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NEW PERSPECTIVES IN BIOLOGY

Proceedings of a Symposium held at the Weizmann Institute of Science on the Occasion of the Inauguration of the Ullmann Institute of Life Sciences, June 10–17, 1963.

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PREFACE

A Symposium on New Perspectives in Biology was held at the Weizmann Institute of Science, Rehovoth, Israel, on June 10–17, 1963, to coincide with the inauguration of the Ullmann Institute of Life Sciences. This building, donated by Siegfried and Irma Ullmann, of New York, in memory of their parents Jacob and Fanny Ullmann, comprises research units in Biochemistry, Biological Ultrastructure, Biophysics, Cell Biology, Chemical Immunology, Genetics and Theoretical Chemical Physics. Thus, several groups interested mainly in molecular and subcellular aspects of biology are gathered under one roof. The creation of the new institute was also supported by the then Chancellor Konrad Adenauer and the Max Planck Gesellschaft in order to commemorate Fritz Haber, Richard Willstaetter, Carl Neuberg, Otto Meyerhof, Max Bergmann and Rudolf Schoenheimer, and those German Jewish scientists who were victims of the Nazi tyranny.

The ceremony of dedication of the Ullmann Institute of Life Sciences was presided over by Meyer W. Weisgal, Chairman of the Executive Council of the Weizmann Institute of Science. Prime Minister David Ben Gurion's greetings on behalf of the Government of Israel were one of his last official acts before resignation. Amos de Shalit, then Scientific Director of the Weizmann Institute, and Ephraim Katchalski, on behalf of the scientists moving into the new building, spoke briefly about the meaning of the new development. Mr. Ullmann responded and Mrs. Ullmann then formally opened the new Institute by cutting the tape across the main entrance.

A group of eminent biologists, biochemists and biophysicists from Europe and the United States joined their colleagues in Israel during the Symposium week to discuss the present state and future prospects of many fundamental problems in biology. Twenty-three papers were presented at the meeting. The full text of 21 of these is contained in this volume. Besides the lecturers, the list of numerous participants at the Symposium included the following distinguished guests from abroad: A. Bendich, Sloan-Kettering Institute, New York; L. Bugnard, Institut National d'Hygiene, Paris; W. V. Consolazio, National Science Foundation, Washington, D.C.; C. P. Huttrer, U.S. National Institutes of Health; I. I. Rabi, Columbia University, New York; D. Rittenberg, Columbia University, New York; H. E. Savely, U.S. Air Force Office of Scientific Research; and H. A. Scheraga, Cornell University, Ithaca.

Most of the sessions took place in Rehovoth. For one day the Symposium moved to the Hebrew University in Jerusalem. The same evening the President of the State of Israel gave a reception for Symposium members. One evening lecture was given in Tel-Aviv. During mid-week the participants left Rehovoth for a two-day trip along the coast, to Caesarea, Haifa and Acre, and then to Galilee and Lake Kinnereth, with an overnight stay at a kibbutz.

The lectures given at the Symposium and published in this volume cover wide areas of research in biological and biochemical sciences. These include various aspects of protein

and nucleic acid chemistry; organization and function of enzyme systems and subcellular units; the problem of differentiation on cellular, subcellular and molecular levels; and new chemical approaches to antibodies and to immunology. While the range of these topics is wide, they contain one central, unifying concept: the effort to understand biological phenomena on a molecular level. Appreciable developments have occurred in recent years in this area, and I venture to hope that the present volume serves as a graphic illustration of the accuracy of this assertion.

I should like to express most sincere thanks to all contributors, and to my colleagues on the Organizing Committee of the Symposium.

Rehovoth, March 1964 Michael Sela

ADDRESS BY PROFESSOR EPHRAIM KATCHALSKI AT THE INAUGURATION OF THE ULLMANN INSTITUTE OF LIFE SCIENCES

Mr. Chairman, Mr. Prime Minister, Ministers of the Government, Mrs. Weizmann, Your Excellency the American Ambassador, Mr. and Mrs. Ullman and family, Distinguished Guests, Ladies and Gentlemen:

We are assembled today for the inauguration on our campus of the Ullmann Institute of Life Sciences, and I would like first of all to express the gratitude of my colleagues and myself to Mr. and Mrs. Siegfried Ullmann for their magnificent contribution to the facilities of research in molecular biology at the Weizmann Institute of Science.

Within the spacious laboratories of this edifice, furnished with the finest instruments and equipment, our scientific community will coordinate its investigations into the structure, properties and functions of the basic matter of life. We shall be able to tackle in these new surroundings some of the inscrutable problems that challenge modern science in the chemical and physical aspects of living processes; in genetics, immunology, biological differentiation, and in the microcosmic world of the bacteria and viruses.

Just as the physicist and chemist try to understand the inanimate world by studying the properties of molecules and atoms of which it consists, so the biologist seeks to explain the structure of living matter by studying the molecules which compose it. Molecular biology thus serves as a natural bridge between chemistry and physics on the one hand, and biology on the other. For that reason it may be regarded as the pinnacle of the natural sciences, and that because there is nothing more challenging than the study of life itself in the conspectus of contemporary science.

By creating living matter Nature achieved the unique and incomparable in the whole of the cosmos. Nothing grips the interest of man more than an intimate knowledge of his own being – or, to use a colloquial expression, to know what makes him tick.

Many of the scientists coming into the Ullmann Institute of Life Sciences will be concerned with molecular biology, and it is our earnest hope that they will eventually offer a modest contribution to basic knowledge in the new field.

Dr. Weizmann's field of research interest was in synthetic organic chemistry at the time when he opened the Daniel Sieff Institute nearly 30 years ago. Later the programme was extended to the mechanisms of bacterial fermentations, and in particular of butanol-acetone fermentation which he developed in England during the first world war.

As an organic chemist, Weizmann became attracted to the micro-organisms with whose fermentations he concerned himself, and he began increasingly to study the complicated and versatile metabolic processes of bacterial cells. When the Weizmann Institute of Science was planned, he favoured the establishment initially of research units in the exact

sciences, and next of those branches of biological sciences which claim support from mathematics, physics and chemistry.

In 1949, therefore, the Sieff Institute was supplemented by the departments of Applied Mathematics, X-Ray Crystallography, Isotope Research and Polymer Research, and the Infra-red Spectroscopy Section. Electronics and Nuclear Physics were added in the two years after Dr. Weizmann's death. Biophysics and Experimental Biology came into being around the same time, as the first biology units, and since then sections of biochemistry, virology, plant genetics and immunology have been added.

Now that the Weizmann Institute is able to extend its activities with the opening of the Ullmann Institute, these expanding units in the Life Sciences will enjoy the benefit of the knowledge and experience of the original departments. I am confident that the day-by-day contact and cooperation of scientists, both young and senior, who are to work in the various disciplines will stimulate not only fresh approaches to the problems of living matter, but will also create new and important fields of interest.

We are off to a good start in this direction in the Symposium on "New Perspectives in Biology" which begins immediately after this ceremony. Some of the foremost world scholars in molecular biology have come to Rehovoth to share our joy on this occasion. I have little doubt that this Symposium will be a source of inspiration to scientists in Israel and will focus attention both on past achievements and future effort, and thereby enable us to come to grips with the many and abstruse problems awaiting solution.

Some impressive achievements in the theoretical aspects of molecular biology in the past few years are to be reviewed at the Symposium. I would mention among them the elucidation of the chemical and physical structure of several proteins; the elucidation of the nature and structure of the nucleic acids; the deciphering of the processes of the cell in conserving and transferring genetic information – or, in other words, the "cracking" of the genetic code; the insight into the mechanisms controlling cellular metabolism; and the work done in elucidating the conservation of solar energy in the processes of photosynthesis, and the utilization of this energy in various biological processes.

We may venture the hope that these theoretical achievements will in due course be transposed into the realm of practical benefits to mankind. Indeed, there is every reason to think that basic research will pave the way to development of new strains of plants and animals, the eventual cure of various hereditary and physiological diseases, and the control – if not the eradication – of viral infections and malignant diseases.

Progress in molecular biology hitherto may be the prelude to the conversion of many branches of biology from inductive to deductive disciplines. Biology as a whole is at a similar stage of development as atomic physics 30 years ago, in that it is developing new general hypotheses as to the mechanism of action of genes, the biosynthesis of proteins and the biological code, which are being tested by experimentation. Without doubt these hypotheses, like those of the atomic physicists, will lead biologists to new laws, new observations and new basic discoveries that may eventually transform biology into a stimulating branch of the exact sciences.

We are happy indeed to welcome into our midst today not only the outstanding men in the various branches of the Life Sciences who have come to us from all parts of the world, but also those who initiated and developed many branches of modern biology. Among them are our teachers and friends who have guided our first faltering footsteps. I know that they will continue to support us in our aim and desire to develop in Israel a new

research centre of basic inquiry and knowledge in the natural sciences. Their presence here furnishes additional proof, if proof were needed, of the international character of science and of the sincere desire of the scientist to understand the secrets of creation and to harness the natural forces for the benefit of men qua men, liberating him from disease, hunger, poverty and suffering.

I pray that in the Ullmann Institute which we are dedicating today we may be privileged to make our own modest contribution to the advancement of the Life Sciences, to learn to live and work in a free academic atmosphere, and to identify ourselves closely with the spiritual values of our renascent people and country.

No finer dispensation can guide us than Dr. Chaim Weizmann's statement at the opening of the first Constituent Assembly of the State of Israel, the first Knesseth of Israel, which was held in Jerusalem on the 14th of February 1949. In the course of his inspiring address Dr. Weizmann said, and I quote:

"First let us strive to strengthen our constructive resources of science and research which are the basis of human achievement. All the scientific capacity which we have displayed in every country of the world must now be mobilised to help build our motherland. Yet, for all the decisive importance of science, it is not by science alone that we shall win through. Let us build a new bridge between science and the spirit of man. 'Where there is no vision the people perish'. We have seen what scientific progress leads to when it is not inspired by moral vision – the atomic bomb threatening to destroy the entire planet.

"All my life I have laboured to make science and research the basis of national endeavour, but I have always known full well that there are values higher than science. The only values that offer healing for the ills of humanity are the supreme values of justice and righteousness, peace and love. 'Zion will be redeemed with judgment and her converts with righteousness'."

CONTENTS*

* Papers by Dr. JAQUES MONOD, *The Role of Allosteric Effects in the Control of Cellular Metabolism*, and by Dr. PAUL DOTY, *Hybrid Molecule Formation in Nucleic Acids*, read at the Symposium, are not represented in this volume.

RECENT ADVANCES IN PROTEIN RESEARCH

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It is a great pleasure and privilege to be here in Israel for the first time. I would like to say, as Professor Monod did yesterday evening, how deeply I was moved and impressed by the dedication ceremonies of the Ullman Institute yesterday. These had a special quality that one seldom feels on such occasions. The sense of dedication pervaded the whole event. I am also particularly happy to be here in Israel at last because of my long association with Ephraim Katchalski. He first came and worked in our laboratory eleven or twelve years ago, and did a most brilliant piece of research with most indefatigable energy and skill. Ever since I have followed with admiration the great work that he, and the remarkable group associated with him, have been doing.

In discussing proteins today I shall try to offer some general perspectives. I shall say a little about some of the work in our own laboratory, but for the most part I want to consider in quite general terms the present state of our knowledge of protein structure and its biological implications. Last year I attempted a brief backward look at the long and arduous development of our understanding of proteins as molecules, from the time of Liebig[1] on. When I began part time research on proteins as a medical student in 1926, it was a major triumph that a few great protein chemists – notably Sörensen, Svedberg and Adair – had determined the molecular weights of a few proteins within perhaps five or ten percent. Following that came the pioneer X-ray work of Astbury in the 1930's which began to lay the foundations of our knowledge of the structure of fibrous proteins. It was only in the late 1930's that we began to have some reasonable idea of the shapes of protein molecules even in a semi-quantitative way. Indeed, von Muralt and I, when I was first starting work in E. J. Cohn's laboratory, had studied myosin, or what is now called actomyosin, and found by its flow-birefringence that it must be an extraordinarily asymmetrical molecule. But apart from a few special cases like this, it was not until about 1940 that semi-quantitative ideas began to emerge for estimating the shapes of protein molecules. Even then these estimates depended chiefly on measurements of hydrodynamic properties in solution, viscosity, sedimentation, diffusion, and other related irreversible processes. These conclusions were complicated and rather bedevilled by the ambiguities that were inherent in discriminating between the effects of solvation on the protein and the effects of asymmetry. Some of the conclusions drawn then had to be substantially revised and refined later.

Out of this long period of development, extending over about a century and a quarter, has come the great flowering of protein chemistry in the last 15 years. The new era began with the extraordinary work of Sanger in working out the structure of the insulin molecule an achievement that paved the way for the flood of developments that have come since in the determination of peptide chain sequences in proteins.

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The discovery of the alpha helix by Pauling and Corey represented another great turning point in protein chemistry, and the work of Kendrew and Perutz on myoglobin and hemoglobin has transformed our picture of protein molecules and revealed their structure with a fineness of detail that would have seemed inconceivable to any of us even ten or fifteen years ago. I am still amazed at these achievements. Since Dr. Kendrew will follow me, I need say no more on the work that they have been doing.

I want to take up some general aspects of the factors that determine the structure of proteins. Later in this symposium Dr. Anfinsen will discuss the factors that determine the three-dimensional conformation of proteins, once the amino acid sequence is specified. I will not attempt to anticipate him. It is, of course, becoming quite clear, especially from his work and that of his associates, that a peptide chain does tend to fold into the correct three-dimensional pattern, if dissolved in a suitable medium, usually an aqueous solution, not far from pH 7, with a little salt present. Once the peptide chain has been synthesized on the ribosome and liberated from it, it will then, apparently spontaneously, find its way into the correct three-dimensional folded structure.

HYDROPHOBIC BONDING AND PROTEIN STRUCTURE

Recently we have begun to appreciate the importance of the non-polar side chains for protein structure. Since the work of the early discoverers of the amino acids, we have known that these side chains were present, and indeed for most proteins in very large numbers; but it was not easy to see what they were doing. The physical chemists who were interested in proteins, and this includes myself, focussed their attention on the acidic and basic side chains, and on the other polar groups that could serve as donors or acceptors in hydrogen bonding. The interactions of these groups were clearly of importance, whereas it was comparatively difficult to understand the role of the hydrocarbon side chains of alanine, valine, leucine, isoleucine, and phenylalanine. Much is indeed still obscure; we do not yet understand why specific hydrocarbon side chains should be found in specific places in the peptide chain sequences of proteins, although the steric requirements for good fitting must obviously be important. What has become clear in general, however, is that these non-polar residues tend to cluster close together in the interior of globular protein molecules, when these assume their stable native configuration. This feature of protein structure, long suspected, has been made manifest in full detail for myoglobin by Kendrew's magnificent work[2]. The interior of the molecule is predominantly a non-polar medium, with contacts between non-polar side chains enormously predominating over those involving the polar groups, which are oriented mostly toward the outside. In some respects, of course, myoglobin may not be at all a typical protein. More than three-quarters of the peptide chain of myoglobin is in the form of α-helix; most proteins are probably less helical than this, and evidence from optical rotation suggests that some native proteins, such as pepsin, γ-globulin, and carbonic anhydrase, contain scarcely any helix at all. It seems highly probable, however, that the tendency for the non-polar side chains to cluster in the interior of the molecule, and for the polar side chains to face outward, will be found to be a nearly universal feature of the structure of globular proteins.

Although mankind has known for a long time that oil and water do not mix, it is only quite recently that we have begun to understand the nature of the interactions between

hydrocarbon chains and water. I remember being very puzzled, some 30 years ago, by some of the strange properties of molecules containing hydrocarbon side chains in aqueous solution. In those days, in E. J. Cohn's laboratory, our prime concern was with the structure of amino acids, peptides, and proteins as ions and dipolar ions. We were concerned with the charged groups, their influence on the dipole moment of the dipolar ions, and their interactions with the surrounding water molecules to produce electrostriction with marked reduction of the volume and heat capacity of the system. However, in looking over the rather scanty data on heat capacity of aqueous solutions of organic molecules, I noticed a striking effect of hydrocarbon side chains in water in *increasing* the heat capacity[3]. In a homologous series of pure organic liquids–alcohols, ketones, fatty acids–each added CH_2 group increased the heat capacity by about 5 or 6 $cal \cdot deg^{-1} \cdot mole^{-1}$; but when the same substances were dissolved in water the effect of an added CH_2 group was 3 or 4 times as great, in increasing the apparent molal heat capacity. The work of Frank Gucker and his associates on the heat capacity of glycine and alanine solutions showed that the extra methylene group in alanine had the same effect. Dr. Cohn and I[4], in 1943, summed up the situation as we saw it then; but the reason for these high heat capacities, produced by hydrocarbon groups immersed in water, was still very obscure to us, and apparently to everyone else as well. It was indeed clear that the introduction of hydrocarbon groups into water was accompanied by a large evolution of heat, and a decrease of entropy – Dr. Scatchard and I[5], in another chapter of the same monograph, summed up much of the evidence available at the time – so that the low solubility of hydrocarbons in water was essentially an entropy effect.

The situation was greatly clarified by the work of Frank and Evans[6], who pointed out that all the evidence favored the view that the introduction of hydrocarbon groupings into water actually promoted hydrogen bonding of the water molecules in the immediate neighborhood. In picturesque language, it led to "iceberg formation." These "icebergs" should not be pictured as having the structure of ordinary ice; they probably differ from that. The essential fact is that the water structure, close to the hydrocarbon group, becomes more ordered. More hydrogen bonds form, as they form when water freezes to form ice; and, as in the freezing of ice, heat is evolved and entropy decreases. The heat capacity also increases, as more energy is required to "melt" the more ordered structure. These ideas were further developed, and their implications for protein structure and denaturation pointed out, by Kauzmann[7]; and from a somewhat different point of view by Klotz[8]. Recently Nemethy and Scheraga[9] have worked out an extremely detailed model for the structure of liquid water and its interactions with hydrophobic groups, both for small hydrocarbon molecules and for proteins. Although their model may be open to some criticism in detail, it does appear to go a very long way toward a quantitative theory of the properties of at least some aqueous solutions.

Tanford[10] has employed a somewhat different approach to calculating the effect of hydrophobic bonding in stabilizing the compact native structure of globular proteins. The work of Cohn, McMeekin, myself, and others (summarized in ref. 4) had shown the systematic influence of added methylene groups or aromatic rings in increasing the relative solubility of molecules in organic solvents as compared to water. If it is assumed that the side chain groups of unfolded peptide chains interact with water in the same way that they do in simpler molecules, then it is clear that the chief stabilizing force that drives the peptide chain to assume the compact globular conformation is the tendency of

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the non-polar side chains to get away from the water and cluster together in the interior of the compact molecule. To achieve this, the molecule must sacrifice the large amount of configurational entropy that is available to it in the relatively flexible unfolded form. To make the globular form stable, it is also important that, if some polar side chains are more or less buried in the interior of the compact molecule, they should form hydrogen bonds with other appropriate donor or acceptor groups. Otherwise the unsatisfied affinities of such groups, in this essentially non-polar interior medium, will make the globular structure unstable. Kendrew's work on myoglobin[2] shows that for this molecule all these requirements are well fulfilled.

Recently Liquori, in work still unpublished, has calculated the internal energy of the peptide chain of poly-L-alanine, as a function of the angles of orientation of the groups on the two sides of the α-carbon atoms in the alanine residues. Since the CO · NH group can be regarded as an essentially planar rigid structure, these angles of orientation around the α-carbon essentially define the conformation of the peptide chain. The internal energy was computed in terms of Van der Waals' interactions between "non-bonded atoms" in the chain, assuming semiempirical potential functions. The method of approach is that previously used by DeSantis, Giglio, Liquori and Ripamonti[11] in calculating the conformational potential energy of chains of polyethylene and various other synthetic polymers. The surprising result emerges that the deepest minimum in the potential energy for poly-L-alanine corresponds to a conformation which is practically that of the right-handed α-helix, with a shallower minimum corresponding to a left-handed helix. In this calculation no account was taken of hydrogen bonding at all; when this is taken into account, there is of course an important additional energy contribution tending to stabilize the helix. These calculations indicate the importance of Van der Waals' interactions as one of the factors to be taken into account in calculating the relative probability of helical and non-helical conformations in peptides and proteins. Liquori's calculations, of course, relate to the *internal* interactions between the methyl and other groups of the alanine residues. They do not apply to the interactions between the alanine residues and surrounding water molecules, if the peptide chain is immersed in water.*

THREE-DIMENSIONAL CONFORMATION OF PROTEINS IN SOLUTION

One of the great future problems will be to determine the three-dimensional structure of protein molecules in solution, and to see how far it corresponds to what can be learned from the X-ray measurements in the crystal. This problem is being continually debated among the protein chemists, and I would make a few remarks about it.

In the first place, the evidence concerning structure in solutions is necessarily at present very scanty and incomplete, compared to the marvellous detail obtained from the X-ray studies in the crystal. Urnes, Imahori and Doty[12] and Beychok and Blout[13] have studied the optical rotatory dispersion of myoglobin in solution. They infer a helical content of 75–80% for the myoglobin molecule, in good agreement with what Kendrew[2] finds for crystalline myoglobin. This is encouraging enough as far as it goes; but, in terms of

* The interested reader should note an important recent paper on the same general theme by Ramachandran, Ramakrishnan and Sasisekharan, in *Aspects of Protein Structure* (G. N. Ramachandran, Ed.), Academic Press, London–New York, 1963, p. 121.

structural detail, it does not take us very far, particularly as the theory of optical rotatory dispersion must still be applied very cautiously to structures as complex as globular proteins.

In considering the evidence from the X-ray studies on crystals, we must remember that the protein molecules in a crystal are bathed in a considerable amount of mother liquor. Usually at least as much water as protein is present in the system. The crystal indeed closely resembles a highly organized, well-ordered, concentrated solution. It is true of course that there are direct protein-protein contacts in the crystal. These must be broken when the crystal passes into solution, with some resulting influence on the protein conformation. One may suspect that in many cases, the dissolution of the crystal structure may produce only minor changes in the indiyidual protein molecules.

Many proteins, of course, undergo reversible transitions of conformation in solution. Serum albumin, indeed, undergoes two really distinct transitions in acid solution, around pH 4 and below [14,15]. These two transitions can be distinguished, since one of them is particularly marked by changes in electrophoretic mobility and the other by alterations in the ultra-violet absorption spectra. Both stages involve changes in viscosity, optical rotation and other properties, and the whole process is quite reversible.

Likewise β-lactoglobulin undergoes a reversible transition at about pH 7.5 (see for instance Tanford and Taggart[16]). There are changes in the sedimentation constant, in optical rotatory dispersion, and also in the titration curve, indicating that certain groups which were previously unreactive had become reactive. Clearly, if the protein on one side of such a transition were to correspond with the protein as it exists in the crystal, the protein on the other side of the transition is obviously different from that in the crystal*.

Doscher and Richards[17] have shown recently that the ribonuclease molecules in the crystal of ribonuclease-S function catalytically when substrate enters the crystal, and with a degree of activity quite comparable to that of the same enzyme molecules in solution. This result would indicate that the active site of the enzyme functions in essentially the same manner, and presumably in virtually the same conformation, in the crystal as in solution. Obviously there may still be some changes of conformation in less vital regions of the ribonuclease molecule, when the molecules that are separate in solution come together to form a crystal. We may suspect, but as yet we certainly cannot prove, that these changes are relatively minor.

EXPLORATION OF PROTEIN CONFORMATION WITH CROSS-LINKING AGENTS

To find out whether two groups in a protein molecule are close together, one may attempt to join them by a suitable cross-linking reagent, a double headed molecule that contains a suitable reactive group at each end, with the general formula X · R · Y, where X and Y are the reactive groups and R the intervening portion of the molecule. By varying the size of R, and thus the distance between X and Y, one may tailor the dimensions of the cross-

* In this discussion I had tacitly assumed that the same kind of crystal would form in equilibrium with the protein solution on either side of the transition point. This assumption is of course too restrictive. In the following paper Dr. Kendrew points out that the workers at the Royal Institution have prepared two different forms of β-lactoglobulin, one on each side of the transition point near pH 7.5. When this is possible, our range of knowledge is obviously greatly increased. Inevitably, however, the question still remains as to the relation between each of these crystalline forms and the state of the molecule in solution, in equilibrium with the crystal, in either form.

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linking agent to join together groups on the protein molecule that are separated by a specified distance. Such agents have been developed for use in protein chemistry particularly by Zahn (see for instance Zahn and Meienhofer[18]). One of them, in which R is a diphenyl sulfone with 2 nitro groups, and X and Y are both fluorine atoms in the p,p′ positions of the diphenyl sulfone, has been used by Wold[19] to cross-link amino groups in serum albumin. Wold's work clearly established the fact that such cross-links are formed about 10 per albumin molecule under the conditions he employed. He has also shown that the cross-linked molecule remains relatively compact under conditions tending to cause expansion of the albumin molecule that lacks cross-links–for instance, exposure to concentrated urea solutions. However, to find out just which groups are cross-linked will require long and arduous studies, involving complete knowledge of the amino acid sequence, and partial or complete hydrolysis of the peptide chain by procedures that leave the newly established cross-links intact. Moreover, even when such evidence is obtained, one must interpret it with caution. Cross-linking of two side chains in a protein molecule does not prove that they are necessarily at a specified distance apart in the most probable conformation of the native protein. The protein may be undergoing transitions from one conformation to another, and the particular conformation that makes cross-linking possible may actually be a somewhat unusual one for the native protein to assume. Nevertheless the potential power of this method for studying three-dimensional conformation of proteins in solution seems very great.

TRYPTOPHYL, TYROSYL, AND HISTIDYL SIDE CHAINS IN PROTEINS: INSIDE OR OUTSIDE?

As we have seen, the evidence is very strong that non-polar groups are generally on the inside of native globular proteins, and the polar groups on the outside. Some side chains, however, cannot be simply classified as polar or non-polar; they partake of some of the characteristics of both. This is notably true of tyrosine and tryptophan. The two tryptophan residues of myoglobin[2] are largely buried in non-polar surroundings, but the polar nitrogen of the indole ring is close to the surface. The benzene ring of tyrosine would tend to make it associate with other non-polar groups, whereas the hydroxyl group is polar and acidic, and can act both as a donor and an acceptor in hydrogen bonding. It is well known that some tyrosyl groups in protein are not available for titration with bases until the protein has undergone a conformation change from its native state–sometimes apparently a rather drastic conformation change. The best known case of this sort is probably ribonuclease, with three tyrosyl groups that titrate normally and reversibly, and three that do not react at all until the protein has undergone alkaline denaturation[20,21]. Hermans[22] has found two tyrosyl residues that are reversibly titratable in native myoglobin and one that is unreactive until the molecule undergoes unfolding at very high pH. The latter presumably corresponds to the tyrosyl residue identified by Kendrew[2] as being hydrogen bonded to the C=O group of a peptide linkage many residues away in the peptide chain, and well buried in the interior of the molecule. The other two tyrosyl groups, from Kendrew's work, are near the outside of the molecule and presumably quite accessible to the solvent.

It is now becoming clear that a portion of the histidyl groups in some proteins, perhaps in many, must be masked and unavailable for titration in the native protein. The most

thoroughly studied case is that of hemoglobin, for which the searching investigations of Steinhardt and his collaborators have established clearly the existence of a large number of histidyl residues that are unreactive in the native protein and become titratable only on acid denaturation (for a recent report of some of this work, see Steinhardt, Ona and Beychok[23]). Breslow and Gurd[24] have made similar observations on myoglobin; approximately half of its 12 histidine groups are unreactive in the native protein. Here, of course, it is possible to correlate the titration data with Kendrew's evidence concerning the three-dimensional structure, but the correlations are not always simple. The two histidine residues adjoining the heme, one firmly bonded, the other closely adjacent on the other side, are rather naturally expected to be unreactive; but the placement of several of the others is such that it is not immediately obvious whether they would be reactive or not. Gurd and his collaborators (see Banaszak *et al.*[25]) have recently been exploring this problem by studying the reactivity of the histidine residues with bromoacetate, and determining the location of the reactive and unreactive residues in the peptide chain sequence. Since this sequence can also be directly related to the three-dimensional structure, this work should lead to important results, but the results are not yet sufficiently complete to permit any simple summary.

Recent work in our own laboratory on carbonic anhydrases from human erythrocytes has shown that at least one of these enzymes, derived from the same cells that contain

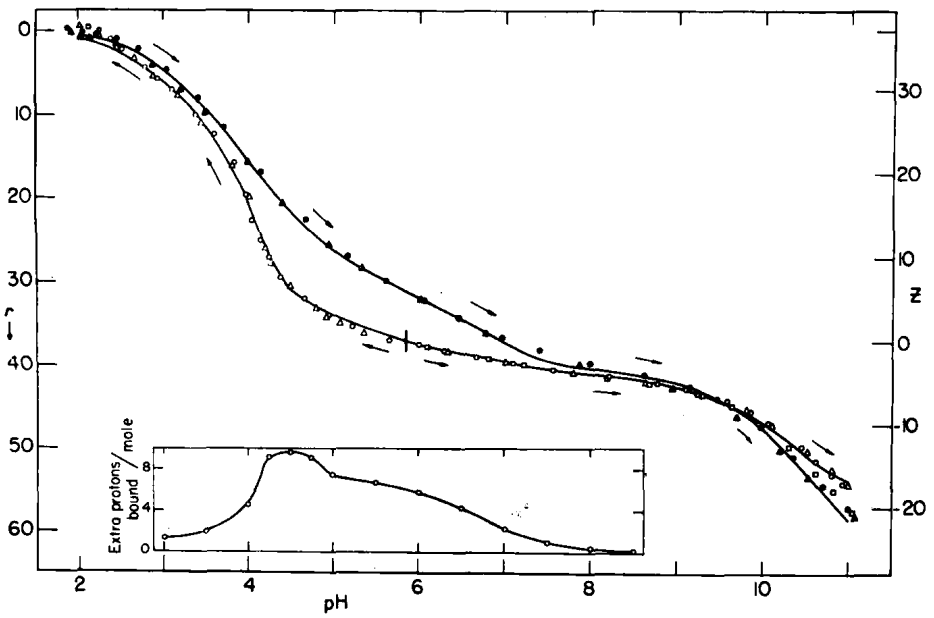

Fig 1. Titration curves of human carbonic anhydrase B, at ionic strength 0.15 and 25°. The abscissa denotes protons removed per molecule, taking the pH of maximum acid binding as the point of reference. Open symbols indicate the initial titration, starting at the isoionic point of the native protein (pH 5.85). Solid symbols indicate back titration with KOH, starting at pH 1.6. The different symbols indicate different protein preparations. Solid lines denote theoretical curves calculated with parameters defined in the paper by Riddiford[28] from which this figure is taken. The inset is the difference curve between the initial and the reverse titration. The left hand ordinate denotes the mean number of protons removed from the enzyme molecules, taking the maximally protonated enzyme as the reference point. The right hand ordinate gives the mean net charge ($\bar{Z}$) on the enzyme, assuming that no ions other than protons are bound.

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hemoglobin, resembles hemoglobin and myoglobin in that most of its histidyl residues, about 7 out of the total of 11 (taking the molecular weight as 30 000) are masked in the native protein. First, a word as to these enzymes. We have separated two distinct enzymes, both with carbonic anhydrase activity, by chromatography on hydroxylapatite[26]. One is present in larger amount, but with relatively low specific activity; the other in smaller amount, but with much higher specific activity. Both contain one atom of zinc per molecule. Nyman[27], by an electrophoretic procedure, separated what appear to be the same two enzymes, plus a third minor component. Most of our work so far has been done on the component present in largest amount, now denoted as carbonic anhydrase B[26]. Dr. Lynn Riddiford has studied the titration curve of this enzyme in detail, and some of her results[28] are shown in Fig. 1. When one starts with the native protein at its isoionic point (pH 5.85) and adds acid, there is a steep rise in the titration curve near pH 4, indicating the sudden release of groups that were unavailable for titration in the native protein. On back titration with alkali from pH 2, the reverse curve (upper curve in Fig. 1) follows quite a different course. The insert in the lower part of Fig. 1 shows the difference in the number of groups titrated at a given pH, for the forward and the reverse titrations. The maximum in this difference curve is 8–9 groups per molecule. From a more detailed analysis of the titration, Riddiford concludes that this difference is due primarily to the release of 7 histidyl groups that were unavailable for titration in the native molecule, but are liberated during the sharp transition that occurs near pH 4.

Figure 2 shows the spectral change that accompanies this transition[26]. The data

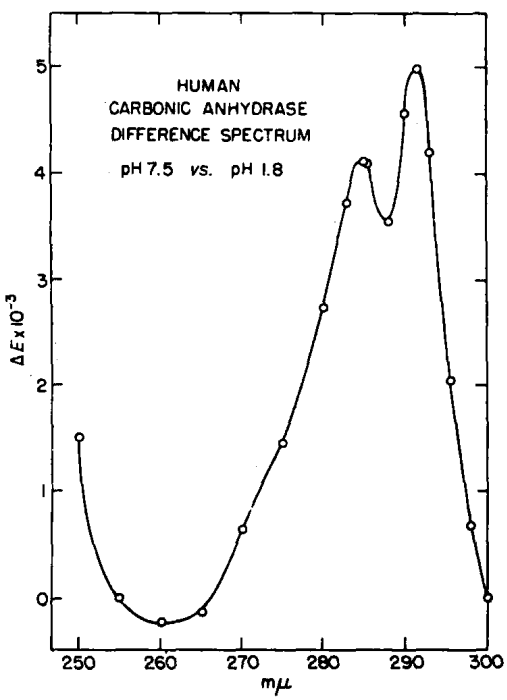

Fig. 2. Acid difference spectrum of human carbonic anhydrase B. Sample solution: native protein at pH 7.5. Reference solution: acid denatured protein at pH 1.8. From work of S.A.S. Ghazanfar (see Rickli, Ghazanfar, Gibbons and Edsall[26]).

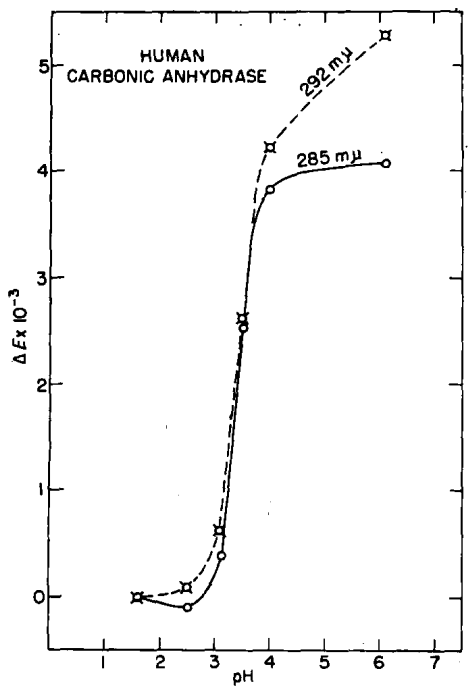

Fig. 3. Carbonic anhydrase B: dependence of acid difference spectrum on pH. From ref. 26.

represent a difference spectrum between the native protein at pH 7.5 and the acid-denatured protein at pH 1.8. There are two peaks, one at 285 mμ and a higher one at 291–292 mμ. The protein contains 6 tryptophan and 7–8 tyrosine residues per molecule. The difference spectrum is characteristic of proteins containing substantial amounts of tryptophan. As is usual in such cases, the native protein absorbs ultraviolet light, in this wavelength range, more strongly than the acid-denatured protein, since the absorption bands undergo a shift to shorter wavelengths when the aromatic chromophores become more exposed to the solvent on partial unfolding of the molecule[29].

Figure 3 shows the acid difference spectrum of carbonic anhydrase B as a function of pH. The transition that occurs is extremely sharp, far steeper than an ordinary titration curve, and the midpoint is close to pH 3.5 at ionic strength 0.15. The change is accompanied by aggregation of the protein, with marked increase in viscosity and sedimentation coefficient. The enzyme activity is lost on the acid side of this pH transition, and we have not found conditions for restoring it.

The tyrosyl groups of carbonic anhydrase B, like the histidyl groups, are of different types. There are 7 or 8 such tyrosyl groups per enzyme molecule–at the moment we cannot definitely say which, until we obtain new analytical data. Spectrophotometric titrations by Riddiford[28] at 295 mμ indicate that only two ionize instantaneously and reversibly; two others ionize slowly, at pH 11 or above, and the other three (or more probably four) ionize very slowly, except at extremely high pH (pH near 13 at 25°, near 14 at 1°). Riddiford carried out measurements at 1°, 10°, and 25°; the data at 10° are shown in Fig. 4. The dashed line (points shown by solid circles) shows the initial readings,

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