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A Final Report on the Activities
of the
Biophysics Research Laboratory
under
Contract N0 118-877 - Dec 1956(04)

October 8, 1967

Submitted by: Bert E. Yallow, M.D.
Scientific Director
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The Biophysics Research Laboratory of the Department of Medicine, Harvard Medical School, and the Peter Bent Brigham Hospital was dedicated on May 17, 1954. The laboratory, which comprises an area of about 7,500 square feet, was completely remodeled within the existing facilities of the hospital. It has been fully equipped with all the instruments necessary for investigation in emission and absorption spectroscopy, intermediary metabolism and physical chemistry. It was established with major grants from the Rockefeller Foundation and the National Institutes of Health. Additional funds were received from private donors and from Harvard Medical School. The first application to the Office of Naval Research was submitted in April 1952 and a contract went into effect in 1953. Up to the time of the dedication of the laboratory at the Peter Bent Brigham Hospital, the work was carried out in the Department of Biology and in the Spectroscopy Laboratory at the Massachusetts Institute of Technology.

The present report constitutes a review of our activities under this joint grant from the Navy, Army and Air Force which was administered by the Office of Naval Research.

Scientific, philosophical and educational considerations were fundamental in the establishment of the Biophysics Research Laboratory:

1. The laboratory was to be concerned primarily with fundamental investigations. Biophysics, physics, physical biochemistry, and quantitative biology were to be emphasized. It was anticipated that the existence of medical facilities would occasion extensions into the clinic.

2. It was envisioned that the study of the function of metals in biology in general, and catalytic phenomena in particular, would become a primary target...
of the scientific effort. The development of quantitative spectrographic methods opened new avenues for a concentrated effort in this field which had not previously been approached systematically. The elucidation of the molecular basis of metalloenzyme action appeared especially promising and was to constitute a specific objective.

Continued fundamental studies in emission and absorption spectroscopy were considered essential to a proper evaluation of such biological interests.

3. The establishment of the laboratory in a hospital of relatively small size was expected to result in closer contact between scientists and the physicians of the hospital. A reorientation of some phases of medical teaching to pre- and postdoctoral students of medicine and of the sciences was anticipated to be a desirable result of such an association. It was hoped that the laboratory would attract individuals from both medicine and the various scientific disciplines.

It is a pleasure to report that these major objectives have been achieved.

This report will emphasize the highlights of these past years' work. Much of it has been published in the scientific literature and copies have been sent to the Armed Forces. A full bibliography is attached.
I. SPECTROSCOPY

A. Porous Cup Electrode Sparking Method: The porous cup electrode technique, repeatedly described in our annual reports, has been standardized and has remained the routine spectrographic technique of the laboratory. It has not required nor undergone any significant changes in the last year and a half. Details of the method, such as internal standardization, control of ashing temperature, plate calibration, and densitometry, have been clarified to a high degree. The experience of the last three years has now measured us that under present working conditions this method is probably as adequate as may that can be designed for biological emission spectroscopy without major alterations in existing instrumentation. Compilation of data on reproducibility and precision indicate that 5 to 7 per cent is a reasonable estimate of the standard deviation at any level of concentration from 0.01 ppm to 500 ppm. We feel that sufficient experience with the method and its performances has now been gained to warrant placing it on record in the scientific literature and therefore several publications arc planned. A summary chapter in "Methods of Biochemical Analysis", Volume 1, is contemplated.

B. Noble Gases and the DC Arc: The study of the effects of helium, argon, neon, and krypton on the DC arc, begun at Massachusetts Institute of Technology, was completed. Early findings of an increased signal to noise ratio and selective enhancement of certain spectral lines were confirmed and extended. A general theory was formulated which adequately predicts and explains the observations. The selective enhancement is apparently due to a resonance phenomenon involving collisions of the second kind between metastable gas atoms and those of the elements to be excited. When the transition of the atom is within the energy range of the excited gas atom, greater efficiency of excitation...

C. The Direct Reading Flame Spectrometer: A direct reading flame spectrometer for the simultaneous analysis of sodium, potassium, calcium and magnesium has been completed and is in routine use in the laboratory. With this instrument it is possible to determine all four elements simultaneously in one sample. The limits of detection are: sodium .01, calcium .1, potassium .3 and magnesium 1 ppm. Thus far, no effort has been made to extend these limits.

The Jarrell-Ash Company, Newtonville, Massachusetts, is under contract to manufacture this instrument, but a commercial model has not yet appeared on the market in spite of heavy demand. The subject matter pertaining to these studies is reported in three articles: (Anal. Chem. 28: 176, 1956, Anal. Chem. 28: 180, 1956; Anal. Chem. 29: 1086, 1958). The general subject of flame photometry has also been reviewed in a chapter in "Methods of Biochemical Analysis", Volume 3, 1955. In addition, a special technical report to the Office of Naval Research was published by the Department of Commerce as "Direct Reading Flamespectrometry. Principles and Instrumentation" (PB 111743), 1958.

D. The Cyanogen-Oxygen Flame: The study of the direct reading procedures with the hydrogen-oxygen flame persuaded us that the extension of emission spectrographic work in biology should not be sought in arc or spark spectroscopy. It seemed that a flame could constitute the "ideal" source, if a particular fuel could be found which would energize the sample sufficiently to excite transitions of atoms having a higher excitation potential than those characteristic of alkali metals and alkaline earths. No such flame was in
existence for spectrophotographic purposes. We were able to utilize the reaction
\[ \text{O}_2 + \text{H}_2 \rightarrow \text{H}_2 \text{O} + \text{I} \]
for this purpose and to demonstrate that spectrometry of the transition metals could be performed (J. Opt. Soc. Am. 46: 77, 1956).

This is the first really new flame source discovered in more than 50 years.

Further, qualitative measurement by photometric means was feasible (Anal. Chem. 18: 1753, 1956). Our studies of the four-channel flame spectrometer indicated the practicality of substituting a direct-reading procedure for photographic recording; hence, a "direct-reading xenon-nitrogen flame spectrometer" has now been developed which presently allows the analysis of copper, manganese, and magnesium. An extension to other transition elements is contemplated.

The sensitivity of detection of these three elements is 50 parts per billion.

The total volume of solution, .04 cc./15 sec., represents a major step forward in lowering the limit of detection and decreasing the volume of solution necessary for analysis. In the course of these studies various physical factors affecting the temperature of the flame have been explored. It has been shown that a high flow rate of an aqueous sample materially cools the flame and decreases sensitivity.

Fundamental processes accounting for this circumstance have been investigated, and a mathematical formulation predicts the events accurately. These investigations are in preparation for publication.

8. The Coenzymester: As a result of our studies in enzymology, it became apparent that a spectrophotometer capable of measuring reactions at 340 m\(\lambda\), the wavelength at which DPNH absorbs maximally, would be extremely useful, both for research investigators and for clinicians interested in adapting DPNH-dependent reactions to clinical diagnosis, as in lactic dehydrogenase and transaminase determinations. A simple instrument was therefore designed which is now in commercial production. It has been given the trade name "Coenzymester".
The instrument has been acclaimed by hospitals and practitioners, since it makes unnecessary an expensive ultraviolet spectrophotometer selling at 15 times the price of this instrument. Details of design and construction are in preparation for publication.

II. METALLOENZYMES

A. Metalloenzymes. The study of metalloenzymes is the major biochemical interest of the laboratory; investigations in this field have led to the discovery of zinc in five enzymes not hitherto known to contain this metal: the ADN of yeast, ADN of equine liver, LDH of bovine liver, LDH of rabbit muscle and carboxypeptidase of bovine pancreatic juice. In all instances, zinc has been found to be an intrinsic part of the enzyme molecule and essential to its catalytic behaviour. These differences have been possible by virtue of compositional, structural and functional studies in which instrumental analyses involving spectrophotometry, ultracentrifugation and electrophoresis have been employed. The functional properties have been tested further by the use of metal binding inhibitors and the study of the kinetics of their interactions with these enzymes. Another group of pyridine nucleotide dependent dehydrogenases (PyN) have also been shown most likely to be metalloenzymes; they have been referred to as pyridine nucleotide-metallodehydrogenases (PMD). This work has been reported in various publications: (Proc. Nat. Acad. Sci. 41, 327, 1955. Chapter in "Advances in Protein Chemistry", Volume 10, 1955. J. Biol. Chem. 221, 491, 1956. J. Am. Chem. Soc. 77, 5196, 1955; J. Biol. Chem. 217, 285, 1955, J. Am. Chem. Soc. 78, 1771, 1956; J. Am. Chem. Soc. 78, 5679, 1956; J. Biol. Chem. 225, 105, 1957) These studies have, for the first time, implicated a metal to be involved
in enzymatic dehydrogenation reactions. The finding of zinc in carbamylpeptidase has reconciled previously discrepant reports in the literature. A further review requested by the Editor will appear in "The Enzymes", Volume I, Part II, 2nd Edition.

8. Kinetics: Extensive kinetic studies have resulted in a model for the action of a metal chelating agent, 1,10-phenanthroline (OP) in bringing about inhibition. Three papers on this subject are in preparation for publication.

C. Mixed Complexes: The interaction of 1,10-phenanthroline with LADH has been studied in detail, it has been possible to show that this agent exerts its effect by binding directly to the zinc of the enzyme protein, forming a 1:1 complex in situ. The studies were reported at the meetings of the Federation of Societies of Biology (Fed Proc 16, No. 11, 1957) and details currently are in press in the J. Am. Chem. Soc.

9. Other Active Groups: Since sulphydryl groups have been stated by other investigators to be active sites in these enzymes which have they been identified as zinc metalloenzymes, studies were undertaken on -SH groups. These groups do not bind the zinc in LADH nor do they seem to bind substrate or enzyme. SH groups do appear to be involved in the maintenance of the structure of the active enzyme. The general interaction of -SH binding reagents with proteins has also been studied. A preliminary report has been rendered (Fed Proc 16, No. 10, 1957). Three papers on these findings are in preparation.

10. Cadmium Protein: We have succeeded in isolating a cadmium protein from equine kidney cortex, it contains 2% of the metal/green dry weight of protein. This is the first instance of the isolation of a natural product retaining cadmium, an element which has not been thought to have biological significance. Studies are currently underway to ascertain the specific nature and
function of this unusual metalloprotein. A preliminary report has been published in the J Am. Chem. Soc. 79: 4813, 1957.

Electron Transport in Mitochondria: An electron transport system in mitochondria, reducing triphenyltetrazolium chloride (TTC) to triphenyl-
formazan, was found to be activated by ATP and further by magnesium, manganese
and nickel ions. Maximum activation was observed when the metals were equimolar
to ATP. It was postulated that a metal-ATP complex is the true activator (Nature
178: 260, 1956). Further factorial studies (unpublished) have shown critical
interactions between different metals modifying this effect. A paper on this
subject is in preparation for publication.

Metal Content of Subcellular Fractions: The above investigations
revealed extensive by metal analysis of subcellular fractions. Connective tissue
and all subcellular fractions of rat liver studied (nuclei, mitochondria, micro-
somes and clear supernatant fluid) were shown to contain metals in substantial
quantities and in characteristic patterns of distribution, giving a new and
objective base line for the interpretation of experiments with these important
constituents of cells (J. Biol. Chem. 229: 811, 1957). The induction of magnesium
deficiency in rats caused significant dislocations not only of magnesium in
connective tissue and mitochondria but also of calcium and iron, changes which
could not have been discerned had liver tissue alone been analyzed. (Proc
of the First Conf. on Biophysics, Columbus, in press). These studies are
currently being extended.

Hibernation: Investigations were carried out on the biochemical,
cellular changes incident to cold adaptation and on hibernation. Freeze liver
mitochondria from animals either hibernating or exposed to cold environments
showed markedly increased succinic oxidase, succinic dehydrogenase - cytochrome
C reductase, and cytochrome oxidase activities. Since oxidative phosphorylation
was normal, or even high, the oxidative efficiency of these animals was much
increased. Rats, a non-hibernating species, showed no such changes in their
adaptation to cold. This work was the basis of an accepted thesis for the
Ph.D. degree (by H.R. Chaffee) and is being prepared for publication.

III. CLINICAL INVESTIGATION

A. Copper in Myocardial Infarction: Early studies had shown that
serum copper is markedly increased subsequent to myocardial infarction. It was
shown that this phenomenon is accompanied by an increase in paraphenylene diamine
(PPD) and benzidine oxidation, allowing the assumption that the rise of serum
copper is the result of an increase in ceruloplasmin. The copper bearing prote-
ins of brown nose which exhibits enzymatic activity toward these two sub-
strates. The increase in benzidine oxidation was greater than could be
accounted for by the increase in copper alone, suggesting the appearance of

B. Lactic and Malic Dehydrogenases in Myocardial Infarction: Our
studies of these pyridine nucleotide dependent metallo dehydrogenases (see II A)
led to their investigation in myocardial infarction. Both enzymes are significantly
increased in this condition and allow simple, accurate and rapid diagnosti-
procedures. The colorometer (see I B) was the result of these studies which
have been reported in the Proc. J. Med. 225: 449 1966. This method has
been used as a routine diagnostic procedure at this hospital and some 500 patients
have been studied to date; their records are undergoing analysis at this moment.

C. Magnesium and Acute Renal Failure. The multichannel flame spec-
rometer was adapted for the measurement of magnesium in serum. It has been
shown that this element is significantly increased in acute renal failure resulting from a variety of causes. The hypermagnesemia contributes greatly to the symptomatology of these individuals and explains clinical manifestations on the bases previously had been obscure. The elevation in serum magnesium roughly follows that of potassium and returns to normal with restoration of kidney function. These findings are in press in the New Eng. J. Med.

D. Zinc in Cirrhosis: Our studies in post-alcoholic cirrhosis led to an exploration of the possible mechanism leading to the establishment of this disease. Serum zinc concentrations were found significantly decreased (New Eng. J. Med. 281: 403, 1969). These studies have now been extended and currently indicate severe metabolic abnormalities which may be summarized as consisting of a decrease in serum zinc, proportionate to the severity of the disease, marked azoturia and marked decrease of liver zinc concentrations. In a series of clinical studies oral zinc sulfate caused a cessation of azoturia and a return of liver function as evidenced by changes in MEP retention. A chemical mechanism to explain these changes has been hypothesized. A preliminary account has been published (J. Clin. Invest. 36: 833, 1957). This material is in press in the New Eng. J. Med - November 24, 1967.

IV MATERIICA

A. The Sequential Probability Ratio Test (SPRT): The clinical studies just reported have led to the development of the sequential probability ratio test as a design for clinical experiments (New Eng. J. Med. 256: 438, 1957). This novel statistical design allows the economical and efficient plan of clinical experiments, terminating them at the very moment a given biochemical, physiological or pathological hypothesis has been validated or rejected. This procedure eliminates a large fraction of previous uncertainty, concerning both the adequacy of data.

2. Stochastic Models: Observations in chemical kinetics have to be made sequentially in time; it is commonly assumed that if all observational methods could be made without error, the observations at any given time would reflect the true state of a given reaction at this particular moment. Irregular fluctuations in the data of well controlled reactions would suggest that certainty in chemical kinetics is no greater than in physics and biology.

Stochastic models have been devised as mathematical constructs for a kinetic theory which would allow the inclusion of a probabilistic measure of the uncertainty in the interpretation of kinetic data. The models do not substitute for, but extend past, deterministic theories. The theory has been set up for unimolecular and bimolecular reactions, and equations have been solved for these systems. A general theory for multimeolecular processes has also been developed but several partial differential equations thus far resist solution. This study was the subject of a Ph.D. thesis entitled "A Stochastic Approach to Reaction Kinetics" by A.F. Bartholomew.

V. ADDITIONAL CHEMISTRY

Aside from the spectrographic methods, a variety of others have been investigated.

A. Determination of Zinc (by Means of Methyl Callosolve and Dithizone): Conventional analytical procedures for zinc call for phase separations, requiring the extraction of the element from an aqueous solution into a carbon tetrachloride or chloroform phase by means of dithizone. Such separation procedures are always time consuming, difficult and compare unfavorably with known monophase procedures.
By dissolving this reagent in methyl Cellosolve, which is miscible with water, a monophase method has been devised which, in terms of precision, accuracy and sensitivity, compares very favorably with the conventional extraction methods. (Anal. Chem. 26: 914, 1954).

B. Zinc in Urine: A new method of zinc analysis in urine has been devised by the simple expedient of extracting urine with dithizone as if it were a simple aqueous solution of inorganic ions, an assumption which has been justified by the results obtained with this method. Time-consuming and tedious ashing procedures are thus avoided with a concomitant improvement of precision as a result of this simplification. The principle of this method should be applicable to the determination of many other metals in urine. (This material is in preparation for publication).

C. Concentration Techniques: Increase of sensitivity of determination of trace elements might be expected by concentration methods. Zinc, iron, copper and cobalt have been separated from biological material by ion exchange procedures resulting in an aggregation of these elements and allowing their precise determination by emission spectrography. A preliminary report has been published (Anal. Chem. 27: 315, 1955). A detailed account of these studies is in preparation for publication.

D. Contamination and Separation: The general interest of the laboratory required a thorough appraisal of contamination hazards. The significance of metal contamination in work of the type here described can best be compared to the need for sterility in bacteriology and virology. The knowledge and viewpoints and experiences of this laboratory have recently been summarized in a chapter entitled "Separation and Concentration of Milligram Quantities of Metals and Contamination Hazards" in "Methods of Biochemical Analysis", Volume 3, 1957.
VI. EDUCATIONAL ACTIVITIES

The educational objectives of the laboratory have received increasing attention during the past year and a half. While their ultimate range cannot be foreseen, their scope has become apparent.

A. Pre- and Postdoctoral Training. Fifteen young scientists have thus far received training in the laboratory, nine are continuing in residence. The diverse educational backgrounds of these gentlemen testifies to the achievement of the stated aims. Opportunities of contact for scholars educated in different though related disciplines: physics, biophysics, chemistry, biochemistry, mathematics, and medicine. There is little doubt that this very diversity of backgrounds has resulted in increasingly profitable exchange of ideas. The changing intellectual viewpoints and mode of procedure of all concerned can be traced readily to such mutual influences. The adaptation of these individuals to each others' different intellectual viewpoints has been one of the most stimulating features of the laboratory's activity, an aspect which has been encouraged wherever possible. It cannot be stated with certainty to what degree this elusive characteristic of the laboratory's operation contributed to achievement, but all participants are convinced that it plays a major and most significant role.

Formal and informal course work was designed to remove barriers of communication and thought. In particular, a brief course in human biology, which constitutes a deviation from tradition, should be mentioned. On request, a member of the staff, trained primarily in medicine, undertook to teach some aspects of this subject to those members of the laboratory whose primary training was in the natural sciences. The course was voluntary and was taught on an informal basis. It was received enthusiastically and achieved the desired objective: improved
effectiveness of the staff's interaction and integration with the existent environment.

The laboratory is in a somewhat anomalous educational position. It harbors individuals aiming primarily at further education and training in the biological sciences, but in an environment which is preoccupied with the practice and teaching of medicine. Thus no formal, concrete program for the recruitment of graduate students has existed.

It was possible, nevertheless, to make arrangements with the Harvard School of Public Health, the Biology Department of Harvard University, the Department of Biology of the Massachusetts Institute of Technology and the University of London to allow five candidates to undertake their thesis work in the Biophysics Research Laboratory. Three of these have now received their doctorate degrees in mathematical biology, biology and biophysics, respectively. The two remaining candidates have just begun their work.

Five of the research fellows hail from overseas: two from Japan; one from Switzerland; one from Great Britain; one from Paraguay.

B. Medical School Teaching and Hospital Activities: The staff of the laboratory has had increasingly profitable, frequent and pleasant contact with the physicians and other research laboratories of the hospital. The evolution of these relationships has been left to chance and interest in the belief that integration will be achieved most effectively without pressure or editorial direction.

In 1964 an experimental teaching program was established to investigate the feasibility of integrating biochemical approaches to internal medicine within conventional medical teaching but without a change in curriculum. Two groups of medical students have now been processed through the courses in second and third
year medicine given by the laboratory staff, and a third group is due to arrive in November. The enthusiastic reception of the course by the students may be an indication of the outcome of this program which cannot be evaluated finally until further data have been accumulated. Several of these students have asked for and received permission for elective work in the laboratory.

Weekly teaching "rounds" for the house staff and fourth year students have been instituted. These "rounds" again stress the relationship of dynamic and physical biochemistry to the basis of medical thinking and practice and attempt to delineate these facets of the sciences which may now serve as a basis for generalization and application.

Doctors Valles, Hoch and Wacker have been appointed Tutors in Medical Science for the freshman medical school class. Dr. Price has been appointed a Tutor in Biochemistry in the tutorial program of Harvard College. Dr. Thiers has assumed the direction of the Chemistry Laboratories of the Peter Bent Brigham Hospital. Dr. Bartholomay, who has been appointed Assistant Professor of Mathematical Biology in the Harvard School of Public Health, informally functions as adviser in mathematics to the Department of Medicine of the Peter Bent Brigham Hospital.

For the fourth year the laboratory has been requested to participate in the teaching of biophysics at Massachusetts Institute of Technology. Doctors Valles and Thiers take part in the formal lecture course. Laboratory sessions are given at the Peter Bent Brigham Hospital. Mr. Donald Ploke, a graduate student in biophysics at the Massachusetts Institute of Technology, has undertaken his doctorate thesis in the laboratory under this program.

Mr. Thomas L. Combe, a graduate of the University of London, has registered there as a candidate for the Ph.D. under that university's overseas program and has been granted permission to complete his thesis in the Biophysics Research Laboratory.
The grant from the Armed Services allowed the establishment of the laboratory and the accomplishment of the objectives detailed above. From the viewpoint of the laboratory the collaboration with the Armed Services was most successful. The administrative quarters could not possibly have been more cooperative or helpful. The administrative demands were kept at an absolute minimum, and the atmosphere created was as favorable to the pursuit of knowledge as any scientist could hope for. The entire staff is deeply appreciative of the interest and consideration which it has been shown at every turn, and it is regretted sincerely by one and all that this contract, like all good things, must come to an end. The association has endeared for all concerned a most receptive attitude toward whatever the future may bring in the way of cooperation with the Armed Services.
PERSONNEL

Present Staff Members

Bert L. Valley, M.D.
Frederic L. Zach, M.D.
Warren E. Weather, M.D.
Ralph E. Thiers, Ph.D.
Anthony L. Bartholomew, B.D.
C. A. Price, Ph.D.
Felicien Pena, Ph.D.
Jerome Kraig, M.D.
Philip J. Boundy, M.D.
Edward S. Reynolds, M.D.
Thomas L. Connors, M.D.
Hans Dijkman, B.S.
Kazuo Schiffman, M.D.

Past Staff Members and Their Present Professional Activities

Stanley J. Lashof, M.D., Ph.D. - Resident, Peter Bent Brigham Hospital
Marvin Eisenstadt, Ph.D. - Spectroscopy Division, National Bureau of Standards, Washington, D.C.
Robert Chaffee, Ph.D. - Research work - U.S. Army Air Force Base, Randolph Field

H. Richard Tyler, M.D. - Neurologist, Peter Bent Brigham Hospital
Kazuo Schiffman, B.S. - Professor of Pathology, Institute for Tropical Research and University of Bremen, Germany


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