Biochemical Applications of Mass Spectrometry

Edited by
GEORGE R.
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BIOCHEMICAL APPLICATIONS OF MASS SPECTROMETRY

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To PROFESSOR DAVID RITTENBERG

PREFACE

The development and application of techniques that allow scientists to penetrate deeper into the chemical basis of life and ultimately help to provide mankind with a better understanding offer a challenging and exciting frontier. Mass spectrometry is such a technique. Mass spectrometers and particularly the combination of the mass spectrometer with the gas chromatograph provide unparalleled opportunities for identifying minute quantities of chemical compounds. Indeed, much attention is given in this book to the combined mass spectrometer-gas chromatograph because of its usefulness in analyzing small amounts (e.g., nanogram quantities when optimum conditions are used) of substances in mixtures of biological origin (that do not require a high degree of prior purification). During the last decade, the application of mass spectrometry to biochemistry and other biomedical areas has emerged as one of the most promising techniques in identifying and elucidating the structure of biologically active compounds. Biochemical applications of mass spectrometry, although generally considered a specialized portion of organic mass spectrometry, is actually broader because of the requirement in the determination of stable isotopes, which are used in tracing both organic and inorganic metabolic pathways.

This book attempts to provide a comprehensive, up-to-date treatment of the areas of biochemical knowledge elucidated by mass spectrometry and is designed for the use of the advanced student and professional worker in the biochemical, chemical, biological, agricultural, and biomedical sciences. Most medical schools and many agricultural schools are now using mass spectrometry. Although the cost of sophisticated instruments remains quite high, some of the newer models are becoming inexpensive enough so that the individual scientist can afford to have his own mass spectrometer.

From a historical viewpoint, biochemistry played an important role in the development of the instrumentation of mass spectrometry. One of the early contributions by biochemists was the development of instruments needed for analyzing stable isotopes. Then a gap of some 15 years occurred during which organic structural analysis emerged. Contributions by biochemists became noticeable again, but this time in the development and application of the mass spectrometer-gas chromatograph and more recently in chemical ionization techniques.

It is desirable to provide scientists with an understanding of the advantages as well as the limitations of mass spectrometry in solving biomedical problems. In the organization of this book, the conventional classification of biochemical compounds is followed for mass spectral interpretation. Several chapters on general interpretation of mass spectra and one on instrumentation are included. A large section on *APPLICATIONS* includes the conventional classes of biochemical compounds and in addition deals with selected new techniques of interest such as chemical ionization and field ionization mass spectrometry. Since computers are being used to an increasing extent in mass spectral data collection, processing, and analysis, a chapter in which mass spectrometrists describe their particular instrumentation is included. A chapter on the use of mass spectrometry in detecting extraterrestial life is included. Although food flavors are not generally considered to be a major biochemical group, a chapter on this subject is included since it is historically significant in the development of mass spectrometry and because it is of widespread interest. There is broad coverage on the use of stable isotopes in biochemistry and their measurement by mass spectrometry. Finally, four appendices describe specialized services available and list manufacturers of mass spectrometers currently used in the biochemical field. For a number of authors, this represents a first effort at writing a comprehensive review of the mass spectral studies in their particular field; however, others have pioneered in the area.

Although my initial contact with the work of David Rittenberg came in an undergraduate course in biochemistry and later when I used his methods and techniques, we first met at the Fifty-Second Annual

Meeting of the Federation of American Societies for Experimental Biology (FASEB) held in Atlantic City, New Jersey, April 15–20, 1968 on a television program sponsored by FASEB on "Mass Spectrometry: New Applications." Later, when I asked Dr. Rittenberg to write the chapter on the use of stable isotopes and to write a historical background on the development of biochemical principles based on the contribution that stable isotopes have made, he most graciously consented. We discussed the contents of these chapters at length on several occasions and he was very much concerned with the details to be covered. Because of his untimely death this material has now been written by Dr. Richard M. Caprioli, who was Dr. Rittenberg's last graduate student to complete his doctoral dissertation, and by Dr. Sarah Ratner, one of Dr. Rittenberg's graduate students during the exciting period when isotopic tracer techniques were being developed.

Dr. Caprioli graciously agreed to write the dedicatory statement which follows.

I would like to express appreciation for help and encouragement from Roger E. Koeppe and James A. Whatley. Special recognition is due Seymour Meyerson and Ragnar Ryhage for their guidance and their role in stimulating my interest in mass spectrometry. The editorial assistance of and frequent consultations with Otis C. Dermer are gratefully acknowledged. I wish to thank Jack Fryrear, Mary F. Reed, and Patricia Kelly for help in preparation of illustrations and to Donna Mitchell for stenographic assistance.

The financial support that the National Science Foundation, Washington, D.C. has made available to me during the past few years for the support of the biochemical mass spectrometry research program is gratefully acknowledged. I am also pleased to acknowledge the National Institutes of Health for support at the Karolinska Institutet in 1963–1964.

Stillwater, Oklahoma
January 1971

GEORGE R. WALLER

DEDICATORY REMARKS FOR DR. DAVID RITTENBERG

For if the trumpet give an uncertain sound,
Who shall prepare himself to the battle?

I Corinthians, xiv, 8.

David Rittenberg was a truly remarkable man. He was very purposeful and dedicated to life. The quotation cited above, which hung above the desk in his office, is particularly fitting. He did things positively and with great gusto, displaying that type of infectious enthusiasm which could motivate colleagues and students alike to become deeply involved. He had the ability to recognize the central theme and the drive and determination to pursue it. Perhaps the most remarkable side of this man was his very strong personal magnetism. He was always surrounded by colleagues eager to engage him in conversation, and his greatest joy was such conversation with friends and especially with students. No matter who made up the group or what the topic of discussion, David Rittenberg was in the mainstream. One could say, and not be in fear of overstating, that he enjoyed discussions.

As a graduate student in his laboratory, I quickly became acquainted with unscheduled meetings which Professor Rittenberg frequently held in the laboratory. Anyone who cared to participate was welcome and any subject was fair game. The only ground rule was that you had to be prepared to say your piece whenever you could get a word in, for David Rittenberg possessed a fascinating, never-ending wealth of information on any subject. I always found it quite amusing during these meetings when others managed to exchange a few words on the subject at hand, causing Professor Rittenberg to turn pathetically to the nearest available sympathetic ear and say, "No one lets me talk!" He was a master at telling stories. He would begin with great relish to unfold a story, thus winning your interest immediately. From then on, you were a captive audience. It was obvious that he got immense enjoyment in relating these tales. His repertoire was enormous. By the end of my graduate work, I was still fascinated by stories which I was hearing for the first time.

The impact of David Rittenberg's thoughts and work in the field of biochemistry were revolutionary. His career began in 1934 when he received his doctorate working with Harold Urey on some of the thermodynamic properties of compounds containing a newly discovered isotope, deuterium. Shortly thereafter, he joined the Department of Biochemistry at Columbia University where he was to serve as a faculty member for 36 years. In collaboration with Rudolf Schoenheimer, also of the same department, he began the study of intermediary metabolism using stable isotopes in 1935. Before this, biochemistry was indeed in a very primitive stage of its development, for although many essential cellular components had been discovered, it was clear that existing methods would not be satisfactory in elucidating the details of the interaction of these compounds. His work with Schoenheimer as well as several other colleagues led to the development of the methodology for isotopic tracers and, more important, to some drastic changes in the theories of cellular reactions. Using the stable isotopes of hydrogen, carbon, nitrogen, and oxygen, Professor Rittenberg was able to study the manner in which cells utilize fats, proteins, and other essential building blocks. This work showed that the cell was in a dynamic state, that it was certainly undergoing catabolic and anabolic processes, and that it was not in a stable state with only a small amount of energy production and repair going on, as previously believed. The basic concepts resulting from this work were instrumental in changing the direction of biochemical research during that period. In 1954, Professor Rittenberg was elected

to the National Academy of Sciences. He was the recipient of many other honors and awards throughout his career and was the author of nearly 200 papers in the field of biochemistry. His interests were diverse, encompassing many aspects of biochemistry.

Professor Rittenberg contributed many pioneering advances in his experiments with isolated systems, at the same time continuously examining the relationship of the part to the whole . This is exemplified by the great deal of work he did with isotopic tracers using whole animals to investigate the chemical reactions of essential dietary constituents. At a Harvey Lecture given on April 28, 1948, entitled "Dynamic Aspects of the Metabolism of Amino Acids," Professor Rittenberg said, "The relative simplicity of the *in vitro* system is obtained by the sacrifice of both geometric and energetic organization, which uniquely characterizes the living system. The problem of summing the various chemical reactions known to the enzyme chemist is equivalent to that which an untutored inhabitant of Central Australia would face who never having seen a plane, were asked to put together the remnants of a plane crash. It is possible he could build a useful and working device of the debris but it would surely not be an aeroplane. The integration into a unified whole of the individual reactions which we observe in systems more or less closely related to the intact organism is the central problem of biochemistry." Today, we are still very much involved with this central problem.

David Rittenberg was, above all else, a teacher. His dedication to this profession was unmatched. He worked constantly for the development of the student, a metamorphosis that would bring a student through study which would produce a competent scientist. But he was frequently subtle, not allowing the student to know that he was being guided through the learning process. He was always interested primarily in the welfare of his students and was determined to execute successfully the responsibility he felt toward them. As a result, a very close relationship developed between him and his students. And when he was through, he had passed on a very precious gift—one of great enthusiasm and devotion toward life. As one who benefited from such a relationship, I know that my life has been immeasurably enriched because of my experiences with this great man. I am among many who knew and loved him. David Rittenberg was a phenomenon which happens just once, but the goodness and light which he has shed will remain eternally.

West Lafayette, Indiana
August 1970

RICHARD M. CAPRIOLI

INTRODUCTION

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During the past fifteen years mass spectrometry has been used in solving most biochemical problems of structural nature. Today it is common to find a paper or two in any biochemical journal which describes the use of mass spectrometry in some problem-solving application.

The pioneering contribution of biochemists in the development of mass spectrometry in the 1930s generally has been overlooked; e.g., the development of instruments for measurement of isotope ratios and the preparation of samples for such instruments. In the late 1940s a few organic chemists began using mass spectrometry to correlate the fragments produced upon electron impact with the structure of the molecules. During the 1950s, when scientists began exploring the mechanistic approach of decomposition under electron impact, the possibility of using mass spectrometry as an aid in identifying a variety of organic and biochemical compounds was realized. Biochemists began using mass spectrometry in the middle of the 1950s for structure studies. By 1960 it was apparent to both the organic chemist and the biochemist that organic mass spectrometry was a tool that could be very useful in solving a wide variety of problems. To a large extent, it is the organic chemist who developed the specialized but systematic organic chemistry of mass spectrometry which is related to the environment in which it was obtained. The biochemist, on the whole, treats mass spectrometry as a tool. As a result, widespread use by the biochemical community did not develop until the last half of the 1960s.

It is generally recognized that a vast amount of information regarding the structure of an unknown compound of biological origin can be obtained from a microgram or nanogram sample through the use of mass spectrometry. However, this instrument cannot solve all of the unknown identification problems; e.g., stereoisomers. When used in conjunction with nuclear magnetic resonance, ultraviolet and infrared spectroscopy, x-ray crystallography and chemical derivatization, mass spectrometry becomes even more useful in accurately solving complex structure problems. The use of computers in acquiring and processing mass spectral data is becoming widespread and in some laboratories small computers are now considered an integral part of the instrument; e.g., gas chromatograph-mass spectrometer-computer. The computer can also be used to control the mass spectrometer and will undoubtedly play a larger role in the future. Most installations described have the mass spectrometry and computer in close proximity to each other. The use of centralized computer facilities remains undeveloped; a recent attempt at centralization was unsuccessful (1).

Within the past few years, a number of new journals either devoted exclusively to mass spectrometry or having a major portion of their papers concerned with it have come into existence: *Organic Mass Spectrometry* (1968), *Archives of Spectral Data* (1970), and *International Journal of Mass Spectrometry and Ion Physics*. The well established abstracting service provided by the *Mass Spectrometry Bulletin* (1966) was joined by *Gas Chromatography-Mass Spectrometry Abstracts* in 1970. This increase in specialized coverage of literature is convincing evidence of the widespread use of mass spectrometry.

The use of stable isotopes, particularly carbon-13, is expected to increase significantly during the decade of the 1970s since the price of this isotope will become very much lower. To assist the biochemist in using this isotope, a section on the synthesis of compounds labeled with stable isotopes has been included in Chapter 27. This represents the first effort to pull

together in a systematic form the information on stable isotopes since the Symposium on the Use of Isotopes in Biology and Medicine (2) held in Madison, Wisconsin, in 1947.

Mass spectrometry is used in many other fields such as the broader field of organic chemistry, catalysis, analytical chemistry, atomic physics, nuclear physics, and geological dating. A number of books and articles describing its use in these fields are available.

The literature is cited through early 1970.

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CONTENTS

CHAPTER 1

A HISTORICAL SURVEY OF MASS SPECTROMETRY

A. APPLICATIONS OF STABLE ISOTOPES TO THE STUDY OF INTERMEDIARY METABOLISM: HISTORICAL SURVEY

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1. Introduction

The value of isotopes as biological tracers was realized as early as 1923 by Hevesy (1). When neither stable nor radioactive isotopes were available, he followed the uptake, distribution, and displacement of lead in plants by adding a small amount of the naturally occurring radioactive isotope thorium B (^{212}Pb). As soon as heavy water became available he also carried out experiments of fundamental importance on water metabolism, and in this connection he estimated that the time a water molecule resides in the human body is about 14 days (2).

Shortly thereafter Schoenheimer and Rittenberg began to apply deuterium as a tracer to the study of organic constituents of living organisms. Their first few papers, called "Deuterium as an Indicator in the Study of Intermediary Metabolism," described the preparation of tetradeuterostearic acid (stearic acid-*6, 7, 9, 10-*d_4) by chemical synthesis and, following its administration to adult mice, the eventual deposition of appreciable amounts of this compound in the fat depots of the body, as judged by the deuterium content of isolated stearic acid (3, 4).

In the early 1930s metabolic studies were carried out as balance experiments and the view generally held was that the main bodily constituents—fats, proteins, and carbohydrates—were metabolically inert. With deuterium it became possible for the first time to tag foodstuffs and suspected metabolites without changing their physical or chemical properties. Isotope labeling thus provided a new and powerful tool to follow administered compounds in the living

organism and to detect their participation in a multiplicity of hitherto unrecognized reactions.

It is of interest to recount the circumstances under which these pioneer studies began. Urey and his coworkers at Columbia University discovered deuterium in 1932 (5) and shortly thereafter methods were developed for increasing the abundance by electrolytic fractionation of water (6). At Clarke's invitation, Schoenheimer came to the Department of Biochemistry at the Columbia College of Physicians and Surgeons from Freiburg in 1933 to continue his studies in sterol metabolism. Rittenberg joined this department a year later under the auspices of the Rockefeller Foundation to exploit the biological potentialities of deuterium. He had been a student of Urey's and was well versed in deuterium techniques. Through acquaintance with Hevesy in Freiburg, Schoenheimer was much interested in enlarging the applications of deuterium. Out of this fortunate association there soon developed the ideas and techniques for applying deuterium, and later ^{15}N, to the study of the intermediary metabolism of fatty acids, sterols, amino acids, and proteins.

Schoenheimer led the way in subjecting new and old observations to rigorous scrutiny and in devising highly imaginative yet beautifully simple experiments to test, establish, and extend the new concepts. In 1941, the year of Schoenheimer's death, the studies had progressed surprisingly far and Schoenheimer had succeeded, with great perception, in disclosing the existence of specific biosynthetic systems which awaited further development. Fortunately these and further aspects continued to be pursued with remarkable results by the group at Columbia: Rittenberg, Bloch, Shemin, and others. When ^{2}H, ^{15}N, and ^{13}C became more easily available, isotope-labeling techniques were applied to metabolism studies in many laboratories. Of particular importance were the pioneer studies with ^{13}C and ^{11}C on CO_2 fixation and various aspects of carbohydrate metabolism in plants and animals and the applications of ^{18}O to problems in photosynthesis and enzymatic mechanism. Space does not permit a comprehensive survey of this large body of work. Thirty-five years have intervened since the introduction of deuterium. The study of intermediary metabolism has rapidly advanced away from animal experimentation and few biochemists are now familiar with the original findings nor can they recall their very great impact. The studies selected for mention are those that have become models for later experimental designs, have served to dispel outmoded concepts, or have laid the groundwork for the elucidation of one or another biosynthetic mechanism of general significance.

2. Studies in Fatty Acid and Sterol Metabolism with Deuterium as Tracer

a. Preliminary Considerations and Techniques

Little was known of the chemical properties of deuterium in 1935 and, from the start, it was necessary to develop many new techniques and critically test the physical, chemical, and physiologic properties of organic compounds containing the isotope.

i. ANALYTICAL PROCEDURES

The first methods for estimating deuterium made use of refractive index measurements or of density measurements with the submerged float (4) and later with the falling drop (7). The samples of water required for these methods were obtained as water of combustion and were purified by repeated distillations over an oxidizing agent (4). Delayed by the Second World War, the procedure later developed by Rittenberg for estimating the abundance of isotopes of hydrogen in the mass spectrometer was not made available until 1952. It overcame the problems of wall hold up and isotope equilibration and had the great advantage of requiring a small sample (3 to 4 mg of water) for analysis (8). This procedure is still in general use.

ii. METHODS OF SYNTHESIS

The methods of synthesis were chosen to conserve the limited supply of isotope and also to avoid reactions that might introduce deuterium into labile positions. The most frequently used procedures involved the catalytic reduction of one or more double bonds in the presence of deuterium gas or the introduction of deuterium into stable linkage with carbon by exchange with heavy water. Compounds such as coprostanone-*4,5*-d_2 (9), stearic acid-*5,6,9,10*-d_4 (4) and butyric acid-*2,3*-d_2 (10) were prepared by the first method. The second method, when used under acidic conditions, introduced deuterium into the α position of aliphatic acids, as in the preparation of palmitic acid-*2,2*-d_2 (11). Under alkaline conditions, deuterium was introduced into aliphatic acids uniformly along the chain (11). The exchange reactions were carried out at elevated temperatures for many hours and the deuterium introduced in this manner did not exchange out under the conditions to which the compound might be exposed in the body or during isolation and purification.

iii. NATURAL ABUNDANCE AND TOXICITY

Despite the large difference in mass between deuterium and hydrogen, the distribution of deuterium in organic compounds isolated from divergent natural

sources, found by Dole (12), indicates that this isotope is not appreciably fractionated in nature. Moreover, after the concentration of deuterium was elevated in animals, no appreciable fractionation was found by Krogh and Ussing (13). It was necessary, however, to avoid concentrations in the body water above 10% (14). The toxic effects observed above this level are probably caused by the effect of the isotope on reaction kinetics.

iv. STABILITY OF THE DEUTERIUM-TO-CARBON BOND

With each of the labeled organic compounds, it was also necessary to ascertain that the deuterium did indeed occupy a stable position and would not be lost by exchange (4). The large body of information gathered in this connection greatly extended the usefulness of deuterium as a tracer. Various theoretical predictions as to the lability of acidic hydrogens were confirmed through tracer application (14). With these crucial points established, the potentialities of tracer labeling could be fully exploited for the study of intermediary metabolism. Moreover, results obtained with living animals were immune to the criticism that the experimental conditions were unphysiological or artificial in any way.

b. Deuterium as Tracer for Hydrogen

i. DESATURATION AND SATURATION OF FATTY ACIDS

One of the earliest studies was concerned with the interconversion of fatty acids. Previously, investigators of fat metabolism had given consideration to the possibility that the process of fatty acid desaturation might take place in the body, but the hypothesis was far from being generally accepted in the absence of definite proof. Evidence came from experiments in which labeled stearic acid was administered to mice for several days. The sample of unsaturated fatty acids subsequently isolated from the body fats contained a high content of deuterium (15). The reverse process, saturation of unsaturated fatty acids, was demonstrated on feeding labeled oleic acid (16). Studies of chain elongation (17), chain shortening (18), and other aspects of lipid and phospholipid metabolism were continued along these lines by Stetten and by others elsewhere.

c. Deuterium as Tracer for the Carbon Chain

Although deuterium could not entirely supplant a carbon isotope in usefulness for labeling the carbon chain, the principles followed by Schoenheimer, Rittenberg, and their collaborators greatly extended the utility of deuterium for this purpose. Essentially this depended on the strategic location of the tracer with respect to the metabolic fate of the compound and on the proven stability of the H—C bonds. When a suitable chemical synthesis was not feasible, biosynthetic procedures were resorted to for the first time. Thus deuterated oleic acid was prepared by isolation from animals which had been given deuterated stearic acid (16).

About half of the deuterated stearic acid fed to mice was deposited in the depot fats (4). The animals were maintained at constant body weight or were allowed to lose weight. The deposition of fat of dietary origin in the tissues, before it was utilized, demonstrated for the first time that fats of *endogenous* and *exogenous* origin must follow the same metabolic fate, since the living organism is unable to distinguish between them. Experiments of this kind were equally instrumental in dispelling the concept that exogenous and endogenous sources of protein and carbohydrates were handled differently in the body.

i PRECURSOR-PRODUCT RELATIONSHIPS IN INTERMEDIARY METABOLISM

The earliest application of deuterium to the study of cholesterol metabolism was concerned with the pathway of conversion of cholesterol to coprosterol. Deuterium-labeled cholestenone and coprostanone were administered to both dogs and humans and the deuterium was found to be present in the excreted coprosterol. These interconversions suggested that the administered sterols might be biological intermediates in cholesterol metabolism. It was made clear that the deuterium could not enter coprosterol from the medium since the isotope liberated by degradative processes would undergo a large dilution. The conversion of cholestenone to coprosterol and of stearic acid to oleic acid established the basic tenet on which all tracer technique depends. To quote the original statement (19), "a substance A, containing an isotopic label in stable linkage, is administered to an animal. After a suitable time a compound is isolated from the tissues or excreta and, by analysis, found to contain the isotope. Results obtained in this way provide conclusive proof that the administered compound A may be converted in the animal to compound B." Countless isotope applications of this principle have since that time reached into the development of all aspects of intermediary metabolism.

Possible precursor-product relationships had previously been explored in animals by balance experiments. One of the obstacles that often made their interpretation extremely difficult was the possibility that one substance could induce the formation of others without itself being involved in the synthesis.

This weakness of the balance study was aptly referred to by Schoenheimer as "the slot machine analogy": "A penny brings forth one package of chewing gum, two pennies brings forth two. The first observation would be taken as an indication of the conversion of copper into gum; the second observation would constitute proof" (19).

ii. SYNTHESIS OF FATTY ACIDS AND STEROLS FROM SMALL COMPONENTS

Perhaps the most far-reaching use of deuterium as a combined tracer for both hydrogen and the carbon chain pertained to the biosynthesis of fatty acids and sterols. When adult animals were injected with heavy water, and the deuterium concentration of the body fluids was maintained at a constant level over a period of time, stably bound deuterium was incorporated into fatty acids. The deuterium concentration reached a steady level within a few days at about 50% of the value in the body fluids. This entirely unexpected finding could be explained only on the basis that fatty acids were being rapidly synthesized *de novo* from low molecular weight components, presumably two-carbon fragments (20). These imaginative experiments provided an extraordinary insight into the mechanism of biosynthesis, stimulating much additional work.

Similar experiments in which the incorporation of deuterium into stable positions in body cholesterol was followed over a longer period indicated that the biosynthesis of cholesterol also involved a large number of small molecules, or fragments, originally possessing labile hydrogens (21). It is difficult for biochemists of the present generation to imagine the puzzlement caused by these findings. Chemists had not even begun to speculate on the mode of synthesis of a structure as complex as cholesterol. Studies continued later by Bloch and Rittenberg with labeled acetate showed that this compound could serve as a precursor for cholesterol in rats (22). Sonderhof and Thomas had found that acetate was also a precursor in cholesterol biosynthesis in yeast (23). This was followed by the ingenious experiments of Bloch and his collaborators with doubly labeled acetate ($C^{13}H_3C^{14}OOH$), which permitted the origin of the carbons in the side chain to be worked out (24). Later similar deductions with regard to the ring skeleton culminating in the entire elucidation of the isoprenoid sequence and of the enzymatic mechanism in squalene and cholesterol biosynthesis.

iii. TURNOVER OF DEPOT FATTY ACIDS

The uptake of deuterium from body water during biosynthesis was exploited by Schoenheimer and Rittenberg in a third way. The rate of uptake of deuterium into the fatty acids displayed a linear increase with time before leveling off. When deuterium in body fat was built up previously, the rate of disappearance of deuterium followed a parallel course. The half-life of body fat determined from these rate curves was less than 3 days (20). The second method has served as the prototype decay curve for innumerable applications of this technique for the estimation of turnover of proteins and other constituents of the body.

3. Studies on the Metabolism of Nitrogenous Compounds with ^{15}N as Tracer

Urey and his collaborators developed the cascade process (25) for concentrating ^{15}N in 1937. Commencing with the earliest operations of the columns, Urey made available, with extraordinary generosity, numerous samples of $(NH_4)_2SO_4$ enriched in ^{15}N. The first gifts were of necessity extremely small and precious. The use of a mass spectrometer became essential at this time and an instrument following the design of Bleakney (26) was constructed by Rittenberg. A second instrument was built several years later, following the improved Nier design (27). With the introduction of ^{15}N as a tracer for metabolic study, many analytical and manipulative techniques were developed for handling organic compounds; many of these convenient and flexible procedures continue in use today.

a. Early Considerations and Techniques

i. ANALYTICAL PROCEDURES

The general method chosen by Rittenberg (28, 29) to convert carbon-bound nitrogen to gaseous N_2 for analysis in the mass spectrometer involves conversion of organic nitrogen to NH_3 by acid digestion under Kjeldahl conditions followed by oxidation to N_2 by alkaline hypobromite. Procedures set at this time for calculating and expressing the abundance of ^{15}N from the mass peak ratios have been widely adopted.

ii. SYNTHESIS OF AMINO ACIDS CONTAINING ^{15}N

The selection of methods of synthesis was determined by the severe restrictions on the amount of ^{15}N available and by the form in which the isotope was received. Two general procedures were adopted, both suited to the synthesis of a variety of amino acids. Thus alanine, phenylalanine, tyrosine, norleucine, and glutamic and aspartic acids were prepared by the catalytic reduction of the corresponding keto acids

in the presence of $^{15}NH_3$. Glycine and leucine were prepared with ^{15}N-phthalimide. These two methods described by Schoenheimer and Ratner (30) afforded good yields in the presence of a limited excess of the reagent, permitted a high degree of recovery of unused ^{15}N, and avoided dilution with ordinary nitrogen.

iii. DOUBLE LABELING OF AMINO ACIDS WITH ^{15}N AND ^{2}H

The technique of double labeling greatly extends the usefulness of isotopes in intermediary metabolism, particularly for the study of amino acids. Doubly labeled leucine was prepared by introducing deuterium into stable positions of the starting material, isocaproic acid, by exchange, before the introduction of ^{15}N (30, 31). Lysine (32) and proline (33) containing deuterium and ^{15}N were synthesized for metabolic study by more complex procedures.

iv. AMINO ACID RESOLUTION AND THE TECHNIQUE OF WASHING OUT

The racemic amino acids obtained by chemical synthesis had to be resolved by the laborious fractionation techniques then available and complete separation of the isomers was not possible. In the case of leucine, for example, the L-form was contaminated with 6% of the D-form. The less soluble salt of the desired L-isomer was recrystallized repeatedly in the presence of unlabeled D-isomer, thus greatly reducing the isotope concentration in the contaminant (31). In the preparation of labeled oleic acids, the deuterium in contaminating stearic acid was also removed in this way (16). This general procedure is capable of great variation and has been extensively applied to remove contaminating tracers.

v. STABILITY OF THE C—N BOND

At this time it was established that chemical exchange of $^{15}NH_3$ with the amino group of glycine or other amino acids did not take place. The stability of the C—N bond was studied with the aid of ^{15}N, and it was found to withstand a variety of conditions to which amino acids might be exposed (34). Thus transfers of nitrogen that would be observed after ingestion of an amino acid could be attributed to metabolic activity. The feasibility of applying ^{15}N to amino acid metabolism was tested with material enough for only one animal in what would now be considered a most cautious experiment, the formation of hippuric acid from ^{15}N-glycine. The glycine administered contained about 2 atom % ^{15}N excess. This concentration permitted a dilution no greater than 100-fold. The analysis indicated that the administered glycine had undergone a three-fold dilution *in vivo* before conjugation with benzoic acid (35).

At this time also it was shown that the natural abundance of ^{15}N in amino acids isolated from tissue proteins varied very little, whether the nitrogen was located in the α-amino group, in the amidine group, or in other locations (36).

b. Amino Group Transfer among Amino Acids

i. AMINO ACID TURNOVER

Unexpected results were obtained when $^{15}NH_3$ or any ^{15}N-amino acid was given to adult rats for several days. The ^{15}N was found in all of the amino acids of tissue proteins, except lysine, whether glycine, tyrosine, leucine, or another amino acid was given. The excreted creatinine, NH_3, and urea also contained ^{15}N (31, 37–40). The extent of ^{15}N incorporation varied, being highest in the amino acid administered and next highest in amide nitrogen and in aspartic and glutamic acids. The diverse and rapid incorporation of ^{15}N indicated that transfer of amino groups were going on continually and also that extensive *de novo* synthesis of amino acids was taking place in adult animals well provided with amino acids in the form of dietary protein.

iii PROTEIN TURNOVER

The evidence for amino group transfer was derived from the analysis of amino acids isolated from tissue proteins. For this purpose the protein served as a trapping agent which took up a mixture of existing and newly synthesized amino acids. The incorporation of labeled amino acids into proteins revealed the presence of an equally important process, the synthesis of new protein. All the tissues examined in steady-state animals showed active protein-synthesizing capabilities; such tissues as liver and intestinal tract exhibited a higher rate of ^{15}N uptake than muscle (31, 40).

The half-life of tissue proteins was estimated from the rate of replacement of ^{15}N after previously raising the concentration by feeding glycine -^{15}N. Specificity was achieved by choosing circulating antibody protein for study since after immunization it could be isolated by selective precipitation with the antigen. The half-life of antibody protein was found to be about two weeks, the same as the average half-life of serum protein investigated in this way by Schoenheimer, Ratner, Rittenberg and Heidleberger (41, 42) with which it was directly compared. This approach has continued to shed light on many aspects of protein synthesis.

c. The Concept of the Dynamic State of Cell Constituents

The evidence gathered with deuterium and ^{15}N in the span of a few years pointed to a rapid synthesis and breakdown of all bodily constituents whether they be fats or proteins (31) or such metabolites as fatty acids, amino acids, creatine, or purines. Schoenheimer realized that this activity was the consequence of already existing mechanisms for synthesis and degradation which are in continual operation even in the adult maintained in the steady state. He developed the concept of the *dynamic state* of bodily constituents with great clarity in three Dunham lectures published after his death (43). It is scarcely possible to overestimate the importance to biochemical thought of this concept and of the allied concept of the *metabolic pool* which it implied, and which was later expanded by Sprinson and Rittenberg (44). By stressing the significance of the continuous operations of biosynthetic processes, the *dynamic state* concept focused a great deal of interest on the biosynthetic pathways and mechanisms involved.

d. Biosynthetic Pathways Studied with ^{15}N as Tracer

i. BIOSYNTHESIS OF CREATINE FROM MULTIPLE PRECURSORS

The previously obscure metabolic origins of creatine were established by judicious choice of ^{15}N-labeled precursors and subsequent location of the isotope in the several positions of excreted creatinine. A great advantage was given by the finding that creatine, once formed, is metabolically inert, i.e., the amidine nitrogens are not subject to turnover as are those of arginine (45). Possible precursors were synthesized so as to contain ^{15}N either in the amino group or in the amidine group and were then tried individually. It was shown that glycine serves as the precursor of the sarcosine moiety and arginine supplies the amidine group. The study by Bloch and Schoenheimer (46) also cast light on the turnover rate of creatine and the relation to creatinine (45). By appropriate labeling, du Vigneaud, Chandler, Cohn, and Brown showed that methionine, by transmethylation, supplied the methyl group for sarcosine and choline (47).

ii. MOBILITY OF AMINO GROUPS IN ESSENTIAL AMINO ACIDS AND THE TECHNIQUE OF DOUBLE LABELING

Simultaneous labeling with two different isotopes has provided one of the most powerful techniques for the study of metabolic reactions involving group transfer. The ability of the respective analogs (α-keto, α-hydroxy, D-isomer) to satisfy the growth requirements for each of the essential amino acids varies greatly and the reason for this was obscure. This problem was investigated with amino acids labeled with deuterium along the carbon chain and ^{15}N in the amino group. Either the L-form or the D-form was administered, and the ratio of ^{2}H to ^{15}N in the amino acid isolated from tissue protein was then compared to the ^{2}H-to-^{15}N ratio in the starting compound. Since the amino acids chosen for study cannot be synthesized by the animal, changes in isotope ratio would reflect independent turnover of the amino nitrogen with respect to the carbon chain.

The results obtained by Ratner, Schoenheimer, and Rittenberg (48) showed that the amino group of both L- and D-leucine are turned over relative to the carbon chain, thus explaining why the analogs mentioned can replace L-leucine (31) and why D-leucine can undergo inversion to the L-form (48). On the other hand, as Weissman and Schoenheimer showed, the α-amino group of D- and L-lysine does not undergo replacement (32); for this reason the D-form does not undergo inversion and therefore cannot replace the L-form (49). With proline containing ^{2}H and ^{15}N, it was possible to show that this nonessential amino acid is converted to glutamic acid and also to ornithine (33); this enlarged on an earlier study in which the conversion of ornithine to proline and glutamic acid was shown (50).

iii. TRACER LABELING AS A METABOLIC PROBE

In connection with an interest in finding a function for D-amino acid oxidase, Shemin and Rittenberg applied ^{15}N as a probe to detect the possible synthesis of D-amino acids *in vivo*. If the route of synthesis was not exclusively asymmetric, this would be indicated by a dilution of the isotope. On testing D-tyrosine and D-glutamic acid under suitable conditions, no dilution of ^{15}N was found in the excreted amino acid (51). Bernhard originally applied deuterium labeling in this way to demonstrate that long-chain dicarboxylic acids are not normal intermediates in fat metabolism (52).

iv. UBIQUITOUS ACTIVITIES OF GLYCINE AS A PRECURSOR

In the course of these intensive studies glycine emerged as a highly reactive amino acid participating in numerous biosynthetic pathways. As mentioned previously, glycine is one of the most effective nitrogen donors in the formation of other amino acids (40); it functions also as a precursor of creatine (46), and is itself synthesized at a rapid rate. Hippuric acid served as a trapping agent in the study of glycine synthesis (35). Making use of this technique somewhat