

APPLICATIONS OF MOLECULAR SPECTROSCOPY TO RELATIONSHIPS BETWEEN BIOLOGICAL ACTIVITY AND STRUCTURE

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I. INTRODUCTION

The study of the physicochemical factors associated with or responsible for biological activity in a variety of molecules has not received the attention that it deserves. In many cases, the complex interplay of various forces makes the mechanism and mode of action difficult to comprehend. Molecular size and surface area, tautomerism, resonance effects and electron distribution, the chemical reactivity of specific groupings, and the intergroup distances, together with the conformation of the molecule, are all factors that must be considered. These properties are usually studied singly, and little or no correlation is found with the physiological activity. However, spectroscopic data, which also result from multiple interactions both within the molecule and with the molecular environment, might be expected to bear some relationship to the pharmacological properties. In addition, in some series of compounds only spectroscopic methods are sufficiently sensitive to reflect the more subtle effects attendant upon slight structural modification. By far the greater part of the research on relationships between biological activity and structure has hitherto been strictly chemical. However, with recent advances in spectroscopy, particularly of infrared spectroscopy, sufficient reference data are now available for these newer and more subtle methods to be extensively applied. This, in fact, is already apparent, for apart from hydrogen-bonding studies and determination of tautomeric structure, spectroscopic methods are now widely used in investigations

of the function of ionic chelates and complexes in biochemical systems.

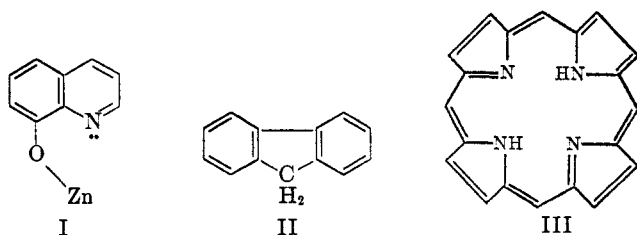
It is the purpose of this review to summarize the spectroscopic data, mainly obtained over the past ten years, that have shed some light on relationships between structure and activity and to draw attention to those aspects of the problem where future work should prove most useful. The steroids are not included, as many summaries of their spectroscopic data already exist.

II. PLANARITY OF MOLECULE AND ELECTRON DISTRIBUTION

Molecular planarity is of considerable importance, as it promotes the full transmission of resonance effects and thereby influences the electron distribution at the extremities of the molecule. Electron distribution, in turn, affects the chemical reactivity of the molecule and, in conjunction with the steric properties, affects the ability to bond at a surface, e.g., to charged subsites of enzymatic active centers.

Fluorescence spectra have been of great value in studies of molecular planarity. The essential difference between fluorescing ring structures and non-fluorescing open structures seems to be the compactness of the closed structure. This atomic compactness induces a rigidity of the molecule as a whole, which is absent in molecules in which parts may vibrate with respect to each other. A molecule whose structure tends to impose rigidity can then be expected to fluoresce, while, conversely, a structure that permits

mutual motion of large parts of the molecule militates against fluorescence (124). Molecular rigidity may be achieved in various ways. It may be inherent in the fundamental molecular structure, if this consists of fused rings, or it may be imposed by diminishing the relative motion of constituent parts of the molecule by adsorption at a surface or by decreasing degrees of freedom in aggregates of mutually interacting molecules. The strong fluorescence of certain metal chelate compounds in comparison with their parent molecules is a result of ring closure; for example, the zinc and magnesium salts of 8-hydroxyquinoline (I) in colloidal suspension are much more fluorescent than 8-hydroxyquinoline (see also Section VI).



The tendency of the compact structure associated with ring closure to increase fluorescence efficiency is also shown in simple compounds whose fluorescence is in the ultraviolet; e.g., the fluorescence efficiency of fluorene (II) approaches unity, while that of biphenyl is about 0.23. Substituent effects vary, but in general hydroxyl and methoxyl groups increase the fluorescence of aromatic compounds and displace the spectra to longer wavelengths, whereas the carboxyl group exerts a depressant action on fluorescence. This can be partially compensated by the introduction of other groups, such as amino or hydroxyl, which can form hydrogen-bonded rings. For example, anthranilic acid fluoresces fairly strongly from the near ultraviolet region to 4900 Å. and 1,4-dihydroxyanthraquinone is fluorescent, whereas the parent compound is not. The planar heterocyclic ring of porphine (III) is conducive to fluorescence (49, 81), and the fluorescence of pyrimidines, purines, and nucleic acids has been utilized in various studies of these groups (50, 59, 62, 103, 113, 206).

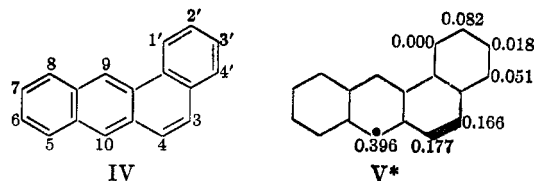
Fluorescence spectra have been used in an investigation of the relationship of carcinogenic activity to structure. A preliminary study of the relative fluorescence intensities of some of the homologs of cholanthrene showed a diminution of fluorescence intensity as the series was ascended (30). Further studies of homologous 20-substituted cholanthrenes showed that the fluorescence intensity increases as does the carcinogenic activity in a comparison of cholanthrene and methylcholanthrene. In the higher homologs, however, the rapid decline in carcinogenic activity

is not accompanied by a corresponding decline in fluorescence intensity (29).

Numerous attempts have been made from many points of view, both chemical and physical, to account for the differences in the carcinogenic activity of aromatic compounds, with varying degrees of success. In most cases it has been assumed that the mobility of the π electrons in certain parts of the molecule is related to the carcinogenic activity. Consequently, absorption spectra, magnetic anisotropy, chemical reactivity, and many other physical and chemical properties which are characteristic of the π -electron system have been investigated. Absorption spectra (97) perhaps give less information than do purely theoretical molecular orbital calculations.

Many of the alkyl-1,2-benzanthracenes are carcinogenically active, and it is interesting to examine whether any relation can be found between the spectra of these compounds and their physiological action. An attempt to discover such a relation implies that the carcinogenic activity can be assessed quantitatively, e.g., in terms of the latent period between the commencement of treatment and the development of tumors. Such studies were not numerous at the time of the earliest spectroscopic investigations (44, 61, 103, 196) but in the case of alkyl-1,2-benzanthracenes (IV) permitted their arrangement in order of increasing carcinogenic potency. The ultraviolet spectra of all the compounds examined contained a peak described as the D band ranging from 2870 Å. for 1,2-benzanthracene to 2970 Å. for 20-methylcholanthrene, and some correlation could be traced between carcinogenic activity and the bathochromic shift of this band. Eight of the twelve monomethyl derivatives were examined; the carcinogenically inactive 1- and 7-methyl compounds showed a very small shift (5 and 10 Å., respectively), whereas the 10-methyl compound, the most active of this group, had the largest shift (45 Å.). The 9,10-dimethyl compound and cholanthrene, both of which are highly active, showed large shifts (95 and 80 Å., respectively), while 20-methylcholanthrene, which is a very potent carcinogen, had the maximum shift of 100 Å. The introduction of a second alkyl group into an alkyl aromatic hydrocarbon causes a further shift of the spectrum to longer wavelengths, yet in the feebly carcinogenic 6,7-dimethyl compound the shift was only 10 Å., which is less than that of many of the monomethyl derivatives. The relative carcinogenic activities of the 5,6- and 6,7-cyclopentano derivatives were also in agreement with their spectral shifts. The correlation, however, was not without exception, for the inactive 10-isopropyl compound showed a large bathochromic shift (103). In this work 1,2-benzanthracene had been considered to be noncarcinogenic (6, 24, 84, 122, 149, 197) but,

according to more recent experiments (204, 205), it has become clear that this compound is in fact carcinogenic. Therefore, some modification may have to be made to theories based on the supposition that this compound is inactive.



* ■ = principal carcinogenophore; ● = subsidiary carcinogenophore.

Molecular orbital calculations of what have been termed frontier electron distributions (67, 68, 69) have indicated that an intimate correlation exists between the carcinogenic activities and electron densities in nonsubstituted aromatic hydrocarbons (145). These authors designate the position corresponding to the phenanthrene double bond and the meso position of anthracene the "principal carcinogenophore" and "subsidiary carcinogenophore," respectively. Frontier electron distributions and the superdelocalizabilities, which are the quantities determining the chemical reactivity, were calculated for about thirty compounds. From a consideration of the data, the value of superdelocalizability at the principal carcinogenophore appeared to be the most important factor determining the magnitude of carcinogenic activity. The frontier electron densities calculated at several carbon atoms (145) are shown for 1,2-benzanthracene (V). The calculated values of superdelocalizability at the principal and subsidiary carcinogenophores indicate that this molecule is carcinogenic, in agreement with the more recent biological data.

Studies related generally to electron densities with reference to biological activity include a theoretical discussion of high-energy bonds from the point of view of electron distribution in the atoms involved (148). Substituents in aromatic systems affect electron distribution in quite remote parts of the molecule and may thus modify both the chemical and the physiological activities in certain types of compound. As it has been shown that σ is a monotonic function of electron density for meta- and para-substituted benzene ring compounds (98), importance has been added to the infrared approach to measuring the Hammett constants. Transmission through the phenylamino group of the effects of substituents in the benzene ring have been studied by infrared spectroscopy (158, 167), and the influence of ortho-substituents, for which there are no Hammett σ values, on the stretching frequencies of side-chain carbonyl groups in substituted benzenes have been investigated (160). Cor-

relation may also be made between Taft's σ^* parameters and infrared frequencies (163).

III. ISOMERISM

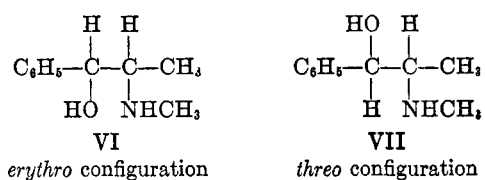
A. Structural isomerism

That the shape of a molecule is highly important in drug action has long been appreciated, and in some instances similarity in the shape of active molecules may be more significant than composition (168). Chemical methods have hitherto been used almost exclusively in investigations of this aspect of the relationship of structure to biological activity. For example, such methods have been utilized to demonstrate that in synthetic analgesics the isomer exhibiting the greater activity is related to D(-)-alanine, i.e., with greater complementarity for the suggested receptor surface; and it is further postulated that 6-methylmethadone, with the correct configurational arrangement of the three essential analgesic groupings, is inert because the methyl group is incorrectly positioned and prevents correct combination between the drug and the receptor surface (15, 16). Many similar studies have been made for other active compounds, but although spectroscopic methods often provide structural information not otherwise obtainable, with few exceptions these studies have been neglected.

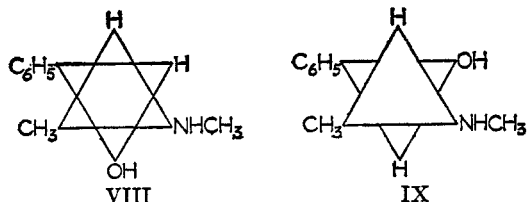
Hexachlorocyclohexane is a mixture of stereoisomers formed by the addition of chlorine to benzene under the influence of actinic light. Seven of the eight expected isomers have been characterized (119); of these the γ -isomer, which represents about 12 per cent of the product, alone is a highly active insecticide. To obtain further information concerning the relation of structure to insecticidal activity, a critical study was undertaken of the infrared and Raman spectral data as they relate to the molecular configurations of the various isomers (221). The results obtained were in good agreement with the theoretical studies. That is, γ -hexachlorocyclohexane is not isosteric with inositol; therefore earlier attempts to explain its insecticidal activity as a possible antagonist to inositol appear unwarranted (12, 215). The γ -isomer, which has the configuration *pppeee* (*p* = a polar substituent and *e* = an equatorial substituent with reference to the chair form of cyclohexane), has a calculated diameter of 8.5 Å. and a thickness of 7.2 Å., i.e., it is nearly spherical, whereas the β -isomer, which has the configuration *eeeeee*, has a molecular diameter of 9.5 Å. and a thickness of 5.4 Å. and is nearly planar. To test the theory that the γ -isomer is less stable than the α -, β -, and δ -isomers, owing to its greater molecular strain, and that therefore addition of energy should convert the γ -isomer to the next more stable isomer, samples of the γ -, α -, and β -isomers were irradiated with ultraviolet light at elevated temperatures and in the

presence of heavy metal catalysts. None of the isomers was transformed into other isomers. It seems, therefore, more likely that the γ -isomer is formed via more highly chlorinated intermediates, such as the heptachloro or octachloro compounds (117).

The relationship between biological activity and configuration of the ephedrine isomers is also an interesting problem. Their conformation, that is, their actual shape in space as indicated by ease of acyl migration (218), by conversion into oxazoline (63, 64), and by reaction with urea (43), is considered to be such that the methyl and phenyl groups are trans in all isomers. Thus the chemical evidence indicates projection formulas VI and VII for (-)-ephedrine and (+)- ψ -ephedrine, respectively.



Such conformation makes the distance between the polar hydroxy group and methylamino group differ considerably in the ephedrine and ψ -ephedrine isomers, a factor which may contribute significantly to the variance in their biological properties. Further information on this topic is now available as the result of spectroscopic studies (110, 111, 112). The infrared spectra from 600 to 3600 cm^{-1} were obtained for *dl*- and *l*-ephedrine and *dl*- and *d*- ψ -ephedrine. Three bands in the 3 μ region near 3300, 2700, and 2400 cm^{-1} were tentatively assigned to the hydroxy, amino, and methylamino groups, respectively. The ψ compounds show a greater frequency separation for these bands than the normal compounds; this difference is attributed to a stronger *intramolecular* hydrogen bond in the ψ compounds. In carbon tetrachloride solution the spectra of ephedrine and ψ -ephedrine are identical from 0.001 *M* to 0.01 *M*, but at high concentrations the intensity of the sharp band near 3600 cm^{-1} decreases, the broad band near 3400 cm^{-1} increases, and a new band appears near 3300 cm^{-1} which is assigned to an *intermolecular* hydrogen bond. The band at 3370 cm^{-1} in ephedrine is assigned to an N—H vibration. In solution a mixture of rotational isomers exists for both ephedrine and ψ -ephedrine, with the latter having a greater amount in the *intramolecularly* bonded form, and it is suggested that the conforma-



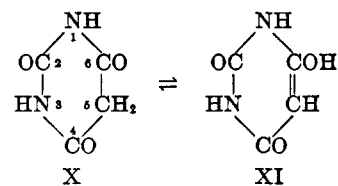
tions of ephedrine and ψ -ephedrine which would account for this are VIII and IX, respectively (110, 111, 112).

B. Keto-enol and lactam-lactim tautomerism

Until recently little systematic work had been undertaken on the infrared spectra of nitrogen-containing heteroaromatic systems, but the series of publications on vibrational frequency correlations in heterocyclic molecules (90, 114, 156, 157, 161, 166) now provides sufficient data to permit formulation of empirical rules correlating the appearance of given bands with known substitution types in molecules based on the indole structure.

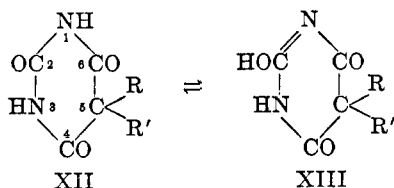
Ultraviolet spectra have been utilized extensively in an investigation of the relationship between the absorption spectra and pharmacological action of tetrazole derivatives. Substituted tetrazoles were grouped according to their pharmacological action, as (a) pure depressants, (b) depressants with some stimulation, (c) substances having no action, and (d) purely stimulant substances, and the wavelengths and the extinctions of the two absorption maxima between 220 and 340 $\text{m}\mu$ were recorded. Curves for five examples [5-diethylaminomethyl-1-(2-naphthyl)tetrazole, 5-(*m*-aminophenyl)-1-propyltetrazole, 1-(*p*-aminophenyl)-5-methyltetrazole, 5-methyl-1-phenyltetrazole, and 1-cyclohexyl-5-ethyltetrazole] were also given, and it was shown that for decreasing depressant action the lower wavelength steadily decreased, while for increasing stimulant action both maxima decreased (193a).

More generally, the association of characteristic absorption frequencies with given structural units has been used to elucidate equivocal structure in the heterocyclic field, particularly for the identification of the predominant form in cases of potential tautomerism. Here, too, ultraviolet spectroscopy has been applied; for example, the similarities shown in the spectra of 1-methylbarbituric acid, 1,3-dimethylbarbituric acid, and the parent compound (X, XI) and in particular the almost identical value of ϵ_{max} at ca. 2600 \AA . in alkaline solution suggest that barbituric acid in aqueous solution undergoes only one enolization involving the active methylene group in position 5 (207).



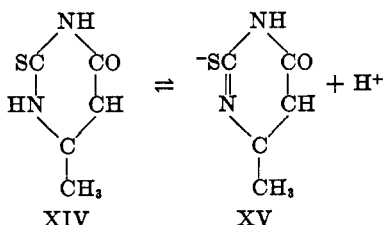
In the case of barbital (XII: R = R' = ethyl) and phenobarbital (XII: R = ethyl, R' = phenyl) the ultraviolet spectra indicate that in aqueous alkaline

solution both undergo lactam-lactim tautomerism involving one hydrogen only at position 1 to give a 2-hydroxy compound (XIII) (208).



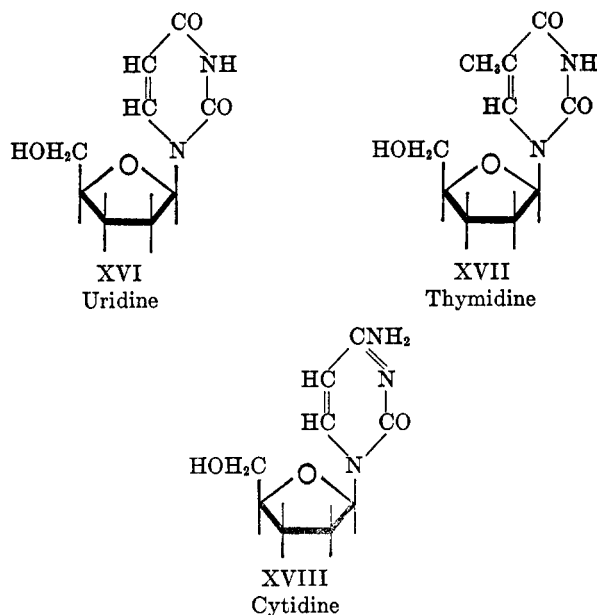
The increase in the ultraviolet absorption (ca. 2600 A.) of barbituric acid which occurs on dilution has been shown to follow the increase in the degree of ionization (209). Several other studies of the absorption spectra and structure of barbituric acid derivatives as a function of pH have been made (65, 178, 179). Certain conclusions drawn by these investigators are not in complete agreement with earlier work. For, although barbituric acid (X) has three potentially dissociable hydrogen atoms, the triketo form is reported to exist between pH 7 and pH 11. Common-ion structures are present as a resonance hybrid in the 1,3-dimethyl derivative at lower pH values, and lactam-lactim tautomerism is said to occur in both barbituric acid and its 1-methyl derivative between pH 11 and pH 14, leading to an enolized structure unsaturated in the 1,2- or 2,3-positions. Comparison of the data with that for other ketopyrimidines confirms that tautomerism in barbital produces an enolized structure in the 2-position. The spectrum of 2,4,6-trimethoxypyrimidine, which has no dissociable hydrogen atoms, remains unchanged from pH 1 to pH 13.

Other tautomeric compounds of biological interest include a number of derivatives of thiourea and especially the substituted thiouracils which inhibit thyroid activity in animals (8, 41). The suggested mechanism of antithyroid activity for these compounds, i.e., the theory of iodine absorption, is supported by the finding that the loss of thyrotropic activity occurring during exposure of a pituitary extract to iodine is restored by treatment of the iodinated hormonal material with 2-thiouracil (2). Further, the production of a disulfide compound incorporating the thiol form of the thiouracil molecule suggests that any mechanism of iodine absorption depends on ionization and subsequent ion tautomerism (XV).



A spectroscopic investigation (210) disclosed a connection between change in pH and change in ultraviolet absorption for 2-thiouracil and 4-methyl-2-thiouracil (XIV), which indicated that changes in absorption are due primarily to ionization and subsequent ionic rearrangement (XV). On the basis of the iodine oxidation theory, the probable ionic nature of the mechanism suggests that thiouracil derivatives which are appreciably ionized at pH 7.3 are most likely to exhibit antithyroid activity.

Among other investigations of substituted pyrimidines are two analyses of infrared spectra of several nucleosides and nucleotides which have been studied in aqueous solution to determine which tautomeric forms are predominant (139, 200). Such information could be of biological interest in connection with hypotheses about the mechanism of reaction of individual nucleotides, and should also be of fundamental importance in formulating ideas of the structure of nucleic acids, just as information on the detailed structure of amino acids has proved useful in investigations of protein structure. In the postulated structure for deoxyribose nucleic acid (DNA) (217) two polynucleotide strands are held together by hydrogen bonding between pairs of purine and pyrimidine bases which form the core of the double helix. It was assumed that the bases occur only in the keto rather than the enol configuration; therefore, it is desirable to have experimental evidence on the tautomeric forms of nucleotide bases in solution to help decide which hydrogen bonds could be formed to hold the bases together. Infrared spectra of numerous pyrimidines and cytidylic acid, deoxycytidine hydrochloride, and 5-methyldeoxycytidine hydrochloride have been reported (21, 22, 27, 28, 48, 138), but with the exception of some simple pyrimidines such as the 2-hydroxy-, the 4-hydroxy-, and probably the 2,4-dihydroxypyrimidines, which are found to be ketonic (199), interpretation of these spectra is still a matter of controversy with regard to keto-enol tautomerism. However, with the aid of model compounds which have structures very closely analogous to those of the 2-nucleosides, but which are incapable of undergoing tautomerism, it is possible to decide which functional groups in the molecule are responsible for particular infrared bands. In this fashion, uridine (XVI) and uridylic acid were found to exist in D₂O in the diketo form. Thymidine (XVII) was found largely present in the diketo form, but probably existed to some extent also in the enolic form in solution. Although a definite assignment of tautomeric structure to cytidine (XVIII) and cytidylic acid was not possible, their existence in the amino form in solution appeared likely (139).



Reference data for such investigations are still rather sketchy, for although carbonyl and imino stretching frequencies in heterocyclic compounds have been fairly widely studied because of their relative simplicity, only an analysis of the whole spectra can provide a better understanding of the structures involved. Spectroscopic studies which may aid future investigations of tautomeric structure, particularly in heterocycles, include infrared spectra of substituted isatins (157), oxindoles (114), tetrahydro-2-oxo-1,4-benzodiazine, dihydro-2- and -3-oxobenzomorpholine, dihydro-6-oxo-4,5-benz-1,3-oxazine, isatoic anhydride and related bicyclic compounds (162), indoxyls (90), and enolic structures related to oxindole and isatin (166). Mention should also be made of an investigation of chelation and enolization of some acyloins (127), a study of keto-enol tautomerism by the method of vibration spectra (198), and enolization in β,γ -unsaturated sulfoxides (118).

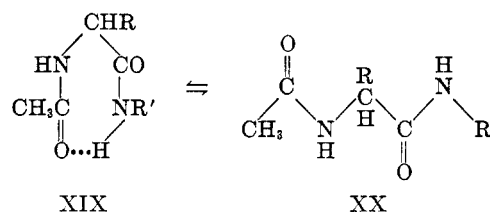
A consideration of tautomeric structure often plays a part in the design of new active compounds, e.g., the possibility that the antimalarial activity of Mepacrine and Paludrine is due to tautomerism led to the preparation of derivatives of 2-aminopyridine which exhibit similar tautomerism (183). However, if this approach to chemotherapy is to be given the opportunity to succeed, there must be no uncertainty as to the actual tautomeric structures involved.

IV. HYDROGEN-BOND FORMATION

Hydrogen bonds, perhaps, are of greatest importance in determining the configuration of the polynucleotide strands which form the core of the double helix of nucleic acids (see Section III) and also of polypeptide chains and proteins. Among the many structural investigations that have been made, in-

frared spectral studies of synthetic polypeptides have been important in the development of the current ideas of the molecular configuration of such materials and their relationship to the structure of certain proteins (169, 170).

The existence of α - and β -forms of synthetic polypeptides has frequently been demonstrated (4, 5, 9, 10); the α -form possesses a carbonyl stretching mode (Amide I)¹ at about 1660 cm^{-1} , whereas the β -form has a stretching mode around 1630 cm^{-1} . Infrared studies of the structure of compounds of the type $\text{CH}_3\text{CONHCHRCONHR}'$, which can be considered as a structural unit of a polypeptide chain, have indicated that such molecules exist in both folded (XIX) and extended forms (XX) (143). Similar studies have been made for molecules of the type $\text{CH}_3\text{CON}(\text{CH}_3)\text{CHRCONHR}'$ containing only one NH group, which enables population studies of the extended and folded forms to be made more easily (142).



The frequency shifts of the carbonyl stretching band in both polypeptides and proteins have shown that in the α - or folded form the carbonyl stretching frequency remains close to 1660 cm^{-1} , whereas the β - or extended form has this frequency near 1630 cm^{-1} . The frequency difference in the two forms has been attributed to the change in the $\text{C}=\text{O}\cdots\text{H}-\text{N}$ angle, the carbonyl stretching frequency being lowered as the $\text{C}=\text{O}\cdots\text{HN}$ bond approaches linearity. An alternative explanation for the amide frequency shifts in polypeptides has been advanced (38). This is based on the observations that there is lack of correlation of carbonyl frequency with $\text{O}\cdots\text{N}$ distance, the carbonyl frequency shifts in α - β transitions without change in NH stretching frequency, the carbonyl shift relative to the NH shift is large on association, and the maximum carbonyl shift occurs when the OCN dipoles are colinear. The deduction is that the dipole-dipole interaction may make a greater contribution to the total interaction energy than the potentially weak hydrogen bonding (38). The assignments of high-frequency absorption bands in the spectra of

¹ Both primary amides and noncyclic *N*-monosubstituted amides give rise to two strong bands between 1710 and 1470 cm^{-1} , which are commonly referred to as the Amide I and Amide II bands; the Amide I band is essentially a $\text{C}=\text{O}$ stretching vibration and the Amide II an N-H deformation vibration.

compounds containing the peptide group have been critically reviewed in the light of new data on *N*-monosubstituted amides. Evidence based on hydrogen-bonding studies and substitution of deuterium for hydrogen in the peptide NH group has confirmed the previous association of several high-frequency bands with peptide group oscillations (82).

Polymerization of γ -benzyl-*N*-carboxy-L-glutamate anhydride yields mixtures of α - and β -forms of polypeptides. The β -polypeptides may be extracted from the mixtures by virtue of their solubility in 98 per cent formic acid. The β -polypeptides show, as expected, a carbonyl (Amide I) infrared absorption band at 1630 cm^{-1} in the solid state, but this is shifted to frequencies as high as 1678 cm^{-1} in dilute solution or by heating more concentrated solutions. This spectral behavior parallels that of low-molecular-weight secondary amides; on this basis, as well as on the low solution viscosities, it was concluded that the β -polypeptides are of very low molecular weight. The fractions insoluble in formic acid exist in the α -form and have higher solution viscosities and higher molecular weights. As in other examples the α -form is characterized by a carbonyl stretching frequency at 1655 cm^{-1} in the solid state. The position of the band in these α -polypeptides does not change in most solvents or upon heating. No evidence was found with poly- γ -benzyl-L-glutamate to indicate an α - β transformation upon treatment with formic acid (20). An infrared spectroscopic method for determining the kinetics of polymerizing systems has been developed (96). This method has been applied to the determination of the kinetics of the primary amine-initiated polymerization of γ -benzyl-*N*-carboxy anhydride. By this method it has been shown that there are at least three products of the polymerization reaction. The two main products are low-molecular-weight (β L) polypeptides which are formed at a slow rate, and higher-molecular-weight (α) polypeptides, which are formed at faster rates (96).

Analogous to the observations made in connection with the effect of optical configuration on the ionization of the functional groups of peptides (54), the spectra of some polypeptides remain unchanged upon complete isomerization: e.g., glycylalanine, alanyl-glycine, and glycylalanyl-glycine, which contain only one asymmetric carbon atom, and trialanine (3L), in which all three centers of symmetry are changed to their isomers (3D) simultaneously. Conversely, isomerization of one residue at a time in trialanine and tetraalanine gives rise to marked differences. Some frequencies are shifted and new ones appear, while others disappear. This is probably due to strains and changes in spatial configuration, as has already been deduced from titration curves (56).

Infrared spectra of peptides containing two amino and two or three carboxyl groups on the whole begin to resemble spectra obtained on crystalline insulin (55). In the tripeptides distinct bands are observed over the entire range scanned. Adding one and then two more alanine residues to the peptide reduces the number of clearly recognizable bands very greatly. Comparison of the spectra with those of alanine peptides leads to the conclusion that the presence of the second functional group gives rise to many more interactions in such a manner as to obscure many expected frequencies. This effect of an additional functional group is well illustrated in the spectra of the glutamyl peptides. The bands of α -glutamylalanine (LL), α -glutamylglutamic acid (LL), and also α -alanylglutamic acid (LD) are well resolved. It might be inferred that the mutual interaction between the functional groups in these peptides is essentially confined to the formation of *intermolecular* hydrogen bonds. The spectra of their diastereoisomers, on the other hand, are quite diffuse, which may be indicative of strong *intramolecular* interactions in addition to the *intermolecular* ones (57). In relatively small peptides both the peptide NH group as well as the free α -amino NH group should contribute to the NH stretching frequencies. The fact that three or four frequencies are observed in certain cases may indicate that different types of hydrogen bonds are involved, the higher frequencies possibly belonging to the weaker hydrogen bonds. Similar studies are now being carried out on thiopeptides (134); related investigations include an infrared spectroscopic study of the hydrogen bonding of the thiol group (3) and the thiopeptide group in cyclic structures (135).

V. REACTIVITY OF ACTIVE COMPOUNDS

A. Oxidation and reduction

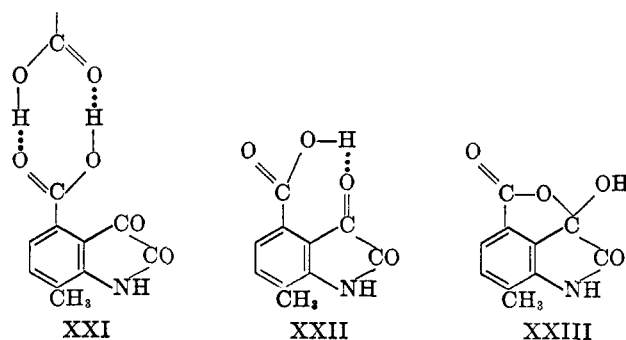
The significance of this factor may be appreciated from the fact that the body is well supplied with systems for oxidation-reduction, including such compounds as ascorbic acid, glutathione, and cysteine. Their interaction with compounds of higher or lower potential may, depending on circumstances, modify the compound either to inactivate or even to activate it. For example, pentavalent arsenic is inactive toward spirochetes until it has undergone reduction to the trivalent state. In addition, it is possible that many antibiotics, especially those derived structurally from quinone, are effective because of their oxidation-reduction possibilities.

Oxidation-reduction potential, effects of structural modification, and carbonyl stretching frequencies are, of course, interrelated; and extensive studies of the spectra of a large variety of quinones have disclosed many valuable correlations. In simpler dialkyl

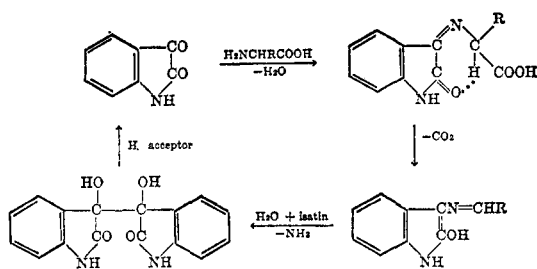
ketones a correlation has been demonstrated between the carbonyl stretching frequencies and the corresponding oxidation-reduction potentials (47, 71), and in a series of substituted benzophenones an approximately linear relationship was found when the carbonyl frequencies of the substituted benzophenones in carbon tetrachloride solution were plotted as a function of the corresponding polarographic half-wave potentials (26, 109). Similar correlations have been made for substituted quinones, in which the variation of the carbonyl frequency is related to the inductive effect of the substituents in the same way as is the variation in oxidation-reduction potential (106, 107, 108). If correlations may be made between oxidation-reduction potentials and antibiotic properties, then absorption spectra should provide valuable information relating to biological activity in quinonoid compounds. From a review of eighty-five titles (132) it appeared that most of the known antibacterial agents are reversibly oxidizable and it was believed that the antibiotic effect might be related to this property. To test this a series of quinones were assayed at varying dilutions and pH values. All inhibited the growth of *Staphylococcus aureus* to some extent, so the quinone structure may be inherently toxic, but the antibiotic effect was not strictly parallel with the oxidation-reduction potential (132, 167a). However, the relatively high oxidation-reduction potential of certain quinones which are believed to interact with bacterial respiratory enzymes still provides a working hypothesis for their antibiotic action. An alternative theory, that the antibacterial properties of quinones are dependent on their ability to react with thiol groups (72), is discussed in Section VI.A. Yet another possibility, that the antibacterial action, at least *in vitro*, results from the removal of essential amino acids from the culture medium by a Strecker reaction, is indicated by the degradation of α -amino acids to the corresponding aldehyde or ketone containing one less carbon atom by the action of carbonyl compounds of the type $-\text{CO}(\text{CH}=\text{CH})_n\text{CO}$. The relationship of dehydrogenase activity to structure of the ketonic compound has been extensively investigated (39, 74, 128, 140, 192), and in the case of isatin much useful information on this topic has been provided by both polarographic (39a) and spectro-

scopic studies (157, 159). The enzymic behavior of isatin in catalyzing the dehydrogenation of amino acids is considered to proceed by a reaction series involving, at different stages, both carbonyl groups of isatin. Chemical reactivity and the stretching frequency of the β -carbonyl group in substituted isatins increase with the σ -values of the substituents; also, out of six examples of substituted isatins studied, five possessed dehydrogenase activity towards DL-alanine which increased steadily with the above properties (157). Further results showed that for nineteen of the twenty-one compounds examined, satisfactory correlation exists between σ -values, β -carbonyl frequencies, and dehydrogenase activities (159). Now as the β -thiosemicarbazones of certain substituted isatins possess antiviral properties (13, 14), it was thought that, provided the substituted isatin thiosemicarbazones undergo hydrolysis *in vivo*, there might be some correlation between antiviral activity and dehydrogenase activity of the parent isatins. However, examination of a large number of derivatives disclosed that the steric effects of substituents were of greater importance than the inductive or mesomeric effects, so this correlation may not be made.

A special case is that of 7-methylisatin-4-carboxylic acid which, along with other isatin-4-carboxylic acids, has very high dehydrogenase activity and several possible structures as a result of both *intermolecular* (XXI) and *intramolecular* (XXII) hydrogen bonding and also lactol formation (XXIII).



It is not possible, in the case of 7-methylisatin-4-carboxylic acid, to relate dehydrogenase activity to the carbonyl stretching frequencies, since the latter do not occur as discrete entities. Nor may the high catalytic activity (140) be compared with the σ -values for the substituents, as the carboxyl group is ortho to the β -carbonyl group. However, attention has already been drawn to the possible relation between the unusual dehydrogenase activity of isatins substituted with acid groups in the 4-position and *intramolecularly* hydrogen-bonded structures which may occur in these compounds (141). This is supported by the spectroscopic data (190), as under conditions approximating to those used in the measure-



ments of catalytic activity, 7-methylisatin-4-carboxylic acid is shown to exist predominantly in an *intramolecularly* hydrogen-bonded form. But there is obviously no simple relation between bonding propensity and the dehydrogenase activity of 4-substituted isatins, as both the simple amide and the propylamide possess *intramolecular* hydrogen bonds, yet there is considerable discrepancy between their relative dehydrogenase activities.

B. Hydrolysis and condensation

In normal biochemical processes, condensation reactions are represented in the biogenesis of macromolecules such as proteins, polysaccharides, and nucleic acids, and in processes of esterification such as fat formation. Foreign carboxylic acids will often combine with amino acids, e.g., as in the excretion of benzoic acid in the form of hippuric acid, and phenols frequently form sulfuric esters, but here the choice of the conjugating molecule is influenced by substituents in the benzene ring (219). The biochemical aspects of these reactions, which are usually described as detoxication mechanisms, have been reviewed (220), and some spectroscopic studies of these reactions have been undertaken (70).

The hydrolytic function of enzymes is somewhat easier to study, and knowledge of the distribution of such systems in cells and tissues has been acquired largely through the use of homogenization-centrifugation processes (53). Also, many ingenious staining methods for enzyme localization (45, 75, 76, 125, 126, 171) have attracted considerable attention because of their potential value, but few have been subjected to sufficiently rigorous analysis for their quantitative potentialities to be adequately assessed. Analyses of model systems typifying cytochemical staining processes for locating enzymes in cells and tissues have demonstrated that certain physicochemical requirements must be fulfilled if the results are to be precise and quantitative (91, 150, 151, 152, 153, 154, 155). In an investigation of indigogenic reactions for the localization of cellular esterases, a series of closely related substrates (i.e., indoxyl acetates) was prepared (93, 185, 186). On enzymic hydrolysis these give free indoxyls which, while diffusing, undergo an oxidative "capture reaction" to the corresponding indigoid dyes.

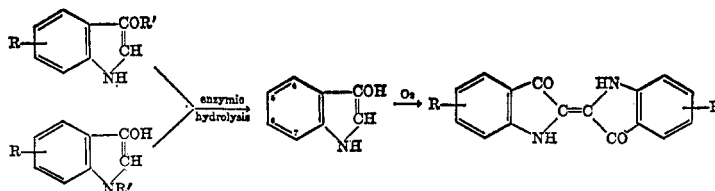
depends upon the rate of oxidation of the free indoxyl, the degree of binding of the latter, and any other reaction intermediate to cellular material, and the solubility and substantivity of the dye produced. In the first place, the effects of changes in oxidation rate on the staining pattern were studied by comparing results obtained with aerial and chemical oxidation. Conditions for maximum oxidation rates were found to be similar in all cases, and these conditions were then adopted during an appraisal of substrates giving rise to intermediates with different capacities for binding to protein, and dyes with different solubilities and substantivities. A clear correlation was found between the staining patterns and the molecular structures of the dyes and their intermediates, as indicated by spectroscopic and other studies (90, 92, 94, 165, 185, 187, 189). These results and ancillary studies have led to the development of a greatly improved indigogenic staining process for esterases, which appears to have a precision of the order of 0.5 μ .

Substrates bearing large substituents in either the 4- or the 7-position give poor results because they prevent the formation of strong *intermolecular* hydrogen bonds, whereas substitution in the 5- or 6-position strengthens this bonding. The latter compounds may bond either to themselves or to receptors on the protein surface, but the addition of a relatively small 4-substituent, e.g., as in 5-bromo-4-chloroindoxyl acetate, gives rise to a dye with the best bonding propensity. A study of the ultraviolet absorption spectra of the derived dye shows that the electron densities in the neighborhood of the carbonyl groups are the highest observed in these compounds, the increase in polarity resulting in irreversible bonding to the protein. This, coupled with its intense absorption in the visible and its general stability, render it highly suitable for the precise delineation of *intracellular* subsites.

VI. ACTIVITY ARISING FROM SPECIFIC CHEMICAL GROUPS

A. Thiol reactors

The sulfhydryl group is of vital biological significance, yet despite the ready availability of pertinent reference data, little direct spectroscopic work has been undertaken to illuminate its reactions in vital processes. Thiol groups play an important role in natural processes because of their capacity for re-

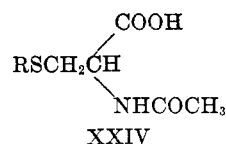


The precision of localization given by this process

versible oxidation to disulfides, their acylation to give

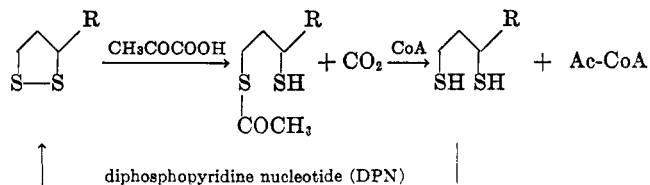
so-called high-energy bonds, and other characteristic reactions. Thiol groups are essential components of many apoenzymes, and they are of vital significance in smaller molecules such as those of coenzyme A and glutathione. It follows that compounds which can react with thiols may be expected, subject to the limitations imposed by their physical properties, to exhibit biological activity. Most important of the purely organic types of compounds which can react with thiols under physiological conditions are those containing the α,β -unsaturated ketonic group, including open-chain compounds and cyclic structures such as unsaturated lactones and quinones. These are commonly found in biologically active natural products, and studies of their reactivity with thiols have been recorded (73). The antibacterial action of synthetic quinones has also been studied extensively from this viewpoint. Although more than one mode of action is feasible, there is considerable evidence that the activity of quinones, against Gram-negative organisms at least, is associated with their ability to react with thiol groups (72). Further studies have shown that many thiols react with and inactivate many types of antibiotics, both quinonoid and otherwise (40); this suggests that many antibiotics depend on their ability to combine with essential —SH groups in bacterial metabolism for their fundamental mode of action. However, although detailed studies, both in the ultraviolet and in the infrared, have been made of α,β -unsaturated ketones (37), lactones (31, 162), and quinones (104, 105, 222), spectroscopic methods have not been extensively used in elucidating the mode of interaction of thiols with such ketonic compounds.

Spectroscopic work which may be of future value in interpreting the activity of thio and thiol-containing compounds include two publications on the infrared spectroscopy of the thiocarbonyl group (80, 137) and one on mercapturic acids (70). The latter is an analytical study of eight of the nine known mercapturic acids (XXIV) and of several *S*-substituted cysteines, the results being of practical value in the identification of these compounds among the metabolites from feeding experiments.



Of especial interest are the spectroscopic studies of 6-thioctic acid and related compounds. With the discovery that 6-thioctic acid (77, 177) is a coenzyme for the oxidative decarboxylation of pyruvate to active acetyl groups, which through CoA feed carbon into

the Krebs cycle (120, 147), it was suggested that the process could be formulated as below (36).



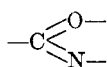
The mechanism involves reductive scission of the S—S linkage to form a dithiol. Isomeric 5- and 4-thioctic acids have respectively 0.3 and 0.1 per cent pyruvate oxidation activity relative to 6-thioctic acid (32), a result which suggests that ring strain in these structures may be an important factor. The ultraviolet absorption spectra of these compounds show a displacement of the absorption peak to progressively longer wavelengths as the size of the disulfide ring diminishes. If the excited states of the disulfides have approximately the same energy curves, then the difference in λ_{max} , 2500 Å. for straight-chain aliphatic disulfides to λ_{max} . ca. 3300 Å. for 6-thioctic acid, implies that the latter experiences a strain of ca. 25–30 kcal.; hence the dissociation energy of the S—S bond will be reduced by a like amount (11). The ultraviolet curve for 4,8-thioctic acid is closely similar to that for a straight-chain disulfide, the very slight bathochromic shift in λ_{max} . indicating negligible ring strain, which probably accounts for its low activity. Further spectroscopic studies of related compounds, e.g., trimethylene disulfide, have been carried out as part of an investigation of the possible function of 6-thioctic acid in photosynthesis (11).

B. Chelates and coordination complexes

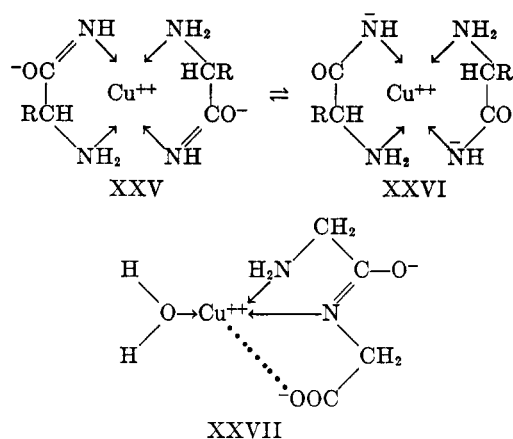
The importance of metal chelates in most branches of chemistry, and particularly medicinal chemistry, is now well recognized. Naturally occurring chelates such as chlorophyll and hemoglobin have significant functions in biological systems, cytochrome and catalase are characteristic heme proteins, and copper enzymes such as the various oxidases are involved in oxidation-reduction systems.

The role of chelation in the action of proteolytic enzymes, which has been extensively studied (201, 202, 203), has created the need for a better elucidation of metal-peptide bond interaction. For example, the role of metal ions in metal-activated enzymes has been explained by assuming a metal chelate bridge between the enzyme and the substrate molecule. Interaction of the peptide group with the metal ion is viewed as resulting in an electron displacement rendering the bond susceptible to nucleophilic attack by hydroxyl ions (203). If this were the case, the infrared frequencies of the amide linkage should be consider-

ably influenced by the metal ion. However, infrared methods of investigation of such complicated compounds could only be successful provided that the behavior of the important chelate-forming groups, such as NH_2 , has been studied in simple molecules with established configurations. Early work included some infrared curves for such compounds (123), and more recently the Cu^{++} and Ni^{++} chelates of glycine have been investigated (66, 195). As a necessary introduction to further studies of complexes of metal ions with peptides, an investigation of amino acid-metal chelates was carried out (180). Pt^{++} , Cu^{++} , and Ni^{++} were used, since these ions form chelates of the same square-planar type (133). If the conclusion that the carboxyl group retains its ionic structure is correct (195), the carboxyl frequency should lie unchanged at approximately 1590 cm^{-1} as in salts with alkali metals. This is the case with Ni^{++} chelates. On the other hand, Cu^{++} and Pt^{++} chelates do show a definite shift: Na^+ glycine, 1590 cm^{-1} ; Ni^{++} glycine₂, 1590 cm^{-1} ; Cu^{++} glycine₂, 1618 cm^{-1} ; Pt^{++} glycine₂ (trans), 1644 cm^{-1} . It is suggested that a shift of this magnitude and direction can be correlated with covalent-bond formation. The chelation of peptides and amides by metal ions has mainly been studied in solution, by methods aimed at the determination of complex constants (46, 51, 174), but in addition, a number of crystalline chelates have been prepared and analyzed and the ratio of metal to ligand thus determined (131, 211). Owing to the complexity of the spectra of amide-copper (II) chelates in the $3\ \mu$ region, this is not really suitable for detailed study, but the $6\ \mu$ region is, as the intense Amide I band can easily be identified. This is usually considered to be a stretching frequency of the amide carbonyl in resonance with the C—N bond. In the chelates the band corresponding to the Amide I is lowered by 100 cm^{-1} and must be the stretching frequency of the resonating structure



A frequency lowering of this order can hardly be due to other effects than to a definite shift of the resonance equilibrium of the amide group to the canonical form (XXV) (181). In the case of the dipeptide-copper(II) chelates, the COO-frequencies show no appreciable trend towards higher wave numbers, so that the interaction between the carboxyl group and the metal ion ought to be of the same kind as in alkali salts of carboxylic acids. The band at 1541 cm^{-1} in glycylglycine copper(II)· H_2O must be ascribed to the Amide I frequency, representing the same mode of vibration of the peptide group as observed in the copper(II) chelates of amino acid amides. This frequency is at a considerably lower wave number than



the normal peptide group vibration or a conjugated CN band frequency, indicating that the resonance structure of the peptide group is preserved but that the equilibrium is shifted towards the canonical structure (XXVII). On the other hand, nickel(II) and zinc(II) chelates have split Amide I maxima, the shifts being only 32 cm^{-1} and 20 cm^{-1} for the mean frequencies. Therefore, the copper chelate of glycylglycine shows a major change in the peptide group resonance, whereas that of $\text{Zn}(\text{II})$ scarcely shows any change. Although it is not quite correct to apply results obtained with solid chelates to aqueous solutions, it is interesting to note that the zinc(II) ion, which activates several proteolytic enzymes, shows practically no tendency to interact with the peptide group. This means that if chelate formation with the peptide group of the substrate molecule with a resulting electron displacement is a necessary step in the mechanism of metal-activated enzymes, the role of the metal ion must be considerably influenced by the protein molecule.

The strength of interaction between DL-2-phosphoglyceric acid (PGA), the substrate of the enzyme enolase, and metal ions activating this enzyme has been determined (129), and Zn^{++} has been found to activate enolase while the Ni^{++} enzyme is inactive (130). Analogously to the concept of metal-ion activation of peptidases (202), it was suggested that the metal ion furnishes one of the points of interaction between enzyme and substrate. Thus, knowledge of the nature of the complexes between PGA and activating ions is important in interpreting the mechanism of the enzymic reaction. In an attempt to obtain this information the infrared spectra of some metal-ion complexes of PGA have been recorded (182). The sodium salt of PGA can be regarded as a normal salt, and it exhibits, as expected, a broad and intense band at approximately 1590 cm^{-1} . If the carboxyl group were to form a bond of more covalent character with Zn^{++} and Ni^{++} , a shift of this band to higher wave numbers would be expected, but the carboxyl

frequency in the complexes of PGA with Zn^{++} and Ni^{++} shows no such trend. On the contrary a small shift towards lower wave numbers can be observed. If this shift has any significance of its own, it is difficult to decide, as the position of the band for the sodium salt is very approximate owing to its broadness; but it is clear, anyhow, that the carboxyl group shows no evidence for covalent-bond formation with Zn^{++} and Ni^{++} . As an explanation for the fact that Zn^{++} activates enolase while the Ni^{++} enzyme is inactive, it has been suggested, on the basis of ultraviolet measurements (130), that the complex of Ni^{++} with the substrate is of a different type. However, the infrared data show that such a difference, if it exists, cannot be the amount of covalent character of the interaction with the carboxyl group, since this is completely ionic in both complexes.

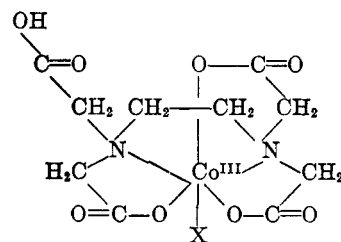
Since the natural metal chelates play a vital role in biological systems, it is not unusual to find that certain unnatural chelating agents will profoundly affect these systems. Such a chelating agent can compete with the enzyme for the metal in a metal-enzyme function so as to kill the organism.

The uses of unnatural chelating agents in biological systems have been classified as follows (133): (1) Destruction of the organism by chelation of essential metals, bacteriocidal and fungicidal action: e.g., 8-hydroxyquinoline. (2) Inhibition of certain metals and metal enzymes for the purposes of studying functions of metals and enzymes in biological media: e.g., cyanide ion, carbon monoxide, amino acids. (3) Removal of harmful metals from living organisms: e.g., ethylenediaminetetraacetic acid, ion-exchange resins.

In recent years infrared spectroscopy has received considerable attention as a tool in the determination of the structures of complex inorganic compounds; but as in the case of the naturally occurring metal chelates, because of experimental difficulties, the application has been restricted for the most part to the study of organic functional groups and the effect which complex formation has on these groups. The carboxyl group has been the subject of several studies of this kind; e.g., the oxalato complexes of several metal ions have received detailed attention (52, 146, 191). As a result of the investigation of the metal complexes of glycine (195) the conclusion was reached that the link between the carboxylate group and the metal ion is largely ionic. A somewhat different view has been expressed by other investigators (34, 35, 116). According to this point of view it is supposed that if a carboxyl group is linked to some group Z

the carboxylate resonance, $\begin{array}{c} \text{O} \\ \parallel \\ \text{—C—O—Z} \end{array}$, will increase as the ionic character of the O—Z link increases.

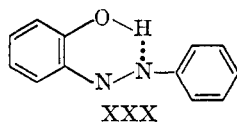
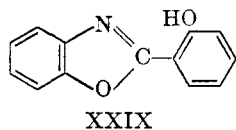
Since an increase in carboxylate resonance imparts enhanced single-bond character to the carbonyl group, it also causes a lowering of the C=O stretching vibration. In consequence, the C=O stretching frequency provides some information relating to the ionic character of the bond. On this basis it is possible to distinguish between a carboxyl group bound to a proton and a carboxyl group bound to a metal ion. Alternatively, it has been suggested that uncomplexed carboxylate groups may be distinguished from complexed carboxyl groups on the basis of the sharpness of the carbonyl absorption band (116). These studies have made possible the assignment of unequivocal structures to several cobalt chelates. For example, the conclusion that the polyfunctional ligand is pentadentate (XXVIII) in the compounds of the general formula $M'[\text{Co}(\text{HY})\text{X}]$, where M' is an alkali metal, Y is the tetranegative anion of ethylenediaminetetraacetic acid, and X is Cl^- , Br^- , or NO_2^- (33, 194), has been substantiated by the study of the C=O stretching vibrations (144). Two other detailed investigations of infrared absorption of complex cobalt compounds have been made, both using data obtained from solid-state spectra (85, 172).



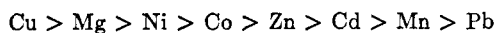
XXVIII

The relation of spectra to stability in series of chelates has been studied in 1,3-diketones, 2-(*o*-hydroxyphenyl)benzoxazole (XXIX), and *o*-hydroxyazo compounds (XXX). In the case of the 1,3-diketones, the position of the perturbed carbonyl band is largely dependent on three factors: (1) the masses of the groups attached at the ends of the ligand molecule to the carbonyl groups; (2) interaction of the carbonyl group with neighboring π or d orbitals; (3) the relative electron density of the σ bonds. The latter factor is, for the most part, controlled by the electronegativity of the groups attached to the carbon atom of the carbonyl group. If more conjugation between the metal ion and the carbonyl group exists in one metal acetoacetate than another, this results in less double-bond character in the carbonyl group and a lowering of the frequency of the perturbed band, as exemplified in the acetylacetonate chelate series: Na, 1630 cm^{-1} ; Ni(II), 1618 cm^{-1} ; Mg, 1615 cm^{-1} ; Pd(II), 1577 cm^{-1} (95). These results are closely similar to those obtained from pH measurements (214), the order of increasing stability of several

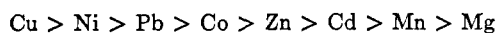
metal chelates of acetylacetonone being $\text{Mg(II)} < \text{Cd(II)} < \text{Mn(II)} < \text{Co(II)} < \text{Zn(II)} < \text{Cu(II)}$. Comparison of these two series indicates, with nickel an exception, a qualitative relationship between the strength of the metal-oxygen bond, as obtained from the stability constants, and the frequency of the perturbed carbonyl absorptions. Related work includes a detailed analysis of the spectra of acetylacetonone, acetylchloroacetone, acetoacetic ester, dibenzoylmethane, benzoylacetone, and their deuterium-substituted equivalents (25) and an infrared study of metal chelates of bisacetylacetonone-ethylenediimine (213).



An attempt to correlate the frequency of the N—H stretching vibrations with the relative stabilities of the metal complexes of 2-(*o*-hydroxyphenyl) derivatives of benzimidazole, imidazoline, benzothiazole, benzothiazoline, and benzoxazole was unsuccessful. Although the spectra of any particular reagent and its chelates were generally similar over the range 2–15 μ , one band centering around 1250 cm^{-1} was found to undergo a regular shift from one metal chelate to another (79). The most noticeable shift occurred for the chelates of 2-(*o*-hydroxyphenyl)-benzoxazole (XXIX), the relative order obtained from a consideration of the frequency shifts being

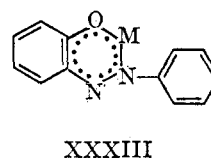
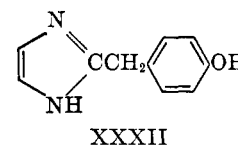
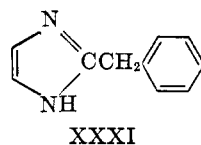


which is practically the same as the usual stability order of divalent metal ions (136):



The most notable difference is the variance in the position of magnesium. Similar shifts have been observed for this peak for the chelates of imidazoline and the other heterocycles quoted. In each series the copper chelate, which is the most stable of the metal-reagent complexes, has its 1250 cm^{-1} peak at the highest frequency, while the zinc chelate, which is generally less stable than the nickel or cobalt chelate, is at the lowest frequency. Spectroscopic studies of the imidazoline structure are of particular importance because of the high degree of pharmacological action found in many synthetic analogs of the type. It is interesting that, although the useful compounds may be considered broadly as cardiovascular drugs, there is little correlation of structure with resultant activity. One compound is adrenergic, whereas its close relative is adrenolytic. Chelation probably plays little or no part in the pharmacological action of these compounds, for Tolazoline (XXXI), which is histaminic

in nature and in general produces vasodilation and increased venous return (1), has no hydroxyl group. Introduction of a hydroxyl group into the para position (XXXII), where it is unlikely to be involved in chelation, converts the compound into a potent pressor (193).



Correlation of frequency shifts with stability of the complex may also be made for the chelates of 8-hydroxyquinoline and derivatives (42, 176). Owing to resonance stabilization of the intramolecular hydrogen bond, a broad band appears at 1610 cm^{-1} in the infrared spectrum of *o*-hydroxyazobenzene (78). In the chelates, this band is observed at 1531–1540 cm^{-1} as the result of involvement of the $\text{C}=\text{O} \cdots$ linkage in the metal chelate ring (XXXIII). For the metals $\text{Cu} > \text{Ni} > \text{Co}$, the order of decreasing C=O frequency is parallel with the order of decreasing stability (212).

Cyanide ion has been extensively used as an inhibitor in tissue metabolism studies for the purposes of studying functions of certain metals and metal enzymes. Investigation of the structures of various cyano coordination complexes by infrared spectroscopy include compounds containing the pentacyanoferrate group (83), the hexacyanodnickelate ion (58), potassium ferrocyanide (23), potassium silver cyanide (100), potassium aurocyanide (102), cyanocobaltate (60), and copper cyanide (173). Copper-containing chelates (17) are of particular interest as they may be responsible for the specific action of certain antiviral compounds against vaccinia virus, as has been demonstrated in both *in vivo* and tissue-culture studies (14).

VII. COMBINATION OF TWO FUNCTIONAL GROUPS IN THE SAME MOLECULE

Molecular size and surface area, tautomerism, resonance effects and electron distribution, and the chemical reactivity of specific groupings, together with the conformation of the molecule, are all factors which may be affected by the introduction of further substituents into an active molecule, and modification of any of these factors may of course profoundly affect the biological activity of the molecule. Substituent effects are often described as being either

physical or chemical. Physical effects arise mainly from changes in solubility or steric properties; chemical effects result if the substituent potentiates the reactivity of another functional group in the molecule. Such a division is of course an oversimplification necessitated by the available means of investigation, but spectroscopic data, which also result from multiple interactions both within the molecule and with the molecular environment, might be expected to bear some relationship to the pharmacological properties. Comprehensive spectroscopic studies of active molecules by the examination of large series of compounds differing in only minor detail might well lead to the production of more active compounds, but as yet little has been done in this respect.

In some cases it is fairly simple to apportion the effects of substitution to either physical or chemical effects. For instance, the halogenation of phenols and quinones raises their bacteriostatic activity, and halogenation in the appropriate positions of phenoxyacetic acid raises the herbicidal activity. The substitution of a nonreactive halogen atom for hydrogen or methyl in certain natural metabolites frequently results in the production of competitive antagonists. Thus, fluoroacetic acid is metabolized to fluorocitric acid, an antagonist of citric acid (175); 6-fluoronicotinic acid competes with nicotinic acid; substitution of chlorine atoms for the methyl groups of riboflavin produces a riboflavine antagonist and 2-methyl-1,4-naphthoquinone, which has vitamin K activity, is antagonized by 2-chloro-1,4-naphthoquinone. In many cases, however, it is not possible to apportion the effect of halogen substitution clearly to physical effects or to effects on chemical reactivity: e.g., the substitution of chlorine in quinoline and acridine (Chloroquine and Mepacrine), in biguanidine antimalarials, and in antibiotics such as Aureomycin and Griseofulvin.

Interruption in the conversion of anthranilic acid to indole in *Escherichia coli*, caused by Chloromycetin, Aureomycin, and Terramycin, has been observed (18, 19), and both 4- and 5-methylantranilic acid inhibit the growth of *E. coli*. The latter effect is reversed by anthranilic acid, indole, and tryptophan, from which it is concluded that the two methyl compounds interfere with the synthesis of tryptophan at the stage of anthranilic acid (184). In accordance with these results, experiments with *E. coli* have shown that 5-methylantranilic acid and 4- and 5-fluoroanthranilic acids are antagonists of the parent substance, anthranilic acid, the magnitude of the inhibition being greater in the case of the fluoro compounds (216). This difference in activity either results from differences in physical properties, e.g., the Van der Waals radii of the substituents which might affect

the degree of complementariness with some enzyme surface, or from differences in chemical reactivity. Spectroscopic investigation of a large series of substituted anthranilic acids (115) has shown that both the amino and the carboxyl groups are affected by the substituents. In the case of the amino group a correlation exists between both the asymmetric and the symmetric NH_2 vibrations and the σ constants of the substituent groups with reference to the amino group. Correlation coefficients (99) for the asymmetric and symmetric NH_2 frequencies are 0.817 and 0.770, respectively. All the anthranilic acids show intense absorption in the 1670 cm^{-1} region, produced by the stretching vibration of the $\text{C}=\text{O}$ group. These frequencies, both in solution and in the solid state, can be correlated with the σ -values of the substituents considered in relation to the carbonyl group. This correlation is better with solutions than with disks, but in view of the extensive hydrogen bonding that occurs it can be considered satisfactory even with the solids; the correlation coefficients for solution and disks are 0.887 and 0.642, respectively. Hence, as there is a definite relationship between the stretching frequencies of carboxyl and amino groups and substituent values in anthranilic acids, the greater inhibitory action of the fluoroanthranilic acids in comparison with the methyl compounds probably results from enhanced chemical reactivity in the former rather than from physical effects alone.

The reverse is true for halogenation effects in isatin thiosemicarbazones, for although the infrared spectra indicate that both the α -carbonyl group and the β - $\text{C}=\text{N}$ linkage are activated (188), the antiviral activity drops to zero (14). The substituent effects in this case are purely physical.

Among the compounds which act as acylating agents, e.g., ATP (adenosine-5'-triphosphate), coenzyme A S-acetate, penicillin, and the modern organophosphorus insecticides, the latter have been most extensively investigated. These compounds exert their effect by acylating various esterases (acetylcholinesterase, butyrylcholinesterase, chymotrypsin, etc.) with the formation of phosphorus-containing enzymes in which biochemical function has been destroyed. Reactivators of the inhibited enzyme usually contain two functional groups, one to provide a point of attachment to the anionic or other subsite of the enzyme and the other to interact with the phosphorylated esteratic site. For example, various 1,2-dione monoximes of the type $\text{RCOC}(\text{R}')=\text{NOH}$ will reactivate esterases inhibited by isopropylmethylphosphonofluoridate (Sarin) (7). A spectroscopic examination of isatin β -oximes, which contain both oximino and α -carbonyl groups essential to this type of reactivator, was made to ascertain whether the carbonyl

group exerted its effect mainly by activating the oximino group or by its hydrogen-bonding propensity. The latter would appear more likely, as although the activities in general vary with the σ -values of the substituents, they are poor reactivators because of the preferential formation of the dimer by intermolecular hydrogen bonding (164) rather than bonding to the enzyme surface. More potent reactivators contain both a quaternary ammonium group and an oximino group, and by a combination of kinetic and spectroscopic studies useful antidotes to organophosphate poisons have been developed (i.e., bisquaternary pyridinium aldoximes). Both the ultraviolet and the infrared spectra of substituted pyridine 2-, 3-, and 4-aldoximes were examined. These generally absorb near 3600 cm^{-1} in carbon tetrachloride owing to the O—H stretching vibration and they exist, therefore, mainly in the enolic form in nonpolar solvents. As the O—H stretching frequency for the 2-isomer corresponds to that of a free O—H group it must have the trans configuration, i.e., it is suitably projected to effect reactivation of the phosphorylated esteratic site. The ultraviolet spectra indicated that the resonance between the zwitterion and the quinonoid form of the quaternary pyridine aldoximes varied throughout the series, and that in the most active monoquaternary compounds the quinonoid structure contributes little to the resonance hybrid, which results in a greater negative charge on the oximino oxygen atom. In the isomeric pyridine aldoxime methiodides both the indicated and the observed orders of activity were $2 > 4 > 3$. As bisquaternary compounds can be obtained only with difficulty from pyridine 2-aldoxime, derivatives of pyridine 4-aldoxime were synthesized in which oximino groups of high intrinsic activity are linked by quaternary groups with optimum spacing (86, 87, 88, 89).

Many similar problems of the relation between biological activity and structure for which solution would usually be sought in the integration of several traditional lines of investigation may be solved by the intelligent application of molecular spectroscopy.

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