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RESPIRATORY CARBON-14 PATTERNS AND PHYSIOLOGICAL STATE

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Carbon-14, the most useful radioactive isotope of carbon, has been very important in chemical and biochemical research since *circa* 1945. The radioactivity from this weak beta emitter (maximum energy of the β-particle is 155 kev) is difficult to detect and it is not possible to measure the radioactivity or determine its location from outside the body. Thus animal studies using this isotope usually require sacrifice of the animal, unless measurements are restricted to excretion products and blood. Partially because of difficulties of this nature, carbon-14 has not yet become a useful tool in clinical medicine.

Although carbon-14 cannot be detected in animals *in situ*, it can be measured as it is excreted. Of the several catabolic end fates of carbon in animal systems, quantitatively the most important is breath excretion as carbon dioxide. In addition, carbon dioxide excretion responds rapidly to changes in the metabolic level of the body — the only carbon excretion product to do so. The breath excretion of carbon-14 following administration of a labeled organic compound can therefore be used in intact animals to study the metabolism of specific compounds and of the particular labeled carbon atom in the compound.

Furthermore, if one has established the respiratory pattern of a given labeled compound in an animal system, variations in this pattern can potentially be interpreted in terms of the physiological state of the animal

on a biochemical level. Since an almost unlimited number of compounds labeled in various positions is presently available or can be synthesized, the excretion of labeled carbon dioxide from specific substrates is potentially one of the most important nondestructive tests that can be made in metabolic studies in intact animals.

Many experiments have been made on respiratory carbon-14 excretion in animals[1,2,3] but the procedures for measuring this excretion have been slow or have required large amounts of radioactivity. In this paper we describe some of our measurement techniques which overcome these difficulties, and present typical experimental data for a number of animal systems. The effects of physiological state are shown in some cases.

Experimental Techniques. - The respiratory excretion of $C^{14}O_2$ is usually measured as follows: After injection with a labeled substance, the animal is placed in a closed cage. The air from the cage is passed through a dilute sodium hydroxide or barium hydroxide solution. These solutions are changed periodically and the collected CO_2 is precipitated, usually as the insoluble barium carbonate, filtered, dried, weighed, and analyzed for radioactivity.[4] This method gives results in terms of the specific activity of the $C^{14}O_2$, and if quantitative measurements are made on the CO_2 excretion, cumulative excretion of C^{14} can also be calculated.

This method of analysis is used by the authors for long-term respiration studies because it may be made very sensitive if special radioactive assay procedures are used.[5,6] It suffers the disadvantage of being slow, or requiring lots of work, and/or permitting only a limited number of samples.

As an alternate procedure we have devised an apparatus that continuously measures the $C^{14}O_2$ excreted by an animal (see Fig. 1).[7] In this system an animal is placed in a small cage. Air is pulled through the cage at a constant rate and is passed through a drying tube and through an ionization chamber. The radioactivity produces a charge on the collecting electrode of the ion chamber; this charge is directly proportional to the radioactivity in the chamber. A vibrating-reed electrometer and potentiometer recorder make a continuous tracing of these radioactivity levels.

For mice, rats, and rabbits 100- to 1000-cc. ionization chambers are used. Air flow rates are from 100 to 400 cc./minute. The sensitivity of this equipment is such that a normal dose of 40 μcuries C^{14}/kg. body weight is used for 24-hour studies using simple body metabolites such as acetate or glycine. The electrical and gas-flow time constants for 50% response are about one minute.

Most of the acetate-coenzyme A (CoA) experiments described in this paper were made using such equipment. This instrument measures the rate at which carbon-14 dioxide is excreted; the cumulative excretion curve may be obtained by integrating this rate curve. However, one does not get the specific activity of the $C^{14}O_2$, such as is obtained by the barium carbonate measurements and which is probably a better measure of the radioactivity in the body bicarbonate pool.

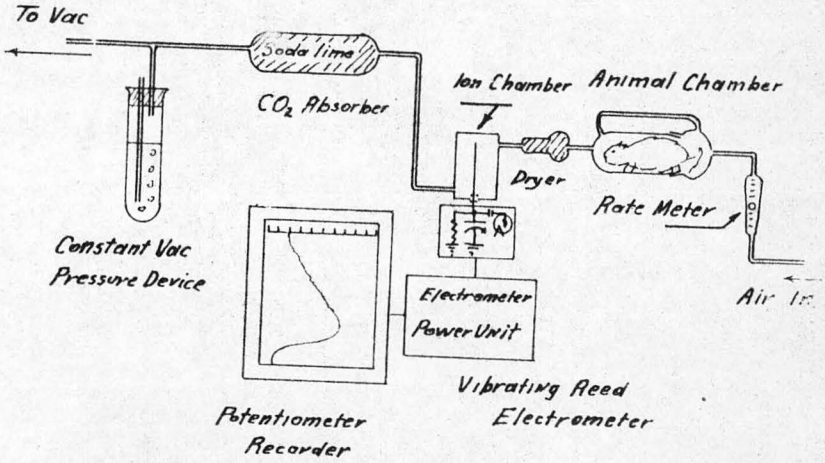

Fig. 1 Apparatus for continuous measurement of breath $C^{14}O_2$ radioactivity

In order to automatically and continuously determine breath specific activity we have constructed a second type of instrument.[8,9] This improved apparatus continuously measures and records (a) the CO_2 excretion by infrared absorption, (b) the C^{14} excretion by an ionization chamber and (c) the specific activity of the $C^{14}O_2$ by a ratio analyzer.

As before, the animal to be studied is injected with a labeled compound and is placed in a cage no larger than necessary for the particular animal's size (see Fig. 2). For humans a transparent helmet is used together with a slightly modified flow system and safe tracer doses are used.[10] (See Fig. 3) Air is provided to the subject from a tank of compressed air which has been aged to allow decay of short-lived naturally occurring α-radioactive gases — mostly radon and thoron.

The air is passed through a sulfuric acid bubbler to remove water and through an ionization chamber. As described above the radioactivity is determined by a vibrating-reed electrometer and potentiometer recorder. The air then passes through a variable-volume chamber and an infrared CO_2 analyzer. The variable-volume chamber adjusts the response time of the CO_2 analyzer to match that of the radioactivity-measuring unit.

The electrical signal corresponding to the % CO_2 in the breath is recorded. This recorder also determines the ratio of the radioactivity signal to the % CO_2 signal and plots this figure. This ratio is directly proportional to the specific activity of the excreted $C^{14}O_2$. An example of the original data is shown in Fig. 4.

For small laboratory animals and 100- to 1000-cc. ion chambers, the radioactivity dose was about 40 μcuries C^{14}/kg. body weight. For humans a normal dose was 10 μcuries of simple organic metabolites, such as acetate or glycine. This corresponds to a dose/weight ratio of only 0.14 μcurie/kg. body weight which is well within the safe tracer dose. The lower figure is made possible by use of larger ionization chambers — from 10- to 20-liters in size.

The time for 50% response of this instrument as used is about one minute. This time constant can be decreased by increasing air flow rates and decreasing the ion chamber sensitivity. A more rapid response does not seem necessary, however, if one considers the biological system. The carbon dioxide produced in a cell becomes part of the body bicarbonate pool. Using known figures for plasma bicarbonate levels, total body water and CO_2 excretion rate, we calculate that the half-time for excretion of CO_2 from the human body is 10 minutes, assuming complete mixing at all times. This agrees well with an early experimental value of 15 minutes for the CO_2 turn over time in rats which can be calculated from the work of Gould et al.[2]

Acetate Metabolism and Coenzyme A. - The acetate fragment has been one of the most extensively studied compounds in biochemistry.[11] A schematic diagram of this compound is shown in Fig. 5, which emphasizes the role of CoA. Acetate is presumed mostly metabolized through the intermediate, acetyl CoA. Oxidation of the acetyl CoA to CO_2 may proceed via the citric acid cycle, or the acetyl CoA may be synthesized into fats, using more acetyl CoA for each two-carbon addition. Acetyl CoA can also be formed from sugars via pyruvic acid oxidation.

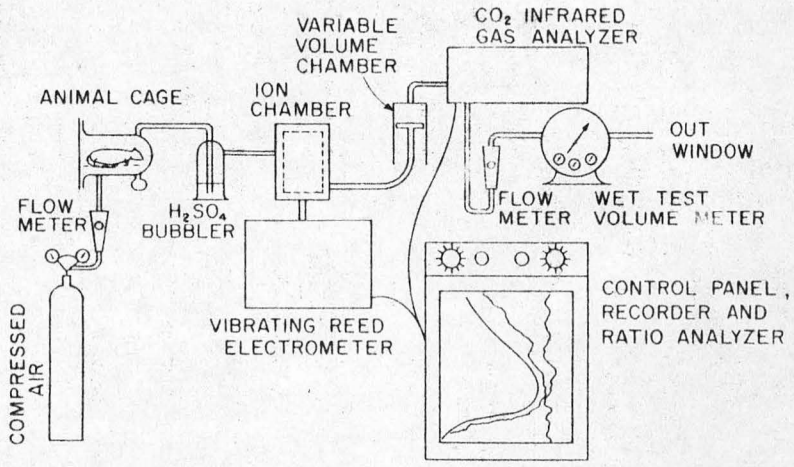

Fig. 2 Schematic diagram of respiratory $C^{14}O_2$ analyzer for small laboratory animals.

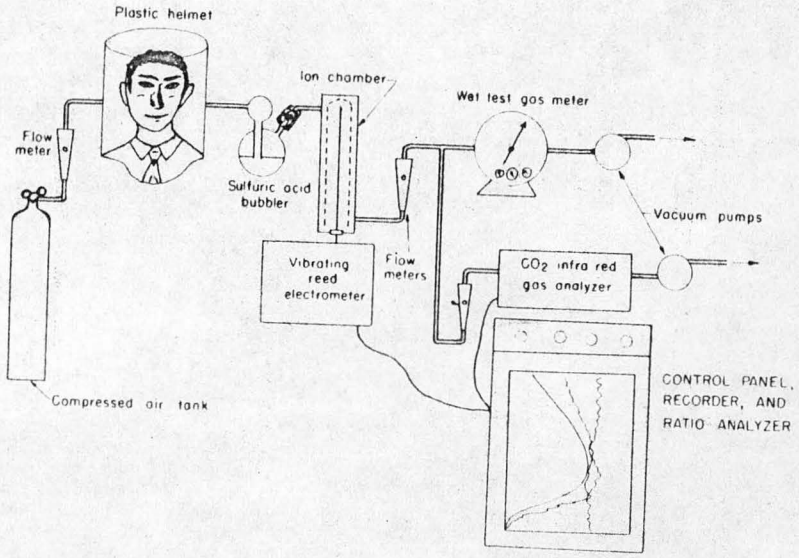

Fig. 3 Schematic diagram of respiratory $C^{14}O_2$ analyzer for humans.

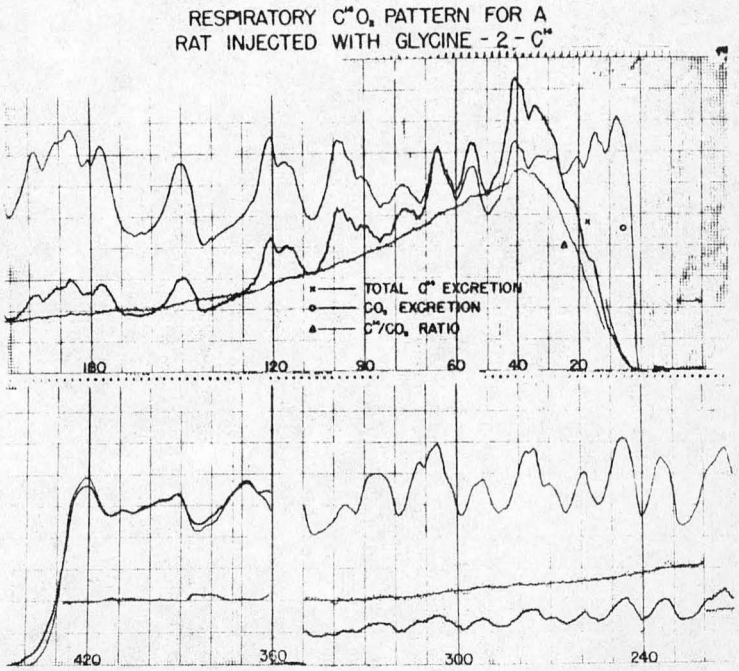

Fig. 4 An example of a respiratory $C^{14}O_2$ pattern as made by the instrument described.

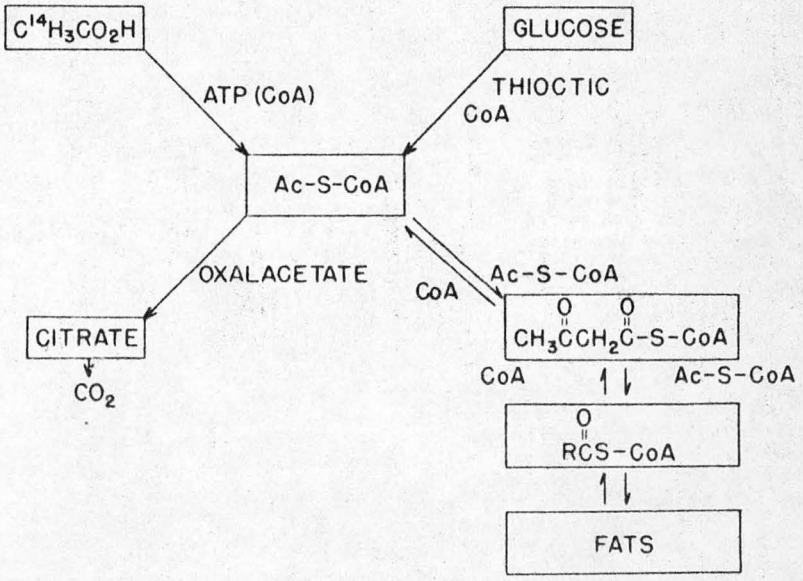

Fig. 5 Schematic diagram of acetate metabolism.

This dynamic equilibrium may be conveniently studied in the intact animal by the equipment described. CoA and labeled acetate are readily available and rats may be made deficient in CoA by feeding them on a pantothenic acid deficient diet (PAD rats).[12] The pattern of excretion of $C^{14}O_2$ can therefore be determined as a function of body CoA levels and the nutritional state induced by pantothenic acid deficiency.

A number of variables that could influence the respiration patterns were briefly investigated. Intraperitoneal (i.p.) injection of the CoA gave more consistent results than intravenous (i.v.) or subcutaneous injection. No differences in patterns were found with air flow rates that gave cage CO_2 concentrations from one to four percent. The effective body pool size of acetate in rats should be quite large since 0.9 to 1.2 g. of acetic acid are formed per day per 100 g. body weight.[13,14] In agreement with this assumption no significant differences were observed with doses of labeled acetate varying from 0.2 to 20 mg.; 2-mg. doses were used. Individual animal variations, especially of the PAD rats, were such that 6 to 20 separate curves were measured for each nutritional state.

Fig. 6 shows the rate of excretion of $C^{14}O_2$ after injection of sodium acetate-2-C^{14} in rats of four different physiological states. Fig. 7 shows the cumulative excretion curves for this same series. From these patterns one sees that labeled acetate is more rapidly and more completely oxidized to CO_2 in PAD rats than in normal rats. One also sees that CoA depresses $C^{14}O_2$ excretion in both normal and PAD rats, and by comparable amounts.

These data are consistent with the acetate metabolism scheme presented. Since PAD rats are deficient in CoA, they should have a smaller acetyl CoA pool than normal rats. Therefore, in PAD rats, after administration of acetate-2-C^{14}, a higher C^{14} specific activity should be observed in products directly derived from this intermediate, such as $C^{14}O_2$ formed via the citric acid cycle. This should be a short-term effect and is indeed seen only in the early part of the rate curves.

Conversely, injected CoA should increase the acetyl CoA pool, and this should decrease the peak CO_2 specific activity, as observed. But this change in pool size should not change the cumulative C^{14} excretion as shown in Fig. 7, unless alternate metabolic fates for the acetate fragment are enhanced by the CoA. A pathway that is probably enhanced is fat synthesis, in which each major step requires an acetyl CoA molecule. Furthermore, an animal deficient in CoA will have a fat deficiency, which should be selectively alleviated when more acetyl CoA is provided.

This hypothesis was further confirmed by making measurements of liver fat radioactivity. PAD rats (average of 4) that were given labeled acetate and 6 mg. of CoA incorporated 5.2% of the injected label into liver lipids in one hour; PAD rats (average of 2) given labeled acetate only, incorporated 1.2% of the injected radioactivity into liver lipids in the same time.

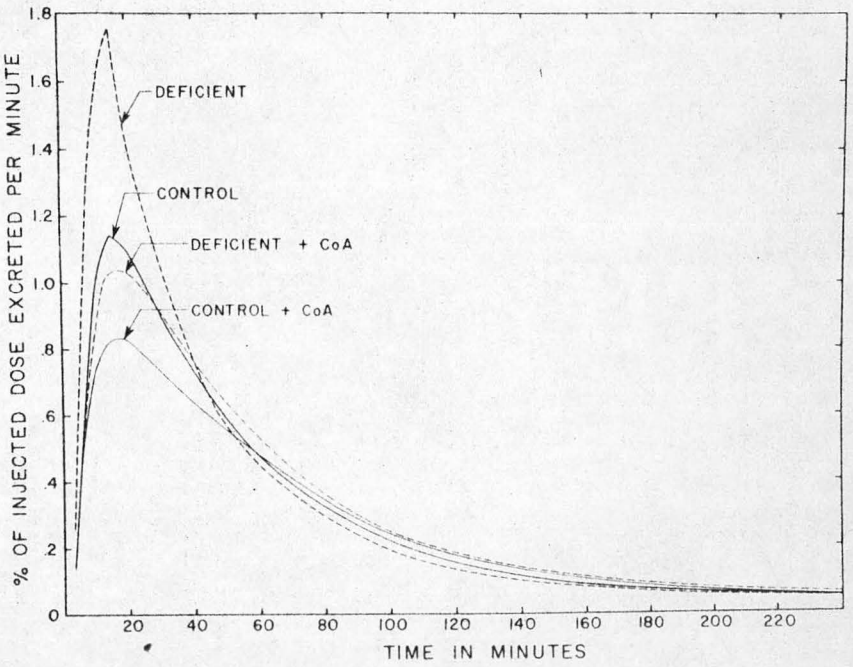

Fig. 6 The effect of pantothenic acid deficiency and CoA on the rate of excretion of $C^{14}O_2$ after injection of sodium acetate-2-C^{14}.

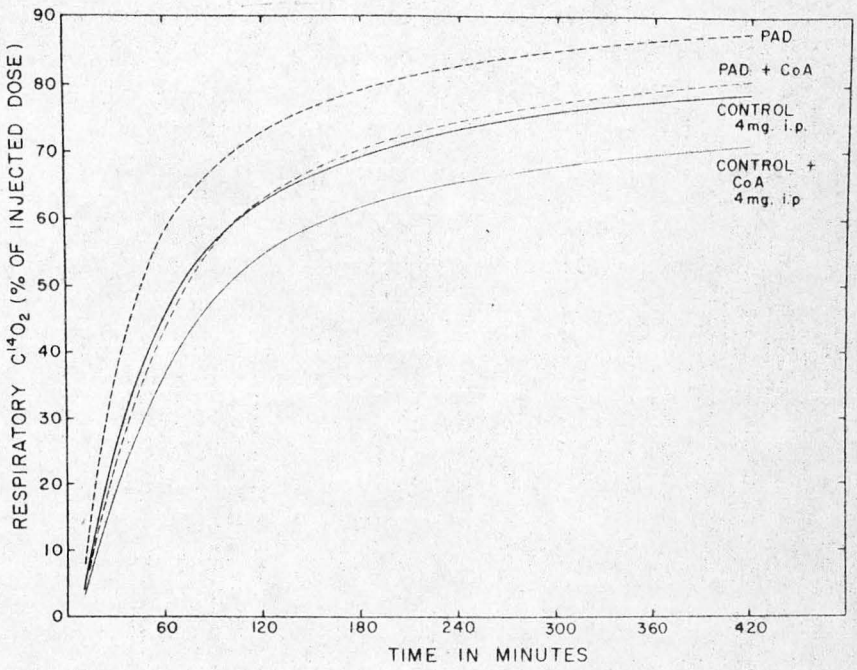

Fig. 7 The effect of pantothenic acid deficiency and CoA on the cumulative excretion of $C^{14}O_2$ after sodium acetate-2-C^{14} injection.

Respiratory Patterns of Single Metabolites. - Preliminary animal studies have been made using the instrument that gives CO_2-, C^{14}-, and C^{14}/CO_2 analysis of the breath.[8,9] The respiratory patterns from rats for radioactivity from sodium acetate-2-C^{14}, glucose-$C^{14}{}_6$, DL-leucine-3-C^{14}, glycine-2-C^{14}, and tripalmitin-carbonyl-$C^{14}{}_3$ are presented as a series of curves in Figs. 8 and 9.

The acetate and glucose are both about 60% oxidized to CO_2 in 7 hours. The glycine and leucine have similar peak excretion specific activities, but the leucine is oxidized at a much higher rate than the glycine in the later part of the curve. This is probably so because half the leucine is the abnormal D-metabolite, which cannot be directly used[15] and is slowly deaminated[16] and oxidized to CO_2. The later part of the leucine rate curve is nearly a straight line when plotted on semi-log paper -- a good confirmation of the above suggestion. The tripalmitin is so slowly oxidized to CO_2 that one can postulate that most of it is stored in body tissues as a fat, perhaps without change.

These respiratory data establish a rapid method for accurate measurement of breath $C^{14}O_2$ excretion, both in terms of specific activity and cumulative excretion. Certain other interesting observations are also possible. At the start of our work an important question was whether specific activity curves were continuously decreasing functions, once a peak was reached. Within the limit of our instrumentation this is true for the glycine, acetate, leucine, and tripalmitin. It is not true for glucose; sharp reversals of $C^{14}O_2$ specific activity were observed in every rat studied and corresponded with periods of marked physical activity. These reversals probably correspond to mobilization of recently deposited glycogen in the liver -- a selective phenomenon observed by Stetten and Stetten.[17] The specific activity curves for adenine-2-C^{14}, adenine-4,6-C^{14} and adenine-8-C^{14} in mice[18] have also been determined on this unit and are not continuously decreasing functions.

As mentioned earlier the half time for response of the $C^{14}O_2$ analysis unit is about one minute. After i.p. or i.v. injection of such simple metabolites as labeled acetate or glucose in animals and man, detectable amounts of radioactivity were observed in the breath in 1 to 2 minutes. This, then, represents an upper limit for the minimum respiratory excretion time for these compounds. For all five compounds maximum excretion rates occurred within the first hour, provided the animal was not in an abnormal physiological state.

Glycine-2-C^{14} in Man. - Respiratory $C^{14}O_2$ excretion studies have been made by use of glycine-2-C^{14} [5,6] and the barium carbonate radioactivity analysis method. We have now repeated the short term respiration studies for a number of patients with advanced cancer or polycythemia vera. Fig. 10 shows the specific activity rate curve for Mr. F., a polycythemia patient in remission. This curve is typical for patients in an apparent normal condition, but it appears to be drastically modified by advanced cancer.

The similarities between human and rat glycine-2-C^{14} curves (see Fig. 8) are very striking. Both the general shape and the time of peak $C^{14}O_2$ specific activity are almost identical. This would indicate that the metabolic pathways are very similar, and are the limiting rate factors, rather than circulation and mixing times.

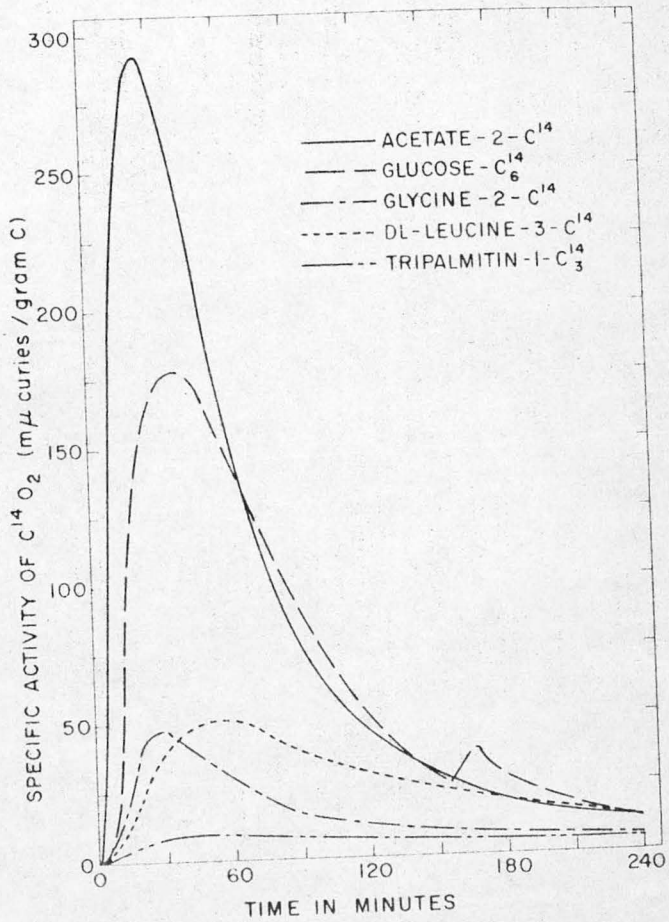

Fig. 8 Specific activity of $C^{14}O_2$ from rats injected with various labeled compounds. The data are normalized to a 250 g. rat and 10 μcurie injected dose.

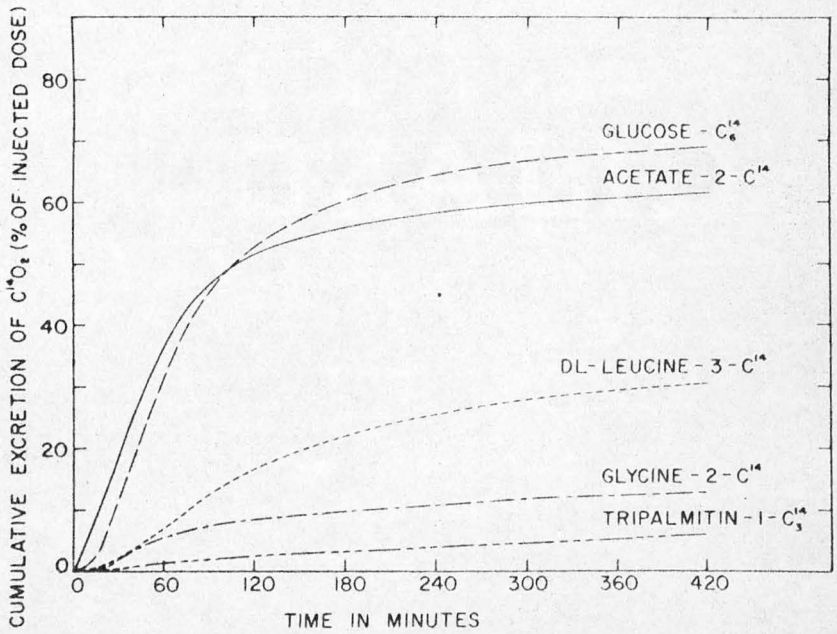

Fig. 9 Cumulative $C^{14}O_2$ excretion patterns from rats injected with various labeled compounds.

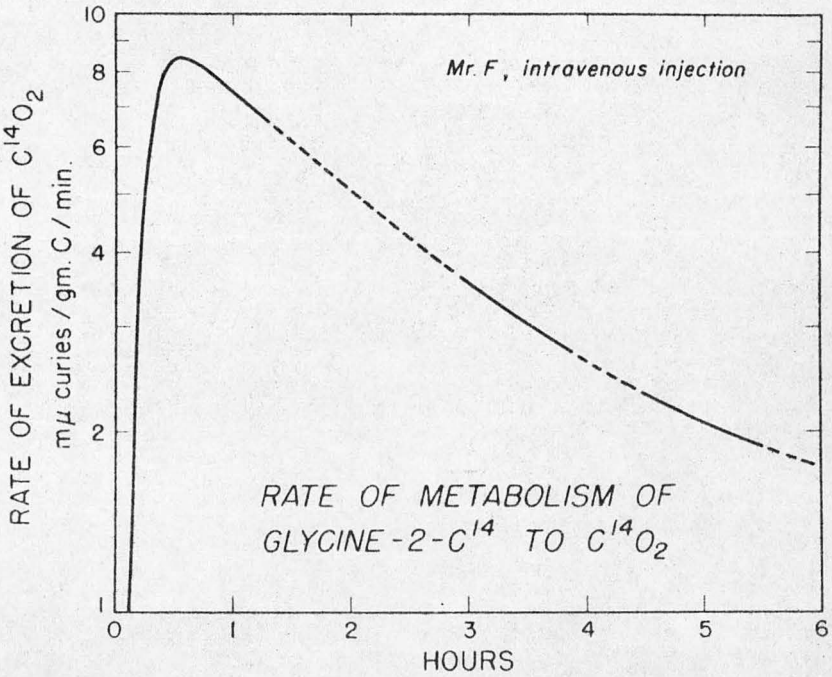

Fig. 10 Respiratory $C^{14}O_2$ excretion of glycine in a patient.

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