CITRIC ACID SYNTHESIS BY THE CONDENSATION OF ACETATE AND OXALACETATE IN RABBIT KIDNEY*

by

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The recognition of acetic acid as the common product in the oxidative degradation of carbohydrates, fats, and proteins is now generally accepted. Acetic acid is the end product of carbohydrate oxidation through oxidation of pyruvate[1, 2]; it is the end product of fat oxidation through the process of successive β-oxidations[3, 4, 5] followed by removal of acetate; and it is the end product of protein degradation through the oxidation of amino acids[6]. A study of the mechanism of acetate metabolism became thus of primary importance for the understanding of the last steps of the complete oxidation of foodstuff and for the understanding of the role of acetate in synthesis reactions. Acetic acid is extremely resistant to the action of oxidizing agents; any theory postulated for enzymatic reaction has therefore to start with the assumption of condensation reactions. The first indication of the existence of such processes was given by the discovery of JOWET AND QUASTEL[7] of malonate inhibition of fatty acid oxidation in animal tissues. This inhibition (provided malonate is a competitive inhibitor of succinate oxidation) meant that acetate in its metabolism passed through succinate. Further evidence was obtained by SONDERHOFF AND THOMAS[8], who demonstrated the formation of labelled citric and succinic acids by yeast on addition of deuterium labelled acetic acid. WEINHOUSE AND MILLINGTON[9] confirmed these observations and demonstrated that the oxidation of acetate by yeast starts with the formation of citric acid. The mechanism of acetate oxidation in animal tissues has been the subject of numerous investigations. With the use of labelled acetic acid it was shown that α-ketoglutaric, fumaric, and succinic acids were formed from acetic acid[10]. Yet, BUCHANAN *et al.*[10], and WEINHOUSE *et al.*[11] were unable to find isotopic citric acid, and eliminated citric acid as an intermediate in the oxidation of acetate by animal tissues. Negative experiments with the isotope technique do not have the force of positive results and they must not be taken as evidence that the postulated mechanism does not take place. The formation of dicarboxylic acids from acetic acid was strong indication that the metabolism of acetate started with a condensation process: either with itself to give succinic acid or with oxalacetic acid to form citric or cisaconitic acid. Such a scheme was postulated by various investigators[12, 13, 14]. We present in this paper evidence for the existence of this condensation process. On incubation of C^{14} carboxyl labelled acetic acid with oxalacetate, there was formation of labelled citric acid with the isotope C^{14} in the primary carboxyl groups.

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EXPERIMENTAL

Acetic acid labelled in the carboxyl C with C^{14} was prepared by the usual GRIGNARD reaction from $BaC^{14}O_3$, the GRIGNARD reagent being methyl magnesium bromide. The acetic acid was distilled by steam distillation after addition of Ag_2SO_4 to remove HBr and was converted into sodium acetate, which was evaporated to dryness. Oxalacetic acid was prepared by the method of KRAMPITZ AND WERKMAN[15]; Ba adenosine triphosphate, by the method of DUBOIS *et al.*[16]. Preparation of rabbit kidney homogenates was similar to the technique used by KALNITSKY AND BARRON[17]. The medium in which the tissue was suspended was of the following composition: KCl, 0.123 *M*; $MgCl_2$, 0.004 *M*; sodium phosphate buffer, p_H 7.46, 0.02 *M*. The homogenate contained 100 mg of rabbit kidney cortex per ml.

For the isolation of citric acid, protein precipitation was carried out by addition of one volume of 5% HCl and one volume of 5% $HgCl_2$. After centrifugation the supernatant was treated with H_2S to precipitate mercury. The HgS was filtered off and the filtrate was steam distilled. The citric acid in the residue was isolated according to WEINHOUSE, MEDES, AND FLOYD[11]. In the experiments with malonic acid this acid was oxidized into acetic acid and CO_2 by boiling with 5 *N* H_2SO_4 for ten hours with a reflux condenser. Acetic acid was separated by steam distillation, and citric acid was isolated as the quinidine salt.

Radioactivity measurements were performed with the Nucleometer to which a scaler unit was attached. All the measurements were made on samples of $BaC^{14}O_3$ spread on aluminum dishes of an area of 1.4 cm^2. The amount of carbonate was such as to give samples of infinite thickness. The specific activites are the counts per minute of these samples.

For the degradation of quinidine citrate, 200 mg were placed in a glass tube carrying a side bulb which contained 2 ml of concentrated H_2SO_4. The procedure of WEINHOUSE *et al.*[11] was followed, except that the CO_2 was liberated without removal of the CO formed on degradation of the tertiary carboxyl groups. The gases, CO_2 and CO, were then passed with the aid of a stream of oxygen through a saturated solution of $Ba(OH)_2$ to trap CO_2. The remaining CO and O_2 were passed through a combustion tube containing a 40 gram platinum gauze. CO was oxidized over this hot platinum gauze (800°) and the CO_2 was trapped into $Ba(OH)_2$. The remaining three noncarboxyl carbons of the molecule were isolated as the acetone DENIGÈS complex, according to VAN SLYKE[18].

I. The Optimum Conditions for Citric Acid Synthesis. If the metabolism of acetate starts with a condensation reaction with oxalacetate to form citric acid or an analog of citric acid, its formation may be increased by inhibition of the tricarboxylic acid cycle of oxidation and by addition of adenosinetriphosphate which this cycle provides on its complete operation. As can be seen in the experiments plotted in Fig. 1, the oxidation of acetate was increased in the presence of ATP. Furthermore, ATP addition in the presence of malonate brought for the first 10 minutes an O_2 uptake similar to that of the kidney homogenates with no malonate or ATP. The experiments were performed at 28° because KALNITSKY AND BARRON[17] found that the enzyme was rapidly destroyed at 38°.

The rate of citric acid formation depends on the rate of condensation and on the rate of its utilization. Maximum formation of citric acid was observed after 20 minutes incubation. After this time the amount of citric acid formed remained stationary, although the O_2 uptake continued. The steady state of the condensation process was

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thus reached promptly (Fig. 2). As expected, citric acid formation increased in the presence of malonate (Fig. 3). Under those conditions, oxidation stopped with succinate formation.

The formation of citric acid from acetate and oxalacetate requires no oxygen, as was shown by PERSKY AND BARRON[19] in experiments with a soluble enzyme from rabbit brain. With kidney homogenates citric acid formation in the absence of oxygen was about 30% of that produced in its presence.

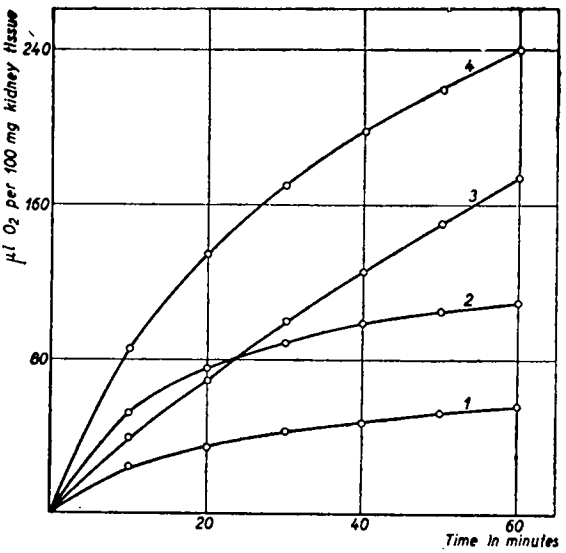

Fig. 1. Effect of ATP on the oxygen uptake of rabbit kidney homogenates. – 1. Acetate + malonate; 2. Acetate + malonate + ATP; 3. Acetate; 4. Acetate + ATP; t = 28° C.

II. Isolation of Labelled Citric Acid. From a study of the experiments above reported it was concluded that isolation of citric acid could be best attempted in the presence of malonate so that the labelled citric acid would not be contaminated with citric acid formed by condensation of labelled acetic acid and labelled oxalacetic acid obtained through the KREBS' cycle. Kidney homogenate (150 ml) was incubated in the presence of 0.01 *M* carboxyl labelled acetate, 0.01 *M* oxalacetate, 0.03 *M* malonate, and 0.002 *M* ATP. The suspension was divided into 35 ml aliquots, which were put into 150 ml. WARBURG vessels (six) and kept shaking for 20 minutes at 28°. The amount of citric acid produced was 19.2 mg. Unlabelled citric acid (38.4 mg) was added to facilitate its isolation. Citric acid was isolated as the quinidine salt. The M.P. of the sample was 127–129°, while another sample prepared directly from citric acid gave a M.P. of 130–132°. The specific activity was calculated from the formula $3C_{20}H_{24}O_2.2C_6H_3O_7$. The M.P. values, as well as the specific activities, were obtained after two recrystallizations of the quinidine citrate. Both increased on second recrystallization. Further recrystallization had no effect on either melting point or specific

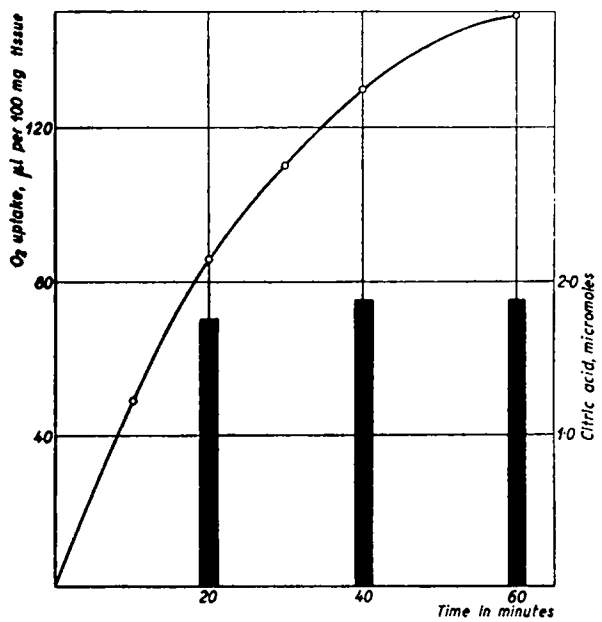

Fig. 2. Oxygen uptake and citric acid formation of rabbit kidney homogenate. Incubation medium contained 0.01 *M* oxalacetate, 0.01 *M* acetate, 0.03 *M* malonate, 0.002 *M* ATP, t = 28° C. Equilibration period of 10 minutes.

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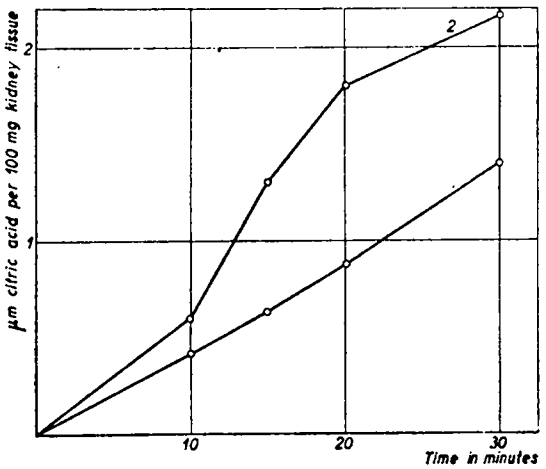

Fig. 3. Citric acid formation by rabbit kidney homogenate with 0.01 *M* oxalacetate, 0.01 *M* acetate, 0.002 *M* ATP. – 1. Control; 2. With 0.03 *M* malonate; t = 28° C.

activity. From the specific activity of the citric acid it can be seen that about 14% of the citric acid isolated came from the $CH_3C^{14}OOH$. The specific activity of the citric acid calculated for one carbon was 6.5×10^4 counts per minute, while the specific activity for the primary carboxyl group as calculated for one carbon was 6×10^4 counts per minute. No radioactivity was found in the three residual carbons isolated as the DENIGÈS complex (Table I).

Incubation of Kidney Homogenate with Added Citrate. In these experiments, to 100 ml of kidney homogenate containing C^{14} carboxyl labelled acetate and non-labelled oxalacetate (0.01 *M*), and ATP (0.002 *M*), there were added 0.01 *M* citrate as a nonlabelled carrier. The suspension was incubated in large WARBURG vessels at 28° for one hour after an

TABLE I

SPECIFIC ACTIVITIES OF CITRIC ACID FORMED BY RABBIT KIDNEY HOMOGENATE INCUBATED WITH OXALACETATE, C^{14} CARBOXYL LABELLED ACETATE, MALONATE, AND ATP

Substances analysed	Specific activity
	Counts per min.
Citric acid	10 980
Tertiary carboxyl	0
Primary carboxyls	30 240
3 residual carbons as DENIGÈS complex	0
Acetate, initial 2.02 mM	230 000
Acetate, after incubation 1.50 mM	245 000

TABLE II

SPECIFIC ACTIVITY OF CITRIC ACID FORMED BY KIDNEY HOMOGENATE IN THE PRESENCE OF OXALACETATE, LABELLED ACETATE, ATP, AND CITRIC ACID AS NON-LABELLED CARRIER

Substances analysed	Specific activity
	Counts per min.
Citric acid	7 800
Tertiary carboxyl	960
Primary carboxyls	21 500
3 residual carbons as DENIGÈS complex	0
Acetate, initial 0.84 mM	260 000
Acetate, after incubation 0.41 mM	275 000

equilibration period of 15 minutes. Citric acid was isolated as previously described and the specific activities were then measured. It can be calculated from the data in Table II that about 8% of the citric acid came from the labelled acetic acid. The primary

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product of the condensation of oxalacetic and acetic acids was in equilibrium with the added citric acid since radioactivity was found in the citric acid isolated from the suspension which contained non-radioactive citric acid. The tertiary carboxyl group contained about one fortieth of the activity of the primary carboxyl groups (calculated on a one carbon basis). This figure is a reasonable one since the radioactive oxalacetic acid formed during the oxidation of radioactive citrate would be greatly diluted by the oxalacetate added initially. The three residual carbons of the DENIGÈS complex contained no radioactive carbon.

In none of the experiments was there dilution of the labelled acetate, since the initial and final activities remained about the same. This may be taken as an indication that the precursor of the remaining citric acid was not in equilibrium with the labelled acetic acid.

III. Incubation of Kidney Homogenates with Oxalacetate and Fluoroacetate. KALNITSKY AND BARRON[17], and MARTIUS[20] found that fluoroacetate increased citric acid formation by kidney suspensions. MARTIUS has postulated that oxalacetate and fluoroacetate may condense to give fluorocitric acid, which would accumulate by lack of oxidation. In order to test this hypothesis, kidney homogenate was incubated for one hour in the presence of oxalacetate, ATP, acetate, and fluoroacetate (0.01 *M*). The citric acid was isolated as the quinidine salt. The M.P. of these crystals was 126–129°; the M.P. of a sample of quinidine citrate was 129–132°. The M.P. of the mixture was 126–129°. The salt was ignited with sodium. The zirconium alizarine test[21] for fluoride ion was negative, an indication that the isolated sample of citric acid contained no fluorine in its molecule.

DISCUSSION

The experiments presented in this paper have shown that in kidney suspensions the metabolism of acetate starts by its condensation with oxalacetate to give citric acid, and proceeds with its oxidation through the tricarboxylic acid cycle of KREBS. In fact, in the presence of malonate, the citric acid isolated on incubation of rabbit kidney homogenates with C^{14} labelled acetate, oxalacetate, Mg, and ATP contained all the C^{14} in the primary carboxyl group. In the absence of malonate, when oxidation proceeded undisturbed through the KREBS cycle, there was some C^{14} in the tertiary carboxyl group. This undoubtedly came by condensation of the two labelled compounds: acetate and the newly formed oxalacetate. The increase in the synthesis of citrate by Mg previously found by KALNITSKY AND BARRON[17] may be taken as an indication that the protein moiety of the enzyme, citrogenase, is a metalloprotein with Mg as the metal. The condensation of acetate with the enol form of oxalacetate (at the p_H value of our experiments all the oxalacetate was in its enol form as shown by spectrophotometric measurements) is probably similar to the acid-base catalysed aldol condensation reactions well known in organic chemistry, such as the PERKINS, and KNOEVENAGEL reactions. For this condensation it is first necessary to labilize the hydrogen of the methylene group of acetic acid. ATP undoubtedly plays this role by the formation of an ATP-acetate complex. This complex is not acetylphosphate. In fact, PERSKY AND BARRON[19] working with a rabbit brain water soluble enzyme which produced citric acid in the presence of acetate, oxalacetate, ATP, and yeast juice, found that acetylphosphate was ineffective. The ATP-acetate complex may be the substance postulated by KAPLAN AND LIPMANN[22]. It needs, however, to be isolated so that its structure may be determined.

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The synthesis of citric acid reported by other investigators on incubation of animal tissues with pyruvate[23] and acetoacetate[24, 25, 26] can be reasonably explained as due to the same condensation process. There is no need to postulate the formation of hypothetical C^7 condensation products.

The citric acid isolated by the previous addition of non-labelled citric acid was in equilibrium with the newly synthesized citric acid. This is an indication that the condensation product is not *cis*-aconitic acid as has been postulated[27]. ATP-acetate on condensing with oxalacetate will give an asymmetrical molecule, which on successive oxidations *via* the KREBS' cycle would produce α-ketoglutarate with labelled carbon next to the keto group. The lengthy controversy which has been going on as a consequence of experiments with isotopic tracers[28, 9, 10] thus become meaningless. Finally, the role of aconitase would be that of rendering the citric acid capable of oxidation through the tricarboxylic acid cycle by the formation of isocitric acid.

SUMMARY

On incubation of rabbit kidney homogenates with C^{14} carboxyl-labelled acetate, oxalacetate, Mg, adenosinetriphosphate, and malonate, there was formation of citric acid labelled with C^{14} in the primary carboxyl carbon. In the absence of malonate there was some C^{14} in the tertiary carboxyl carbon. These experiments demonstrate that the metabolism of acetate starts with its condensation with oxalacetate to form citrate, and subsequent oxidation through the KREBS cycle. The role of ATP is that of rendering labile the H of the CH_3 group of acetate, a process necessary for the condensation. This probably takes place by the formation of an ATP-acetate complex.

RÉSUMÉ

L'incubation d'un homogénat de rein de lapin avec de l'acétate marqué au groupe carboxyl par C^{14}, de l'oxalacétate, du Mg, de l'ATP et du malonate donne naissance à de l'acide citrique marqué par C^{14} au groupe carboxyle primaire. En absence de malonate on trouve un peu de C^{14} dans le groupe carboxyl tertiaire. Ces expériences démontrent que le métabolisme de l'acétate débute par sa condensation avec l'oxalacétate en donnant du citrate et continue par une oxydation suivant le cycle de KREBS. Le rôle de l'ATP consiste à rendre labile le H du groupe CH_3 de l'acétate, ce qui est nécessaire pour la condensation. Cela se produit probablement par formation d'un complexe ATP-acétate.

ZUSAMMENFASSUNG

Lässt man auf ein Kaninchennierenhomogenat in der Carboxyl-Gruppe mit C^{14} markiertes Acetat, Oxalacetat, Mg, ATP und Malonat einwirken, so wird Zitronensäure mit C^{14} in der primären Carboxylgruppe gebildet. In Abwesenheit von Malonat war etwas C^{14} im tertiären Carboxylkohlenstoff. Diese Versuche zeigen, dass der Acetatmetabolismus damit beginnt, dass sich das Acetat mit Oxalacetat zu Citrat kondensiert und dann über den KREBS'schen Cyklus oxydiert. Die Rolle des ATP ist es, das H der CH_3-Gruppe im Acetat labil zu machen, was zur Kondensation nötig ist. Dies geschieht wahrscheinlich durch Bildung eines ATP-Acetat-Komplexes.

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