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Method for Determining Incorporation of Acetate into Citrate (38065)

PADMAKAR K. DIXIT AND ALLEN M. STERN

Department of Anatomy, University of Minnesota, School of Medicine, Jackson Hall 272, Minneapolis, Minnesota 55455

In a previous communication we have reported a radiometric technique for the determination of citrate synthetase activity (1). The method was based upon the ability of citrate condensing enzyme (CCE) to synthesize citrate by combining oxaloacetate with 1-^{14}C acetyl coenzyme A (acetyl CoA). The citrate formed was labeled at carbon-1 position and following its oxidation to pentabromacetone, the labeled carboxyl group was evolved as $^{14}CO_2$ and collected. This method of assaying citrate synthetase compared favorably with the well known colorimetric method described by Srere *et al.* (2), in which CoA released during the condensation of acetyl CoA with oxaloacetate was combined with Ellman's reagent [5-5′ dithio-bis (2-nitrobenzoate)] (DTNB), to form a yellow colored product. The radiometric method was also used for the determination of citrate synthetase activity of liver and skeletal muscle homogenates and also for isolated liver mitochondrial suspensions.

Assay using acetyl CoA provides a measure of the activity of CCE but does not furnish a true index of the citrate synthesizing capacity of the tissues. Acetyl CoA (or also called active acetate) is formed from 3 precursors, acetate, pyruvate and fatty acids. There are relatively small quantities of acetic acid normally present in mammalian tissues. However, the formation of acetyl CoA from acetate has been widely studied by biochemists because this reaction is known to require a single enzyme and ATP, CoA and Mg^{2+} as cofactors (3).

In the formation of acetyl CoA from pyruvate three enzymes and four cofactors are closely involved and have to be present in the tissues while four enzymes and three cofactors are known to be required when acetyl CoA is formed during fatty acid degradation. We have, therefore, selected the uptake of acetate by tissues in the formation of citrate as an indicator of the citrate synthesizing capacity of the tissues.

In the present communication we report a simple radiometric method to determine the incorporation of labeled acetate into citrate. This method offers the advantage in that the labeled citrate which is formed does not have to be separated from labeled acetate by chromatographic or other means prior to its determination.

Materials and Methods. Reagents. Sodium acetate 1-^{14}C (New England Nuclear Corp. sp. act. 2 mCi/m*M*). Monofluoroacetic acid, sodium salt (Sigma Chemical Co., St. Louis, Mo.) Citric-1,5 ^{14}C acid (New England Nuclear Corp. sp. act. 5.8 mCi/m*M*) Sodium pyruvate 2-^{14}C (New England Nuclear Corp. sp. act. 8.2 mCi/m*M*) Fluoropyruvic acid, sodium salt (Sigma Chemical Co.) Hydroxide of Hyamine 10-X (Packard Instrument Co., Inc.)

Principle of the assay. The assay depends upon the ability of the tissues to incorporate acetate and form acetyl CoA, which is subsequently condensed with oxaloacetate by tissue citrate condensing enzyme to form citrate. The reactions involving labeled acetate are shown below:

$$\text{1.}\quad CH_3 - {}^*COOH + ATP + CoA \rightleftharpoons CH_3 - {}^*C(=O) - S - CoA + AMP + PPi$$

(Acetyl CoA) (Adenylic Acid) (Inorganic Pyrophosphate)

2. $CH_3 - {}^*C(=O) - S - CoA + O = C(-H_2C - COOH) - COOH \xrightarrow[\text{(i.e. citrate synthetase)}]{\text{condensing enzyme}} H_2C(-{}^*COOH) - C(-OH) - CH_2 - COOH + HS\text{-}CoA$

(Acetyl CoA) (oxaloacetate) (citric acid) (coenzyme A)

The amount of labeled citrate thus formed can be determined by its oxidation to pentabromacetone as reported earlier (1). The labeled carboxyl group of citrate which is synthesized is evolved as $^{14}CO_2$ and can be collected quantitatively. It is believed that the citric acid is oxidized to pentabromacetone as follows (4).

3. $H_2C(-{}^*COOH) - C(-OH) - CH_2 - COOH + 5\,Br_2 \longrightarrow HC(-Br_2) - C(=O) - C(-Br_3) + 2CO_2 + 5HBr + {}^*CO_2$

Pentabromacetone

When monofluoroacetate (MFA) is substituted for acetate, fluorocitrate is formed which is accumulated (5). Advantage was taken of this property, as will be explained later.

Quantitative recovery of labeled citrate in the presence of an excess amount of 1-^{14}C acetate. The simplicity of this technique is based on the direct measurement of newly formed labeled citrate from a large amount of labeled acetate contained in the incubation medium. For the successful use of the method, it is incumbent that tiny quantities of labeled citrate formed from labeled acetate as substrate should be quantitatively recovered without any release of radioactivity from acetate.

We, therefore, added 0.1 μCi or 0.5 μCi of 1-^{14}C acetate in a number of specially designed flasks with side arms to which various amounts of 1,5-^{14}C-citrate (0.025–0.25 μCi) were added in duplicates. Following the oxidation of citrate to pentabromacetone as described earlier (1), the labeled carbon dioxide evolved was absorbed in 0.5 ml hydroxide of hyamine which was then transferred quantitatively with scintillation fluid to a counting vial and the radioactivity was measured at an efficiency of 80%. The blank flasks contained only 0.1 or 0.5 μCi 1-^{14}C acetate which were similarly treated. The radioactive counts obtained from the blanks were subtracted from the values obtained with various amounts of 1,5-^{14}C citrate.

In vitro incorporation of 1-^{14}C acetate into labeled citrate by the liver tissue. Liver tissue was rapidly excised from normal rats and placed in 50 ml chilled beakers. The tissue was minced with scissors to a fine pasty consistency. Approximately 50 mg of the minced tissue was weighed on small glass cover slips and placed gently in the incubation flask having a side arm. The flasks contained 0.25 μCi 1-^{14}C sodium acetate plus 2 ml of a medium described by Dixon and Perkins (6) for the determination of citrogenase activity. In brief, the final concentration of constituents in the medium were (1.) 0.01 *M* sodium acetate or monofluoroacetate, (2.) 0.1 *M* potassium chloride, (3.) 0.003 *M* magnesium sulfate, (4.) 0.01 *M* cysteine hydrochloride, (5.) 0.02 *M* adenosine triphosphate, (6.) 0.001 *M* oxaloacetic acid, (7.) 0.01 *M* phosphate buffer pH 7.4.

The pH of the medium was adjusted to 7.4 using 0.1 *N* sodium hydroxide.

In experiments where citrate synthesis from pyruvate was studied, pyruvate or monofluoropyruvate was substituted for acetate or monofluoroacetate, and 2-^{14}C sodium pyruvate was added in place of 1-^{14}C sodium acetate.

Using a magnetic stirrer and magnetic flea the tissue was completely dispersed in the medium and the flasks were placed in a Dubnoff shaking bath at 70 oscillations per min for 30 min at 27°. At the end of the incubation period 1 ml of 9 *N* sulfuric acid

(containing 1 mg anhydrous citric acid per ml as carrier) was added to the flasks through the side arm. The flasks were then partially evacuated prior to the oxidation of labeled citrate that was formed as described earlier (1). As explained before, this step was necessary to preclude a blow out of the stoppers during the subsequent steps of citrate oxidation. The labeled citrate formed was oxidized to $^{14}CO_2$ and collected quantitatively.

In vivo incorporation of 1-^{14}C acetate into citrate. Three normal young male rats weighing about 200 g were injected by intravenous route a dose of sodium monofluoroacetate (MFA) (5 mg/kg body wt) in 0.85% saline. Two control rats were administered saline solution. Ten minutes after the injection of saline or MFA, 1-^{14}C acetate was injected intravenously into the rats at a dose of 0.05 μCi/g body weight. Fifteen, 30 and 60 min after the injection of labeled acetate, the nembutalized animals were bled by cardiac puncture and blood was placed in heparinized centrifuge tubes. One tenth ml of blood was quickly pipetted in replicate samples and transferred into the side arm vessels containing 1 ml of 9 *N* sulfuric acid plus carrier citric acid. Blood was centrifuged and serum was separated. One-tenth ml serum samples were likewise pipetted into 1 ml of 9 *N* sulfuric acid (containing carrier citrate). The animals were sacrificed and liver, spleen, kidney, heart and lungs were removed and rapidly frozen in liquid nitrogen. The frozen tissues were quickly weighed on an analytical balance and homogenized in glass homogenizers with 9 *N* sulfuric acid containing carrier citrate (approximately 1 g tissue per 2.5 ml acid). Five-tenths ml of replicate samples of each tissue homogenate were added to vessels with side arms which contained 0.5 ml of the citrate containing 9 *N* sulfuric acid. Following oxidation, the radioactivity contained in the body fluids and tissue homogenates was collected as $^{14}CO_2$ and represented the amount of labeled citrate synthesized *de novo* from 1-^{14}C acetate injected into the animal.

Results. Quantitative recovery of labeled citrate in the presence of an excess amount of 1-^{14}C acetate. As shown in Fig. 1, the recovery of various amounts of citrate added to the flasks containing either 0.1 or 0.5 μCi of 1-^{14}C acetate was excellent. The recovery of added citrate ranged between 92 and 99% with 0.1 μCi of labeled acetate and 88 and 102% with 0.5 μCi of 1-^{14}C acetate.

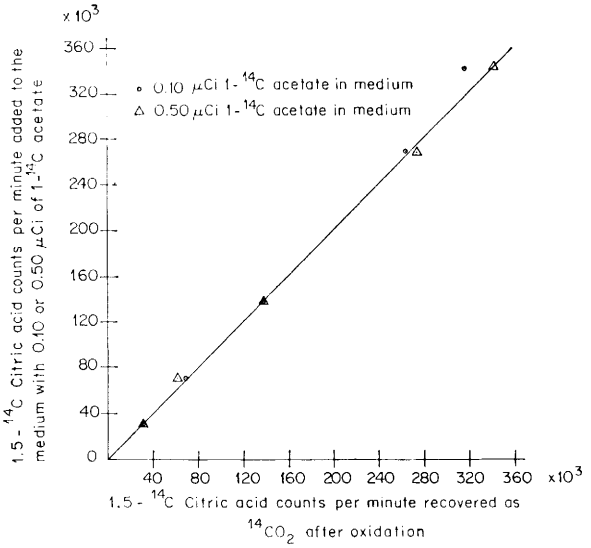

FIG. 1. Recovery of various amounts of labeled citrate in the presence of excess amounts of 1-^{14}C acetate.

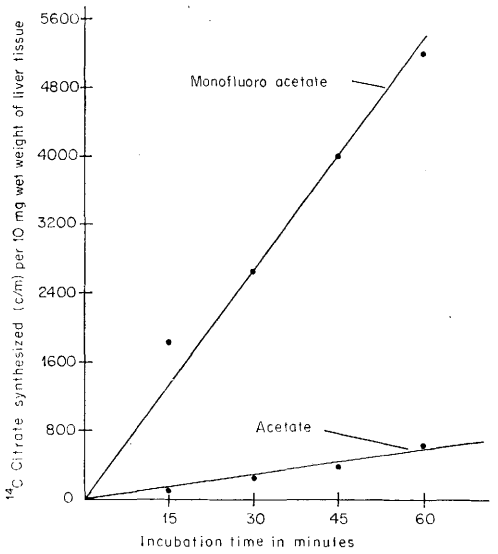

FIG. 2. Citrate synthesis by rat liver tissue incubated at 28° for various periods of time in media containing either acetate or monofluoroacetate (MFA) as substrates. [1000 counts per min of ^{14}C-citrate was equivalent to 0.08 μM citrate synthesized.]

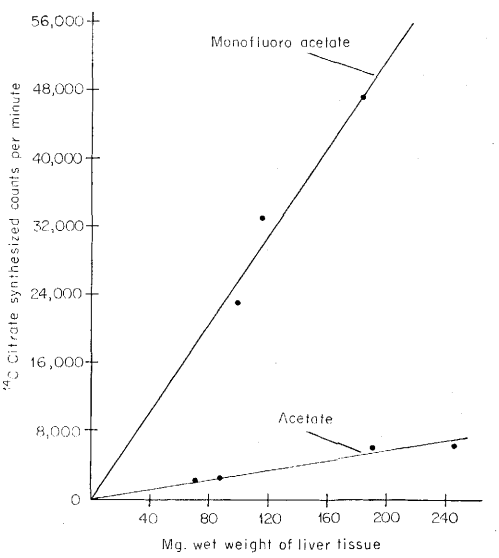

FIG. 3. Relationship between citrate synthesis and the amount of liver tissue. [1000 cpm ^{14}C-citrate = 0.08 μM citrate synthesized.]

Synthesis of citrate from acetate by liver tissue. Figure 2 depicts the synthesis of labeled citrate from 1-^{14}C acetate when the liver tissue was incubated for various periods of time in acetate containing medium. With increased time of incubation, greater amounts of citrate were synthesized as indicated by increased $^{14}CO_2$ collected following its oxidation. When monofluoroacetate (MFA) was added into the reaction medium instead of acetate as the substrate, in the presence of the same amounts of 1-^{14}C acetate, considerable amounts of labeled citrate were formed. (Ideally it would have been more appropriate to use labeled monofluoroacetate along with MFA.)

With both acetate and MFA as substrates, there was a linear increase in citrate formation with time of incubation. However, with MFA the amount of citrate recovered, at any particular time period, was about ten-fold greater than with acetate as substrate.

Figure 3 shows the results of an experiment in which the amount of liver tissue which was incubated in the MFA or acetate containing media was varied, while the incubation period was kept at 30 min. With the increase in tissue concentration, a proportionate increase in citrate synthesis occurred. Again it was evident that in the presence of MFA there was a ten-fold increase in citrate synthesis as compared to when acetate was added as substrate.

Effect of temperature on the in vitro incorporation of acetate into citrate by liver tissue. As is evident from Fig. 4, the incorporation of 1-^{14}C acetate into citrate was minimal when liver tissue was incubated at 0° in medium containing MFA as substrate. The maximum incorporation of acetate into citrate occurred at an incubation temperature of 27°. At 0° the citrate synthesized was equivalent of 1900 cpm by 10 mg liver tissue, whereas at 27° the radioactive citrate count was 11,000. The amount of citrate synthesized at 38° was about 50% of that at 27°.

Using acetate as the substrate in the medium, the amount of citrate which was synthesized was considerably less at all the 3 temperatures. At 0° and 38° the amount of citrate synthesized was approximately one-third of that synthesized when MFA was used as the substrate. At 27°, the citrate synthesized in the presence of acetate

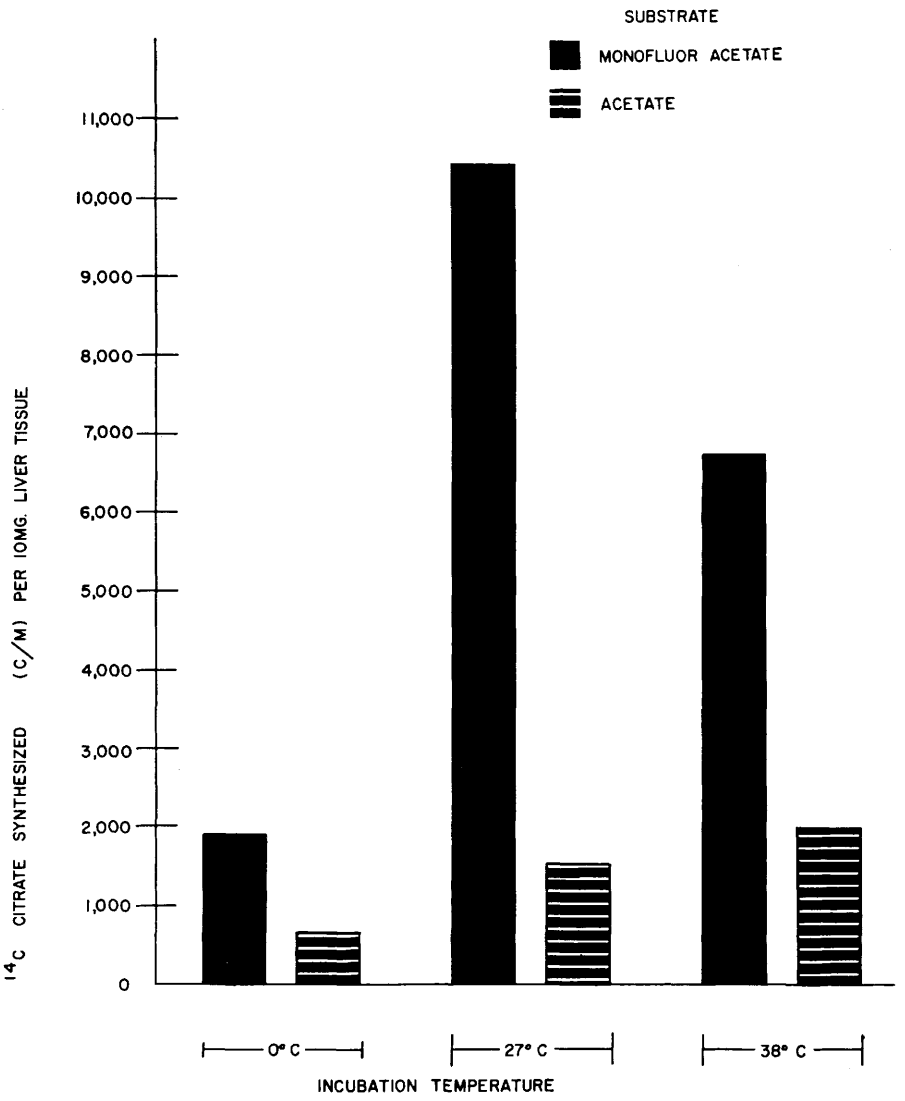

FIG. 4. Effect of temperature on citrate synthesis by rat liver tissue from acetate or MFA as substrates. [1000 cpm ^{14}C-citrate = 0.03 μM citrate synthesized.]

as the substrate was about one-tenth of that synthesized in the MFA containing medium.

In vitro synthesis of citrate by various rat tissues in the presence of MFA and acetate as substrates. The ability of various rat tissues to synthesize citrate varies as shown in Fig. 5. Kidney, liver and heart—in that order—synthesized large amounts of citrate, whereas skeletal muscle, pancreas and lungs appeared to synthesize moderate amounts of citrate. Using acetate as substrate, the net citrate which was synthesized by most of the tissues was considerably less than when MFA was present in the medium. In most tissues the net amount of citrate determined with acetate as substrate was approximately 10% of that synthesized with MFA in the medium. In the skeletal muscle the amount of citrate synthesized with acetate as substrate was about one-fourth of that which was synthesized with MFA. The amount of citrate synthesized by pancreatic tissue with acetate as sub-

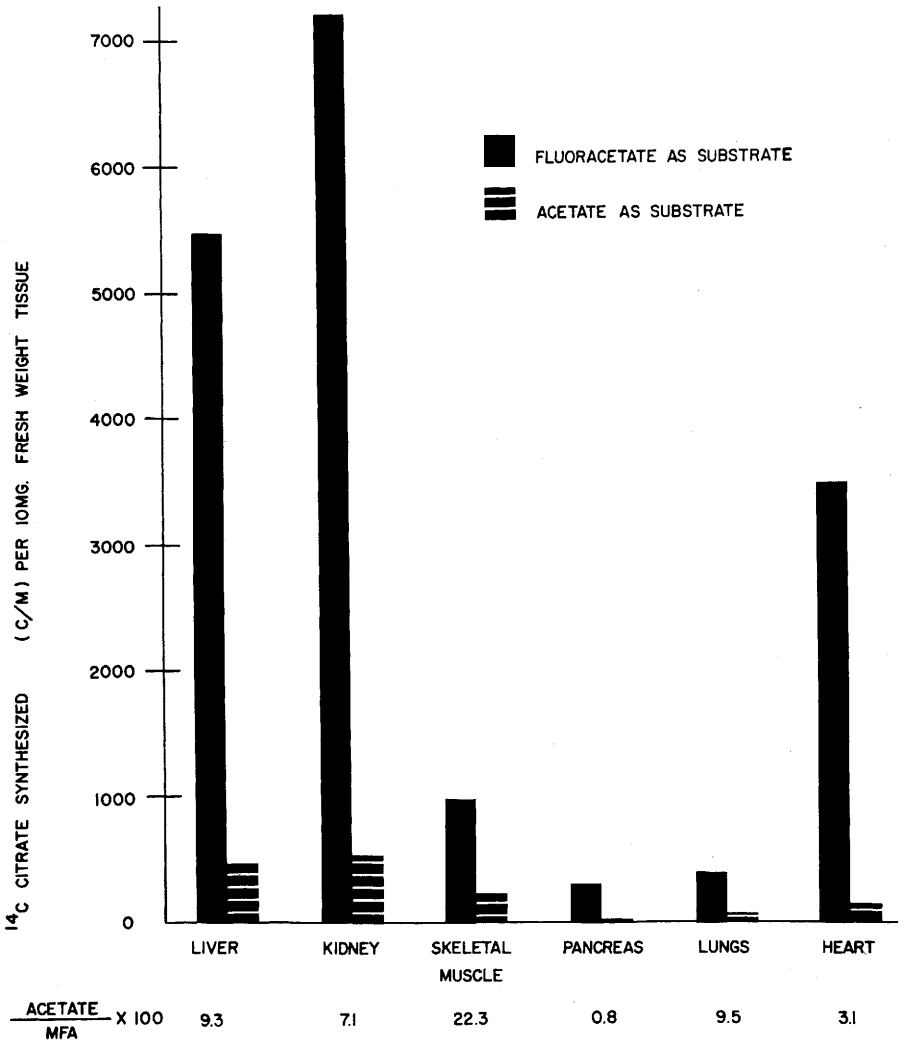

FIG. 5. *In vitro* synthesis of citrate by various tissues of rat. [1000 cpm ^{14}C-citrate = 0.07 μM citrate synthesized.]

strate was almost negligible (about 1%).

Synthesis of citrate from labeled pyruvate. In another series of experiments, pyruvate and fluoropyruvate were used as the substrates and citrate synthesized by liver tissue was determined. When 2-^{14}C labeled pyruvate was added along with the two substrates, the label was located in the 1-^{14}C acetyl position and subsequently incorporated into acetyl CoA as shown below.

Labeled acetyl CoA formed from pyruvic acid then condenses with oxaloacetate to form labeled citrate.

The amount of citrate synthesized from fluoropyruvate (FP) was considerably less than that formed when pyruvate was added as substrate (Fig. 6). Although with both the substrates there was a linear increase in citrate synthesis, with pyruvate as substrate the linear increase in citrate synthesis occurred

4. $CH_3 - {}^*C(=O) - COOH + SH\text{—}CoA + DPN^+ \rightarrow CH_3 - {}^*C(=O) - S - CoA + CO_2 + DPNH + H^+$

(2-^{14}C Pyruvic acid) (CoA) (1-^{14}C Acetyl CoA)

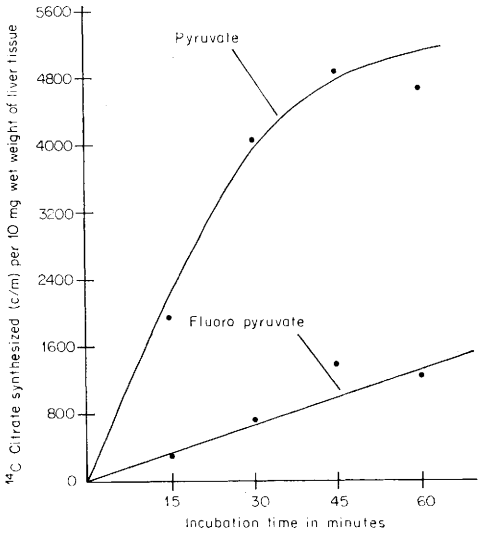

FIG. 6. Synthesis of citrate from pyruvate. [1000 cpm ^{14}C-citrate = 0.06 μM citrate synthesized.]

up to 30 min of incubation time. At subsequent time periods the rate of synthesis was markedly slowed down. With FP as the substrate, rat liver tissue synthesized approximately 20% of citrate synthesized when pyruvate was used as the substrate. Thus, after 30 min of incubation period, 10 mg of liver tissue synthesized citrate in pyruvate containing medium which on oxidation gave a count of 4000. The corresponding count in FP containing medium was about 800.

In vivo incorporation of 1-^{14}C acetate into labeled citrate. As shown in Fig. 7, when 1-^{14}C acetate was injected into rats, radiocarbon was incorporated into citrate molecules by various tissues of rat. In the rats which were previously treated with MFA, 15 min after labeled acetate injection, a large amount of the radioactivity as newly synthesized citrate was located in the blood, most of which was in the serum. At this time period some tissues appeared to contain appreciable radioactivity, but the levels in the tissues (namely spleen and kidney) never exceeded those present in the whole blood. Thirty minutes after 1-^{14}C acetate injection in the MFA treated rat, all of the radioactivity of blood appeared to be present in the serum. The amount of radio-

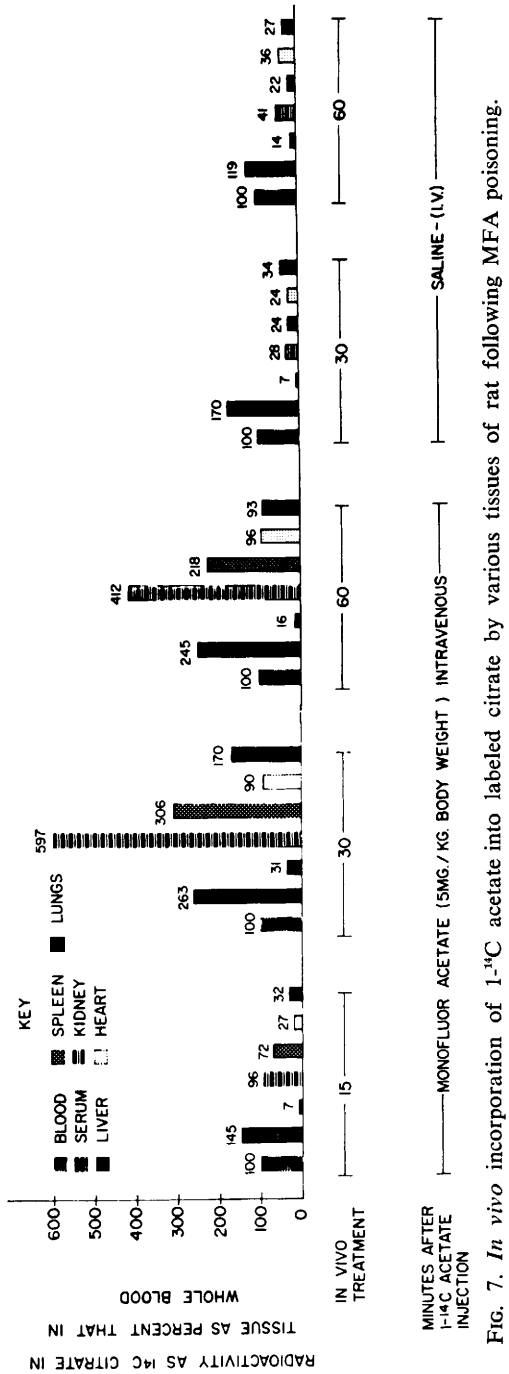

FIG. 7. *In vivo* incorporation of 1-^{14}C acetate into labeled citrate by various tissues of rat following MFA poisoning.

activity contained in the spleen, kidney and lungs was considerably greater than in the whole blood, although the heart and liver also exhibited marked elevation in the radioactive counts. At 60 min after the injection of labeled acetate, the radioactivity in spleen and kidney continued to be much greater than in the whole blood. Lungs and heart tissues contained radioactivity close to that of the blood levels.

By contrast the control animals which were treated with saline followed by 1-^{14}C acetate, the radioactive citrate recovered from the various tissues was much less than that found in blood or serum. It would seem that the labeled citrate synthesized by the tissues from 1-^{14}C acetate was quickly disposed of by the tissues by way of (i) oxidation and (ii) diffusion into the blood fluids, where it was accumulated.

We had earlier reported (1) that when 1-^{14}C citrate was injected into rats, 30 and 60 min later the kidney contained over three-fold the amounts of radioactivity present in blood.

Discussion. The method described here determines the incorporation of acetate into citrate. The technique takes advantage of the fact that when citric acid, formed from labeled acetate, is oxidized to pentabromacetone, the carbon-14 in the carboxyl groups of citric acid is quantitatively evolved as $^{14}CO_2$. Although all the three carboxyl groups, namely 1, 5 and 6, of citric acid are released as CO_2 during oxidation, the citric acid formed from 1-^{14}C acetate is labeled only at one carboxyl position.

Presence of a large quantity of labeled acetate does not interfere with the quantitative recovery of the labeled citrate that is formed *in vitro*. This is evident from the fact that the recovery of known amounts of 1,5-^{14}C citrate added to the medium containing 0.1 or 0.5 μCi of 1-^{14}C acetate was excellent (Fig. 1). When 1-^{14}C acetate alone was oxidized, the radioactive counts recovered as $^{14}CO_2$ represented less than 3% of the total counts added to the medium. Thus, the method described here offers the advantage in that a prior separation of small quantities of newly synthesized labeled citrate from large amounts of labeled acetate in the medium is not essential.

It is known that tissues like liver, kidney, heart, skeletal, muscle, etc., contain the condensing enzyme (CE) which combines the oxaloacetate with acetyl CoA (active acetate) to form citrate (7). This enzyme is found in high amounts, particularly in those tissues which have a rapid rate of aerobic metabolism. The utilization of acetate and formation of citrate involves the close cooperation and participation of two enzymes—one which concerns with acetate activation to acetyl CoA (i.e., active acetate) formation and the other is the condensing enzyme (CE). This complex was referred to as the "Citrogenase" enzyme system by Dixon and Perkins (6).

Using labeled acetate in Dixon and Perkins enzymatic procedure, it has been possible to study this "Citrogenase" enzyme system. However, as shown in Figs. 2 and 3, if the further oxidation of newly synthesized citrate is not blocked, the total amount of labeled citrate found at the end of the incubation period would be considerably lower. By using unlabeled fluoroacetate instead of acetate as the substrate, in addition to labeled acetate in tracer quantities, it is possible to prevent further oxidation of *de novo* synthesized citrate, because according to Peters (8) fluorocitrate is a competitive inhibitor of aconitase. Furthermore, Liebecq and Peters (9) found a marked accumulation of citrate when homogenates of the cortex of guinea pig kidney were incubated with fluoroacetate, which was attributed to the fact that "the condensing enzyme responsible for combining acetyl CoA with oxaloacetate was not distinguishing between acetate and monofluoroacetate." With unlabeled fluoroacetate and labeled acetate as the substrates, fluorocitrate which was formed, accumulated because the citrate utilizing enzymes were unable to oxidize this substrate, and thus the radioactive citrate count was considerably higher than when unlabeled and labeled acetate were present as substrates. In the method described here we have taken advantage of these properties of the citrate synthesizing and utilizing enzymes.

Dixon and Perkins (6) measured the enzymatic activity of citrogenase at a temperature of approximately 28°. We have likewise found that the synthesis of citrate using MFA as substrate was much greater at 27° than at 38°, which is generally accepted as the optimal temperature for most enzyme reactions (see Fig. 4).

The ability of different tissues to synthesize citrate from MFA varied markedly (see Fig. 5). Thus, kidney, liver and heart can incorporate acetate into citrate to a much greater extent than the skeletal muscle, pancreas and lungs. With acetate as the substrate, the citrate synthesized by these tissues was considerably less. The difference between the amount of labeled fluorocitrate and citrate synthesized from MFA and acetate as substrates, respectively, represents the amount of citrate which was added to or removed from the tissue citrate pool during the incubation time.

Pyruvate, formed during glycolysis, provides 2-carbon fragments to generate acetyl CoA, which is subsequently incorporated into the citrate molecule. The formation of citrate from pyruvate by way of acetyl CoA requires the presence of a number of enzymes and cofactors in the tissues, which together constitute the complex termed pyruvic acid dehydrogenase. We have demonstrated in Fig. 6 that citrate can be synthesized from pyruvate by the rat liver tissue. However, unlike fluoroacetate, the fluoropyruvate is not properly utilized by the tissue enzyme system as a substrate, and considerably less citrate (as fluorocitrate) was synthesized when this substrate was added to the medium. This is reflected in the decreased labeled citrate formation from this substrate. Obviously, the pyruvic acid dehydrogenase enzyme complex exhibits a high substrate specificity.

By injecting labeled acetate as a tracer dose in animals administered sub-lethal doses of monofluoroacetate, it is possible to determine *in vivo* citrate synthesis in animals. Accumulation of citrate in tissues of MFA poisoned rats has been reported by several workers (10–12). Buffa and Peters (10) found that citrate accumulation varied with tissues. The data presented by these authors appear to indicate a close correspondence between the initial tissue citrate levels and those obtained 1 hr after poisoning with MFA. In certain tissues, e.g., kidney, spleen, lungs and heart, considerable amounts of citrate were accumulated following poisoning with MFA. We have also shown (Fig. 7) that there was a marked accumulation of labeled fluorocitrate in the spleen and kidney and moderate amounts in lungs and heart when 1-^{14}C acetate was administered 30 and 60 min. prior to sacrifice. There was very little citrate accumulation in the liver, which could be ascribed either to the known detoxifying ability of the liver or rapid synthesis and diffusion from this organ. Spencer and Lowenstein (13) also reported that fluoroacetate poisoning had little effect on the citrate content of male rat liver, whereas there was a seven to eight-fold increase in the kidney citrate content of poisoned rats.

The technique of using MFA poisoned animals in conjunction with labeled compounds may provide an appropriate tool for studying the *in vivo* citrate synthesis and/or oxidation. A study of the fate of citrate in diabetic animals has been recently concluded in these laboratories (Cuestas and Dixit, 14).

Summary. A simple radiometric method is described to determine the incorporation of acetate or pyruvate into citrate. Following *in vitro* incorporation of these substrates by the tissues, they are converted to 'active acetate' which then condenses with oxaloacetate to form citrate. When 1-^{14}C acetate or 2-^{14}C pyruvate were added as tracers, the citrate was labeled at 1-^{14}C carboxyl position which on oxidation was released as $^{14}CO_2$. Labeled acetate in the medium did not interfere with the quantitative recovery of small quantities of citrate formed. When monofluoroacetate (MFA) instead of acetate was added with labeled acetate as the substrate, monofluorocitrate was formed which accumulated in the tissues, perhaps due to block in further oxidation. There was an eight-fold increase in the labeled CO_2 evolution by liver tissue when MFA was used as the substrate.

Of the three temperatures at which the

reaction was studied, the maximum amount of citrate was formed at 27°. The ability of the different tissues of rats to synthesize citrate was varied. Kidney, liver and heart synthesized far more citrate than the skeletal muscle, pancreas and lungs. *In vivo* incorporation of acetate into citrate was studied by injecting 1-^{14}C acetate into rats who were poisoned with MFA before hand, and then sacrificing the animals 30 and 60 min after the labeled injection. Spleen, kidney and lungs contained much greater radioactivity than that present in the whole blood. Heart and liver also exhibited marked elevation when compared with corresponding tissues from saline-injected control rats treated similarly.

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