Amino Acids, Proteins and Cancer Biochemistry

Papers presented at the
JESSE P. GREENSTEIN
MEMORIAL SYMPOSIUM
Division of Biological Chemistry
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With a biographical article on Dr. Greenstein and a bibliography of his writings

Edited by JOHN T. EDSALL

Preface by SIDNEY W. FOX *and* JULIUS SCHULTZ

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Preface

AFTER THE DEATH of Jesse P. Greenstein in February 1959, the Executive Committee of the Division of Biological Chemistry of the American Chemical Society decided unanimously to schedule a memorial symposium. A full day was to be devoted exclusively to this purpose, in view of the magnitude of Dr. Greenstein's contributions to biochemistry and his close association with the Division. It was also unanimously agreed that Dr. John Edsall would be the appropriate chairman. His willingness to accept the assignment and the ready response of invited participants are testimony to the warmth of feeling for the late Dr. Greenstein. All of the contributions in this volume reflect the influence of Greenstein's work. Some of the contributors were his close associates and collaborators over many years, whereas others have worked quite independently but in their work were deeply influenced by him. We believe that this book, which includes all of the papers given at the Symposium, will make an important contribution in portraying some of the major current developments in amino acid and protein chemistry and in the biochemistry of cancer—the two great fields in which Greenstein's work was preeminent. The volume opens with an account of his life and work, written by his long-time friends and associates, Drs. Edsall and Meister. This tribute is a combined and extended version of the earlier obituary articles by Alton Meister in the *Archives of Biochemistry and Biophysics* **82,** i (1959) and by J. T. Edsall in *Science* **130,** 83 (1959). The volume is concluded with a comprehensive bibliography of Greenstein's writings.

Dr. Greenstein's contributions, both scientific and professional, were made in large part through this Division of which he was chairman in 1954–1955. His research was oriented toward problems of great biochemical and medical interest and was characterized throughout by skill in the use of the principles and methods of physical, inorganic, and organic chemistry. His close affiliation with the American Chemical Society was therefore most appropriate.

No one can measure the full impact of the contributions of an outstanding scientist. It is hoped, however, that the contents of this volume will serve to illuminate some of the many fields of research which were enriched by Greenstein's ability and insight, and that

the problems here set forth will stimulate further active research by outstanding investigators. If this volume promotes the advancement of our knowledge of amino acids and proteins and of cancer biochemistry it will serve at least in part as a fitting memorial to Jesse P. Greenstein.

SIDNEY W. FOX, Chairman,
1958-59
JULIUS SCHULTZ, Secretary

Division of Biological Chemistry,
American Chemical Society
December, 1959

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JESSE PHILIP GREENSTEIN
1902–1959

JOHN T. EDSALL and ALTON MEISTER

The Biological Laboratories, Harvard University, Cambridge, Massachusetts, and Department of Biochemistry, Tufts University School of Medicine, Boston, Massachusetts

JESSE GREENSTEIN died suddenly on February 12, 1959. At the time of his death he was actively engaged in research and in writing; he was the scientific and administrative leader of a group of distinguished scientists. He will long be remembered and honored for his contributions to science and to his fellow men. His passing brought to an untimely close one of biochemistry's most active and productive careers; he had achieved eminence in his extensive work on the chemistry of amino acids, peptides, proteins, and the biochemistry of cancer. The bibliography of his published work, which is printed at the end of this volume, will convey to the reader some idea of the extraordinary breadth and scope of his activities. Here we shall attempt a brief portrayal of his character and personality and of the major events of his career and will discuss some of the chief landmarks in his work.

He was born on June 20, 1902 in New York. His scientific interests developed early, while he attended public elementary and high schools in New York, and while he worked in the Food and Drug Laboratory of the New York City Department of Health. He received the B.S. degree, *cum laude* in chemistry, from the Polytechnic Institute of Brooklyn in 1926, and the Ph.D. degree in 1930 from Brown University, where he worked with C. A. Kraus and P. H. Mitchell on the dissociation constants of glycine and certain simple peptides containing glycine (see reference 1).* The character of this first study foreshadowed much of his later work during the following decade. In 1930-1931 he was a National Research Council

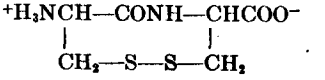
* References to the bibliography of Dr. Greenstein's work at the end of this volume are cited by number in parentheses throughout the remainder of this article.

fellow at Harvard, under Edwin J. Cohn, where he continued and extended his studies on the ionization constants of peptides (2-4). This was followed by a year's work in Dresden under Max Bergmann, who was just developing the carbobenzoxy method of peptide synthesis. Greenstein's experience in Bergmann's laboratory profoundly influenced his subsequent career. With Bergmann and Zervas he was the first to apply the new methods to the synthesis of lysylglutamic acid and lysylhistidine (5), and he developed a mastery of both organic and physical chemistry, particularly in relation to the study of peptides and proteins, which was exceptional among the biochemists of his generation.

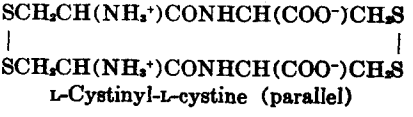
After a year in Berkeley in the laboratory of C. L. A. Schmidt, where he carried out further studies on the peptides of trivalent amino acids (6), he returned to Harvard for six years (1933-1939). During this period he was active as a tutor in Biochemical Sciences at the college and was also research associate at Harvard Medical School, in Cohn's laboratory. His zeal and devotion to both teaching and research, and his extraordinary energy and enthusiasm, were strikingly displayed then as later. As tutor in Biochemical Sciences he looked after a larger number of students than any other member of the board has ever been responsible for, and served as an inspiring and effective teacher in frequent personal conferences with students. He also served as head assistant to L. J. Henderson in the latter's course in biological chemistry; indeed, as Henderson's major interests were shifting from biochemistry toward sociology, Greenstein for several years was largely responsible for the general management of the course. At the same time he was astonishingly productive in research. He continued to synthesize new peptides and to determine their ionization constants and correlate these with their structure, and, in collaboration with J. Wyman, T. L. McMeekin, and others, he studied their dipole moments, solubility, and other physical properties (8-15, 19, 24, 26, 27). During these years he began those important studies of the peptides of cystine and cysteine (20, 21, 23, 25, 28, 33, 35) to which he frequently returned, with new points of view, and new techniques, in the course of the next twenty years (see for instance 98-101, 114, 224, 225).

In 1937 he published the first synthesis of L-cysteinyl-L-cysteine (23). This unique peptide was prepared by condensing dicarbobenzoxycystyl chloride with two molecules of cysteine ethyl ester to give dicarbobenzoxycystyldicysteinyl ethyl ester, which was cleaved

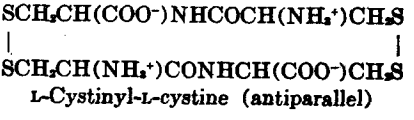
with phosphonium iodide to cysteinylcysteine ethyl ester hydroiodide. The latter compound was converted in ethanol saturated with ammonia to the crystalline diketopiperazine anhydro-L-cysteinyl-L-cysteine, which on treatment with concentrated hydrochloric acid gave the hydrochloride of L-cysteinyl-L-cysteine. Oxidation of the L-cystine. The relationship of these studies on oxidation of cysdipeptide at weakly alkaline reaction gave crystalline L-cystinylteinylcysteine to later work on proteins and naturally occurring peptides (such as insulin or oxytocin) led Greenstein to return to the problem in 1956. With Wade and Winitz (236) he investigated the oxidation of L-cysteinyl-L-cysteine at various values of pH and isolated a number of products of oxidation including the cyclic disulfide derivative of L-cysteinyl-L-cysteine, which he designated "cyclo-L-cystinyl":



The crystalline dimeric product was shown to consist mainly of parallel cystinylcystine, with the two free amino groups adjoining one disulfide linkage, and the two free carboxyl groups adjoining the other:



L-Cystinyl-L-cystine (parallel)



L-Cystinyl-L-cystine (antiparallel)

Although the formation of small amounts of the antiparallel isomer could not be unequivocally excluded, these studies clearly demonstrated preferential formation of one isomer, and represent a potentially important model system for the study of this type of cyclic structure. In one of his last papers (269) he studied the difficult problems involved in preparing unsymmetrical open-chain derivatives of cystine, and the complications introduced by disulfide interchange reactions; as a simple example of such a derivative, pure monoglycyl-L-cystine was prepared for the first time.

About 1937 he turned also to the study of protein structure and denaturation, making use of porphyrindin, which had recently been

synthesized by Richard Kuhn, for the determination of protein sulfhydryl groups, in native and denatured proteins. He discovered the powerful action of guanidinium salts as denaturing agents for proteins and made extensive use of them and of concentrated urea solutions in studying the titratable sulfhydryl groups of egg albumin, edestin, excelsin, globin, myosin, and other proteins (29, 31, 34, 36, 37).

In 1939 his career entered a new phase when he became a member of the National Cancer Institute at Bethesda. Here again his extraordinary energy and vitality displayed themselves. He threw himself into an extremely active program for the study of the biochemistry of tumors, and in particular set about the determination of the activities of a variety of enzymes—arginase, amylase, catalase, xanthine dehydrogenase, and many others—in normal and cancerous tissue. This long series of studies, which extended over many years and involved numerous collaborators, represents probably the most extensive work of its kind yet carried out on cancer tissue. From this he evolved the concept of the "biochemical uniformity of tumors," the near-uniformity of tumors being due to the loss in a normal tissue, when it becomes malignant, of those specific functional characteristics which distinguish it from other normal tissues. The tumors which so emerge bear little or no physiological resemblance to their normal tissues of origin and appear to converge to a common type of tissue. Thus, normal hepatic and gastric tissues are very distinctly different, but biochemically the hepatoma and the gastric adenocarcinoma are closely similar. These researches were summarized, together with a comprehensive and critical survey of the whole field, in Greenstein's outstanding monograph, "Biochemistry of Cancer," first published (154) in 1947 and extensively revised in a second edition (221) which appeared in 1954. The findings and general concepts set forth in this monograph are discussed by Sidney Weinhouse later in this volume, and in the other papers which follow Weinhouse's contribution.

During his first years in Bethesda he also devoted himself vigorously to the study of nucleoproteins in normal and cancer tissue. This work (48-52, 55, 56, 58, 75, 77, 83 and later papers) was begun at a time when relatively few biochemical studies on nucleic acids were in progress. He published a number of papers on the enzymatic deamination and dephosphorylation of RNA and DNA (105, 106, 121, 122, 124). He was very much aware of the potential significance of nucleic acids for the structures of viruses and chromosomes

and of their probable relationship to transforming factor. In his paper at the Cold Spring Harbor Symposium on Nucleic Acids in 1947 (143) he pointed out that "knowledge of the metabolic fate of the nucleic acids within tissues is of fundamental importance in the understanding of the phenomena presumed to be elicited by these substances." He also carried out work on the effect of radiation on DNA (144, 151) on the cation activation of deoxyribonuclease, and on the very interesting and remarkable effect of DNA in protecting ovalbumin and serum albumin against heat coagulation (117, 118, 178).

His interest in the study of protein denaturation remained unabated. With Neurath, Putnam, and Erickson he published in 1944 a review of this subject which has become one of the classics in the field (84).

In 1946 he was appointed chief of the newly created Laboratory of Biochemistry of the National Cancer Institute, a position which imposed upon him an increasing burden of administrative responsibility. His breadth of vision, his enthusiasm, and his patient good humor and understanding of many diverse types of people were of great importance in the development of the research activities of the Institute and in attracting workers to it from all over the United States and from the rest of the world.

These heavy administrative responsibilities, however, did not prevent him from continuing with an extraordinarily active program of research; indeed, the last dozen years of his career were probably the most productive of all. He made intensive studies of dehydropeptides and dehydropeptidases (see 161), of many cellular peptides, of the enzymatic desulfuration of cystine peptides, of the deamidation of glutamine and asparagine, and of other problems too numerous to mention here.

His outstanding contribution to the chemistry of the amino acids, and thereby to biochemistry and nutrition, lay perhaps in his superb researches on the preparation of optically pure L- and D-amino acids, in which he utilized the asymmetric hydrolytic or oxidative specificity of the amino acid acylases and oxidases to prepare the optical enantiomorphs in better than 99.9% purity.

Greenstein's work on the resolution of amino acids emphasized the problem of the optical purity of amino acids. In his classic review (223) in 1954 on the resolution of racemic α-amino acids, he summarized in elegant and thorough fashion the literature of the development of this area of research as well as his own important

contributions to the problem up to that time. He pointed out that polarimetric measurement was not a sufficiently sensitive test of optical purity, and emphasized the value of employing enzymes—for instance the D- and L-amino acid oxidases and decarboxylases—as test reagents to detect minute amounts of optical isomers present as impurities, and also for obtaining preparations of high optical purity by destruction of the undesired isomer (267, 269). It is evident that this work has an important bearing on the problem of optical homogeneity of synthetic peptides. The work on resolution led directly to a major series of studies on the stereochemical configuration of diasymmetric amino acids. Greenstein and his collaborators devised procedures for the preparation of the possible isomers of isoleucine (192, 229), threonine, hydroxyproline (202), β-phenylserine, γ-hydroxyglutamic acid (259), β-methylaspartic acid (270), and β-hydroxy-β-methylaspartic acid (271). The studies on the three isomeric forms of α, ϵ-diaminopimelic acid (232, 254) gave much impetus to microbiological and enzymatic studies of this amino acid. The preparation (226) of the four isomers of α-aminotricarballylic acid (Greenstein (11, 19) had prepared one of the racemic diastereoisomeric forms of this amino acid in 1935) and their conversion to the corresponding isocitric acids (227), made it possible to conclude that the α-carbon atom of the natural form of isocitric acid has the L-configuration. Greenstein and his collaborators succeeded in correlating information derived from rotatory dispersion studies, enzymatic work, and chemical investigations, and by these means were able to clarify in a most elegant way important aspects of the stereochemistry of the naturally occurring amino acids. An approach to the problem of the stereochemistry of α-substituted amino acids (e.g. isovaline) was made using enzymatic techniques.

In the last years of his life he initiated an extensive series of nutritional researches with diets containing mixtures of optically pure amino acids (272-274 and earlier papers). His work also demonstrated the toxic effect of injecting amino acid mixtures lacking in arginine, and the protective effect of the addition of arginine to these mixtures, which was clearly shown to be due to the function of arginine in the Krebs urea cycle, whereby the toxic ammonia released from other amino acids was rapidly converted to urea (266). A further discussion of the progress of this work will be found in the immediately following paper by Winitz *et al.* in this volume.

At the time of his death he was engaged, with M. Winitz, in the writing of a comprehensive treatise in three volumes on the chemistry of the amino acids (281). This great work has now been completed and will shortly be published. Several experts who have had the opportunity of examining the manuscript in advance of publication believe that it will stand for many years as the most authoritative discussion of the amino acids in existence.

Greenstein was awarded the Neuberg medal in 1950, the Distinguished Service award of the Department of Health, Education, and Welfare in 1952, and the Hillebrand award in 1958. In 1954 he served as chairman of the Division of Biological Chemistry of the American Chemical Society. He was for several years an editor of the *Archives of Biochemistry and Biophysics,* and, with A. Haddow, was editor of *Advances in Cancer Research.* He served as visiting professor of biochemistry at the University of California in 1948, and in 1957 he became a member of the Committee on Biochemistry of the National Research Council and served as the chairman of the Subcommittee on Amino Acids. In 1949 he was a member of the American delegation of the Cancer Colloquium called by Pope Pius XII at the Vatican, and in 1956 he was a visiting lecturer at several Japanese universities and became an honorary member of the Japanese Chemical Society and the Japanese Foundation for Cancer Research.

He led an arduous life as an investigator, administrator, and writer. In the course of his career he collaborated with more than 60 scientists. His influence on those with whom he worked was profound and lasting. He took a constant and active part in every piece of research that was published under his name and was personally responsible for writing all of the papers to which his name was attached. His hours of work were long. After dinner at home he would, several times each week, return to the laboratory quite early in the evening, where he could write or experiment uninterruptedly until one or two o'clock in the morning. Yet with all of this activity he was accessible, friendly, and relaxed when anyone came to see him and talk things over with him.

His interests were broad and ranged far beyond the sciences. He was always a prolific reader. One of us can well recall how, in his Harvard days, he delighted in Dickens, and especially in "Pickwick Papers." He read widely in philosophy, theology, and biography and was something of an expert on the history of the Civil War —its battles, issues, and great men. Undoubtedly the breadth of his

reading and his appreciation of literature had much to do with the high quality of his own writings.

He married Lucy Mitchell in 1933. They had two children, a daughter, Louise Brill, and a son Michael. The latter had already worked during his school days for a summer in his father's laboratory on nutritional problems relating to the amino acids (268). During the last few years of his life Jesse Greenstein had become a boating enthusiast. Starting originally with a small outboard motor he had purchased in 1958 a 22-foot cabin cruiser. With his usual enthusiasm and intensity of purpose he had engaged in a thorough study of navigation and had received several certificates from the Washington Area Power Squadron. In this activity also his son Michael joined him.

Jesse Greenstein was outstanding in scientific skill, learning, energy, and devotion to his work. He inspired numerous young men, who came to work at the National Cancer Institute, with much of his own ardent enthusiasm for science. He was proud to be a servant of the United States Government, in a position of great responsibility, and his service went far beyond the allotted duties of his post. He was a loyal friend, whose wisdom and courage were a source of inspiration to his colleagues. Men with his inspiring qualities are rare, even unique. He will be sorely missed, but the inspiration which he and his work have imparted lives and continues.

Quantitative Nutritional and *In Vivo* Metabolic Studies with Water-Soluble, Chemically Defined Diets

MILTON WINITZ, SANFORD M. BIRNBAUM, TAKASHI SUGIMURA, and M. CLYDE OTEY

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OVER THE PAST DECADE, a sizeable portion of Jesse P. Greenstein's research program was devoted to the development of resolution procedures (Greenstein, 1954; Greenstein and Winitz, 1960) which would permit the large-scale preparation of optically pure α-amino acids for primarily two reasons: first, to make the optical antipodes of amino acids readily available as starting materials for the synthesis of peptides to be employed as substrates in *in vitro* studies of peptidase specificity; second, to provide the amino acid components for a completely synthetic diet which would be suitable for long-term nutritional studies with experimental animals and appropriate for parenteral administration to human beings. It is the aspect of synthetic diets with which the present discussion will be primarily concerned. However, before embarking on this subject, some of the methods customarily employed to procure the optically active amino acids, which constitute a major portion of these synthetic diets, will be briefly reviewed.

Large-Scale Preparation of Optically Pure Amino Acids

At the present time, optically active amino acids in large amount are obtained essentially by three different techniques, namely, by isolation from acid or enzymatic hydrolyzates of proteins, by microbial synthesis, or by enzymatic resolution of the racemic form of the amino acid. With respect to the first technique, it should be noted that a given protein hydrolyzate may contain some eighteen or nineteen different amino acids. Although each of these amino acid components should, theoretically, be isolable in chemically pure form, the large-scale isolation of each of these un-