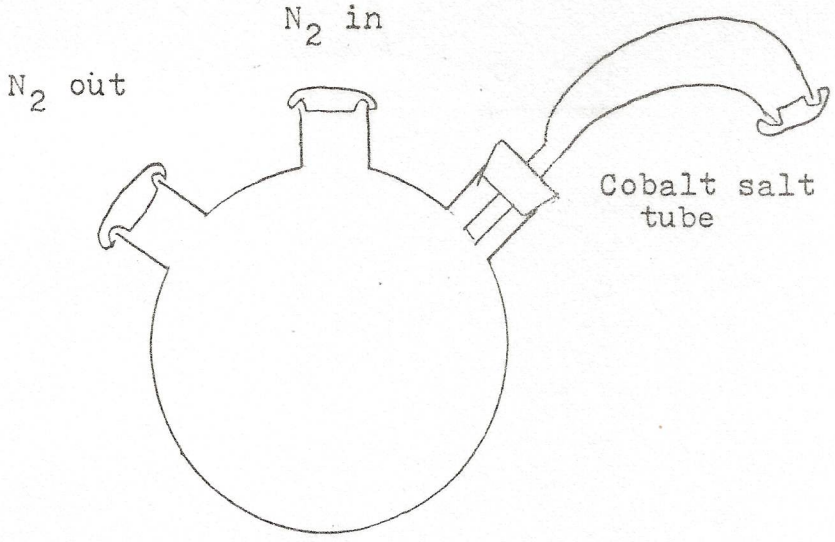


Fig. 11.

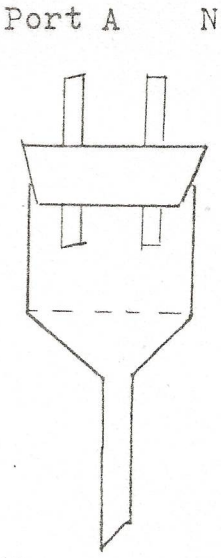


Fig. 12.

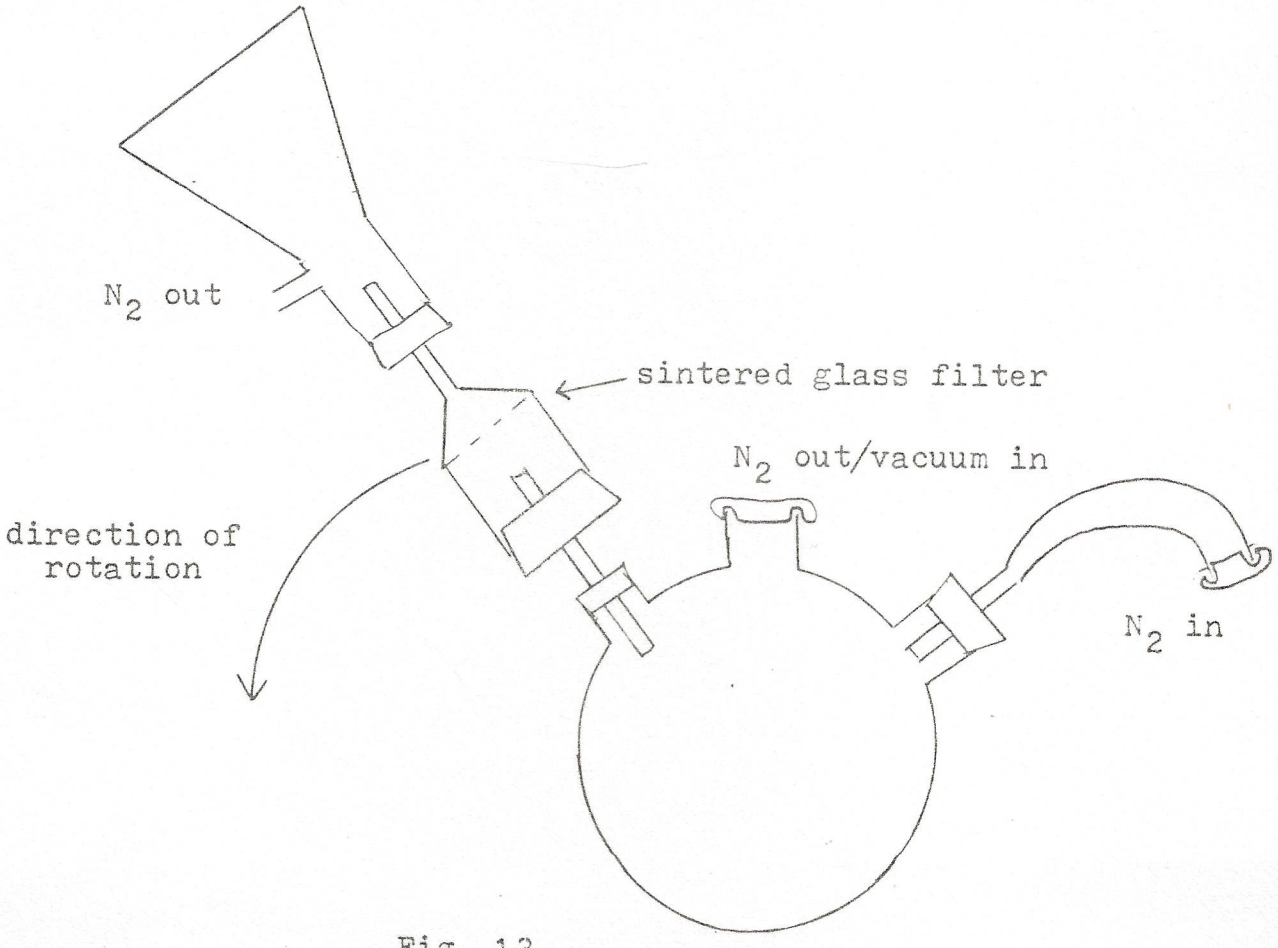


Fig. 13.

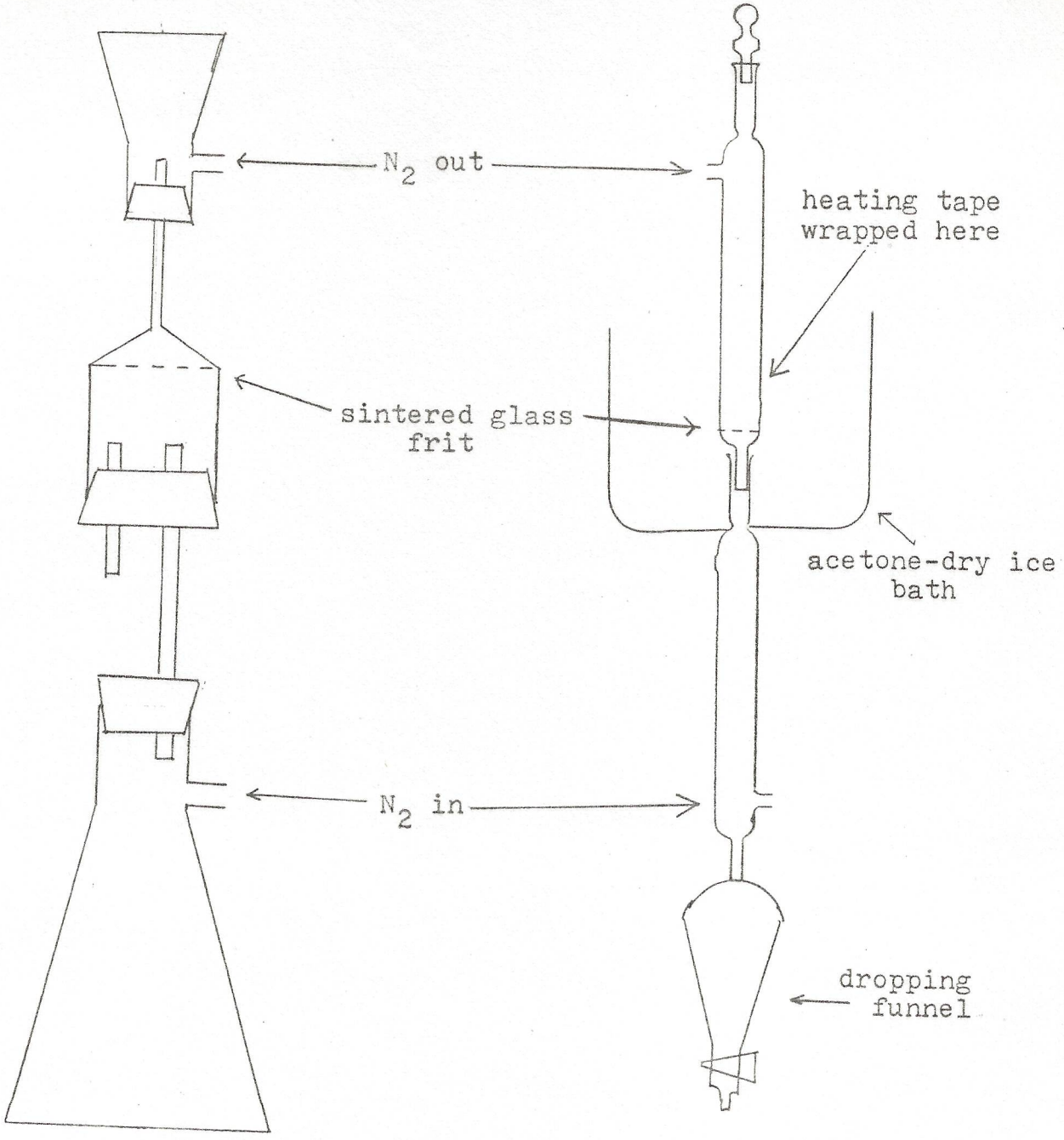


Fig. 14. Recrystalization apparatus. This was inverted twice.

Fig. 15. Low temperature recrystalizer. After the first filtration, the dropping funnel was quickly placed at the top for the second filtration.

The fault was attributed to contact with oxygen during transfer of the reaction mixture to the filtering apparatus by syringe. The apparatus was modified to eliminate this step (figure 13).

The butanol was not filtered off, but evaporated in our second attempt. Hot toluene, degassed by bubbling N_2 through for one to two hours, was added. Filtration was accomplished by inverting the flask. During this operation, the reaction mixture turned black. Dark orange crystals which became darker upon exposure to air, were recovered.

Since the crystals were darker than those reported by Basolo, we made further efforts to exclude oxygen. The reaction was run a third time, in a glove bag, in addition to the N_2 purge used previously. The orange precipitate was simply filtered. The result was a fine orange powder which decomposed at 150-153°C. (see Runge 3).

For the recrystallization, we employed the apparatus in figure 14, as well as toluene which was degassed by distilling under N_2. The result was 1 gm. of bright orange crystals in the form of thin needles. They decomposed at 150-153°C., in an open tube. The yield was 28%.

Synthesis of the complexes with n=3-6 was then attempted. Since no synthesis for these complexes was known to us, we began by following the procedure we had evolved for the n=2 complexes. The complexes were synthesized and filtered in a glove bag, and recrystallized with the apparatus in figure 15.

B. Results and Discussion

The results are presented in Table 1:

N	Color of Reaction Mixture	Color of Filtered Precipitate	After Recrystallization
3	Dark Blue	Blue solid became light blue on drying	no crystals
4	Dark Orange	light green	no crystals (even with cooling to -70°C.)
6	Opaque green	light gray	no crystals (cooled to -78°C.)

We chose to attempt synthesis of the hexa complex first hoping the greater flexibility afforded by the longer chain would make it easiest to fabricate. Upon failure to observe crystals during recrystallization, the toluene containing the dissolved product was removed, leaving a gray solid. This solid was placed in a 1:4 pyridine-toluene solution, in the hope of observing a change in color. This change in color would indicate that oxygenation had taken place, and we did indeed have the complex, but somehow failed to recrystallize it. No color change was noted.

The n=3 complex was attempted next. Interestingly the filtrate, initially clear, became dark. This was an indication that the complex might not be in the precipitate, but might be soluble. Similar results were obtained for the n=4 complex. To test the theory that the higher homologs of Co(acacen) were soluble, the filtrates were evaporated and flame tests performed.

FLAME TESTS

Material	Flame Color
$CoBr_2$	purple
Cobalt Salt	purple
Ethyl complex	
oxygenated	orange
unoxygenated	red orange
Propyl complex	
precipitate	purple-blue
filtrate	gas-type flame
Tetra complex	
filtrate	gas-type flame
Hexa complex	
precipitate	purple
gray material	gas-type flame

These tests indicated no cobalt in the filtrate. The purple color in all the precipitates suggested that the cobalt, in one form or another, had indeed precipitated. The observed color changes in the filtrate may be attributed to some unknown side reaction, possibly an oxidation.

It seemed likely that the preparation of Co(acacen) could not be easily applied to the preparation of its higher homologs. We began to think of modifying the procedure with respect to choice of solvent, source of cobalt, temperature, length of reaction, etc. Since there was no obvious reason for our inability to extend the synthetic procedure for Co(acacen) to its homologs, the modifications of procedure would have to be made arbitrarily.

Faced with the dilemma of investing a large amount of time testing a large number of arbitrarily chosen possible modifications or to shift our attention to a similar, but new, series of molecules, we restricted ourselves to only two modifications.

We chose to vary both the solvent and the source of cobalt ion. In one experiment, we replaced the tetraethylammonium cobaltate (II) with cobalt (II) acetate dissolved in methanol. The ligand was dissolved in methanol and triethyleneamine (Matheson). The apparatus in Fig. 16 was used. This approach lead to no precipitate. When the reaction mixture was placed on a rotary evaporator to remove the MeOH, a flaky purple material was recovered. This did not darken in air and was presumed to be cobalt (II) acetate crystals.

Our second endeavor was simply to replace butanol with N,N-dimethyl formamide. Using the apparatus in figure 11, we obtained for the n=3 ligand a precipitate that underwent a color change (blue to green) but would not crystallize. No further attempts were made to synthesize these complexes.

The isopropylsalicylaldehyde molecules were now chosen for study, despite the lack of proof for reversible oxygen binding. These molecules were nevertheless useful for our purpose because they exhibited the two essential properties of 1) sensitivity to oxygen and 2) varying degrees of distortion.

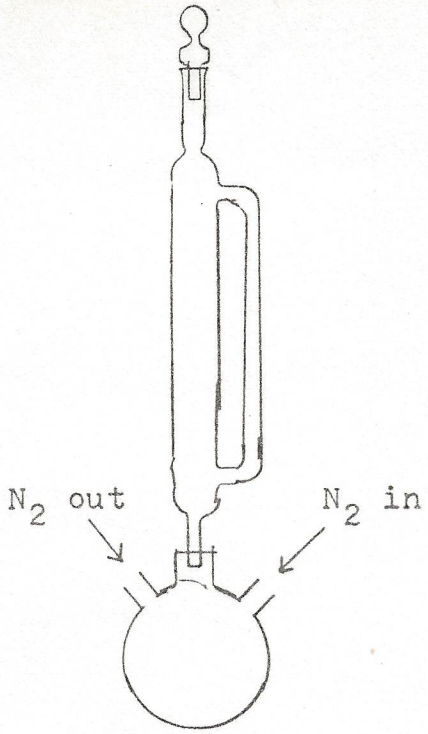


Fig. 16.

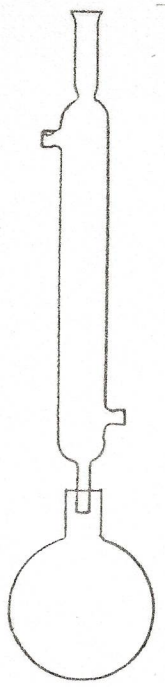


Fig. 17.

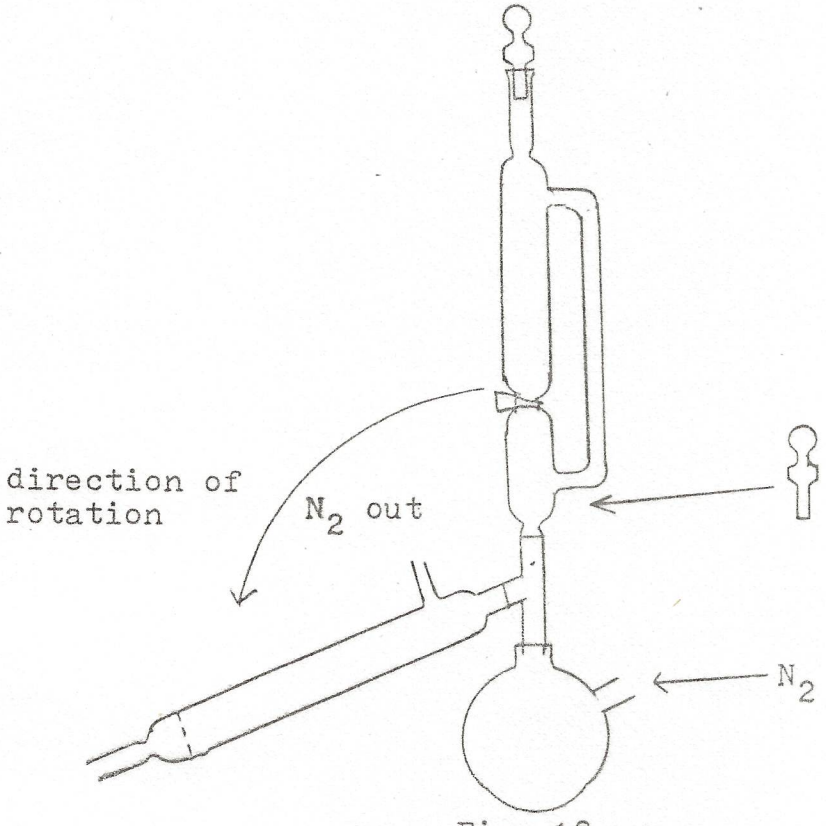


Fig. 18.
The dropping funnel was replaced with a glass stopper before rotation.

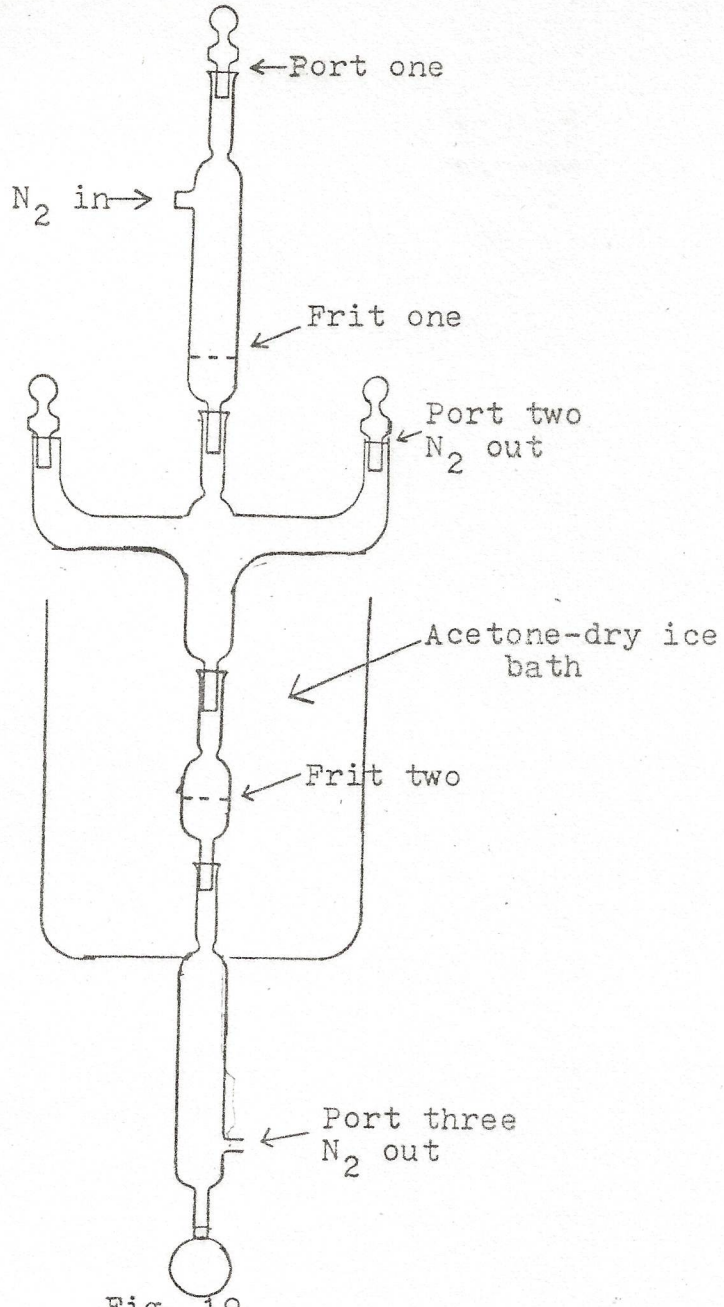

←Port one
N_2 in→
Frit one
Port two
N_2 out
Acetone-dry ice
bath
Frit two
Port three
N_2 out

Fig. 19.

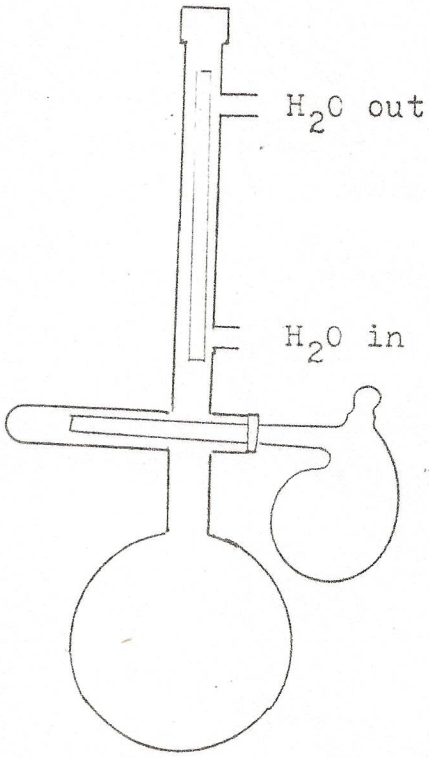

H_2O out
H_2O in

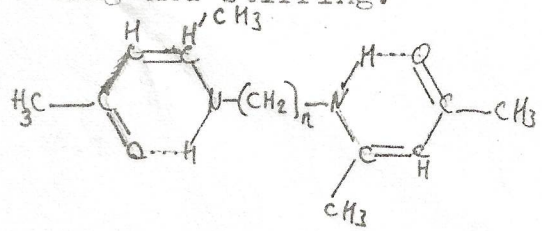
Fig. 20

III. SYNTHESIS OF N,N' ETHYLENEBIS(ACETYLACETONIMINATO)POLYMETHYLENEDIAMINE COBALT (II) COMPLEXES

. Material and Methods

a. Synthesis of ligands. To 20.0 gm. of acetylacetone (Baker) dissolved in 25 ml. of absolute ethanol, 6.0 gm. of the appropriate ligand* diluted with an equivalent volume (6.7 ml) of ethanol, was added dropwise, with cooling and stirring.

$$NH_2(CH_2)_n NH_2 + 2CH_3COCH_2COCH_3 \longrightarrow$$



$n = 2\text{-}7$

The yellow solutions were evaporated to dryness and the crystals recrystallized from toluene yielding lemon yellow crystals. The ligands were characterized by NMR spectroscopy.

b. Synthesis of tetraethylammonium tetrabromocobaltate. Tetraethylammonium tetrabromocobaltate (II) was prepared by reaction of:

$$CoBr_2 + 2(C_2H_5)_4N^+Br^- \longrightarrow [(C_2H_5)]_2\ [CoBr_4]$$

Five grams of $(C_2H_5)_4NBr$ (2.4×10^{-2} moles) was dissolved in 50 ml. ethanol. Four grams of $CoBr_2$ (Fischer) was also dissolved in

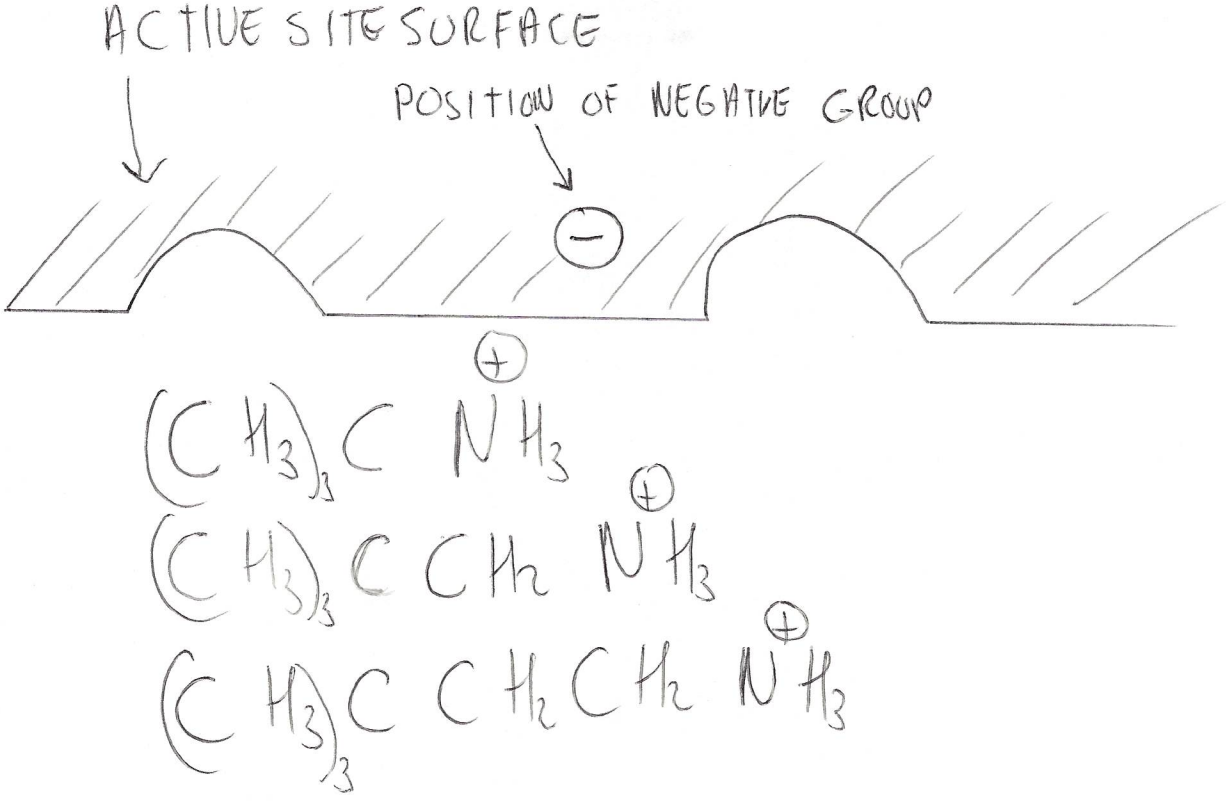


Fig. 3. t-Butyl Amine and neo-Pentyl Amine have K_i s that are about equal, and neo-Hexyl Amine has a K_i that is about 2.5 times greater. This indicates that an anionic group may exist in the active site in approximately the position shown.

The alternate explanation for the amide results is that the three K_i values are essentially the same. The error in the determination of a K_i can be as high as 100%, thus, it is not possible to attach too much significance upon a difference of 2.5 times. This explanation implies that the electrostatic interaction is not important, or that the anionic group of the enzyme is so placed or so dispersed that each of the amides can take commensurate advantage of the electrostatic force.

Why are the butyl and pentyl acetamides less effective inhibitors than the amines? The most obvious difference between the amines and the acetamides is that the acetamides do not have a positive charge. This interpretation supports the existence of an anionic group on the active site. Furthermore, the binding of the amides may be further impeded because they are better solvated that the amines.

However, the hexyl acetamide shows an inhibition ability that is better than the butyl or pentyl acetamides, indeed, it is approximately as effective an inhibitor as the hexyl amine. This observation can be explained by the presence of a methyl group in the proper position to interact with a methyl site on the enzyme; the hydrophililic contribution to the binding of this methyl site interaction is thus approximately equal to the electrostatic interaction possible in the amines. See Figure 4.

The butyl and pentyl acatamides show a noncompetitive component to the inhibition at the higher inhibitor concentrations. (See Tables 4 and 5) The Hexyl acetamide was not tested at high concentration and thus may or may not follow the same behavior.

Noncompetitive inhibition in acetylcholinesterase is usually explained by the formation of an enzyme-substrate-

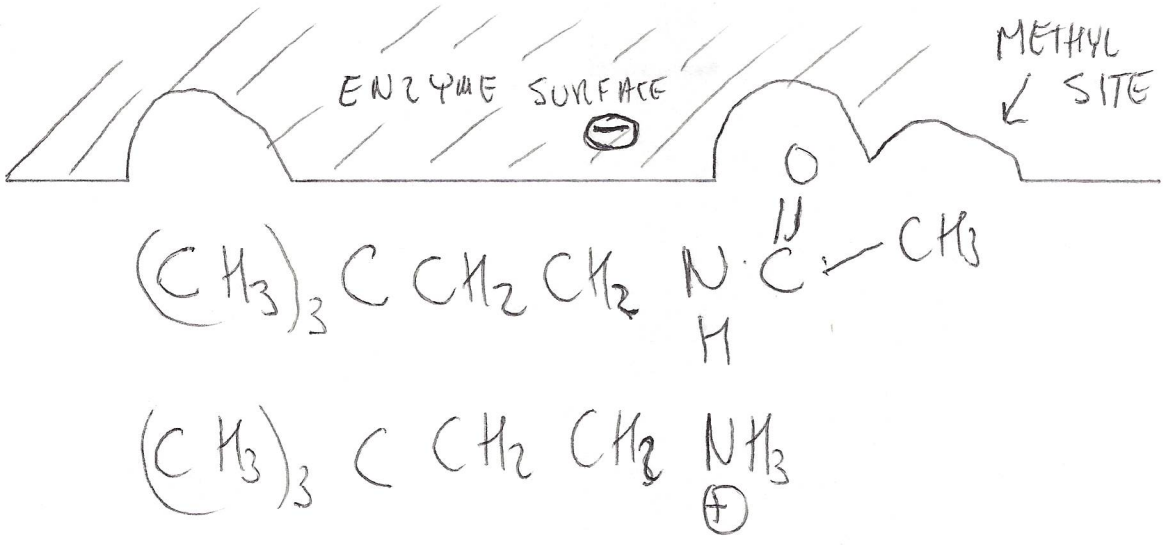


Fig. 4. The hexyl acetamide may bind better than the butyl and pentyl acetamides because the methyl group is in the proper position to interact with a methyl site. This interaction is approximately equal to the electrostatic interaction postulated for the hexyl amide.

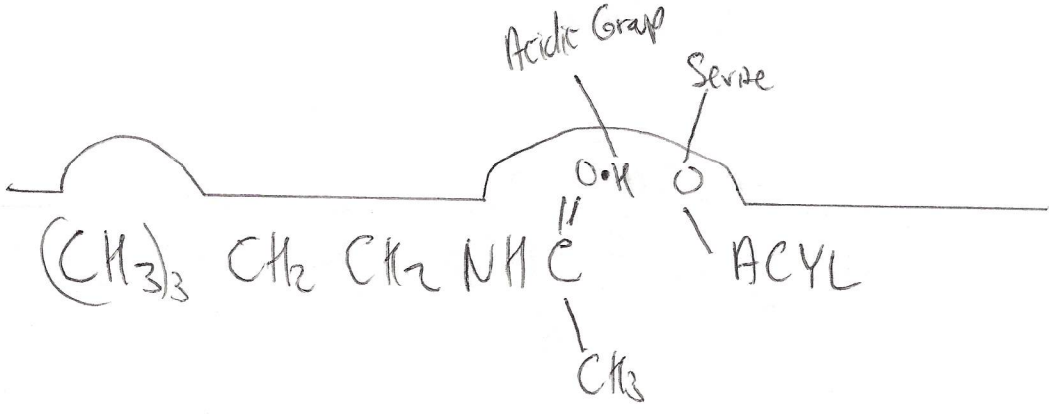


Fig. 5. The acidic group necessary for deacylation may hydrogen bond to the carbonyl of the inhibitor, thus preventing deacylation and introducing a noncompetive component to the inhibition type.

inhibitor (ESI) complex or an acylenzyme-inhibitor (EAI) complex.

An ESI complex could be formed by the adsorbtion of the inhibitor at an allosteric site. Presumeably, a comformation change makes the ESI complex inoperative. Desorption of I from ES could be slow or nonexistent.

If an EAI complex is formed, noncompetiveve inhibition is caused by prevention of deacylation of the enzyme. This may be due to the interaction of the electronegative carbonyl oxygen with the acidic group in the active site which is postulated to be important in deacylation. (See Figure 5.) Alternatively, the deacylation could be prevented by misaligning essential groups in a conformation change. Formation of an ESI or an EAI complex can be distingushed by noting the relation between K_i and V_m. See Biochem 3: 1749.

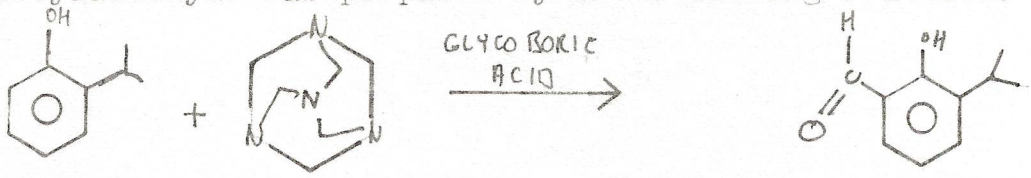
The introduction of a noncompetitive component at high inhibitor concentration may also be a nonspecific effect. The high concentration of the inhibitor may partially denature the enzyme. This possibility may be tested with coumpounds such as n-propyl amide, which lacks the t-bytyl group, and would presumeably not bind to or act at the the active site.

Finally, it should be noted that the possibility that the acetamides are hydrolized by the enzyme was not investagated.

IV. SYNTHESIS OF N,N'-BIS(3-ISOPROPYLSALICYLIDENE)POLYMETHYLENEDIAMINE COBALT(II)

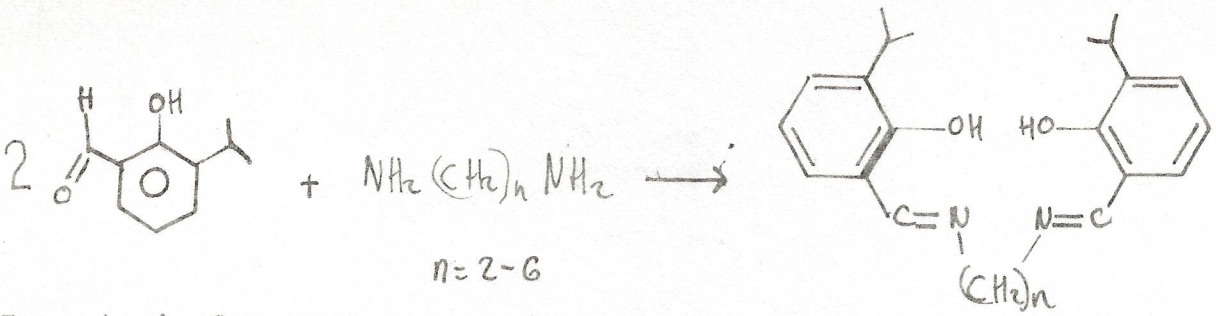
A. Materials and Methods

a. Preparation of 3-isopropylsalicylaldehyde. 3-isopropylsalicylaldehyde was prepared by the following reaction:



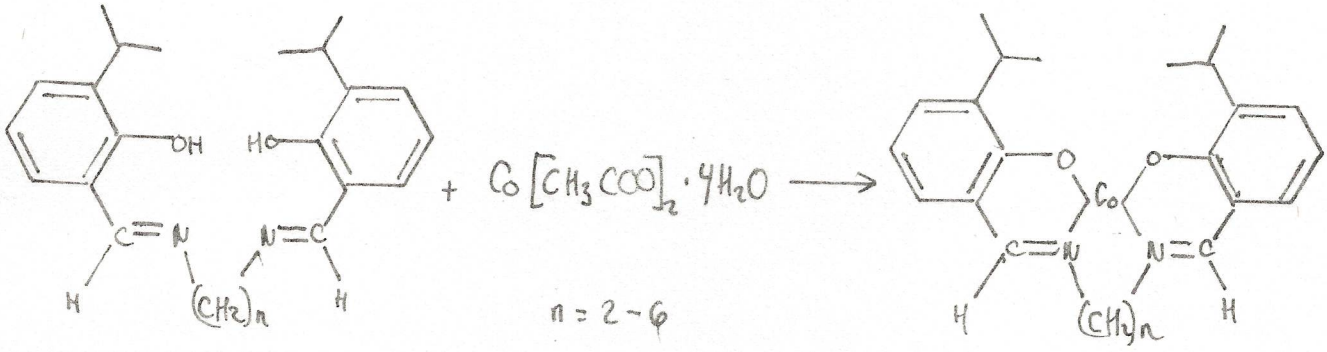
In a 1000 ml 3 neck round-bottom reaction flask, 240 ml of glycerine (Fisher) was heated for 1.5 hours and the temperature was then held at 170°C for 0.5 hours, at which time the resulting glycerol borate was cooled to 160°C. A total of 50 gm. of hexamethylenetetramine (Matheson,Coleman,&Bell) and 50 gm. of 2-isopropylphenol(Fisher) was added rapidly and alternately in small portions. To moderate the violently exothermic reaction, the flask was cooled in an ice bath. The reaction mixture was maintained at 150°C for 7 min., and then rapidly cooled to 110°C by running a stream of tap water over the outside of the flask. When the temperature reached 110°C, a mixture of 45 ml. concentrated sulfuric acid(Fisher) and 150 ml. water was added and the mixture was immediately steam distilled. Approximately 200 ml. of a cloudy distillate came over at 100-104°C. The distillate had a yellowish layer on top. The distillate was placed on a rotary evaporator, leaving 3 gm. of a sweet smelling light yellow oil. This was characterized by NMR spectroscopy.

b. Preparation of the ligands. The ligands were prepared according to the following general reaction:



In a typical reaction, 0.018 moles of the 3-isopropylsalicyaldehyde and 0.008 moles of the appropriate diamine were dissolved in 10 ml. of methanol each, and poured into a 50 ml. round bottomed flask. This was heated under reflus for 20 minutes, employing the apparatus in Fig. 17. The reaction mixture was then placed on a rotary evaporator. The di-, tri-, and pentamethylene ligands were yellow oils, and the tetra- and hexamethylene ligands were yellow crystals. The crystals were recrystalized from methanol. The oils were examined by thin layer chromatography and found to consist of two components in each case. They were therefore purified by column chromatography, on neutral alumina with benzene and 10-20:1 ether-benzene as eluents. A light yellow component was eluted first, and an orange-yellow component was left behind on the column. Approximately 1.5 gm. of the oil was collected from the various fractions. Thin layer chromotography was employed on several fractions to check on the purity. The oils were characterized by NMR spectroscopy.

c. Preparation of the complex. The cobalt complexes were prepared according to the following reaction:



The typical procedure was: 1.8 gm. of the appropriate ligand and a slight excess of $Co(CH_3COO)_2 \cdot 4H_2O$ (Fisher) were dissolved in

separate portions of boiling methanol. The ligand solution was placed in a 100 ml. round bottomed flask and the cobalt acetate solution in a 60 ml. dropping funnel with a pressure equalizing sidearm.(Fig. 16). The apparatus was flushed with N_2 for 15 minutes. The acetate was then allowed to drop slowly, with stirring, at room temperature, into the solution of ligand. After 15 minutes, the apparatus was modified by quickly replacing the dropping funnel with a glass stopper, under positive N_2 pressure. The entire apparatus was then rotated to the left, allowing the reaction mixture to be filtered under N_2 pressure. Pertinent observations are summarized in the following table:

COMPLEX REACTION OBSERVATIONS

N	Reaction Mixture	Precipitate	Remarks
2	Dark brown red	Dark red	
3	Red	Red	Filtrate went from pink to dark green
4	Dark red, then dark green	Light green	
5	Orange yellow	yellow	Ppt. became green upon exposure to air
6	Orange	orange-brown	

d. Recrystallization of the Complexes. The apparatus is in Fig. 19 was used. The complex was placed on the first frit and heating tape was wrapped around the area. The apparatus was flushed with N_2 for 15 minutes. Toluene, distilled under nitrogen, was introduced through port one. The toluene was heated to boiling to dissolve the complex. Port two was opened next, leaving port three closed. The hot toluene

TABLE TWO
Results of Oxygen Sensitivity Test
(glove bag & deoxygenated solvents)

COMPLEX / SOLVENT & CONDITIONS		DI	TRI	TETRA	PENTA
TOLUENE	IN GLOVE BAG	light orange	very light yellow	light yellow	color-less
	WITH PYRIDINE	no change*	dark yellow	no change	no change
METHYLENE CHLORIDE	IN GLOVE BAG	orange red	yellow green	yellow green	color-less
	IN AIR	no change	dark green	no change	no change
	WITH PYRIDINE	darker	still dark green	no change	no change

for the di- and trimethylene complexes but not for the tetra- and pentamethylene complexes.

b. Redox Potentials. The set of ligands was examined on a cyclic voltammeter built in the Chemistry department. A 0.01M solution of $CH_3(CH_2)_3N^+PF_6^-$ in methylene chloride(Fisher) was used as the solvent. The concentration of the ligands was 0.01M. The half-wave potentials for oxidation and reduction were found to depend on such factors as whether N_2 or air was bubbled through the solution, whether the solutions were made up in N_2 or in air, whether or not pyridine was added, and the order in which these operations were performed. A large number of scans were taken, and the following pattern emerged.

The di- and hexamethylene ligands showed neither oxidation nor reduction waves. The solvent showed no waves after bubbling with N_2, except for a peak at -1.75v, which was outside of the range I concentrated on.

The dimethylene complex exhibited no waves after bubbling with N_2. If pyridine or oxygen were introduced, a reducing peak at -.7v and an oxidizing peak at +.2v appeared. Oxygen bubbling resulted in a color change and displaced the reducing peak to lower values, but subsequent N_2 bubbling could restore the reducing peak to -.7v. The reducing peak was more pronounced than the oxidizing peak.

The trimethylene complex exhibited no waves if prepared under N_2. Addition of pyridine produced reducing peaks at -.75v, and an oxidizing peak in the region of -.65v. These peaks were not displaced by N_2 bubbling. Air bubbling turned the solution dark green, and two sets of peaks were observed; the one noted above for pyridine alone and a reducing peak at -.5v and a much smaller oxidizing peak at -.3v.

No lasting peaks were observed when the tetra- and penta-

does.

One enigma arises from our observations. Hariharan and Urbach reported that all complexes would have no differences in electronic structure when pyridine is present. Yet the dimethylene complex binds oxygen after addition of pyridine whereas the tetra- and pentamethylene complexes do not. This observation is evidence that some factor other than strain may be implicated in oxygen binding. A condition for further study is that the ligand is not entirely equatorial in the tetra- and pentamethylene complexes, but may be deployed as shown below.

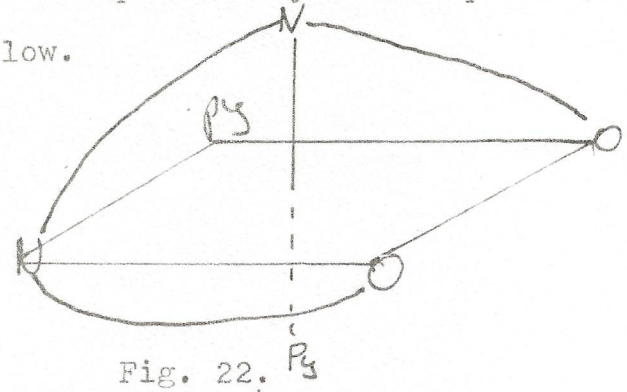


Fig. 22.
Possible Structure of n=4,5 Complexes (CIS)

The importance of a strong electron donor in the axial position was discussed above, in section II F. The geometry shown above would prevent an axial electron donor, such as pyridine, from donating electrons to the d_{xz} orbital of cobalt, and thus prevent binding by not enhancing the flow of π electrons to oxygen. See Fig. 8.

V. CONCLUSION

The n=2-6 N,N' bis(isopropylsalicylidene)polymethylenediamine cobalt(II) complexes show a sensitivity to oxygen, and thereby an increase in electronic structure distortion (strain) in the order $3 > 2 > 4=5$. These results are consistent with the entatic state

and also in methylene chloride; the color was noted. The solutions were removed from the glove bag and color changes noted. The results are given in Table Two.

TABLE ONE

Results of Oxygen Sensitivity Test

SOLVENT & CONDITIONS \ COMPLEX		DI	TRI	TETRA	PENTA	HEXA
TOLUNE	AIR ALONE	red-orange	dark yellow -got darker	light yellow	colorless	light yellow
	PYRIDINE ADDED	purple	no change	no change	no change	no change
HEXANE	AIR ALONE	amber	light yellow	yellow green	colorless	light yellow green
	PYRIDINE ADDED	Dark purple	Dark yellow	no change	no change	no change

The color of the complexes matched the colors reported by Hariharan and Urbach. IR spectra were recorded on a Cary Model 14 Spectrophotometer. We were able to reproduce the reported spectra

methylene complexes were scanned. Air bubbling produced varying peaks, but in all cases these could be obliterated by N_2 bubbling.

c.Discussion. It appears that the tetramethylene and pentamethylene complexes do not react with oxygen. No color change was noted for these complexes under any conditions and no oxidation or reduction waves were measured. This observation is consistent with the entatic state hypothesis because these molecules are expected to have the least amount of strain imposed on the cobalt by the ligand.

Alternatively, two pieces of evidence for formation of an oxygen adduct exist for the trimethylene complex. A dramatic (light yellow to black) color change was noted upon exposure to atmospheric oxygen, even in the absence of pyridine. Moreover, the complex shows two strong reducing peaks. Since Basolo has postulated that Co(II) becomes Co(III) upon oxygenation, these reducing peaks also seem to indicate that it did react with oxygen; it is the Co(III) that is being reduced. The appearance of two peaks are consistent with either two separate, one electron reductions of a single species or perhaps the reduction of two species. Two possible species which give rise to the double peak may be a monomeric complex with pyridine coordinated axially above the plane and dioxygen below it; and a bridged species.

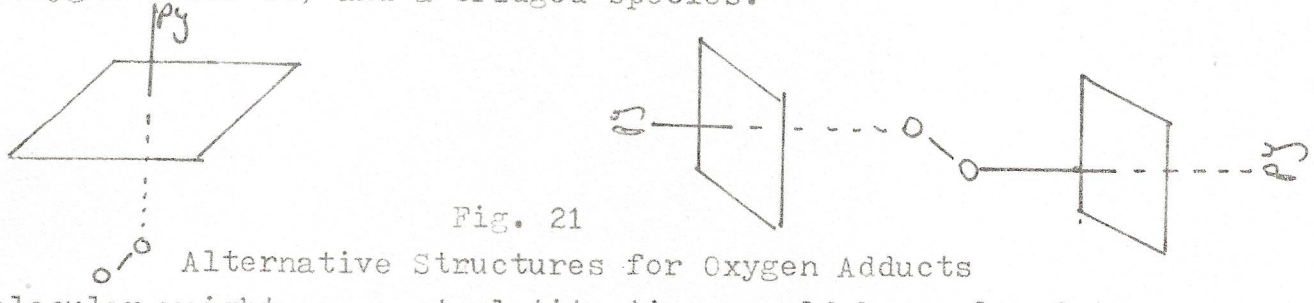


Fig. 21
Alternative Structures for Oxygen Adducts

Molecular weight or spectral titrations could be employed to distinguish between the two possibilities.

The comparitively high reactivity of the trimethylene complex

can be accounted for by recalling that its electronic structure was postulated to be a severly flattened tetrahedron. The dioxygen can make the flattened tetrahedron five-coordinate or possibly six coordinate, thus placing the molecule in a condition of lower energy (less strain).

The dimethylene complex did not become dark in air merely upon exposure, as the trimethylene complex did; pyridine was always required. Since the tetra methylene and pentamethylene complexes show no color change upon complexing with pyridine, the color change in the dimethylene complex is evidence for the formation of an oxygen adduct. Further evidence for the formation of an oxygen adduct comes from the observed reduction peak. The dependance on the presence of pyridine indicates a greater reluctance to bind oxygen. From this it can be inferred that the low spin, square planar complex is more stable than the high spin distorted tetrahedron, but less stable than the pseudotetrahedral structures of the tetra- and pentamethylene complexes

The single set of redox potentials indicates that only one species was formed, probably in a manner analogous to Co(acacen). The pyridine combines axially on one side of the plane initially, creating a five coordinate situation and preparing the way for an octahedral arrangement to be attained upon oxygen binding. The five coordinate dimethylene complex-pyridine adduct is an intermediate with a great tendency to become six coordinate. The pyridine donates electrons to the cobalt, and thus the cobalt can more readily bind to oxygen. Apparently, pyridine is not necessary in the trimethylene complex because the distorted tetrahedrom presents a condition closer to an intermediate than the square planar complex

hypothesis, if the n=4 and n=5 complexes do not have ligands which are equatorially deployed. The failure to reproduce IR spectra for the n=4 and n=5 complexes may cast some doubt on these conclusions.

This study has shown that continued work with this series of molecules, such as determining the stoichoimetries of the adducts, K_{O_2}, and reversibility of the reactions, is warrented.

was forced through the second frit, leaving any crystals to dry under a nitrogen stream on the second frit.

No crystals were recovered after repeated attempts for the di- and pentamethylene complexes. The tetramethylene complex left a crystaline light green material after recrystalization, which appeared to be identical with the starting complex. We could not ascertain how the apparatus or procedure could be ameliorated in order to produce crystals. It was decided to proceed with the unrecrystallized complexes. Careful purification of the ligands noted above was undertaken to insure that the unrecrystallized complexes would be reasonably pure.

B. DETERMINATION OF REACTIVITY

a. Reactions with oxygen. Up to this point the complexes were kept under nitrogen untill dry. Hariharan and Urbach reported marked sensitivity to oxygen in solution. To determine the sensitivity of these complexes to air, they were dissolved as noted below. Pyridine was also added because Balolo reported that a strong donor base was necessary for oxygen addition.

Therefore, between 0.001 and 0.002 gm. of the complexes was dissolved in toluene and in hexane; the color was noted. Than one or two drops of pyridine was added and the color noted. The results are given in Table One(next page).

In a second experiment, the complexes were first dried under vacuum using the apparatus shown in Fig. 20. Xylenes (Malinckrodt), b.p. 137-144°C, was refluxed to maintain a high temperature. All operations were carried out with deoxygenated toluene and in a glove bag. A spatula tip of the complex was dissolved in 5 ml. of toluene

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*METALLOENZYMES: THE ENTATIC NATURE OF THEIR ACTIVE SITES**,†*

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Communicated by F. Albert Cotton, December 20, 1967

Selective chemical modifications of specific amino acid side chains have been shown to affect the function of a large number of enzymes and constitute an apparent link between their chemical and catalytic properties.[2-4] The modifications indicate that particular side chains are exceptionally reactive toward certain reagents. This unusual reactivity has led to the hypothesis that side chains present in active, enzymatic sites are chemically atypical when compared to similar residues in analogous model substances or in catalytically inert regions of the protein. This view suggests the search for unusual physical features of these functional enzyme side chains which would define "atypical" character in terms of structural or electronic abnormalities of the groups or of their environment. In the case of most (nonmetallo-)enzymes this search has proved difficult since *alterations of catalytic activity* attending chemical modifications have usually served as the relative indices of the condition of the side chains. In certain cases anomalous pK_a values have also been reported but the difficulties associated with their interpretations are well known.[5] Thus, with this class of enzymes it has not been feasible to examine the possibility that enzymes might be *poised for catalytic action in the absence of substrate*, i.e., are in an *entatic state*.[1]

Metalloenzymes would seem exceptionally well suited for the examination of the physicochemical basis of enzymatic properties, for the physical and chemical characteristics of certain metals constitute *intrinsic* probes of their protein environments.[6-8] In fact, the systematic examination of the absorption, optical rotatory dispersion (ORD), and electron paramagnetic resonance (EPR) spectra, magnetic properties, redox potentials, and ligand binding has increasingly revealed that the properties of the intrinsic catalytically active metals of metalloenzymes are quite unusual when compared to those of well-defined model coordination complexes.[7, 8] Importantly, these properties are discernible *in the absence of substrate*. On the other hand, the features of the vast majority of metalloproteins presently thought to be enzymatically inert are comparable to those of known models emphasizing the unusual nature of metalloenzymes.[7, 9] The following discussion will summarize pertinent characteristics of some of the metals of metalloenzymes, here viewed as probes, and draw deductions as to their relevance to the catalytic potential of metalloenzymes.

Spectral Properties of Metalloenzymes.—Both the absorption and EPR spectra of copper and nonheme iron enzymes are readily accessible and reveal unusual properties when compared with the simple complexes of the same metals (Tables 1 and 2). Their features indicate an unusual environment, either by virtue of their intensities or by the location of the maxima and fine structure, jointly reflecting the unusual geometry of the metal site. In particular, the spectra of

the numerous typical "copper blue enzymes" have no known parallel with the spectra of any type of copper compounds, including all copper proteins currently known not to be enzymes, copper protein complexes, and complex ions (models).[9, 10] The absorption bands differ strikingly in intensity, i.e., transition

TABLE 1. *Spectral characteristics of copper (A), iron (B), and cobalt (C) complexes and enzymes.*

	Compound	λ, mμ (molar absorbance, M^{-1} cm^{-1})			Notes	Source
A.	*1. Simple Cu(II) Complexes:*					
	$[Cu(NH_3)_4]^{2+}$	600(~20)	660(~40)	750(~25)	Tetragonal	(1)
	Bis(3-phenyl-2,4 pentanedionato) copper(II)	490(~50)	520(~25)	580(~50) 650(~50)	Twofold axis	(2)
	$[Cu(H_2O)_2]^{2+}$	700(~5)	790(~10)	950(~5)	Tetragonal	(3)
	2. Cu-Protein Complexes:					
	Cu(II) carbonic anhydrase		760 broad	(120)	Unknown low symmetry	(4)
	Cu(II) serum albumin		570 broad	(90)	?	(5)
	3. "Inert" Cu Proteins:					
	Erythrocuprein		655 broad	(280)	Unknown symmetry, probably tetragonal	(6)
	Cerebrocuprein		655 broad	(400)		(7)
	4. "Active" Cu Proteins:					
	Plastocyanin	460(500)	597(4400)	770(1600)		(8)
	Laccase	450(970)	608(4000)	850(700)	Unknown but low symmetry	(9)
	Pseudomonas aeruginosa blue	500(weak)	625(~3000)	725(weak)		(10)
	Pseudomonas dentrificans blue	450(weak)	594(~3000)	800(1000)		(11)
B.	$[Fe(CN_6)]^{3-}$	General rise in absorption with but one or two discrete bands			Octahedral	(12)
	$[Fe(H_2O)_5OH]^{2+}$					
	Ferredoxin (spinach)*	325(5160)	420(5160)	463(4650) 570(shoulder)	?	(13)
C.	$[Co(H_2O)_6]^{2+}$	510(10)	1200(2)		Octahedral	(14)
	$[CoCl_4]^{2-}$	685(700)	1700(100)		Tetrahedral	(15)
	Co carbonic anhydrase	510(280)	550(380)	615(300) 640(280)	Irregularly tetrahedral or five coordinate	(16)
	Co alkaline phosphatase	515(265)	555(350)	605(180) 640(230)		(17)

* Exemplifying plant and bacterial ferredoxins and adrenodoxin.

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TABLE 2. *EPR parameters of Cu(II) and Fe(III) models, "copper blue enzymes" and ferredoxins.*

Compound	High field (g)	Low Field g	Low Field A_{cm}^{-1}	Source
$Cu(NH_3)_4^{2+}$	2.05	2.4–2.15	0.015	(1)
Cu(II) EDTA (1:10)	2.09	2.40	0.015	(2)
Pseudomonas aeruginosa blue protein	2.055	2.26	0.006	(3)
Ceruloplasmin	2.06	2.21	0.008	(4)
Laccase (*Polyphemus*)	2.05	2.20	0.009	(5)
Laccase (*Rhus*)	2.09	2.27	0.004	(6)
Rhus vernicifera (stellacyanin)	2.04	2.30	0.004	(6)
Plastocyanin	2.05	2.23	0.006	(7)
	g_z	g_y g_z		
Fe(III)	1.7,	2.1, 2.8		(8)
Ferredoxin (spinach)*	1.89,	1.96, 2.04		(9)

* Exemplifying plant and bacterial ferredoxins and adrenodoxin.

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probability, and in fine structure, i.e., energy distribution of transitions (Table 1, *A*). In instances studied so far, extremes of pH, but without removal of copper, followed by neutralization, reversibly remove and restore these spectral or EPR features concomitantly with activity. Hemocyanin, which functions in oxygen transport, also has an abnormal though different absorption spectrum.[9, 10]

The spectra of nonheme iron enzymes also differ from those of iron complex ions.[11] In this instance the spectra consist of at least four intense but discrete absorption bands between 320 and 600 mμ, a pattern not yet observed in other iron complexes known to us (Table 1, *B*).

The zinc ion, also common in metalloenzymes, has neither intrinsic color nor unpaired electrons, rendering it a poor probe in this context. However, cobalt has been substituted for zinc in carboxypeptidase,[12] carbonic anhydrase,[13] and alkaline phosphatase of *E. coli*,[14] resulting in enzymatically active products which now exhibit visible spectra. Again, these differ significantly from those of model cobalt(II) complexes[8] in that there are as many as *four* bands of high intensity, spread over the region of energy from 450 to 700 mμ (Table 1, *C*).[12–14]

Alteration of the coordination sphere by reaction with an added ligand, e.g., inhibitor anions like cyanide, sulfide, and sulfonamides, converts the irregular spectra of cobalt(II) carbonic anhydrase to one recognizably that of a tetrahedron.[13] Similarly, addition of azide, cyanate, and thiocyanate to "copper blue enzymes" removes the unusual features of their spectra.[15, 16] Modification of the absorption spectra through substitution of ligands at the metal ion therefore helps to demonstrate an unusual condition of the active metal-ligand site, an experimental approach which is capable of considerable expansion.

Electron spin resonance spectra of copper and iron enzymes are similarly

unusual (Table 2). Again, prototypes for the EPR spectra of the "copper blue enzymes" have not as yet been found. The small g values, just at the extreme limit of what has been observed in conventional models, together with the unique, small values of A, the nuclear hyperfine splitting constant, have been interpreted to denote an unusual distortion around the cupric ion.[9, 10, 15, 17] In the ferredoxins, the three g values, centered on $g = 1.94$, are equally unusual in iron chemistry.[18] High g and low A values, which could well denote a low symmetry environment, have also been seen in the few molybdenum enzymes examined so far, but their detailed interpretation has not been recorded as yet.[19] Thus, jointly the absorption and EPR spectra of virtually all metalloenzymes containing a suitable metal are unusual and characteristic of low symmetry, apparently a feature common to their metal binding sites.

Prosthetic Groups.—Metal-containing heme or corrin prosthetic groups can be valuable probes also, though the physical characteristics of the metals become intertwined with those of the organic moiety. Hence, the spectral contribution of the metal probe can be reduced considerably or even obscured completely. Unusual features could appear now either in the physical properties of the metal or in those of their organic moiety, but not necessarily in both.

On the basis of a comparison with model heme complexes the spectra of cytochromes cc',o, and P_{450} deviate markedly both in the wavelength and intensity of the Soret band region, reflecting an unusual condition of the organic moiety.[20] Though not so markedly anomalous, the cytochrome oxidase spectrum has a split Soret band, and an unusual position and intensity of the α-band, showing that its heme moiety, too, differs considerably from those of model compounds.[20, 21] Although other cytochromes have often been reported to have conventional hemochrome absorption, spectral evidence is accumulating that some of their properties are also unusual. In addition to the absorption bands of other heme proteins, the cytochromes c in the oxidized state exhibit one band at 695 mμ of unknown origin and have weak absorption bands beyond 800 mμ. The long-wavelength bands and the studies of magnetic susceptibility measurements have shown that these cytochromes are in fact about 5 per cent high spin, implying that their "low spin" $\rightleftharpoons$ "high spin" equilibrium is finely balanced in a manner unusual in models. Electron spin resonance and magnetic methods have recently shown that cytochromes a_3 and b are spin-state mixtures rather than simple hemochromogens, as had long been supposed.[22]

Redox Potentials.—A number of atypical thermodynamic and kinetic properties of these same metalloenzymes are also indicative of unusual sites of the metal ions. Thus, the redox potentials of the "copper blue enzymes" are much higher than those of all copper complexes except those which are strongly distorted from tetragonal symmetry, viz. +0.3 to +0.4 volt, as compared with −0.5 to +0.2 volt.

The redox potentials of a number of cytochromes are illustrated in Figure 1 and compared with model heme complexes. While the potentials of most model heme complexes are below 0.0 volt, those of cytochromes b, c, and a all lie higher, many of them by 0.3 to 0.4 volt. These data indicate that in the electron-transfer proteins the environment destabilizes the Fe(III) with respect to the Fe(II)

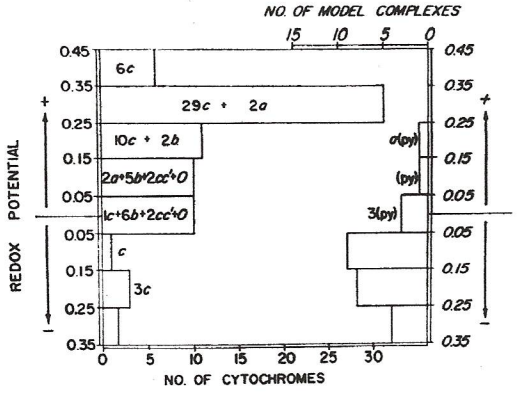

FIG. 1.—The redox potentials of cytochromes (*left-hand side*) compared with those of models (*right-hand side*). The cytochromes are given their conventional labels and the numbers in the boxes indicate the number of examples of a particular cytochrome type.

On the right, the lower boxes give the number of model heme complexes with imidazole bases, while the higher three boxes refer to pyridine heme complexes. The top box is for a pyridine heme *a* complex.

state to a degree unknown in models. It has been conjectured that the absorption and EPR spectra and the spin-state equilibria jointly suggest that in these instances the axial bonds to the iron from the proteins are longer than is normal in model-heme complexes (*vide infra*). This is entirely consistent with the high redox potentials observed. In corrin enzyme complexes a similar situation seems to pertain, since there is now evidence that the nominally cobalt(III) atom can be 5-coordinate, bringing the three valence states of cobalt, i.e., (I), (II), and (III), closer together in energy than is conventional.[23]

Implications to Enzymatic Catalysis.—The principal biological functions of the metalloenzymes studied so far relate either to electron transfer or hydrolysis. Intuitively, one might conjecture that the atypical properties are connected to these catalytic functions. Present treatments of the mechanism of enzymatic catalysis postulate an active intermediate, and the rate of a reaction depends on the ease with which the related transition state is reached. In our terms, this would be facilitated by *a state of entasis: the existence in the enzyme of an area with energy, closer to that of a unimolecular transition state than to that of a conventional, stable molecule, thereby constituting an energetically poised domain.* On this basis the differences between the physical properties of metals in metalloenzymes and models could be understood, since conventional metal complexes, which generally serve as models, do not have geometries of the proposed unstable intermediates of chemical reactions but relax to conventional, stable geometries. It appears that the future design of suitable models should incorporate the emerging structures of metal complexes in enzyme centers, which may deviate significantly from simpler systems currently known.

Electron Transfer.—It is generally assumed that the intermediates of electron transfer reactions involving complex ions require a compromised geometry between those normally assumed by the two valence states, i.e., the transition state is thought to be a distorted complex. Copper(I) and copper(II) demand different site symmetries, i.e., tetrahedral and tetragonal. Therefore, a suitable intermediate of irregular symmetry would result in an entatic state. Precisely such geometry would appear to exist in the "copper blue enzymes" (*vide supra*). On the other hand, the two valence states of iron, Fe(II) and Fe(III), demand the same, i.e., octahedral, symmetry but different bond distances. Thus, a compromise would result in lengthened bonds to iron(III), as postulated for the

cytochrome (*vide supra*). Thus, in many enzymes concerned with biological electron transfer the properties of the catalytically active metal are consistent with those to be expected if the metal had the distorted geometry approximating that of the plausible transition state for the very reaction in which it is involved. The active enzymatic site itself would thus be designed to achieve a condition energetically favorable to catalysis.

Hydrolytic Reactions.—When a metal participates in a catalytic step involving transfer of an atom, radical, or a group rather than of an electron, ligand substitution is required. The active intermediates in substitution reactions of simple metal chelates either are presumed to have an open coordination position[24] or a distorted coordination sphere. Just such energetically unfavorable, distorted environments seem to exist, for example, in cobalt (II) carbonic anhydrase, carboxypeptidase, and phosphatase prior to substrate entry. Among the elements of the first transition series, Co^{2+} and Zn^{2+} atoms readily accept distorted geometries in model complexes. Strikingly, they are most effective in enzymes catalyzing substitution reactions as for instance in carbonic anhydrase,[13] carboxypeptidase,[12] and alkaline phosphatase.[14] Nickel and copper (II) both have more regular geometries in model complexes and are either less active or inactive when substituted into these same proteins. In model systems, by contrast, both of these cations are more active catalytically than either cobalt or zinc.

Thus in these hydrolytic enzymes the particular metal present and its structural condition would appear to be suitably chosen to assist reaction, for they are such as to permit addition (or substitution) with very low activation energy. Thus the entatic state is again closely allied to the required intermediate of a conventional chemical reaction.

Entatic Active Sites and Active Centers.—Now it might be asked in what manner a protein might generate a center resulting in irregular metal geometries and whether or not analogous centers might be encountered in enzymes other than metalloenzymes. The identities of the amino acid side chains of metalloenzymes which serve as ligands for the metal atom are not known in their entirety at present in any instance. Moreover, on the basis of values derived from models it has not been possible to assign readily the apparent pK's to specific amino acid side chains of the apoenzyme which become the ligands of the metalloenzymes in the few instances which have been examined with this objective in mind. This contrasts with the relatively extensive knowledge on the identity of metal binding side chains of catalytically inactive peptides or proteins; these correspond quite well with those of amino acid and peptide models.[4, 25]

The difference between the properties of metalloenzymes and simple metal complexes might be thought to reflect the constraints imposed in the former by the specific secondary and tertiary structure on amino acid side chains serving as their *multidentate* metal ligands. Further, in metalloenzymes unusual disposition of the ligands may force on the metal atoms irregular geometries which may or may not be encountered in nonfunctional metalloproteins. Hence, in metalloenzymes the metal and its ligands should be considered to generate the entatic state jointly. For example, the metal-ligand pair could well be an extremely effective acid-base system for cooperative attack—both being in a condition close

to that of the intermediate state for their normal reactions and removed from a position of minimum free energy, as seen in models. Similar considerations might be pertinent for substrate binding sites as well.

The ligand-metal interaction of metalloenzymes might be compared with, e.g., that of reactive histidyl, seryl, tyrosyl, or sulfhydryl residues of certain enzymes with protons, the proton being the simplest cation. This unusual reactivity has been related to unusual microscopic, chemical, and conformational environments which might predispose the ligands of apometalloenzymes to the formation of metal complexes of distorted geometries and the organic residues of other (non-metallo-) enzymes to selective reactivity with organic reagents. At present any abnormalities of proton-ligand conditions would be hard to demonstrate by physical methods since the proton is a relatively poor probe, being at present accessible largely to kinetic or chemical methods.

In view of the curious properties of metals at active sites it is important to search for other systems and methods which might permit the demonstration of the presence or absence of "anomalous" properties of the groups at an active site. Thus, the spectral alterations which are seen on interaction of coenzymes, e.g., FAD, NADH, PPL, etc., with their respective apoenzymes might be indicative of an entatic state in such systems, and hence, coenzymes might be employed as probes in these instances. Already it is known that many of these coenzymes exhibit unusual spectroscopic properties when bound to enzymes. Only when more searching experiments on these and other systems are performed will it be clear to what degree metalloenzymes are a very special class of enzymes or examples of a very general state of biological catalysts which happen to be demonstrable in this group by virtue of the properties of the metal probe. These considerations should further stimulate the search for new complex ion models and others which might exhibit features suggested by the entatic state of enzymes. Additional kinetic, thermodynamic, and physical-chemical approaches for the characterization of this state might also be discerned.

A substrate entering this poised domain would find itself under attack by unusually activated groups, and, consequently, the activation energy for reactions would be small.[26] The view presented in no way precludes, of course, that the entry of the substrate itself would lower the energy of activation further. The ideas here suggested rest upon the properties of the *enzyme itself* and are not intended to bear upon the role of substrate; hence, they make no contribution to the manner in which the substrate will subsequently affect its complex with the enzyme or lower the activation energy further, a problem which has been considered.[3, 27–29]

* For the purposes of our discussion the designation "active site" will refer specifically to atom(s) or groups essential to the catalytic step, i.e., the metal atoms, their ligands, and amino acid side chains demonstrably involved in activity. "Entatic," from the Greek "entasis," *ἔντασις*, literally meaning in a stretched state or under tension, implies a catalytically poised state *intrinsic to the active site.* "Active centers" will refer to all those features of primary, tertiary, and quaternary structure of enzymes—including the active site—which are required for substrate binding, specificity, and catalysis.

† This work was supported by grant-in-aid HE-07297 from the National Institutes of Health of the Department of Health, Education and Welfare, Bethesda, Md. Reprint requests should be sent to 721 Huntington Avenue, Boston, Massachusetts.