ON THE STUDY OF CELLULAR METABOLISM USING CARBOHYDRATES ENRICHED WITH STABLE ISOTOPES

By

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ABSTRACT

ON THE STUDY OF CELLULAR METABOLISM
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Part I.

An evaluation of the potential of gas chromatography-mass spectrometry (GC-MS) in studying cellular intermediary metabolism was undertaken using a fermentation of glucose by Escherichia coli as the metabolizing cell system.

Ion exchange techniques necessary for subsequent GC-MS analysis of the bacterial cell extracts are described. Microscale columns of 500 µl Dowex 50 [H^+, Li^+] resin and 75 µl AG MP-1 [$acetate^-$] resin were used to remove three compounds (glucose, HEPES buffer, perchlorate) that interfere with the GC-MS analysis of the cell metabolites. Recoveries of four representative metabolites from the ion exchange system were found to be greater than 96%.

Gas chromatography of the pertrimethylsilylated metabolites was performed using a 2m X 2mm packed column of 3% OV-101 on Gas Chrom Q and a hydrogen flame ionization detector. GC-MS analysis was used to identify 14 of the extracted metabolites.

Two of these metabolites, α-hydroxyisovalerate (valic acid) and α-hydroxyisocaproate (leucic acid), have not been reported previously as products of bacterial glucose fermentation or as E. coli metabolites. The analytical system

developed in this study was then used in conjunction with 1- and 2-^{13}C enriched glucose to explore the metabolic origin of leucic acid and to show that, while its production is triggered by glucose fermentation, only a small proportion is derived from glucose.

The merits and limitations as well as possible extensions of the analytical system are discussed.

Part II.

Methods are described for the synthesis of aldoses enriched at specific positions with oxygen isotopes. The general applicability of these methods is illustrated by their use in the synthesis of a group of 22 different ^{18}O labeled aldoses of 4,5, and 6 carbons. This group includes most of the biologically important aldoses and is listed as follows: [1-, 2-, 3-, 4-, and 6-^{18}O] glucose, [1-, 2-, 3- 4-, and 6-^{18}O] mannose, [1-, 2-, 3-, and 5-^{18}O] arabinose, [1-, 2-, 3-, and 5-^{18}O] ribose, [1- and 2-^{18}O] erythrose, and [1- and 2-^{18}O] threose.

These sugars were characterized with respect to the position and degree of ^{18}O enrichment by GC-MS methods.

The merits and disadvantages of synthetic approaches to ^{18}O enrichment are discussed.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

acetyl CoA	acetyl coenzyme A
AMP	adenosine 5'-monophosphate
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
CoA	coenzyme A
DMSO	dimethylsulfoxide
FAD	flavin nucleotide
$FADH_2$	reduced flavin nucleotide
FDP	fructose-1,6-diphosphate
GC	gas (-liquid partition) chromatography
GC-MS	gas chromatography-mass spectrometry
HEPES	N-2-hydroxyethylpiperazine-N'-ethane sulfonic acid
HPLC	high pressure liquid chromatography
LC-MS	liquid chromatography-mass spectrometry
MS	mass spectrometry
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced NAD^+
NMR	nuclear magnetic resonance spectroscopy
TFA	trifluoroacetic acid
TMS	trimethylsilyl
TPP	thiamin pyrophosphate

I. INTRODUCTION

A. The Study of Metabolism

The study of metabolism is perhaps the oldest endeavor in the field of biochemistry. It became evident in the 19th and 20th centuries that living creatures were able to effect an amazing array of chemical transformations. For example, plants were observed to transform the simple compounds CO_2 and inorganic forms of nitrogen, phosphorous, and sulfur into dozens of different carbohydrates, hydroxy acids, amino acids, fatty acids, phosphate esters, homo- and heterocyclic aromatics, alkaloids, and a variety of giant polymers or non-aqueous phases of these smaller compounds. Many microorganisms were observed to effect similar transformations when given a single organic substrate such as glucose or succinic acid.

The study of metabolism arose from the desire to understand how these living "black boxes" were able to perform such remarkable synthetic feats and obtain the energy necessary to maintain and reproduce their structures. This understanding is essential for understanding life itself.

B. The Use of Isotopes in Studying Metabolism

The use of isotopic tracers has contributed much to the understanding of metabolism, and indeed has often provided information that would be impossible to obtain by other means.

An isotopic tracer is an uncommon form of an element which differs from the common form(s) in atomic mass but not in electronic configuration. The tracer will thus, (disregarding isotope effects), undergo chemical transformations identical to those of the common elemental form. The tracer can still be distinguished from the common form experimentally, however, due to its possession of a distinctive nuclear magnetic moment or atomic mass (in the case of stable isotopes) or its radioactive decay (in the case of radioactive isotopes).

These characteristics allow isotopic tracers to be uniquely suited for the study of metabolism in the following instances: 1. determining the fate of an isotopically labeled exogenous substrate, i.e. determining what general chemical transformations take place in a living system; 2. determining the specific mechanisms by which these transformations take place by comparing the positions of tracers in reactants and products; 3. determining the absolute amounts of metabolites using isotope dilution; and 4. determining reaction rates in situations such as the dynamic steady state of metabolism.

The choice between using radioactive or stable isotopes in metabolic studies at different times in this century has been dictated mainly by two considerations:

1. availability of necessary analytical methods
2. availability of isotopically enriched substrates.

The first tracer experiment carried out with a living subject was Hevesy's study of lead distribution in plants in 1923 (1). The radioactive isotope of lead used in this study was available at that time as a product of radium decay. Although this study was not very interesting from a strictly biological standpoint, it did illustrate the basic principles of the tracer technique and also the fact that isotope availability can dictate the kind of experiments that are done, i.e., at that time only the naturally occurring radioactive isotopes of heavy metals were available for tracer work.

The first generally useful isotopic tracer to become available for metabolic studies was the stable isotope of hydrogen of mass 2, discovered in 1932 by Urey who named the isotope deuterium (2). Shortly after this discovery, Urey's laboratory developed methods for producing substantial amounts of highly enriched deuterium gas and deuterium oxide (heavy water). This supply of deuterium soon made possible the first metabolic tracer experiment utilizing an isotopically enriched organic substrate which was performed by Schoenheimer and Rittenberg in 1935 (3).

The result of this first experiment was a quite spectacular contribution to the understanding of the nature of metabolism. Prior to 1935 it had been intuitively felt by many biochemists that exogenous and endogenous foodstuffs would be metabolized differently in the adult organism. For example, it was thought that lipid and amino acids from dietary sources would be oxidized to provide energy for the adult and would not be incorporated significantly into the lipid and protein fractions of the body which were regarded as being essentially inert. Consequently, when Schoenheimer and Rittenberg synthesized partially deuterated linseed oil and fed this to an adult mouse, they expected most of the deuterium tracer to appear rapidly in the urine as the labeled fat was oxidized. Contrary to expectation, however, the major fraction of the deuterated fat was found to be incorporated into the body fat of the animal while only a small fraction of the tracer was recovered from the urine.

A short time later the stable isotope of nitrogen, ^{15}N, became available through the efforts of Urey and coworkers. Schoenheimer and Rittenberg subsequently synthesized amino acids enriched with ^{15}N and found that upon feeding these to adult animals, a large fraction of the tracer was incorporated into body proteins that had previously been regarded as inert (4). The results of these two tracer experiments and others led to the realization that much of an adult

organism's body is not metabolically inert, but is maintained by synthetic and degradative reactions of approximately equal rates.

During this pre-World War II period deuterium was also used to study the mechanism of synthesis of fatty acids (e.g., 5,6), cholesterol (e.g., 7), and tyrosine (8) while ^{15}N was used in studying the metabolism of amino acids and protein (e.g., 9,10)

The first biologically useful radioactive isotopes became available soon after the discovery of artificial radioactivity in 1934 by Irene Curie and her husband, Frederic Joliot (11). In 1935 Hevesy used radioactive ^{32}P to study phosphate incorporation in the rat (12). Since that time ^{32}P has become one of the most widely used isotopes in biochemistry.

A radioisotope of carbon (^{11}C) was first used in 1939 in a study of photosynthesis (13). This isotope was subsequently used during the early 1940's in studying the mechanism of glycerol fermentation (14), urea formation (15), and gluconeogenesis (16). Use of ^{11}C was restricted, however, by its short half-life of 20 minutes which necessitated it being used in close proximity to the particle accelerators in which it was made and which also prevented its position in molecules from being determined by any but the fastest procedures.

The first reported use of the stable carbon tracer, ^{13}C, was in 1940 (17). In this study Wood *et al.* used ^{13}C

enriched CO_2 to study the mechanism of CO_2 fixation by heterotrophic bacteria. The study allowed them to confirm that CO_2 was fixed into the carboxyl carbon of succinic acid - a mechanism they had previously proposed but had been unable to confirm without the use of an isotopic tracer. At approximately the same time ^{13}C was used in studying the formation of urea from CO_2 (18), the functioning of the citric acid cycle (19), and glycogen formation (20).

A stable oxygen isotope, ^{18}O, was first used as a metabolic tracer again by Hevesy and associates in 1938 (21). In this study, ^{18}O labeled sulfate was used to follow the metabolic fate of sulfate in a rabbit and it was concluded that almost all the sulfate left the rabbit's body unchanged.

After World War II, the use of radioactive isotopes came to dominate the area of metabolic tracing due mainly to the introduction of radioactive ^{14}C and the important position that carbon tracers hold in metabolic studies.

If one had to choose a single element to use in general metabolic tracing studies, that element would probably be carbon. Isotopes of carbon would be preferable to those of other elements because carbon is a constituent of almost all metabolites while elements such as phosphorous are not, and carbon isotopes, unlike those of oxygen and hydrogen, are rarely subject to chemical exchange losses. In a remarkably short time following World War II, the U.S. government began making available for research purposes, radioactive isotopes

which were by-products of atomic fission research. One of these isotopes was ^{14}C and it quickly became one of the most used tracers in studying intermediary metabolism. Its long half-life of 5100 years resulted in it almost completely replacing radioactive ^{11}C. Its greater sensitivity to detection and more facile production made its use preferable to that of the stable isotope ^{13}C.

In addition, consideration of the analytical instrumentation available at that time made the use of ^{14}C more attractive than ^{13}C. At that time the mass spectrometer was the only generally useful instrument for detecting stable isotopes. This was (and still is) an expensive and complex instrument that requires a good deal of skill and expense for operation and maintenance. In contrast, simple and inexpensive proportional and Geiger-Muller counters were available for routine detection of radioactive tracers, such as ^{14}C and ^{32}P. The subsequent availability of liquid scintillation counters for radioisotope determination made sample preparation easier and also allowed routine determination of the weak β-emitter, tritium. Scintillation counters were more expensive and complex than gas ionization counters, but it remained true that more laboratories could afford their own scintillation counters than could afford their own mass spectrometers.

However, three important developments in the 1960's and 70's have made the use of stable isotopes an increasingly attractive alternative to radioactive tracers in many

metabolic studies. One of these developments was the dramatic increase in the supply of highly enriched stable isotopes since 1970. Programs such as those at the Los Alamos Scientific Laboratory have made available large amounts of rare stable isotopes such as ^{13}C, ^{15}N, and ^{18}O at greater than 90 atom % enrichment and at moderate cost.

The other two developments were advances in analytical instruments for detecting stable isotopes. These two advances were the introduction of the combined gas chromatograph-mass spectrometer (GC-MS) in 1957 by Holmes and Morrel (22) and the Fourier Transform NMR spectrometer by Ernst and Anderson in 1966 (23). The principal advantage of GC-MS was that it allowed the analysis of complex mixtures of compounds by mass spectrometry. Fourier Transform (FT) NMR provided great gains in instrumental sensitivity over the older continuous wave instruments, and thus made ^{13}C NMR studies feasible. The great advantage of using stable isotopes and ^{13}C NMR or GC-MS analysis in metabolic studies is that quantitative and tracer data can be obtained for complex metabolite mixtures without tedious mixture separations and chemical degradations of metabolites that are required when radioactive tracers are used.

C. The Relative Merits of Radioactive Versus Stable Isotopes In Metabolic Studies

It should be mentioned at this point that in tracer work involving certain elements, there is little choice in whether to use stable or radioisotopes. For example, since

the radioisotopes of oxygen and nitrogen have half-lives of less than 2 minutes, one must use the stable isotopes of these elements. On the other hand, no stable isotopic tracer exists for phosphorous and one would be forced to use the radioisotope, ^{32}P. However, in view of the low rate of oxygen exchange from phosphate, it should be possible to use ^{18}O enriched phosphate as a phosphorous tracer in many cases.

The main advantages of using radioactive isotopes are:

1. their high sensitivity to detection
2. the relative low cost of analytical instrumentation.

The major disadvantages in using radioisotopes are:

1. the somewhat cumbersome handling precautions required by their radioactivity
2. when dealing with complex metabolite mixtures, the mixture must be separated into pure components and the components then degraded by tedious chemical methods in order to obtain important data such as the position and relative amount of tracer in the components.

The principal disadvantages in using stable isotopes are:

1. their relatively low sensitivity to detection
2. the high cost of required analytical instruments (NMR and mass spectrometers).

The major advantages in using stable isotopes are:

1. freedom from handling precautions
2. in dealing with metabolite mixtures, the relative amount and position of tracers in individual components can often be determined with NMR or GC-MS with much less effort than is required with radiotracers.

The area of metabolism of interest in this study was cellular intermediary metabolism. A study of cellular intermediary metabolism requires analysis of a complex mixture of metabolites. Consideration of this fact and the last mentioned advantage of stable isotopes led to their being used in this work.

D. NMR, GC-MS and the Study of Metabolism

NMR spectroscopy is a true form of spectroscopy which is based upon the interaction of atomic nuclei having magnetic moments with radiofrequency radiation. It is not a particularly sensitive spectroscopy; but it is noninvasive and amazingly versatile, having the potential to supply information such as molecular structure, (and positive identification of compounds), molecular conformation, relative amount and position of isotopic tracers in molecules, molecular motion in solution, rates of chemical reactions, solution viscosity, and solution pH.

The advent of Fourier Transform instruments, high field superconducting magnets, and improved probe designs have

dramatically increased NMR sensitivity in recent years and allowed application of its varied potentials to biological problems. Two of the nuclei used most for *in vivo* NMR metabolic studies are ^{31}P (natural abundance 100%) and ^{13}C (natural abundance 1.1%).

Because the chemical shift of inorganic phosphate is pH sensitive, ^{31}P NMR has been used to measure intracellular pH and transmembrane pH gradients in *E. coli* (24), yeast (25), erythrocytes (26), and rat muscle (27). In these living samples it was also possible to simultaneously detect and estimate important phosphate metabolites such as nucleoside phosphates, creatine phosphate, and sugar phosphates. The estimation of these phosphorylated metabolites was facilitated by the known isotope content (100% ^{31}P), but since no other stable isotope of phosphorous exists, ^{31}P NMR has not been used for metabolic tracer work.

Much of the potential of ^{13}C NMR in studying biological problems is due to the great resolution of ^{13}C NMR signals. This resolution is a consequence of the intrinsically large range of chemical shifts (~200 ppm) and the relatively long relaxation times of ^{13}C nuclei of small molecules which result in signals of narrow line width.

For many years, the problem of low sensitivity prevented the extensive application of ^{13}C NMR to biological problems, but the advances in instrumental sensitivity mentioned above and the increasing availability of ^{13}C enriched biomolecules have done much to offset this problem.

^{13}C Enrichment of biological substrates has made possible ^{13}C metabolic tracer experiments which are similar in many respects to those using radioactive ^{14}C.

One area in which such ^{13}C tracer studies have proved of great value is the biosynthesis of complex natural products. Compounds such as antibiotics, steroids, porphyrins, and bile acids are large and complex, and it has often been difficult to develop degradative procedures for locating radioactive tracers in such compounds.

However, no chemical degradations are required to locate the positions of ^{13}C tracers in these compounds using ^{13}C NMR. The ^{13}C NMR spectrum will often reveal the position and relative amount of ^{13}C labels incorporated from specifically ^{13}C labeled precursors. Provided the ^{13}C signals of the spectrum have been correctly assigned to the corresponding carbon atoms of the natural product, the biosynthetic mechanism involved can usually be deduced from a comparison of the positions and relative amounts of ^{13}C label in both the precursor and final product. Examples of such studies include those involving rifamysin S (28), vitamin B-12 (29), cephalosporin (30), penicillin (31), and bile acids (32).

The most elegant applications of ^{13}C NMR in metabolic studies have been the <u>in vivo</u> studies of cellular metabolism. In these experiments, a thick suspension of living cells is fed a substrate which has been specifically and highly enriched with ^{13}C. Subsequent NMR spectra of the

suspension will reveal a diminution of the substrate's ^{13}C signal as it is metabolized by the cells and the appearance of the ^{13}C tracer in various positions of intermediary metabolites and end products. Examples of such studies are those involving blue-green algae (33), E. coli (34,35), rat hepatocytes (36,37), erythrocytes (38), and Propionibacterium shermanii (39). Similar studies of living tissue instead of cell suspensions include those of perfused mouse liver (40,41), perfused mouse heart (42), and soybean (43).

In these studies, determining the position of ^{13}C label in the metabolites is relatively easy provided enough labeled metabolite is present. However, determination of the percent isotope-labeled species of these metabolites (percentage(s) of metabolite containing one or more atom(s) of isotopic tracer) can present problems in complex mixtures. Observation of the single bond spin coupling of a ^{13}C nucleus to an adjacent ^{13}C or ^{1}H atom is necessary in order to obtain the relative amount of ^{13}C at any particular position of a metabolite. In the case of an adjacent ^{13}C nucleus, the coupling can be difficult to observe if the adjacent position contained ^{13}C only at the natural abundance level of 1.1%. Spin coupling to an adjacent ^{1}H nucleus using proton NMR would be much easier to observe in terms of sensitivity. However, the inherently small chemical shift range of protons often produces signal overlap in the spectrum of a complex mixture. In addition, if

the ^{13}C nucleus of interest was not bonded to a ^{1}H atom, this coupling could not be observed.

In spite of these potential problems, the convenience, noninvasiveness, and ability to determine the position of ^{13}C label in many metabolites simultaneously, make such *in vivo* ^{13}C NMR studies quite attractive.

The gas chromatograph-mass spectrometer is a combination of two instruments that were more or less developed and used independently before being joined together in the late 1950's. In their most sensitive forms, both the GC and MS detect compounds of interest as gaseous phase ions. As with most analytical systems based upon ion detection, GC and MS have the potential to be very sensitive.

Liquid-liquid partition chromatography was developed by Martin and Synge in 1941 as a means of separating amino acid derivatives (44). In their report of this work, they predicted that the moving liquid phase could be replaced with a moving gas phase. Although this paper was widely read, the prediction was not tested until 10 years later when Martin and James used gas-liquid partition chromatography (GC) for the first time to separate and quantitate mixtures of volatile fatty acids (45) and alkyl amines (46). Since that time GC has become one of the most versatile and widely used analytical methods in the physical sciences.

The columns used in GC continue to undergo changes in design, solid supports, and liquid phases but the most important innovations had been introduced by the early

1960's. These innovations include the many relatively inert solid supports and liquid phases, particularly the diatomaceous supports and polysiloxane phases for packed columns. The introduction of open tubular (so-called capillary or Golay columns) GC columns by Golay in 1958 (47), has made possible much greater resolutions than are available from packed columns.

Sensitive GC detectors were very important to the overall development of GC, and many different detectors have been introduced over the years. Perhaps the most important of these continue to be the argon ionization and related detectors of Lovelock (48) and the flame ionization detector of McWilliam and Dewar (49).

The scope of GC (or GC-MS) application in metabolic studies is determined mainly by the stability and volatility of the metabolites to be analyzed. Some metabolites, such as volatile alcohols and carboxylic acids, can be analyzed in an underivatized form. However, many important metabolites such as hydroxy acids, polyols, phosphate esters, amino acids, etc., are quite polar and are not sufficiently volatile for GC analysis. A large number of derivatization procedures have been developed, however, to increase the volatility and stability of polar metabolites, making them amenable to GC analysis. These derivatizations include different acylations and alkylations, but perhaps the most versatile and widely used derivatization is silylation, whose value was demonstrated in a classic paper by Sweeley,

Bentley, Makita, and Wells (50). Strong silylating reagents can produce stable derivatives of alcohols, amines, carboxylic and phosphoric acids and enolizable ketones. This makes possible the single step derivatization, under mild conditions, of a complex mixture of metabolites of diverse chemical classes.

The first crude mass spectrometer can be considered that developed and used by J.J. Thomson in 1912 to demonstrate the multiplicity of stable isotopes of neon. In this simple apparatus the sample was ionized in the gas phase, the ions accelerated by an electric field and then deflected by magnetic and electric fields before being recorded. Most of these basic features are still found in modern mass spectrometers. Many instrumental design improvements were introduced by Aston, Dempster, and Nier among others. Today, the most commonly used spectrometers in biochemical work are the magnetic sector and quadrupole types, similar to those designed by Nier (51) and Paul and Raether (52), respectively.

The gas chromatograph and the mass spectrometer are both instruments for the analysis of small amounts of organic compounds in the gas phase. Contemplation of this fact led to the realization that these instruments might be coupled together and that such a combined instrument would indeed be very powerful. For the gas chromatographer, a mass spectrometer used as a GC detector would offer

unparalleled opportunities for identification and characterization of separated components. On the other hand, a GC inlet would offer the mass spectroscopist a convenient and rapid way of analyzing complex mixtures. The first combined GC-MS analysis was reported by Holmes and Morrel in 1957 (22).

Because the operating pressure of a packed column GC is ~760 mm Hg while that of a MS is ~$1x10^{-6}$ mm Hg, it was necessary to develop devices that would preferentially remove carrier gas from the GC effluent without undue sample loss before GC-MS could be routinely used for complex mixture analysis. The most important of these devices is the fritted glass tube introduced by Watson and Biemann in 1964 (53) and the jet separator reported about the same time by Ryhage (54). These efficient separators concentrate samples of interest and provide great sensitivity gains for GC-MS. They were the last major technical innovations necessary for the routine application of this powerful instrument to biological problems.

In the analysis of a complex mixture by GC-MS, the mixture components are first separated on the GC column. As each purified component leaves the GC, it is concentrated, if necessary, at the GC-MS interface and then enters the ionization chamber of the mass spectrometer. Here approximately 0.1% of the component is converted to positive ions by electron impact or chemical ionization. These ions then fragment in a way that is dependent on their potential

energy content and structure. The fragment ions are accelerated into the mass analyzer of the instrument where they are separated according to mass (or more accurately, according to their mass-to-charge ratio, m/z). The separated ions are finally recorded and the data presented as a function of relative intensity versus m/z value which is called the mass spectrum of the compound.

The mass spectrum can provide very important information concerning the compound. The GC retention time and mass spectrum of a compound are usually sufficient to unambiguously confirm its identity. In the case where the mass spectrum cannot be matched to that of a known substance, further analysis of the spectrum can often solve the unknown's identity. First of all, a molecular weight for the compound can usually be determined, at least by using chemical ionization. Secondly, if a double focusing mass spectrometer is used, an elemental composition can also be obtained. Finally, the fragmentation pattern of the mass spectrum is a function of molecular structure, and an experienced person can often deduce a structure from a mass spectrum.

The mass spectrum of a compound can also furnish information about its isotope content. Since isotopically labeled or unlabeled ions are detected with equal facility, the relative isotope content of the fragment ion is usually readily measurable. In addition, if the fragmentation of

the molecule can be interpreted in a straightforward way, information concerning the positions of isotopic labels in the molecule can also be obtained.

Finally, as in NMR, the intensity of a signal measured in mass spectrometry is proportional to the amount of material producing the signal. This, in principle, makes possible quantitative determination of compounds.

GC-MS analysis has been used extensively in a number of areas of biochemistry. Some of the most important of these areas include analysis of complex mixtures of natural products such as food flavors (e.g., 55), fatty acids (e.g., 56), and terpenoids (e.g., 57); analysis of body fluids for endogenous metabolites such as urinary acids (e.g., 58, 59), urinary steroids (e.g., 60) and plasma steroids (e.g., 61); analysis of drug metabolites in body fluids (e.g., 62); and analysis of the primary structure of polymers, such as peptides (e.g., 63) and oligosaccharides (64). Stable isotopes, such as ^{2}H, ^{15}N, ^{18}O, and ^{13}C, have been used in many GC-MS studies both in metabolic tracer experiments and in the form of internal standards for the quantitation of metabolites using isotope dilution.

E. The Relative Merits of Using ^{13}C NMR Versus GC-MS in Metabolic Tracer Studies

It was the ultimate aim of this research to study cellular intermediary metabolism using carbohydrates labeled with stable isotopes. The use of cell-free systems in studying cellular metabolism has provided invaluable

information, such as the mechanism of individual reactions and the factors that control the activity of the enzyme catalysts of these reactions. It is still necessary, however, to study the integrated whole of cellular metabolism, i.e., the metabolism of intact cells. This is necessary not only to confirm that metabolic control factors detected *in vitro* also obtain *in vivo*, but also to determine if additional factors are operative which escaped *in vitro* detection.

In studying the metabolism of intact cells, measurement of the following three entities are often needed:

1. the position of isotopic tracers in metabolites
2. the percent isotope-labeled species (analogous to specific activity in radioisotope work) of metabolites enriched with stable isotopes
3. the absolute amounts of metabolites at various points in time.

In principle, both ^{13}C NMR and GC-MS can provide these three measurements for a number of metabolites in a complex mixture.

As discussed above, the advantages of *in vivo* ^{13}C NMR studies are convenience, non-invasiveness, and a generally unrivaled ability to determine the precise position of ^{13}C tracer in metabolites. The principle disadvantage of ^{13}C NMR is that it is a relatively insensitive method for detecting ^{13}C. This can make difficult the detection of minor

and unlabeled metabolites and also the determination of the percent isotope-labeled species and absolute amounts of labeled metabolites.

The relative disadvantages of using GC-MS in such studies are that more sample manipulations are usually required and the position of ^{13}C tracers in metabolites can not usually be determined with the precision available with ^{13}C NMR. The principle advantage of GC-MS is that it is inherently much more sensitive than ^{13}C NMR in detecting either ^{13}C enriched or unenriched metabolites. In many cases as little as 10 ng of an unlabeled metabolite can be analyzed with GC-MS while over 100 times this amount is required for ^{13}C NMR analysis. In the mass spectral analysis, unenriched and isotopically enriched metabolite ions are detected with equal facility (e.g., 65); making the percent isotope-labeled species of metabolites more readily measurable.

It was mainly the sensitivity advantage of GC-MS which would facilitate the detection of minor and unlabeled metabolites and the measurement of the percent isotope-labeled species of labeled metabolites which led to GC-MS being chosen as the analytical method of this study of cellular metabolism.

F. Statement of the Problem - Part I.

GC-MS has in the past been used to analyze the pertrimethylsilylated derivatives of a variety of important metabolites in both synthetic mixtures and body fluids. These metabolites are of such diverse chemical classes as citric acid cycle acids (e.g., 66, 67), sugar phosphates (e.g., 68, 69), and inorganic ions, such as phosphate and sulfate (70).

These studies suggest that GC-MS analysis could have great potential in studying cellular intermediary metabolism. However, a search of the literature failed to provide an evaluation of this potential. It was for this reason that the study reported here was undertaken.

The necessary first step was to determine if GC-MS could be used to obtain mass spectra of important intermediary metabolites from a metabolizing cell system. The cell system used in this study was an E. coli fermentation of glucose.

Early in the course of this study, it became evident that extraneous compounds interfered with the GC-MS analysis of the metabolite mixture. Compounds such as free sugars or buffer salts resulted in samples which were difficult to dry before their derivatization with a water sensitive silylating reagent. As a consequence, these samples required large excesses of derivatizing reagent with attendant dilution and loss of sensitivity. In addition, these compounds often formed volatile derivatives which obscured

metabolite signals during analysis. Perchloric acid (used initially to extract the cells) causes decomposition of the metabolite derivatives.

The subject of this thesis is the ion exchange techniques that were developed to remove compounds (glucose, HEPES buffer, perchlorate) which interfered with the GC-MS analysis of the bacterial fermentation. Also described is the GC-MS analysis of the intermediary metabolism of *E. coli* these ion exchange techniques made possible and an application of the analytical system to a study of the production of leucic acid using [1-^{13}C] and [2-^{13}C] glucose. Possible extensions of the analytical system as well as its merits and disadvantages are discussed.

G. Statement of the Problem - Part II.

In performing metabolic tracer experiments, it is necessary to have not only an analytical system but also isotopically enriched substrates. In recent years much of the work of this laboratory has been directed toward developing improved syntheses of monosaccharides enriched with stable isotopes of carbon (71, 72, 73), hydrogen (74), and oxygen (74). These isotopically enriched monosaccharides were used mainly in NMR studies of solution conformation of carbohydrates, but they can equally well be used as substrates in metabolic tracer experiments. Recently, it appeared likely that the synthesis of aldoses

enriched with oxygen isotopes (^{17}O,^{18}O) could be improved, and an account of this study constitutes the second part of this thesis.

Aldoses labeled with oxygen isotopes have value as substrates in metabolic tracer experiments (75), in obtaining such basic information as mass spectral fragmentation mechanisms (76), and NMR parameters (77, 78) of sugars, and as internal standards in the quantitation of sugars using isotope dilution.

To date, two of the most extensive studies (76, 77) of labeling monosaccharides with oxygen isotopes relied on synthetic methods that are not generally applicable to the 4-6-carbon aldose family.

Serial application of the cyanohydrin synthesis (74) can be used to produce aldoses labeled at any or all positions with oxygen isotopes. However, this general applicability is offset by low product yields for a particular aldose after two or more serial applications of the synthesis.

Recently, an unusual rearrangement of aldoses was discovered (79) which involves the molybdate catalyzed inversion of the fragment comprising the first two carbons of a 4-6-carbon aldose. This fragment inversion results in a 2-epimerization of the starting aldose as illustrated for the interconversion of [1-^{13}C] mannose and [2-^{13}C] glucose in Figure 1.

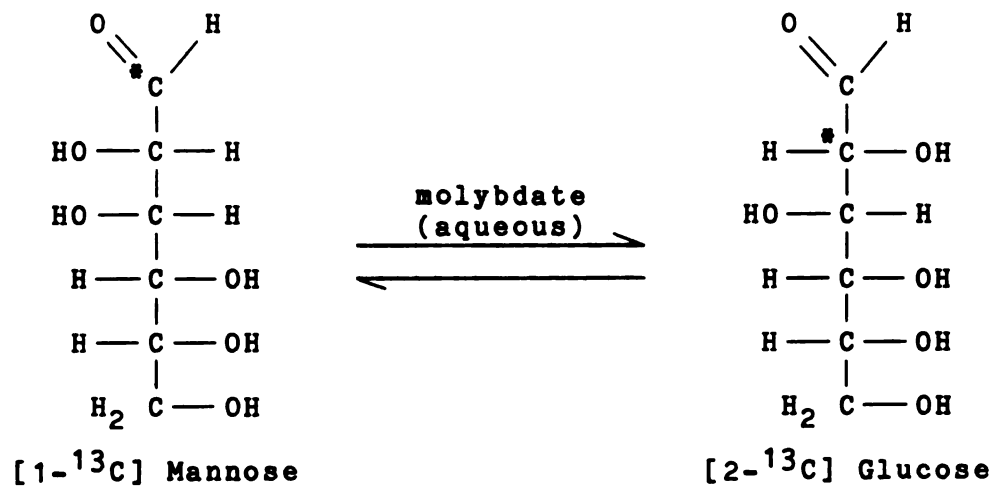


Figure 1. Interconversion of [1-^{13}C] mannose and [2-^{13}C] glucose by molybdate epimerization.

Asterisk denotes ^{13}C atom.

Since it had been established (79) that carbon and hydrogen isotopes at position-1 of a starting aldose are transferred to position-2 of its 2-epimer in this epimerization, it seemed likely that this would also be the case for oxygen isotopes at position-1. This being true, both aldoses would become labeled at position-2 if the reaction were carried out in $H_2{}^{18}O$, as shown in Figure 2.

These considerations gave rise to a synthetic scheme in which the molybdate epimerization could be used in conjunction with the cyanohydrin synthesis and readily available starting materials to produce aldo-tetroses, pentoses, and hexoses labeled with ^{18}O as shown in Figure 3.

This synthetic scheme has advantages over the previously mentioned approaches (74,76,77) in terms of either general applicability or product yields as will be discussed.

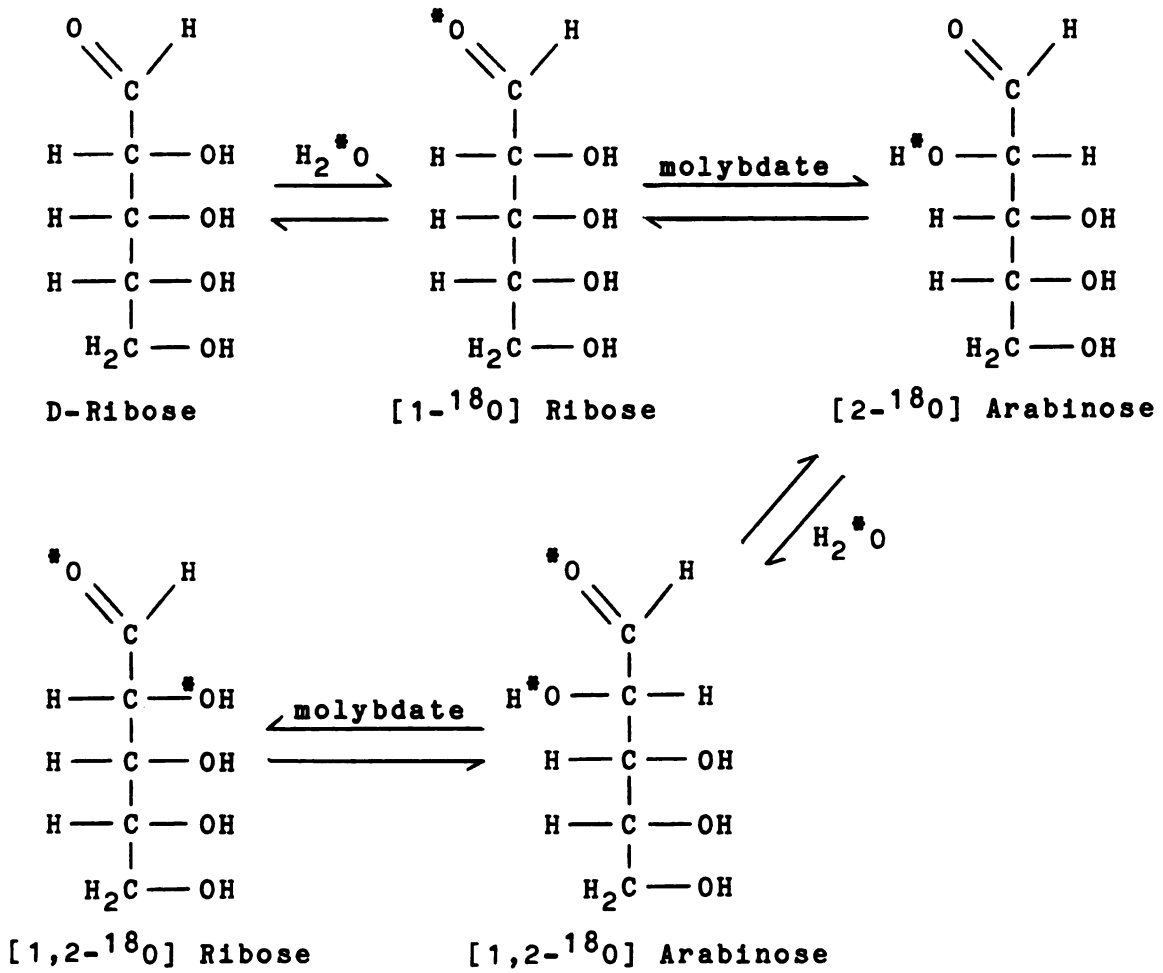


Figure 2. Proposed mechanism for enriching both epimeric aldoses with ^{18}O at position-2 during molybdate epimerization.

Asterisk denotes an ^{18}O atom

Figure 3. Synthetic scheme for the synthesis of 22 different ^{18}O labeled aldoses.

Abbreviations: Ery = erythrose, Thr = threose, Rib = ribose, Ara = arabinose, Glc = glucose, Man = mannose. Mo = molybdate epimerizations in $H_2{}^{18}O$ except in the case of [5-^{18}O] ribose in which the reaction medium was $H_2{}^{16}O$. CN^- = cyanohydrin synthesis (these reactions were carried out in normal $H_2{}^{16}O$ but the reaction steps are the same as those discussed in section III, Part II, C; and illustrated in Figure 16 where the cyanohydrin synthesis is carried out in $H_2{}^{18}O$ as a method of producing [2-^{18}O] aldoses). Astrisk denotes ^{18}O atom.

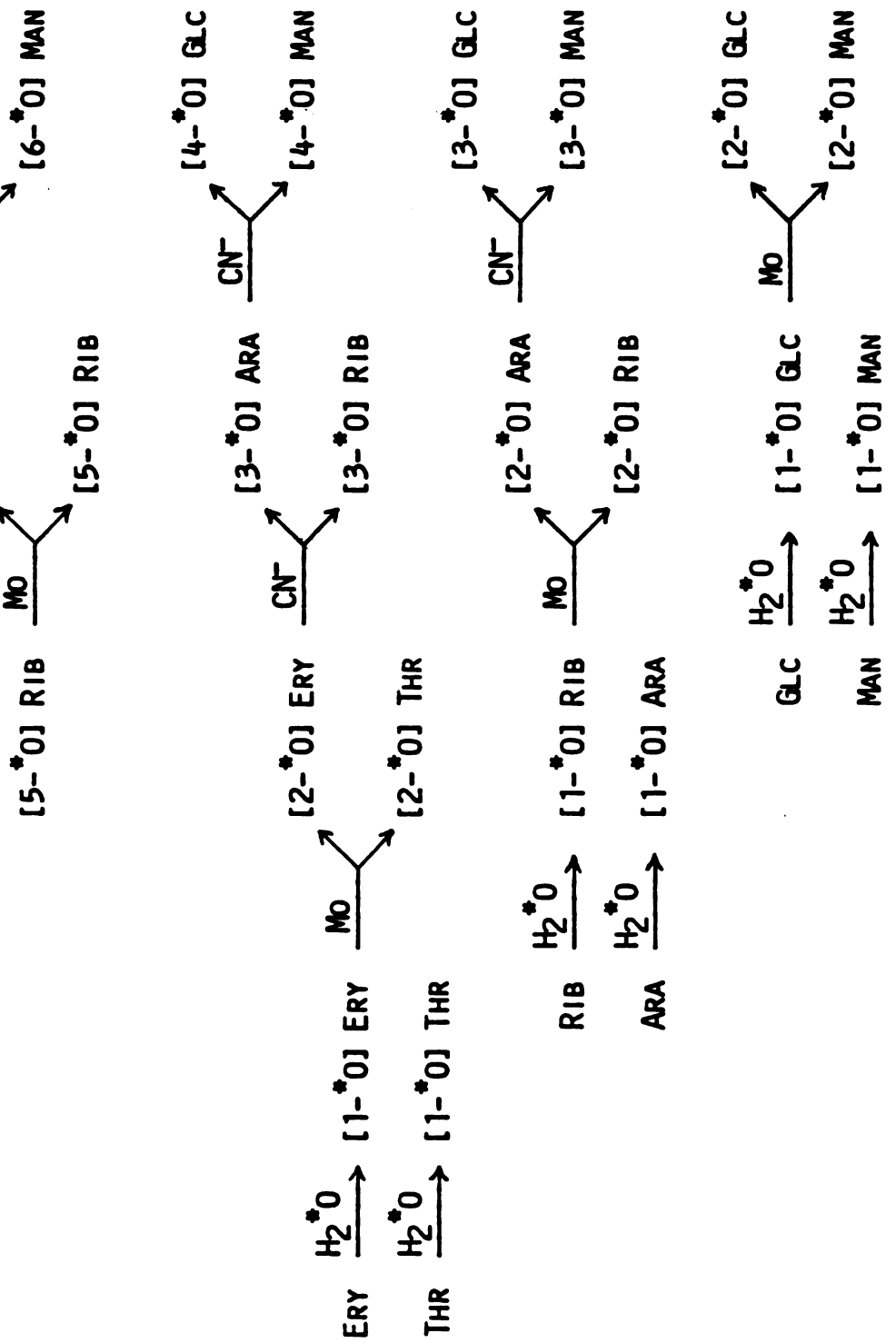

ERY
THR
RIB
ARA
GLC
MAN
H2*O
MO
CN⁻
[1-*O] ERY
[1-*O] THR
[1-*O] RIB
[1-*O] ARA
[1-*O] GLC
[1-*O] MAN
[2-*O] ERY
[2-*O] THR
[2-*O] ARA
[2-*O] RIB
[2-*O] GLC
[2-*O] MAN
[3-*O] ARA
[3-*O] RIB
[3-*O] GLC
[3-*O] MAN
[4-*O] GLC
[4-*O] MAN
[5-*O] RIB
[5-*O] ARA
[5-*O] RIB
[6-*O] GLC
[6-*O] MAN

Figure 3

II. EXPERIMENTAL

Part I. GC-MS Analysis of Cellular Metabolism

A. Reagents

All deionized water used in these procedures, including resin preparation, was "HPLC grade", i.e., purified by an ion exchange-reverse phase chromatography system similar to the Millipore "Milli-Q" system.

Glacial acetic acid, hydrochloric acid, potassium hydroxide, and sodium hydroxide were Mallinckrodt AR reagents. Acetonitrile, sodium bicarbonate, potassium bicarbonate, and 70 % perchloric acid were Fisher reagent grade. Lithium hydroxide and trifluoroacetic acid (TFA) were from Aldrich. Dowex 50x8, 200-400 mesh, [H^+] resin, and N-2-Hydroxyethylpiperazine-N'-ethane sulfonic acid (HEPES) were from Sigma. AG MP-1, 200-400 mesh, [Cl^-] resin was purchased from Bio Rad. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was a product of Pierce, and glucose used in the bacterial fermentation was from MCB Corporation. ^{14}C Labeled glucose-6-posphate, fructose-1,6-diphosphate, succinic acid, and citric acid were ICN Corporation products. [1-^{13}C] Glucose (99 atom % ^{13}C) was synthesized by the method of Serianni *et al.* (71) and [2-^{13}C] glucose (99 atom %^{13}C) by the method of Hayes *et al.* (79).

B. Resin Preparation

Dowex 50x8, 200-400 mesh [H^+] cation exchange resin was de-fined by three successive resuspensions and decantations in water and then de-gassed under water for 10 min on an aspirator. The resin was packed into an all-polyethylene column and washed with two cycles of 1 M NaOH, water, 2 M HCl, and water. The lithium form of the resin was obtained by eluting a column of the purified hydrogen form with 1 M LiOH and then rinsing to neutrality with water. The resin was stored at room temperature under chloroform-saturated water in glass bottles with well-sealing, polyethylene lined screw caps.

AG MP-1, 200-400 [Cl^-] anion exchange resin was de-fined and de-gassed as above and then converted to the bicarbonate form in a column using 0.8 M $NaHCO_3$ at 4° until the effluent gave a negative chloride test with 5 % $AgNO_3$ in 5 M HNO_3. The resin was rinsed to neutrality with water, transferred to a filter flask, and converted to the acetate form at room temperature by adding 2 M acetic acid with swirling until an excess of acid had been added and CO_2 evolution had ceased. The resin was further degassed by gentle magnetic stirring under an aspirator vacuum. The resin was packed in a column, eluted with 2 M acetic acid, rinsed to neutrality, and stored under chloroform-saturated water.

C. Column Preparation for Metabolite Isolation

1. Cation exchange column

The cation exchange column was packed with both the hydrogen and lithium forms of Dowex 50 resin. This column had the dual function of first removing HEPES buffer on the hydrogen resin and then neutralizing the extract with the lithium resin before it was transferred to the anion exchange column.

The column (see Figure 4) was constructed from a 5.5 cm long piece of 5 mm o.d. Pyrex tubing; two 1.5 cm long plastic luer fittings cut from disposable Becton-Dickinson 1 ml tuberculin syringes; two resin supports of Whatman GFD glass fiber filter cut with a #3 cork borer; and two "glass needles" made from Yale brand (Beckton-Dickinson) disposable hypodermic needles by pulling the steel cannula from the plastic hub, enlarging the cannula hole with a #49 drill bit, and press fitting a fire polished, 1.3 cm length of a 100 µl Pyrex disposable micro sampling pipet (Corning #70995-100) into the hub, using a paper or tape covered surface for support and water for lubrication. The "glass needles" provided a convenient connection to the anion exchange column through a short piece of polyethylene tubing.

The column was assembled by first filling a needle and luer fitting with water and then pressing a glass fiber

Figure 4. Ion exchange system used for cell extract purification prior to GC-MS analysis.

Cation exchange column (containing Dowex 50 resin), and anion exchange column (containing AG MP-1 resin), are shown in cross section. Details concerning dimensions, construction, and operating conditions are given under section II, Part I, C & E.

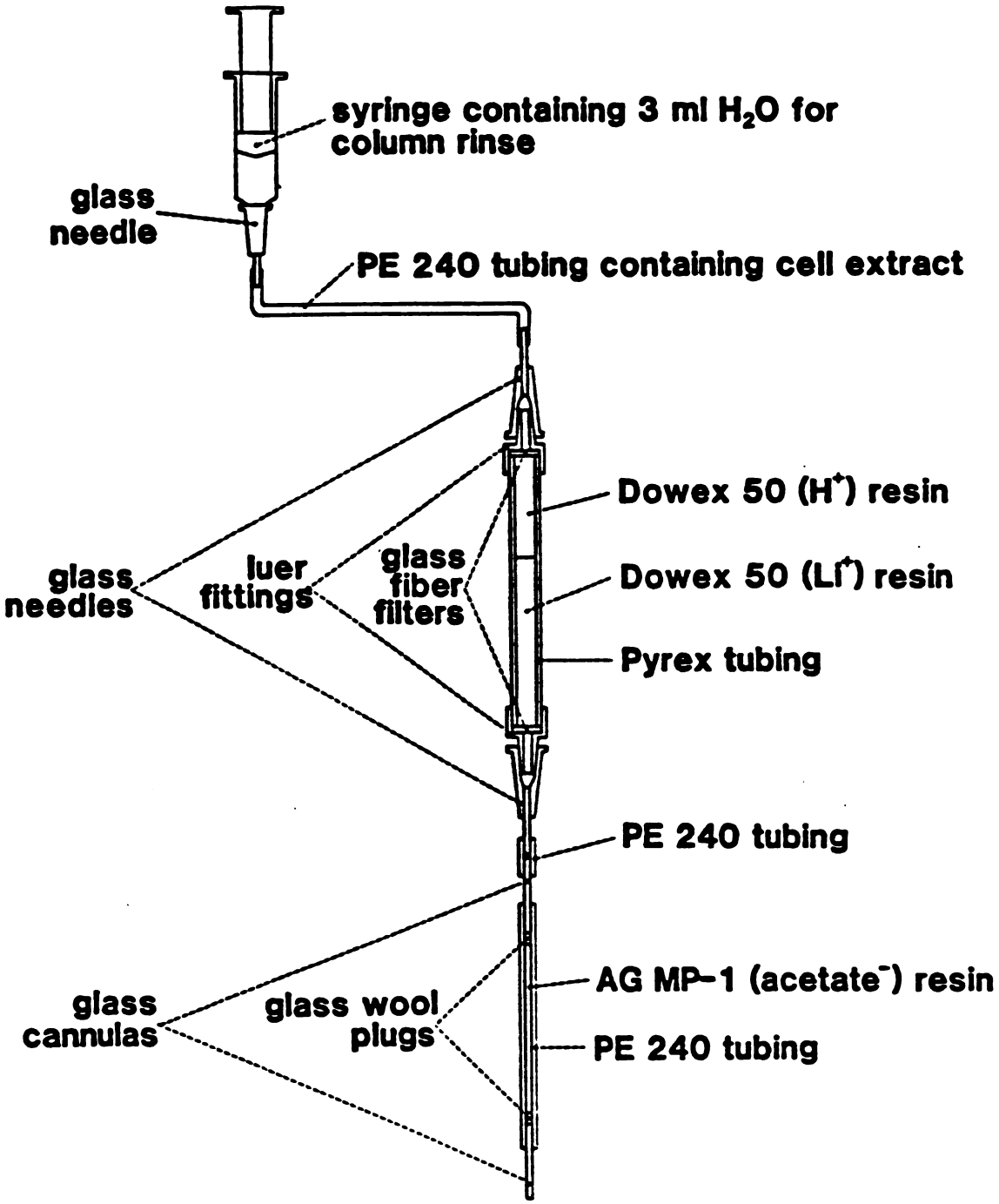

syringe containing 3 ml H_2O for column rinse
glass needle
PE 240 tubing containing cell extract
Dowex 50 (H^+) resin
Dowex 50 (Li^+) resin
Pyrex tubing
PE 240 tubing
AG MP-1 ($acetate^-$) resin
PE 240 tubing
glass needles
luer fittings
glass fiber filters
glass cannulas
glass wool plugs

Figure 4

filter to the bottom of the luer fitting, being careful to exclude air bubbles. The Pyrex tubing was then pressed into the luer fitting, trapping the filter between the glass and plastic pieces. (The ends of the glass tubing should be fire polished so they will collapse just enough to provide a satisfactory, leak-free press fit.) After washing broken glass fibers from the column, it was packed to a depth of 3.4 cm with 310 µl of Dowex 50 [Li^+] resin. The remainder of the column was packed with about 190 µl of Dowex 50 [H^+] resin, and the inlet end of the column was closed with another water-filled assembly of glass needle, luer fitting, and glass fiber filter. The Dowex 50 column was rinsed with 5 to 10 ml of water to remove color throw just before the anion exchange column was attached to its outlet.

2. Anion exchange column

The anion exchange column containing 75 µl AG MP-1 [$acetate^-$] resin was used to bind anionic metabolites and perchlorate, separating them from excess glucose of the fermentation.

The column was constructed by first fire polishing one end of a 1.5 cm length of 100 µl Pyrex sampling pipet until the end collapsed, leaving an opening of about 0.5 mm. This end was inserted into an 8 cm long piece of PE-240 Intramedic polyethylene tubing (Beckton-Dickinson), and the assembly filled with water. A resin support consisting of a twisted, wetted thread of glass wool about 1 cm long was

then inserted into the plastic tubing using a large sewing needle with the end of its eye cut off. The glass wool was pressed into a firm, even layer at the bottom of the column using the plunger of a 100 µl Hamilton syringe. After rinsing broken glass fibers from the column, it was packed with a suspension of MP-1 [acetate$^-$] resin to a depth of 3.4 cm and rinsed with 2 ml of water using a syringe fitted with a glass cannula needle. The resin bed was then immobilized with a second glass wool plug. After cutting off the excess tubing about 0.5 cm above this plug, a second water-filled piece of glass tubing was pressed tightly into the plastic tubing and against the glass wool. It is important that air bubbles be excluded during all of these steps.

The completed column was held upright by inserting it into a 4.3 cm length of 4 mm o.d. Pyrex tubing whose ends had been fire polished just enough to give a snug fit. The MP-1 [acetate$^-$] column was attached to the outlet needle of the Dowex 50 column using a short length of PE-240 tubing. The tandem columns were allowed to equilibrate at 4°, 0.5-1 h before applying a cell extract.

D. Bacterial Fermentation

E. coli (strain K-12, auxotrophic for proline and rifampicin resistant) used in these studies were the gift of Dr. Robert P. Mortlock.

The basic salts growth medium (80) was supplemented with 5 g of glucose, 0.2 g L-proline, 0.2 g $MgSO_4$ and 2 mg thiamine·HCl per liter. The various additions and the salts medium were autoclaved separately. Cells were grown as a 1600 ml batch in a 2800 ml Fernbach flask on a New Brunswick model G-76 gyrorotary shaker with a bath temperature of 37° and shaking speed of 11. The cells were harvested at mid-log phase (OD_{660}=0.3-0.4 or $4x10^8$ to $5x10^8$ cells per ml) by rapidly cooling the culture to 10° in a dry ice-2-propanol bath, while maintaining magnetic stirring and efficient aeration using compressed air. The cells were then collected by centrifugation at 4° and washed three times with 25 ml volumes of cold, 0.1 M HEPES-KOH, pH 7.5. The washed cell pellet was resuspended to a final volume of 4.0 ml with cold, 0.2 M HEPES-KOH, pH 7.5 in plastic γ-counting vial (Wheaton). A magnetic stirring vane was added, and the stirred cell suspension allowed to warm to room temperature (22-25°).

Fermentation was started by adding 100 µl of 0.5 M glucose to the stirred cells. At predetermined times, 0.5 ml samples were taken for analysis and added to plastic γ-counting vials containing 50 µl of 70 % perchloric acid and a magnetic flea. The suspension was stirred rapidly for 10-20 s, then frozen for 5 min in a dry ice-2-propanol bath. After thawing, the suspension was immediately centrifuged at 39,000 x g at 4° for 5 min. The

supernatant was transferred with a plastic tipped pipet to a clean γ-vial in an ice bath. Immediately, 0.5 µl n-octanol was added to prevent permanent foaming, and a predetermined amount of $KHCO_3$ (in this case 46 mg) was then added to neutralize the rapidly stirred extract. The neutralized extract was allowed to stand an additional 10 min on ice, and the precipitated $KClO_4$ was removed by centrifugation at 39,000 x g at 4° for 5 minutes. The supernatant was again transferred to a clean γ-vial and purified by ion-exchange or frozen at -20° until it was convenient to process it.

Carbon-13 tracer experiments were performed as above except that the 4.0 ml cell suspension was divided into equal parts and 50 µl of either 0.5M [1-^{13}C] glucose or [2-^{13}C] glucose was added to the 2.0 ml cell suspensions to initiate fermentation. Aliquots (0.5 ml) from each fermentation were taken for GC-MS analysis at 40 min after glucose addition.

E. Ion Exchange of Cell Extract

The neutralized cell extract was purified by ion exchange at 4°. It was first drawn into a 35 cm length of PE-240 tubing using a 3 ml plastic disposable syringe fitted with a glass needle. The γ-vial was rinsed with 0.25 ml water and this volume drawn into the tubing behind the extract. The outlet end of the tubing was then attached to the inlet needle of a Dowex 50 column. The syringe was

detached from its needle, filled with 3 ml cold water as a rinse volume, and reattached to its needle. An Orion syringe pump was used to load and rinse the ion exchange columns at a flow rate of 1 drop per 30 seconds (0.07 ml/min). The effluent of the columns (3 ml volume) containing unconsumed glucose and any other neutral species can be saved for further analysis or discarded. Figure 4 is an illustration of ion exchange system used in the loading and rinse of a 0.5 ml extract volume. After rinsing, the MP-1 [acetate$^-$] column containing sorbed metabolites and perchlorate was detached from the Dowex 50 column. A 1 ml plastic, disposable tuberculin syringe fitted with a glass needle and a short piece of PE-240 tubing was filled with cold 0.35 M TFA and fitted to the inlet cannula of the MP-1 [acetate$^-$] column. The syringe pump was used to elute the MP-1 column with a total of 0.45 ml, 0.35 M TFA at 4° at a rate of 1 drop/20 seconds (0.1 ml/min). The eluted metabolites were collected in a translucent plastic γ-vial and immediately shell frozen in a dry ice-2-propanol bath. The vial was tightly fitted with a polyethylene quick disconnect tubing connector (Mallinckrodt), and the frozen metabolite volume was taken to dryness using a lyophilizer fitted with a KOH trap. The residue was left under vacuum 5-10 min after ice removal appeared complete. The residue was immediately dissolved in 0.25 ml H_2O and transferred to a 300 µl "Reacti Vial" (Pierce), leaving an air space of 50 µl in the tip of the conical vial. A 200 µl plastic pipet tip was held in

the metabolite solution with the tip just touching the air space. The solution was frozen (dry ice), the pipet tip removed, and the vial closed with a specially constructed cap and lyophilized to dryness as before. The dry residue was derivatized immediately by adding 7 µl of dry acetonitrile (stored over 4 Å molecular sieves) and 10 µl BSTFA. The vial was sealed quickly with a screw cap and teflon-lined silicone septum and heated at 60° for 3 h with occasional vortexing until the residue was completely dissolved.

F. Gas Chromatographic Analysis

The derivatized metabolites were analyzed using a Varian 2100 gas chromatograph equipped with hydrogen flame ionization detectors and 2 m x 2 mm i.d. U-shaped packed columns. The GC column was packed with 3 percent OV-101 on Gas Chrom Q, 100-120 mesh (Applied Science), to levels just below the injection and detector ports. The minimum amount of silylated glass wool necessary to immobilize the packing was used. The packed column was conditioned by first attaching its inlet end in the gas chromatograph and purging the column with helium (30 ml/min) at room temperature for 30 min. The oven temperature was then raised to 280°, and the column was left at this temperature for 8-12 h.

Operating conditions of the instrument during extract analysis were: injection port temperature = 250°, detector temperature = 300°, helium flow rate = 25 ml/min, column

oven temperature programs of 75 to 280° at 4°/min or 175 to 280° at 4°/min, electrometer range and attenuation settings at 10^{-10} and 1, respectively, and recorder attenuation about one third full scale with a chart speed of 40 cm/hr (Linear model 285 recorder).

Combined GC-MS analysis of extracts were performed on a Finnigan 3300 instrument using a similar GC column and an ionization potential of 70eV.

G. Recoveries of 4 Representative Metabolites from the Ion Exchange System

Sorption and elution efficiencies of the ion exchange system were tested by adding 4 radiolabeled metabolites to 0.5 ml neutralized extracts (taken 5 min after glucose addition) and re-isolating them using the ion exchange procedure described above. The amounts of radioactivity added were approximately 0.25 µCi ^{14}C-succinic acid (16 mCi/mmol), 0.10 µCi ^{14}C-citric acid (8 mCi/mmol), 0.10 µCi ^{14}C-FDP (180 mCi/mmol), and 0.15 µCi glucose-6-phosphate (225 mCi/mmol). Radioactivity in the initial extract volumes and in their corresponding TFA elution volumes of the MP-1 [acetate$^-$] columns were determined by liquid scintillation counting.

Part II. Synthesis of ^{18}O Enriched Aldoses

A. Materials

D-Erythrose and D-threose were prepared by the methods of Perlin (81) and Ball (82), respectively. Methyl-2,3-O-isopropylidene-β-D-ribofuranoside, D-ribose and D-mannose were from Phanstiehl Laboratories, Inc. D-Glucose was from MCB Corporation. D-Arabinose and $NaBH_4$ were from Sigma Chemical Co. Potassium tert-butoxide, methyl iodide, and chromium trioxide were Aldrich Chemical Co. reagents. 2-Propanol (Mallinckrodt) was distilled after refluxing with BaO and stored over 4Å sieves. AG MP-1, 200-400 mesh [Cl^-] resin was purchased from Bio Rad Laboratories and ammonium molybdate ($(NH_4)_6$ Mo_7 O_{24}) was a Mallinckrodt reagent. DMSO (Fisher Scientific) was refluxed with BaO and distilled at 5 mm Hg and then stored under dry argon over 4 Å sieves. Oxygen-18 enriched water (> 95 atom% ^{18}O) was provided by the Los Alamos Scientific Laboratory, University of California, Los Alamos, NM.

Molybdate AG MP-1 resin was prepared by eluting a column of the chloride form resin with 0.1 M $(NH_4)_6Mo_7O_{24}$ until the column effluent gave a negative Cl^- test with 5% $AgNO_3$ in 5 M HNO_3. The $AgNO_3$ reagent also produces a white precipitate with $(NH_4)_6Mo_7O_{24}$, but this dissolves with vigorous shaking. After the resin conversion was complete,

the column was rinsed well with distilled H_2O. The unpacked resin was first dried at 60° for 3 h and then stored in a sealed vial over Drierite.

B. General Methods

The progress of molybdate epimerizations was followed by GC analysis of the alditol acetates of the reaction products. The reaction volume was centrifuged at ~1/2 speed on a clinical centrifuge and an aliquot (0.5 µl) containing ~ 0.5-1 µmol total sugar was injected into a 100 µl Reacti-Vial (Pierce) containing 60 µl of a 2 mg/ml solution of $NaBH_4$ in dry 2-propanol. After sealing the vial and mixing the contents thoroughly, the reaction mixture was kept for 30 min at room temperature. Excess $NaBH_4$ was destroyed by adding 25 µl, 1.7 M acetic acid. Volatile reactants were removed at 65° under a N_2 stream. Boric acid was removed by mixing the dry residue with 100 µl methanol and evaporating to dryness as above. After repeating the last step twice more, the dry residue was derivatized with 7 µl dry pyridine and 10 µl acetic anhydride and heated at 80° for 30 min. One µl volumes were injected for GC analysis and a 2mm x 2m column of 3% OV-225 on Gas Chrom Q (Applied Science) was used to separate the alditol acetates using a temperature program of 170 to 225° at 2°/min.

The ^{18}O enrichment in the aldose products was determined by GC-MS analysis of their permethylated alditols. Approximately 0.5 µmol of each aldose was reduced to its alditol as described above. After removal of boric

acid, the dry residue was dissolved in distilled H_2O and passed through ~100 µl Dowex 50 x 8, 200-400 mesh [H^+] resin in a Pasteur pipet. The column effluent was evaporated 2-3 times from water to remove acetic acid and the alditol sirup was mixed with absolute ethanol in a 1 dram screw cap vial and dried in a 90° oven for several h. The vial was then tightly capped and stored over Drierite.

Potassium dimsyl reagent was prepared by dissolving 1 g potassium tert-butoxide in 3 ml dry DMSO under argon, in a tightly sealed screw cap vial for 1 h at room temperature. The clear yellow reagent containing a small amount of white solid was stored at 4° over Drierite and thawed and centrifuged before use. Samples of dried alditol were permethylated by a modification of the method of Finne *et al.* (83).

Pentoses were quantitated by the anthrone method of Bailey (84). Hexoses were assayed by the anthrone method of Bartlett (85) and tetroses were estimated by weighing desiccated sirups.

C. General Syntheses

Cyanohydrin syntheses and separation of epimeric aldoses were performed according to Serianni *et al.* (71). Total aldose yields were typical of those observed for these reactions (71).

Epimerizations were carried out by dissolving a dry sample of the starting aldose in a screw cap vial in enough $H_2{}^{18}O$ to give a 1 M solution. The vial contained a magnetic flea for stirring and was tightly sealed with a screw cap

and teflon-faced silicone rubber septum. The starting aldose was pre-exchanged with $H_2{}^{18}O$ for varying times as detailed below. Molybdate resin (17 mg dry resin per mmol starting aldose) which had been rehydrated with a small amount of $H_2{}^{18}O$ was then added. The epimerization was initiated by heating the stirred solution in an oil bath at 90° for hexoses and pentoses or 80° for tetroses for varying times given below. The reaction progress was followed by removing the reaction from the oil bath and cooling and centrifuging it. A micro syringe was then used to withdraw 0.5-1 µl of the clarified solution through the silicone septum of the vial for alditol acetate analysis as described above.

When the reaction was complete, $H_2{}^{18}O$ was recovered by lyophilization. The dry residue was redissolved in distilled H_2O and filtered through a pad of glass wool or GFD glass fiber filter (Whatman) in the tip of a Pasteur pipet. After carefully rinsing the filtered resin with H_2O, the combined filtrate and rinsing was stirred with Dowex 1x8 [HCO_3^-] resin and then passed through a column containing successive layers of Dowex 50 [H^+] and Dowex 1x8 [HCO_3^-] resin to remove ions. The epimeric aldoses were separated on Dowex 50 [Ca^{++}] columns (71).

To be certain that residual ^{18}O label in the carbonyl oxygen of the separated aldose epimerization products was removed, each aldose was dissolved in normal $H_2{}^{16}O$ to give a 0.5 M solution whose pH was adjusted to 1.95 with HCl.

These solutions were heated in sealed vessels at 60° for 8 h. Most of the H_2O was then removed on a rotary evaporator at 40°. The residue was restored to its original volume with fresh H_2O and the solution was heated an additional 6h at 60° . After cooling, the HCl was removed by neutralization with Dowex-1 [HCO_3^-] resin. The aldose could then be used in subsequent cyanohydrin syntheses.

Total aldose recoveries after molybdate epimerizations were typically greater than 92%.

[1-^{18}O] Aldoses for GC-MS analysis were made by dissolving dry aldose samples in enough $H_2{}^{18}O$ to give a 0.5 M solution, using $H_2{}^{18}O$ that had been adjusted to pH 1.95 with concentrated HCl. The aldose solutions in tightly capped vials were heated in an oil bath at 60° for 24 h. Samples (1 µl) were taken directly for GC-MS analysis of the permethylated alditols as described above.

D. Specific Syntheses

1. Aldoses derived from [5-^{18}O] ribose

Methyl 2,3-O-isopropylidene-β-D-ribo-pentadialdo-1,4-furanoside was synthesized from methyl 2,3-O-isopropylidene-β-D-ribofuranoside by the CrO_3-dipyridine method of Arrick et al.(86). The crystalline aldehyde was stored over a desiccant if it was not to be exchanged in $H_2{}^{18}O$ immediately. In a sealed vial, 400 mg of the dialdo furanoside was dissolved in 2 ml $H_2{}^{18}O$ and 0.6ml dry tetrahydrofuran. After 24 h at room temperature, the exchanged aldehyde was reduced by careful addition of 75 mg $NaBH_4$ with stirring. After 2 h

excess $NaBH_4$ was destroyed by careful addition of 50 µl glacial acetic acid and the [5-^{18}O] methyl 2,3-O-isopropylidene-β-D-ribofuranoside was extracted 3 times with equal volumes of $CHCl_3$. The remaining $H_2{}^{18}O$ was recovered by lyophilization. The $CHCl_3$ extract was evaporated at 40° to yield the [5-^{18}O] ribose derivative as a colorless sirup. The isopropylidene group was removed by stirring the sirup with 5 ml, 0.04 M HCl at 100° for 2 h. The resulting [5-^{18}O] ribose solution was deionized with Dowex 50 [H^+] and Dowex 1 [HCO_3^-] resins.

[5-^{18}O] Ribose (1 M) in $H_2{}^{16}O$ was treated with molybdate resin as described under General Syntheses to produce a mixture of [5-^{18}O] ribose and [5-^{18}O] arabinose. After 12 h at 90°, GC analysis of the alditol acetates showed the reaction had reached its equilibrium of 2:1 arabinose : ribose with about 0.5% xylose side-product. The [5-^{18}O] pentoses were separated as described above and a portion of the [5-^{18}O] arabinose was used to produce [6-^{18}O] glucose and [6-^{18}O] mannose via the cyanohydrin synthesis (71).

2. Aldoses derived from [2-^{18}O] tetroses

A desiccated sirup of D-erythrose was dissolved in $H_2{}^{18}O$ to give a 1 M solution and the solution was kept at 80° for 3 h in a sealed vial. Molybdate resin, prehydrated in a small volume of $H_2{}^{18}O$, was then added and the stirred solution was kept at 80° for 12 h. After recovery of $H_2{}^{18}O$ by lyophilization, the mixture was deionized, separated and

the tetroses incubated with $H_2{}^{16}O$ to remove exchangeable ^{18}O from the anomeric center. A portion of the [2-^{18}O] erythrose was used to produce [3-^{18}O] ribose and [3-^{18}O] arabinose via the cyanohydrin synthesis (71). After purification and separation of the pentose mixture, the cyanohydrin synthesis was applied to a portion of the [3-^{18}O] arabinose to produce [4-^{18}O] glucose and [4-^{18}O] mannose.

3. Aldoses derived from [2-^{18}O] pentoses

D-Ribose (1 M) in $H_2{}^{18}O$ was pre-exchanged at 60° for 12 h. After addition of molybdate resin, the stirred solution was heated at 90° for 8 h . At this time GC analysis of the alditol acetates showed an arabinose : ribose ratio of 1.6 : 1 and about 0.8% xylose side-product. After recovery of $H_2{}^{18}O$, the [2-^{18}O] pentoses were isolated in the usual fashion. After exchanging the purified pentoses with $H_2{}^{16}O$, a portion of the [2-^{18}O] arabinose was used to produce [3-^{18}O] glucose and [3-^{18}O] mannose by the cyanohydrin synthesis.

4. [2-^{18}O] Hexoses

D-Glucose (1 M) in $H_2{}^{18}O$ was incubated at 90° for 24 h, and then treated with molybdate resin at 90° for 16 h. The final ratio of glucose to mannose was 2.5 as determined by alditol acetate analysis. The mixture of [2-^{18}O] glucose and [2-^{18}O] mannose was purified, separated, and exchanged with $H_2{}^{16}O$.

III. RESULTS AND DISCUSSION

Part I. GC-MS Analysis of Cellular Metabolism

Before this project was undertaken it seemed likely that GC-MS could be a powerful tool for studying cellular metabolism since, in principle, it could provide an isotopic and quantitative analysis for a number of metabolites in a single run and also afford an opportunity for detecting small amounts of unknown or unexpected metabolites.

However, it was not known if a GC-MS analysis of cellular metabolism was feasible and it thus became very important to demonstrate the practicality of such an analysis. The presence of extraneous compounds in the cell extract presented formidable technical problems which were eventually overcome using the purification techniques described in the EXPERIMENTAL, Part I. section.

The first part of the present section will be devoted to the purification techniques and GC-MS analysis these techniques subsequently made possible. The second part will discuss an application of the GC-MS analysis in solving a specific biological problem. This problem was to determine the metabolic origin of leucic acid, a previously unreported metabolite in *E. coli* which was discovered in the GC-MS analysis. In the final part of this section, possible extensions of the analytical system will be discussed as well as how the system can complement other techniques for studying cellular metabolism.

A. Technical Aspects of the Ion Exchange Purification and GC Analysis of the Cell Extracts

The ion exchange procedures described in the EXPERIMENTAL, Part I. section effectively removed substances that interfere with the GC-MS analysis of bacterial metabolites.

HEPES buffer in a neutralized cell extract exists primarily as a zwitterion, but it has a tertiary amine group that allows its quantitative sorption to Dowex 50 [H^+] resin in a neutralization reaction.

After neutralization by passage through a layer of Dowex 50 [Li^+] resin, anions in the extract were sorbed to AG MP-1 [$acetate^-$] resin while glucose and other neutral species were washed from the column with water. The metabolites were then separated from the more tightly bound perchlorate by selective elution with 0.35 M TFA. It was found that metabolites were more efficiently eluted from the macroporous AG MP-1 resin than from a gel-type resin such as Dowex 1.

The sorption and elution efficiencies of the ion exchange system were checked by using radiolabeled metabolites added to the extracts before their purification by ion exchange. Percentage recoveries were 99, 97, 99, and 96 for glucose-6-phosphate, succinate, FDP, and citrate, respectively.

The entire ion exchange procedure was carried out in a cold room to minimize possible hydrolysis of the more acid-labile metabolites such as FDP. Operating the columns in

series allowed the steps of buffer removal, extract neutralization, and metabolite sorption to be performed simultaneously. The small amount of resin used in metabolite sorption allowed small volumes of TFA to be used in eluting the column, thus minimizing the time spent lyophilizing samples. It was found possible to do all volume reductions of cell extracts by lyophilization including the final reduction in the derivatization vessel. Trifluoroacetic acid was used to elute the anion exchange column because of its high affinity for quaternary ammonium exchangers (three times that of chloride ion (87)), and because it is rapidly and quantitatively removed by lyophilization. The metabolites were eluted from the anion exchange column in their free acid forms which are easily derivatized with BSTFA. It was possible to process 4 cell extracts simultaneously by binding the rinsing and elution syringes together before installing them in the syringe pump.

As can be seen in Figures 5 and 6, the conditions used in this study produced generally well resolved gas chromatograms with good signal-to-noise ratios. Two temperature programs were necessary to obtain a more complete GC analysis. With the program of low initial temperature (75^{o}), the larger metabolite derivatives, such as that of FDP, were seen at reduced intensity or not at all. This phenomenon is illustrated in Figure 5, with the arrow indicating the position where pertrimethylsilylated FDP is sometimes seen at reduced intensity. A program with an initial temperature

Figure 5. Gas chromatogram of a 0.5 ml *E. coli* fermentation sample taken 5 min after glucose addition.

The cell extract had previously been purified by ion exchange as described in the EXPERIMENTAL, Part I section. One µl of the 17 µl volume of pertrimethylsilylated metabolites was injected and a temperature program of 75 to 280° at 4°/min was used. Other instrumental conditions were as described in the EXPERIMENTAL, Part I section. The arrow at the far right of the figure indicates the position where the FDP derivative is sometimes seen at reduced intensity under these operating conditions.

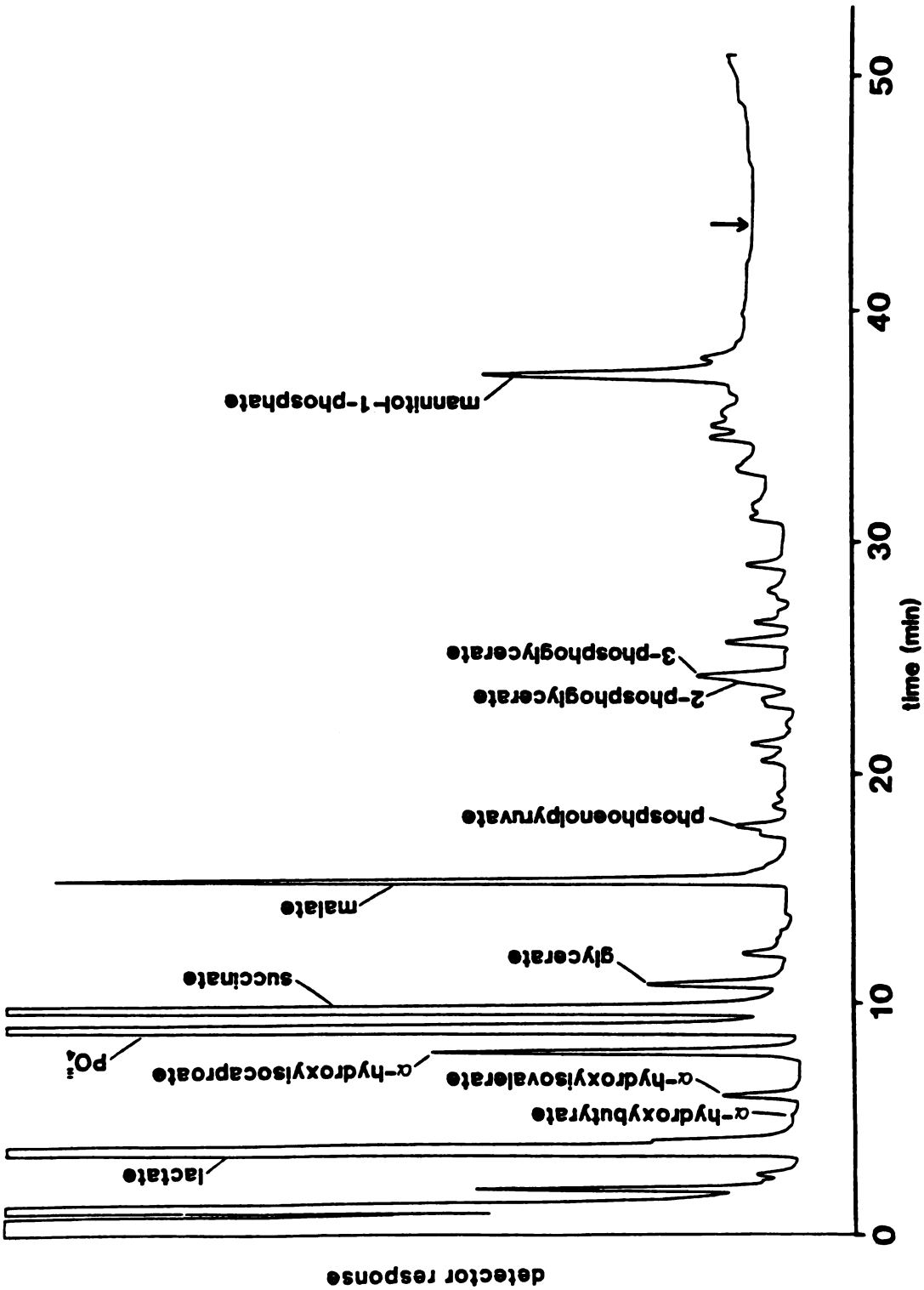

detector response
lactate
α-hydroxybutyrate
α-hydroxyisovalerate
α-hydroxyisocaproate
PO_4^{3-}
succinate
glycerate
malate
phosphoenolpyruvate
2-phosphoglycerate
3-phosphoglycerate
mannitol-1-phosphate
0
10
20
30
40
50
time (min)

Figure 5

Figure 6. Gas chromatogram of a 2 µl injection of the same derivatized metabolite sample used in Figure 5.

Instrumental conditions were the same as for Figure 5 except a temperature program of 175 to 280° at 4°/min was used. The consistently greater intensity for the FDP derivative is readily evident by comparison of its intensity with that of mannitol-1-phosphate in Figures 5 and 6. Large and small doublet peaks eluting after FDP probably represent the persilylated derivatives of nucleoside monophosphates (AMP, etc.) but this has not been confirmed with mass spectrometry.

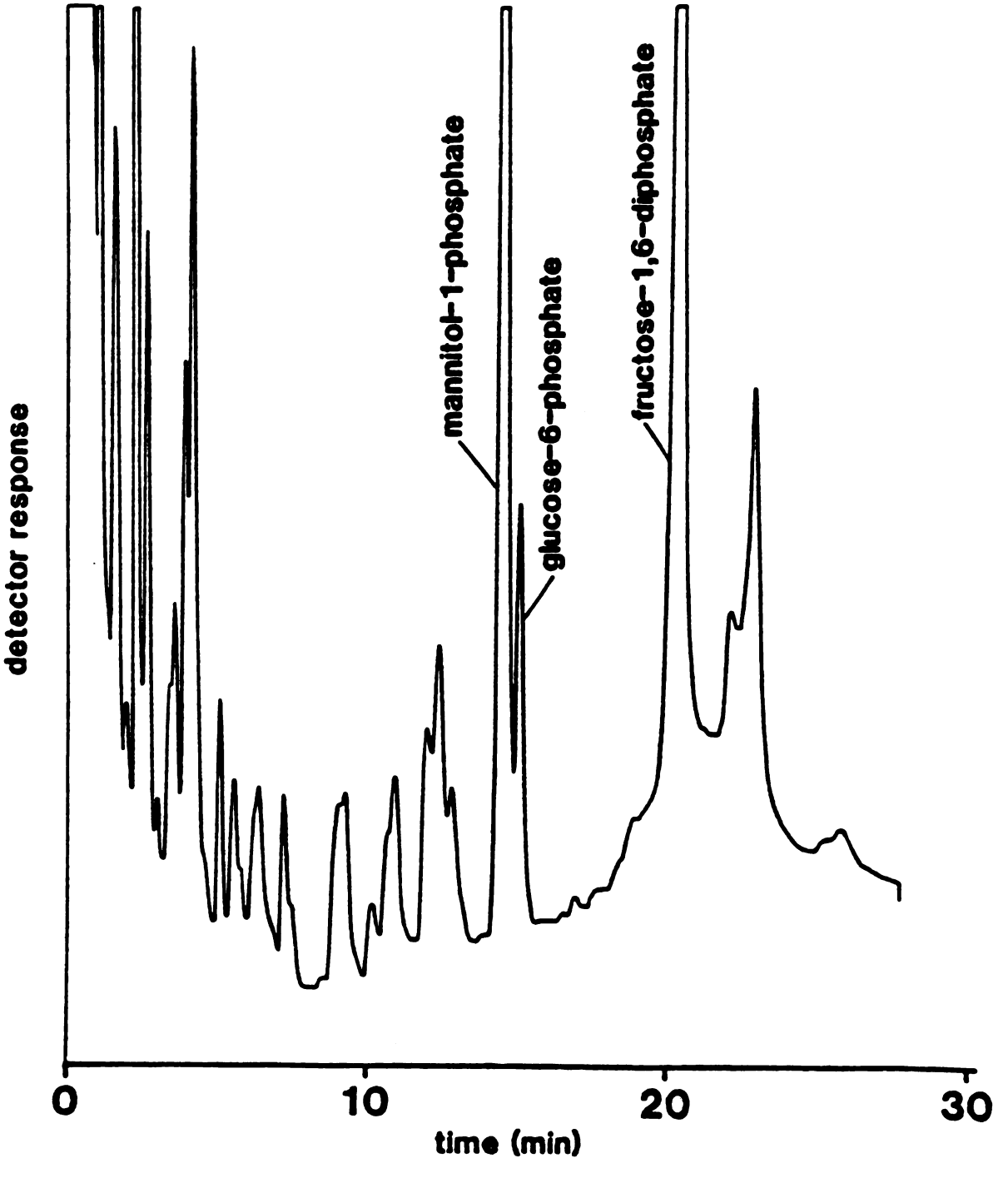

detector response
mannitol-1-phosphate
glucose-6-phosphate
fructose-1,6-diphosphate
0
10
20
30
time (min)

Figure 6

of 175° was then tried which consistently allows the FDP derivative to be seen at good intensities as illustrated in Figure 6.

Metabolite derivatives from *E. coli* extracts were identified by comparing their GC elution positions and 70 eV electron impact mass spectra with those of authentic compounds (obtained from Sigma). The metabolites so far identified as their pertrimethylsilylated derivatives are lactate, α-hydroxybutyrate, α-hydroxyisovalerate, α-hydroxyisocaproate, inorganic phosphate, succinate, glycerate, malate, phosphoenolpyruvate, 2-phosphoglycerate, 3-phosphoglycerate, mannitol-1-phosphate, glucose-6-phosphate, and fructose-1,6-diphosphate.

This list includes fundamental intermediary metabolites from three major chemical classes: sugar phosphates, citric acid cycle acids, and α-hydroxyacids. The list makes it evident that the crucial question which motivated this study can be answered in the affirmative: GC-MS analysis of cellular intermediary metabolism is practical.

B. Study of the Origin of Leucic Acid Using GC-MS and ^{13}C Labeled Glucose

A major motivation for developing a GC-MS analysis of cellular metabolism was the possibility of detecting unexpected metabolites. This possibility was realized with the discovery that valic and leucic acids are produced during the fermentation of glucose by *E. coli*.

The production of these acids from growth in complex media has been observed for Proteus vulgaris (88), Bacillus subtilus (88), Bacillus granulobacter pectinovorum (89), Lactobacillus casei (90), and Lactobacillus plantarum (91). However, valic and leucic acid have apparently not been reported as metabolites of E. coli or as products of a bacterial glucose fermentation.

In determining the significance of valic and leucic acid in the metabolism of E. coli, one can ask the following questions:

1. Where did they come from?
2. How did they get there?
3. What are they doing there?

The mass fragmentation pattern of leucic acid was such that useful information could be obtained from ^{13}C tracer experiments. Such experiments were undertaken to answer the first question in the case of leucic acid.

1. The source of leucic acid

a. 1-Source model

From gas chromatograms taken before and after the fermentation of glucose by E. coli, the amount of leucic acid present was seen to increase approximately 10-fold. A priori, one might assume from this observation that leucic acid was derived directly from glucose, although this would not necessarily have to be true. If leucic acid were

synthesized directly from glucose, one would expect that the pathway followed would be the same as that for its amino acid analogue, leucine.

This pathway has been well-established by isotopic tracer, mutant and enzyme studies in E. coli and other organisms and is illustrated in Figures 7 and 8.

In step II of Figure 7, a molecule of pyruvate becomes bonded to the thiamin pyrophosphate (TPP) cofactor of the enzyme, acetolactate synthetase, and is decarboxylated. The resulting acetaldehyde remains bonded to TPP and in this form the aldehydo hydrogen is sufficiently acidic to allow its carbon to attack (as a carbanion) the carbonyl carbon of a second pyruvate. The acetolactate thus formed is then released from the enzyme.

In step III, acetolactate undergoes a simultaneous pinacol-like rearrangement and reduction to give α,β-dihydroxyisovalerate. The rearrangement in this step can be thought of as occurring by a mechanism commonly observed in organic chemistry, namely, rearrangement of a carbonium ion through a 1-2 alkyl shift.

The dihydroxy acid is subsequently dehydrated in step IV in a way that yields an enol tautomer of α-ketoisovalerate. The keto acid can be transaminated to provide the amino acid, valine or, as suggested by this study, reduced to give the hydroxy acid, valic acid.

The α-ketoisovalerate can also condense with acetyl coenzyme A (acetyl CoA) as shown in reaction VIII of Figure 8,

Figure 7. Pathway for the synthesis of α-ketoisovalerate from pyruvate in *E. coli*.

Superscript (a,b,c) labeling of carbon atoms denotes their correspondance to the carbon atoms of pyruvate. Step I denotes the reactions of the hexosediphosphate pathway. Broken arrow in step II represents attack of the 1-carbon of the acetaldehyde adduct on the carbonyl carbon of pyruvate. Broken arrows with acetolactate represent electron pair movements in the pinacol-like rearrangement of that compound. Step V shows the conversion of α-ketoisovalerate to valine by transamination. Step VI represents the presumed reduction of α-ketoisovalerate to valic acid.

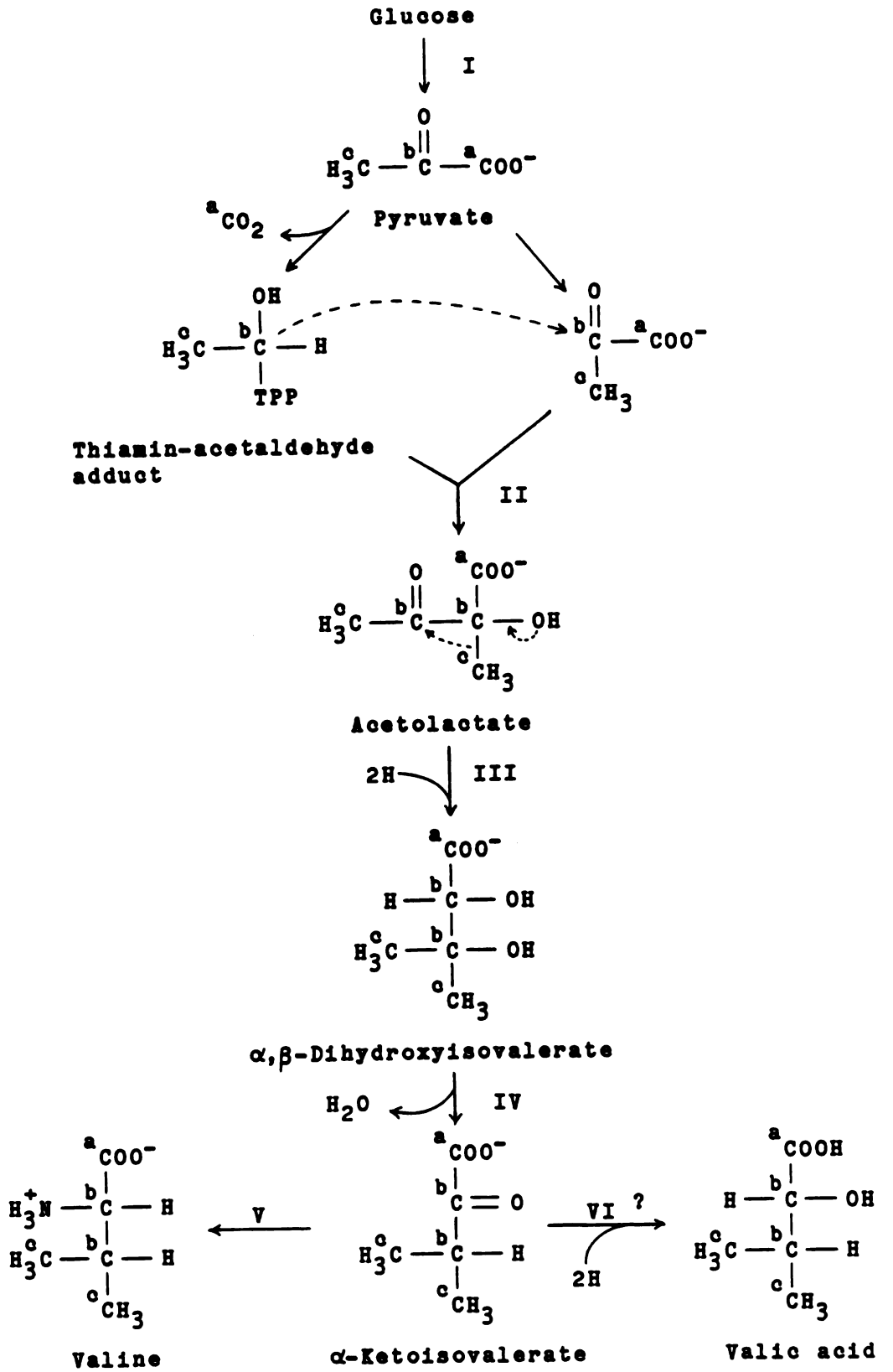

Glucose
I
Pyruvate
TPP
Thiamin-acetaldehyde adduct
II
Acetolactate
2H
III
α,β-Dihydroxyisovalerate
H2O
IV
V
VI ?
2H
Valine
α-Ketoisovalerate
Valic acid

Figure 7

Figure 8. Pathway for the synthesis of leucine from acetyl CoA and α-ketoisovalerate in *E. coli*.

Broken arrow in step VIII represents attack of methyl carbon of acetyl CoA on the carbonyl carbon of α-ketoisovalerate. Step XIII shows a transamination converting α-ketoisocaproate to leucine. Step XIV represents the presumed reduction of α-ketoisocaproate to leucic acid.

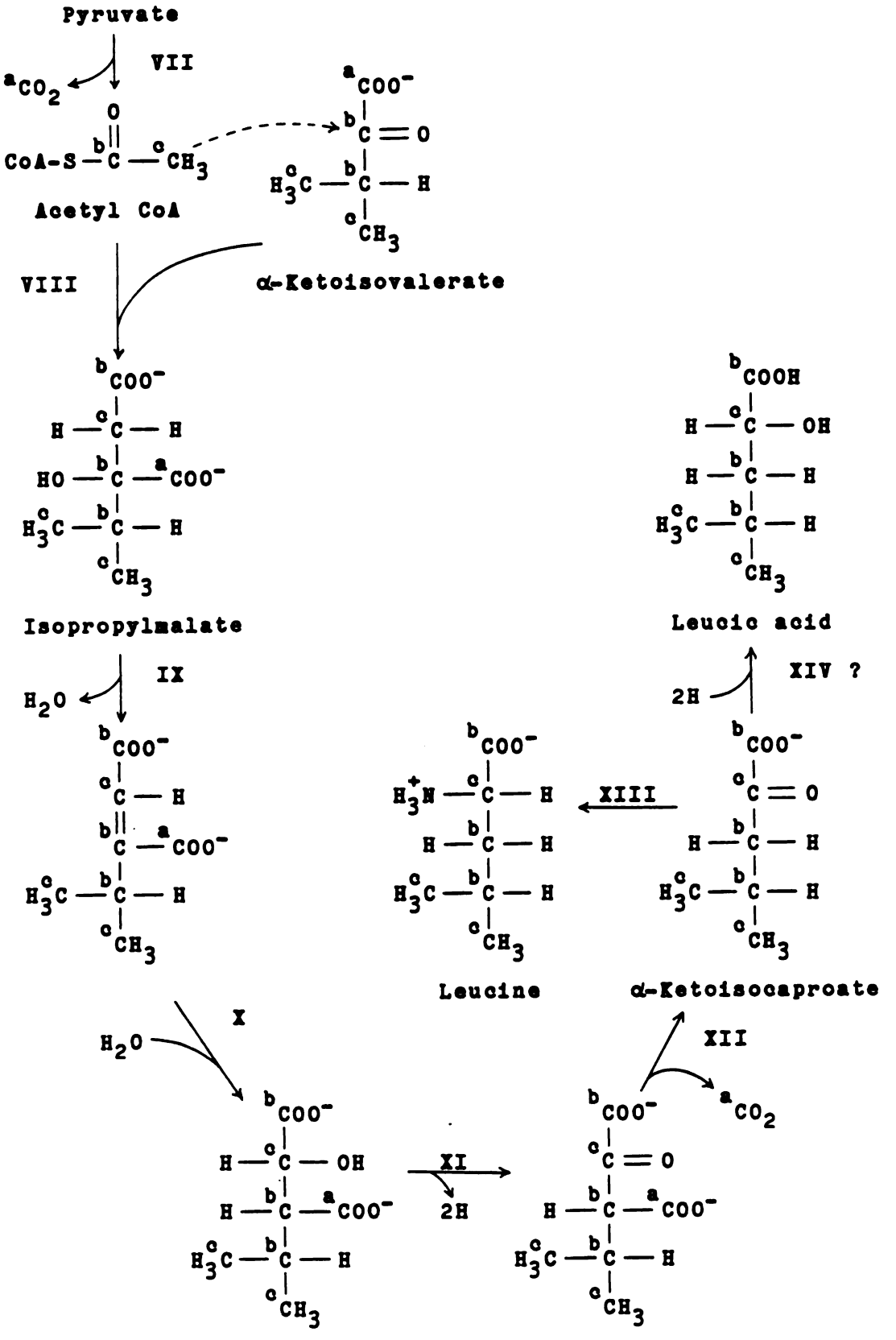

Pyruvate
VII
Acetyl CoA
α-Ketoisovalerate
VIII
Isopropylmalate
IX
X
XI
2H
XII
XIII
XIV ?
Leucine
α-Ketoisocaproate
Leucic acid

Figure 8

yielding isopropylmalate. In a series of reactions (IX-XII) that are remarkably analogous to those converting citrate to α-ketoglutarate in the citric acid cycle, the isopropylmalate is converted to α-ketoisocaproate which can yield leucine by transamination or presumably be reduced to leucic acid.

In a ^{13}C tracer study of the leucine pathway using ^{13}C glucose as substrate, the derivation of the pyruvate and acetyl CoA carbons from glucose must be considered. In E. coli glucose is fermented to pyruvate via the hexosediphosphate ("typical glycolysis") pathway. The correspondence of the glucose carbons with those of pyruvate and acetyl CoA in this pathway is illustrated in Figure 9 where the corresponding carbons are aligned horizontally across the page.

As can be seen in Figure 9, one mole of glucose gives rise to two moles of pyruvate in which glucose carbons 3 and 4, 2 and 5, 1 and 6 correspond to pyruvate carbons a,b,c, respectively. The carboxyl carbon (labeled "a") of pyruvate can be lost by either the pyruvate-formate lyase reaction or the pyruvate dehydrogenase reaction to provide acetyl CoA in which the b and c carbons correspond to the b and c carbons, respectively, of the pyruvate. By this scheme [1-^{13}C] glucose containing 99 atom % ^{13}C at C-1 should give rise to pyruvate and acetyl CoA containing ^{13}C only in their "c" (methyl) carbons. However, only 0.99/2 or 0.50 of these methyl carbons should contain ^{13}C since one unlabeled pyruvate will be produced for every labeled one.

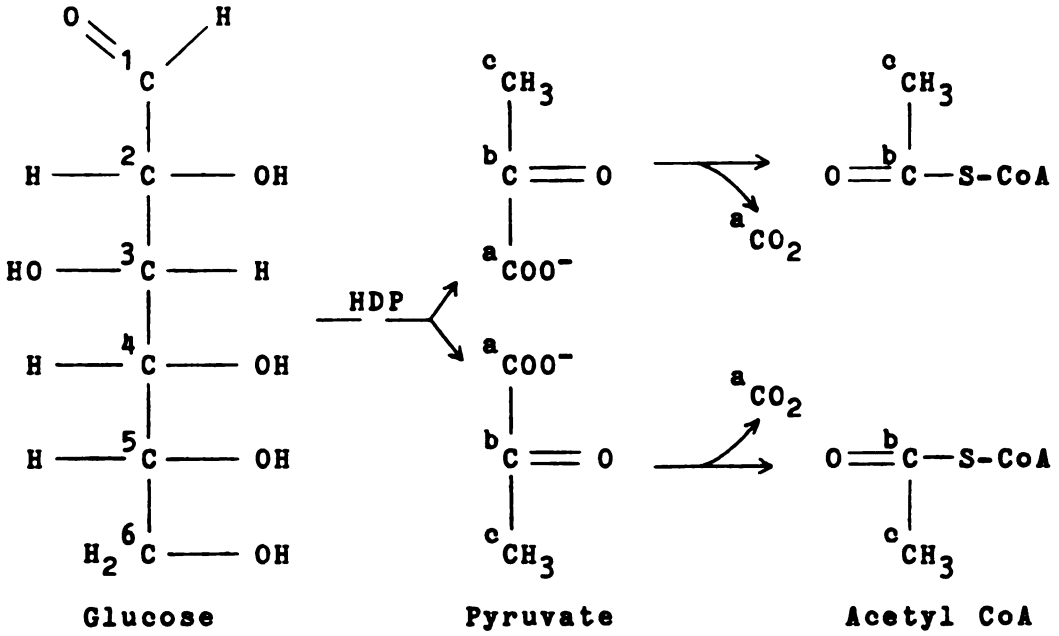


Figure 9. Correspondence of carbons of glucose, pyruvate, and acetyl CoA when glucose is fermented by E. coli.

Corresponding carbons of the three compounds are aligned horizontally. The labeling of the pyruvate and acetyl CoA carbons (a,b,c) corresponds to that used in Figures 7 and 8. HDP denotes the reactions of the hexosediphosphate ("typical glycolysis") pathway.

Leucic acid with its carbon correspondence to pyruvate was illustrated in Figure 8 and is reproduced below in Figure 10.

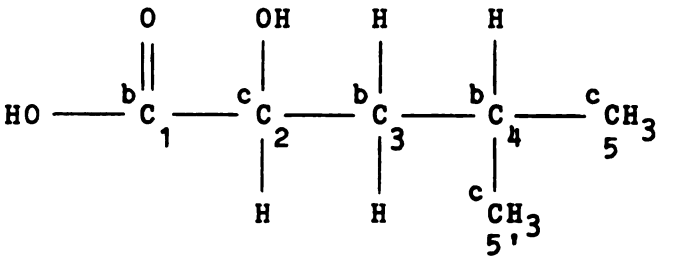


Figure 10. Correspondence of leucic acid and pyruvate carbons.

Lower case letters (b,c) denote carbon derived from the 2 and 3 carbons respectively, of pyruvate as in Figures 7 and 8.

From the foregoing discussion it can now be seen that leucic acid produced from [1-^{13}C] glucose will become labeled with ^{13}C at carbons 2, 5, and 5', while that from [2-^{13}C] glucose will become labeled at carbons 1, 3, and 4.

The expected ^{13}C labeling patterns for pyruvate and leucic acid derived from [1-^{13}C] glucose as discussed above would give rise to a characteristic labeling pattern for the diagnostic ions of the TMS derivatives of lactate and leucic acid as illustrated in Figure 11.

Since lactic acid arises from a direct reduction of pyruvate, its ^{13}C content will be the same as that for the pyruvate produced during the fermentation. The ^{13}C content of the pyruvate will thus be the same as that of the 117 ion of $(TMS)_2$ lactate. If the fraction of m/z 117 containing

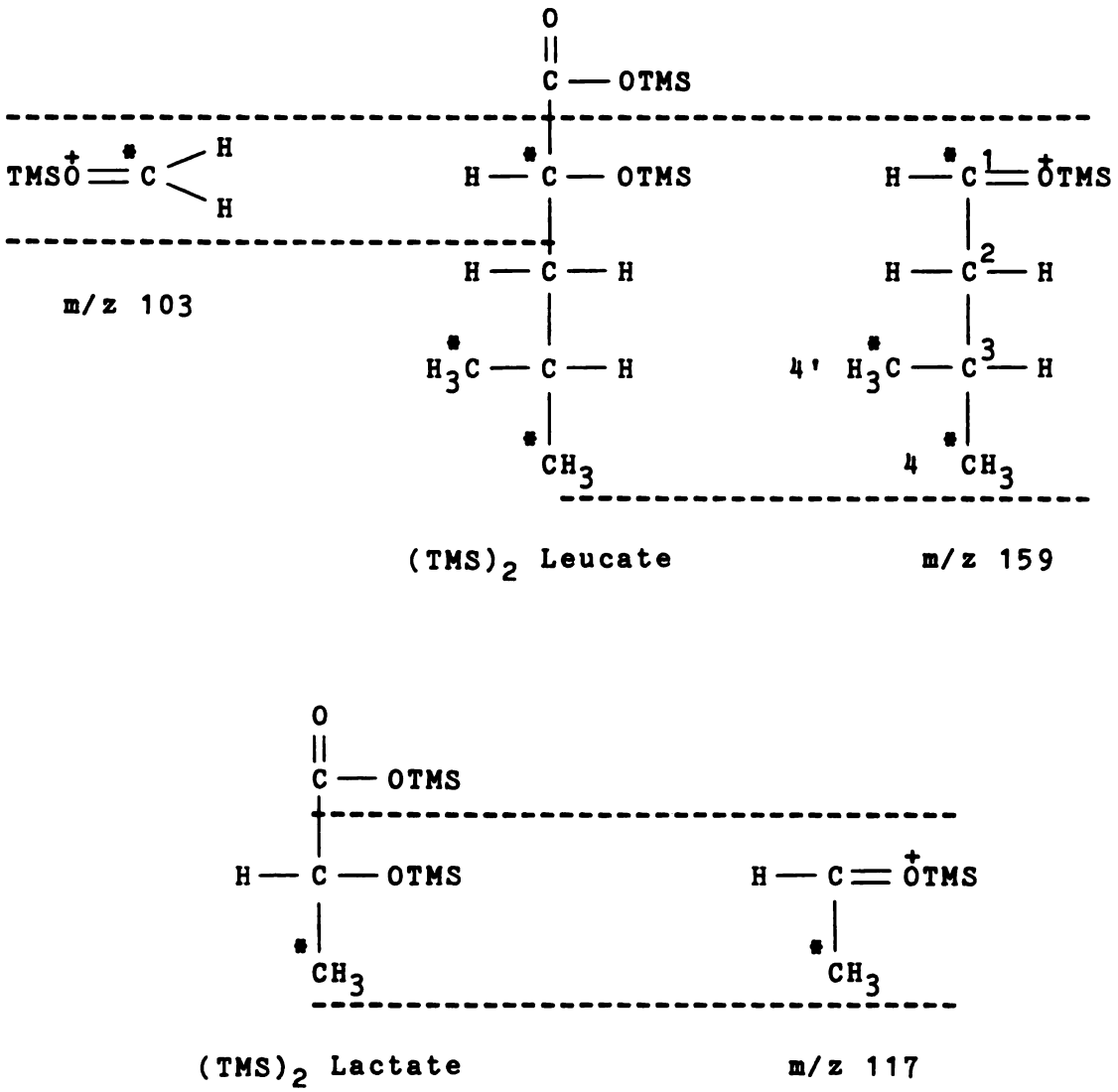


Figure 11. The carbon correspondence between the TMS derivatives of lactic and leucic acid and their diagnostic fragment ions.

Corresponding carbons are aligned horizontally. Asterisk denotes positions of ^{13}C enrichment expected for the derivation of lactic and leucic acid from [1-^{13}C] glucose.

one excess ^{13}C atom is defined to be x, then upon feeding [1-^{13}C] glucose (99 atom % ^{13}C), we would expect that x = 0.99/2 = 0.50.

Because the 2-carbon of leucic acid is derived from the methyl carbon of pyruvate, the m/z 103 ion of $(TMS)_2$ leucate which comprises this carbon should become labeled with ^{13}C and the fraction of 103 ions containing ^{13}C should be x.

Considering the facts that the 159 ion of $(TMS)_2$ leucate would contain the methyl carbons of 3 different pyruvates and that unlabeled as well as ^{13}C labeled pyruvate would arise from [1-^{13}C] glucose, it becomes evident that a heterogenous population of 159 ions should be produced with species containing 0,1,2, or 3 excess ^{13}C atoms. The fractions that should be observed for each of the 4 different 159 ions can be calculated with equations 1-4:

1. fraction containing 0 excess ^{13}C atoms = $(1-x)^3$
2. " 1 " = $3x(1-x)^2$
3. " 2 " = $3x^2(1-x)$
4. " 3 " = x^3

where x = fraction of 117 ions of $(TMS)_2$ lactate containing one excess ^{13}C atom.

Equation 1 gives the probability that the 159 ion will contain 0 excess ^{13}C atoms. There is only one way such an event can occur and that is that carbons 1,4, and 4' (see Figure 11) contain only ^{12}C at these positions.

The probability of finding ^{12}C at each position is 1-x and of course the final probability of finding ^{12}C at carbons 1 and 4 and 4' is $(1-x)(1-x)(1-x) = (1-x)^3$.

The 159 ion containing one ^{13}C atom can arise in 3 different ways. For each way we must have that the first position contains ^{13}C and the second and the third positions ^{12}C. The probability for each way is the product of $x(1-x)(1-x) = x(1-x)^2$. The final probability of having the 1st or the 2nd or the 3rd way obtain is the sum of the probabilities for each way or $3x(1-x)^2$.

As with the 159 ion containing one ^{13}C atom, there are 3 different ways the ion could contain two ^{13}C atoms. For each way we must have ^{13}C at two positions and ^{12}C at the remaining one with an associated probability given by $x^2(1-x)$. The final probability as given in equation 3 is the sum of the probabilities for each way which is $3x^2(1-x)$.

Finally, there would be only one way for the ion to contain 3 ^{13}C atoms and that is by each of the 3 possible positions containing ^{13}C. The probability of this event would be x^3.

The ^{13}C tracer experiment was performed using [1-^{13}C] glucose (99 atom % ^{13}C) as substrate. At the same time, a separate portion of the bacteria was allowed to ferment [2-^{13}C] glucose (99 atom % ^{13}C) under similar conditions. This was done for the purpose of confirming that the m/z 103 ion of leucic acid from [2-^{13}C] glucose should not contain ^{13}C while that from [1-^{13}C] glucose should. Such a result

would confirm that leucic acid was produced from glucose by the expected pathway of leucine biosynthesis.

The results of these tracer experiments are given below in Table 1.

A quick glance at the table allows two notable observations. The first is that, as indicated by labeling of the 103 ions, leucic acid from [1-^{13}C] glucose became labeled at C-2 while leucic acid from [2-^{13}C] glucose did not. This indicates that the labeled leucic acid was produced from glucose by the leucine pathway. The second observation is that lactate derived from either [1-^{13}C] or [2-^{13}C] glucose contained essentially the same amount ^{13}C (as indicated by their 117 ions) and both these amounts were significantly smaller than the predicted value of 0.99/2= 0.50. This interesting and unexpected result will be discussed later.

Further examination of Table 1 shows the ^{13}C content of leucic acid to be far less than was predicted for its synthesis from [1-^{13}C] glucose alone. For example, the fraction of 103 ion containing ^{13}C was expected to be 0.43 while the observed value was only 0.16. The deviations are also serious for the 159 ion, especially for the case of 159 containing 0 excess ^{13}C atoms. Here the predicted fraction containing no ^{13}C atoms is 0.18 while the observed value was 0.75. This particular disparity strongly suggests that in addition to being produced from [1-^{13}C] glucose by the leucine pathway, the major portion of the leucic acid was derived from an endogenous, unlabeled carbon source.

Table 1. ^{13}C Labeling of the TMS derivatives of lactic and leucic acid following fermentation of [1-^{13}C] and [2-^{13}C] glucose.

Source[a]	Ion[b]	Fraction of ions containing ...					excess ^{13}C atoms
		.. 0	1	2	3	4	
lac from [1-^{13}C] glc	117	0.57	0.43	0	-	-	
lac from [2-^{13}C] glc	117	0.58	0.42	0	-	-	
leu from [1-^{13}C] glc	103	0.84	0.16	-	-	-	
leu from [2-^{13}C] glc	103	>0.99	<0.01	-	-	-	
leu from [1-^{13}C] glc	159	0.75	0.16	0.07	0.02	0	
expected values for leu from [1-^{13}C] glc	159	0.18	0.42	0.32	0.08	0	

[a] Abbreviations: lac = lactic acid, leu = leucic acid, glc = glucose
[b] fragment ions of the TMS derivatives of lactic and leucic acids as illustrated in Figure 11.

b. 2-Source model

The most plausible candidate for such an unlabeled carbon source is the amino acid, leucine. If glucose fermentation could trigger deamination of endogenous leucine, the resulting α-ketoisocaproate could then be reduced to provide leucic acid containing no excess ^{13}C. To test this 2-source model, a relative contribution of 75% was assumed for unlabeled leucine. Since we are only interested in the relative contributions of the pathways, we can arbitrarily assign a value of 1.00 to the amount of leucic acid derived from [1-^{13}C] glucose. The value of 1.00 was chosen since in this case the amounts of the different ^{13}C containing ions remain identical to their original fractional values. A value of 3.00 for the amount of unlabeled leucic acid derived from leucine is then added to the amount (0.18) of unlabeled leucic acid derived from [1-^{13}C] glucose. The fractional values for the different ions are recalculated on the basis of the total amount of leucic acid being 4.00 instead of 1.00. These values are presented in Table 2 with those of the 1-source model and the observed values for comparison.

Table 2. Comparison of ^{13}C labeling patterns generated by 1-source and 2-source models with the experimentally observed values.

Model	Ion	Fraction of ions containing ... excess ^{13}C atoms			
		..0	1	2	3
1-Source	103	0.57	0.43	-	-
	159	0.18	0.42	0.32	0.08
2-Source	103	0.89	0.11	-	-
	159	0.79	0.11	0.08	0.02
Observed values	103	0.84	0.16	-	-
	159	0.75	0.16	0.07	0.02

As is readily evident from Table 2, the 2-source model provides a good first approximation to the observed values while the 1-source model does not come close to doing so. However, significant disparities still exist between pairs of observed and predicted values, which are very unlikely to be due to experimental error. Furthermore, simple algebraic analyses showed that it would be impossible to choose any value of the endogenous leucine contribution which would allow this 2-source model to reproduce the experimental results with a high degree of approximation.

In attempting to improve the 2-source model, the only conceivable modification that could be made would be to assume that the ^{13}C content of the 3 constituent pyruvate

molecules of leucic acid is different from the value of 0.43 used initially. Figure 7 shows that two of these pyruvates are incorporated directly enroute to leucic acid (step II) and their ^{13}C contents should not be different from 0.43. However, the third pyruvate is incorporated indirectly as acetyl CoA (Figure 8, step VIII). Since acetyl CoA and acetate cannot be detected with the present analytical system, the cells might have contained endogenous, unlabeled acetate and acetyl CoA which could effectively lower the ^{13}C content of the acetyl CoA incorporated into leucic acid. Such an isotope dilution by acetyl CoA would appear unlikely since the cellular amount of CoA (and thus, acetyl CoA) are very small, as is the case with other vitamin cofactors. Endogenous acetate could conceivably be phosphorylated and the resulting acetyl phosphate converted to acetyl CoA. However, this would seem unlikely since one would think that the cellular CoA would be preempted by glucose metabolism during the fermentation.

Despite these reservations, this modification was subjected to a brief test using the following definitions and equations.

Definitions:

x = fraction of acetyl CoA containing ^{13}C

0.57 = fraction of both directly incorporated pyruvates containing ^{12}C

y = amount of leucic acid from endogenous leucine

1.00 = amount of leucic acid from [1-^{13}C] glucose

Since the 103 ion of $(TMS)_2$ leucate comprises the methyl group of acetyl CoA, the total amount of 103 ion containing ^{13}C would be x(1.00). The total amount of 103 ions is y + 1.00, and so the fraction of 103 ions containing ^{13}C will be x/(y + 1.00) which is equated to the observed value of 0.16 from Table 1:

$$\frac{x}{y + 1} = 0.16$$

A similar approach shows that the fraction of 159 ions containing only ^{12}C (no ^{13}C) should satisfy the relation:

$$\frac{(0.57)^2(1-x) + y}{y + 1} = 0.75$$

The unique solution of this pair of equations is x = 0.55, y = 2.42. This solution is obviously not admissible since the ^{13}C content of the acetyl CoA (x=0.55), significantly exceeds the ^{13}C content of the pyruvate (0.43) from which it was derived. It appears, then, that the 2-source model cannot be improved.

c. 3-Source model

The 2-source model provided a good first approximation to the experimentally observed results. This strongly suggests that endogenous leucine was deaminated to provide the major portion of the observed leucic acid. Such a deamination of endogenous leucine during fermentation makes a similar deamination of valine seem a not unlikely event. The resulting α-ketoisovalerate could then condense with acetyl

CoA to provide a third source of leucic acid as illustrated in steps VIII-XIV of Figure 8. Leucic acid from this third source would be labeled with ^{13}C only at C-2.

Using the following definitions:

x = fraction of acetyl CoA containing ^{13}C

0.43 = fraction of pyruvate containing 1 excess ^{13}C atom

1.00 = amount of leucic acid derived from [1-^{13}C] glucose

w = amount of leucic acid derived from endogenous valine + acetyl CoA

z = amount of leucic acid derived from endogenous leucine

the relative ion intensities predicted by the 3-source model are shown equated to the observed values in equations 5-10 below:

ion

103 5. $$\frac{(1-x)(1.00)+(1-x)(w)+z}{z+w+1} = 0.84$$

103 6. $$\frac{(x)(1.00)+(x)(w)}{z+w+1} = 0.16$$

159 7. $$\frac{(1-x)(0.57)^2(1.00)+(1-x)(w)+z}{z+w+1} = 0.75$$

159 8. $$\frac{(x)(0.57)^2+2(0.43)(0.57)(1-x)+(x)(w)}{z+w+1} = 0.16$$

159 9. $$\frac{2(x)(0.43)(0.57)(1.00)+(0.43)^2(1-x)(1.00)}{z+w+1} = 0.07$$

159 10. $$\frac{(0.43)^2(x)(1.00)}{z+w+1} = 0.02$$

where equation 5 represents the fraction of m/z 103 containing 0 excess ^{13}C atoms; equation 6, the fraction of m/z 103 containing 1 excess ^{13}C atom; equation 7, the fraction of m/z 159 containing 0 excess ^{13}C atom; equation 8, the fraction of m/z 159 containing 1 excess ^{13}C; equation 9, the fraction of m/z 159 containing 2 excess ^{13}C; and equation 10, the fraction of m/z 159 containing 3 excess ^{13}C.

In solving for the unknown parameters (x,w,z), we need only use three of these equations. Noticing that we can equate the left hand sides of 6 and 8, we readily find x=0.42. Substituting this value of x in 6, we find w=0.615z-1. Substituting this value for w in 7 we find z=2.65 and finally, w=0.63. Using these values of x,w, and z in the left hand sides of equations 5-10, relative intensities for the leucate ions are generated which are given in Table 3 with the observed values for comparison.

Table 3. Comparison of ^{13}C labeling patterns generated by 3-source model with the experimentally observed values.

Model	Ion	Fraction of ions containing ... excess ^{13}C atoms			
		..0	1	2	3
3-Source	103	0.84	0.16	-	-
	159	0.75	0.16	0.07	0.02
Observed values	103	0.84	0.16	-	-
	159	0.75	0.16	0.07	0.02

Obviously the numerical values for x,w,z and the 3-source model can produce an excellent match to the experimentally observed results. Furthermore, the numerical values obtained for x,w, and z appear quite reasonable. A value of x = 0.42 for the ^{13}C content of acetyl CoA was obtained, which is almost exactly the expected value of 0.43. An endogenous amino acid contribution to leucic acid of 0.75 with the 2-source model allowed a good approximation to the observed data (see Table 2). This would lead one to expect a similar value for the endogenous amino acid contribution in the 3-source model, and indeed this contribution was $(z+w)/(z+w+1)=0.77$.

The two key reactions proposed as the basis of the 3-source model were the deaminations of valine and leucine. Experimental support for the occurrence of these reactions under the fermentation conditions was obtained by allowing E. coli to ferment glucose in the presence of either 12.5 mM L-valine or 9.0 mM L-leucine. In the presence of leucine approximately 5 times more leucic acid was produced than from fermentation of glucose alone. Similarly, 5 times more valic acid was produced in the presence of valine than in its absence. These results leave no doubt that the key reactions proposed as the basis of the 3-source model can occur under the physiological conditions producing leucic acid.

It appears then, that the question "Where did it come from?" can be answered for leucic acid as follows:

23% from [1-^{13}C] glucose by the leucine biosynthetic pathway

15% from endogenous valine + acetyl CoA

62% from endogenous leucine.

2. The intermediate reactions producing leucic acid

Regarding the question "How did it get there?", an immediate partial answer is that 23% of the leucic acid was synthesized from glucose via the leucine biosynthetic pathway. The remaining 77% was apparently derived from endogenous valine and leucine. The amino acids would have to have been deaminated, most probably to the α-keto analogues of the amino acids. α-Ketoisocaproate could then be reduced to yield leucic acid while α-ketoisovalerate could condense with acetyl CoA to yield leucic acid via reactions of the leucine biosynthetic pathway.

Deaminations of amino acids generally occur through the action of 3 different enzyme types:

1. oxidases
2. dehydrogenases
3. transaminases.

Amino acid oxidases are flavin enzymes which catalyze the following general reaction:

$$\text{amino acid} + O_2 \xrightarrow{\text{oxidase}} \text{keto acid} + NH_3 + H_2O_2.$$

One would not expect the operation of such reactions in the present study since oxygen was not present. However, a

"pseudotransamination" (92) catalyzed by amino acid oxidase could conceivably occur if a second keto acid was available to combine with NH_3 liberated from the amino acid:

$$\text{amino acid}_1 + \text{FAD} \rightleftharpoons \text{keto acid}_1 + NH_3 + FADH_2$$
$$\text{keto acid}_2 + NH_3 + FADH_2 \rightleftharpoons \text{amino acid}_2 + \text{FAD}$$

Amino acid dehydrogenases are NAD-linked enzymes which catalyze the following reaction:

$$\text{amino acid} + NAD^+ \rightleftharpoons \text{keto acid} + NH_3 + NADH$$

Dehydrogenations of valine and leucine under the conditions of this study would seem unlikely for two reasons. It is difficult to visualize how the fermentation could trigger the dehydrogenation of endogenous amino acids and in addition, the equilibrium of such reactions markedly favors synthesis of the amino acid rather than the keto acid.

Transaminases are pyridoxal-linked enzymes which catalyze a reversible amination between an amino acid and keto acid:

$$\text{amino acid}_1 + \text{keto acid}_1 \rightleftharpoons \text{keto acid}_2 + \text{amino acid}_2$$

Transamination would appear to offer the best mechanism for the deamination of valine and leucine for two reasons.

It is known from enzyme and mutant studies of *E. coli* that transaminations are involved in the last step of valine and leucine biosynthesis (see step V, Figure 7 and step XIII, Figure 8). Secondly, transamination would provide a simple explanation of how glucose fermentation could trigger the deamination of valine and leucine.

This explanation would be that glucose fermentation produces α-keto acids which can undergo transamination with endogenous amino acids. As a mixed acid bacterium, *E. coli* is known to produce significant amounts (transiently, i.e. they do not accumulate) of two such acids, oxaloacetate and pyruvate, during glucose fermentation.

In vitro enzyme studies (93) have revealed that the transaminases of *E. coli* can be separated into 3 fractions (A,B,and C) with the following substrate specificities: fraction A utilizing leucine, methionine, glutamate, aspartate, phenylalanine, tyrosine, tryptophan and their respective α-keto acids; fraction B utilizing leucine, valine, isoleucine, phenylalanine, tyrosine, glutamate and their α-keto acids; and fraction C using only valine, alanine, α-aminobutyrate and their corresponding keto acids. The deaminations of valine and leucine observed in the present study can be rationalized with the results of these *in vitro* transaminase studies in a number of ways as illustrated in Figure 12.

Although amino acids are not present in the fraction of metabolites examined in this study, it would be interesting

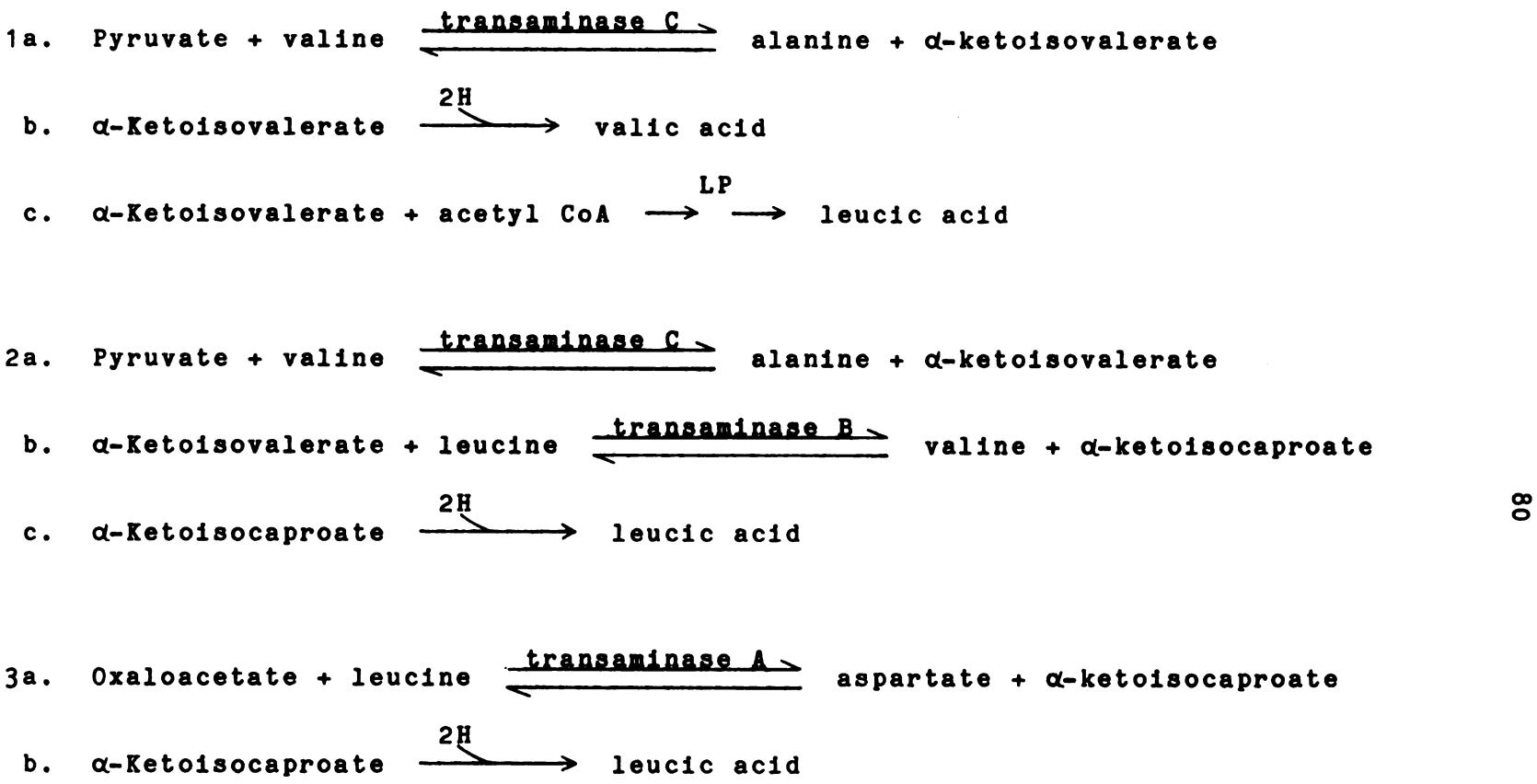


Figure 12. *In vitro* transaminase reactions of *E. coli* which could explain how glucose fermentation converts endogenous valine and leucine to valic and and leucic acids.

Abbreviation: LP = reactions of the leucine biosynthetic pathway illustrated in Figures 7 and 8.

(as discussed later) if the present analytical system could be extended to include a GC-MS analysis of the cellular amino acids as a separate fraction. GC-MS analysis of the intracellular amino acids might provide a means of determining which, if any, of the above transaminations occur during glucose fermentation.

For example, if glucose was fermented in the presence of ^{15}N labeled valine (instead of the normal ^{14}N valine) one would expect ^{15}N label to appear in alanine via reaction 1a of Figure 12 with perhaps a smaller amount appearing in leucine by a reversal of reaction 2b. Smaller amounts of ^{15}N might also appear in other amino acids via secondary reactions catalyzed by transaminase B.

If α-ketoisocaproate is produced indirectly from pyruvate via reactions 2a + 2b of Figure 12, glucose fermentation in the presence of ^{15}N leucine should yield ^{15}N labeled valine by reaction 2b with perhaps a smaller amount of ^{15}N in alanine and other amino acids via secondary reactions. If, however, α-ketoisocaproate is generated only through reaction 3a., fermentation in the presence of ^{15}N leucine should result in aspartate containing the largest amount (besides leucine) of ^{15}N with smaller amounts of ^{15}N appearing in other amino acids through secondary reactions.

The operation of transamination could also explain the unexpected isotope dilution of the lactate (and by inference, the pyruvate) produced from either [1-^{13}C] glucose or [2-^{13}C] glucose (see Table 1). The ^{13}C

content of lactate from both sources was expected to be ~0.50 while the observed values were 0.43 and 0.42. Gas chromatograms taken before glucose addition demonstrated a virtual absence of endogenous, unlabeled pyruvate or lactate, thus excluding these as a possible cause of the isotope dilution. However, this isotope dilution could result if the ^{13}C labeled pyruvate underwent transamination with endogenous, unlabeled alanine:

$$^{13}C\text{-pyruvate} + \text{alanine} \underset{\text{transaminase C}}{\rightleftharpoons} {}^{13}C\text{-alanine} + \text{pyruvate}$$

$$\text{pyruvate} \xrightarrow{2H} \text{lactate (unlabeled)}$$

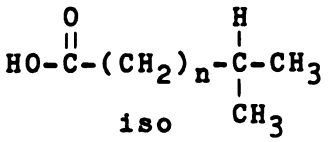
Other possible explanations of the isotope dilution would be operation of certain combinations of pentose phosphate pathway reactions or perhaps fermentation of unlabeled glucose from an endogenous store of glycogen.

3. The significance of leucic acid in the metabolism of *E. coli*

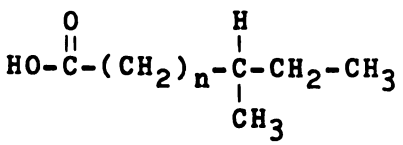
To answer the question "What is leucic acid doing in *E. coli*?", both the biosynthesis and degradation of its amino acid analogue, leucine, should be considered.

The ^{13}C tracer data indicate that leucic acid was derived either directly from endogenous leucine or by the established biosynthetic pathway for leucine (see Figures 7 and 8). Thus, it would seem unlikely that leucic acid represents an intermediate in a previously unknown pathway for leucine biosynthesis.

The ability to degrade the carbon chains of the branched chain amino acids (leucine, valine, isoleucine) is widely distributed in nature. For example, many bacteria can oxidatively decarboxylate valine, leucine, and isoleucine to yield the CoA thioesters of isobutyrate, isovalerate, and 2-methylbutyrate, respectively. Serial additions of malonyl CoA to these esters then provides branched chain "iso" and "anteiso" fatty acids which are the major fatty acid constituents of the membrane phospholipids of many bacteria (94):



iso



anteiso

Many microorganisms and mammals can also use the branched chain CoA esters as energy sources, degrading them further to acetic, acetoacetic, or succinic acid (95).

However, branched chain fatty acids are not found among the lipids of *E. coli* (96) and the carbon skeletons of the branched chain amino acids are considered to be metabolically inert as an energy source in *E. coli* (97).

In view of these facts, it is proposed as a working hypothesis that valic and leucic acid represent the entire

catabolic pathways for valine and leucine in *E. coli* under anaerobic conditions.

One can speculate that the ability of *E. coli* to produce valic and leucic acid under anaerobic conditions could be of great importance. Under anaerobic conditions, *E. coli* must reoxidize its reduced flavin and nicotinamide nucleotides by reducing organic compounds rather than O_2. It is important that this be done efficiently since the cellular amounts of these nucleotides are relatively small. If α-ketoisocaproate was produced from leucine via transaminations 2a,b or 3a of Figure 12, then pyruvate or oxaloacetate might become unavailable as oxidants for reduced nucleotides. If *E. coli* did not then have the option of reducing α-ketoisocaproate to leucic acid, its store of oxidized nucleotides might become quickly exhausted with a consequent cessation of glucose fermentation and anaerobic growth.

The production of valic and leucic acid might also function as a "safety valve" in a different way, namely, as a way of removing valine and leucine from the intracellular amino acid pool under anaerobic conditions. The presence of excess amounts of certain amino acids in many living systems is poisonous and can result in complete cessation of growth. Probably the best known example of this phenomenon is the growth inhibition of *E. coli*, K-12 by excess amounts of valine (98).

Whatever significance valic and leucic acid prove to have in *E. coli*, further studies of the amino acid

metabolism of *E. coli* under anaerobic conditions should certainly take into account its ability to produce these two acids.

C. Extensions of the Present Analytical System and Discussion of its Merits and Limitations

The GC-MS analysis developed in this study could be extended in a number of directions.

For example, greater resolution of the metabolite derivatives could be obtained using gas capillary columns instead of packed columns. Packed columns were used in this study since they are more durable and much less expensive than capillary columns. However, the large number of metabolites encountered in complex mixtures often results in overlapping of some metabolite derivatives when these are chromatographed on packed columns. For example, the TMS derivatives of citrate, isocitrate, and 3-phosphoglycerate are difficult to separate using chromatographic conditions similar to those of this study. The open tubular design of capillary columns often allows spectacular increases in the resolution of mixtures over those obtained with packed columns. It would be interesting to see what resolution enhancement could be obtained for the *E. coli* metabolites by chromatographing them on a capillary column.

As mentioned above, it would also be useful to extend the analysis to include cellular amino acids. Amino acids have not been detected among the metabolites examined thus far and they presumably were sorbed to the Dowex 50 [H^+]

resin with HEPES buffer during purification of the cell extract. These amino acids could probably be separated from HEPES and then analyzed as their N-TFA amide, n-butyl ester derivatives (99) by GC-MS. It would be an advantage to obtain the amino acids as a separate fraction since the addition of the amino acids to the present metabolite mixture could result in peak overlaps during gas chromatography. In addition, as a separate fraction, the amino acids could be derivatized by a method other than silylation. This would be advantageous because the TMS derivatives of amino acids are fairly unstable.

In extending the analytical system as a quantitative analysis, a promising course to follow would be to quantitate the metabolites using GC-MS and the isotope dilution method. Metabolites labeled with stable isotopes (e.g., ^{18}O) could be added in known amounts to the cell sample just after perchloric acid denaturation. The labeled metabolites, being chemically indistinguishable from cell metabolites, would serve as internal standards which would obviate the tedium and uncertainty involved in determining metabolite recoveries at all subsequent analytical steps. In the final step of analysis, the mass spectrometer could determine the ratio of amount of cell metabolite to internal standard, and the absolute amount of cell metabolite initially present in the perchloric acid extract could then be calculated. Examples of compounds that could be tried as internal standards include [2-^{18}O] lactate, [2-^{18}O] leucic

acid, and [2-^{18}O] valic acid. These could be synthesized by exchanging the keto group of the readily available pyruvate, α-ketoisovalerate, and α-ketoisocaproate in $H_2{}^{18}O$. The exchanged keto acids could then be reduced with $NaBH_4$ to afford [2-^{18}O] labeled lactic, valic, and leucic acids, respectively. Other examples of possible ^{18}O labeled internal standards are the ^{18}O labeled aldoses as discussed later.

The results of this study indicate that GC-MS can be a very valuable tool in the study of cellular metabolism. A single GC-MS run allows the simultaneous detection and isotopic analysis of a large number of metabolites of diverse chemical classes. A comparable analysis using radioisotopes and classical methods would require much larger amounts of labor, time, and material. GC-MS analysis also affords the opportunity of detecting unexpected or previously unknown metabolites as was illustrated by the discovery of valic and leucic acid in this study of E. coli. When an unknown metabolite is detected, mass spectral analysis can bring a great deal of information to bear in solving its identity.

The large number and diversity of metabolites that can be analyzed in a GC-MS run is impressive and this gives GC-MS an aspect of being a "wide window" for viewing metabolism. However, there are a number of important metabolites that cannot be detected with GC-MS. These include the nucleoside di- and triphosphates (ADP, ATP, etc.), nucleoside diphosphate sugars, pyridine nucleotides, and

flavin nucleotides. The derivatives of these large phosphate anhydrides are too large and/or labile to be eluted from a GC column. Although technical advances in combined liquid chromatography - mass spectrometry (LC-MS) might someday make mass spectral analyses of these compounds possible, it would appear that classical enzymatic and spectrophotometric methods or modern HPLC instruments will provide the best determinations of these compounds for some time to come. Proteins and nucleic acids are also "invisible" to GC-MS analysis, and it is unlikely that the rapid and convenient radioactive assays of protein and nucleic acid synthesis will soon become obsolete.

In performing ^{13}C metabolic tracer experiments, GC-MS offers greater sensitivity than ^{13}C NMR and facilitates the determination of % ^{13}C content of metabolites. As mentioned previously however, GC-MS usually cannot provide the position of ^{13}C enrichment with the precision available from ^{13}C NMR. The availability of substrates labeled at different positions and some knowledge of the metabolic pathways involved can often offset this shortcoming as was the case with the present study of leucic acid. However, in a situation where the positions of ^{13}C enrichment in a molecule were needed with great precision, ^{13}C NMR would become the analytical method of choice.

In concluding, it should be emphasized that a great deal more work must be done to gain a complete understanding of cellular intermediary metabolism. This is true despite

the impression one would get from most biochemistry texts. Although many of the individual cellular reactions have been studied thoroughly *in vitro*, it is still necessary that the sum of these reactions be studied as they occur in the intact cell. In such studies it would be desirable to quantitate a large number of metabolites at various points in time. It would also be important to determine the position and amount of isotopic tracers in metabolites. A good reason for obtaining such isotopic data on a fairly routine basis is that many metabolites can have multiple origins. This fact was well illustrated in the present ^{13}C tracer study which indicated that leucic acid arose from not one, but three different sources and lactic acid had not one, but two or possibly more origins. Obtaining this information for a large number of metabolites on a routine basis using radiotracers and classical techniques would require large amounts of time, labor, and material, while these disadvantages are not shared in using GC-MS and stable isotopes. The purpose of this study was to illustrate the practicality of GC-MS analysis of cellular metabolism and it is hoped that application of GC-MS and stable isotopes will make possible other studies of the metabolism of intact cells which had previously been considered impractical.

Part II Synthesis of Aldoses Enriched with ^{18}O

A. The Use of Molybdate Resin in Conducting Epimerizations

When the idea of using molybdate epimerization as a method for incorporating oxygen isotopes into aldoses was first conceived, a serious technical problem delayed implementation of the idea. The problem was that epimerizations to that time (see 79) employed aqueous solutions of molybdate and such epimerizations with pentoses produced substantial amounts of unwanted C-3 epimers in addition to the desired C-2 epimer products. For example, epimerization of xylose to lyxose also produced arabinose and ribose totaling 42% of the pentose mixture at equilibrium (79). Conversion of arabinose to ribose was accompanied by the side-products lyxose and xylose totaling 19% of the mixture, while conversion of ribose to arabinose produced lyxose and xylose totaling 10% of the pentose mixture. These side-products not only lowered the yield of the desired C-2 epimers, but they also seriously complicated their purification.

The production of these C-3 epimer side-products can be substantially reduced if the epimerization is carried out in an aprotic solvent such as DMF, using dioxobis(2,4-pentanedionato-O-O') molybdenum (VI) as catalyst (100). However, these epimerizations reach equilibrium after about a week instead of the 6-8 h typically required by aqueous reactions. The aprotic epimerization would also not have been convenient for ^{18}O incorporations since it was desired to carry out these reactions in $H_2{}^{18}O$.

Acting on the idea that if the molybdate catalyst were sorbed to an anion exchange resin, that it might sorb in a form that would catalyze the desired 2-epimerization and not the 3-epimerizations, an epimerization of an aqueous ribose solution was performed using an anion exchange resin in the paramolybdate form as catalyst. The reaction using molybdate resin was found to be consistently superior to those using aqueous solutions of molybdate. Soluble molybdate reactions starting with ribose produced a total of ~10% xylose + lyxose side-products at equilibrium, while with those utilizing molybdate resin only 1.5% total side-product was formed at equilibrium. Consequently, all epimerizations for incorporation of ^{18}O into aldoses were catalyzed with molybdate resin.

After completion of this study, a further refinement of the resin catalysis was introduced which was based on the idea that residual side-product formation during resin catalysis was due to soluble molybdate leached from the resin (E.L. Clark, Jr., M.L. Hayes, and R. Barker - manuscript submitted). Anion exchange resin in the formate form was added to the resin catalyzed epimerizations to act as a scavenger of soluble molybdate, and in these reactions a further 5-fold decrease in the amount of side-products formed was observed. This latter method would be the one of choice for future ^{18}O incorporations into aldoses as discussed below.

B. Characterization of the ^{18}O Aldose Products

Total ^{18}O incorporation into aldoses was determined by analysis of their permethylated alditols with ammonia chemical ionization mass spectrometry. Exchange of $H_2{}^{18}O$ into the aldehydo group of monosaccharides produced [1-^{18}O] aldoses and aldoses derived from [5-^{18}O] ribose containing greater than 95 atom % ^{18}O.

Molybdate epimerizations in $H_2{}^{18}O$ produced aldoses with total ^{18}O enrichments given in Table 4.

Table 4. ^{18}O Enrichments of aldoses achieved by molybdate epimerizations.

Aldose	Atom % excess ^{18}O
[2-^{18}O] erythrose	96
[2-^{18}O] threose	96
[2-^{18}O] arabinose	96
[2-^{18}O] ribose	67
[2-^{18}O] mannose	97
[2-^{18}O] glucose	93

As can be seen in Table 4, high enrichments were achieved for these aldoses except in the case of [2-^{18}O] ribose where ^{18}O incorporation was about 70% that of the other five. This result can be understood by consideration

of Figure 2 which describes the reaction in which [2-^{18}O] ribose was made. Preincubation of the ribose in $H_2{}^{18}O$ before the epimerization insured that the arabinose produced from it would be highly enriched at position-2, while a similar preincubation of the arabinose from which the [1,2-^{18}O] ribose was derived was not performed. Probably a more important factor was that the epimerization was terminated when the arabinose : ribose ratio was 1.6 instead of the equilibrium value of 2.0. This could have left a significant fraction of the ribose not having undergone any epimerization.

Although a longer epimerization would have produced more highly enriched [2-^{18}O] ribose, this might have led to a significant accumulation of xylose and lyxose side-products which totaled about 1.2% in this particular case. After completion of this study, a more refined method (see above) for conducting molybdate epimerizations in aqueous media was developed. This method employs a mixture of molybdate and formate resins and should allow longer reaction times and higher enrichments to be achieved without significant side-product formation.

In verifying that molybdate incorporated ^{18}O label only into the 2-position of aldoses, the best course to follow would be to chemically remove the 2-oxygen in the Table 4 aldoses and demonstrate that these derivatives contain no excess ^{18}O by ammonia chemical ionization mass spectrometry.

This might best be done by preparing the peracetylated phenylosotriazoles (101) of the Table 4 aldoses as illustrated for [2-^{18}O] ribose in Figure 13.

Time constraints have not allowed such analyses to be performed, however, other evidence strongly suggests that molybdate incorporated ^{18}O only into the 2-position of aldoses. An example of such evidence is the electron impact mass spectra of the permethylated mannitols derived from the various ^{18}O labeled mannoses that were synthesized. The diagnostic ions of these mass spectra are the m/z 89 and m/z 133 ions derived from α-cleavage of the carbon chain as shown in Figure 14.

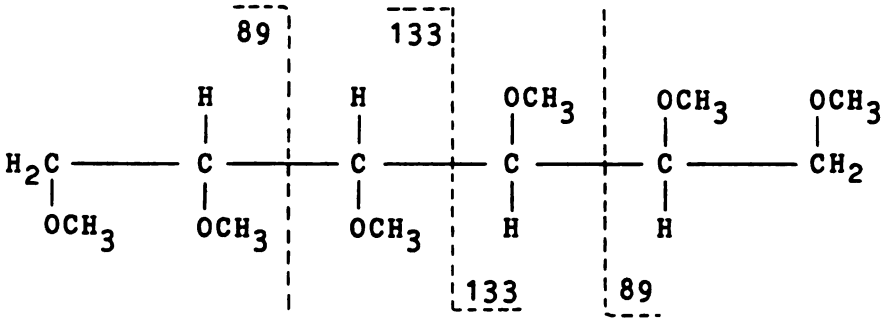


Figure 14. α-Cleavage fragments of permethylated mannitol

Broken lines intersect carbon-carbon bonds broken in the formation of the ions.

The symmetry of permethylated mannitol is such that these ions should arise from either end of the molecule with equal probability. The fraction of α-cleavage ions containing one excess ^{18}O atom for the various ^{18}O labeled mannitols is shown below in Table 5.

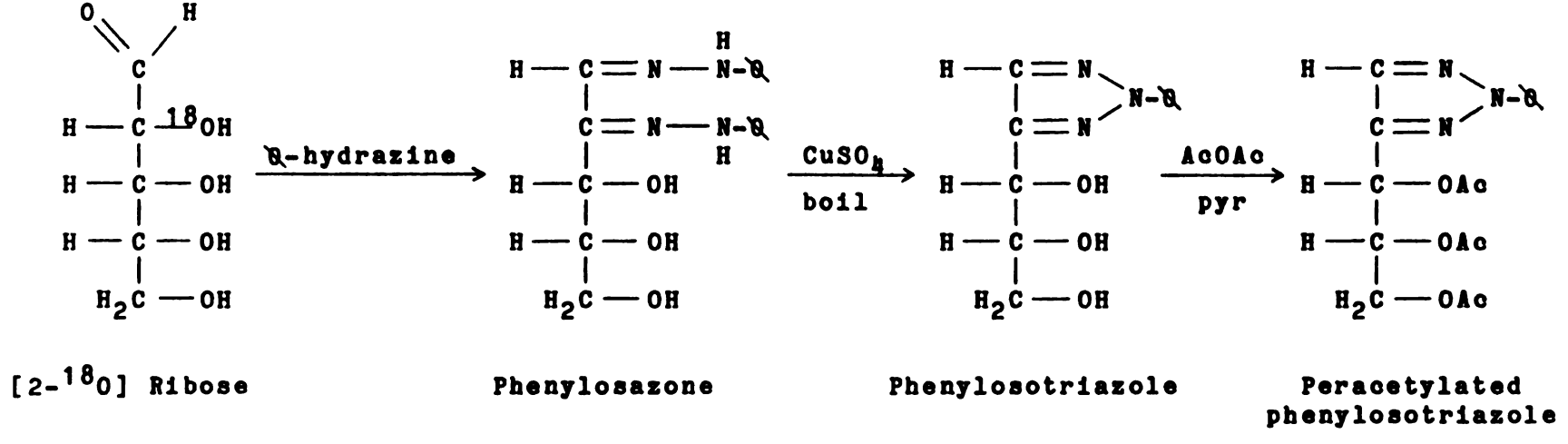


Figure 13. Proposed conversion of [2-^{18}O] ribose to its peracetylated phenylosotriazole for GC-MS analysis.

Abbreviations: Ø = phenyl, AcOAc = acetic anhydride, Ac = acetyl, pyr = pyridine.

Table 5. ^{18}O Labeling of m/z 89 and 133 fragment ions of various ^{18}O labeled permethylated mannitols

Source	Ion	Fraction of ions containing one excess ^{18}O atom
[1-^{18}O] mannitol	89	0.44
	133	0.45
[6-^{18}O] mannitol	89	0.44
	133	0.45
[3-^{18}O] mannitol	89	0.06
	133	0.34
[4-^{18}O] mannitol	89	0.06
	133	0.33

[1-^{18}O] Mannose and [6-^{18}O] mannose were synthesized in ways that leave no doubt as to the position of their ^{18}O enrichment. Because of the symmetry of permethylated mannitol, we would expect the mass spectra of the mannitol derivatives of these two mannoses to be identical and the data of Table 5 shows this to be the case.

[3-^{18}O] Mannose and [4-^{18}O] mannose were ultimately derived from aldoses that were enriched with ^{18}O through molybdate epimerizations. [3-^{18}O] Mannose was derived from [2-^{18}O] arabinose while [4-^{18}O] mannose was derived from [2-^{18}O] erythrose (see Figure 3). If molybdate epimerization incorporated ^{18}O only into the expected 2-positions of erythrose and arabinose, then the symmetry of the resulting

[3-^{18}O] and [4-^{18}O] mannitol derivatives should result in identical mass spectra for the two. The data of Table 5 shows this to be the case.

In addition, ammonia chemical ionization mass spectra of the permethylated alditols derived from ^{18}O aldoses showed that only one excess ^{18}O atom was present in the ^{18}O aldoses. This would be the expected result if molybdate epimerization incorporated ^{18}O only into the single, expected 2-position of aldoses. These data indicate that it is very unlikely that molybdate epimerizations incorporate ^{18}O label into aldose positions other than the expected 2-position.

C. The Relative Merits and Disadvantages of the Methods and Their Application in Synthetic Problems

The methods described in this paper have advantages in terms of general applicability over the previously mentioned approaches of Caprioli and Seifert (76) and Gorin and Mazurek (77).

The objective of the first study (76) was to produce samples of glucose labeled at different positions with ^{18}O and this work relied heavily on the commercial availability of enzymes which would act upon derivatives of glucose and fructose. Although this approach produced such rare glucoses as [4-^{18}O] glucose in good yield, its general applicability to other aldoses is restricted by a lack of commercially available enzymes that will act upon these sugars.

The objective of Gorin and Mazurek's study was to produce a variety of ^{17}O labeled monosaccharide derivatives for ^{17}O NMR studies. Their syntheses relied heavily on $H_2{}^{17}O$ exchange with the keto group of a specifically oxidized monosaccharide derivative (most commonly a cyclic acetal derivative). The isotopically exchanged compound was reduced to afford epimeric, isotopically enriched aldose derivatives. Such a synthesis with 1,2 : 5,6-di-O-isopropylidene-D-ribo-hexofuranos-3-ulose as the starting compound is illustrated in Figure 15.

Although the general applicability of producing isotopically enriched aldoses by similar approaches or others such as the opening of epoxides (102) in isotopically enriched H_2O has not been evaluated, this approach would appear to be hampered in some cases by a lack of substrates for the isotope exchange step. In addition, problems could be encountered in the subsequent reduction of an isotopically exchanged keto derivative. For example, attempts to obtain significant yields of [3-^{18}O] glucose (76), [3-^{17}O] glucose (77), or [3-^{2}H] glucose (103) using the synthesis of Figure 15, failed owing to the very stereoselective reduction of the keto function which yields the allo-epimer almost quantitatively.

The methods described in this thesis have advantages in terms of product yields over the cyanohydrin synthesis (74) for producing ^{18}O enriched aldoses. In the cyanohydrin synthesis, the carbonyl oxygen of an aldose is exchanged

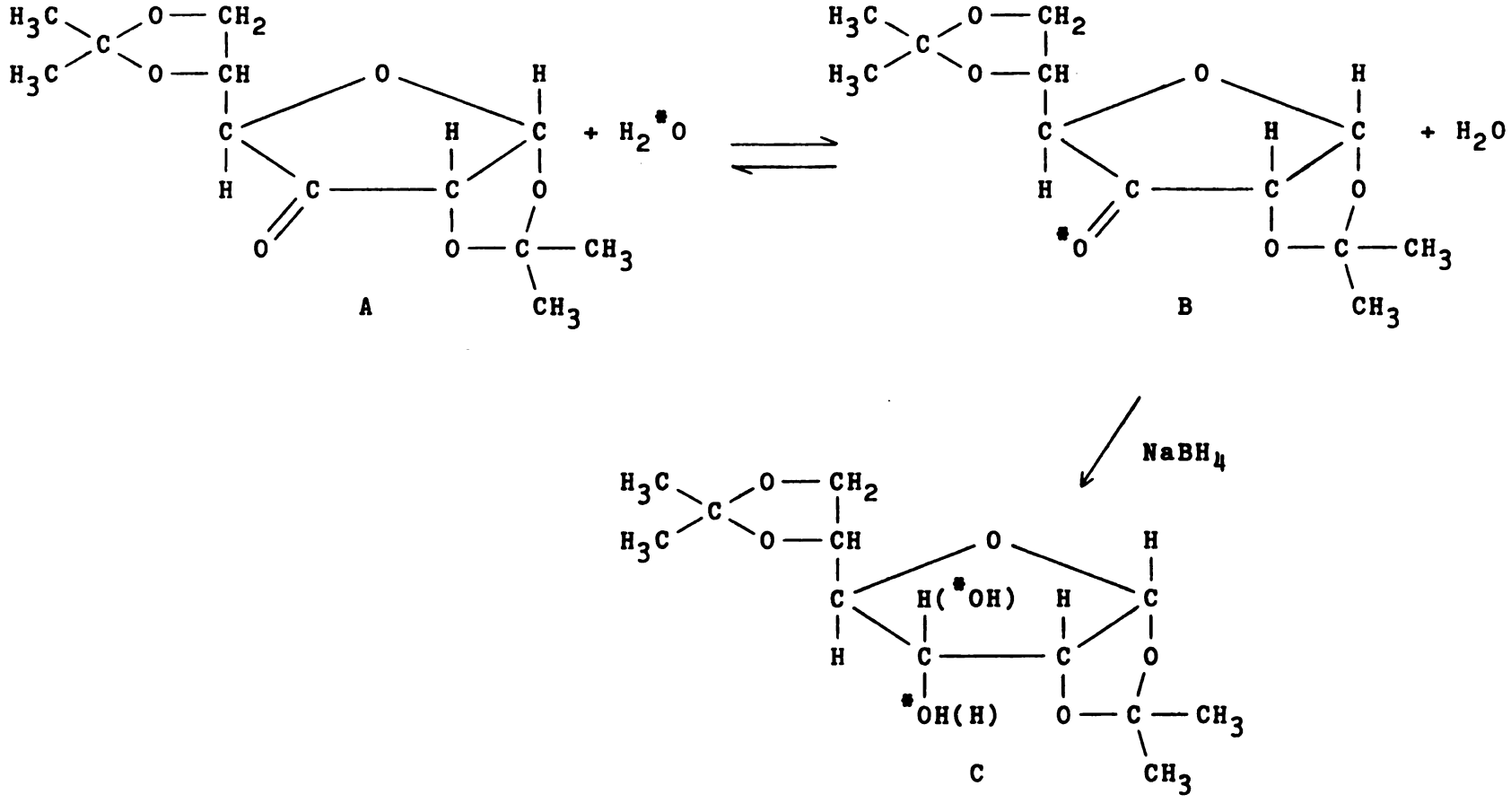


Figure 15. A synthesis of ^{18}O labeled monosaccharides using a specifically oxidized cyclic acetal derivative.

A = unlabeled starting compound. B = ^{18}O labeled starting compound after exchange with $H_2{}^{18}O$. C = epimeric ^{18}O labeled monosaccharides. Asterisk denotes ^{18}O atom.

with isotopically enriched H_2O; and cyanide is added to the exchanged aldose to trap the oxygen isotope and produce two epimeric cyanohydrins that are reduced to epimeric aldoses one carbon longer than the starting aldose. The general steps of the cyanohydrin synthesis are illustrated in Figure 16.

Serial application of the cyanohydrin synthesis could be used to produce all 17 aldoses from formaldehyde through the hexoses with oxygen label at any or all desired positions. However, this synthesis can become very inefficient in producing a particular ^{18}O enriched aldose since product losses as high as 30% can occur through the production of aldonate and amine side-products and large losses occur due to the formation of an unwanted epimeric aldose at each application of the synthesis. The two types of product losses incurred in the cyanohydrin synthesis are ameliorated by use of the molybdate epimerization approach of the present study. For example, the synthesis of [2-^{18}O] glucose was not accompanied by side-product formation as would have been the case in producing [2-^{18}O] glucose from [1-^{18}O] arabinose in the the cyanohydrin synthesis. In addition, the molybdate epimerization produces an equilibrium between the pair of 2-epimers that generally favors the more biologically important (and thus, more desirable) of the two. This is often not the case with the cyanohydrin synthesis. For example, the production of [2-^{18}O] glucose via the

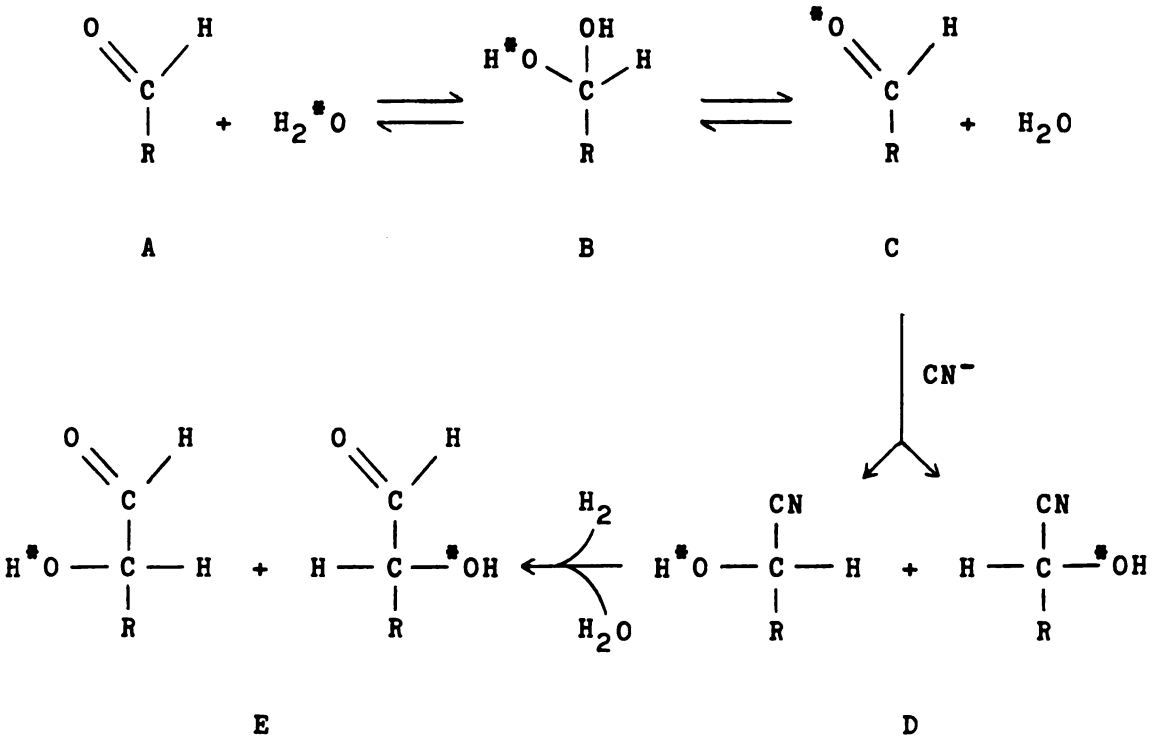


Figure 16. The general cyanohydrin synthesis for labeling aldoses with ^{18}O.

A = unlabeled starting aldose. B = gem diol intermediate in exchange reaction. C = ^{18}O labeled starting aldose. D = epimeric, 2-^{18}O labeled cyanohydrins. E = epimeric, 2-^{18}O labeled aldoses one carbon longer than starting aldose. Asterisk denotes ^{18}O atom.

cyanohydrin synthesis yields glucose and mannose in a ratio of 1:2 while the molybdate epimerization gives a ratio of 2.5:1.

The methods described in this study could be used in a similar fashion to produce the other aldo-hexoses and -pentoses not synthesized in this study. For example, various ^{18}O labeled alloses and altroses could be synthesized from the ^{18}O enriched riboses made in this study, as illustrated in Figure 17.

The methods could also be used in a reaction scheme similar to that of Figure 3 to produce xylose, lyxose, galactose, talose, gulose, and idose enriched at various positions with ^{18}O as illustrated in Figure 18. The [5-^{18}O] xylose of Figure 18 could be obtained by first exchanging 1,2-O-isopropylidene-D-xylo-dialdopentofuranose (104) with $H_2{}^{18}O$ and then reducing with $NaBH_4$ to afford [5-^{18}O] 1,2-O-isopropylidene-D-xylofuranose. After hydrolysis of the monoacetone xylose, the resulting [5-^{18}O] xylose could be epimerized with molybdate to give a mixture of [5-^{18}O] lyxose and xylose.

The methods discussed in this study could also be used to produce aldoses with multiple enrichments. For example, a [2-^{18}O] aldose produced by molybdate epimerization could be pre-exchanged with $H_2{}^{18}O$ at the carbonyl position and then converted into epimeric [2,3-^{18}O] aldoses via the cyanohydrin synthesis. Aldoses enriched simultaneously with oxygen, carbon, and hydrogen isotopes could also be

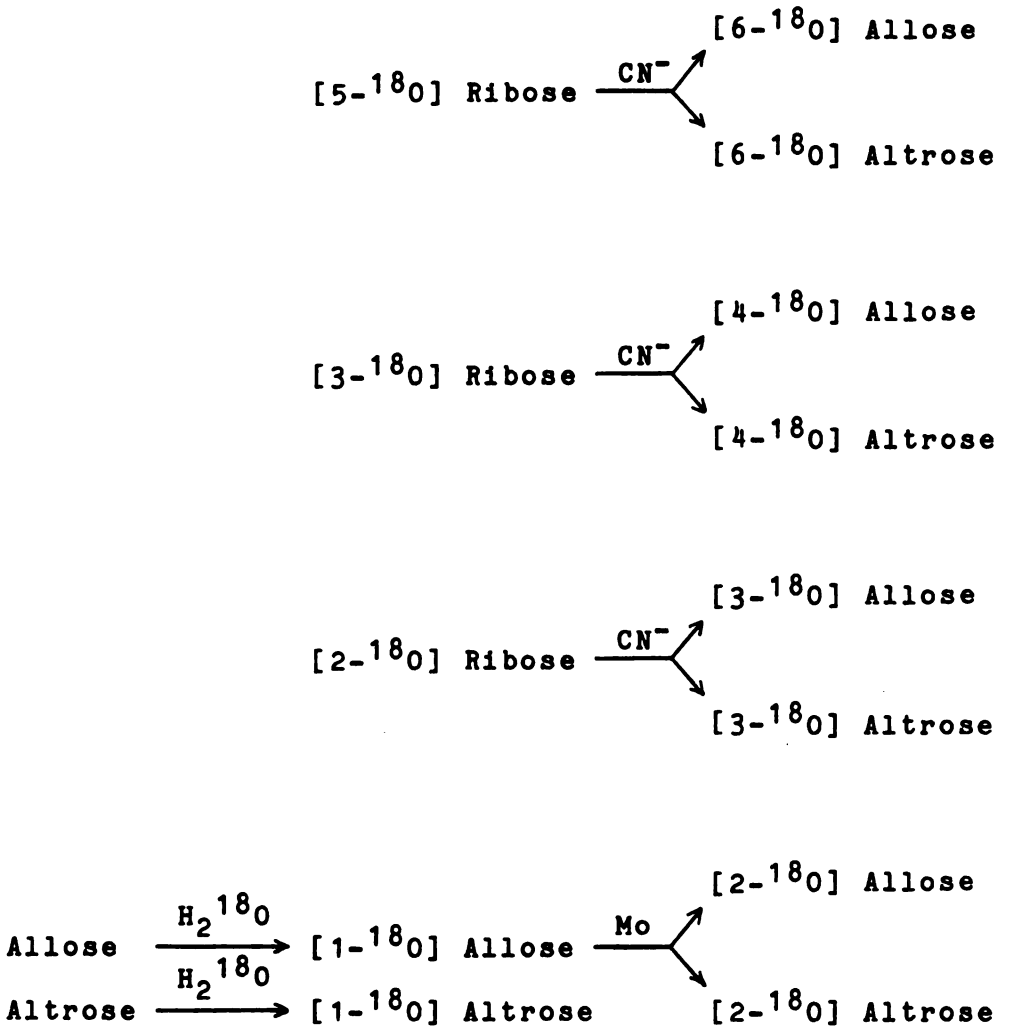


Figure 17. Application of ^{18}O synthetic methods in making various ^{18}O labeled alloses and altroses.

CN^- = cyanohydrin synthesis, Mo = molybdate epimerizations in $H_2{}^{18}O$.

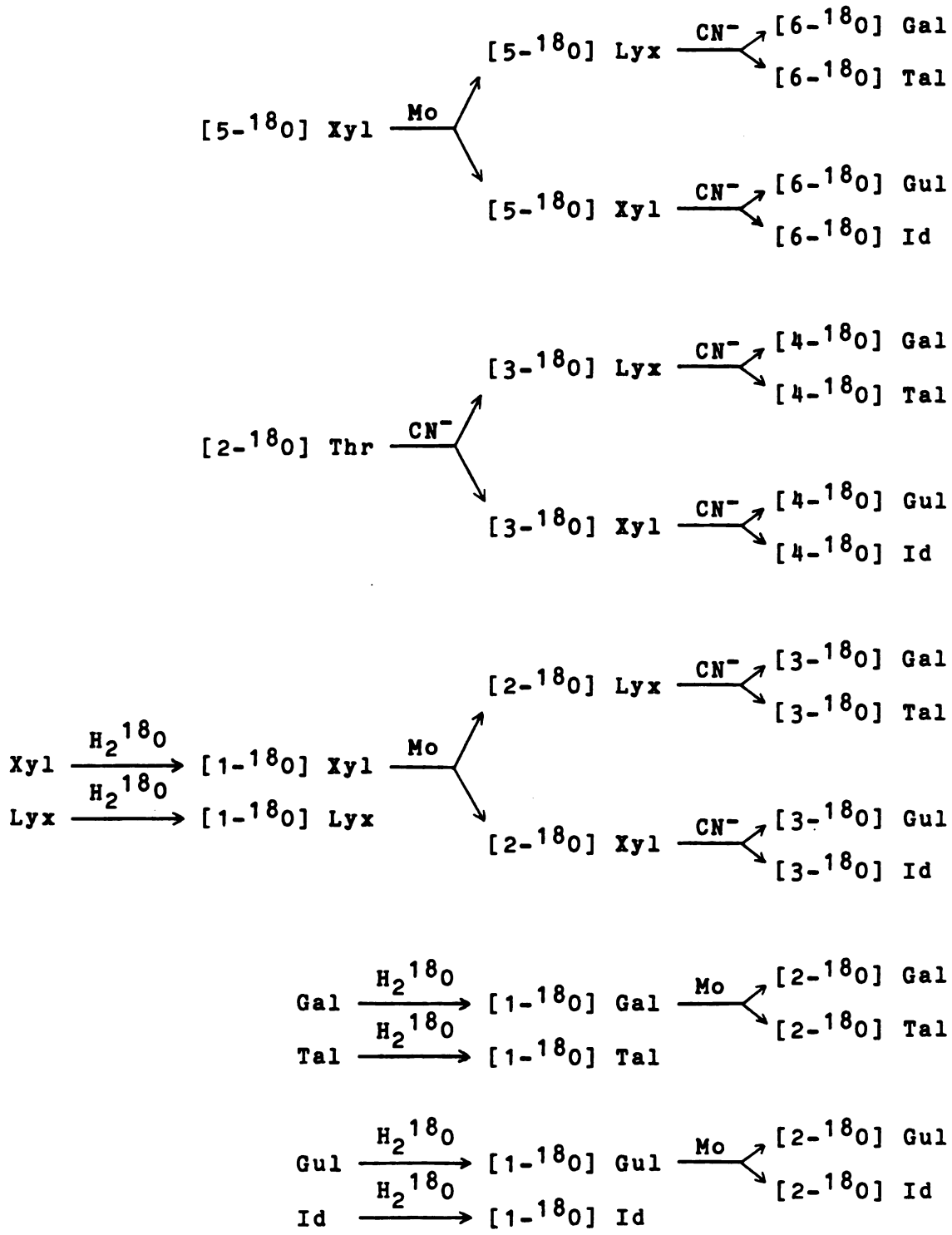


Figure 18. Application of ^{18}O synthetic methods to lyxose (lyx), xylose (xyl), galactose (gal), talose (tal), gulose (gul), and idose (id).

synthesized. For example, [1-^{13}C, 1-^{2}H] glucose would yield [1-^{13}C, 1-^{2}H, 2-^{18}O] glucose and [2-^{13}C, 2-^{2}H, 2-^{18}O] mannose after epimerization by molybdate in $H_2{}^{18}O$. Many other labeling combinations could be obtained by combining the oxygen labeling methods of this study with cyanohydrin methods (71,74) for introducing carbon and hydrogen isotopes into aldoses.

It is concluded that the synthetic methods of this study are good, general approaches for producing [1-, 2-, 3-, or 6-^{18}O] hexoses and [1-, 2-, 3-, or 5-^{18}O] pentoses. [4-^{18}O] Hexoses were synthesized to illustrate the fullest applicability of the methods. However, the two cyanohydrin syntheses required by this route (see Figure 3) mean that a final yield of a particular [4-^{18}O] hexose will be no better than 20%. Other synthetic routes to [4-^{18}O] hexoses and the [5-^{18}O] hexoses and [4-^{18}O] pentoses not available by the present methods, should be sought.

A possible use of the ^{18}O aldoses synthesized in this study would be as internal standards for the quantitation of sugar metabolites by isotope dilution. The enzymes and reagents necessary for converting ^{18}O aldoses to important ^{18}O sugar phosphates are commercially available and inexpensive. For example, [2-^{18}O] glucose could be converted to [2-^{18}O] glucose-6-phosphate by the action of hexokinase. [6-^{18}O] Glucose could be converted to [6-^{18}O] fructose-1,6-diphosphate by the enzymes hexokinase, hexose phosphate isomerase, and phosphofructokinase.

The [6-^{18}O] FDP could also be converted to [6-^{18}O] fructose-6-phosphate by mild acid hydrolysis.

Although time did not permit the application of the ^{18}O aldoses as internal standards, it is hoped that future work in that area as well as in metabolic tracing can make use of the synthetic methods described in this thesis.

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LIST OF REFERENCES

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APPENDIX

APPENDIX

List of Publications

1. A.S. Serianni, E.L. Clark, Jr. and R. Barker, Carbon-13 Enriched Carbohydrates. Preparation of Erythrose, Threose, Glyceraldehyde and Glycoaldehyde with ^{13}C Enrichment in Various Carbon Atoms, Carbohydr. Res. 72 (1979):79.

2. R. Barker, E.L. Clark, Jr., H.A. Nunez, J. Pierce, P.R. Rosevear and A.S. Serianni, The Synthesis of Mono- and Oligosaccharides Enriched with Isotopes of Carbon, Hydrogen, and Oxygen, Stable Isotopes (1982):719.

3. A.S. Serianni, E.L. Clark, Jr. and R. Barker, Chemical Synthesis of Aldoses Enriched with Isotopes of Hydrogen and Oxygen, Methods Enzymol. 89 (1982):64.

4. E.L. Clark, Jr. and R. Barker, A Gas Chromatographic Approach to the Study of Cellular Intermediary Metabolism, Anal. Biochem. submitted for publication.

5. E.L. Clark, Jr. and R. Barker, General Methods for Enriching Aldoses with Oxygen Isotopes, Carbohydr. Res. submitted for publication.

6. E.L. Clark, Jr., M.L. Hayes and R. Barker, Paramolybdate Form, Catalysis of the C-1, C-2 Rearrangement, C-2 Epimerization of Aldoses, Carbohydr. Res. submitted for publication.