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Reactions of 3-Ethoxy-2-oxobutyraldehyde Bis(N^4-dimethylthiosemicarbazonato)-Zinc(II) with Tumor Cells and Mitochondria

DANIEL T. MINKEL, CHRISTINE CHAN-STIER, AND DAVID H. PETERING[1]

Department of Chemistry, University of Wisconsin – Milwaukee, Milwaukee, Wisconsin 53201

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SUMMARY

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Physicochemical and biological properties of bis(thiosemicarbazonato)-zinc complexes have been investigated. Respiration of Ehrlich ascites cells is inhibited by 3-ethoxy-2-oxobutyraldehyde bis(N^4-dimethylthiosemicarbazonato)-zinc(II). Zinc ion is much less effective, but the thiosemicarbazone ligand initially stimulates, then depresses, oxygen uptake. State 3 oxygen uptake of tumor cell mitochondria is more sensitive to this zinc complex than is state 4. In bovine heart mitochondria respiratory inhibition occurs almost exclusively in state 3. The insensitivity of the electron transport chain to the complex was confirmed with Keilin-Hartree particles. Formation constants and partition coefficients for several bis(thiosemicarbazonato)-zinc complexes are used to interpret the different behavior of zinc ion, thiosemicarbazone ligand, and zinc complex with tumor cells and mitochondria.

INTRODUCTION

In the study of the antineoplastic properties of bis(thiosemicarbazonato)-copper(II) complexes, a striking structure-function correlation has been observed with respect to their ability to inhibit tumor cell respiration (1). As peripheral alkyl substitution increases on the structure (Fig. 1), the effective inhibition of tumor cell respiration is lost. However, in this progression the corresponding zinc complexes become active. To explain the behavior of this series of copper complexes, linear free energy correlations have been demonstrated for some of their kinetic and thermodynamic properties (2). These relationships separate the complexes into groups which also correspond qualitatively to their biological effectiveness. Furthermore, a similar grouping of these complexes is observed when their effects on mitochondrial respiration are analyzed (3). It is currently thought that the active copper complexes are highly specific for the oxidation of thiols (4, 5).

Some basic observations of the chemistry and biochemistry of bis(thiosemicarbazonato)-zinc complexes have been made which apply to this structure-function question (6). Here the activation by alkyl substitution is related in part to the increase in stability of the zinc complexes, for the simple reason that this increase permits the complex to exist in the presence of competing biological ligands such as amino acids.

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[1] To whom correspondence should be addressed.

A more detailed investigation of the chemistry and biochemistry of bis(thiosemicarbazonato)-zinc complexes has been undertaken to complement work on the associated copper complexes, with the primary aim of elucidating the chemical basis of the biological structure-function relationships.

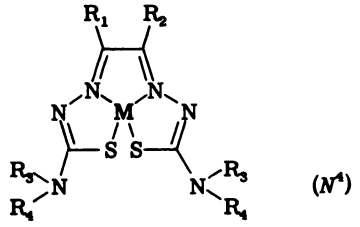
METHODS

Table 1 sets forth the abbreviations used to describe the bis(thiosemicarbazone) ligands and zinc complexes.

Formation constants for zinc complexes. The bis(thiosemicarbazone) ligands may be described as H_2L, containing two slightly acidic protons on the N^2 nitrogens of the thiosemicarbazone moiety. These are dissociated in the chelation reaction

$$Zn^{2+} + H_2L \rightleftharpoons ZnL + 2\,H^+ \qquad (1)$$

It has been previously shown for 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazone) that titrations of these protons occur at high pH, at which the ligand itself is unstable (4). Hence pK_a values for the ligands have not been determined, and only pH-dependent equilibrium constants are reported.



(N^4)

FIG. 1. *Bis(thiosemicarbazonato)-metal ion complex*

$$K(\mathrm{pH}) = \frac{[ZnL]}{[Zn^{2+}]\,[\text{ligand}]_{\text{all forms}}} \qquad (2)$$

Given the equilibria for the ligand, $K_1 = [H^+][HL]/[H_2L]$ and $K_2 = [H^+][L^{2-}]/[HL^-]$, the pH dependence of K(pH) is

$$K(\mathrm{pH}) = \beta\left[1 + \frac{[H^+]}{K_1} + \frac{[H^+]^2}{K_1K_2}\right] \qquad (3)$$

in which $\beta = [ZnL]/[Zn^{2+}]\,[L^{2-}]$. For these complexes K(pH) and its pH dependence are determined spectrophotometrically by competition between H^+ and Zn^{2+} for ligand as described previously (6). The form of the pH dependence of K(pH) is

$$\log K(\mathrm{pH}) = 2\ \mathrm{pH} + C \qquad (4)$$

in which $1 + [H^+]/K_1 \ll [H^+]^2/K_1K_2$ and $C = \beta/K_1K_2$.

The zinc complexes were made up in 0.1 M KNO_3 and 0.01 M Tris, pH 7.2. Adjustments in pH were made with HNO_3. Data were taken at 25.0°, and pH readings have a range of ±0.05.

Stability of ZndAcTS in plasma. As described previously for other zinc complexes, the behavior of 12 μM ZndAcTS in human outdated plasma was observed spectrophotometrically using an Acta V spectrophotometer balanced with plasma in sample and reference cells (6). The plasma pH was 7.15, and the temperature was controlled at 25°.

Partition coefficients of $ZnKTSM_2$. The partition of $ZnKTSM_2$ between 1-octanol and water, pH 6.7, was accomplished by periodic gentle shaking of the mixture, interspersed with equilibration, in a temperature bath at 25°. Changes in absorb-

TABLE 1

Abbreviations of materials

Refer to Fig. 1 for general structure. The ligands were gifts of Drs. Harold G. Petering and Eugene Coats, University of Cincinnati.

Compound or derivative	R_1	R_2	R_3	R_4	Ligand	Zinc complex
3-Ethoxy-2-oxobutyraldehyde bis(thiosemicarbazone)	$CH(OEt)CH_3$	H	H	H	H_2KTS	ZnKTS
N^4-Methyl	$CH(OEt)CH_3$	H	CH_3	H	H_2KTSM	ZnKTSM
N^4-Dimethyl	$CH(OEt)CH_3$	H	CH_3	CH_3	H_2KTSM_2	$ZnKTSM_2$
Pyruvaldehyde bis(thiosemicarbazone)	CH_3	H	H	H	H_2PTS	ZnPTS
N^4-Methyl	CH_3	H	CH_3	H	H_2PTSM	ZnPTSM
N^4-Dimethyl	CH_3	H	CH_3	CH_3	H_2PTSM_2	$ZnPTSM_2$
Diacetylbis(thiosemicarbazone)	CH_3	CH_3	H	H	H_2dAcTS	ZndAcTS

ance of the aqueous phase were followed until equilibrium had occurred. The partition coefficient was then defined as $\rho = [ZnKTSM_2]_{oct}/[ZnKTSM_2]_{aq}$ for equal volumes of each solvent. Volumes used were 8.0 ml of H_2O and 0.20 ml of 1-octanol. The initial and final concentrations of $ZnKTSM_2$ in water were determined spectrophotometrically. The final concentration of Zn^{2+} was measured by back-titration with excess H_2KTSM_2.

Tumor cell studies. Ehrlich ascites tumor cells were obtained from a line maintained in Swiss mice. Cells were suspended in Eagle's minimal essential medium plus Earle's salts and exposed to agents. Rates of respiration of treated and control cells were compared simultaneously, using a Yellow Springs oxygen analyzer as previously described (3). Protein determinations were made by the biuret method. Then Fig. 3 was developed by plotting the percentage of control rate of oxygen consumption for the treated sample against time.

Mitochondria and mitochondrial particles. Tumor mitochondria were prepared by the method of Thorne and Bygrave (7). Average state 4 and state 3 rates of oxygen uptake for five preparations were 4 and 19 ng atoms of oxygen per minute per milligram. The average P:O ratio using the substrate glutamate was 1.85.

Heart mitochondria were isolated by a minor modification of the procedure described by Smith (8). The P:O ratios for at least 10 preparations averaged 2.5, 2.5, and 2.0, respectively for the substrates glutamate, pyruvate-malate, and succinate. Average respiratory control ratios were 3.5, 3.2, and 1.8 for these substrates. The average rate of oxygen consumption in state 3 was 30 nmoles/min/mg.

Mitochondrial respiration was measured in a medium containing 2.5 ml of the mixture 0.025 M Tris, 0.015 M KCl, 0.03 M KH_2PO_4, 0.045 M sucrose, and 0.005 M $MgCl_2$ adjusted to pH 7.5, plus 0.1–0.2 ml of mitochondrial suspension. State 4 respiration occurs in the absence of the phosphate acceptor ADP; state 3, in the presence of ADP.

Keilin-Hartree particles were prepared according to method 3 of King (9). Average rates of oxygen consumption were 34 and 21 nmoles/min/mg in the presence of 0.52 mM NADH and 11 mM succinate, respectively.

The titration experiments summarized in Figs. 4–8 are compilations of data from several preparations of mitochondria and indicate the reproducibility of experimental effects between batches of mitochondria in this work. To normalize differences in rate of oxygen consumption per milligram of protein among the preparations, each point on the titration curves is the ratio times 100 of respiration rates in experimental and control samples, which were measured simultaneously. Hence ordinates in the graphs represent percentages of control rates of oxygen uptake for drug-treated mitochondria and are plotted against nanomoles of $ZnKTSM_2$ per milligram of mitochondrial protein.

RESULTS

Formation constants. The pH-dependent formation constants for a set of bis(thiosemicarbazonato) - zinc(II) complexes, differing in R_1–R_4 substitution (Fig. 2 and Table 2), have been determined. The pH dependence of the formation constants is shown in Table 2, together with the least-squares equations which describe the linearity of these relationships. In general, the equations follow the derived form of the equilibrium constant for the case in which virtually all free ligand exists as the neutral, diprotonated species. From the values at pH 7.4, it is clear that the formation constants increase with peripheral alkyl substitution of the ring.

In previous work with the corresponding copper complexes, the reduction potential, $E_{1/2}$, determined at pH 9.1, was employed as a measure of substituent effects seen at the metal center. The more negative the potential, the more electron density is released to the metal by the substituent (2). Using this index of substituent effect, a satisfactory linear free energy correlation is obtained (Fig. 2). In parentheses are indicated the percentage of complex undissociated in human plasma (6). The value

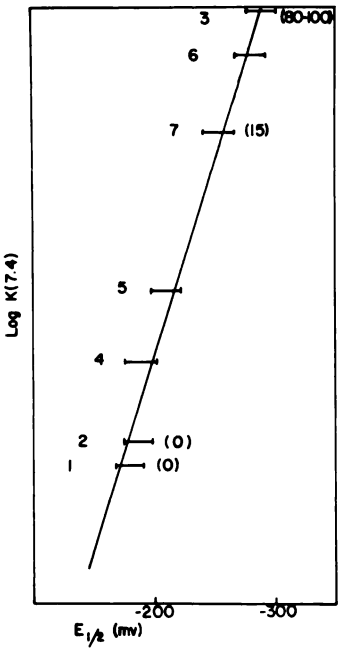


FIG. 2. *Linear free energy correlation for bis(thiosemicarbazonato)-zinc complexes*

Reduction potentials were taken from ref. 2, and log K(pH) for *1–3*, from ref. 6. The range of $E_{1/2}$ is ±10 mV. Numbers in parentheses refer to percentage of complex undissociated in plasma (this work and ref. 6).

for ZndAcTS is from the current work and is the average for two trials. As with the other complexes, the absorbance maximum for ZndAcTS shifts from 400 to about 406 nm in going from aqueous solution to plasma. This indicates that the environment of the complex has some lipophilic character (4, 6). It is evident that only the most stable complexes can exist at all in a biological medium containing typical competing ligands for zinc, such as amino acids.

Partition coefficients (ρ) of $ZnKTSM_2$ and H_2KTSM_2. The distribution of $ZnKTSM_2$ between 1-octanol and water, pH 6.7, has been analyzed in terms of the following equilibria:

$$ZnKTSM_{2(aq)} \overset{\rho_1}{\rightleftharpoons} ZnKTSM_{2(oct)} \quad (5)$$

$$Zn^{2+}_{(aq)} + H_2KTSM_{2(aq)} \overset{K(pH)}{\rightleftharpoons} \cdot ZnKTSM_{2(aq)} \quad (6)$$

$$H_2KTSM_{2(aq)} \overset{\rho_2}{\rightleftharpoons} H_2KTSM_{2(oct)} \quad (7)$$

The results of two determinations at 25° are summarized in Table 3. $ZnKTSM_2$ is distributed preferentially into the nonpolar solvent. However, the solubility of the complex in water at pH 6.7 is also substantial, about 80 μM. Hence it is expected that in biological systems $ZnKTSM_2$ can move easily across lipid membranes and also develop significant concentrations in the aqueous phase. One of the interesting results here is that, because of the extremely large partition coefficient for H_2KTSM_2, $ZnKTSM_{2(aq)}$ can be partially dissociated

TABLE 2

pH-dependent binding constants of bis(thiosemicarbazonato)-zinc complexes

Complex	pH	log K(pH)
ZnPTS[a]	7.27	6.71
	6.64	5.23
	6.53	4.99
	6.45	4.90
	6.33	4.70
	6.21	4.52
	5.97	3.99
	log K(pH) = 2.06 pH − 8.47	
ZnPTSM[b]	6.66	6.81
	6.47	6.12
	6.37	5.99
	6.24	5.56
	6.10	5.30
	6.00	5.26
	5.80	5.04
	log K(pH) = 2.04 pH − 7.71	
$ZnPTSM_2$[c]	6.45	8.58
	6.05	7.97
	5.73	7.47
	5.62	7.15
	5.37	6.85
	5.21	6.52
	log K(pH) = 1.70 pH − 3.09	
ZndAcTS[d]	7.07	7.87
	6.46	6.45
	6.07	5.88
	5.92	5.53
	5.75	5.26
	5.66	5.11
	log K(pH) = 2.00 pH − 6.31	

[a] Molar extinction coefficient (ϵ) at 416 nm = 10,120 ± 400 M^{-1} cm^{-1}.

[b] ϵ (418 nm) = 11,400 ± 400 M^{-1} cm^{-1}.

[c] ϵ (436 nm) = 11,690 M^{-1} cm^{-1}.

[d] ϵ (400 nm) = 7200 ± 500 M^{-1} cm^{-1}.

as H_2KTSM_2 is removed from the equilibrium in aqueous solution described by Eqs. 6 and 7.

Reaction of $ZnKTSM_2$, Zn^{2+}, and H_2KTSM_2 with Ehrlich ascites cells. Data in Fig. 3 describe the effect of $ZnKTSM_2$ upon Ehrlich cells *in vitro*. There is a slow, time-dependent inhibition of respiration.

TABLE 3

Partition coefficient for $ZnKTSM_2$

Parameter	Value
$[ZnKTSM_2]_{initial,\ aq}$ (C_T)	259 μM
$[ZnKTSM_2]_{final,\ aq}$	14.5 μM
$[Zn^{2+}]_{aq}$[a] = $[H_2KTSM_2]_{oct}$	134 μM
$[ZnKTSM_2]_{oct}$[b]	111 μM
$[H_2KTSM_2]_{aq}$[c]	312 ρM
ρ_1	3.10×10^2
ρ_2	1.7×10^7

[a] Determined by addition of H_2KTSM_2 to aqueous solution to react with free Zn^{2+} to form $ZnKTSM_2$; $[Zn^{2+}]_0 \cong [H_2KTSM_2]_{aq} \sim 0$.

[b] $[ZnKTSM_2]_{oct} = C_T - [ZnKTSM_2]_{final,\ aq} - Zn^{2+}_{aq}$.

[c] Determined from $K(pH) = [ZnKTSM_2]/[Zn^{2+}][H_2KTSM_2]_{all\ forms}$, in which $\log K(6.7) = 8.54$.

Nearly complete inhibition is observed when these experiments are carried on for long periods. As shown in the inset, the process is concentration-dependent. To obtain 75% inhibition at 1 hr, a concentration ratio of 75 nmoles of $ZnKTSM_2$ per milligram of cells was employed. When 70 nmoles/mg of $ZnSO_4$ are added to cells, substantial, though less pronounced, inhibition of respiration is seen.

Of particular interest is the dynamics of the respiratory response of ascites cells to the ligand H_2KTSM_2. Initially there is stimulation, followed by progressive inhibition of respiration. The percentage of control respiratory stimulation in four different tumor cell populations treated with 65–196 nmoles of ligand per milligram of protein is 134 ± 17 (SD). No obvious concentration dependence is observed. As indicated below, H_2KTSM_2 itself has no effect upon isolated heart mitochondria. A possible explanation for this observation is outlined under DISCUSSION and is based on the hypothesis that some Zn^{2+} is normally bound to mitochondria in the Ehrlich cell.

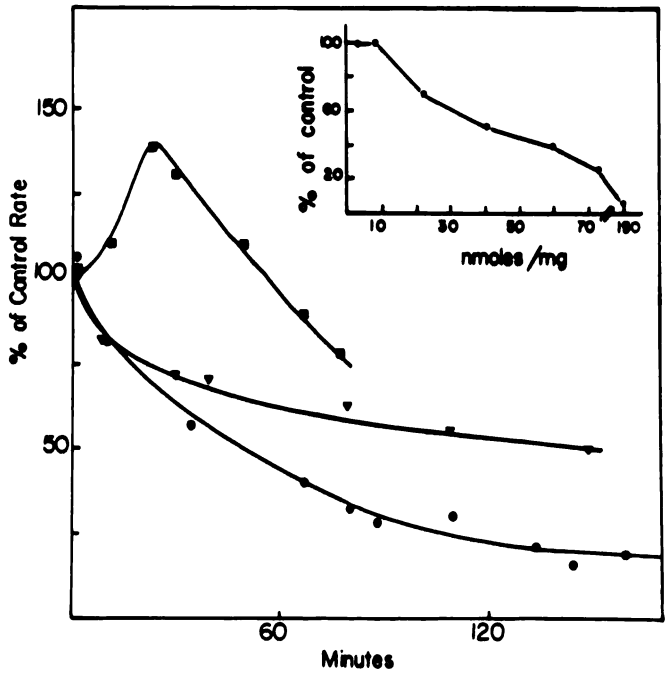


FIG. 3. *Time dependence of tumor cell respiration in the presence of $ZnKTSM_2$ (●, 75 nmoles/mg), $ZnSO_4$ (■, 70 nmoles/mg), and H_2KTSM_2 (■, 65 nmoles/mg)*

The inset shows concentration dependence of inhibition of cell tumor respiration by $ZnKTSM_2$ after 60 min of incubation, expressed as rate of respiration (sample)/rate of respiration (control) × 100.

Reaction of $ZnKTSM_2$ with Ehrlich tumor mitochondria. Figure 4 summarizes the effect of $ZnKTSM_2$ upon respiration in tumor mitochondria after incubation at 0° for 30 min. Both respiratory states show progressive inhibition, with state 3 somewhat more sensitive than state 4. The initial plateau is due to the presence of EDTA in the mitochondrial medium. Because EDTA is necessary to stabilize these mitochondria in the final stages of preparation, it cannot be deleted. As shown in other work, this chelating agent competes successfully with H_2KTSM_2 to bind zinc.[2] Hence, below the EDTA concentration in these preparations, as shown in Fig. 4, little if any $ZnKTSM_2$ is present. Once all the EDTA has become complexed with Zn^{2+}, extensive inhibition occurs upon the addition of more $ZnKTSM_2$. Because of this problem and the relatively greater availability of heart mitochondria, other experiments were done with beef heart mitochondria.

Reaction of $ZnKTSM_2$ with beef heart mitochondria. $ZnKTSM_2$ reacts with phosphorylating mitochondria to inhibit the synthesis of ATP. Figures 5–7 show the extent of state 4 and state 3 inhibition at various ratios of complex and mitochondrial protein, for different substrates, after at least 30 min of incubation at 0° to assure complete reaction.[3] Several different mitochondrial preparations exhibit titration-like behavior, in which respiratory stimulation by ADP is completely abolished at about 25–30 nmoles of $ZnKTSM_2$ per milligram with glutamate, α-oxoglutarate, or pyruvate-malate as substrate. The inhibition may be somewhat less effective for succinate, with which complete inhibition of state 3 should approach 40% of the control rate at 30 nmoles of $ZnKTSM_2$ per milligram, given an initial respiratory control ratio of 1.8 and approximately 70% of control for state 4. In no case is inhibition reversed by 2,4-dinitrophenol.

[2] D. Minkel and D. H. Petering, unpublished observations.

[3] A reviewer has suggested that the lengthy incubation may lead to indirect effects upon the mitochondria as a result of damage of lysosomes by $ZnKTSM_2$.

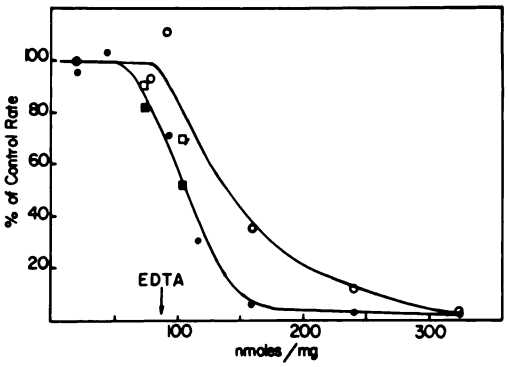


FIG. 4. *Degree of inhibition of tumor mitochondrial respiration vs nmoles $ZnKTSM_2$/mg mitochondrial protein.*

Substrates in state 4 are: ○, succinate; □, glutamate; in state 3, ●, succinate; ■, glutamate. The concentration of EDTA in the assay mixture is shown.

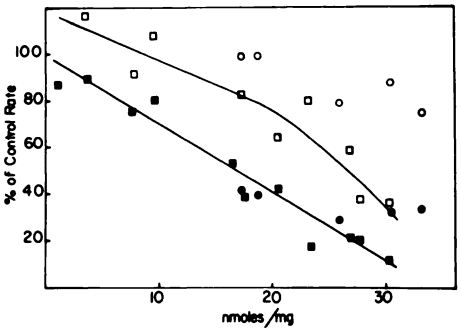


FIG. 5. *Degree of inhibition of heart mitochondrial respiration vs nmoles $ZnKTSM_2$/mg mitochondrial protein.*

Substrates in state 4 are: ○, α-oxoglutarate; □, glutamate; in state 3, ●, α-oxoglutarate; ■, glutamate.

Small effects on state 4 are seen most clearly with pyruvate-malate, and possibly with succinate and α-oxoglutarate. In contrast, at higher concentration ratios $ZnKTSM_2$ presumably can inhibit the oxidative deamination of glutamate to α-oxoglutarate (Fig. 5).

Although $ZnKTSM_2$ may react to a small extent either with the dehydrogenases generating NADH or with components of the electron transport chain to depress state 4 rates, the major sites of inhibition are located in the coupling mechanism between the chain and ATP. The reaction does not uncouple the oxidation-reduction process from phosphorylation, for in the region of partial inhibition

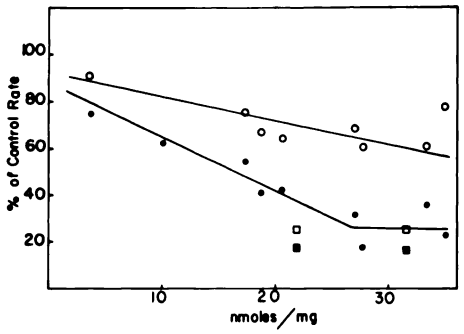


FIG. 6. *Degree of inhibition of heart mitochondrial respiration in states 4 and 3 vs nmoles of Zn or Zn^{2+}/mg of mitochondrial protein.*

Pyruvate-malate was the substrate. State 4: ○, $ZnKTSM_2$; □, Zn^{2+}. State 3: ●, $ZnKTSM_2$; ■, Zn^{2+}.

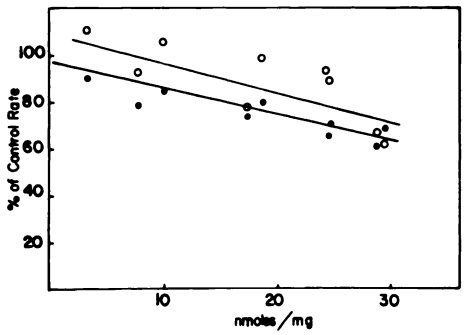


FIG. 7. *Degree of inhibition of heart mitochondrial respiration vs nmoles $ZnKTSM_2$/mg mitochondrial protein.*

Succinate was the substrate. ○, state 4; ●, state 3.

of state 3 using any of the substrates $ZnKTSM_2$ has no effect upon the P:O ratios relative to controls.

In other experiments amounts of H_2KTSM_2 as large as 65 nmoles/mg did not influence oxidative phosphorylation. In contrast, as observed by others, Zn^{2+} is a strong respiratory inhibitor (10). For instance, Fig. 6 shows the effect of two $ZnSO_4$ concentrations upon state 4 and state 3 processes. However, electron transport is much more sensitive to Zn^{2+} than to $ZnKTSM_2$. Hence the characteristics of inhibition by $ZnKTSM_2$ are distinct from those of Zn^{2+}.

This difference between Zn^{2+} and $ZnKTSM_2$ can also be seen using Keilin-Hartree particles, which directly oxidize NADH as well as succinate but do not synthesize ATP. In Fig. 8 the parallel inhibition of NADH- and succinate-dependent oxygen consumption by Zn^{2+} follows a much steeper titration curve than does inhibition by $ZnKTSM_2$. In both cases the similar degrees of inhibition of NADH- and succinate-dependent respiration are consistent with the site of inhibition lying beyond the entrance point into the chain of succinate electron equivalents, perhaps between cytochromes *b* and c_1, as previously hypothesized (10, 11).

DISCUSSION

Van Giessen *et al.* (1) have described the effect of combinations of metal ions and peripheral ring substitution upon the cytotoxicity of bis(thiosemicarbazones) *in vitro*. In that study the index of cytotoxicity was the degree of inhibition of cellular respiration. In the present work two approaches were taken to increase the understanding of these events. Formation constants of several zinc complexes were determined to solidify the structure-function analysis. Second, properties of the inhibition of respiration of cells and mitochondria were examined as a basis for consideration of the mechanism of cytotoxicity of $ZnKTSM_2$.

Reviewing the results of the first approach, Fig. 2 shows the linear free energy correlation between log K(pH) of the var-

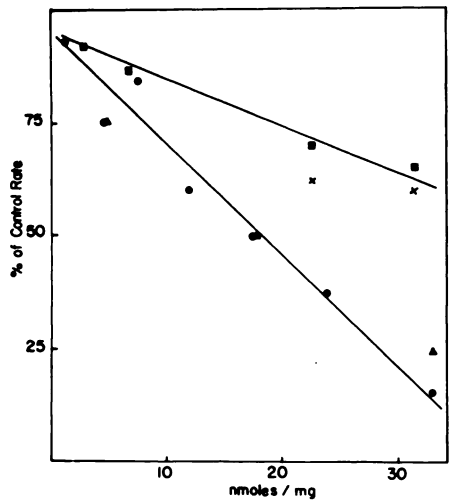


FIG. 8. *Degree of inhibition of Keilin-Hartree particles vs nmoles of $ZnKTSM_2$ as Zn^{2+}/mg of particle protein.*

Zn^{2+}: ●, NADH; ▲, succinate. $ZnKTSM_2$: ■, NADH; ×, succinate.

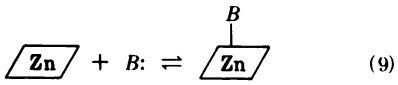
iously substituted zinc complexes and $E_{1/2}$ for the corresponding copper complexes. The observed linear relationship is reasonable on the basis that changes in $E_{1/2}$ with substituent, reflecting changes in electron density on the copper, will also be seen in ligand binding processes which involve other metal ions or proton, particularly if the mode of ligand binding is predominantly electrostatic. The plausibility of this correlation is discussed thoroughly elsewhere (2).

Included in this correlation are indications of the stability of several complexes in human plasma. Plasma stability has been used as an empirical measure of the stability of metal complexes in biological media containing a variety of competing ligands. Since ZndAcTS is only marginally stable, a log $K(7.4)$ approaching 9.7, the value for $ZnKTSM_2$, is necessary to ensure the existence of zinc complexes in this common medium.

Having established that $ZnKTSM_2$ may exist in biological systems, one may turn to possible mechanisms of reaction of this complex with cellular constituents. Two general modes may be suggested on the basis of the typical chemical behavior of zinc complexes, ligand substitution:

$$ZnKTSM_2 + H_2L_{cell} \rightleftharpoons ZnL + H_2KTSM_2 \quad (8)$$

and adduct formation:



$ZnKTSM_2$ (9)

Reaction 9 may include a charge transfer complex between the ligand system of $ZnKTSM_2$ and an aromatic site of the cell. Although adducts between $ZnKTSM_2$ and Lewis bases have not been detected, present information does not rigorously exclude this reaction. An additional qualification applies to either mechanism. Since data on the partition of $ZnKTSM_2$ between aqueous and lipid phases shows that solubility of the ligand in lipid can shift the equilibrium concentration of zinc, ligand, and complex, the physical process of solvent partitioning may also play a role in the interaction of the complex with cellular components. Nevertheless, the first mechanism seems likely, and subsequent discussion is based upon its presumed operation.

Recently several articles have shown that Zn^{2+} is a chain inhibitor in heart mitochondria from various sources (10–12). At least one site of inhibitory binding of the ion appears to be between cytochromes *b* and *c* (10, 11). It is striking, therefore, to observe the characteristics of inhibition of respiration by $ZnKTSM_2$. The electron transport chain is relatively insensitive to the chelate, whereas the coupling systems are the primary sites of reaction.

A qualitative explanation for the differing results with metal ion and metal complex depends on the assumption of at least two different types of binding sites for Zn^{2+} located in the chain and in coupling sites, which have markedly different binding constants for zinc. The former does not compete well, thermodynamically, with H_2KTSM_2 for Zn^{2+}; the latter does. Nichols and Malviva (11) have determined an equilibrium constant of 1.2×10^4 for the association of Zn^{2+} with the electron transport chain in Keilin-Hartree heart muscle particles. In whole mitochondria from rat liver a binding constant of 1×10^6 has been reported (12). This is too small to cause much dissociation of $ZnKTSM_2$. If the affinity of zinc-binding ligands for Zn^{2+} in the coupling system is large enough to remove the metal from $ZnKTSM_2$, they must also, of course, bind zinc ion well. Previous authors have focused on the interaction in the chain and have not examined this question to see whether Zn^{2+} also influences energy-coupling reactions.

At a higher level of organization, $ZnKTSM_2$ also inhibits respiration of Ehrlich tumor cells. The complex is readily taken up by cells because of the favorable partition of $ZnKTSM_2$ between lipid and aqueous media. Then, as in the studies with isolated tumor mitochondria, inhibition of respiration takes place. However, an additional factor is operative. H_2KTSM_2 is not simply a lipophilic carrier for zinc to facilitate its movement across membranes. It also serves to prevent wide-

spread, nonspecific binding of zinc by the variety of cellular materials which have metal binding capacity. That is, with a log K(7.4) of 9.7, $ZnKTSM_2$ is a thermodynamically stable complex which will not be dissociated by typical cellular ligands such as amino acids. Its reactions are then restricted to the metal-binding ligands with very large affinities for zinc.

Considering the explanation of the kinetics of respiratory effects of H_2KTSM_2 with tumor cells (Fig. 3), the initial stimulation followed by inhibition may be interpreted using results gained with heart and tumor mitochondria. Zinc appears to bind to H_2KTSM_2 more firmly than to the chain. Yet there are sites which react with $ZnKTSM_2$, leading to depression of the rate of respiration. Furthermore, H_2KTSM_2 does not directly affect mitochondrial respiration. Assuming some that zinc is bound to mitochondrial electron transport chains (ETC) in the cells as isolated, the initial observed rate of oxygen consumption will be less than maximal.

$$\underset{\text{respiring}}{\text{ETC}} + Zn^{2+} \rightleftharpoons \underset{\text{inhibited}}{\text{ETC}\cdot Zn} \qquad (10)$$

When H_2KTSM_2 is added, $ZnKTSM_2$ forms, leading to transient increases in active ETC.

$$\text{ETC}\cdot Zn + H_2KTSM_2 \rightarrow \text{ETC} + ZnKTSM_2 \qquad (11)$$

Then $ZnKTSM_2$ reacts with the second site to abolish state 3-dependent respiration.

If this is a correct analysis, there is now preliminary evidence, at least in this cell line, for the physiological operation of the zinc-binding site in electron transport chains in the control of electron flow.

Of general interest in this work is the finding that appropriate ligands bound to biologically essential metals such as Zn^{2+} can generate potent cytotoxic entities. There is no need to restrict attention in the design of pharmacological agents to the use of exotic toxic metals such as platinum. In fact, with either essential or nonphysiological metal ions, the mode of complexation largely determines the degree of toxicity to the organism. Additionally, given the unpublished finding[4] that $CdKTSM_2$ is highly toxic to tumor cells and that copper and iron complexes of 5-substituted 2-formylpyridine thiosemicarbazones and 1-formylisoquinoline thiosemicarbazone also have substantial cytotoxic properties, we suggest that a variety of metal chelates of both essential and nonessential toxic metals may show effects which warrant a general inquiry into the biological activities of metal complexes, a rich, relatively unexamined class of chemical compounds (13).

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[4] D. Solaiman and D. H. Petering, unpublished information.