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ABOUT THE COVER

Our chemist-collector seems to enjoy nothing more than to correct or determine attributions of old paintings and to figure out just what an artist had in mind.

This rather lascivious and wildly colored, large (42x59 inches) work on canvas by Aert de Gelder, signed and dated 1681, was auctioned at Christie's some years ago, and was then called Ahasuerus and Esther. De Gelder was particularly fond of the Book of Esther, and more of his works depict that story than any other. Our chemist believes, however, that this represents Tamar and Judah, rather than Esther and her uncle. Tamar had been married to Judah's two older sons, who had died, and Judah had refused to let his youngest son marry her as was required by custom later formulated in the levirate marriage law of Deuteronomy XXV, made so familiar through the story of Ruth. Tamar then pretended to be a prostitute who attracted Judah and kept his staff, signet ring and cord—clearly shown in this painting—as a pledge for payment of services rendered. When Judah heard later that Tamar was pregnant, he condemned her to death, until she sent him his own pledges saying "By the man whose these are, am I with child." No other history of a people is as honest as the Bible: David and the Messiah are the descendants of Ruth the Moabitess and Tamar the Canaanite pretending to be a whore.

If our collector is correct, one wonders why Tamar is unveiled; almost all other paintings of this subject show her heavily veiled. De Gelder is unlikely to have known of the Talmudic commentary that Tamar had been heavily veiled while in Judah's house—so that he had never seen her there—and did remove her veil after she had enticed him. Probably the artist just couldn't resist the temptation to show Tamar's eager expression—impossible to show through a veil, and, though a bachelor, de Gelder may have guessed that it is difficult to make love through a veil.

Tamar's expression is strongly reminiscent of that of a girl painted by de Gelder's master, Rembrandt, thirty six years earlier—the painting of a girl at an open door now at the Chicago Art Institute, and the painting is also related to one of Rembrandt's iconographically most puzzling works, the so-called 'Jewish Bride' in Amsterdam.

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Macrocyclic Polyethers for Complexing Metals

Dr. Charles J. Pedersen
Elastomer Chemicals Department
E. I. Du Pont De Nemours & Company, Inc.

Discovery of "Crown"

Certain macrocyclic polyethers, nicknamed crown compounds, have aroused considerable interest in several branches of chemistry in recent years because they are the first neutral synthetic compounds to form stable complexes with the alkali metal ions. It is my purpose in this short article to tell you about the preparation and properties of these compounds and their complexes. To start with, this is how I discovered the crown compounds.

For years, I had studied the autoxidation of petroleum products and rubber, and its retardation by antioxidants. Autoxidation is greatly catalyzed by trace-metals, such as copper and vanadium, which also accelerate the destructive oxidation of antioxidants. Hence, I had developed compounds known as "metal deactivators" which suppress the catalytic activity of the metal salts by converting them into inactive, multidentate complexes.[1] For example, N,N'-(1,2-propylenebis) (salicylideneimine) is an excellent deactivator for copper and has been used industrially for this purpose for many years. (See Figure 1.)

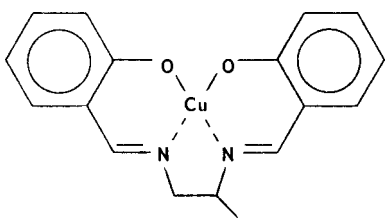


Figure 1. Copper Complex of N,N'-(1,2-Propylenebis)(Salicylideneimine)

When I transferred my interest to vanadium catalysts for the polymerization of olefins, I decided to study the effects of uni- and multidentate phenolic ligands on the catalytic properties of the vanadyl group, VO. The quinquedentate ligand I selected was bis [2-(o-hydroxyphenoxy)ethyl] ether whose synthesis is depicted in Figure 2.

The partially protected catechol[2] (see Figure 2, I) was contaminated with about 10% unreacted catechol but I decided to use this mixture for the second step, since purification would be required anyway at the end. The reactions were carried out as outlined and gave a product mixture in the form of an unattractive goo. Initial attempts at purification gave a small quantity (0.4% yield) of white crystals which drew attention by their silky, fibrous structure and apparent insolubility in hydroxylic solvents.

It was fortunate that I used an ultraviolet spectrophotometer to follow the reactions of the phenols. These compounds and their ethers, in neutral methanol solutions, absorb in the region of 275 mμ. On treatment with alkali, the absorption curve is not significantly altered if all the hydroxyl groups are covered, but it is shifted to longer wavelengths and higher absorption if one or more hydroxyl groups are still free.

The unknown product was very little soluble in methanol and the neutral solution gave an absorption curve characteristic for a phenolic compound. The solution was made alkaline with sodium hydroxide with the expectation that the curve would either be unaffected or shifted to longer wavelengths. The resulting spectrum, however, showed neither effect but the one shown in Figure 3. At the same time, I noticed that the fibrous crystals were freely soluble in methanol in the presence of sodium hydroxide. This seemed strange since the compound did not contain a free phenolic group, a fact confirmed by its infrared and NMR spectra. I then found that the compound was soluble in methanol containing any soluble sodium salt. Thus, the increased solubility was not due to alkalinity but to sodium ions, but there was no obvious explanation for this property of the compound because its elementary analysis corresponded with that for 2,3-benzo-1,4,7-trioxacyclononane,

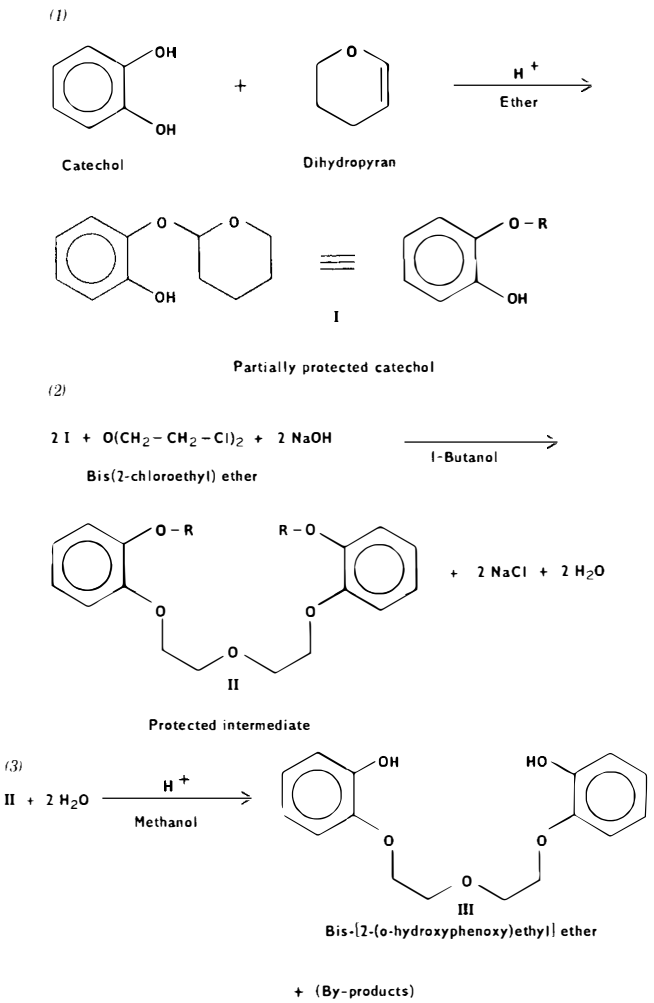
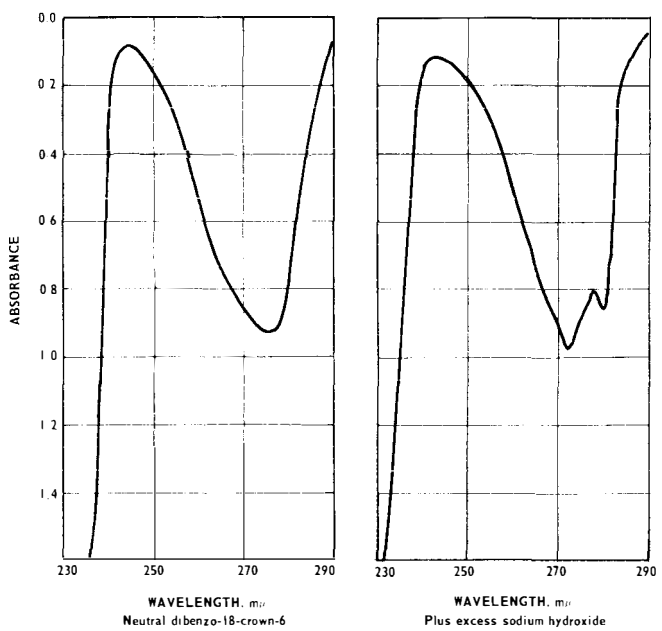


Figure 2. Synthesis of Bis[2-(o-Hydroxyphenoxy) Ethyl] Ether

(see Figure 4) a plausible product from the reaction of catechol and bis(2-chloroethyl) ether in the presence of sodium hydroxide. Its molecular weight, however, was exactly twice that of the above compound and revealed the true structure of dibenzo-18-crown-6, the first and most versatile of the aromatic crown compounds. (see Figure 5)

It seemed clear to me now that the sodium ion had fallen into the hole in the center of the molecule, and was held there by the electrostatic attraction between its positive charge and the negative dipolar charge on the six oxygen atoms symmetrically arranged around it in the polyether ring. Tests showed that other alkali metal ions and ammonium ion behaved like the sodium ion so that, at long last, a neutral compound had been synthesized which formed stable complexes with alkali metal ions. My excitement, which had been rising during this investigation, now reached its peak and ideas swarmed in my brain. I applied the epithet "crown" to the first member of this class of macrocyclic polyethers because its molecular model looked like one and, with it, cations could be crowned and uncrowned without physical damage to either, just as the heads of royalty.

Another aspect of this discovery filled me with wonder. A ring of eighteen atoms had been formed in a single operation by the reaction of two molecules of catechol, which was present as a minor impurity, with two molecules of bis(2-chloroethyl) ether. Further experiments revealed that dibenzo-18-crown-6 can be synthesized from these intermediates in a 45% yield without resorting to high dilution techniques. This was most unexpected and some good reason must exist for such an unusual result. I concluded that the ring-closing step, either by a second molecule of catechol or a second molecule of bis(2-chloroethyl) ether, was facilitated by the sodium ion which, by ion-dipole interaction, "wrapped" the three-molecule intermediates around itself in a three-quarter circle and disposed them to ring closure. Later experiments appear to support this hypothesis. The yields of dibenzo-18-crown-6 are higher when it is prepared with sodium or potassium hydroxide than when lithium or tetramethylammonium hydroxide is used. Lithium and the quaternary ammonium ions are not strongly complexed by the polyether. The best complexers, rings of 15 to 24 atoms including 5 to 8 oxygen atoms, are formed in higher yields than smaller or larger rings, or rings of equal sizes with only 4 oxygen atoms. Finally, open-chain polyethers such as 3,4,12,13-dibenzo-2,5,8,11,14-pentaoxapentadeca-3,12-diene, (see Figure 6) were found to form complexes with sodium and potassium ions.[3]



Concentration of polyether: 0.000183 mole/liter Cell path: 1 cm.

Figure 3. Effect of Sodium Hydroxide on the Ultraviolet Spectrum of Dibenzo-18-Crown-6 in Methanol

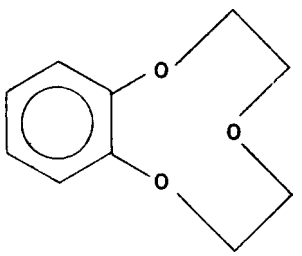


Figure 4. 2,3-Benzo-1,4,7-Trioxacyclononane

Preparation and Properties of Macrocyclic Polyethers

Spurred by curiosity regarding the factors involved in the stability of the salt complexes, such as the relative sizes of the hole and the cation, and the number and symmetrical arrangement of the oxygen atoms in the polyether ring, I initiated an extensive program of syntheses. Ultimately, about 60 macroyclic polyethers were prepared containing 12 to 60 atoms in the polyether ring including 4 to 20 oxygen atoms.[4,5] Many of these compounds were found to be useless as complexing agents but they served to define the effective ones which are compounds containing 5 to 10 oxygen atoms in the ring each separated from the next by 2 carbon atoms.

Since the official names of the macrocylic polyethers is too cumbersome for convenient use, a system of abbreviated names has been devised solely for their ready identification.[4] The examples in Figure 7 illustrate how the name is made up of the side-ring substituents, the total number of atoms in the polyether ring, the word "crown", and the number of oxygen atoms in the main ring.

The aromatic macrocyclic polyethers are neutral, colorless compounds with sharp melting points, and are little soluble in water and alcohols, fairly soluble in aromatic solvents, and very soluble in methylene chloride and chloroform. They undergo substitution reactions characteristic for aromatic ethers (halogenation, nitration, etc.), and form formaldehyde resins when treated with paraformaldehyde under acid conditions. They are decomposed by reactions which cause the scission of aromatic ethers.

The saturated macrocyclic polyethers are obtained most simply by catalytically hydrogenating the aromatic compounds using ruthenium catalyst. Bridge-bond isomers are obtained from compounds containing two or more aromatic side-ring substituents. For example, dibenzo-18-crown-6 gives two isomers of dicyclohexyl-18-crown-6. The saturated polyethers are colorless, viscous liquids or solids of low melting points. They are thermally stable but, like the aromatic compounds, must be protected from oxygen at high temperatures. They are, as a group, very much more soluble than the aromatic compounds in all solvents, and

most of them are even soluble in petroleum ether. They cannot be easily substituted but certain substituted compounds can be obtained by hydrogenating the appropriate aromatic precursors.

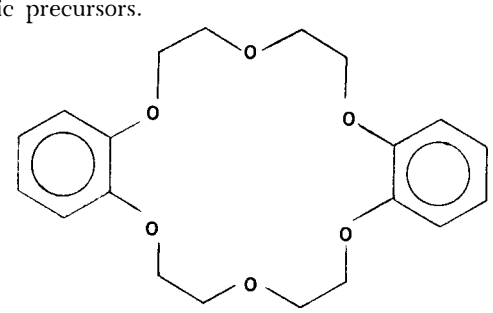


Figure 5. 2,3,11,12-Dibenzo-1,4,7,10,13,16-Hexaoxacyclooctadeca-2,11-Diene (Dibenzo-18-Crown-6)

Salt Complexes of Macrocylic Polyethers

The importance of macrocyclic polyethers as complexing agents is due to their preference for alkali metal ions which do not form complexes with many of the numerous ligands for the transition metal ions. The crown compounds form stable crystalline complexes and solutions of the complexes with some or all of the cations of Li, Na, K, Rb, Cs, NH_4, RNH_3, Ag(I), Au(I), Ca, Sr, Ba, Ra, Zn, Cd, Hg(I & II), La(III), Tl(I), Ce(III), and Pb(II).[4] Some of them, e.g., dicyclohexyl-18-crown-6, also form complexes with Co(II) and some other transition metal ions.[6] The saturated compounds are better complexing agents than the corresponding aromatic compounds.

Three criteria have been used for the formation of complexes between macrocyclic polyethers and salts: (a) isolation of the complexes as crystals; (b) characteristic changes in the ultraviolet spectra of the aromatic compounds; and (c) changes in the solubilities of the polyethers and salts in different solvents.

As is evident from the diagrams in Figure 7, these compounds have holes of different diameters in the center of the polyether rings. The uncomplexed cations also differ in size: sodium 1.90A, potassium 2.66A, ammonium 2.84A, rubidium 2.96A, and cesium 3.34A. Depending, therefore, on the relative sizes of the hole and the cation, crystalline complexes with polyether/cation ratios of 1:1, 3:2, and 2:1 have been prepared.[7] For example, benzo-15-crown-5 forms 1:1 with sodium, 2:1 with potassium, ammonium and cesium; dibenzo-18-crown-6 forms 1:1 with potassium, ammonium and rubidium, 3:2 with cesium, 2:1 with rubidium and cesium; dibenzo-21-crown-7 forms 2:1 with cesium; and dibenzo-24-crown-8 forms 2:1 with cesium.[6] The aromatic macrocyclic polyethers tend to give high melting complexes which are not readily soluble in aprotic solvents, while the saturated compounds give lower melting complexes which are more soluble. Most of the pure complexes are decomposed by water, the rate and extent of decomposition depending on the proportion of water and the temperature.

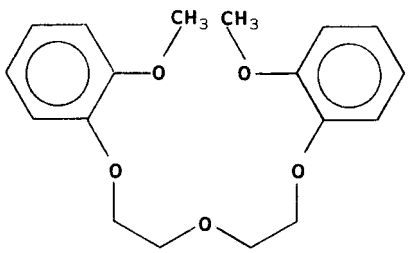
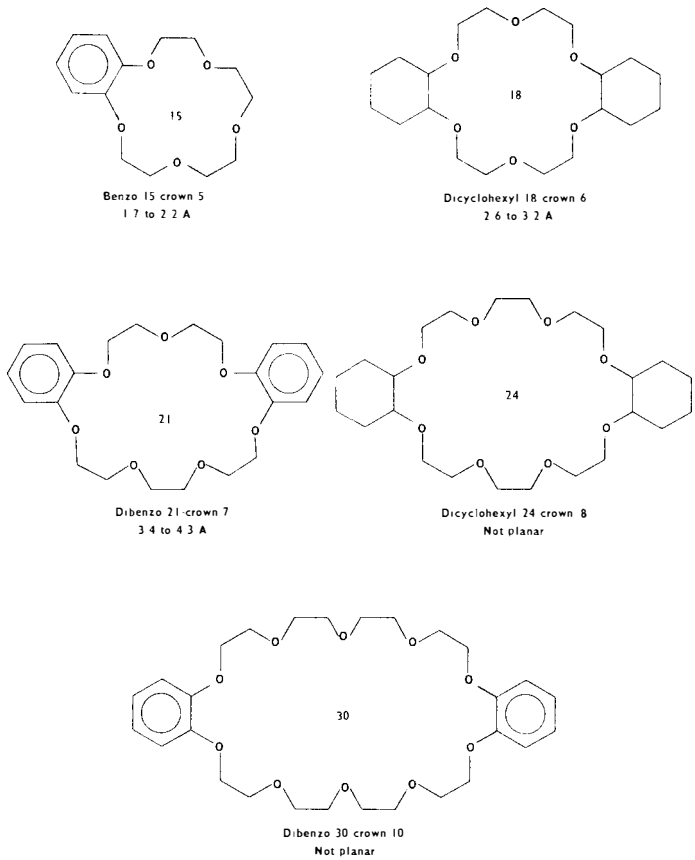


Figure 6. 3,4,12,13-Dibenzo-2,5,8,11,14-Pentaoxapentadeca-3,12-Diene

It was postulated from the beginning that complexes of macrocyclic polyethers containing less than 7 oxygen atoms consisted of a cation surrounded by the oxygen atoms arranged symmetrically in a single plane.[4] The essential correctness of this view of the structure has been confirmed by Professor M. R. Truter and her collaborators who have been the first to determine the structures of a number of crystalline salt complexes of crown compounds.[8,9]

All macrocyclic polyethers containing one or more benzo groups have a characteristic absorption maximum at 275 mμ in methanol, and the shapes of the curves are altered by the addition of complexable salts as shown in Figure 3. The spectral evidence is nearly always confirmed by the other two criteria. This is a convenient test but it cannot be applied to the saturated polyethers which do not absorb in a useful range.

Macrocyclic polyethers and complexable salts mutually increase their solubilities in solvents wherein the complexes are soluble. Sometimes these effects are spectacular, for instance, the solubility of dibenzo-18-crown-6 in methanol is 0.001 mole per liter, but the solubility of the potassium thiocyanate complex is 0.107 moles per liter, a 100-fold increase. Many other examples are given in the original publication.[4] Some of the saturated polyethers, such as dicyclohexyl-18-crown-6, have the useful property of solubilizing alkali metal salts, particularly those of potassium,



The numbers within the diagrams are the numbers of atoms in the polyether rings.

The numbers under the names are the estimated diameters of the holes in A.

Figure 7. Some Macrocyclic Polyethers

in aprotic solvents. Crystals of potassium permanganate, potassium *tertiary*-butoxide, and potassium palladous tetrachloride ($PdCl_2$ + 2 KCl) can be made to dissolve in liquid aromatic hydrocarbons merely by adding dicyclohexyl-18-crown-6. Benzylpotassium is rendered soluble in n-heptane by this polyether, but the polyether ring is gradually decomposed by this organometallic compound. A strongly alkaline (ca. 0.3 normal) solution is obtained by dissolving an equimolar mixture of potassium hydroxide and dicyclohexyl-18-crown-6 in methanol, and replacing as much as possible of the methanol with benzene or toluene.[4] This solution will readily saponify the hindered esters of 2,4,6-trimethylbenzoic acid which are resistant to ordinary saponifying agents. The solution also is a powerful anionic catalyst and induces the polymerization of anhydrous formaldehyde and the trimerization of aromatic isocyanates. The solubilizing power of the saturated macrocyclic polyethers permit ionic reactions to occur in aprotic media. It is expected that this property will find practical use in catalysis, enhancement of chemical reactivity, separation and recovery of salts, electrochemistry, and in analytical chemistry.

When a large quantity of a salt solubilized with dicyclohexyl-18-crown-6 is used in stoichiometric rather than catalytic proportion, it is frequently possible to recover the polyether for further use after the desired reaction has been completed. If the reaction mixture is warmed in a large amount of water, the polyether will separate and can be extracted by a solvent, such as toluene.

When an aqueous solution of an alkali metal hydroxide or salt containing a very low concentration of the picrate of the same cation is mixed with an equal volume of an immiscible organic solvent, such as methylene chloride or toluene, nearly all the picrate is present in the yellow aqueous phase and the organic phase remains substantially colorless. If a cyclic polyether is added to the system, the complexed picrate transfers to the organic phase, the extent depending on the effectiveness of the polyether as a complexing agent for the cation. If the polyether is ineffective, the organic phase will be colorless; if the polyether is very powerful, most of the color will be in the organic phase. The efficiencies of the polyethers will lie between these two limits, and can be expressed as percentage extracted. This method is simple, it can be applied to saturated compounds, and it makes it possible to numerically rank the efficiencies of complexation.[10]

Dr. H. K. Fresdorff has determined the stability constants for 1:1 complexes of many macrocyclic polyethers with alkali metal ions by potentiometry with cation-selective electrodes.[11] Selectivity toward the different cations varies with polyether ring size, the optimum ring size being such that the cation just fits into the hole, i. e., 15–18 for sodium ion, 18 for potassium ion, and 18–21 for cesium ion.

The structures of macrocyclic polyethers are similar to those of certain naturally-occurring macrocyclic antibiotics, such as valinomycin, which affect cation transport across biological and artificial membranes. The polyethers, therefore, have created great interest among biologists for studying the mechanism of transport of sodium and potassium ions across cell membranes, one of the fundamental processes in both animal and vegetable kingdoms.

Limitations and Toxicity

Although salts with high lattice energy, such as fluorides, nitrates, sulfates and carbonates, form complexes with macrocyclic polyethers in alcoholic solvents, they cannot be isolated in the solid state because one or the other uncomplexed component precipitates on concentrating the solutions. For the same reason, these salts cannot be rendered soluble in aprotic solvents by the polyethers.

Dicyclohexyl-18-crown-6 possesses unusual physiological properties which require care in handling. It is likely that other cyclic polyethers with similar complexing power are also toxic, and should be handled with equal care. The approximate lethal dose for ingestion by rats was 300 mg./kg. It should be mentioned, however, that no difficulty whatever was encountered with this compound during seven years of handling in the laboratory.

REFERENCES

1. C. J. Pedersen, *Ind. Eng. Chem.*, **41,** 924 (1949).
2. W. E. Parham and E. L. Anderson, *J. Am. Chem. Soc.*, **70,** 4187 (1948).
3. C. J. Pedersen, unpublished results.
4. C. J. Pedersen, *J. Am. Chem. Soc.*, **89,** 7017 (1967).
5. C. J. Pedersen, *J. Am. Chem. Soc.*, **92,** 391 (1970).
6. A. C. L. Su and J. F. Weiher, *Inorg. Chem.*, **7,** 176 (1968).
7. C. J. Pedersen, *J. Am. Chem. Soc.*, **92,** 386 (1970).
8. D. Bright and Mary R. Truter, *Nature*, **225,** 176 (1970).
9. M. A. Bush and Mary R. Truter, in press.
10. C. J. Pedersen, *Federation Proceedings*, **27,** 1305 (1968).
11. H. K. Frensdorff, *J. Am. Chem. Soc.*, in press.
12. Four articles by D. C. Tosteson; H. Lardy; B. C. Pressman; G. Eisenman, S. M. Ciani, and G. Szabo, *Federation Proceedings*, **27,** 1269–1304 (1968).

Dr. Charles J. Pedersen

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The beautiful alchemical painting depicted on our cover is discussed in the article on the opposite page.

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Cornelis Bega's Alchemist

A Problem in Art History

Alfred Bader

One of the best known works in the collection of alchemical paintings of the Fisher Scientific Company in Pittsburgh, is a painting by Cornelis Bega called *The Chemyst* (fig. 1), familiar to most chemists through more than 9000 reproductions distributed by Fisher Scientific.[1]

Fisher describes this painting as "the gem of all alchemist paintings because of its modern rendition of highlights, the meticulous attention to all details, the flesh-tones of the alchemist, and the unusual heliotrope tint of the entire picture. In fact, the former director of fine arts of Carnegie Institute, Pittsburgh, stated that this picture would do justice to any collection in America for its artistic merit alone—without even considering its scientific interest."

Figure 1

1 I would like to thank Mr. John C. Pavlik, the Director of Public Relations at the Fisher Scientific Company for allowing me to reproduce the cover of *The Laboratory* and the Fisher paintings, and for his generous help in comparing the paintings.

"This particular painting was the favorite picture of the late Sir William Jackson Pope, professor of chemistry at the University of Cambridge in England, who attained just about all the honors available to a chemist, including an honorary membership in the American Chemical Society, for his contributions to the winning of World War I."

Figure 2

Certainly it is one of the two best paintings in the Fisher Collection. Unfortunately, the greatest artists—Rembrandt, Titian, Vermeer, Velasquez, for instance—did not paint alchemical subjects, and so most alchemical paintings are of greater historical than artistic interest. The Fisher Collection's other really fine alchemical painting is a study by Adriaen van der Venne (fig. 2) which unfortunately has never been reproduced.

Two years ago there appeared at Christie's a small canvas (fig. 3) which of course must be related to the Fisher painting.

The painting in London came from the collection of one of England's greatest 19th century collectors, John Sheepshanks, is signed and, except for a yellowed varnish which was easily removed, is in a fine state of preservation.

The great English auction houses have a simple way of indicating their opinion of a painting's authenticity. If it is called "Cornelis Pietersz Bega" they believe it to be a work by the artist. If called "C. P. Bega" they believe it to be a work of the period, which *may* be the work of the artist. If just called "Bega," they don't think that it could possibly be a work by Bega, but the owner says it is. The Sheepshanks painting was called Cornelis Pietersz Bega, and because of its beauty and provenance brought an auction record for a work by Bega, and perhaps also for an alchemical painting.

Professor Pope had purchased the Fisher painting from the collection of J. C. W. Sawbridge–Earle–Drax which was auctioned also at Christie's, on May 10, 1935, was then described as a work by C. P. Bega and brought the rather unsubstantial price of 50 guineas.

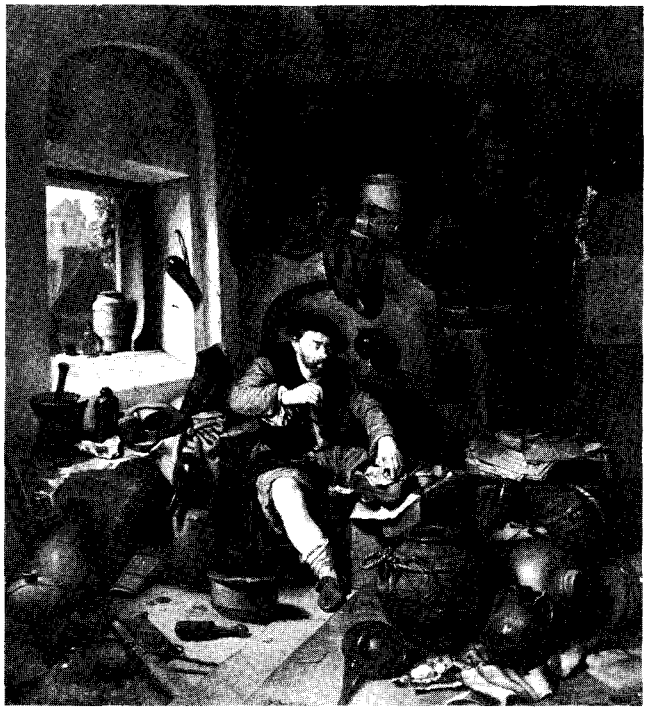


Figure 3

Knowing that, it seemed possible that the Fisher painting was a copy after the Sheepshanks original. A direct comparison of the paintings side by side in Pittsburgh made it clear however that both paintings are of such fine quality, and there are so many variations of details that one cannot think of one being someone else's copy of the other, no matter how skillfully done. The Fisher painting is somewhat smaller (14½ x 13⅜ inches against 16¾ x 15 inches), on panel and signed and indistinctly dated. The Sheepshanks painting is on canvas and signed on the lower right.

Some years ago, Fisher's publication, The Laboratory (Vol. 23, Number 1; fig. 4), featured on its front cover one of the world's foremost authorities on the conservation of paintings, The Mellon Institute's Dr. Robert L. Feller, examining the Fisher painting.

When asked about the signature of the Fisher painting, he said that he had not really cleaned the painting, but only posed for the photograph; Dr. Eric C. Holmer, the restorer who really did clean the painting, is certain that the signature in the Fisher painting sits firmly on the letter in the center of the painting. Similarly, the signature on the painting on canvas was immovable by acetone during cleaning, and is certainly contemporary with the painting.

Thus the logical conclusion is that both paintings are by Cornelis Bega. It is surely conceivable that a customer had greatly admired the first work and had commissioned the artist to paint a work much like it.

But which is the first and which the second version? There certainty is much harder to attain. Comparing details, there are some in each painting better than the corresponding detail in the other. This is subjective and elusive. But I believe that there are some changes which only an artist would make when 'improving' a second version after a first. Take the pestle, for instance, at the foot of the step. In the Sheepshanks painting it is small (fig. 5) and the spatial delineation of the steps is not quite clear. In the Fisher

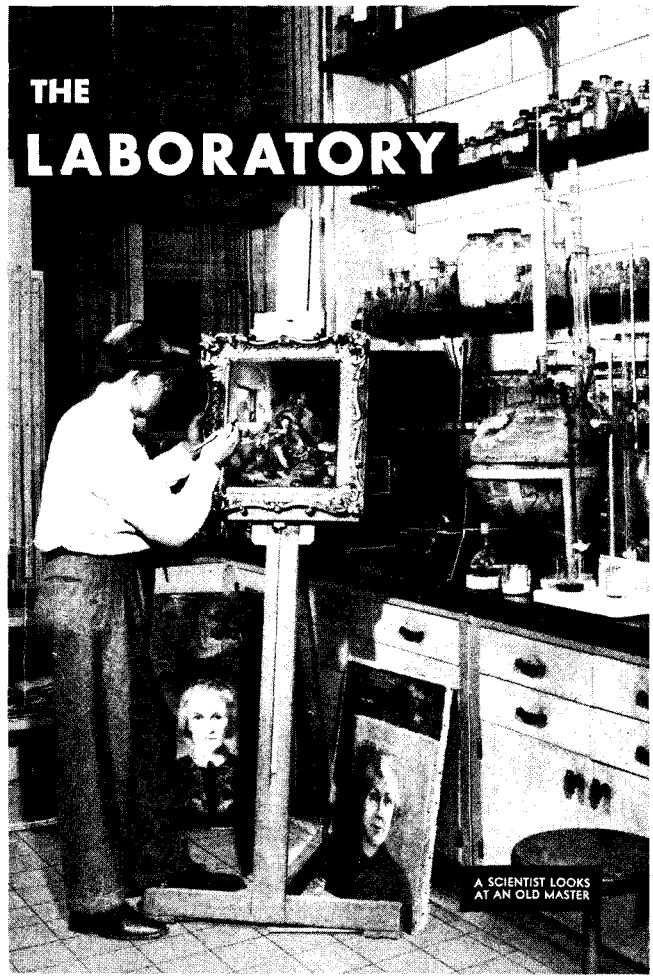


Figure 4

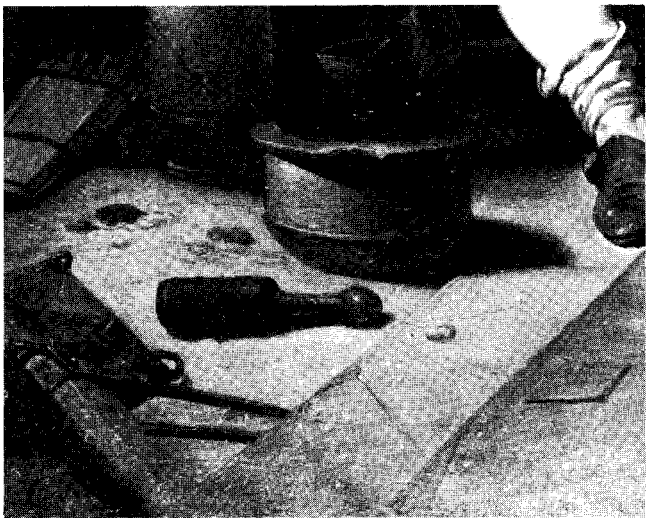


Figure 5

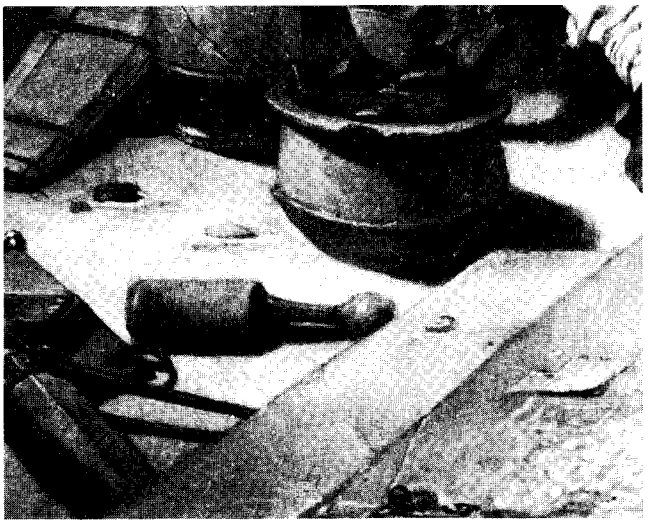


Figure 6

painting, the pestle (fig. 6) is much larger and serves to give spatial clarity to the steps. The larger pestle is, I believe, the sort of 'improvement' an artist would make when working on a replica of his own work. This is, of course, a double edged argument: if I thought the Sheepshanks painting to be a copy after the Fisher painting, I would argue that the copyist simply did not understand the purpose of the large pestle—to aid in delineating the steps. Clearly, art historical analysis is not as straightforward as chemical analysis. Perhaps the most convincing argument for the priority of the Sheepshanks painting lies in the comparison of size and support. When Bega painted these around 1660, canvas was relatively inexpensive and can, of course, be cut to any size. Panels were much more expensive, were planed towards the edges and hence of fixed size. If Bega had painted the panel painting first, he could easily have cut the canvas to the same size as the panel. The painting on canvas contains some beautiful details which are partly or fully missing in the panel painting: presumably Bega only had a panel smaller than the canvas, and so had to leave them out.

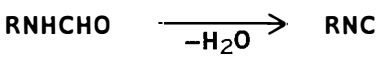
I think that Bega would be amused and intrigued if he could know that his two alchemical paintings after 300 years are in the collections of chemical companies and enjoyed by thousands of chemists.

Isonitriles

Dr. Ishak Ferosie, New Product Development Department, Aldrich Chemical Company, Inc.

STRUCTURE AND SYNTHESIS

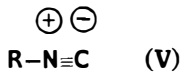
The discovery of isonitriles by Gautier[1] and Hofmann[2] dates back nearly one hundred years. The long delay in the thorough investigation of compounds with this functional group, as evidenced by scarcity in early publications, can be attributed to lack of a convenient synthesis method as well as the considerable stench common to isonitriles. The general method for the preparation of isonitriles by dehydrating N-monosubstituted formamides was just developed in the last ten years.



The first structural formula was postulated by Gautier[1] for ethyl isonitrile (I) differentiating it from the isomeric propionitrile (II).

The terminal carbon could be divalent or tetravalent. Therefore structures (III) and (IV) were proposed by Nef who discussed these further in a series of subsequent papers.[3]

Lindemann and Wiegrebe[4] proposed polar structure (V) which, in analogy to the structure of carbon monoxide, obeys the octet rule.

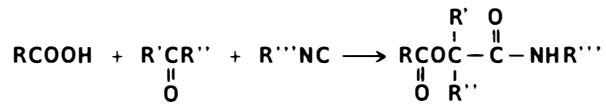


Extensive microwave studies twenty years later confirmed the linearity of the C-N-C bond system thus proving conclusively Lindemann and Wiegrebe's triple-bonded structure. The equivalent structural representation (VI) is now commonly used.

REACTIONS OF ISONITRILES

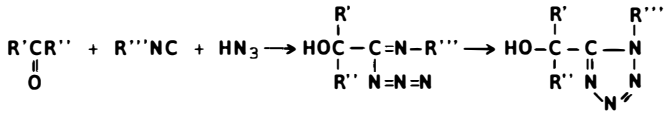
I. The Passerini[5] and Related Reactions

The reaction of isonitriles in a three-component system (addition with carboxylic acids and aldehydes or ketones) was discovered by Passerini in 1921.

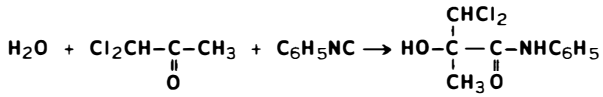


a) *α-Acyloxycarbonamides*[6]

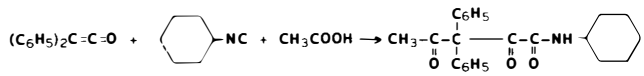
b) *Tetrazoles*[7]



c) *α-Hydroxyamides*[6]



d) *α, γ-Diketoamides*[8]

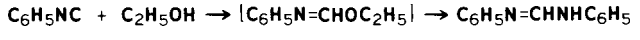


In addition to the aforementioned examples of tri-component reactions involving isonitriles there are many others in the recent literature.

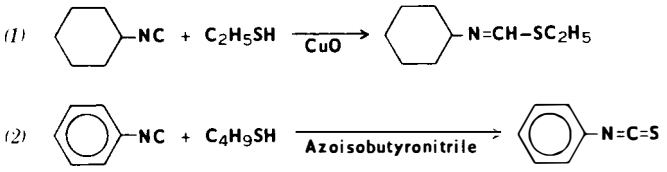
Isonitriles are the only stable representative of organic compounds which contain a formally divalent carbon. This carbon accounts for the wide variety of reactions that isonitriles undergo such as α-addition and multi-component reactions.

II. α-Addition Reaction of Isonitriles

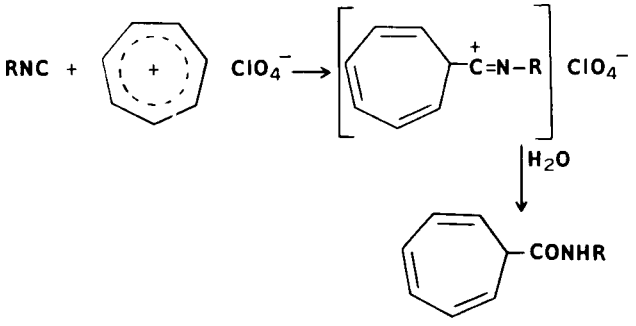
a) *N,N'-Diarylformamidine*[9]



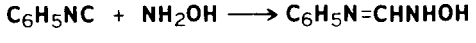
b) *Thioformimidate and Isothiocyanates*[10, 11]



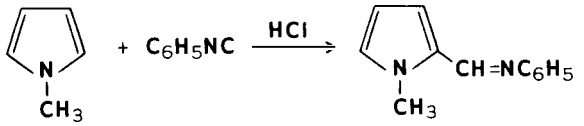
c) *N-Substituted Cycloheptatriene Carboxamide*[12]



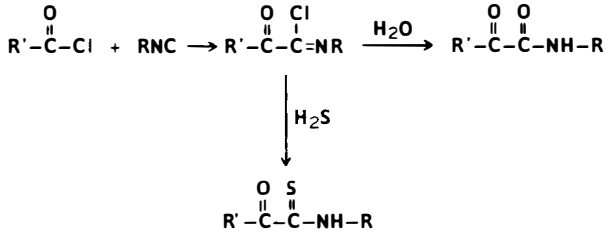
d) *Formamidine Derivatives from Phenyl Isocyanide and Hydroxylamine*[13]



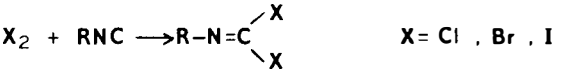
e) *Isonitriles react with pyrroles at position 2 in the presence of HCl and yield the Schiff base derivatives.*[14]



f) *Reaction with Acid Chlorides*[15]



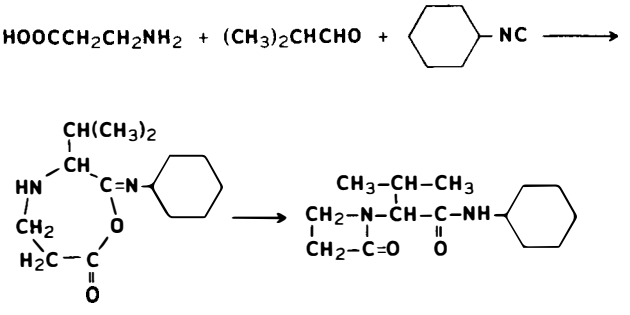
g) *Reaction with Halogens*[9]



III. The Multi-component Reactions

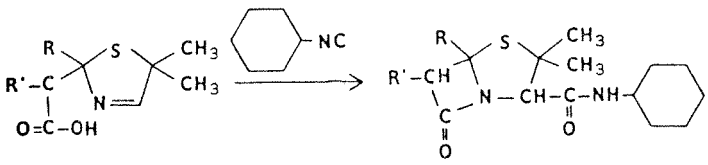
A. β-Lactams and Penicillanic Acid Derivatives

a) *Simple β-lactams can be prepared by condensing β-amino acid, with isonitriles and carbonyl compounds*[16]



b) *Penicillanic Acid Derivatives*

The Δ^3-thiazolines Ia or Ib react with alkyl isocyanides to yield penicillanic amides IIa or IIb.[17]

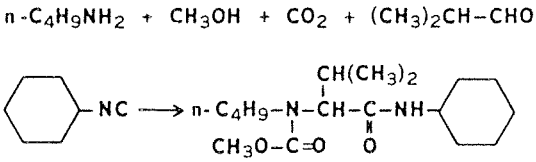


I II

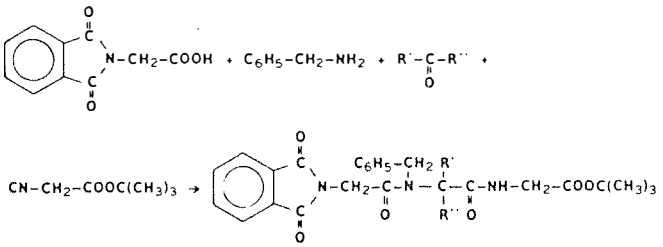
Ia R= CH_3, R'= H
Ib R= H, R'= CH_3

IIa R= CH_3, R'= H
IIb R= H, R'= CH_3

B. Urethanes[18]



C. Peptide Syntheses[18]



For further information the reader is directed to a monograph on Isonitrile Chemistry edited by Dr. Ivar Ugi.[19]

References

1. Gautier, A., Ann. Chim. (Paris) [4] 17, 218 (1869). Gautier, A., Justus Liebigs Ann. Chem., 142, 289 (1867).
2. Hofmann, A. W., Justus Liebigs Ann. Chem., 144, 114 (1867).
3. Nef, I. U., Justus Liebigs Ann. Chem., 270, 267 (1892).
   Nef, I. U., *ibid.*, 280, 291 (1894).
   Nef, I. U., *ibid.*, 287, 265 (1895).
   Nef, I. U., *ibid.*, 298, 202 (1897).
   Nef, I. U., *ibid.*, 298, 368 (1897).
   Nef, I. U., *ibid.*, 309, 126 (1899).
4. Lindemann, H. and Wiegrebe, L., Chem. Ber., 63, 1650 (1930).
5. Passerini, M., Gazz. Chim. Ital., 51, II, 126 (1921).
6. Passerini, M., Gazz. Chim. Ital., 54, 529 (1924).
7. Passerini, M., and Ragni, G., Gazz. Chim. Ital., 61, 964 (1931).
8. Ugi, I. and Rosendahl, F. K., Chem. Ber., 94, 2233 (1961).
9. Nef, I. U., Justus Liebigs Ann. Chem., 270, 267 (1892).
10. Cairns, T. L., Larchar, A. W. and McKusick, B. C., J. Org. Chem., 17, 1497 (1952).
11. Saegusa, T., Kobayashi, S., Hirota, K., Okumura, Y. and Ito, Y., Bull. Chem. Soc. Japan, 41, 1638 (1968).
12. Ugi, I., Betz, W. and Offermann, K., Chem. Ber., 97, 3008 (1964).
13. Nef, I. U., Justus Liebigs Ann. Chem., 280, 291 (1894).
14. Treibs, A. and Dietl, A., Chem. Ber., 94, 298 (1961).
15. Ugi, I. and Fetzer, U., Chem. Ber., 94, 1116 (1961).
16. Steinbrückner, C., Ph.D. Thesis, University of München, (1961).
17. Ugi, I. and Wischhöfer, E., Chem. Ber., 95, 136 (1962).
18. Ugi, I. and Steinbrückner, C., Chem. Ber., 94, 2802 (1961).
19. Ugi, I., Ed. "Isonitrile Chemistry," Academic Press, N.Y., N.Y. (1971).

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Volume 4, Number 3, 1971

Special Thiol Reagent Issue

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ABOUT THE COVER

Our collector-chemist purchased this long (50 x 88 cm, oil on panel) painting by Moses van Uyttenbrock in Holland some months ago, and was then told that it depicts Tobias and the angel. This our chemist does not believe, but thinks that it shows Jacob fighting with the angel at Peniel; the river would then be the Yabok, and the ferry dividing Jacob's property in anticipation of Esau's attack.

This was of course *the* most important moment in Jacob's life: the fight with the evil within himself. Up to then Jacob had often acted shabbily and even dishonestly: in buying Esau's birthright, in deceiving his father, in his dealings with Laban. Now he resolved that if his life was spared the next day, he would become a truly good person, worthy of the name Israel.

This fight saved Jacob's life. Had he not been injured, and limping, Esau would surely have killed him; it is difficult even for an Esau to kill a man who can hardly walk.

Volume 4, Number 3
1971

Published by
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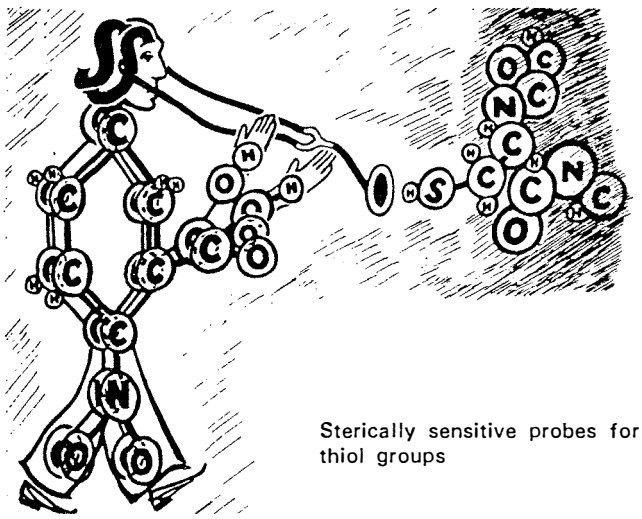


Sterically sensitive probes for thiol groups

In 1963 we made a new reagent for thiol groups, 5,5′-dithiobis-(2-nitrobenzoic acid) or bis-(3-carboxy-4-nitrophenyl)-disulfide which is now widely known as DTNB or Ellman's reagent. At the time we could hardly have known that we were in effect starting a revolution in the methodology of assaying sulfhydryl groups and, indirectly, in studies of the tertiary structure of proteins and particularly of enzymes. Nevertheless this revolution did take place and was the direct result of making DTNB commercially available. DTNB was specifically designed as a chromogenic reagent for thiol or potential thiol groups which would be soluble in aqueous buffer solutions.[1,2] It gained popularity so rapidly that it may now be considered to be the standard reagent for thiol groups. Several new reagents based on the principle of disulfide-thiol interchange but causing spectral changes at other wave-lengths were recently reported and have also been produced commercially by Aldrich. It is the intention of this review to survey various methods used for the assay of thiol groups and to discuss some of their applications.

I. ASSAY OF THIOL GROUPS WITH DTNB

1. *Compounds of Low Molecular Weight*

Soon after the appearance of the original papers describing DTNB,[1,2] an improved method for the assay of blood glutathione was described.[3] Measurement of enzymically produced glutathione with DTNB was used as an indirect assay of nicotinamide-adenine dinucleotide phosphate in blood.[4] A more sensitive method for assaying nanogram amounts of total and oxidized glutathione in blood and other tissues depends on the catalytic action of glutathione or oxidized glutathione in the reduction of DTNB by a mixture of reduced nicotinamide adenine dinucleotide phosphate and yeast glutathione reductase.[5] A less sensitive method also permits the assay of reduced and oxidized glutathione in deproteinized extracts of blood or plasma.[6]

A specific and sensitive assay for disulfides depends on their reduction with dithiothreitol or dithioerythritol; excess of the reducing dithiol is then bound with sodium arsenite, while the newly formed monothiols are determined with DTNB.[7] An autoanalyzer procedure for the assay of glutathione with DTNB has been reported.[8] DTNB has also been used for the assay of sulfite[9] and sulfide[10] and as a reagent for the detection of various thiols on paper or thin-layer chromatograms.[11]

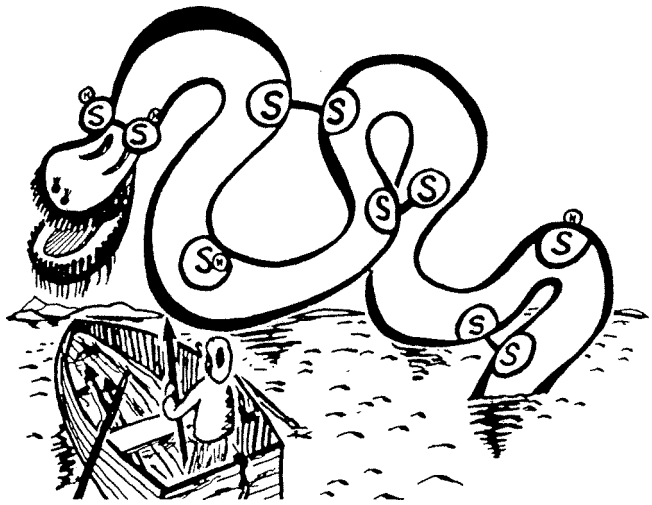
2. *The Assay of Acetylcholinesterase*

The assay of acetylcholinesterase has been revolutionized by the introduction of the substrate acetylthiocholine and the determination of thiocholine liberated by the enzyme with DTNB.[12] Many studies of acetylcholinesterase resulted from the development of the new method;[13,14] thus the existence of isoenzymes in tissues was recognized.[15] A detailed study of variables influencing the assay of thiocholine liberated from acetyl-, propionyl- or butyryl-thiocholine in the presence of various buffers, salts, or inhibitors resulted in the development of a rapid manual or automatic method for distinguishing the typical, atypical and heterozygous forms of the enzyme.[16,17] Butyrylthiocholine was also found to be a good substrate in another investigation.[18]

3. *The Assay of Thiols and Disulfides in Proteins*

The importance of disulfide linkages in maintaining the tertiary structure of protein molecules has been reviewed by many authors[19,20] and studies of the position of disulfide links,[e.g.21] of their biological cleavage by exchange[22] or by photo-reduction [23,24] and of their reformation in partially or completely reduced proteins are essential prerequisites to the preparation of fully synthetic proteins.

Disulfide groups maintain the tertiary structure of even the Loch Ness monster



Thus the intact structure of muramidase,[25] pancreatic ribonuclease[26] and its derivatives,[27], trypsin,[28] taka-amylase A,[29] ribonuclease T_1,[30] human chorionic gonadotrophin[31] and soy-bean tryspin inhibitor[32,33] could be reformed by oxidation of the fully reduced molecules with partial or complete restoration of their biological activities. With more complex molecules such as insulin, only a small proportion of biological activity could be recovered in early experiments of the same kind,[34–36] but later work described the recovery of a substantial proportion of the theoretical biological activity.[37] Total syntheses of insulin were reported from 3 different laboratories[38–40] and there have been many improvements since then, particularly by the use of the solid phase peptide synthesis.[41]

Early studies showed that the thiol groups of proteins reacted considerably more slowly with DTNB than those of soluble compounds of low molecular weight.[1] The sulfhydryl contents of normal and pathological erythrocytes and erythrocyte membranes were investigated using DTNB.[42] The sulfhydryl groups of avian ovalbumins, bovine β-lactoglobulin and bovine serum albumin were determined with p-chloromercuribenzoate, iodine, N-ethylmaleimide and DTNB; of these reagents DTNB was found the most useful.[43] A modification of the DTNB method for the estimation of protein thiol groups involves the formation of the TNB-protein mixed disulfides, their isolation and subsequent liberation of the colored TNB anion.[44] A method of assaying disulfide groups of proteins consists of reduction with sodium borohydride, destruction of excess of reducing agent and estimation of thiol groups with DTNB.[45] A method of assaying both total and protein thiol groups uses different pH values.[46] Total as well as protein thiol groups can be assayed manually or automatically, again using different values of pH.[8] A different procedure assays total thiol groups and non-protein thiol groups after precipitation of protein with trichloroacetic acid.[47] We shall deal later with the extensive applications of these methods.

II. *OTHER PRINCIPAL METHODS OF DETERMINING THIOL GROUPS*

N-Ethylmaleimide was originally described as a color reagent for thiols and thiol esters.[48] Two spectrophotometric assays for sulfhydryl groups using N-ethylmaleimide were reported almost simultaneously from 2 laboratories.[49,50] Some problems connected with the procedure were examined[51] and attempts were made to increase the sensitivity by using ^{14}C-labeled reagent.[52] Like DTNB,[9,10] N-ethylmaleimide will react with other sulfur-containing compounds.[53] The related reagent N-(4-dimethylamino-3,5-dinitrophenyl)maleimide, Tuppy's maleimide, is used to prepare colored derivatives of cysteinyl residues to permit their identification at a later time.[54–59] Another reagent widely used for the assay of sulfhydryl groups is p-chloromercuribenzoate.[60] An attempt to increase the sensitivity of this reagent was made by the use of ^{14}C-labeled p-chloromercuribenzoate,[61] while another method permits the reaction to be followed in the visible part of the spectrum.[62] p-Chloromercuribenzoate and N-ethylmaleimide have been applied to the assay of thiol groups in β-lactoglobulin in the absence and presence of denaturing agents.[63] An interesting procedure converts cysteinyl residues by reaction with 4-vinylpyridine into S-β-(4-pyridylethyl)cysteinyl residues which are liberated on acid hydrolysis and may be estimated in the autoanalyzer.[64,65] A related method converts cysteinyl residues by reaction with acrylonitrile into S-β-cyanoethylcysteinyl residues which give rise, after acid hydrolysis, to S-carboxyethylcysteine.[66] Mention should be made of the older methods using amperometric titration of sulfhydryl groups with various heavy metal ions—e.g. with Ag^+-tris complex.[67] Both picrylsulfonic acid[68] and 1-dimethylaminonaphthalene-5-sulfonyl (DANSYL-) chloride[69] can react with thiol groups. The situation is more complex, however, in the case of the reaction of DANSYL chloride with the reactive thiol groups of creatine kinase.[70]

III. *DITHIODIPYRIDINES AND RELATED REAGENTS FOR THIOL GROUPS*

2,2′-Dithiodipyridine (ALDRITHIOL-2) and 4,4′-dithiodipyridine (ALDRITHIOL-4) were reported as new reagents for thiol groups, which after reaction formed 2- and 4-thiopyridones with new absorption peaks at 340 and 324 nm, respectively.[71] ALDRITHIOL-2 was used for the assay of glutathione or of nicotinamide adenine dinucleotide phosphate using appropriate enzymic coupling with glucose-6-phosphate, glucose-6-phosphate dehydrogenase and glutathione reductase.[72] ALDRITHIOL-4 was used for the determination of the thiol groups of porcine, ovine, bovine and human thyroglobulins,[73] while the rate of reaction of the β-93 sulfhydryl group of hemoglobin with ALDRITHIOL-2 and ALDRITHIOL-4 was found to be much slower when the hemoglobin was bound to haptoglobin than when it was free.[74] Both DTNB and ALDRITHIOL-4 were used as reagents for sulfite,[9] while the same reagents as well as ALDRITHIOL-2 were used for the assay of sulfide.[10] DTNB and ALDRITHIOL-4 were used to study the thiol groups of mercaptalbumins and whole plasma.[75] ALDRITHIOL-2 was found to penetrate and oxidize the thiols of Ehrlich ascites tumor cells with inhibition of respiration and glycolysis,[76,77] but these could be partly restored by treatment with dithiothreitol.[78] In contrast 6,6′-dithiodinicotinic acid (DTDNA) reacted only with the thiol groups on the surface of Ehrlich ascites tumor cells and changed the electrophoretic mobility of the cells.[79,80] A preliminary report suggests that such a modification may affect the ability of these cells to metastasize.[81] 2,2′-Dithiobis-(5-nitropyridine) (DTNP) was developed as a selective reagent for thiol groups particularly suitable for paper and thin-layer chromatograms.[82] [U.S patent 3597160 (August 3, 1971 to Dr. D. R. Grassetti) covers the use of DTNP as a thiol reagent, and Aldrich is not licensed under that patent. Anyone wishing to use DTNP as a thiol reagent should apply to Dr. Grassetti for a license.] ALDRITHIOL-4 proved more suitable than DTNB for the assay of thiol groups of colored heme proteins.[83,84] Other dithiobis(heterocyclic derivative) compounds have been cited as potential reagents for thiol groups.[85]

IV. *THE APPLICATION OF DTNB AND OTHER REAGENTS FOR THIOL GROUPS TO STUDIES OF THE STRUCTURE OR FUNCTION OF ENZYMES*

We have already mentioned some enzyme reactions which are readily followed by means of DTNB.[4,5,12–18] DTNB has also been used for studies of the cystationine cleavage enzymes[86–88] and of thiogalactoside transacetylase.[89] But perhaps the most interesting applications of DTNB and related reagents have been to the charting of

sulfhydryls of proteins and especially of enzymes by dividing them into groups with different reactivities and accessibilities, requiring varying levels of denaturing agents for exposure to the reagent. A further extension of such studies has been to protect otherwise accessible thiol groups by the presence of a substrate or coenzyme and to modify accessibility by change of pH or temperature.

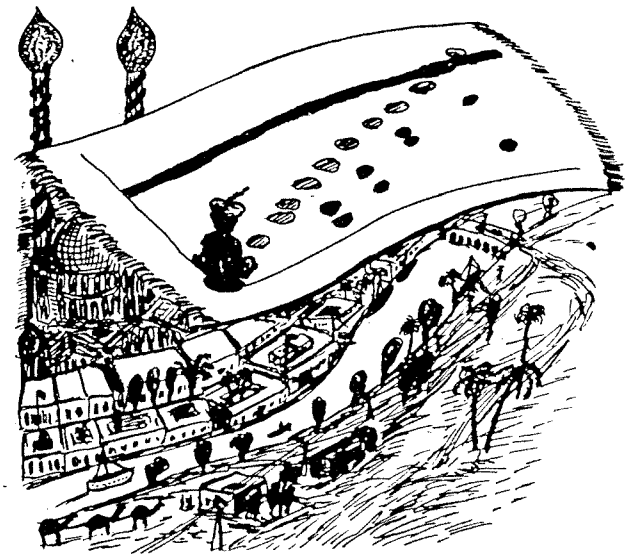
Clostridial ferredoxin required the presence of 4M guanidine hydrochloride before 14 moles of DTNB reacted with the protein; there was little reaction in 6.4M urea.[90] The gradual unmasking of thiol groups of porcine pancreatic α-amylase by ethylenediaminetetraacetate and sodium dodecylsulfate to permit reaction with DTNB, N-ethylmaleimide or p-chloromercuribenzoate reveals a complex situation.[91] Reaction of thiol groups of dihydrofolate reductase with DTNB in the presence of salts or urea leads to irreversible activation.[92] The reaction of DTNB with the thiol groups of ATP:guanidine phosphotransferases is also complex: After 20 min. in tris-acetic acid buffer, pH 7, the following numbers of SH-groups/mole enzyme reacted in the absence (presence) of 8M urea: creatine kinase 2.0 (5.0), arginine kinase 6.0 (6.0), lombricine kinase 1.2 (6.1), taurocyamine kinase 2.0 (14.0) and glycocyamine kinase 6.0 (16.0). The effect of changes of pH and of the presence of arginine or Mg-ATP on the reaction of SH-groups and on the enzyme activity is complex.[93] But different results were obtained with an arginine kinase of different origin.[94] Generally 4-5 SH-groups/mole of fructose diphosphate aldolase of yeast reacted with DTNB without loss of activity, while another 4-5 thiol groups/mole reacted with irreversible inactivation.[95] Six SH-groups of crystalline phosphofructokinase from rabbit skeletal muscle reacted immediately with DTNB (per protomer of 93,200 daltons), while the 7th and 8th; 9th and 10th; 11th and 12th SH-groups reacted with decreasing rates, the remaining 4-6 very slowly. In 2M guanidine hydrochloride all 16-18 SH-groups reacted immediately.[96] But somewhat different results were reported by another group.[97] Thiol groups of different reactivities were also found in heart phosphofructokinase of sheep.[98] DTNB reacts fast with 4 thiol groups/mole of aldolase, prepared from rabbit muscle, slowly with the next 8; another 16 react only in 4M urea, 4M guanidine, or on heating the enzyme to 40°C.[99] Isocitrate dehydrogenase provides an interesting example of substrate protection against inactivation by reaction with DTNB.[100] A single thiol group of the TPN^+-specific isocitrate dehydrogenase of *Azotobacter vinelandii* reacted with DTNB with loss of activity, most of which could be recovered by reaction with dithiothreitol; both substrates protected the thiol group and slowed the reaction.[101] Similar protection of 6–8 reactive SH-groups by Mg-citrate was noted with citrate cleavage enzyme.[102] Inactivation of guinea pig liver transglutaminase by DTNB in the absence of Ca^{++} has been shown to be due to the formation of a single intramolecular disulfide bridge; treatment with dithiothreitol reactivated the enzyme.[103] DTNB proved a suitable substrate for the disulfide reductase of *Achromobacter starkeyi*.[104] Titration of the SH-groups of the glyoxylic acid reductase of spinach leaf indicated the presence of 1 rapidly reacting and 3 slowly reacting groups per molecule consisting of 2 identical or similar units; the thiol groups could be protected by the presence of substrates.[105] Titration of alanine aminotransferase of rat liver with p-chloromercuribenzoate or DTNB showed that 7–8 SH-groups reacted without loss of activity, but rather with slight activation; the reaction of the remaining 16 SH-groups/mole resulted in progressive loss of activity.[106] In D-serine dehydratase of *Escherichia coli* only 1 of 5 thiol groups was titrated with DTNB in the holoenzyme, while 3 were titrated in the apoenzyme and reacted with markedly different rates.[107] In rabbit muscle aldolase, 3 of 7 thiol groups/subunit of molecular weight 40,000 could be titrated with DTNB, iodoacetamide or N-ethylmaleimide and their modification led to loss of enzymic activity; one thiol group/subunit could be protected from reaction by substrate with retention of enzymic activity.[108] Chemical modification of either of two sulfhydryl groups of myosin led to the same change in enzymic properties.[109] But different sulfhydryl groups are involved in the activation and transfer reactions of isoleucyl transfer ribonucleic acid synthetase, as indicated by titration with p-chloromercuribenzoate and DTNB.[110] Fructose-1,6-diphosphatase is stimulated by limited reaction with DTNB,[111] sphingomyelin synthetase by reaction with N-ethylmaleimide, DTNB or ALDRITHIOL-2.[112] DTNB split off a light chain fraction from myosin prepared from the skeletal muscle of rabbits.[113] DTNB also modified the metabolism of rat liver mitochondria.[114] A remarkable variation in reactivity of SH-groups was uncovered in the case of the calf brain isoenzyme of ATP-creatine transphosphorylase: of 10 SH-groups/mole demonstrated as cysteic acid only 8 were titrated with DTNB or p-chloromercuribenzoate in the presence of denaturants, but at pH 3.2 and 30°C 2 more thiol groups reacted with ALDRITHIOL-4.[115] DTNB or ALDRITHIOL-2 titrated 14–16 SH-groups/mole of χ-isopropylmalate synthase of *Salmonella typhimurium* and 2 types of thiol groups could be identified; enzymic activity was lost after 4–5 SH-groups had been titrated, but incubation with dithiothreitol partially restored activity.[116] Both species of porcine pancreatic lipase were found to contain 2 SH-groups/mole, one of which is accessible to the reagent, the second of which requires the presence of denaturant; reaction of the first thiol group did not inactivate the enzyme.[117] A differential inactivation towards its 2 substrates, histidinol and histidinal, was observed during titration of the histidinol dehydrogenase of *Salmonella typhimurium* with p-chloromercuribenzoate or with N-(4-dimethylamino-3,5-dinitrophenyl)maleimide (Tuppy's maleimide).[118] The reaction of p-hydroxymercuribenzoate or of DTNB with pyruvate carboxylase of chick liver leads to complete inactivation with reaction of about 36 of a total of 55 SH-groups per tetramer of 655,000, but the situation is rather complex, as there is partial dissociation of tetramers to monomers and aggregation to species of high molecular weight.[119] N-Ethylmaleimide or DTNB titrated 26–28 SH-groups/mole of Jack bean urease rapidly without loss of activity; the next 7–9 SH-groups reacted more slowly and the enzyme lost activity; a further 45–50 SH-groups/mole reacted only after exposure to 6M guanidine hydrochloride.[120] p-Hydroxymercuribenzoate or DTNB titrated a total of 24 thiol groups/mole of methionyl transfer ribonucleic acid synthetase prepared from *Escherichia coli* K12, but in the presence of a substrate analogue 8 were protected from reaction with the first reagent. Different sulfhydryl groups could be shown to be involved in the two activities of this enzyme.[121] Polyribosomes of rat liver on treatment with p-chloromercuribenzoate or DTNB were rapidly deaggregated to monomeric ribosomes and some rapidly labeled redioactive RNA was released.[122]

Continued on Page 46

(Continued from Page 35)

V. *THE LOCATION OF DISULFIDE BRIDGES IN THE SEQUENCE OF PROTEINS*

A diagonal electrophoresis method,[123] widely used for the identification of cystine peptides from partial enzymic hydrolysates of proteins,[e.g.124] involves paper electrophoresis in one dimension, oxidation of the separated peptides on paper with performic acid and electrophoresis in the second dimension under the same conditions.

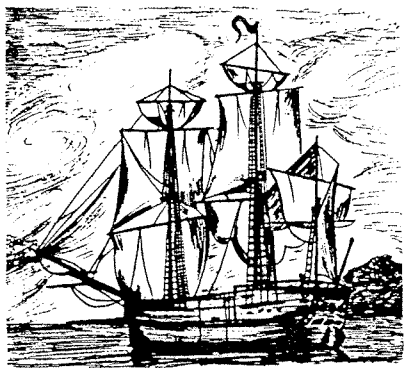


Diagonal electrophoresis locates dithio links

Cysteic acid peptides lie off the diagonal line.[123] Performic acid may, however, hydrolyse peptide bonds.[125] In a new procedure, two-dimentional finger-prints of a partial enzymic hydrolysate of lysozyme were strained with ninhydrin.[126] Cystine peptides were then stained by reduction with sodium borohydride in ethanol, dipping in acetic acid-hydrochloric acid-acetone reagent, adjustment of pH to about 8.4 (phenol red) with ammonia and spraying with 0.1% DTNB in ethanol-0.45M tris HCl buffer, pH 8.2 (1:1 v/v).[126] The cystine peptides were cut out, hydrolysed and analysed quantitatively for amino acids.

VI. *CONCLUSION*

A survey of the publications cited shows that often different reagents for sulfhydryl groups will react with varying numbers of thiol groups of the same protein or enzyme molecule. One may conclude that this variation in reactivity of the thiol groups is due to the steric environment of these groups, such as the shape of the molecule and the surrounding groups of atoms and their charges, as well as to the size, shape and charge of the reagent molecule or ion. Frequently the presence of adequate concentrations of denaturants such as sodium dodecylsulfate, urea or guanidine hydrochloride will cause sufficient unfolding of the protein molecule to permit equal access to all of the reagents for thiol groups. By varying the concentration of denaturant, the degree of unfolding can be controlled and in this way thiol groups with different degrees of reactivity may be revealed. Although up to date most of the work reported has been done with p-chloromercuribenzoate, N-ethylmaleimide and DTNB, it is likely that the other more recently developed reagents may prove equally useful. Because of their varying structures, it seems likely, in fact, that they may respond differently to changes in the steric environment of the thiol groups.



As Endeavour charted the coast of Australia, so our reagents chart the thiol groups of proteins

References

1. G. L. Ellman, Arch. Biochem. Biophys. **82,** 70 (1959).
2. G. L. Ellman, Arch. Biochem. Biophys. 74, 443 (1958).
3. E. Beutler, O. Duron and B. M. Kelly, J. Lab. clin. Med. **61,** 882 (1963).
4. R. H. Thompson, Clin. chim. Acta **12,** 198 (1965).
5. F. Tietze, Analyt. Biochem. **27,** 502 (1969).
6. G. L. Ellman and H. Lysko, J. Lab. clin. Med. **70,** 518 (1967).
7. W. L. Zahler and W. W. Cleland, J. biol. Chem. **243,** 716 (1968).
8. P. C. Jocelyn, Biochem. J. **85,** 480 (1962).
9. R. E. Humphrey, M. H. Ward and W. Hinze, Analyt. Chem. **42,** 698 (1970).
10. R. E. Humphrey, W. Hinze and W. M. Jenkines III, Analyt. Chem. **43,** 140 (1971).
11. C. B. Glaser, H. Maeda and J. Meienhofer, J. Chromat. **50,** 151 (1970).
12. G. L. Ellman, K. D. Courtney, V. Andres, Jr., and R. M. Featherstone, Biochem. Pharmac. **7,** 88 (1961).
13. B. W. O'Malley, C. E. Mengel, W. D. Meriwether and L. G. Zirkle, Jr., Biochemistry **5,** 40 (1966).
14. C. E. Mengel, B. Hyman and B. W. O'Malley, Blood **24,** 451 (1964).
15. G. A. Davis and B. W. Agranoff, Nature, Lond., **220,** 277 (1968).
16. P. J. Garry, Clin. Chem. **17,** 183 (1971).
17. P. J. Garry, Clin. Chem. **17,** 192 (1971).
18. G. Szász, Clin. chim. Acta **19,** 191 (1968).

19. J. T. Edsall, J. cell. comp. Physiol. 47, Suppl. 1, 163 1956).
20. G. V. Samsonov, R. B. Ponomareva and L. P. Shandalova, Doklady Akad. Nauk SSSR 154, 1448 (1964).
21. F. Miller and H. Metzger, J. biol. Chem. 240, 4740 (1965).
22. L. Lumper and H. Zahn, Adv. Enzymol. 27, 199 (1965).
23. T. Asahi, Agr. biol. Chem. 27, 734 (1963).
24. T. Asahi and S. Masaki, J. Biochem., Tokyo, 60, 90 (1966).
25. K. Imai, T. Takagi and T. Isemura, J. Biochem., Tokyo, 53, 1 (1963).
26. M. Sela, F. H. White, Jr., and C. B. Anfinsen, Science 125, 691 (1957).
27. C. J. Epstein and R. F. Goldberger, J. biol. Chem. 239, 1087 (1964).
28. C. J. Epstein and C. B. Anfinsen, J. biol. Chem. 237, 2175 (1962).
29. T. Isemura, T. Takagi, Y. Maeda and K. Yutani, J. Biochem., Tokyo, 53, 155 (1963).
30. K. Kasai, J. Biochem., Tokyo, 57, 372 (1965).
31. H. D. Schlumberger and R. Blobel, Z. Naturforsch. 24b, 54 (1969).
32. R. F. Steiner, Nature, Lond., 204, 579 (1964).
33. R. F. Steiner, Biochim. biophys. Acta 100, 111 (1965).
34. Du Yu-cang, Zhang Yu-shang, Lu Zi-xian and Tsou Chen-lu, Scientia sinica 10, 84 (1961).
35. Tsou Chen-lu, Du Yu-cang and Xü Geng-jun, Scientia sinica 10, 332 (1961).
36. G. H. Dixon and A. C. Wardlaw, Nature, Lond., 188, 721 (1960).
37. Du Yu-cang, Jiang Rong-qing and Tsou Chen-lu, Scientia sinica 14, 229 (1965).
38. Anonymous, Kexue Tongbao, Academia Sinica, 17, 241 (1966).
39. H. Zahn, J. Meienhofer and E. Schnabel, Acta chim. hung. 44, 109 (1965).
40. P. G. Katsoyannis, A. Tometsko and C. Zalut, J. Amer. chem. Soc. 88, 166 (1966).
41. A. Marglin and R. B. Merrifield, J. Amer. chem. Soc. 88, 5051 (1966).
42. A. Szeinberg and L. Clejan, Biochim. biophys. Acta 93, 564 (1964).
43. M. J. F. Diez, D. T. Osuga and R. E. Feeney, Arch. Biochem. Biophys. 107, 449 (1964).
44. P. H. W. Butterworth, H. Baum and J. W. Porter, Arch. Biochem. Biophys. 118, 716 (1967).
45. D. Cavallini, M. T. Graziani and S. Dupré, Nature, Lond., 212, 294 (1966).
46. S. Gabay, A. M. Cabral and R. Healy, Proc. Soc. exp. Biol. Med. 127, 1081 (1968).
47. J. Sedlak and R. H. Lindsay, Analyt. Biochem. 25, 192 (1968).
48. R. Benesch, R. E. Benesch, M. Gutcho and L. Laufer, Science 123, 981 (1956).
49. E. Roberts and G. Rouser, Analyt. Chem. 30, 1291 (1958).
50. N. M. Alexander, Analyt. Chem. 30, 1292 (1958).
51. J. Leslie, Analyt. Biochem. 10, 162 (1965).
52. C. C. Lee and E. R. Samuels, Can. J. Chem. 42, 164 (1964).
53. R. J. Ellis, Nature, Lond., 211, 1266 (1966).
54. A. Witter and H. Tuppy, Biochim. biophys. Acta 45, 429 (1960).
55. H. Tuppy, Mh. Chem. 92, 992 (1961).
56. T. Yamashita, Y. Soma, S. Kobayashi, T. Sekine, K. Titani and K. Narita, J. Biochem., Tokyo, 55, 576 (1964).
57. K. Narita and M. Akao, J. Biochem., Tokyo, 58, 507 (1965).
58. E. Wintersberger, Biochemistry 4, 1533 (1965).
59. E. Wintersberger, H. Neurath, T. L. Coombs and B. L. Vallee, Biochemistry 4, 1526 (1965).
60. P. D. Boyer, J. Amer. chem. Soc. 76, 4331 (1954).
61. V. G. Erwin and P. L. Pedersen, Analyt. Biochem. 25, 477 (1968).
62. I. Fridovich and P. Handler, Analyt. Chem. 29, 1219 (1957).
63. J. Leslie, L. G. Butler and G. Gorin, Arch. Biochem. Biophys. 99, 86 (1962).
64. M. Friedman and L. H. Krull. Biochem. biophys. Res. Commun. 37, 630 (1969).
65. M. Friedman, L. H. Krull and J. F. Cavins, J. biol. Chem. 245, 3868 (1970).
66. L. Weil and T. S. Seibles, Arch. Biochem. Biophys. 95, 470 (1961).
67. R. E. Benesch, H. A. Lardy and R. Benesch, J. biol. Chem. 216, 663 (1955).
68. A. Kotaki, M. Harada and K. Yagi, J. Biochem., Tokyo, 55, 553 (1964).
69. B. S. Hartley and V. Massey, Biochim. biophys. Acta 21, 58 (1956).
70. C. S. Brown and L. W. Cunningham, Biochemistry 9, 3878 (1970).
71. D. R. Grassetti and J. F. Murray Jr., Arch. Biochem. Biophys. 119, 41 (1967).
72. D. R. Grassetti and J. F. Murray, Jr., Analyt. Biochem. 21, 427 (1967).
73. R. Pitt-Rivers and H. L. Schwartz, Biochem. J. 105, 28C (1967).
74. B. Malchy and G. H. Dixon, Can. J. Biochem. 47, 1205 (1969).
75. P. Fantl, Aust. J. exp. Biol. med. Sci. 48, 631 (1970).
76. D. R. Grassetti, J. F. Murray, Jr., M. E. Brokke and A. D. Gutman, J. mednl. Chem. 13, 273 (1970).
77. D. R. Grassetti and J. F. Murray, Jr., Biochem. Pharmac. 16, 2387 (1967).
78. D. R. Grassetti and J. F. Murray, Jr., Biochem. Pharmac. 17, 2281 (1968).
79. J. N. Mehrishi and D. R. Grassetti, Nature, Lond., 224, 563 (1969).
80. D. R. Grassetti, J. F. Murray, Jr., and H. T. Ruan, Biochem. Pharmac. 18, 603 (1969).
81. D. R. Grassetti, Nature, Lond., 228, 282 (1970).
82. D. R. Grassetti and J. F. Murray, Jr., J. Chromat. 41, 121 (1969).
83. R. S. Ampulski, V. E. Avers and S. A. Morell, Analyt. Biochem. 32, 163 (1969).
84. F. Taketa and S. A. Morell, Analyt. Biochem. 32, 169 (1969).
85. D. R. Grassetti and J. F. Murray, Jr., Analyt. chim. Acta 46, 139 (1969).
86. M. Flavin, J. biol. Chem. 237, 768 (1962).
87. M. Flavin and C. Slaughter, J. biol. Chem. 239, 2212 (1964).
88. M. Flavin and A. Segal, J. biol. Chem. 239, 2220 (1964).
89. D. H. Alpers, S. H. Appel and G. M. Tomkins, J. biol. Chem. 240, 10 (1965).
90. R. Malkin and J. C. Rabinowitz, Biochemistry 6, 3880 (1967).

91. M. Schramm, Biochemistry 3, 1231 (1964).
92. P. Reyes and F. M. Huennekens, Biochem. biophys. Res. Commun. 28, 833 (1967).
93. R. Kassab, L. A. Pradel, E. der Terrossian and N. v. Thoai, Biochim. biophys. Acta 132, 347 (1967).
94. G. Lacombe, N. v. Thiem and N. v. Thoai, Europ. J. Biochem. 9, 237 (1969).
95. J. M. Ingram, Can. J. Biochem., 47, 595 (1969).
96. E. S. Younathan, V. Paetkau and H. A. Lardy, J. biol. Chem., 243, 1603 (1968).
97. R. G. Kemp and P. B. Forest, Biochemistry 7, 2596 (1968).
98. H. C. Froede, G. Geraci and T. E. Mansour, J. biol. Chem. 243, 6021 (1968).
99. P. A. M. Eagles, L. N. Johnson, M. A. Joynson, C. H. McMurray and H. Gutfreund, J. molec. Biol. 45, 533 (1969).
100. R. F. Colman, Biochemistry 8, 888 (1969).
101. A. E. Chung and J. S. Franzen, Biochemistry 8, 3175 (1969).
102. G. L. Cottam and P. A. Srere, Arch. Biochem. Biophys. 130, 304 (1969).
103. J. M. Connellan and J. E. Folk, J. biol. Chem. 244, 3173 (1969).
104. J. Ruiz-Herrera, R. Amezcua-Ortega and A. Trujillo, J. biol. Chem. 243, 4083 (1968).
105. W. A. Warren and L. D. Kohn, J. biol. Chem. 245, 3840 (1970).
106. T. Matsuzawa and H. L. Segal, J. biol. Chem. 243, 5929 (1968).
107. W. Dowhan, Jr., and E. E. Snell, J. biol. Chem. 245, 4618 (1970).
108. P. J. Anderson and R. N. Perham, Biochem. J. 117, 291 (1970).
109. J. C. Seidel, Biochim. biophys. Acta 180, 216 (1969).
110. T. Kuo and M. DeLuca, Biochemistry 8, 4762 (1969).
111. C. Little, T. Sanner and A. Pihl, Europ. J. Biochem. 8, 229 (1969).
112. M. Sribney, Can. J. Biochem. 49, 306 (1971).
113. A. G. Weeds, Nature, Lond., 223, 1362 (1969).
114. N. Haugaard, N. H. Lee, R. Kostrzewa, R. S. Horn and E. S. Haugaard, Biochim. biophys. Acta 172, 198 (1969).
115. K. Okabe, H. K. Jacobs and S. A. Kuby, J. biol. Chem. 245, 6498 (1970).
116. G. Kohlhaw, Biochim. biophys. Acta 212, 58 (1970).
117. R. Verger, L. Sarda and P. Desnuelle, Biochim. biophys. Acta 207, 377 (1970).
118. R. E. Wolf, Jr., and J. C. Loper, J. biol. Chem. 244, 6297 (1969).
119. E. Palacián and K. E. Neet, Biochim. biophys. Acta 212, 158 (1970).
120. A. T. deB. Andrews and F. J. Reithel, Arch. Biochem. Biophys. 141, 538 (1970).
121. F. J. Lawrence, Europ. J. Biochem. 15, 436 (1970).
122. A. J. Dunn, Arch. Biochem. Biophys. 136, 203 (1970).
123. J. R. Brown and B. S. Hartley, Biochem. J. 101, 214 (1966).
124. G. M. Edelman, Biochemistry 9, 3188 (1970).
125. A. J. Finlayson, Can. J. Biochem. 47, 31 (1969).
126. H. Maeda, C. B. Glaser and J. Meienhofer, Biochem. biophys. Res. Commun. 39, 1211 (1970).

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Volume 4, Number 4, 1971

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ABOUT OUR COVER

This large (116 x 96 cms., oil on canvas) painting, Grace before Meal, is one of our chemist-collector's favorites. Painted around 1660 by one of Rembrandt's little known followers, the Amsterdam artist Abraham van Dyck, it depicts true devotion and the love and care of the mother for her son. The enchanting still life of the simple meal of bread and milk is as beautiful as can be. Our chemist believes that the long hair of the boy and the rapport between mother and son suggest that the painting does not just represent a genre scene, but Hannah and Samuel before Samuel began his service with Eli in Shiloh.

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Lanthanide Chemical Shift Reagents

John R. Campbell, Aldrich Chemical Company, Inc.

Nuclear magnetic resonance (nmr) spectroscopy is one of the most powerful structural tools available to a chemist as it enables a detailed evaluation of the chemical and stereochemical features of many molecules. However, in order to measure coupling constants, chemical shifts and nuclear ratios easily, the peaks of a nmr spectrum must be clearly separated. In complex molecules (e.g. steroids) the overlap of proton resonances is a serious problem, and the information which can then be obtained from a nmr spectrum is severely restricted. Solvent shifts, spin-decoupling or INDOR[1] experiments, deuterium substitution, derivatisation and the use of higher frequency (100, 220 or 300 MHz) spectrometers are all useful aids in resolving complex or second-order nmr spectra; however, these techniques exhibit a varying degree of success and are time-consuming or require sophisticated and expensive instrumentation.

The application of paramagnetic complexing reagents is also an attractive method of increasing chemical shift differences. For example, nickel (II) and cobalt (II) diacetylacetonates have been used[2] to induce changes in chemical shifts, but the induced shifts are small and line broadening is a serious problem. Recently, a series of new paramagnetic complexes of trivalent rare earth metals with the 2,2,6,6-tetramethyl-3,5-heptanedionato [thd or dpm (dipivalomethanato)][3] and 1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-octanedionato (fod)[4] ligands have been used to induce remarkable changes in the chemical shifts of protons close to an electronegative substituent such as amino or hydroxyl where co-ordination with the paramagnetic complex occurs. The ability of these rare earth complexes to induce large changes in chemical shifts with relatively small line broadening effects has led to a large number of applications; for example, Figure 1[5(b)] illustrates the use of $Eu(dpm)_3$ in the carbohydrate field.

Hinckley[6] first realized the potential of lanthanide complexes as "chemical shift" reagents in inducing changes in chemical shifts in the nmr spectrum of cholesterol. The addition of the dipyridine adduct of $Eu(dpm)_3$ caused shifts to lower field for the protons close to the hydroxyl substituent and the magnitudes of the induced shifts were found to vary linearly as the reciprocal of the cube of the distance of the proton from the site of co-ordination (hydroxyl). The complex without the two moles of pyridine, commonly written $Eu(dpm)_3$ (this may not be the species present in solution[7]) has been shown to be a superior reagent[8] as the observed shifts to lower field are approximately four times as large as those observed with the dipyridine adduct. The magnitude of the shifts again decreases with increasing distance of the proton from the site of co-ordination. The analogous complexes of praseodymium,[9] neodymium, samarium, terbium, dysprosium and holmium[10] induce shifts to higher field; whereas, the ytterbium, erbium[10] and thulium[5(b)] complexes induce shifts to lower field. Although the complexes of these lanthanides, excluding samarium and neodymium do give changes in chemical shift which are greater in magnitude than those observed with the praseodymium and europium complexes, their effectiveness is hampered by increased line broadening and, consequently, the europium and praseodymium complexes have been most widely used. $Eu(dpm)_3$ appears to be more useful than $Pr(dpm)_3$ because the latter reagent tends to increase the complexity of a nmr spectrum by shifting low field resonances to a high field region which may already be complex. In some cases, the use of the $Pr(dpm)_3$ reagent has proven advantageous in moving the resonances of methyl groups to still higher field.[11] The effectiveness of europium (III) and praseodymium (III) is increased by substituting the partially fluorinated ligand, the anion of 1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4, 6-octanedione (fod)[7] for the dipivalomethanato ligand. $Eu(fod)_3$ and $Pr(fod)_3$ are more soluble than the dpm complexes and also co-ordinate with less basic substituents because the electron-withdrawing fluorines increase the residual acidity of the cation. The usefulness of $Eu(fod)_3$ as a shift reagent is hampered only slightly by ligand resonances at 8-9τ in the nmr spectrum of $Eu(fod)_3$ plus substrate.[7] $Eu(dpm)_3$ and $Pr(dpm)_3$ ligand resonances appear at 11-12 and 5-7τ respectively in the presence of an alcohol (typical substrate) and are therefore, well separated from the main regions of interest for both reagents. The complexes of europium (III) with other ligands such as acetylacetonate and dibenzoylmethanate are of little value because of the hydroscopic nature of the former and the low solubility of the latter.[12]

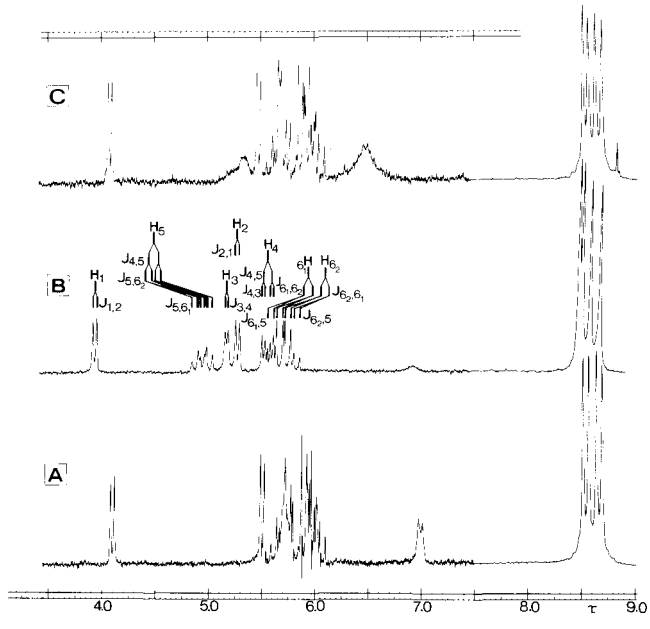
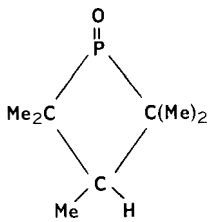


Figure 1[5]: ^1H Nuclear magnetic resonance spectra (100 MHz) of 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (0.0842g) in deuterochloroform (0.5 ml.). [A]. The normal spectrum: [B]. The spectrum after the addition of (tris (dipivaloylmethanato) europium) (9.84×10^{-2} molar equivalents): [C]. As for [B] but with the further addition of water (0.01 ml: 1.55 molar equivalents per mole of Eu $(dpm)_3$. A diagrammatic representation of the first-order assignment is given above the spectrum shown in [B].

The magnitude of the induced shifts depends not only on the nature of the shift reagent but also on the type of co-ordination site,[12] the position of the hydrogen relative to the lanthanide in the substrate-shift reagent complex[6] and various experimental considerations.

Williams,[12] recently, attempted to assess the dependence of the relative magnitudes of the induced chemical shifts upon functionality. A plot of induced chemical shift versus the molar ratio of the concentration of $Eu(dpm)_3$ and substrate concentration gave straight lines whose slope in parts per million per mole of $Eu(dpm)_3$ per mole of substrate were considered to be characteristic of the functional group. These values are the same as Demarco's[13] Δ_{Eu} values, which are defined as the induced chemical shift at a 1:1 mole ratio of shift reagent to substrate, obtained from extrapolation of a plot of induced chemical shift versus $Eu(dpm)_3$ concentration, minus the chemical shift in the free substrate. The slopes of William's plots varied significantly, however, (±15%) in the concentration range reported (0.2-0.5M solutions in carbontetrachloride) with the slope increasing with increasing concentration of substrate. Other plots of this type have also been observed to vary in slope and to deviate from linearity in applications of $Eu(dpm)_3$ to sulfoxides,[14] amines[15] and alcohols.[16] Consequently, the errors in Δ_{Eu} values may be large and Δ_{Eu} values then are useful mainly in determining a qualitative order of the magnitude of induced chemical shifts associated with different substituents.

Functional Group	Ppm per mol of $Eu(dpm)_3$ per mol of substrate in CCl_4
RCH_2NH_2	~150
RCH_2OH	~100
$R_2C{=}NOH$[a]	~40
RCH_2NH_2	30-40
RCH_2OH	20-25
$RHC{=}NOH$[a]	14-30
$RCH_2RC{=}NOH$	14-19
RCH_2COR	10-17
RCH_2CHO	19
RCH_2CHO	11
RCH_2SOR[b]	9-11
$RCH_2{-}O{-}CH_2R$	10 (17-28 in $CDCl_3$[c])
RCH_2CO_2Me	7
$RCH_2CO_2CH_3$	6-5
RCH_2CN	3-7
	2-8[d] ($CDCl_3$)
RCH_2NO_2	~0
halides, indoles, alkenes	0
RCO_2H and phenols	decompose reagent[e]

a Reference 15
b Reference 14
c Reference 18
d Reference 18
e Reference 8

Table 1 Variation of the magnitude of induced shift with functionality.

$Eu(dpm)_3$ is the only shift reagent for which a large range of substrates has been examined. Δ_{Yb} values, using the ytterbium reagent, $Yb(dpm)_3$, have been reported[20] and indicate that the magnitude of the induced shifts are in the order amines > ketones and aldehydes ~ amines.[23]

A comparison of the induced shifts for different substituents has been obtained from intra and intermolecular competition experiments.[18] For example, when equimolar amounts of acetone and tetrahydrofuran (THF) were allowed to compete for $Eu(dpm)_3$ the Δ_{Eu} values obtained were 1.2 and 24.9 (α protons) respectively as compared to values of 11.1 and 28.0 (α protons) obtained when only one substrate was present. THF is, therefore, more effective than acetone at co-ordinating with $Eu(dpm)_3$ by a factor of about 8:1. In a similar manner, dimethyl ether co-ordinates more effectively than acetone (7:3) and dioxane more effectively than 1, 4-cyclohexanedione (3:2) or methylacetate (5:1). The results of intramolecular comparisons are less clear, however, since it is not certain whether differences in Δ_{Eu} values result from differences in binding or from an intrinsically lower bound chemical shift. A better understanding of differences among substituents can be obtained by determining bound chemical shifts (Δ_B) and equilibrium or binding constant (K_B) for the exchange process represented below.[21]

$$La(y)_3 + n(R{-}X) \rightleftarrows (y)_3La \cdots (X{-}R)n$$

(n can be one or two as crystalline complexes of both types have been isolated[22] but probably is one for low concentrations of substrate).

The nmr spectrum of the substrate in the presence of lanthanide shows only one set of peaks for substrate, even for mole ratios of $Eu(dpm)_3$ and substrate approaching one. Consequently, the association-dissociation of substrate with shift reagent is occurring in the fast exchange limit and the chemical shifts of protons in the substrate are then a weighted average of their chemical shifts in the free and complexed substrate. Information about the effectiveness of various reagents, in co-ordinating with different substrates in different solvents requires the measurement of Δ_B and K_B in order to quantitatively assess these factors. Δ_{Eu} values have been considered as a measure of Δ_B; however, Δ_{Eu} values are sensitive to substrate concentration as recently, it has been shown that the slope (Δ_{Eu}) of the plot of induced chemical shift (δ) versus the ratio of the concentrations of lanthanide (Lo) and substrate (So) will approach Δ_B only at relatively high substrate concentrations.[21] In the concentration range [So]>>[Lo] a plot of [So] versus $.1/\delta$ at constant [Lo] gave a straight line from whose intercept and slope the values $-([Lo]+^1/K\beta)$ and $[Lo]\Delta_B$ were obtained (Δ_B rather than Δ_{Eu} is the more accurate measure of the bound chemical shift). Experiments performed on n-propylamine and neopentanol indicate that for $Eu(dpm)_3$ the induced shift magnitudes depend on changes in binding constants [12.3 and 6.2 (liter-mole)$^{-1}$ respectively] and on changes in bound chemical shift (38.7 and 23.7 p.p.m. respectively for $C_1{-}H$). Clearly, by measuring K_B and Δ_B values for a range of substrates, solvents and metals a better understanding can be achieved of the relative importance of steric hindrance, substituent basicity, solvent, ligand and metal.

The magnitude of induced chemical shift (δ) has been observed to depend not only on substrate functionality, ligand and metal, but also on the position of the hydrogen relative to the lanthanide metal in the substrate. Hinckley[6] initially observed that the changes in chemical shifts of hydrogens in the spectrum of cholesterol induced by adding $Eu(dpm)_3(pyridine)_2$ decreased as the cube of the distance of the proton from the coordination site (hydroxyl). This observation, and similar results in other steriods[13] were considered to indicate a pseudocontact (through space) interaction rather a contact (through bonds) interaction was the major contributor to the observed paramagnetic shifts. Observed shifts using $Eu(dpm)_3$ have been correlated with $1/R_i^3$ (R_i is the distance of the hydrogen to the site of co-ordination) in photodieldrin[25] and thioamides[26] but less exact correlations of this type have been observed in nearly every application of a shift reagent. In some cases, a correlation of δ with R_i^{-n} ($n=2-3$)[13,27] was observed, but, values of n not equal to 3 are unexpected since a $1/R_i^3$ dependency for pseudocontact shifts is well established.[13] In solution, the pseudocontact shift (P.S.) may be expressed[28] as follows:

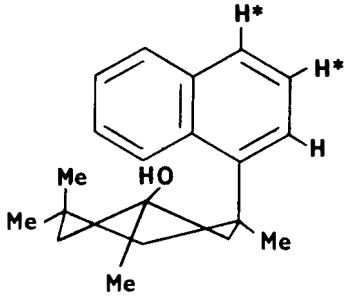
$$\text{P.S.} = k\langle(3\cos^2\theta_i - 1)/R_i^3\rangle_{av}$$

(k is a constant, θ_i is the angle between the crystal field axis of the complex and the radius vector from the metal to hydrogen i and R_i is the distance between the metal and hydrogen i.)

Consequently, in addition to the distance relationship, an angular dependence should apply. In 2,6-di-2-propylacetanilide[29] and compounds (1)[30] (2)[31] an (3)[32] upfield shifts were observed using $Eu(dpm)_3$ for the hydrogens, H*, while the normal shift to lower field was found for all other resonances. [$Pr(dpm)_3$ induced shifts to lower field for H* in (1); but induced shifts to higher field for all other hydrogens.] The abnormal shift of resonances to high field by $Eu(dpm)_3$ and to low field by $Pr(dpm)_3$ has been rationalized in terms of the angular dependence of the pseudo-contact shift arising from the $(3\cos^2\theta_i - 1)$ term. considered to be manifestations of contact interactions; however, in this study, the angular dependence of P.S. was assumed constant. Studies on the 1 and 2-hydroxyadamantane system[33] were considered to indicate both contact and pseudocontact interactions as the magnitude of induced chemical shifts were rationalized in terms of the number of intervening bonds and R_i. Recently, a correlation of δ and R_i^{-2} (the expression describing the pseudocontact interaction requires R_i^{-3}) was considered to indicate that the pseudo-contact interaction is dominant in this system.[27]

The shift reagents, $Eu(dpm)_3$, $Pr(dpm)_3$, $Tb(dpm)_3$ and $Tm(dpm)_3$[34,35] have been shown to induce changes in ^{13}C chemical shifts in the same direction as those observed for hydrogen chemical shifts. Calculated values of P.S. shift combining *both* angular and distance relationships for the ^{13}C spectrum of borneol[34] agree very well with the experimental values with the exception of the C_2 value, and consequently this agreement appears to indicate that the pseudocontact interaction is the major contributor for these ^{13}C chemical shifts except C_2. Preliminary experiments in ^{13}C spectra with a reagent which can act only through a contact interaction[35] indicate that the contact interaction is unimportant in this system. Ambiguities which arise in other studies, because of assumptions made about the geometry of the substrate-lanthanide complex, are absent when this approach is used.

In addition to the importance of substrate, metal and ligand discussed above, it is necessary to give careful attention to details of experimental technique, such as shift reagent solubility, solvent, temperature, and both purity and dryness of all chemicals used, in order to obtain optimum results. For example, $Eu(dpm)_3$ is soluble to the extent of $\sim$ 100 mg per ml of benzene and $\sim$ 200-300 mg per ml



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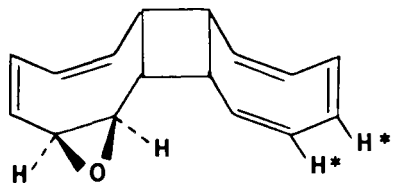

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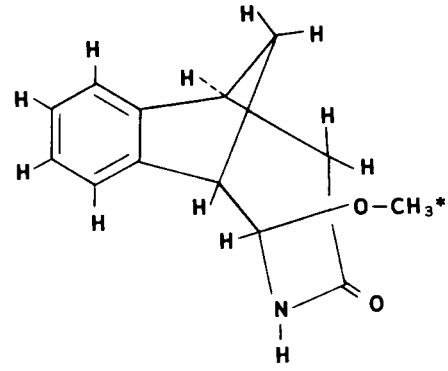


(3)

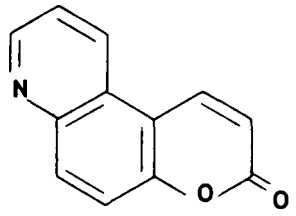
Correlations of induced chemical shifts (usually Δ_{Eu} values) with $1/R_i^3$ for $Eu(dpm)_3$ and $Yb(dpm)_3$[20] combined with their regular dependence have established the pseudocontact interaction as the major contributor to the observed shifts.

Several attempts have been made to assess the importance of contact interactions in applications of lanthanide shift reagents. Hinckley[24] plotted the logarithm of induced shift versus -3 log R_i in an application of the $Eu(dpm)_3(pyridine)_2$ shift reagent to a steroid system. Discrepancies between experimental points and the straight line plot were of carbon tetrachloride and chloroform[12] (in the presence of polar substrates[35]). In benzine and chloroform, the observed shifts are 90%[12] and less than 75%[5,12] respectively, of that observed in carbontetrachloride for the same concentration of $Eu(dpm)_3$.

The deleterious effect of water upon the ability of $Eu(dpm)_3$ to induce downfield shifts is shown in Figure 1(c). The addition of only 0.01 ml (moles of water: $Eu(dpm)_3 = 1.55$) of water to a solution of 1,2:5,6-di-*O*-isopropylidine-α-D-glucofuranose (0.0842 g) in deuterochloroform (0.5 ml) containing $Eu(dpm)_3$ (9.84×10^{-2}

molar equivalents) has almost entirely eliminated any induced shift. The above result implies that water associates more strongly than the substrate with the shift reagent. Clearly, careful drying of solvents, and removal of traces of acid (molecular sieve)[10] which decompose the lanthanide reagents, is necessary for best results. Failure to remove water from solvents or substrate can be detected[5] by tailing of δ versus [Lo]/[So] plots at low [Lo]. Freshly sublimed reagent[5] gives superior results by removing water and traces of insoluble metal oxide, which may prove troublesome by lowering the resolution of a nmr spectrometer.

The effectiveness of lanthanide shift reagents also appears to be increased by observing the spectrum at low temperatures.[5,16,36] This effect may reflect changes in K_B and may be used to increase the amount of induced shift without the increase in line broadening which might be observed if more shift reagent was used at a higher temperature. Finally, the compounds under study may be recovered by thin-layer chromatography of the complex-substrate solution on silica gel.[8]



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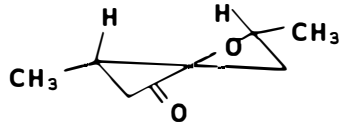

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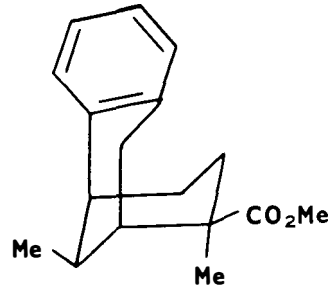

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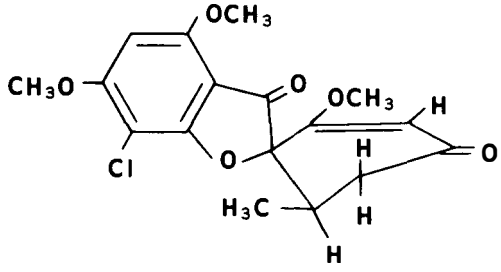

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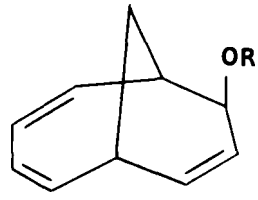

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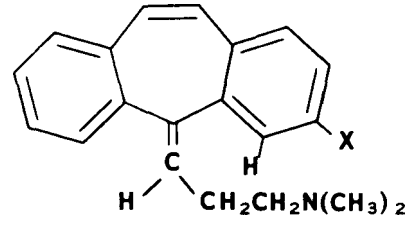


(9)

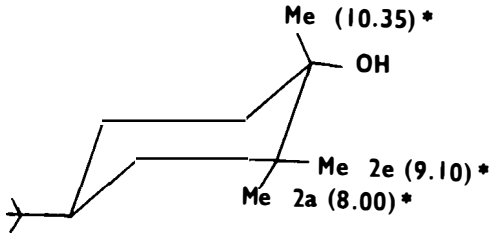
Lanthanide shift reagents have been applied to structural and stereochemical problems in two ways. Most commonly, lanthanide shift reagents have been used to obtain a first order spectrum in order to measure coupling constants and chemical shifts previously hidden by overlapping resonances. Secondly, information about substrate geometry has been obtained indirectly by correlating the orientation of a particular hydrogen relative to the lanthanide nucleus in the substrate-metal complex with an induced shift-distance relationship. The particular substrate-complex geometry which is most consistent with the variation of induced shift as a function of $1/R_i^3$ is then used to deduce the geometry of the substrate itself. The first approach is the simpler and less ambiguous as it assumes that the shift reagent affects only chemical shifts. Examples from the carbohydrate field[5] indicate that lanthanide shift reagents do not affect coupling constants and hence coupling constants, measured in the presence of a reagent, may be taken as good approximations to the corresponding values for the free substrate (especially in aliphatic systems, where contact interactions are rapidly attenuated[24]); however, rotational isomer ratios in 2,6-di-2-propylacetanilide are changed from 2:1 = endo:exo to 1:2 = endo:exo by adding one mole equivalent of $Eu(dpm)_3$[29].

Hinckley[6] and Demarco[13] have shown the potential for applications of shift reagents, in an empirical approach, to steriods containing an hydroxyl in ring A or D. By a combination of lanthanide shift reagent and 220 MHz nmr techniques, it was possible to obtain information about the geometries of rings A, B, C, and C, D respectively, by relating coupling constants to a Karplus-type curve.[37] Applications of shift reagents also enabled a first-order spectral analysis of several carbohydrates[5,38] and isotope analysis in deuterated borneols.[39] In the same way, conformations and structures were assigned using $Eu(dpm)_3$ to compounds (4),[40] (5),[41] isomeric bis (4-aminocyclohexyl) methanes,[15] (6),[42] griseofulvin (7)[43(a)] (8),[31] (9),[43(b)] and substituted pyrazines.[43(c)]

Polymers also have been shown to be amenable to nmr analysis using lanthanide shift reagents. In the case of atactic poly (methyl methacrylate), the addition of $Eu(dpm)_3$ in deuterochloroform caused the separation of three carbomethoxy and C-methyl absorptions corresponding to the respective groups in isotactic, heterotactic and syndiotactic triads.[44]

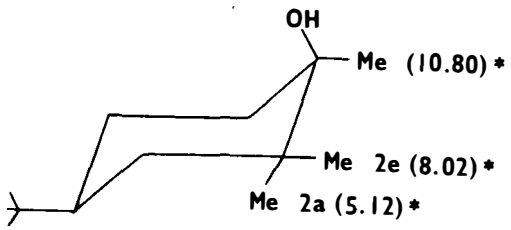
The second approach to the use of lanthanide shift reagents in stereochemical studies relates substrate geometry or configuration to an observed induced chemical shift-distance variation. Assignments of configuration based on this $1/R_i^3$ relationship must be treated cautiously, however, as this relationship ignores the angular dependence of pseudocontact shifts and also requires assumptions about the geometry of the substrate-complex. In some cases, the substrate-lanthanide orientation is not well defined; for example, it is difficult to decide where the europium atom complexes to a carbonyl. Moreover, reversals of the relative magnitudes of induced shifts for different protons in a

glucofuranose[5] have been noted simply by changing from $Eu(dpm)_3$ to $Tm(dpm)^3$. This approach may be useful, however, in assigning configuration qualitatively, where a clear cut choice is possible, especially if the assignment is supported by data from other techniques. For example, Richer used[11] such an approach in assigning configuration to isomeric alcohols (10) and (11), where the shift reagent used was $Pr(dpm)_3$. A comparison of induced shifts of the methyl absorptions indicates a marked decrease in the value observed for one methyl group. This methyl group furthest from the hydroxyl is assigned Me_{2a}, in (11) on the basis of the $1/R_i^3$ induced shift relationship. The angular dependence of pseudocontact shifts was ignored; however, the large difference in R_i for Me_{2a} in (9) and (10) probably causes $1/R_i^3$ to be the dominant term. In addition, θi and R_i are nearly the same for Me_{2e} in both isomers and the induced shifts of Me_{2e} should be nearly the same for both isomers; however, both angular and distance dependences should be considered in order to establish which factor is the dominant term, and in order to assign configuration less ambiguously in cases where both factors are significant.



$$* \Delta\delta = \delta_{100\ mg\ Pr(dpm)_3} - \delta_{0\ mg\ Pr(dpm)_3}$$

(10)

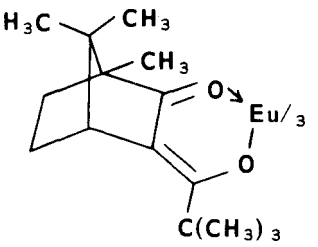


$$* \Delta\delta = \delta_{100\ mg\ Pr(dpm)_3} - \delta_{0\ mg\ Pr(dpm)_3}$$

(11)

Correlations of the induced chemical shift distance relationship with configuration have been noted in sulfoxides[28] and triterpenoids.[36] This relationship has been used to assign configuration, or geometry, in d-camphor,[45] photodieldrin,[25] trachyloban-19-oic acid,[46] ketones,[21] [Yb $(dpm)_3$], oximes,[17] amides[21] [$Yb(dpm)_3$], phosphetan oxides,[19] compounds (2) and (8),[31] nucleosides,[47(a)] thioamides,[26] 1-propenyl-3-cyclohexanones,[47(b)] vinyl aldehydes [$Yb(dpm)_3$[47(c)]] and nitrosamines.[47(d)] In 2-hydroxyl-1-(2-hydroxyethyl) adamantane, the average position of the lanthanide metal relative to the two hydroxyl substituents was assigned using the distance-induced shift relationship.[27]

An elegant approach to the problem of determining optical purity has been reported using an optically active shift reagent (12). This reagent, derived from d-camphor,[49] may be a useful supplement to the use of optically active solvents.[48]



(12)

In the case of α-phenylethylamine, the maximum frequency differences between the resonances of corresponding protons of (R)- and (S)-substrates range from ~0.5 ppm for the C*H* NH_2 proton to 0.07 ppm for the para hydrogen of the aromatic ring. Other amines exhibit the same phenomena, but less basic substances, such as 2-octanol, α-phenylethanol, cyclohexyl-methylcarbinol or benzymethylsulfoxide do not show distinguishable shift differences.

In summary, this discussion has attempted to present, in a general fashion, the important features of the use of lanthanide shift reagents in nmr spectroscopy. General points of experimental technique and of theoretical importance have been included to enable application of these reagents to a wider range of problems on more than an empirical basis; however, in some cases, definitive experiments remain to be performed in order to firmly establish the theoretical basis of some applications. Nevertheless, lanthanide shift reagents represent a useful supplement to existing nmr techniques.

Dr. John Campbell

REFERENCES

1. R. Burton, L. D. Hall and P. R. Steiner, *Can. J. Chem.*, **47,** 1 (1969).
2. W. D. Horrocks, R. C. Taylor, and G. N. La Mar, *J. Amer. Chem. Soc.*, **86,** 3031 (1964).
3. K. J. Eisentraut and R. E. Sievers, *ibid*, **87,** 5254 (1965).
4. C. S. Springer, Jr., D. W. Meek and R. E. Sievers, *Inorg. Chem.*, **6,** 1105 (1967).
5. (a) I. Armitage and L. D. Hall, *Chem. Ind.* (London), 1537 (1970).
   (b) I. Armitage and L. D. Hall, private communication, *Can. J. Chem.*, **49,** 2770 (1971).
6. C. C. Hinckley, *J. Amer. Chem. Soc.*, **91,** 5160 (1969).
7. R. E. Rondeau and R. E. Sievers, *ibid*, **93,** 1522 (1971).
8. J. K. M. Sanders and D. H. Williams, *Chem. Comm.*, 422 (1970).
9. J. Britts, G. H. Frost, F. A. Hart, G. P. Moss and M. L. Staniforth, *ibid*, 749 (1970).
10. (a) D. R. Crump, J. K. M. Sanders and D. H. Williams, *Tet. Letters*, 4419 (1970).
    (b) N. Ahmad, N. S. Bhacca, J. Selbin and J. D. Wander, *J. Amer. Chem. Soc.*, **93,** 2564 (1971).
11. (a) P. Belanger, C. Freppel, D. Tizane and J. C. Richer, *Chem. Comm.*, 266 (1971).
    (b) P. Belanger, C. Freppel, D. Tizane and J. C. Richer, *Can. J. Chem.*, **49,** 1985 (1971).
12. J. K. M. Sanders and D. H. Williams, *J. Amer. Chem. Soc.*, **93,** 641 (1971).
13. P. Demarco, T. K. Elzey, R. B. Lewis and E. Wenkert, *ibid*, **92,** 5734, 5737 (1970).
14. K. K. Andersen and J. J. Uebel, *Tet. Letters*, 5253 (1970).
15. H. van Brederode and W. G. B. Hysman, *ibid.*, 1695 (1971).
16. L. Tomic, Z. Majerski, M. Tomic and D. E. Sunko, *Chem. Comm.*, 719 (1971).
17. Z. W. Wolkowski, *Tet. Letters*, 825 (1971).
18. H. Hart and G. M. Love, *ibid*, 625 (1971).
19. J. R. Corfield and S. Trippett, *Chem. Comm.*, 721 (1971).
20. C. Beauté, Z. W. Wolkowski and N. Thoai, *Tet. Letters*, 817 (1971); Z. W. Wolkowski, *ibid*, 821 (1971).
21. I. Armitage, G. Dunsmore, L. D. Hall and A. G. Marshall, private communication, *Chem. Comm.*, in press.
22. J. Selbin, N. Ahmad and N. Bhacca, *Inorg. Chem.*, **10,** 1383 (1971).
23. C. Beauté, Z. W. Wolkowski and N. Thoai, *Chem. Comm.*, 700 (1971).
24. C. C. Hinckley, M. R. Klotz and F. Patil, *J. Amer. Chem. Soc.*, **93,** 2417, (1971).
25. L. H. Keith, *Tet. Letters*, 3 (1971).
26. W. Walter, R. F. Becker and J. Thiem, *ibid*, 1971 (1971).
27. A. F. Cockerill and D. M. Rackham, *ibid*, 5149, 5153 (1970).
28. R. R. Fraser and Y. Y. Wigfield, *Chem. Comm.*, 1471 (1970).
29. T. H. Siddall, *ibid.*, 452 (1971).
30. B. L. Shapiro, J. R. Hlubucek, G. R. Sullivan and L. F. Johnson, *J. Amer. Chem. Soc.*, **93,** 3281 (1971).
31. M. R. Willcott and J. F. M. Oth, J. Thio, G. Plinke, and G. Schröeder, *Tet. Letters*, 1579 (1971).
32. P. H. Mazzocchi, H. J. Tamburin and G. R. Miller, *ibid*, 1819 (1971).
33. G. H. Wahl and M. R. Peterson, *Chem. Comm.*, 1167 (1970).
34. J. Briggs, F. A. Hart, G. P. Moss and E. W. Randall, *ibid*, 364 (1971).
35. I. Armitage, J. R. Campbell and L. D. Hall, unpublished observations.
36. D. G. Buckley, G. H. Green, E. Ritchie and W. C. Taylor, *Chem. Ind.* (London), 292, (1971).
37. M. Karplus, *J. Amer. Chem. Soc.*, **85,** 2870 (1963).
38. P. Girard, H. Kagan and S. David, *Bull. Chim. Soc.* (France), 4515 (1970).
39. K. Tori, Y. Yoshimura and R. Munejuki, *Tet. Letters*, 333 (1971).
40. K. J. Kiska, A. F. Fentiman and R. L. Foltz, *Tet. Letters*, 4657 (1970).
41. F. J. Carroll and J. T. Blackwell, *ibid*, 4173 (1970).
42. L. F. Johnson, J. Chakravarty, R. Dasgupta and U. R. Ghatak, *ibid*, 1703 (1971).
43. (a) S. G. Levine and R. E. Hicks, *ibid*, 311 (1971).
    (b) D. C. Remy and W. A. Van Saun, *ibid*, 2463 (1971).
    (c) A. F. Bramwell, G. Riezebos and R. D. Wells, *ibid*, 2489 (1971).
44. A. R. Katritzky and A. Smith, *ibid*, 1765 (1971).
45. C. C. Hinkley, *J. Org. Chem.*, **35,** 2834 (1970).
46. O. Achatowicz, A. Ejchart, J. Jurczak, L. Kozerski and J. St. Pyrek, *Chem. Comm.*, 98 (1971).
47. (a) R. F. Butterworth, A. G. Pernet and S. Hanessian, *Can. J. Chem.*, **49,** 981 (1971).
    (b) C. P. Casey and R. A. Boggs, *Tet. Letters*, 2455 (1971).
    (c) C. Beauté, Z. W. Wolkowski, J. P. Merda and D. Lelandais, *ibid*, 2473 (1971).
    (d) R. R. Fraser and Y. Y. Wigfield, *ibid*, 2515 (1971).
48. M. Raban and K. Mislow, *Top. Stereochem.*, **2,** 199 (1967).
49. G. M. Whitesides and D. W. Lewis, *J. Amer. Chem. Soc.*, **92,** 6979 (1970).

Improving Nature's Catalysts

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WATER-INSOLUBLE ENZYMES

Of all recent advances in bio-organic technology, perhaps the most significant is the development of artificially insolubilised enzymes. Unlike the native, soluble enzymes, these novel derivatives can be recovered from reaction mixtures and repeatedly re-used either in batch reactors or in packed beds. Moreover, properties of the enzyme such as specificity and heat stability may be dramatically improved.

The exciting prospect of improving nature's catalysts stimulated us to undertake an intensive programme of research in this area. Working in collaboration with colleagues within the University of Birmingham, we have developed a unique series of copolymers, purpose-designed for the insolubilisation of enzymes. Based on acrylamide and acrylamide derivatives, these copolymers are commercially available under the Koch-Light trade mark, 'Enzacryl.'*

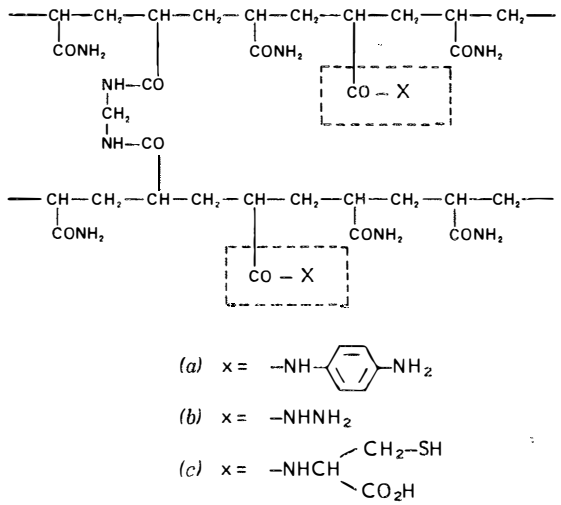

Figure 1. Major structural features of Enzacryls based on polyacrylamide.

DEVELOPMENT OF 'ENZACRYL'

The Enzacryl copolymers are shown in figures 1 and 2. Before we consider them in detail let us briefly examine some of the ways in which enzyme molecules may be water-insolubilised. There are three, fundamentally different, approaches. These are, chemical cross-linking, molecular entrapment within a gel matrix, and binding the enzyme to an insoluble carrier. Chemical cross-linking and gel entrapment are poor methods for enzymes active against substrates unable to penetrate the cross-linked network of enzyme molecules or the gel matrix. On the other hand, binding the enzyme to a pre-formed carrier surface makes for easy access of substrate and is much more economic in use of enzyme. Most important, by judicious choice of carrier, the micro-environment of the enzyme and consequently its properties may be varied. We decided to adopt this method of enzyme insolubilisation.

Our choice of matrix and method of enzyme binding was influenced by several factors. Organic carriers for enzyme insolubilisation must be hydrophilic overall and devoid of prominent hydrophobic or polyionic features if the stability of the bound enzyme is to be ensured.[1] Carriers such as polystyrene and cross-linked polymethacrylic acid can actually de-stabilise the bound enzyme. Insolubilisation procedures involving covalent coupling are preferable to physical methods of binding such as adsorption or ionic binding. Physical methods are unreliable because the enzyme is often resolubilised on contact with its substrate in solution.[2]

When we embarked on our programme, most carriers for enzyme insolubilisation were based on cellulose and other polysaccharides. Unfortunately, cellulose physically adsorbs many enzymes. Not only does this complicate covalent coupling but, if physically bound enzyme is to be detached, rigorous washing procedures must be employed which may damage the covalently bound enzyme. Since biological polymers in general may interact with specific enzymes and substrates, we decided to devise completely synthetic carriers.

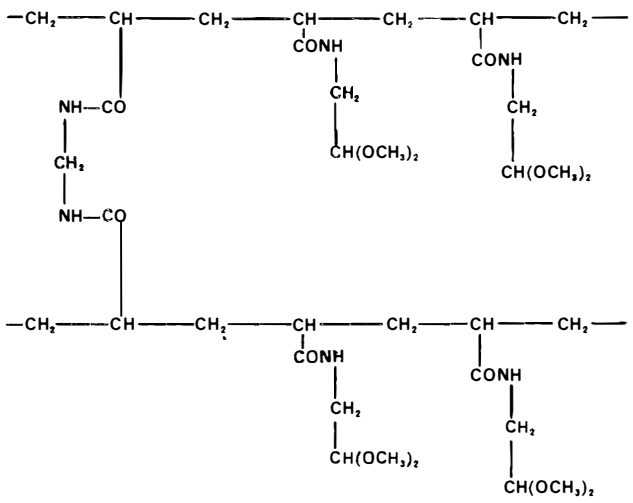

Figure 2. Structural features of Enzacryl Polyacetal.

Almost inevitably we came to consider carriers based on cross-linked polyacrylamide. This neutral, intensely hydrophilic matrix had been used by others, notably Drs. Hicks and Updike[3] to entrap D-glucose oxidase and lactate dehydrogenase. The appropriate enzyme, together with acrylamide and N,N′-methylene-bis-acrylamide was dissolved in aqueous buffer, after which gelation was induced by addition of potassium persulphate. The immobilised

* "Enzacryl" is a trademark of Koch-Light Laboratories, Ltd. and is specifically licensed to Aldrich Chemical Company, Inc. U.S.A. registration has been applied for.

enzymes had excellent stability to storage and lyophilisation. Although we were not directly interested in entrapment, the success of these experiments convinced us that particulate copolyacrylamide would be an ideal matrix onto which to link our enzymes.

Then, as now, perhaps the most popular methods of enzyme binding involved aryl diazo or acid azide groups which were generated on the carrier immediately prior to enzyme coupling. This was achieved by nitrous acid treatment of aryl amino and acid hydrazide residues respectively. At first we thought that these methods would not be applicable to polyacrylamides since it appeared that nitrous acid treatment would destroy the primary amide residues of the carrier. However, we soon realised that polyacrylamide, in contrast to monomeric amides, was refractory to nitrous acid. Polyacrylamides carrying aromatic amino and acid hydrazide groups became our first enzyme carriers.[4, 16]

To prepare a copolyacrylamide derivative substituted with aryl amino groups we first copolymerised acrylamide, 4-nitroacrylanilide and N,N′-methylene-bis-acrylamide. Selective reduction of nitro groups on the copolymer was then carried out with titanous chloride. We called the reduced copolymer (see figure 1a) 'Enzacryl AA', 'AA' indicating that it was the aromatic amino group which was potentially active in enzyme binding.

An important feature of carriers depending on the aromatic amino group for enzyme binding, is that there are two methods by which they may be activated (see figure 3).

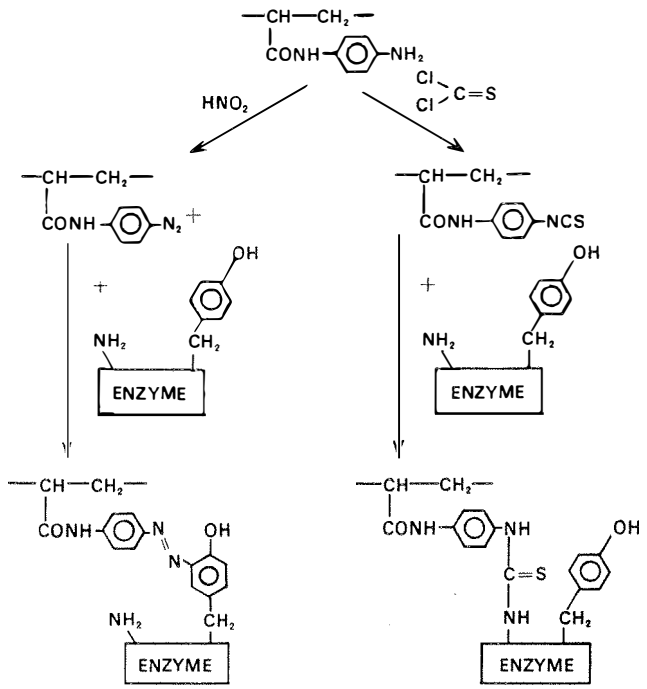

Figure 3. Activation of Enzacryl AA and coupling with enzymes.

Whereas nitrous acid effects diazotisation, treatment with thiophosgene in carbon tetrachloride leads to reactive aryl isothiocyanato groups. Diazonium groups react with enzymes mainly at the phenolic residues of tyrosine although it is probable that coupling with the indole nucleus of tryptophan and the imidazole nucleus of histidine also takes place. On the other hand, enzyme binding by means of isothiocyanato groups takes place at the side chain amino groups of lysine, the enzyme being attached through a substituted thiourea linkage. Although we have found that several enzymes, for example alpha- and beta- amylase, may be successfully insolubilised by both procedures, this adaptability is important because, sometimes, a particular mode of coupling may lead to inactivation of the enzyme. We found this to be the case with derivatives of carboxypeptidase A and gamma-amylase prepared by the isothiocyanato coupling procedure. Diazonium coupling was successful in both instances. It is possible that isothiocyanato coupling may be damaging the active site of the enzymes or, alternatively, coupling the enzymes in such a way that the approach of substrate is hindered.

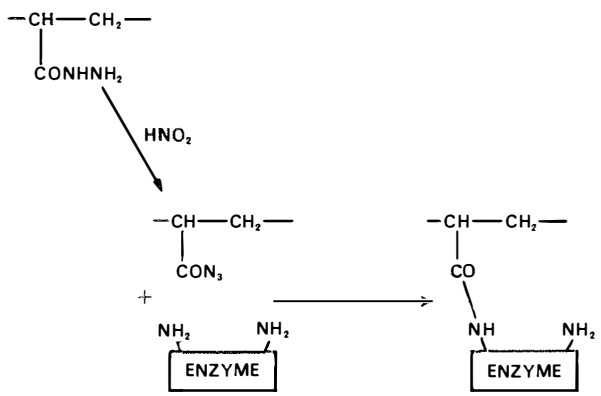

Figure 4. Activation of Enzacryl AH and coupling with enzymes.

In spite of its versatility, the aromatic amino group possesses a disadvantage in that the necessary benzenoid ring is hydrophobic. Enzacryl AA therefore has hydrophobic features. This led to our interest in the acid hydrazide residue as the potentially reactive group. We felt that a purely aliphatic copolyacrylamide derivative, being more hydrophilic, would probably be better from the point of view of enzyme stabilisation. The desired carrier was obtained by copolymerising acrylamide, N-acryloyl-N′-tert-butoxycarbonyl hydrazine and N,N′-methylene-bis-acrylamide and removing the tert-butoxycarbonyl group with dilute acid. We called this carrier Enzacryl AH, 'AH' being indicative of the acid hydrazide group. After activation of Enzacryl AH with nitrous acid, the carrier is effective in coupling enzymes through the side chain amino groups of lysine (see figure 4). In this respect Enzacryl AH is similar to the isothiocyanato derivative of Enzacryl AA.

It is relevant to compare the effectiveness of the acid azide derivative of Enzacryl AH with the diazo and isothiocyanato derivatives of Enzacryl AA in the case of a single enzyme. We have coupled alpha-amylase to all three derivatives. Protein bound per gram of Enzacryl AA on diazo coupling (10.6mg) and isothiocyanato coupling (6.2mg) was rather higher than that coupled by Enzacryl AH (2.2mg). The activity retained by the diazo coupled (6.1%), isothiocyanato coupled (9.5%) and acid azide coupled (16.0%) enzymes bore an inverse relationship to the quantity of protein coupled. This may well reflect the degree to which enzyme molecules are crowded together on the carrier surface. Steric effects probably account for the lower binding capacity of Enzacryl AH since, during coupling, the enzyme molecules have to approach closer to the carrier than is the case of Enzacryl AA. This is because the acid azide groups of Enzacryl AH are closer to

the hydrocarbon backbone than the diazo or isothiocyanato groups of Enzacryl AA.

All three water-soluble alpha-amylase derivatives were more stable to heat denaturation than the free enzyme (see figure 5). As anticipated, the derivative based on the most hydrophilic carrier, Enzacryl AH, was the most stable. Also this derivative had superior stability both to storage and lyophilisation.

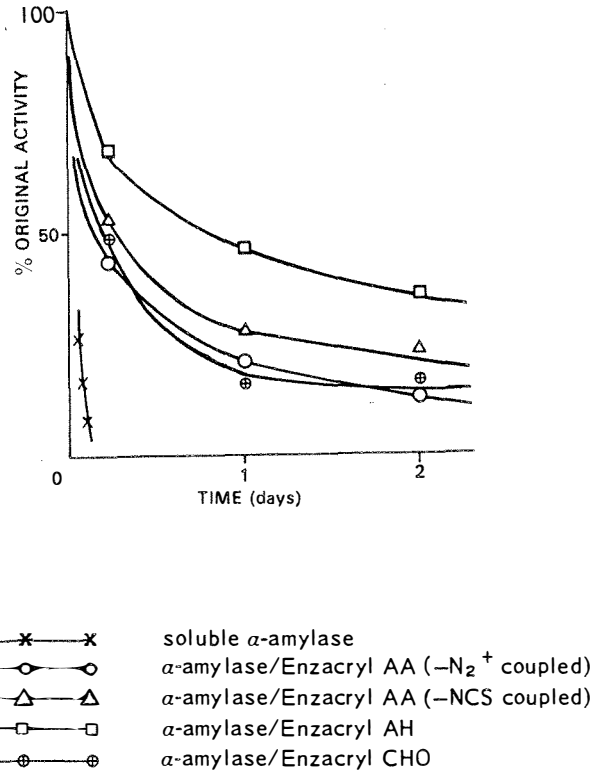

Figure 5. Heat stabilities of free and Enzacryl bound alpha-amylase.

We have compared Enzacryl bound alpha-amylase with water-insoluble preparations obtained by coupling the enzyme to both 3-(p-diazophenoxy)-2-hydroxypropyl cellulose and 2-hydroxy-3-(p-isothiocyanatophenoxy) propyl cellulose.[5] Such properties as the activity retained on coupling and stability to heat, storage and lyophilisation were broadly similar for both types of carrier. The great difference between the Enzacryl and cellulose carriers lay in the ease with which Enzacryl was washed free of unreacted enzyme. In the case of cellulose derivatives, strong physical adsorption occurred which necessitated prolonged washing with buffer and buffered sodium chloride solution. Even then, physically adsorbed alpha-amylase was always re-solubilised on contact with starch solution.[2] This is reflected in the inferior recovery of enzyme activity with cellulose bound alpha-amylase on repeated re-use (see figure 6). The higher activity losses are due to re-solubilisation rather than denaturation.

Numerous coupling procedures have been employed to insolubilise enzymes. Unfortunately, most of these are similar in that they involve the same functional groups on the enzyme. For example, carriers bearing pendant acid azide, anhydride, isothiocyanato and 2,4-dinitrofluorophenyl groups all bind enzymes through the side-chain amino groups of lysine. Recently our colleagues, Professor Barker, Dr. Gray, and their associates within the University of Birmingham have devised a new type of acrylamide copolymer incorporating sulphydryl groups as the functional sites active in enzyme binding.[6] This carrier, Enzacryl Polythiol, was derived from a copolymer of acrylamide, N-acryloyl-S-benzylcysteine and N,N′-methylene-bis-acrylamide. Free sulphydryl groups were exposed following removal of the benzyl protecting groups with sodium in liquid ammonia. Enzacryl Polythiol couples spontaneously with compounds containing disulphide bridges. The cyclic disulphide, lipoic acid and the small protein, insulin, may be insolubilised in this way. For proteins deficient in cystine or for enzymes which are inactivated on disruption of their disulphide bridges, prior enrichment with free sulphydryl groups is

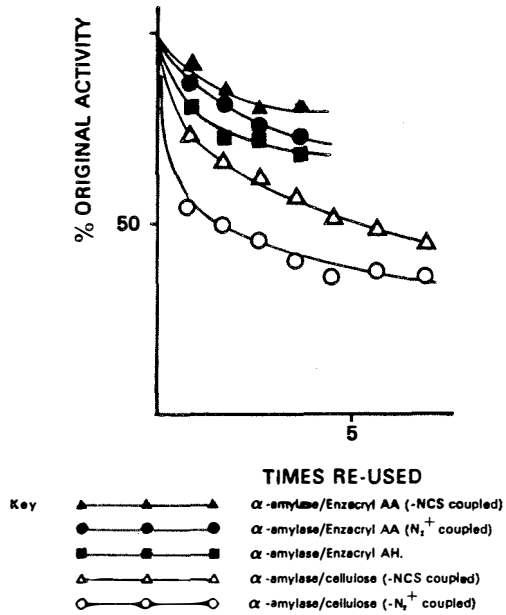

Figure 6. Re-use of Enzacryl/alpha-amplase and cellulose/alpha-amylase.

necessary. This is best achieved with Immunothiol (N-acetylhomocysteine thiolactone). In this case protein insolubilisation is brought about by oxidative coupling with potassium ferricyanide, sulphydryl groups on the carrier being coupled with similar groups on the enzyme. Using this procedure derivatives of human serum albumin and trypsin have been prepared.

A novel feature of conjugates based on Enzacryl Polythiol is that they may be dissociated by treatment with cysteine or mercaptoethanol (see figure 7). This is important because once an enzyme has become denatured the carrier may be regenerated. For example, the Immunothiol derivative of trypsin has been detached by washing with cysteine and fresh enzyme coupled. Regenerable enzyme carriers have obvious commercial potential.

Another feature of Enzacryl Polythiol is that it may also be activated for enzyme coupling with dicyclohexylcarbodiimide. The activated copolymer, Enzacryl Polythiolactone may be stored indefinitely under dry conditions. It

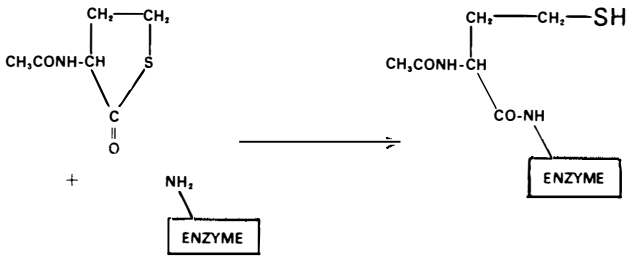

Figure 7a. Prior enrichment of enzymes with Immunothiol.

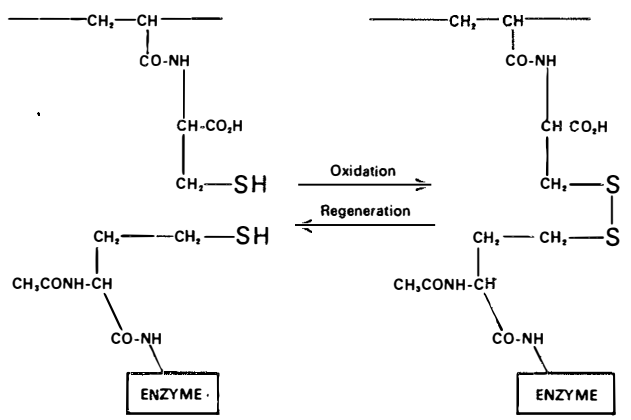

Figure 7b. Coupling and decoupling with Enzacryl Polythiol.

is possible that the reactivity of the thiolactone is sufficient to involve reaction at the hydroxyl groups of serine and tyrosine in addition to the usual side-chain amino groups of lysine.

So far all the Enzacryl copolymers we have described have been polymeric primary amides. We have recently devised a carrier which provides for enzyme insolubilisation within a hydrophilic environment of acetal, hemi-acetal and aldehydrol groups.[7] This carrier, Enzacryl Polyacetal was prepared by copolymerising N-acryloylaminoacetaldehyde dimethylacetal and N,N′-methylene-bis-acrylamide. Prior to enzyme coupling Enzacryl Polyacetal is activated by treatment with dilute (0.25M) hydrochloric acid which generates aldehydrol groups. The activated copolymer, Enzacryl CHO, may be stored suspended in distilled water, for up to a month without serious deterioration. Enzacryl CHO readily coupled enzymes.

By controlled hydrolysis of Enzacryl Polyacetal, we prepared samples of Enzacryl CHO of differing aldehydrol content and swelling properties. The relationship between aldehydrol content and swelling was not a simple one, but maximum aldehydrol content was consistent with maximum swelling. Model coupling reactions with trypsin revealed that good swelling properties were almost as important as aldehydrol content in determining binding capacity. To date we have used Enzacryl CHO to prepare water-insoluble derivatives of alpha-amylase, dextranase, papain, trypsin and urease.

Aldehydrol/enzyme binding involves reaction with primary amino groups on the enzyme. Previous workers have used simple dialdehydes (glutaraldehyde) to cross-link enzymes[8] and also to couple them to carriers such as aminoethyl cellulose.[9] It has been suggested[10] that, before enzyme binding takes place, the aldehyde molecules first undergo condensation to give a random mixture of unsaturated aldehydes. Amino groups on the enzyme then attack either the conjugated double bonds (Michael addition) or the aldehydrol groups. The comparatively low reactivity of the active methylene groups in the acylaminoacetaldehyde side chains of Enzacryl CHO makes preliminary condensation rather more difficult than in the case of a simple aldehyde.

Direct reaction of primary amino groups on the enzyme with aldehydrol groups on Enzacryl CHO leads mainly to the formation of aminol and azomethine linkages. Enzyme binding is thus an equilibrium process lying heavily in favour of the insolubilised enzyme. In contrast to Michael addition the binding reaction should be readily reversible (see figure 8). Although we have been unable to dissociate Enzacryl CHO/enzyme conjugates by repeated washing with buffer, salt and even dilute hydrochloric acid, we have demonstrated that dissociation may be brought about on contact with a macro-molecular substrate. For example, partial dissociation of trypsin derivatives could be brought about by repeated washing with casein solution. The same preparation could be repeatedly used to digest small substrates such as N α-L-benzoylarginine ethyl ester without re-solubilisation of enzyme. Apparently the affinity of the enzyme for the macro-molecular substrate is sufficient to cause significant partitioning of the enzyme with the aqueous phase. We have similarly brought about partial dissociation of Enzacryl CHO/alpha-amylase and Enzacryl CHO/dextranase by repeated washing with starch and dextran solution respectively.[11] Slight re-solubilisation could be valuable in industrial situations when dealing with partially soluble substrates. Further, the re-generated aldehydrol groups could be used to couple fresh enzyme, thereby periodically boosting enzyme activity.

Selection of the best Enzacryl carrier for a particular application is often a matter of trial and error. Enzymes with a high proportion of tyrosine residues are likely to be readily insolubilised by diazo coupling whereas enzymes rich in lysine will best be coupled by an acylative route. If high binding capacity is required Enzacryl Polythiol and Enzacryl CHO are preferable. However, enzyme conjugates based on Enzacryl CHO are best avoided in certain applications, for example degradation studies on proteins or polysaccharides where slight re-solubilisation of enzyme is undesirable. On the other hand, for simplicity of coupling together with good binding capacity, Enzacryl CHO is probably the carrier of choice for enzymes active against small substrates. We have recently used Enzacryl CHO to prepare several urease derivatives, none of which showed any tendency to dissociate on continuous use.

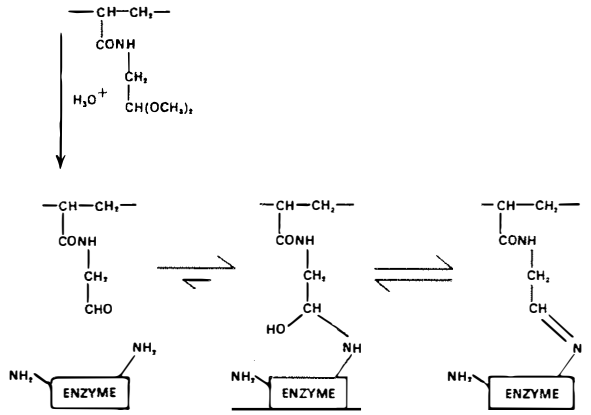

Figure 8. Activation and coupling with Enzacryl Polyacetal.

APPLICATIONS—PRESENT AND FUTURE

Water-insoluble enzymes, mostly based on polysaccharide carriers, are already being applied industrially. Thus gamma-amylase derivatives are being used in experimental reactors to catalyse the hydrolysis of starch to glucose.[12] Insoluble forms of α-L-amino-acid de-N-acylase are now preferred catalysts in the large scale selective hydrolysis of L-N-acetylamino-acids in DL-N-acetylamino-acid mixtures.[13,14] This facilitates the resolution of D and L forms of the amino-acid since the L-amino-acid and D-N-acetyl-amino-acid are easily separated by fractional crystallisation. Water-insoluble derivatives of papain are being used to degrade soluble protein during beer production thereby preventing the development of chill-haze.

The application of proteolytic enzymes, such as papain and trypsin, is invariably accompanied by self-digestion of the enzyme. This is not possible with water-insoluble derivatives. For example, at pH 7.5 and 35°C Enzacryl CHO/trypsin is quite stable whereas the native enzyme is rapidly inactivated. Enzacryl CHO/papain is similarly stabilised and this is useful in that the enzyme is preserved during the rather slow, but necessary, pre-activation with cysteine.

Because the number of enzyme molecules which may be crowded together on a carrier surface is small compared to the number of functional groups active in enzyme binding, many of these groups do not take part in the coupling reaction. Those remaining react readily with suitable small molecules. For example, it is common practice to destroy aryl diazo groups by coupling with phenol or ß-naphthol. This sort of procedure is potentially applicable to insolubilising the enzyme within a pre-selected micro-environment. Insoluble conjugates based on Enzacryl CHO should be particularly suited to coupling a variety of small amines such as peptides, amino-acids and amino-sugars.

Because of their obvious conservation potential, water-insoluble enzymes are likely to be employed increasingly in clinical analysis. Derivatives of glucose oxidase, urease and lactate dehydrogenase have already found application. Insoluble derivatives of choline esterase and creatine phosphokinase, enzymes which are routinely estimated in body fluids, should have considerable advantages over soluble enzymes as clinico-chemical standards.

It is envisaged that Enzacryls will find application as carriers for proteins and peptides, in addition to enzymes. Enzacryl insolubilised antigens would be useful in the isolation of specific anti-bodies. Similarly, water-insoluble analogues of enzyme substrates could be employed in the single stage isolation of enzymes from crude biological fluids.

Enzymes have also been localised in solution by entrapment within collodion or nylon microcapsules.[15] Substrate and products are able to diffuse through the capsule wall. This technique has advantages for certain *in vivo* applications because immuno-evasive properties are conferred on the enzyme. However, because the enzyme is still in solution there is no possibility of enhanced stability or other improved properties. This could be overcome by micro-encapsulation of Enzacryl/enzyme conjugates.

CONCLUSION

Most enzymes, acting in their natural micro-environment, are regimented within biological membranes. Artificially insolubilised enzymes are in an analogous situation. As insolubilisation techniques improve and become more predictable it is inevitable that such derivatives will become preferred to soluble enzymes in most analytical and industrial applications. The use of Enzacryl and similar carriers will then become routine practice.

REFERENCES

1. I. H. Silman and E. Katchalski, *Ann. Rev. Biochem.*, 873 (1966).
2. S. A. Barker, R. Epton and P. J. Somers, *Carbohyd. Res.*, **14**, 323 (1970).
3. G. P. Hicks and S. J. Updike, *Anal. Chem.*, 38, 726 (1966).
4. S. A. Barker, R. Epton, J. V. Mc'Laren and P. J. Somers, *Carbohyd. Res.*, **14**, 287 (1970).
5. S. A. Barker, R. Epton and P. J. Somers, *Carbohyd. Res.*, 8, 491 (1968).
6. S. A. Barker, C. J. Gray, A. M. Khoujah and C. M. J. Law, *Birmingham University Chemical Engineering Journal*, in press (1971).
7. R. Epton, J. V. Mc'Laren and T. H. Thomas, *Biochem. J.*, **123**, 21P (1971).
8. F. A. Quiocho and F. M. Richards, *Proc. Nat. Acad. Sci. Washington*, **52**, 833 (1964).
9. A. F. S. A. Habeeb, *Arch. Biochem. Biophys.*, 119, 264 (1967).
10. F. M. Richards and J. R. Knowles, *J. Molec. Biol.* **37**, 231 (1968).
11. R. Epton, J. V. Mc'Laren and T. H. Thomas, *Carbohyd. Res.*, in press.
12. A. N. Emery and J. Novias, *personal communication*.
13. T. Tosa, T. Mori, N. Fuse and I. Chigata, *Enzymologia*, 31, 214 (1966).
14. M. D. Lilly and P. Dunnill, *Process Biochem.*, p. 29, August (1971).
15. T. M. S. Chang, *Science Tools*, **16**, 3 (1969).
16. S. A. Barker and R. Epton, *Process Biochem.*, p. 14, August (1970).

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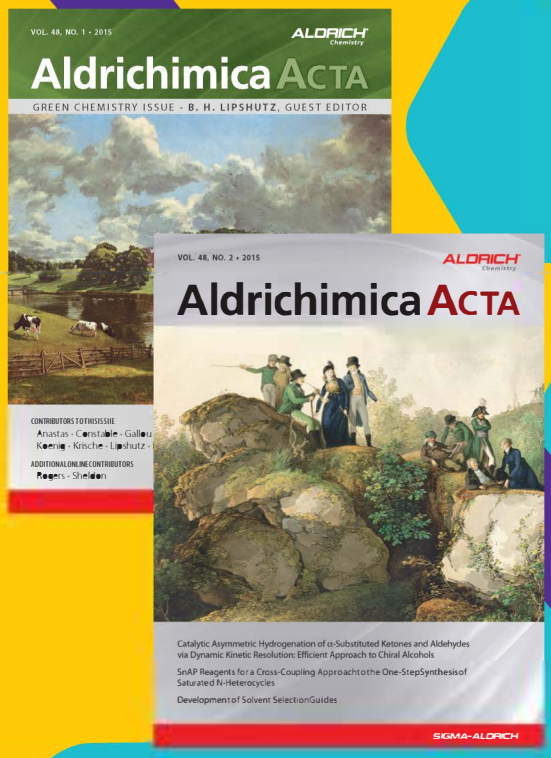

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