PROMISING RESEARCH AREAS – I.

A STUDY OF
THE BIOLOGICAL EFFECTS OF CHEMICAL SUBSTANCES
EMPLOYING THE CONCEPTS AND TECHNIQUES
OF PHYSICAL CHEMISTRY

NOVEMBER 1968

Prepared for

LIFE SCIENCES DIVISION, ARMY RESEARCH OFFICE
OFFICE OF THE CHIEF OF RESEARCH AND DEVELOPMENT,
DEPARTMENT OF THE ARMY
WASHINGTON, D. C. 20310

CONTRACT NO. DA HC 19-68-C-0001

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OFFICE OF BIOMEDICAL STUDIES
FEDERATION OF AMERICAN SOCIETIES FOR EXPERIMENTAL BIOLOGY
BETHESDA, MARYLAND
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This is a technical report prepared for the Life Sciences Division, Army Research Office, Office of the Chief of Research and Development, Department of the Army, by the staff of the Life Sciences Research Office, Office of Biomedical Studies, Federation of American Societies for Experimental Biology (FASEB), in accordance with the provisions of U.S. Army Contract No. DA-HC19-68-C-0001. The text of the report reflects the opinions of an ad hoc study group that met at Beaumont House, FASEB, on June 18, 1968. The report has been approved by the majority, not necessarily by all, of the participants.
SUMMARY

This report was prepared in response to a request to identify promising areas in the life sciences for future emphasis in Army research and development programs. It is based upon a review of the evolving knowledge in physical chemistry and the application of the concepts and techniques of the physical sciences to the study of biological problems of military interest. After a critical examination had been made of technological forecasting methods, it seemed more appropriate to apply the existing fundamental knowledge in the physical sciences to the study of selected biomedical problems. This selection was based upon a survey of the Army life sciences research programs and this report suggests a number of research opportunities related to Army research interests.

The research administrator faces a recurring demand in allocating and justifying funds, staff, and research facilities. In addition, he must identify the most favorable areas for special emphasis in new studies and evaluate the productivity of current programs. Extensive experimentation has been undertaken with forecasting techniques in an attempt to improve and codify methods that will enhance the recognition of research opportunities. The goal of forecasting in the sciences is to make more objective what is essentially a subjective decision by the administrator who must make difficult choices based upon fragmentary data.

An analysis has been made of the numerous technological forecasts of the past five years. A critical appraisal of the methods employed and the content of these forecasts revealed little of value in identifying promising research in the biomedical sciences. Technological forecasts have been most profitable in planning the evolution of devices, machines, and armaments. They permit a good prediction to be made when a specific piece of "hardware" may be expected to reach a certain phase of development. These forecasts do not embrace a consideration of discoveries made by chance observation or even those made from intuitive knowledge. They do not permit the prediction of fundamental discoveries that may have an overriding impact on the biomedical sciences.

To explore the value of a novel approach to uncover new ways to make predictions and expose promising areas in biology and medicine, a small number of experienced biomedical scientists met with experts on the history and philosophy of science. Several broad subjects were identified for future review. However, a more restricted scope of study was selected because it offered goals that may be realized within a shorter period of time with existing knowledge. Such an approach was found in the recent studies of the physical phenomena at the molecular level that have broadened our understanding of many biological cellular events.
Research investigations that have related physical changes in macromolecular structures to biological functions were reviewed by scientists working in these fields. This report summarizes the discussions and includes suggestions for future investigations. The topics discussed involved the nature of the biological changes induced by chemical substances at cell surfaces, enzyme substrate interactions, novel concepts of charge transport through biological structures, and protein fine structure measurements that relate to living systems.

The review discussions emphasized the biological significance of changes in the van der Waals forces in protein structure produced by chemical compounds and the "inert" anesthetic gas xenon; model cell membrane systems that measure the function of the enzyme permeases; the application of nuclear magnetic resonance, electron spin resonance spectra, and spin-labeling techniques to the study of macromolecular protein conformations; fluorescence spectra, polarization and decay times, and absorption spectroscopy as indicators of biological events; and the significance of geometric changes in a series of biologically active compounds as these relate to their chemical structures.

The applications of these concepts to Army research interests are considered in the review discussions. The report presents promising new approaches to a variety of biomedical military problems. The suggested areas for study are specified and the advantages and limitations are outlined for evaluation. It is recognized that Army scientists are aware of the concepts and methods reviewed in this report and the ideas are not presumed to be unique. Rather, the examples cited are highlighted to accelerate the rate of development of these relatively new cooperative research ventures between the physical and biological sciences.
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I. BACKGROUND - A CRITIQUE OF TECHNOLOGICAL FORECASTING

This study is an extension of a 1964 report by the Life Sciences Research Office, Federation of American Societies for Experimental Biology (FASEB), entitled A Study of the Rationale and Techniques for Long-Range Technological Forecasting in the Biological and Medical Sciences (1). The present report has been prepared in response to a request to identify the most promising areas in the life sciences for future emphasis in Army research and development programs.

The 1964 report (1), with 11 references, reviewed the history of forecasting in the biological and medical sciences and emphasized the hazards of forecasting and predicting future discoveries. It was concluded that in the biomedical sciences the value of forecasting has not been proved and it was not possible to make an accurate forecast of the nature, timing, and circumstances of discoveries or the results of specific research projects. However, it was reasonable to expect that, based on existing knowledge and on modest estimates of future capabilities in research and development, forecasts might be useful in accelerating the progress to scientific and technical objectives.

Recently, there has been a major increase in the literature on technological forecasting. One 1967 report written by Jantsch (2) entitled Technological Forecasting in Perspective includes an annotated bibliography of over 400 references. The 1967 joint Army Materiel Command, Navy Materiel Command, and Air Force Systems Command, Report on Technological Forecasting (3) outlined and critically evaluated the following forecasting techniques and aids:

Forecasting Techniques
(a) Intuitive Forecasting
(b) Trend Extrapolation
(c) Trend Correlation
(d) Analogy
(e) Predictive Models

Forecasting Aids
(a) Matrices
(b) Contextual Mapping
(c) Morphological Research
(d) Network Construction
(e) Systems Analysis
(f) Demand Assessment
(g) Analysis of Theoretical Limits and Barriers
(h) Prediction of Technological Changeover Points
No attempt has been made to consider the advantages and disadvantages of these forecasting techniques and aids inasmuch as they have been reviewed (3). However, these concepts were scrutinized to appraise their value in forecasting discoveries in the biomedical sciences. In addition, an extensive bibliography of the forecasting literature was prepared. Certain selected references considered useful are cited in the bibliography of this report.

The large number of forecasting techniques and aids suggests that no one is entirely satisfactory. Indeed, the prediction of fundamentally new developments in any field largely remains more of an art rather than a science, and de Jouvenel (4) prefers the term "conjecture" to imply only a very limited certitude in forecasting. Although these methods may be used successfully in planning the technical evolution of new devices, machines, and armaments, and to predict when a specific apparatus may be expected to reach a development phase, it is not possible to predict new discoveries or even future scientific achievements except in the most general terms.

A small number of experienced biomedical scientists met with experts on the history and philosophy of science to explore new ways to make predictions and expose promising research areas in biology and medicine. Several broad subjects were identified for future FASEB ad hoc study group meetings. However, with the present state-of-the-art of the biomedical sciences, the opinions of experts knowledgeable in specific scientific areas were considered most likely to be of greatest value. Moreover, it was recognized that the problems to be considered should be clearly identified and defined. The assumptions made and the logic used by the experts should be reliable, unbiased, and comprehensive. The topic selected for the FASEB ad hoc study group meeting as a promising research area of military importance is presented in the following section.
II. SELECTION OF THE TOPIC FOR STUDY AS A PROMISING RESEARCH AREA

Throughout this review of technological forecasting methodologies, the life sciences research and development program of the Army was surveyed in order to select those areas of greatest promise. Two significant facts were recognized and accepted: (a) it is more profitable for the military to apply known skills and methods to solve problems than to place emphasis on the search for basic discoveries and (b) there is the opportunity to apply the evolving new knowledge in physical chemistry to the solution of many biological problems. This is a realistic approach to meet the demands of biology and medicine, and ultimately may lead to fundamental discoveries. Specific topics in the life sciences were identified for research emphasis based upon recognized needs and the technical methods are discussed that may be applied in meeting these requirements. This somewhat arbitrary selection of a finite group of subjects for review has the attractive advantage of providing sound reasons for embarking on specific research projects. Therefore, the biological effects of chemical substances employing the concepts and techniques of physical chemistry were selected for review.
III. SPECIFIC OBJECTIVES OF THE FASEB AD HOC STUDY GROUP MEETING ON THE APPLICATION OF THE CONCEPTS OF PHYSICAL CHEMISTRY TO SELECTED BIOMEDICAL PROBLEMS

An understanding of the biological effects of many chemical substances, for example, normal biochemical compounds, drugs, and toxic agents may be obtained by using the tools and the concepts of physical chemistry. In recent years, the methods of the physical sciences and physical chemistry have been applied successfully in interdisciplinary studies in biophysics, biochemistry, and physiology. Studies at the molecular level have assisted in elucidating the nature of the biological changes induced by chemical substances at cell surfaces, membranes, enzyme substrate reactions, and many similar vital life processes. It was proposed to review the work in these emerging areas to exploit the special interests of the Army life sciences research programs.

The scientists who participated in the review were invited to consider the following topics:

To review the recent work on the biological significance of the weak binding forces known as the London, Kaesom, or Debye effect (van der Waals forces) on protein structure modification produced by chemical compounds, particularly anesthetic gases.

To apply the concepts of solid state physics to the study of the patterned macromolecular aggregates of ordered protein structures. Energy exchange at the cell membrane may be correlated with the measurable fine structure of proteins and some quantum chemical fast transfer of energy via charged particles.

To consider new biological applications of the concept of extremely fast reaction rates. These reactions may be measured in certain instances and they may have wide biological significance.

To explore the importance of the excited molecular states of charge transfer complexes in biology. Quantum chemical methods are now available for a broad study of these supra-molecular states in relation to electron transport in many biological changes. In addition, the study of the formation of free radicals may lead to an understanding of the electronic interactions between some large molecules of biological systems.
To consider the future applications to biological problems of the methodologies of nuclear magnetic resonance, electron spin resonance, mass spectroscopy, polarization optical analyses, optical rotary dispersion, x-ray diffraction, high resolution electron microscopy, electroluminescence, absorption spectrophotometry, analytical ultracentrifugation, and other analytical tools of the physical sciences.
IV. **AD HOC STUDY GROUP AGENDA**

The agenda for the ad hoc study group meeting held at Beaumont House, Federation of American Societies for Experimental Biology, Bethesda, Maryland, on June 18, 1968, included the following topics:

- Bacterial membranes and drug effects,
- Fluorescence spectra, polarization and fluorescence lifetimes as indicators of the molecular environment in living systems,
- Examples of protein structural characteristics as revealed by electron spin resonance and spin labeling studies,
- Molecular forces and the binding of anesthetics to protein,
- Novel biological applications of physical and chemical methods,
- Correlations of physicochemical measurements and quantum chemical calculations in a series of volatile anesthetics and convulsants,
- Molecular structural parameters and functional enzyme inhibition, and
- Novel concepts of charge transport through biological structures.
V. LIST OF ATTENDEES

AD HOC STUDY GROUP MEETING, JUNE 18, 1968
ON
PROMISING RESEARCH AREAS - I. A STUDY OF
THE BIOLOGICAL EFFECTS OF CHEMICAL SUBSTANCES
EMPLOYING THE CONCEPTS AND TECHNIQUES OF PHYSICAL CHEMISTRY

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VI. APPLICATION OF THE CONCEPTS OF PHYSICAL CHEMISTRY TO SELECTED BIomedical PROBLEMS

In planning this study an attempt was made to cite successful examples of the use of physicochemical approaches to the solution of biological problems. This work is at the forefront of biophysics today (5, 6, 7, 8). Oncley's 1959 review (9) gave an extensive bibliography and a background summary of the physical and chemical approaches to problems in biology, including studies of muscle and nerve, sensory and integrated neuronal behavior, genetics, and the fine structure of protein macromolecules. On the other hand, comprehensive surveys of the metabolic aspects of transport across cell membranes (10), cellular functions of membrane transport (11), and structure and function of the membranes and surfaces of cells (12) give essentially no consideration to the measurement of the precise physical nature of the membranes or the physical changes that might be induced by drugs or other chemicals. For many years there has been an overriding concern among research investigators about the biochemical aspects of ion transport, nutrient exchange, and enzyme-substrate energy transfer in biological systems.

This review entitled A Study of the Biological Effects of Chemical Substances Employing the Concepts and Techniques of Physical Chemistry emphasizes the physical phenomena at a macromolecular level that may be more significant than the study of classical biochemical events. Indeed, a recent conference (13) on applications of newer physical techniques to the study of drug metabolism stressed the importance of this field in detecting and measuring biologically significant chemical substances.

Selected examples of particularly relevant research investigations are given in the following paragraphs. These brief reviews include a number of research possibilities related to the Army research programs in the life sciences. It was not possible to include all of these examples in this study. However, a representative cross-section was considered in the review discussions and these are presented in Section VII of this report.

The biological effects of the low energy exchange of the weak van der Waals forces have been studied for the "inert" gases xenon and helium. The central nervous system effects of xenon acting as a general anesthetic, and the convulsive actions of helium at high pressures, are membrane phenomena according to recent theories based upon experimental evidence of protein binding through these forces (14). The change in the physical structure of myoglobin and hemoglobin as induced by xenon was studied by x-ray crystallography and x-ray
diffraction. Similar investigations have been made with the more chemically reactive local anesthetics. It has been suggested that these chemical substances may exert their biological effects by changing the protein mosaic either in the cellular membranes or intracellular and hence, induce a marked response on the cell. The local anesthetics presumably exert a more profound effect on nerve cells by covalent and van der Waals forces. Essentially, these are the first definitive experiments in this specific field (15). Conceivably, these concepts afford a new approach to the study of such subjects as: the short or long-term radiation effects on the physical structure of enzymes; single cell cultures or transplants; the effects of botulinal toxin or other purified toxins on enzyme structures; the action of drugs on membranes; olfaction as a physical cellular protein phenomenon that initiates a neuronal excitation; membrane permeability controlled by the structural changes induced by chemical agents known to modify the permeability of cell membranes; and hydrogen-ion and water transport through gastrointestinal "membranes".

High-resolution electron microscopy has made possible effective resolution to 10Å and it is anticipated that the level of < 3Å may be reached. This development has provided new insights into the ultrastructure of such membranes as lamellar systems in myelin and single cells. This technique will display the physical structure of macromolecular aggregates of nerve cells. Perhaps the energy transfer at a molecular level associated with metabolic and electronic changes is reflected in the states of aggregation of the macromolecule. These changes can now be measured and may be equally important or even more fundamental than the widely studied ionic and electrical potential changes. Morphologic studies with the electron microscope at present, must be interpreted to eliminate fixation and drying artifacts. However, the data from fresh tissues can be correlated with many of these structures and polarization optical analysis can be used with fresh cells (16). X-ray diffraction analysis is also available to assist in defining cellular ultrastructure (17, 18). The challenge is the measurement of the precise geometry of the molecules that enter into or reflect biological reactions. This geometry should be mapped without disturbing the delicate nature of living systems.

Electron microscopy has been employed to reveal differences in the ultrastructure of different types of the malaria parasite. Subtle physical changes in the protein architecture may prevent red blood cell invasion by the plasmodium. Likewise, distinctive structural modifications may encourage phagocytic engorgement. Such measurements of the fine structure of the malaria parasite may provide significant new knowledge for the control of malaria.

It has been suggested that some important biological changes at membrane surfaces and during enzymic actions include reactions that
take place within extremely short times. Methods are available to study reaction rates of very low orders, e.g., 10^-10 seconds or less (19, 20). For example, the binding of certain carotenoids to the opsin protein molecule is altered by light (21). It is possible that the success associated with the understanding of the molecular conformational changes that occur in the visual pigment, rhodopsin, after a quantum of light is absorbed, may be extended to other types of sensory and neuronal modalities. Similar fast electron transfer has been suggested as a form of "electroluminescence" in information storage and transfer in brain cells (22). The neuronal actions of the bacterial toxins may be mediated by similar mechanisms.

The quantum mechanical characteristics of the electron systems of the molecules involved in biological changes have only recently been studied. The molecular orbital theory of quantum chemistry has been applied successfully in chemistry to understand reactions involving polyatomic high energy compounds, e.g., boranes, and to predict and explain certain molecular properties of other chemical compounds. One of the more impressive applications has been the study of the electron transport reactions of the respiratory-chain system involving protein-protein interactions (23, 8). Attention has been directed to the analyses of quantum mechanics of three-dimensional structures, the concepts of the charge transfer complex, and free radicals in chemical reactions. These ideas appear to be useful to explain electronic interactions and charge distributions within molecules, especially large molecules, taking part in chemical reactions.

A charge transfer complex has been suggested as the mechanism involved in interactions of drugs with biologically significant chemical structures. The unpaired electron species characterized as free radicals, represent a change in electronic attractions in a molecule and may be formed as a result of interactions between molecules. Nuclear magnetic resonance (NMR) spectroscopy techniques make possible the measurement of these short-lived intermediates. For instance, chlorpromazine may serve as an electron donor and the skin pigment melanin as the electron acceptor in the skin hyperpigmentation produced by chlorpromazine. The positive ion radicals of chlorpromazine have been studied and attempts have been made to elucidate the free radical spectra of this class of drugs in terms of the drug-enzyme interactions. The photoproduction resulting from the transfer of energy from a light-excited molecule to an unexcited molecule offers an explanation of the oculocutaneous hyperpigmentation induced by a number of drugs including the phenothiazines, sulfonamides, tetracycline, chloroquine, and other antimalarial agents (24, 25, 26, 27). Microprobe techniques have been developed to measure electron spin resonance (ESR) signals in situ and in vivo. This is a revolutionary development with applications in physiology, biochemistry, pharmacology, and indeed, in biology in general (28, 29, 30).
Closely akin to the subject of physical changes in relation to biological functions is the nature of the states of aggregation of water in biological systems. The existence of specific temperature-dependent states of liquid water and their energy content, the significance of clathrates of anesthetics in decreasing conductance in biological fluids, and associated studies of hydrate formation in living systems is polemic. A review of this field may be rewarding in developing a number of novel approaches to the solution of biological problems.
VII. REVIEW DISCUSSIONS AND APPLICATIONS TO ARMY RESEARCH INTERESTS

A. MEASUREMENT OF MEMBRANE SURFACE PHENOMENA

The aim of biological ultrastructure studies is to measure the molecular arrangements that account for the physiological properties associated with a particular structure. Physical methods are now available that are capable of providing precise information on the dimensions and orientations of some protein structural units. These methods give data reliable for the identification of macromolecular components and the techniques are now employed in the study of polypeptide chains, fibrous proteins, plasma proteins, globular proteins, and similar large proteins. Protein membrane surfaces are believed to be delicately oriented for the biological function they perform and these macromolecular aggregates control the events on the cell surface and at a microsomal intracellular level. Thus changes in the absorption spectra (31), nuclear magnetic resonance spectra (32, 33), and electron spin resonance (ESR) spectra (34) have been used to study macromolecular structures related to protein conformation.

1. Reporter Groups at Specific Positions in a Protein Molecule.

Information about active sites and conformational changes can be determined by attaching reporter groups at specific positions in a protein molecule. Thus, 2-bromoacetamido-4-nitrophenol has been attached to the active sites of chymotrypsin or glyceraldehydephosphate dehydrogenase and the characteristic spectral changes that are generated by the binding of substrates to the active enzyme sites can be measured. A refinement of this technique was developed by Conway and Koshland (31) by using a different but closely analogous compound attached to precisely the same amino acid. In this case, the geometry of the interaction with the protein surface and the ligands are changed only slightly. The 4-bromoacetamido-2-nitrophenol was compared with 2-bromoacetamido-4-nitrophenol. In these two compounds the bromoacetamido group and the

\[
\begin{align*}
\text{Br-CH}_2-C-NH & \quad \text{O} \\
\text{OH} & \quad \text{NO}_2
\end{align*}
\]

4-bromoacetamido-2-nitrophenol

\[
\begin{align*}
\text{Br-CH}_2-C-NH & \quad \text{O} \\
\text{OH} & \quad \text{NO}_2
\end{align*}
\]

2-bromoacetamido-4-nitrophenol

chromophoric nitrophenol group are the same, but the relative positioning of the chromophoric nitrophenol to an active protein site is different. When 4-bromoacetamido-2-nitrophenol reacts with glyceraldehydephosphate
dehydrogenase the maximum absorption wavelength shifts from 425 m\(\mu\) for the free reagent, to 436 m\(\mu\) after binding to the protein. This absorption spectrum shift for the protein was greater with the 4-bromoacetamido compound as compared to the 2-bromoacetamido derivative when diphosphopyridine nucleotide (DPN) was added in the system. The absorption maximum of the 4-bromoacetamido compound changed to a lower wavelength while that of the 2-isomer was shifted to a higher wavelength. With chymotrypsin the reaction was 10 times faster for the 4-bromoacetamido derivative than for the 2-bromacetamido compound and the spectrum difference of the molecule with benzoyl-L-phenylalanine was the inverse of that obtained with the 2-bromoacetamido derivative. From the results obtained, these authors suggest that these reagents are particularly useful to probe the active reaction sites, and especially allosteric protein combining sites. The spectra in the two cases are qualitatively different and, in addition, give data on two reporter groups in parallel studies. In this way these compounds complement each other in probing the nature of changes that occur at known locations in protein molecules.

2. Nuclear Magnetic Resonance Studies of Proteins. Halide ions can be used as chemical probes for NMR studies of macromolecules in solution. For example, a mercury atom labels specific sites in a macromolecule and the binding of chloride ions by this mercury-protein complex with a rapid exchange of the bound chlorine with the chloride ions of the solvent produces measurable changes in the \(^{35}\text{Cl}\) NMR line width at protein concentrations as low as \(10^{-6}\text{ M}\) (32). The nature of the structure of the labeled site can be inferred from the \(^{35}\text{Cl}\) NMR spectrum. The mercury atoms are usually considered to be bound to proteins at sulfhydryl sites.

A novel application of this approach to a protein active-site study was developed in an investigation of antibody-hapten interactions (32). Broadening of the \(^{35}\text{Cl}\) NMR line width was related to the accessibility of the mercury-protein complex to the chloride ions in the solvent. The magnitude of the broadening depends on the rotational mobility of the bound chloride. The degree of "flexibility" or vibration of a substrate molecule attached at an active site and the extent of its exposure to the solvent can be measured by \(^{35}\text{Cl}\) NMR spectroscopy. This idea was tested with a mercury-labeled hapten interacted with a previously characterized anti-dinitrophenyl (anti-DNP) antibody using \(^{35}\text{Cl}\) NMR spectroscopy. Information was provided on three facets of the hapten-antibody interaction: (a) accessibility of the mercury atom in the hapten-antibody complex to chloride ion in the solvent; (b) binding affinity and stoichiometry of the hapten-antibody complex; and (c) rotational mobility of the bound hapten.

Information can be obtained from the detailed study of this chloride ion probe technique in many protein-substrate interactions. Therefore, the large number of enzyme inhibitors and related physical changes in proteins induced by chemicals or drugs can now be investigated.
3. Electron Spin Resonance-Spin Labeling for Enzyme Substrate Interactions. Berliner and McConnell (34) reported the use of the spin label technique to determine the steric fit of the substrate-enzyme interaction. They used the paramagnetic nitroxide radicals, RR'NO, where R and R' are bonded to the NO nitrogen atom through tertiary carbon atoms. These radicals are most suitable because they are unreactive and show a simple nuclear hyperfine structure that is sensitive to molecular motion. A nitroxide spin-labeled substrate was used to study the activity of the proteolytic enzyme alpha-chymotrypsin. The spin-labeled acyl group is immobilized at the active site, a finding that is in accord with the idea of an induced fit of the substrate to the enzyme. These results are indicative of the requirement for an acyl group environment at the active site, rigid enough to immobilize the paramagnetic acyl group in space, yet sufficiently flexible to admit the substrate for the acylation reaction. These early attempts to employ the spin-label technique for steric conformational studies stimulated more extensive investigations in this field.

Electron spin resonance (ESR) and spin-labeling have been used to determine the dimensional characteristics and the nature of the combining site for rabbit anti-DNP antibodies with various DNP-haptens (35). To obtain this information a nitroxide free radical was required with a half-life long enough to measure, plus an attachment site on the macromolecule to be measured. A spin-label containing a protected nitroxide group attached to the antigen-specific hapten group was used. The free radical nitroxide can serve as a reporter group reflecting its environment as measured by electron resonance absorption.
The nature of one type of antibody combining site was studied by the ESR spin-labeling technique and was extended by Haia and Piette (36) to study the shape of the combining site. The active site was reported to be quite flexible and to behave as if it were shaped in the form of a slit with a finite depth. Thus, the structural characteristics of antibody-hapten combining sites can be examined. By systematically increasing the chain length in a model compound that separated the hapten moiety from the ESR reporting group, it was possible to estimate the depth of the combining site to be between 10-12 Å. In these measurements the specific set of spin-labeled haptens was designed to allow an accurate measurement of the depth of the antibody combining site to ± 1 Å.

In a study of the details of protein structure modification by drugs using spin-label probes, Sandberg and Piette (37) were able to show a direct interaction of certain phenothiazines with erythrocyte membranes. Using hemoglobin-free ghosts of red blood cells labeled with a spin-label reagent, N-(1-oxy)1-2, 2, 6, 6-tetramethylpiperidinylo)maleimide (MSL), it was possible to demonstrate that the reagent was co-valently bonded to sulphydryl groups of the membrane protein. Electron spin resonance spectra of the spin-labeled ghosts, treated with three typical phenothiazine drugs, clearly illustrated that protein modification was induced by the drugs and that there was a preferential physical reaction between the labeled membrane and each drug molecule. The alkyl nitroxide label by reacting with the membrane structure differentiated between conformational changes on the surface of the proteins and deeper sub-surface binding sites as illustrated by the changed patterns of the ESR spectra. A correlation was demonstrated between these physical chemical changes and the different behavioral effects of these drugs. It was suggested that the free radical moiety may be responsible for the psychotropic activity in the central nervous system.

The unique aspects of these studies suggest the following opportunities: (a) active sites on enzyme proteins could be probed
using substrates or specific inhibitors with appropriate labels, (b) membrane surface effects of single cell organisms, or (c) the structural changes of the high molecular weight tissue proteins may be investigated by conformational measures of this character.

The ability to analyze for subtle conformational changes that proteins undergo in solution is a major advantage of NMR spectroscopy. Combinations of amino acid sequence analyses and high resolution (≤ 3Å) x-ray crystallography of proteins have established the conformations of certain myoglobins, hemoglobins, lysozyme, ribonuclease, and chymotrypsin. Unfortunately, many of the proteins of great interest do not lend themselves to optical or x-ray crystallography and it is impractical to apply these analytical techniques. In these situations other tools will be required. ESR and NMR are among the most promising. The ability to make measurements on proteins in solution is an attractive aspect of ESR spin-labeling studies. The possible application of the ESR technique to single cells should be explored.

APPLICATION TO ARMY RESEARCH INTERESTS

Research studies of the type reported in this review can be applied to the elucidation of numerous biological phenomena of military interest. The weak energy exchanges that are now recognized as significant in cellular systems produce protein conformational changes that can be measured by present physical analytical methods. Examples of applications of these studies to Army research interests include: modifications of the protein geometry of the types of botulinal food toxins, or other toxins, as these may reflect the toxicity of these compounds; the physical structure changes of neuronal proteins as these may be influenced by chemical and biological agents; and a more satisfactory explanation of the mechanism of olfaction may be provided by the measurement of ultrastructure changes in the sensory macromolecular protein aggregates of the nerve terminals. The significance of a precise measurement of the surface structure of antigens is obvious. Binding affinity and the nature of the antigen-antibody complex, as related to surface structure, may be more precisely determined by physical conformational analysis. The three-dimensional arrangement of the determinant groups of native proteins may signify the specificity and immunogenicity of antigens. Knowledge
of the protein active sites as provided by chemical probes and nuclear magnetic resonance spectra could materially assist in the solution of the problems of transplantation histocompatibility. The same approach could solve a number of problems in immunochemistry: the heterogeneity of the immunoglobins, the role of the antigen in antibody formation, and the development of superior immunizing substances.
B. MODEL MEMBRANE SYSTEMS

Microbial cultures may serve as useful models for the study of membrane transport phenomena. Extensive investigations have been concerned with the mechanism of action of antibacterial agents as these relate to bacterial cell-wall penetration and synthesis and enzyme inhibition. However, many chemical substances and non-growth inhibitory agents will influence the life processes of bacterial cells and may be studied at the biochemical level to elucidate mechanisms of action that may function in higher organisms. Although drug-induced effects in microorganisms need not reveal the mechanism of the effects in mammalian systems, they may contribute to the understanding of significant and universal features of the interaction of the drug with biological tissues (38).

Using such a model system with Bacillus cereus, it has been possible to study the effect of a barbiturate, e.g. amobarbital, on the conversion of orotate into the ribonucleic acid (RNA) pyrimidines of the exponentially growing cells. Amobarbital at non-growth inhibitory concentrations produces a selective effect on the conversion of the pyrimidine precursor, orotic acid, into the polynucleotides of the microorganisms. Orotic acid and the barbiturate are pyrimidine derivatives and this suggests

\[ \text{orotic acid} \quad \text{(uracil-6-carboxylic acid)} \quad \text{amobarbital} \quad \text{(5-ethyl-5-isopentyl barbituric acid)} \]

an antimetabolic action by the amobarbital. The details of the mechanisms at the cellular level were explored to identify the precise locus of action of the drug.

Amobarbital did not influence oxygen uptake of the cells, the incorporation of adenine or uracil into polynucleotides, the conversion of amino acids into protein, or the incorporation of diaminopimelate into the cell wall. In addition, it was found that the drug did not modify the conversion to deoxyribonucleic acid (DNA), the interconversion
of RNA pyrimidines, the decarboxylation of orotate, the de novo biosynthesis of pyrimidines, or the flavin-mediated interconversion of orotate and dihydroorotate. By using orotate-7-14C in combination with 6-azauridine to block orotidylate decarboxylase, it was demonstrated that the amobarbital effect was localized at the step of entry of orotate from the medium into the cell. This finding suggests that the drug depressed the cellular uptake of orotate by competing with the orotate in the carrier system.

Bacterial cell membranes apparently utilize a permease to control the entry of essential elements into the cell for growth and reproduction (39). The permeases are not concerned with the osmotic properties of bacteria and are functionally distinct from metabolic enzymes. They are related to the permeation of organic molecules with a spatial configuration that is highly selective. The stereospecificity of the permease system for transport of compounds across cell membranes is known. Therefore, it is possible that the closely related chemical configurations of orotate and amobarbital constitute a competitive relationship for such a carrier system. This model is a means of studying a large number of chemical substances that may exert their biological effects on many types of cell membranes. The universality of the concept remains to be explored, but the evidence suggests this system is associated with membrane function and may be useful in studying one parameter that can be measured in a quantitative manner.

The microbial cell system model as it relates to the function of the permeases has been described in detail by Cohen and Monod (39). A large number of permeases have been identified in numerous microorganisms (40). However, the molecular transport mechanism underlying cellular growth and cellular permease regulation is not completely understood. One of the more intensively studied permeases is β-galactoside permease in Escherichia coli. This permease is indispensable along with β-galactosidase to permit the growth of this organism on lactose as a sole source of carbon. In addition, amino acid permeases are known and an α-methylglucoside permease has been demonstrated to act in the induced synthesis of isomaltase in yeast (41). Although these systems in the cell wall have not been isolated and characterized, they appear to involve specific proteins that provide regulatory mechanisms for the utilization of substrates and for growth, enzyme induction, repression, and feedback inhibition of enzyme activity.

Evidence that this membrane carrier effect extends to a large number of other barbiturates confirms the fundamental finding. However, some pyrimidine substituted compounds that do not act by central nervous system depression but by stimulation, had an inhibitory effect on the cellular uptake of orotate. Clearly, one can not extend the basic measurement of cellular uptake of orotate to central nervous system
tissue in a gross pharmacologic sense. All the evidence points to a structural specificity and not to such factors as pH, lipid partition, or metabolite formation. Many other chemical compounds were examined to explore the limits of the model system. It was concluded that this is an active membrane transport phenomenon. The permease factor is stereospecific and it is used by other microorganisms. In addition, the effects are not related to such factors as electrolyte leakage from the cell. The significance of these studies to the animal organism will depend upon the demonstration of similar permeases in the mammalian cell.

APPLICATION TO ARMY RESEARCH INTERESTS

The permeases have been studied most extensively in bacteria and investigators are now searching for similar mechanisms in mammalian cells. As a model cell membrane system that can be manipulated for the basic study of transport phenomena, the permeases may prove to be useful to shed light on biochemical events of fundamental importance. For example, the subtle effects of ionizing radiation may be manifested in living cells through this system. The infectivity of arthropod vectors of disease may be related to permease factors. Susceptibility of man to viral, rickettsial, or bacterial infections or the development of useful vaccines may be correlated with a better understanding of the permeases. Any process so fundamentally involved in the control of the life of the cell warrants careful study.
C. BIOLOGICAL APPLICATIONS OF PHYSICAL ANALYTICAL TOOLS

The structural pattern of amino acid polymers can be measured by NMR spectroscopy if these polyamino acids are complexed with monovalent cations. Amino acid polymers are known to have a high affinity for lithium, sodium, or potassium ions and the degree of complexing has been shown to be related to the ordered structure of the amino acids. High resolution NMR is an excellent tool for this analysis and concentrations of potassium as low as $10^{-9}$ gm per liter can be detected when complexed with these polymers. Crystalline polyproline lithium bromide is an example of this type of polyamino acid - monovalent cation complex. While they have not been studied, presumably large polarizable systems like xenon should behave in a similar fashion with these amino acid polymers.

The antibiotic valeomycin is a polyamino acid complex that binds selectively with potassium, presumably through the unique arrangement of the carbonyl groups in a donut-shaped pattern. This antibiotic is toxic to mammals and its toxicity is very likely related to this potassium-binding property. For this same reason the antibiotic makes a barrier for potassium ions when it is incorporated in a lipid film. In this case, the antibiotic is not charged but the molecule is polarizable and the potassium binding is through electrostatic forces. Some synthetic cyclic poly-ethers exhibit these selective cation binding actions. These properties of amino acid polymers may be of biological significance and cellular ion transfer may be determined by these binding forces.

With present techniques of gas chromatography (GC), fluorescence and phosphorescence spectrometry, radioactive derivative formation, NMR, ESR, and related physical analytical tools it is now possible to identify and quantify extremely low concentrations of biologically active compounds in animals, and more importantly, in man. There is an urgent need to evaluate drugs in man, and these new techniques permit such an approach. For example, the new GC techniques will enhance the determination of the mechanism of action, metabolic products, and possible toxic effects of drugs (13). Moreover, this information could be made available from human drug studies, after the administration of extremely small, non-toxic doses of drugs, prior to the development of overt symptoms.

APPLICATION TO ARMY RESEARCH INTERESTS

The precise determination of extremely low concentrations of biologically significant compounds will have many advantages in future research in biology. The detection and quantitative determination of polyamino acids is much more difficult than the analysis of low molecular weight substances. However, physical analytical tools have been used successfully to meet the more difficult problems.
of analysis of specific proteins and their precise physical structure modifications of biological significance. The methods can provide the biophysical data that are so necessary in developing the penetrating insight that is required for new approaches to the study of proteins, nucleic acids, and similar cellular constituents in biology. The structures of amino acid polymers and the quantitative determination of minute quantities of biologically significant substances are but two examples of a host of applications of these analytical tools to such fields of Army research interests as immunochemistry, pathology, microbiology, pharmacology, and biochemistry.
D. MOLECULAR STRUCTURAL PARAMETERS AND FUNCTIONAL ENZYME INHIBITION

Synthetic organic compounds of known chemical structure have been employed in the study of the molecular structures of receptor functional groups of cells or subcellular elements in the nervous system. The neuromuscular junction paralytic effects of the curariform drugs are well known and active compounds in this series were generally believed to require two quaternary nitrogen atoms separated by the equivalent of 10 carbon atoms spacing, or approximately 14 Å. Thus, decamethonium and structurally equivalent compounds with essentially the same space between the two cationic charged nitrogen atoms are all active curariform compounds. This approach to the study of receptor surfaces by structure modification and the subsequent testing for neuromuscular blocking has proven to be rewarding. A large number of chemical compounds have been studied in a search for potent drugs and to elucidate the nature of the spatial geometry of the active sites involved in motor end-plate depolarization. It is now recognized that the neuromuscular blocking agents may act competitively as in the case of d-tubocurarine, or as depolarizing agents, such as decamethonium. The structure of these compounds, and their relationship to acetylcholine and their relative potencies have been the subject of intensive study for many years. Although many exceptions can be cited, a few generalizations can be drawn. These details and general concepts form the basis of a new approach to the study of the nature of the receptor sites.

The essential factors that determine the activity of competitive or depolarizing agents have been established as the coulombic bonding forces, steric effects, and the lipophilic-hydrophilic balance of the molecule (42, 43, 44). The bis-quaternary structure that has been described, has been accepted as satisfactory evidence for the attachment of the cationic centers of the drug molecule to the anionic groups of the receptor site. The steric factors are of prime importance in regulating the proximity of the reacting groups or the closeness of the fit. The secondary binding forces, e.g., van der Waals, will also influence the approximation of the blocking agent to the receptor site. Recognizing these well established facts, it was surprising to learn that a compound such as hexafluorenium, with only 6 carbon atoms separating the two cationic centers, could exhibit a potency approximately equivalent to d-tubocurarine. Subsequently, another active compound was synthesized with only 3 atoms separating the cationic charged centers. These findings and other developments led to the concept of a post-junctional membrane receptor. These receptors may be a mosaic of polyanionic groups that are attractive to compounds with centers of a highly charged onium nitrogen associated with one or two cationic groups in the molecule. The nitrogen mustards exert their biologic effects through at least initial involvement of a similarly charged positive nitrogen.

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A large number of cholinesterase inhibitors have been studied and several important drugs are classed as anticholinesterase agents. The pharmacologic effects of these substances are due to the inhibition or inactivation of acetylcholinesterase at sites of cholinergic transmission. The toxic chemical warfare agents, the organophosphates, exert their actions by irreversible inactivation of acetylcholinesterase. By contrast, relatively few cholineacetylase inhibitors have been made and none are of therapeutic significance (45).

The opportunities for future research on the details of the site of action of botulinal toxin on the cholinergic portions of the peripheral nervous system have been reviewed (46). These crystalline toxins are high molecular weight proteins possessing remarkable potency with precise loci of action in the cholinergic synapse. The details of the mechanism of action in preventing the release of acetylcholine are not known. The large polypeptide structure of the toxin may block the release of acetylcholine from terminal nerve endings because they are accessible to the neurohumoral storage or donor sites (42). The use of reporter groups, halide ion bindings, or spin-labeling techniques may provide the necessary structure information about these proteins to correlate their physical structure with the proposed chemical structures and related knowledge about the receptor binding sites. Unfortunately, only certain portions of the amino acid composition of the toxins are known. For this reason, it is impossible to approach the precise chemical structure on the same basis as the cholineacetylase inhibitors. However, the concepts developed for these latter compounds may be useful in exploring this subject (45, 47).

In a study of a series of compounds evaluated for their potency as cholineacetylase inhibitors coplanarity in the molecule appeared most important (45, 47). A variety of molecular modifications in this group of styrylpyridines provided insight as to the steric and electronic features of the type of compound that imparted cholineacetylase inhibitory properties. Thus, enzyme inhibitory activity was favored by an aromatic ring system conjugated to a pyrido-ring through an exocyclic unsaturated bond to yield an overall coplanar molecule with minimal three-dimensional geometry. The structures of these compounds should reflect information about the nature of the cholineacetylase enzyme receptor at the molecular level. For example, the optimum size appears to be provided by linkage of a fused bicyclic and monocyclic ring system through either a double or triple bond. One of the cyclic structures should contain at least a weakly basic moiety and quaternization generally increased enzyme inhibitory potency. In this series of compounds there is a direct relationship between the chemical structure and the related physical shape of the molecule, and it is the physical structure that determines the ability to inhibit, or the degree of binding to, the enzyme active site.
Similar examples could be cited for numerous classes of chemical compounds that have been synthesized for their activity in biological systems. In general, compounds were previously synthesized and tested without due consideration to the precise physical shape of the molecules.

In recent years, there has been an increasing use of three-dimensional "space filling" models of large proteins to study the precise geometry of the molecule. These models are useful in understanding the interrelationships of the various parts of the molecule and how they enter into the chemical reactions of biological processes.

Models of large molecules provide detailed information about the reactive characteristics of the outer surface as well as the inner portions of the molecule. In these macromolecules both the intra-molecular forces and inter-molecular reactions are biologically significant. The classical example is the enzyme substrate steric fitting. In this case the matching of the surface structures of both molecules appears to be the major controlling factor in the enzyme-substrate interaction. Moreover, the deep clefts that are characteristically identified with probable active sites of biologically active molecules, e.g., lysozyme, ribonuclease, and chymotrypsin may represent important features of the molecular fit.

To enhance the use of models and the study of molecular configuration a stereophotographic technique has been explored (48). Three-dimensional pictures of molecular models have been made using the full color parallax panoramagram technique. The illustrations convey spatial impressions on the printed page without the use of auxiliary devices. In this first report (48) a skeletal model of lysozyme was used as a guide to the construction of a space-filling model of the enzyme. A model of the substrate was shown to fit well into the cleft surrounding the active site. These pictures give a vivid illustration of the elements of the active site and assist in comprehending how the cleft is involved in the union of the main body of the enzyme with the substrate.

APPLICATION TO ARMY RESEARCH INTERESTS

Many factors have been demonstrated to be responsible for neuromuscular blockade. These, in addition to the spatial geometry of the active sites of the motor end-plate, have led to a revision of the concepts of the action of curariform drugs. The study of the spatial geometry of the active sites of the motor end-plate as revealed by chemical structure modification of neuromuscular blocking agents, has led to a modification of these concepts. Spatial distances between the cationic charged centers in this class
of compounds are important in determining potency, but the distances can vary over wide ranges depending on the nature of the nitrogen substituents. In addition to the cationic centers, secondary binding forces will influence the approximation of the blocking agent to the receptor site. The physical shape of the inhibitor molecule may prevent or facilitate the degree of binding through these forces in addition to the more widely recognized ionic interactions. With methods available to measure these various factors an attempt should be made to develop drug screening programs based upon intermolecular space filling concepts. Preliminary determination of the steric configuration of a series of compounds and their correlations with other physical factors could assist in guiding a chemical synthesis screening program. It is recognized that prior research has included these ideas but the recent use of reporter groups, halide ion binding and spin-labeling techniques, for example, give a new dimension to the measurement of the fine structure of proteins. This knowledge of cell reactor sites has been demonstrated to be useful and might be applied to Army chemical synthesis screening programs.
E. NOVEL CONCEPTS OF CHARGE TRANSPORT THROUGH BIOLOGICAL STRUCTURES

Electron transfer through biological structures may be achieved by a process described as "tunneling" (49). Similar suggestions for electron transport in biological reactions have been made for nerve membranes. Charge transport through a biological membrane may occur by an electronic wave motion in an energy band; by a "hopping" process whereby an electron acquires sufficient energy to surmount and go over the top of an energy barrier; by quantum mechanical "tunneling" of an electron through the barrier; by charge transport involving hydrogen bonds; or by an ionic transport mechanism. On the other hand the proton involved in the hydrogen bond may serve as an electron carrier by virtue of the electron cloud that surrounds the proton (50).

However, biological structures do not exhibit the degree of long-range order required to yield an electron energy band system. In addition, the "hopping" process is unlikely because while it is temperature dependent, the Christov Characteristic Temperature calculated for a typical biological organic system result in an unrealistically high temperature (51). Therefore, charge transport at room or body temperatures strongly favors "tunneling". It is suggested that while the actual charge transfer is by electrons, the rate-determining process is most likely the sterically favorable alignment of a donor and an acceptor site. Thus, the cellular "active patch" idea is supported as the place for the favorable alignment of the electron donor and the electron acceptor.

APPLICATION TO ARMY RESEARCH INTERESTS

It may be possible to apply these concepts of energy transfer to model systems of isolated cells or subcellular elements by quantitative physical measures. The effects of certain energy sources such as microwaves, lasers, and flash or ionizing radiation on biological systems may be examined to explore the nature and extent of injury that is produced by exposure to these forms of energy. The changes in the biological membranes at the cell surface and at the cytoplasmic level may be related to electron transfer through or over energy barriers in a manner that has not been adequately explained. Such a novel approach to the study of cellular exchange or cell injury may prove to be profitable.
F. CYTOCHEMICAL STUDIES WITH FLUORESCENCE PHENOMENA AND ABSORPTION SPECTROSCOPY

Fluorescence spectra are excellent indicators of biological events in living systems. Sensitive fluorescence techniques are capable of detecting quantitatively low concentrations of specific chemical substances. In addition, a complete fluorescence spectrum yields experimental data related to excitation states, binding energies, and molecular configuration as these may be reflections of biological interactions. With analytic scanning microspectroscopy selected portions of a single cell may be studied (52, 53). (See Figure 1).

Relatively few workers have utilized fluorescence scanning microscopy to study transient spectral changes in living cells. Numerous technical difficulties remain to be resolved but the fundamental tools are available. The intriguing challenge for the biologist offered by this approach, is the opportunity to record the biochemical and morphologic events that take place within a 3 to 5 microns area of a single cell. The total spectrum can be scanned within 0.5 to 1 second and may be repeated continuously for days. The methods are applicable to single cell cultures as well as rat lung or rabbit ear tissue in anesthetized or unanesthetized animals. The recent emphasis on fluorescence spectroscopy and fluorescent probes in the study of biological phenomena has been coupled with the knowledge of physicochemical changes that many dye-polymer complexes undergo in response to biological events.

By using cationic dyes as fluorochromes that combine with nucleic acid constituents it is possible to study in situ the physical and chemical nature of the interaction of the dye with cellular components by the technique of image scanning television fluorescence microspectrophotometry (54). Other fluorochromes, such as chlorpromazine, may be detected in cultured cells or after systemic administration and their spectral behavior and distribution observed. Molecular alterations on the binding of carcinogens have been studied in living rabbit ear chamber tissues (55) as shown in Figure 2. These are examples of a new way to study intracellular fluorochromes at low concentrations. The primary interest in these studies is the application of various scanning techniques to measure normal or abnormal molecular events in an uninjured living cell.

The possibilities of using other fluorescent measurements, especially polarization and decay times, should be studied in living systems. Each can be a sensitive indicator of molecular interaction and environment. For example, the decrease in fluorescence polarization from anilinonaphthalenesulfonate absorbed upon bovine serum albumin reveals electronic energy transfer among the ligand molecules and the mutual orientation of the electronic oscillators participating in the transfer (56). Also, the organization of DNA in dipteran polytene chromosomes, stained with acridine orange, has been studied using polarized fluorescence
"A molecule excited to an upper electronic state (such as S₂) can return to the ground state (S₀) in a number of ways (---). First, the molecule very rapidly goes from S₂ to the lowest excited state (S₁) without emitting a photon. Some of the possible fates of S₁ are: (i) fluorescence, a transition of S₀ accompanied by emission of a photon; (ii) internal conversion, a return to S₀ without radiation; and (iii) intersystem crossing, a transition to an excited triplet state (T₁) in which the electron spins are no longer paired as in the singlet states. T₁ may return to the ground state in a radiative process termed phosphorescence, or it may return without emitting radiation. Alternatively, S₁ and T₁ may transfer their excitation energy to other chromophores or participate in photochemical reactions.

Excited-state processes. Straight arrows denote processes in which a photon is emitted or absorbed; wavy arrows denote transitions which do not emit radiation." Used with permission of the author: Stryer, L., Fluorescence Spectroscopy of Proteins, Science 162: 526-533 (1968).
Normalized averaged fluorescence emission curves, intensity vs. wavelength (µm). These curves from above down represent spectral changes that occur in the area of the implanted 3,4-benzpyrene particle in the living rabbit ear. As the particle is absorbed over a period of days, the disappearance of the shortest wavelength (probably the first excited pi-singlet state) is noted.
from the microscope image (57). If fluorescence polarization is studied as a function of wavelength, it can indicate in proteins, the different extent to which the several electronic transitions in the molecule are excited by light absorption, and the degree of local freedom of tyrosine residues (58). In living cells the fluorescence polarization of specially prepared amino acids has been studied and compared with other compounds to detect any interaction of the small molecular weight substances with cellular macromolecules (59).

Fluorescence decay times can also reveal charge transfer with no change in configuration of the molecule in the classical chemical sense. Chen (60) has studied a number of compounds of biological interest in aqueous solutions. These measurements can be made in living cells (61).

The combination of polarization and decay data provides information about total molecular volumes and viscosity of large complexes. Studies of this type give information about living cell systems that should be explored. Subtle differences in the nature of molecular interactions in living systems by the use of these fluorescence techniques may be revealed.

APPLICATION TO ARMY RESEARCH INTERESTS

Fluorescent probes may be used to examine conformational changes in a number of biologically interesting macromolecules. The remarkable fact that these fluorochromes undergo fluorescent changes as a result of noncovalent interaction with proteins suggests applications to the study of biological processes. These basic studies have been made with proteins of known structure such as heme globin and lysozyme, with supportive correlations by chemical and x-ray structural analyses. From this secure foundation many types of macromolecules and proteins may be studied. The immune globulins, chymotrypsin, ovalbumin, and a few enzyme proteins have been examined by the fluorescent probe method to relate conformational changes with loss of biological activity. Bacterial enzyme proteins should be studied for possible correlations with such factors as pathogenicity. Mammalian tissue enzymes involved in hormonal or neurohumoral control, immunoproteins, and connective tissue mucopolysaccharides are excellent subject materials for fluorospectroscopy study. The effects of antibiotics on bacterial cell wall proteins have been studied only superficially by this technique. Thus, any cellular biochemical event that is reflected in some fluorescence change, however slight, may be evaluated by these methods.
G. MOLECULAR FORCES AND THE BINDING OF ANESTHETICS TO PROTEINS

The gases in the helium group of the periodic system of the elements include helium, neon, argon, krypton, and xenon. These elements generally have been considered to be pharmacologically inactive. In addition, these gases were described chemically as "inert", i.e., they do not form covalent or hydrogen bonds although xenon fluorides have been described. However, following the earlier observations that the helium-group gases produced a "depressive" effect in animal and man (62, 63), Cullen and Gross (64) demonstrated that contrary to the general belief, xenon gas was capable of producing surgical anesthesia in man. Although xenon is a weak anesthetic and approximately 80 per cent is required in the inspired air for anesthesia in man, it must be classed as a chemical substance that will induce the degree of central nervous depression required for anesthesia — an easily documented biological effect.

In a search for an explanation of the anesthetic effects of xenon, it was discovered that this substance provides an excellent model for the study of the physical actions of chemical substances that elicit biological effects. The rare gas atoms consist of a dense positive nucleus surrounded by a more diffuse cloud of electrons and the centers of positive and negative electrical charges coincide. The atoms are spherical and do not possess a permanent electric dipole. Thus, xenon is spherically symmetrical and does not have permanent dipoles. Under biological conditions it does not exert covalent, ionic, or hydrogen bonding forces; presumably therefore, the biological effects must originate from the changes produced in the physical structures of the cell protein matrix (14). For these reasons xenon was studied by x-ray diffraction (65) to localize its specific site(s) of association in myoglobin and hemoglobin and to observe if any changes were produced in the three-dimensional protein structures. The structure of the protein must be known in detail in order to apply these techniques to the study of physical binding of the gas to a protein. For this reason, myoglobin and hemoglobin were excellent substances to be studied. In addition, much significant information has been obtained to support the concept of conformational changes in other proteins and some anesthetic gases appear to bind to proteins through London interactions and entropy diminishing effects.

It has been pointed out that while complex formation between proteins and anesthetic agents do not explain the phenomenon of anesthesia, it does offer a new approach to the study of those changes in protein structure that correlate with a biological effect. It is not clear why these effects occur when the partial pressure of these inert gases is raised or why anesthesia in lower animals requires 2 to 3 atmospheres of xenon. The general biological effects of the helium-group of elements have been studied by Schreiner (66) including such diverse measurements.
as the importance of the thermal properties of the gaseous environment on the level of oxygen consumption of rabbits, performance of simple motor tasks by men, growth rate and protoplasmic streaming in fungal hyphae, the growth of mammalian cell tissue cultures at elevated gas pressures, and the influences of these gases on isolated enzyme activity. From these studies, the helium-group gases and nitrogen were arranged in the following order of increasing narcotic potency in man:

\[ \text{He} < \text{Ne} < \text{N}_2 < \text{Ar} < \text{Kr} < \text{Xe}. \]

This relative order of activity is substantiated, in general, by the numerous studies that have been made in several biological systems (67). It is of interest that a number of physical properties of these elements can be correlated with this rank order, e.g., atom size and polarizability. However, it appears that the interatomic relationships with biological structures at the bonding level are most important.

Other anesthetics have been studied for their effects on protein crystals in an attempt to determine the degree of uptake. There is a limit to the size of the organic molecule that will be taken up by the hemoglobin lattice matrix and large anesthetic molecules are therefore not able to penetrate the crystal structure (68). Thus, the protein crystal is a model for study because the size of the anesthetic molecule limits its potency. Only substances with relatively small molecules can serve as anesthetics. A similar size limitation would undoubtedly be of significance in the action on the intracellular membranes of mitochondria. As these structures are the main source of cell energy, this action might be related to the phenomenon of cellular anesthesia. In general, occlusion of any critical portion of the membrane free space might interfere with the permeability of essential molecules or ions and in this way modify the function of the cell.

Significant evidence suggests that these gases produce their pharmacologic actions by modifying cellular functions through interactions at the molecular level. It is important to correlate changes in protein conformation with the pharmacologic effects of the substances investigated. The binding of chemical substances to serum albumin is a well recognized process that takes place in the blood. This phenomenon is not the same as the selective attachment of a molecular species to a special geometrically acceptable locus on a living cellular membrane. The latter biophysical event may change the rate of cellular life processes or indeed, may control the life of the cell.

As a result of the close scrutiny of the relationship between the surface configuration of molecules and their interactions at a physical level, it is recognized that energy shifts or other subtle changes may
occur in macromolecules remote from the point of apparent attachment of the reacting chemical substance. Thus, inhibitors of enzyme proteins may not fit into the obvious specific space on the protein surface but may attach to the molecule at some other position. This combination in turn may modify the entire protein macromolecule by creating an electronic shift across the molecule, or through some other intraatomic forces, thus modifying the steric configuration. Obviously, the forces that are incited to change must be those that are related to the weak binding attractions or repulsive energies, the Keesom, Debye, London, or Born forces. This concept of enzyme inhibition is not the same as the classical competitive inhibition by substrate simulation.

Membrane proteins may be considered as the probably site of action of anesthetic agents. Anesthetic combination with the cell membrane proteins modifies intracellular functions or secondary passive ion transport. Physical combination of the anesthetic with the membrane is influenced by the molecular size and shape of the membrane active site. This growing body of knowledge will increase our understanding of the fundamental process of anesthesia.

Illustrative of the specific chemical structural requirements for biological effects on neuronal membranes is the work that correlates the shape of dichlorodiphenyltrichloroethane (DDT) and its isomers with insecticidal action. Only the chlorine analogs of DDT are active; the iodo or bromo derivatives are inert. In addition, a specific positioning of the chlorine atoms on the benzene rings are significant in determining the potency of the resulting compound. An enormous amount of work has been devoted to the study of the covalent, ionic, or hydrogen bonding effects of the DDT molecule as these might relate to its insecticidal potency. None of these actions are known to be biologically significant. It would appear that only the physical structural shape of the molecule must be important in exerting the characteristic effect on the nervous system of the insect. Considering these actions on a neurophysiological basis the effects of the poison may be manifest by influencing the nerve impulse at a membrane level. In some respects this may be equivalent to the actions of anesthetics on membranes (68). The structure of DDT is such that it properly fits the nerve cell protein cavity and this in turn modified the flow of sodium ion currents so that channels for transport are essentially physically blocked for ion mobility. The available evidence supports the idea that sodium efflux or the "extrusion mechanism" is responsible for generating the bioelectric activity of the nerve impulse and this is controlled by the membrane. Thus, DDT may be equated with an anesthetic-like action on this type of nerve membrane because it possesses the proper physical structure.
Thus, chemically inert molecules inserted into protein structures may be viewed much as wedges - they alter structure perhaps far away from their point of insertion and thus control catalytic activity of the macromolecule.

APPLICATION TO ARMY RESEARCH INTERESTS

The most significant fact to emerge from the study of the "inert" gases and their effects on the molecular conformation of large protein molecules, is the demonstration that pharmacologic effects can be produced without chemical interactions in biological systems. Therefore, a large number of drug effects should be re-examined for similar mechanisms of action. The critical size of so-called "active patches" on cell membranes may be the precise locus of the space that is filled or reshaped by a drug molecule. The modification of the surface configuration of cellular membrane proteins, even remote from the point of attachment of the chemical substance, presents a new view of drug-cell interaction. These remote effects may be mediated by conventional intra-atomic forces or through a series of energy exchanges across the protein macromolecule with a subsequent steric configurational modification. The demonstration that chemicals such as DDT can sterically fit the protein mosaic of insect nerve cells and thereby exert an effect, illustrates the character of biological changes controlled by the precise physical structure of a toxic molecule. Covalent and ionic interactions predominate in the life of the cell but such energy exchanges as charge transfer and polarization interactions now have been demonstrated to be biologically significant. These fundamental facts may be applied to the study of the proteins of many types of cells, cell cultures, bacteria, protozoa, and the toxic proteins. Protein turnover in the animal body may be correlated with the fine structure of these macromolecules, and this could open a number of new opportunities for study of biological problems of interest to the Army.
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X. GLOSSARY OF TERMS

Born force . . . . . A repulsive force between two atoms when the
electron clouds overlap (Ref. 14). (See van
der Waals forces.)

Charge transfer complex . . . . . A molecular complex formed between two compounds
one of which is comparatively rich in electrons
and the other comparatively electron-deficient.

Chelate . . . . . . A type of coordination compound with a central
atom (most frequently a metal) joined to two
or more other atoms of other molecules or ions
(called ligands) forming heterocyclic rings,
with the central atom as a part of each ring.
The term is derived from claw to signify the
two or more bonds holding the central atom
(metal) to the ligand. Metal chelates of
biological systems include the iron-binding
porphyrin of hemoglobin and the magnesium-
binding of chlorophyll. Ethylenediaminetetraacetic
acid (EDTA) is a common chelating agent.

Clathrates . . . . . "Clathrates are physical associations of two
(or more) types of molecules in which there may
be very little actual attraction of the molecules
of one species for those of the second. The
molecules are held in association by the for-
mation of a cage-like structure by one of the
components around an atom or molecule of another
species. The cages are formed by covalent com-
pounds which, because of their chemical config-
uration, cannot close-pack. These compounds
tend, instead, to form lattice structures con-
taining cavities in which other molecules of
critical sizes may be entrapped. Formation of
these cages is a step-wise phenomenon resulting
from the intermolecular hydrogen-bonding of the
lattice-forming molecules. Bonds of this nature
are relatively weak and easily broken. Although
the presence of an entrapped molecule in the
interior of the cage stabilizes the clathrate
structure through van der Waals interaction with
the molecules forming the cage structure, solution
of the crystals is sufficient to disrupt the
lattice and release the trapped molecule" (Ref. 56).
Coordination compound . . . . .

A compound with a molecular structure containing a central atom bonded to other atoms by coordinate (Dipolar) covalent bonds. A coordinate bond is a covalent bond in which both electrons of the new bond comes from the reactant molecules, e.g., \( A + B \rightarrow A:B \). Chelate compounds are a special kind of coordination compounds.

Coulombic bonding . . . . .

At infinite dilution the distribution of ions in an electrolytic solution can be considered to be completely random because the ions are too far apart to exert any attraction to each other. But, at higher concentrations the ions are closer together and according to Coulomb's law the Coulombic attractive and repulsive forces become evident. Because of this interaction of ions the concentration of negative ions is slightly higher near a positive ion, and vice versa, than in the bulk solution.

Debye force . . . . .

A force of attraction caused by a delocalization, or shift, in the electron cloud of a nonpolar atom or molecule due to the electric field of an adjacent dipolar molecule. Such a shift creates a new dipole and the two molecules are attracted to each other depending on the strength of the permanent dipole and the polarizability, or ease of shifting the electrons away from the nucleus, of the nonpolar atom (Ref. 14). (See van der Waals forces.)

Electron spin resonance spectroscopy (ESR) . .

A method for the detection and study of free radicals because they contain unpaired electrons. A measure of electron magnetic moments. The mean lifetimes of free radicals can be determined by this method and conclusions can be drawn about the location of the unpaired electron on a specific atom.

Free radical . . . .

Uncharged compounds, atoms or groups of atoms with at least one unpaired electron or electrons. Most free radicals are very short lived and difficult to detect. Certain free radicals are stable and can be prepared in the free state. They embody high reactivity and high energy. Free radicals may be useful in theoretically illustrating chemical reactions of biological significance.
Keesom force . . . . A force of attraction between two dipolar molecules or molecules that are electrically neutral but have asymmetry in charge distribution, or a finite distance between the centers of electronegative and electropositive charges (Ref. 14). (See van der Waals forces.)

Ligand . . . . . . . A molecule, atom, or ion that is attached to the central atom of a coordination compound, a chelate, or other complex.

London force . . . . A force of attraction between two atoms that have no permanent dipole moments. (Ref. 14). (See van der Waals forces.)

Nuclear Magnetic resonance spectroscopy (NMR) . . A method for the detection and study of the structure of molecules relating to their nuclear magnetic moments. The number and position of protons in the molecule determines the behavior in strong magnetic fields and is characteristic for the compound.

Steric effects . . . . The influence of the geometric shape of a molecule on its reactivity. Chemical reactivity is influenced by steric assistance or steric hindrance. The former will accelerate or facilitate a reaction, the latter will retard a reaction as a result of the bulk or physical shape of reacting atomic groups within the molecule. The field of stereochemistry deals with the spatial arrangements of atoms and groups of molecules, including geometrical isomerism, optical activity, and restricted rotation. Allosteric substances have the same spatial configuration of form; they may not have exactly the same atomic chemical composition.

Stoichiometry . . . . The quantity of chemical substances that enter into and are produced in a chemical reaction. The combining amounts are determined by the laws of chemistry and physics.

van der Waals forces . . A group of weak bonding forces between molecules. The magnitude of the forces determine the state of aggregation of the molecules and is related to the polarity and polarizability of the molecules (Ref. 14). Some reports refer to the Keesom, Debye, and London forces collectively as van der Waals forces.
An analysis has been made of the numerous technological forecasts of the past five years. A critical appraisal of the methods employed and the content of these forecasts revealed little of value in identifying promising research in the biomedical sciences. Technological forecasts have been most profitable in planning the evolution of devices, machines, and armaments. They permit a good prediction to be made when a specific piece of "hardware" may be expected to reach a certain phase of development but they do not permit the prediction of fundamental discoveries that may have an overriding impact on the biomedical sciences.

This report summarizes recent investigations that relate physical changes in macromolecular protein structures to biological functions. It is based on a review by scientists studying protein structure changes induced by van der Waals forces; model cell membrane systems that measure the functions of the enzyme permeases; the biological applications of nuclear magnetic resonance and electron spin resonance spectra and spin-labeling techniques; fluorescence spectra, polarization and decay times, and absorption spectroscopy as indicators of biological events; and the significance of molecular geometric changes in a series of biologically active compounds as related to their chemical structure. The topics include the nature of changes produced by chemicals at cell surfaces, enzyme-substrate interactions, novel concepts of charge transport through biological substrates, and their relationship to fine-structure changes in living systems.

The applications of these techniques and concepts to Army research interests are considered and the report presents promising new approaches to a variety of biomedical military problems. The suggested areas for study are specified and the opportunities and limitations are outlined for evaluation.
Charge transport through biological structures

Binding of anesthetics to proteins

Biological Applications of:

- ESR spectroscopy
- Fluorescence phenomena and absorption spectroscopy
- Fluorescent probe techniques
- Membrane surface phenomena
- NMR spectroscopy
- Polarization and decay times
- Protein structural geometry

Forecasting in biomedical sciences

Solving biomedical problems using physical chemistry

Xenon anesthetic action