergothioneinecobalt(2+) titration the solution became blue and deepened with subsequent addition of base. However, as the titration neared completion the color changed to emerald green. If the solution was titrated rapidly it remained blue throughout the titration. The conversion to green could also be prevented if the blue solution was placed in a vacuum. A preliminary study using manometric techniques suggests this color change is correlated with a pH-dependent reversible interaction of the complex with O_2. For this reason constants for the ergothioneinecobalt(2+) complexes are calcd from data obtained in titrations of sufficient rapidity to prevent a color change in the pH region from which [A] and $\bar{n}$ were calcd.

At pH 7.0 the completely titrated 2:1 ergothioneinecopper(2+) species is present. Further addition of base caused a rapid increase in pH which was accompanied by the distinct odor of Me_3N. At this point in the titration the pH slowly fell and the evolution of the volatile base continued. Possibly, Cu^{2+} exerts more of an electron-withdrawing effect on the thiolimidazole ring, thereby increasing the leaving ability of an H on the β-C of ergothioneine. This is not unreasonable in light of the E_2 value for Cu^{2+}, but it does not explain the complete specificity for Cu^{2+}. The odor of Me_3N was not detected during the titration studies with the three other metal ions, although the metal complexes are fully formed at pH 9.5.

Enzyme Studies.—Ergothioneine exerts a decidedly selective inhibition on metalloenzymes (see Table II).

TABLE II

EFFECT OF ERGOTHIONEINE ON THE ACTIVITY OF METALLOENZYMES[a]

Enzyme	Metal moiety	Source	% inhibition
Alcohol dehydrogenase	Zn	Yeast	0
Glutamic dehydrogenase	Zn	Bovine liver	0
Alkaline phosphatase	Zn	Calf liver	0
Uricase	Cu	Porcine liver	0
Ascorbic oxidase	Cu	Summer squash	0
Monoamine oxidase	Cu	Bovine blood	0
Polyphenoloxidase	Cu	White potato	90
Polyphenoloxidase	Cu	Meal worm	72
Polyphenoloxidase	Cu	Mushroom (*Coprinus*)	75
Polyphenoloxidase	Cu	Mushroom (*Psalliota*)	55

[a] Assays were performed as described in the Experimental Section. Concn of ergothioneine in the assay medium was 5×10^{-4} *M*.

None of the 4 Zn metalloenzymes were inhibited by 5×10^{-4} *M* ergothioneine in the assay mixture. Preincubation of the Zn enzymes with 10^{2-} *M* ergothioneine for up to 6 hr prior to assay also had no effect on activity. This observation is interesting in light of the fact that YADH, LADH, and GDH are inhibited by the planar chelator, 1,10-phenanthroline, with a Zn^{2+} intrinsic 1:1 formation constant of $10^{5.86}$ which is slightly greater than that of ergothioneine.[22] The finding is not unique, however; Vallee[23] mentions a number of metalloenzyme systems, containing Zn as the metal moiety, that are selectively inhibited by planar chelators. The most reasonable explanation of the inability of ergothioneine to inhibit Zn dehydrogenases must also lie in steric factors, although one cannot rule out the possibility of electrical repulsion involving charged groups of the enzymes and the side chain of the ergothioneine molecule.

Of the 4 Cu enzyme systems investigated, only PPO's are inhibited by ergothioneine. Apparently, this results from some structural feature common to many species of PPO molecule, since a number of sources,

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both plant and animal, were used and all are extensively inhibited (Table II). The absence of inhibition of the other Cu oxidases cannot be accounted for on the basis of differences in the degree of substrate saturation. The K_m for *Psalliota* PPO is 3.9×10^{-4} *M* (*vide infra*), making the enzyme 94% saturated in the assay system. According to the literature reports K_m values for AAO, uricase, and MAO are 5×10^{-4} *M*, 1.7×10^{-5} *M*, and 4×10^{-4} *M*.[24,14,25] Our measurements were made under the same conditions of buffer and temp used to obtain these K_m values. Therefore, considering the concn of substrate in each case, per cent saturation is 42, 65, and 90 resp. The degree of saturation is greatest in the mushroom PPO system, yet ergothioneine inhibits this system extensively and the other oxidases not at all.

In the group of Cu enzymes investigated, only PPO's maintain the metal moiety of the active sites in the Cu^+ form while Cu at the active sites of uricase, MAO, and AAO is partly or wholly in the Cu^{2+} state.[26] At present one cannot be sure that ergothioneine inhibition is a characteristic of Cu^+ enzymes in general or is a special feature of PPO systems; however, the usefulness of ergothioneine and other thiolimidazoles as specific probes for cuprous metalloenzymes is being studied.

The kinetics of ergothioneine inhibition were investigated in some detail using mushroom (*Psalliota campestris*) enzyme. Preincubation of PPO with 10^{-3} *M* ergothioneine for up to 1 hr at 25° prior to assay resulted in no more inhibition than that which could be accounted for on the basis of the amount of ergothioneine added to the assay mixture along with the enzyme. This indicates that the reaction between enzyme and inhibitor is instantaneously reversible and that Cu is not removed from PPO during prolonged contact with ergothioneine.

Both the maximum velocity (V_{max}) and the Michaelis constant (K_m) for *Psalliota* PPO are changed in the presence of ergothioneine (Figure 1). Such findings could mean that inhibition is partly competitive and partly noncompetitive. This is at least reasonable since there are noncatalytic Cu sites in highly purified[27] and partially purified PPO preparations.[28] Ergothioneine inhibition data fit a specific "mixed type" inhibition scheme offered by Freidenwald and Maengwyn-Davies.[29] Their expression

$$V_{max/v} = [1 + (K_m/S)]\{1 + (I/\alpha K_i)[(S + \alpha K_m)/(S + K_m)]\} \quad (1)$$

measures the influence of inhibitor on the dissociation of the enzyme–substrate complex in terms of a constant, α. When inhibition is competitive, α = infinity; when noncompetitive, α = 1.0. K_m and K_i are treated as equilibrium constants in this scheme. We show that ergothioneine inhibition is reversible and therefore K_i must be an equilibrium constant. K_m becomes an equilibrium constant when the contribution of the catalytic rate constant [k(cat)] to the numerator decreases to 0; practically speaking, however, when the numerical value of k(cat) comprises less then 10% of the numerator term, K_m can be considered as functionally being an equilibrium constant. In this regard, data presented by Duckworth and Coleman[30] indicate that K_m for L-dopa in the mushroom PPO system is essentially an equilibrium constant.

A K_i for ergothioneine inhibition can be calcd from a plot of the reciprocal of velocity against the concn of inhibitor using the method of Freidenwald and Maengwyn-Davies. This K_i value, equal to 1.05×10^{-4} *M*, is a weighted average of competitive and noncompetitive binding. K_m for L-dopa was calculated as 3.9×10^{-4} *M* using velocity data obtained in the absence of ergothioneine. A value of 4.7 for α was obtained using the method shown in Figure 1. If the appropriate series of substitutions are made in eq 1, one finds that, for an α = 4.7, the relative degree of competitive and noncompetitive inhibition is 3:1. Since both K_i and K_m are equilibrium constants and inhibition is predominantly competitive, one can surmise that the binding affinity of ergothioneine for the catalytic sites of mushroom PPO is nearly 4 times that of L-dopa. It is likely that the aromatic nature of the thiolimidazole group as well as its Cu-binding ability is responsible for the success of ergothioneine as a competitive inhibitor since Duckworth and Coleman[29] state that CN^- inhibits the mushroom PPO catalyzed oxidation of catechol oxidation noncompetitively, while benzoic acid inhibition is strictly competitive.

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