TRACER TECHNIQUES

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The understanding of metabolic processes has advanced at a fantastic rate since the advent of tracer methods. Prior to the discovery isotopes of the elements of concern to biochemists, some crude tracer experiments were done. Oleic acid, for example, was converted to 9,10-dichlorostearic acid by adding chlorine across the double bond. This crude type of label could only trace the general fate of the fatty acid molecule by measuring chlorine but could not lead to information regarding the molecular basis for the metabolic processes in which this fatty acid is involved.

The discovery by Hevesy in 1911 of an unstable isotope of lead (Pb^{210}) which had been assumed to be contaminating radium, marked the beginning of tags or tracers within the nucleus of an atom. The chemical properties of the tagged atom are not significantly altered in these isotopic tracers. First elements such as nitrogen and hydrogen, which occur in nature as mixtures of two or more isotopes, were exploited by separating the stable isotopes of these elements. The stable isotopes usually differ from the predominant isotope by one mass unit. While the permissible dilution of such isotopes is far below that for radioactive isotopes, deuterium, (H^2) and N^{15}, detectable by mass spectrometry, were sufficiently useful to provide data which revolutionized our concepts of metabolism. Schoenheimer and coworkers at Columbia presented an hypothesis which stated that body constituents were in a dynamic state of equilibrium with each other and with the nutrients provided to the organism.

With the production of unstable isotopes of the lighter elements, labels for most of the elements involved in biological processes became available. The necessity of having an accelerator or a neutron source to produce isotopes, limited the number of laboratories using radioisotopes until the close of World War II. At that time, nuclear reactors began to produce the unstable isotopes of biologically important elements in sufficient amounts and at a cost low enough to bring these precious tagged atoms to the aid of scientists in most laboratories in this country. With this increased availability of radioisotopes, great improvement in instrumentation also came. Today a vast array of mass produced instruments for measuring various isotopes present in many compounds is on the market. Because of the ease of measuring isotopic carbon (C^{14}), this method has frequently replaced more conventional methods of following the course of a reaction in the laboratory. The availability of most common nutrients and metabolites labeled in specific carbon atoms with C^{14} has also enhanced the usefulness of this technique.

While the specific biochemical reaction can be studied without isotopically labeled substrate <u>in vitro</u>, the role of this reaction in the intact organism can only be evaluated by in vivo experiments and usually this requires isotopic substrate. Some examples of this application of isotopes will be cited in the remarks which follow. In many cases the understanding

of the metabolism of a compound has come from enzyme experiments designed after isotopic studies in the intact organism had indicated the general nature of the metabolic processes involved.

I. GENERAL PRINCIPLES

A. Chemical properties and hence the name we give an element are determined by the number of extranuclear electrons. Chemical reactions are largely independent of the mass of the nucleus.

B. The rays or particles emitted by unstable isotopes are (a) electrons, (b) positrons, (c) α-particles or (d) γ-rays, each with its own specific properties.

C. The rate of decay of unstable isotopes is independent of temperature, pressure, etc., and of the chemical combination of the element. Thus the half life of C^{14} is 5,700 years regardless of the conditions or the chemical form in which it is found.

D. The isotope must be incorporated into the compound which you wish to study, usually into a specific position in the molecule.

E. There must be a suitable detecting system for measuring the isotope concentrations under the conditions of the experiment. For stable isotopes the maximum dilution tolerance is about 100 fold. For radioisotopes dilution of approximately 100,000 is permissible. Radioisotopes can be determined on material in the gaseous, liquid or solid state.

A list of the isotopes most frequently used to study biochemical processes is shown in Table I:

TABLE I

SOME CHARACTERISTICS OF THE ISOTOPES
USED IN STUDYING LIVING PROCESSES

Element and Mass Number	Type of Radiation	Half-Life	Comments or Energy of Radiation	
			Particles	γ-Rays
H^2 (deuterium)	None	-----	0.02% in Nature	
H^3 (tritium)	β^-	12.1 yr.	0.017 mev	
C^{13}	None	-----	1.1% in Nature	
C^{14}	β^-	5,700 yr.	0.154 mev	
N^{15}	None	-----	0.38% in Nature	
O^{18}	None	-----	0.20% in Nature	
Na^{22}	β^+, γ	2.6 yr.	0.58 mev	1.3 mev
Na^{24}	β^-	14.8 hr.	1.4 mev	1.38, 2.7 mev
P^{32}	β^-	14.3 days	1.7 mev	
S^{35}	β^-	87.1 days	0.17 mev	

TABLE I (Continued)

Element and Mass Number	Type of Radiation	Half-Life	Comments or Energy of Radiation	
			Particles	γ-Rays
K^{40}	β^-	4.5×10^8 yr.	1.3 - 1.9 mev	1.46 mev
K^{42}	β^-	12.4 hr.	2.0 mev (25%) 3.6 mev (75%)	
Ca^{45}	β^-	180 days	0.26 mev (50%) 0.46 mev (50%)	
Fe^{59}	β^-	45.1 days	0.257, 0.460 mev	
Co^{60}	β^-, γ	5.3 yr.	0.31 mev	1.16, 1.32 mev
Zn^{65}	γ	250 days		1.120 mev
Sr^{90}	β^-	19.9 yr.	0.61 mev	
I^{131}	β^-, γ	8 days	0.6 mev	0.080 - 0.722 mev

Tracer techniques as the name implies permits following the fate of atoms or molecules by the mass or radioactivity of the isotopes. Because of the great sensitivity of the methods of measuring radioisotopes they are much preferred when available. Whereas stable isotopes are prepared by separation from natural sources the radioisotopes are prepared with a reactor or an accelerator. The simple procedure of tracing an element such as sodium or iron while useful, does not present the elegant power of tracing a specific carbon or hydrogen atom through the complexities of metabolic reactions. Examples of the various types of application of isotopes to physiology and biochemistry will be presented, attempting to classify these applications.

II. APPLICATIONS OF ISOTOPES TO THE STUDY OF LIVING PROCESSES

A. Requirements and Limitations of the Tracer Technique

The tracer technique impinges upon biology chiefly at the biochemical and physiological level. Its greatest contribution has been in the area of intermediary metabolism, an understanding of which is essential to the development of all areas of biological science. The advent of isotopic tracers has made it possible to follow the wanderings of a specific atomic grouping within the organism.

Before an isotope can be used as a tracer, one must make certain that the initial concentration of isotope is high enough to tolerate the dilution which occurs during metabolism and still remain detectable in the products separated at the close of the experiment. For radioisotopes, this condition can usually be met, but for stable isotopes, it often presents a problem. The labeled atom must remain attached to the molecule or portion of a molecule during the metabolic processes under study. The presence of the isotope must not affect the metabolic process. This becomes of some

importance for isotopes of hydrogen where "isotope effects" are observed. The half-life of the radioisotope must be long enough to permit the experiment to be done. Unstable isotopes of oxygen and nitrogen have half-lives too short for most purposes, but fortunately the stable isotopes O^{18} and N^{15} are available in nature and are separated and used for tracer experiments.

B. Physiological Applications of Isotopes

1. Permeability, Transport, Cellular Uptake, and Distribution

Before the use of isotopes, physiological processes were often studied using "unphysiological concentrations" of various metabolites. Radioactive metabolites can often be used at normal or less than normal physiological concentrations to measure permeability under conditions where there is no net transfer of a metabolite. For example, the passage of Na^+ back and forth across a cell membrane can be measured conveniently using Na^{24}. Transport of compounds into axones, across the intestinal wall and placenta and uptake of compounds by bacterial cells have all been studied effectively using isotopes. The kinetics of such processes can be readily evaluated by frequent analysis of the medium for the isotopic tracer.

2. Intracellular and Extracellular Space Determination by Isotope Dilution

Frequently the fluid volume, or space available for solution of electrolytes, both inside and outside the cells of an organism must be measured. Isotopes are useful for such measurements because the indicator can be a normal component of the space being measured. For total body water, H_2^2O and H_2^3O have been used. For extra-cellular space Na^+, Cl^- and Br^- may be used. For plasma volume, serum albumin, iodinated with I^{131}, and more recently P^{32} di-isopropylphosphorofluoridate, which combines with plasma proteins and with cellular components of blood, have been used. The water space of the brain has been measured using S^{35}-sulfate. Cardiac output and the pattern of blood flow through the heart are examples of similar applications of isotopes in the clinical field.

3. Hormone Studies

Because of the minute quantities required, hormones have been difficult to study. Without isotopes, the analytical methods demanded that massive doses of hormone be given. This left considerable doubt as to whether the findings were related to the true metabolic fate of the hormone in question. The selective uptake of hormones by the target organ can be investigated using isotopically labeled hormone at physiological concentrations. A good example of such experiments has been presented by Glascock (1) who observed that hexoesterol was concentrated more in the reproductive organs than in any other organ except the kidney, in 5.5 hours after being given at a level of 1 μg/kg of body weight.

Iodine-131 has been very useful in labeling the thyroid hormone and related compounds at a specific activity sufficiently high for experiments in the whole animal using near physiological concentrations of the hormone.

4. Mineral Metabolism

The metabolism of trace minerals, as in the case of hormones, can be studied most readily as radioisotopes, to make the trace amounts susceptible to analytical attack. Fe^{55} and Fe^{59} have been used to establish the rate of iron absorption and incorporation into heme. Isotopic studies have shown that red blood cells live about four months and platelets about 1 week. Isotopes of cobalt and copper have been useful in metabolism studies of these trace metals (1). Ca^{45} and P^{32} have been extensively used to study the deposition and mobilization of bone ash.

C. Biochemical Applications

The earliest experiments with isotopes revolutionized the existing views regarding the general nature of metabolic processes. The partitioning between exogenous and endogenous metabolism, a concept developed by Folin on the basis of the effect of dietary components on the concentration urinary constituents, was abandoned when the deuterium and N^{15} experiments of Schoenheimer and coworkers demonstrated the dynamic nature of metabolic processes. The more exciting contributions with isotopes have been those in which entire metabolic sequences and cycles have come to light.

A number of examples of these contributions will be presented briefly. Where appropriate, I will draw these examples from my own area of research.

1. Isotope Dilution

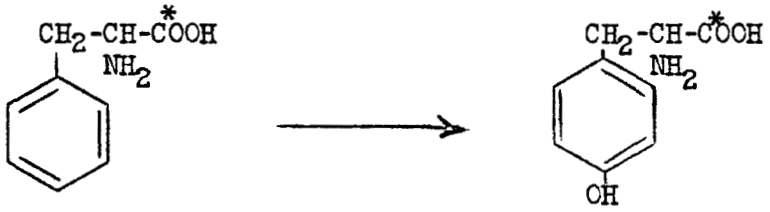
A fundamental concept, essential to the understanding of many applications of isotopes, is the isotope dilution principle. Analytically, this method is precise to about 1%. It depends upon the fact that when a known quantity (x_1) of a substance, S, labeled with an isotope, e.g., C^{14}, is added to a complex mixture containing an unknown amount (x_2) of S, the specific activity (A_1) of S will be reduced to a new figure (A_2) to the degree that the radioactive S is diluted with unlabeled S. The following equation expresses this relationship:

$$A_1 \; x_1 = A_2 \; (x_1 + x_2)$$

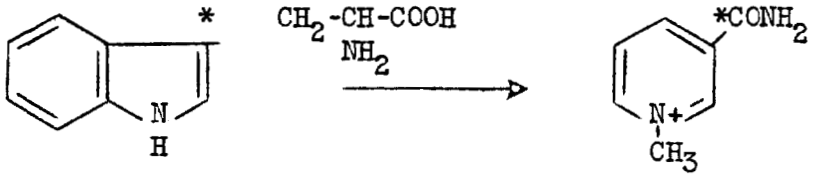
Since A_1 and x_1 are known, if a small sample of S from the mixed, labeled and unlabeled S can be isolated and its specific activity, A_2, determined this equation can be solved for x_2, the quantity desired. Many variations in the application of this principle are used. When a trace of a highly labeled compound is present in a biological material, it can be recovered and the total quantity of isotope ascertained by adding unlabeled compound (cold carrier) in a known amount and isolating a portion of the compound. If a micro-assay is available for the substance, the specific activity of the labeled compound arising from a metabolic process can be calculated.

2. Precursor-Product Relationship

One of the simplest questions often asked is whether compound A is converted to compound B. To answer this question, frequently only qualitative results are needed. For example: the conversion of phenylalanine to tyrosine (2).



and tryptophan to N^1-methyl nicotinamide (3).



In cases where isotope from A is found in substance B, but with so much as to cast doubt on the directness of the relationship, other approaches will be needed to establish a precursor-product relationship. Examples of this type of application are: (1) the formation of the steroid nucleus of cholesterol from acetate and isopentanyl pyrophosphate, (2) CO_2 fixation into certain simple molecules during photosynthesis, (3) the formation of porphyrin from succinate and glycine, (4) the formation of purines from CO_2, formate, glycine, aspartate N and glutamine N.

3. Method of Isotope Competition

This method is applicable specifically to bacterial systems. If a metabolic sequence, represented by $A \longrightarrow B \longrightarrow C \longrightarrow D$, exists in a cell then isotopically labeled A will lead to labeling of D. Addition of unlabeled B or C would then be expected to suppress the labeling of D.

4. Metabolite Overloading

In animals many metabolic processes cannot be studied with isotopes because the end product of one sequence of reactions (C) becomes the reactant of another sequence of reactions:

$$A \longrightarrow B \longrightarrow C \longrightarrow C_1 \longrightarrow C_2 \longrightarrow C_3 \longrightarrow CO_2.$$

If it is suspected that "C" is involved in the conversion of "A" to CO_2, the hypothesis might be tested by administering a small dose of isotopically

labeled "A" and simultaneously a dose of "C" of such magnitude that it appears in the urine. If the exogenous "C" equilibrates with the "C" formed from "A", it should be labeled and the label should be present in the urinary "C" which is isolated. A positive result implicates "C" as an intermediate in the degradation of "A"; but a negative result does not eliminate "C" as an intermediate.

5. Labeling Patterns in Tissue Components

When any compound, labeled in a specific carbon atom is degraded through simple 2, 3 and 4 carbon compounds, these small molecules are labeled in a single carbon atom and lead to characteristic labeling patterns in the glucose of liver and muscle glycogen, in fatty acids and/or in non-essential amino acids. Perhaps the most useful indicator of the identity of degradation products are the amino acids alanine, aspartic acid, glutamic acid, glycine and serine. Compounds leading to labeling in any position in pyruvate or acetate give characteristic labeling patterns especially in glutamic acid and alanine. The experiment is thus very simple. The compound of interest, uniquely labeled, is fed or injected into an animal and the animal is sacrificed about twelve hours later. The liver glycogen and liver or carcass protein are isolated, hydrolyzed and the fragments degraded, carbon by carbon, to CO_2 which is examined quantitatively for C^{14} activity. Table II illustrates the labeling pattern in the most indicative amino acids when acetate-1-C^{14}, acetate-2-C^{14} are fed to rats. From Table III (4) it is evident that the labeling pattern is glutamate and alanine from liver and carcass of a rat receiving tryptophan-7a-C^{14} is essentially the same as that observed when acetate-1-C^{14} is administered (5). Likewise the pattern in these two amino acids is similar to that from acetate-2-C^{14} when tryptophan-5-C^{14} was given (6). These results clearly indicate that

C-5 of tryptophan ——————————→ C-2 of acetate

C-7a of tryptophan ——————————→ C-1 of acetate

6. Trapping Techniques

A small molecule suspected to be a product of degradation might exist in the metabolic pool in quantities too small to detect and it might not be excreted in the urine normally. If a sample of the fragment can be withdrawn from the pool into the urine by detoxification of some foreign compound, it can be degraded and its labeling pattern determined. Acetate (7) and glycine are good examples of molecules which can be trapped in this way, glycine as hippuric acid and acetate as an acetylated amine.

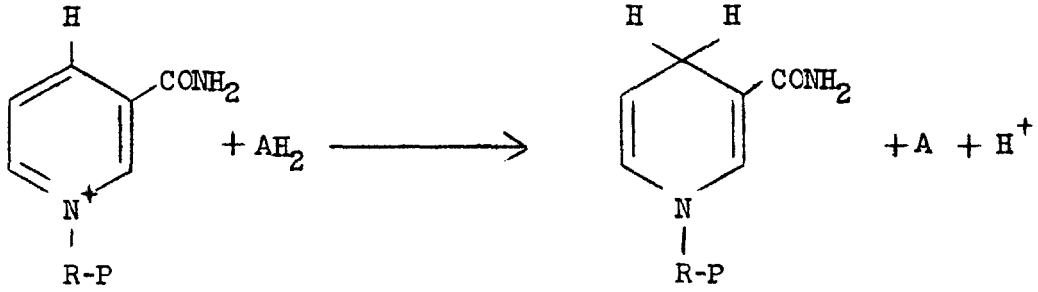
When tryptophan-C^{14} was injected into the rat together with cyclohexylalanine, the acetylcyclohexylalanine was excreted. It was isolated from the urine, hydrolyzed and the resulting acetate degraded stepwise to reveal a labeling pattern in acetate consistent with the amino acid labeling pattern. Table IV shows the results obtained (6, 8).

7. Evaluation of the Extent to Which Alternate Pathways Operate

Glucose metabolism is recognized to proceed by two pathways; glycolysis to 2 molecules of pyruvate and via the pentose pathway by which C_4, C_5 and C_6 also give rise to pyruvate while C_1 yields CO_2. Thus, the finding of C_{14} from C-1 of glucose in lactate reflects glycolysis and a comparison of the labeling in lactate from C-1 of glucose in one experiment and C-6 of glucose in another experiment has been used to determine the extent to which glucose metabolism in the bovine, corneal epithelium proceeds by glycolysis (9). Another method proposed by Blumenthal *et al.* (10)is based on the failure of C-1 of glucose to label acetate when the pentose pathway prevails.

8. Reaction Mechanisms

Both in organic chemistry and biochemistry, isotopes present a powerful tool for determining the mechanism by which a reaction proceeds. Perhaps the best known example of the solution of a reaction mechanism problem with isotopes was the elucidation of the stereospecific addition to, and removal of hydrogen from, carbon-4 of the pyridine nucleus of nicotinamide-adenine dinucleotide (NAD) (11).



Time will not permit the detailed discussion of these experiments, but it was shown that most NAD requiring enzymes are specific for one of the spatially distinct hydrogen atoms and that the atom is transferred directly (not via a proton in the medium) to the reduced product.

Another aspect of the mechanism of reactions brought to light by isotope experiments was the concept of a meso-carbon atom such as that found in glycerol or citric acid: (12, 13)

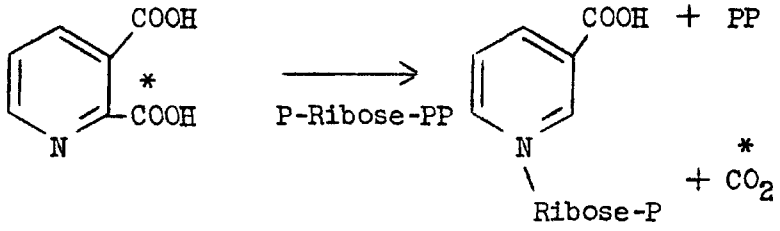
The carbon atom marked * has been considered as a symetric carbon atom by organic chemists. These molecules have a plane of symetry for they are
b
a-C-a type structure. A closer examination shows that they are in reality
d

b
a_1-C-a_2 type molecules, where a_1 and a_2 are related to each other as are
d
our right and left hands. It is not surprising in retrospect that an enzyme or some other optically active reagent should react at a different rate with a_1 than with a_2. Thus the two "a" groups are different to the enzyme and only one group reacts as has been clearly established with specifically labeled citrate and glycerol.

Oxygen-18 has been used to oxidize various substrates and a number of oxygenases shown to incorporate molecular oxygen not oxygen from water into the product.

9. Isotopes in Enzyme Assays

Rapid methods of following an enzyme catalyzed reaction are often based upon the use of radioisotopes. For example, Dr. Gholson and coworkers (6) have been studying the conversion of quinolinic acid to nicotinic acid monoucleotide by following the release of $C^{14}O_2$ from the 2-carboxyl group of the substrate



When isotope methods can be develoved, they are frequently more precise and rapid because of the ease of estimating radioactivity.

We have felt that isotopic techniques are just one more tool which should be understood and used by all who work in biochemistry. All of our students in laboratory courses receive some experience with C^{14} and our graduate courses provide repeated contact with this technique.

This hasty exposure has been intended only to tell you of some possibilities and limitations of the isotopic methods in solving biochemical problems. It is manifestly unsuitable for problems not involving dynamic properties of matter. For static or relatively stable situations, the application of isotopes is restricted largely to their analytical use. I should like to close by discussing briefly a use of isotopes which has been the subject of discussions in these conferences in past years. I hesitate to discuss this application because many of you are working actively in this field. I refer to the use of K^{40} measurements for estimating the composition of live animals or cuts of meat.

D. K^{40} Methods for Carcass Composition

There is great need for a method which would permit one to ascertain the body composition of a meat animal at intervals during the growth period without sacrificing the animal. Even a rapid measure of the mass of muscle in a cut of meat would be useful. Body composition of human

subjects and experimental animals has concerned the physiologist for over 100 years and a great deal of effort has been devoted to this problem recently. Skin fold thickness and height-weight data, used to assess the nutritional status of human populations, is of no value for other species. More precise methods are needed if the energy or protein stored during a period of metabolic study is to be determined without replicating the animals so that samples can be taken at the desired intervals. Even if a sample of the population can be sacrificed, the methods available are not ideal, chiefly because of the difficulty of obtaining a representative small sample of a carcass or a large cut of meat.

Potassium analysis by the usual means as an indication of the mass of muscle would not be considered seriously when samples of the tissue are available for lipid or protein determination. The recognition of K^{40}, a γ-ray emitter, as a normal constituent of natural potassium and the development of very sensitive and efficient liquid scintillation methods of detecting radiation suggested that whole animals or cuts of meat could be analyzed for K^{40} which reflects the potassium content, which in turn reflects the lean body mass.

The use of K^{40} measurements to assess the composition of live animals, including man, as well as cuts of meat has been described by many workers since the measurement of potassium in the body by its radioactivity was first described (15). A brief review of this work up to 1961 was published by Anderson and Langham (16), and the problem was discussed by Andrews and Christian (17) at the Mexico City Conference late in 1961. The method agrees well with other methods of determining lean body weights of man (18).

In 1959, the value of 73 milliequivalents of potassium per kg of lean body weight was adopted in the place of a value of 63 used in the earlier studies. Anderson and coworkers at Los Alamos have worked on this method for approximately ten years. It has been applied to live hogs (19), to hams (20), and to live sheep (21). Because the results indicated that the errors of prediction of body composition were too high for satisfactory use in most animal breeding programs, the K^{40} method was checked against the flame photometry method by Kirton and Pearson (22). They observed that there was a significant correlation between carcass composition of lambs and the K content as measured by flame photometry, but not when estimated by K^{40} content. On separable lean and fat the two methods agreed. On ground lamb and ground pork the K^{40} measurements agreed with the flame photometry method, though the latter method gave values for K which were much more closely related to % water, % fat, and % protein. This relationship was closer for pork than lamb. They concluded that "A degree of precision at least comparable to that obtained by flame photometry is needed for a non-destructive method (such as K^{40}) before the accuracy is great enough to be useful."

E. Some Recent Developments

Isotopes can be expected to make many more important contributions to the field of biological sciences. Three exciting developments of recent years will be mentioned briefly here. The development of density gradient centrifugation has made it possible to separate macromolecules containing

heavier isotopes from their normal counterparts. For example, deoxyribonucleic acid from bacterial cells grown with a N^{15} nitrogen source can be separated from N^{14} DNA using a cesium chloride solution as a medium (23). This technique makes it possible to trace DNA from each parent of bacterial cells among progeny molecules.

A similar application of radioisotopes is exemplified in the use of tritiated thymidine to locate within chromosomes the DNA formed during a given period just after administration of the labeled thymidine (24). Tritium has a distinct advantage in such experiments in that its B^- is so low in energy that the silver grains in a photographic emulsion activated by tritium lie clustered within one micron of the labeled locus.

Finally, mass spectrometry is finding new use in establishing the identity of compounds separated by the rapid, gas chromatographic technique. Since flavor compounds are frequently volatile, their separation by gas chromatography has proved feasible. Drs. Waller and Mason in our laboratories have separated the volatile flavor components from peanuts in this way and are examining the separated compounds by mass spectrometry.

Biemann (25) has described the use of mass spectrometry for establishing the structure of substances which were not previously considered volatile enough. The substances are sublimed directly into the ionizing electron beam. The method is very sensitive and rapid, and requires no chemical pretreatment.

The illustrations given may serve in a small way to indicate the wide variety of applications of isotopes. If tracing an atom or group of atoms will provide a better understanding of a dynamic process or if you need precise analytical data, you might find isotopes useful.

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TABLE II

LABELING PATTERN IN NON-ESSENTIAL AMINO ACIDS FROM CERTAIN METABOLITES

COMPOUND ADMINISTERED	COMPOUND ISOLATED	LABELING PATTERN
$CH_3C^{14}OOH$	GLUTAMATE	$C_5 + C_1 > 95\%$ $C_5/C_1 = 2$
	ASPARTATE	$C_1 + C_4 > 95\%$ $C_1 = C_4$ and $C_2 = C_3$
	ALANINE	$C_1 = 90 - 95\%$
	SERINE	$C_1 = 90\%$
$C^{14}H_3COOH$	GLUTAMATE	$C_4 \gg C_5$, $C_2 = C_3$
	ALANINE	$C_3 = C_2$
$CH_3C^{14}OCOOH$	GLUTAMATE	$C_5 + C_1 = 40 - 66\%$ $C_3 > C_2$
	ALANINE	$C_2 \gg C_3$ or C_1

TABLE III

DISTRIBUTION OF C^{14} IN GLUTAMIC ACID AND ALANINE FROM DL-TRYPTOPHAN-C^{14}*

GLUTAMIC ACID	Tryptophan-7a-C^{14}		Tryptophan-5-C^{14}	
	Carcass	Liver	Carcass	Liver
Sa muc/mmole	28.7	16.9	23.8	60.4
Percent of total C^{14} in				
C_1	24.3	15.9	12.6	12.4
C_2			23.5	23.3
C_3	2.0	.8	24.5	20.7
C_4			38.8	39.2
C_5	74.1	83.5	2.3	2.4
ALANINE				
SA muc/mmole	4.2		7.7	20.3
Percent of total C^{14} in				
C_1	94		22.7	20.4
C_2	6		38.5	39.6
C_3			38.6	39.7

*Mean values for two animals

TABLE IV

DISTRIBUTION OF C^{14} FROM POSITIONS 7a AND 5 OF TRYPTOPHAN IN TRAPPED ACETATE

	Tryptophan-7A-C^{14}	Tryptophan-5-C^{14}
	muc/mmole	muc/mmole
Acetate	22.1	25.5
C-1	19.9	1.4
C-2	.3	24.5

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MR. PEARSON: It is almost noon, but we have time for just one or two questions -- we don't have very much time.

If there are no questions now, if you should have any later that you would personally like to ask the speakers about, you may talk to them individually. I am sure most of them will be available for a little while, anyway.

At this time I will turn the program back to our Chairman, Dr. Kemp.

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CHAIRMAN KEMP: Thank you, Al.

Just one thing before we go. You see the ten names here, we have put them back out there for you to see if you don't remember them. Please put the name on a sheet of paper and pass them down, and, Harold Hedrick, if you will pick them up on this side, and I'll ask Carroll Schoonover to pick them up on the other side.

We will now recess for lunch, and reconvene here at 1:15 sharp.

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