HOPPE-SEYLER'S Z. PHYSIOL. CHEM.
Bd. 350, S. 1367—1376, November 1969

Intramolecular Nucleophilic Catalysis on the Hydrolysis of Citryl-CoA*

By WOLFGANG BUCKEL and HERMANN EGGERER

*Institut für Biochemie der Universität München***

(Received 28 July 1969)

Summary: 1) The hydrolysis of citryl-CoA under physiological conditions is facilitated by neighbouring group participation. Intramolecular nucleophilic catalysis, exercised by the carboxylgroup in β-position to the thioester, yields a water labile anhydride as an intermediate. The otherwise stable thioester group therefore appears as labile and fast hydrolysis of citryl-CoA is observed.

2) Proof for the formation of the asymmetric anhydride was obtained by aminolysis in the presence of glycine: In neutral medium two products, α- and β-citryl glycine, were formed from the thioester *via* the anhydride. In alkaline solution however, the direct aminolysis of the thioester predominated and only α-citryl glycine was formed.

3) The formation of the anhydride and the direct aminolysis of the thioester are pH-dependent competing processes. Their study revealed an increase in reactivity of the carboxylate anion in the intramolecular reaction of ten to the fifth power as compared to that of an intermolecular reaction.

4) Attempts to demonstrate the intermediate formation of citryl anhydride on the enzyme citrate synthase by application of the principle underlying the chemical model, have failed.

5) The chemical preparation of several α- and β-citryl derivatives is described.

Zusammenfassung: *Intramolekulare nucleophile Katalyse bei der Hydrolyse von Citryl-CoA.* 1. Die Hydrolyse des Citryl-CoA wird unter physiologischen Bedingungen durch einen Nachbargruppeneffekt erleichtert. Die zum Thioester β-ständige Carboxylgruppe liefert in intramolekularer nucleophiler Katalyse ein wasserlabiles Zitronensäureanhydrid als Zwischenprodukt. Die andernfalls stabile Thioestergruppierung erscheint daher als labil und es resultiert die rasche Hydrolyse des Citryl-CoA.

2. Der Beweis für die Bildung des asymmetrischen Anhydrids wurde durch Aminolyse erbracht: Im neutralen, glycinhaltigen Medium entstanden aus dem Thioester über das Anhydrid zwei Produkte, α- und β-Citryl-glycin. Im alkalischen Medium dagegen erfolgt die direkte Aminolyse des Thioesters zu nur einem Produkt, α-Citryl-glycin.

3. Die Bildung des Anhydrids und die direkte Aminolyse des Thioesters sind pH-abhängige Konkurrenzreaktionen. Ihr Studium ließ erkennen, daß die Reaktivität des Carboxylat-Anions in der intramolekularen Reaktion gegenüber der einer intermolekularen Reaktion hunderttausendfach vergrößert ist.

4. Auf dem Prinzip der chemischen Modellreaktion aufbauende Versuche, Zitronensäureanhydrid auch als Zwischenstufe am Enzym Citrat-Synthase nachzuweisen, sind mißlungen.

5. Die chemische Darstellung einiger α- und β-Citryl-Derivate wird beschrieben.

* W. BUCKEL, Thesis, Universität München, 1968.

** Address: Dr. W. BUCKEL and Prof. Dr. H. EGGERER, D-8 München 2, Karlstr. 23.

Enzymes:

Citrate synthase, citrate oxaloacetate-lyase (CoA-acetylating) (EC 4.1.3.7)
Citrate lyase, citrate oxaloacetylate-lyase (EC 4.1.3.6)

It has been demonstrated that the hydrolysis of dicarboxylic acid monoesters of appropriate structure is facilitated by neighbouring group participation of the carboxyl group[1]. This process may follow three distinct mechanisms:

a) Intramolecular general acid catalysis (the protonated carboxyl group assists the attack of a hydroxyl ion); b) intramolecular general base catalysis (the carboxylate anion assists the attack of water); and c) intramolecular nucleophilic catalysis (the carboxylate anion attacks the ester carbonyl and forms the anhydride). Chemical proof for the formation of the anhydride seems to be restricted to the salicylic acid type[2,3].

Chemical hydrolysis of citryl-CoA

(*S*)-Citryl-CoA, formed on aldol condensation between acetyl-CoA and oxaloacetate as an enzyme bound intermediate on citrate synthase[4,5], is a substituted succinyl monothioester. In contrast to the lower and higher homologues (i. e. malonyl-CoA and glutaryl-CoA), citryl-CoA, like succinyl-CoA[6], undergoes fast chemical hydrolysis in aqueous solution at neutral pH and 25°C (Fig. 1). These results indicate that the hydrolysis of both compounds is subject to anchimeric assistance[7,8]. It was therefore assumed that citryl-CoA is transformed on the enzyme from the chemically more stable thioester to the water labile anhydride[4,5]. This paper deals with the chemical and enzymatic mechanism of hydrolysis of citryl-CoA. The results show that the chemical hydrolysis under physiological conditions is indeed facilitated by intramolecular nucleophilic catalysis of the carboxylate anion. No proof could be obtained, however, for a like mechanism in the enzyme catalyzed process.

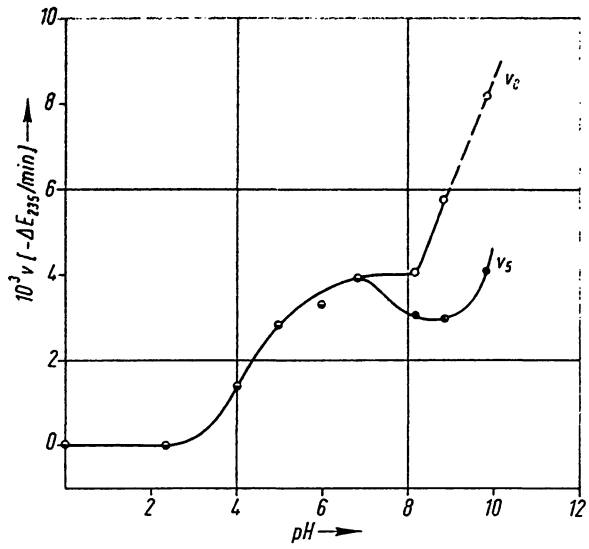


Fig. 1. Rate of hydrolysis of citryl-CoA as a function of pH. The initial concentration of citryl-CoA was 0.14mM. $\lambda = 235$ mμ; $d = 1$ cm; 25°C. v_0 represents the rate obtained by extrapolation to time zero, v_5 represents the rate at time = 5 min. For details see Experimental Section, p. 1372/1373.

Results

Proof for the formation of an anhydride

If the hydrolysis of citryl-CoA is facilitated by intramolecular nucleophilic catalysis, an asymmetric anhydride will be formed as an intermediate. This asymmetry and the difference in reactivity between a thioester and an anhydride group when used as acylating agents for amino acids, provided the basis for the present investigation, in which *S*-citryl-*N*-capryloylcysteamine (I) was used instead of citryl-CoA*. It has been observed previously that thioesters in neutral aqueous medium at room temperature are stable in the presence of amino acids[9,10]. Anhydrides, however, are reactive enough to acylate amino acids under these conditions even though only a small percentage of the reactive free amino group is present.

If the hydrolysis of the citryl-α-monothioester proceeds *via* the anhydride II, two different products

[1] T. C. Bruice and S. J. Benkovitch, in W. H. Benjamin, Bioorganic Mechanisms, New York 1966, Vol. 1, p. 119.

[2] M. L. Bender, Y.-L. Chou and F. Chloupek, J. Amer. chem. Soc. **80**, 5380 [1958].

[3] A. R. Fersht and A. J. Kirby, J. Amer. chem. Soc. **90**, 5833 [1968].

[4] H. Eggerer and U. Remberger, Biochem. Z. **337**, 202 [1963].

[5] H. Eggerer, Biochem. Z. **343**, 111 [1965].

[6] E. J. Simon and D. Shemin, J. Amer. chem. Soc. **75**, 2520 [1953].

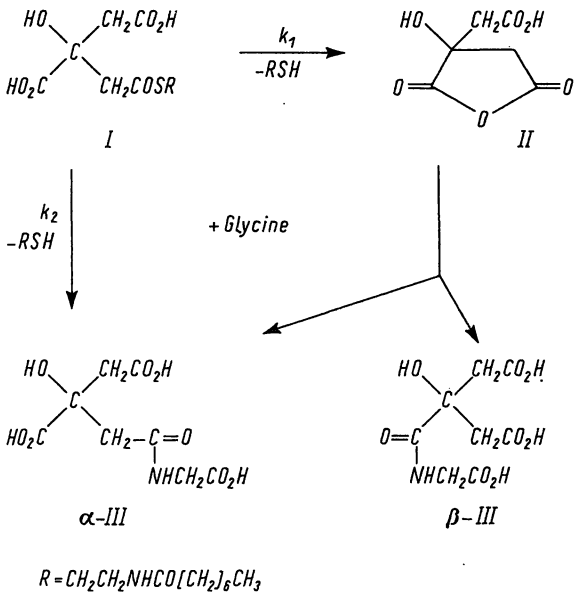
[7] M. L. Bender, Chem. Reviews **60**, 53 [1960].

[8] H. Eggerer, Liebigs Ann. Chem. **666**, 192 [1963].

* As was checked in orienting experiments, citryl-CoA on reaction with [^{14}C]glycine qualitatively yielded the results obtained with I.

[9] R. Schwyzer and C. Hürlimann, Helv. chim. Acta **37**, 155 [1954].

[10] H. Eggerer, E. R. Stadtman and J. M. Poston, Arch. Biochem. Biophysics **98**, 432 [1962].



Scheme 1. Mechanisms of glycinolysis of I.

should be observed in the presence of glycine, namely approximately equal amounts of α- and β-citrylglycine (α-III and β-III respectively; Scheme 1). α- and β-citrylglycine can readily be separated by paper chromatography or -electrophoresis. If the hydrolysis of the thioester is conducted in the presence of ^{14}C-labelled glycine, the formation of both products can be followed quantitatively. As is obvious from the reaction sequence, the formation of β-citrylglycine in this experiment would provide proof for the intermediate formation of the anhydride.

At pH 7, where only a fraction of the total concentration of glycine is susceptible to acylation, the reaction does indeed occur as outlined. Nearly equal amounts of α- and β-citrylglycine were formed as the products. The control of this result was provided as follows: As the pH was increased in parallel experiments, the concentration of the reactive glycine anion was also increased. Under these conditions (high concentration of the reactive amino group) not only the anhydride but also the thioester undergoes aminolysis. An increased formation of α-citrylglycine compared to the β-derivative was consequently observed. At about pH 10, the aminolysis of the thioester is fast enough to suppress the anhydride formation completely and only α-citrylglycine was formed.

These results provide firm proof of the formation of a citric anhydride on hydrolysis of the thioester and consequently for the intramolecular nucleophilic action of the carboxylate anion.

Competition between direct thioester aminolysis and anhydride formation

Depending on pH, the reactivity of the citryl thioester follows two mechanisms which compete for each other: One is the intramolecular reaction of the poorly nucleophilic carboxylate anion with the thioester carbonyl to yield the anhydride. The anhydride in a pseudo-monomolecular reaction with glycine yields the α- and β-citryl derivatives. The other is an intermolecular second order reaction between the thioester group and the strongly nucleophilic amine and yields only α-citrylglycine. The relative amount of α-citrylglycine formed in these experiments thus indicates which mechanism is preferred (Fig. 2).

At pH 7, 40% α-citrylglycine (and 60% of the β-derivative) were formed, thus indicating that all of the thioester was decomposed *via* the anhydride

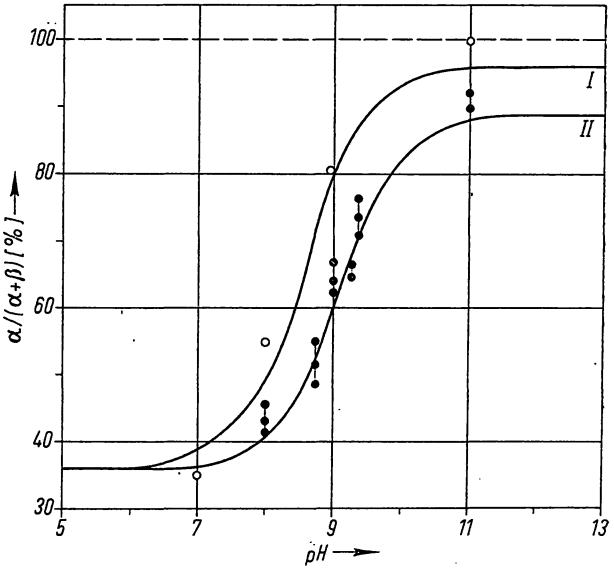


Fig. 2. The relative yield of α- and β-citryl derivatives in glycinolysis of I as a function of pH. The points are experimental, the solid lines were calculated.

Curve I: The initial concentrations were 50mM thioester I and 2M unlabelled glycine. The experimental points were estimated from the intensity of methyl red labelling.

Curve II: The initial concentrations were 45mM thioester I and 0.57M [1-^{14}C]glycine. The experimental points were determined from the radioactivity.

For details see Experimental Section, p. 1373/1374.

under these conditions. With increasing pH, the second order reaction predominates, as is indicated by the increased formation of α-citrylglycine and by the experimental points in Fig. 2. Assuming the formation of the anhydride as the rate determining step in the reaction sequence which yields both isomers, an equation can be derived from the kinetics of the competing processes (*c. f.* Experimental). k_1/k_2, the competition constant of both reactions is unknown in this equation. It can, however, be determined from the equation by fitting a curve to the experimental points. This is demonstrated by the solid lines in Fig. 2. From the competition constant it can be calculated under which conditions both reactions occur at the same rate (see legend to Fig. 2 and Experimental). From these results it appeared that the poorly nucleophilic carboxylate anion could compete equally well with the strongly nucleophilic amine. Due to its suitable steric position, the carboxylate anion in the intramolecular reaction is as good a nucleophile as is the amine in the intermolecular reaction. In an intermolecular reaction, the carboxylate anion, e. g. that of acetate, is a 10^5 times weaker nucleophile than the glycine anion[11].

The following results indicate that the formation of the anhydride from the thioester I can also be demonstrated with other nucleophiles, as expected:

a) I on reaction with 1M hydroxylamine solution, pH 7 (corresponding to 96% free amine) at 25°C yields only α-citrylhydroxamate[8]. On dilution of the reagent to 0.03M, however, the formation of the anhydride is favoured and both, the α- and β-derivatives are formed.

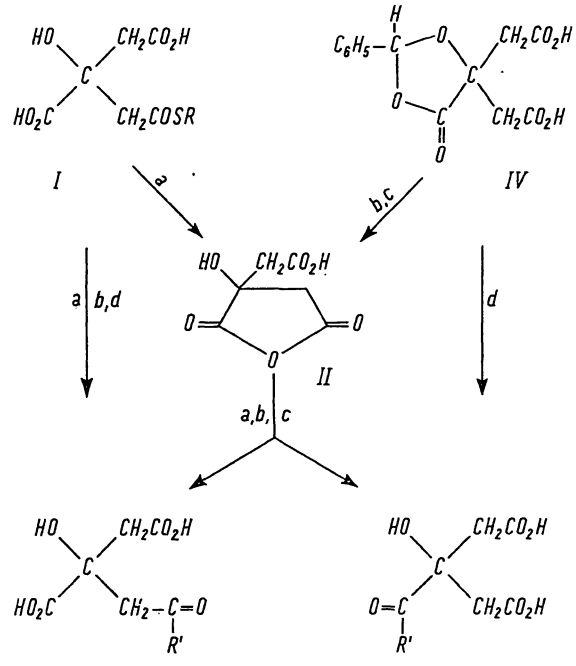
b) Reaction of I with aqueous 1M cyclohexylamine yielded only citric α-cyclohexylamide (α-V). Under the less basic conditions of ethanolic 1M cyclohexylamine, however, both citric α- and β-cyclohexylamides (α-V and β-V, respectively) were formed as products. The citric cyclohexylamide described previously[8] could be identified as the β-derivative. Both compounds were obtained preparatively by reaction of the dioxolanone IV with cyclohexylamine.

In all cases, the α- and β-citryl derivatives could be separated by paper electrophoresis. The β-derivative migrated approx. 20% faster than the corresponding α-derivative.

[11] W. P. JENCKS and J. CARRIUOLO, J. Amer. chem. Soc. **82**, 1778 [1960].

Preparation of α- and β-derivatives of citric acid

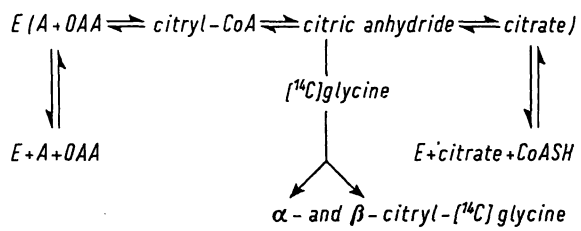
In order to confirm the results obtained with glycine, the two marker substances were synthesized on a preparative scale as follows: α-citrylglycine (α-III) was obtained quantitatively by glycinolysis of the thioester I. For the synthesis of β-citrylglycine, the dioxolanone IV, useful as a shielding group for the preparation of the thioester I[8], was used as an acylating agent for glycine ethylester. The main product isolated (60%) was the expected β-citrylglycine ethylester (β-VI), which on alkaline hydrolysis yielded β-citrylglycine (β-III). In addition, α-citrylglycine ethylester (α-VI, 20%) was also formed. This result indicates that the aminolysis of IV involves the intermediate formation of the anhydride II. α- and β-methylcitrates (α-VII and β-VII respectively) were prepared from sodium methoxide and I and IV respectively. Scheme 2 summarizes these results.



Scheme 2. Preparation of α- and β-citryl derivatives.
R = CH_2-CH_2-NH-CO-[CH_2]$_6$-CH_3
a) Reaction with glycine: α-III, β-III;
R′ = NH-CH_2-CO_2H.
b) Reaction with glycine ethylester: α-VI, β-VI;
R′ = NH-CH_2-CO_2-C_2H_5.
c) Reaction with cyclohexylamine: α-V, β-V;
R′ = NH-C_6H_{11}.
d) Reaction with sodium methylate: α-VII, β-VII;
R′ = OCH_3.

Enzymatic hydrolysis of citryl-CoA

If the enzymatic hydrolysis of citryl-CoA follows the pattern of the chemical model reaction described above, the synthesis of citrate from acetyl-CoA and oxaloacetate may be described as outlined in Scheme 3.



E = enzyme; A = acetyl-CoA; OAA = oxaloacetate.

Scheme 3. Possible trapping of II on the enzymatic synthesis of citrate.

Continuous back and forth reaction of substrates and products under equilibrium conditions[12] might involve the formation of a small concentration of the anhydride II. This, in the presence of ^{14}C-labelled glycine, could yield labelled α- and β-III and thus demonstrate its intermediate existence. The obvious advantage of using equilibrium conditions for the trapping experiments is the fact, that a small concentration of the anhydride, if formed, can be kept constant over a long period of time.

The best conditions for the mobile equilibrium were established by labelling experiments in tritiated water[5]. It was found that glycine had no inhibitory effect on the equilibration and that the enzyme was stable under the conditions employed.

No loss of enzymatic activity was observed during incubations as follows: In typical experiments, the incubation mixture at 25°C in a total volume of 0.10 m*l* contained (mM concentrations in parentheses): Phosphate buffer, pH 7.0 (150); acetyl-CoA (6.6); citrate (between 2 and 50); CoA-SH (2.5); [1-^{14}C]glycine (between 10 and 750; spec. activity: between $4.0 \cdot 10^6$ and $5.4 \cdot 10^4$ dpm/μmole) and 0.2 mg (7 U) citrate synthase.

Parallel experiments were performed with labelled citrate (spec. activity: $3.8 \cdot 10^5$ dpm/μmole) and unlabelled glycine, and also with labelled citrate and unlabelled methanol (end concentration: 20 vol. %). After incubation times between 15 and 157 h, 1 μmole of unlabelled α- and β-citrylglycine (or unlabelled α- and β-methyl citrate respectively) was added as a carrier. The enzyme was inactivated by the addition of 10 μ*l* 5N HCl and the reaction mixture was analyzed by paper electrophoresis.

Result: In no case could any trace amount of ^{14}C-labelled α- and β-citryl derivatives be detected.

Discussion

Two possibilities are to be considered with respect to the nature of the "intermediates" on the enzyme citrate synthase. a) They are real intermediates, b) they represent transition states. Assuming that the active site of the enzyme is freely accessible to nucleophilic agents, the failure to demonstrate the formation of the anhydride by trapping experiments may indicate the latter type of conversion.

The results obtained in several lyses of the thioester I clearly indicate that the observed instability of this compound in neutral aqueous medium is due to neighbouring group participation. Intramolecular nucleophilic catalysis, exercised by the carboxylate anion in the β-position facilitates the hydrolysis of the otherwise stable thioester group. This reaction requires that both participating groups are arranged in a *cis*-position, which in enzyme free solution is disturbed by rotation. In the enzyme catalyzed reaction it may be expected that these groups would be held in the proper steric position, which consequently would increase the rate of reaction. Additionally, the thioester carbonyl may be polarized more strongly in the enzyme catalysis by the action of *e.g.* a metal ion, which would further facilitate the reaction and provide a rationale for the high speed of reaction. The hydrolysis of the water-labile anhydride itself may be catalyzed by covalent catalysis, *e.g.* through intermediate formation of an acyl imidazole enzyme.

Whereas these results and interpretations provide a reasonable chemical basis for the transformation of the β-hydroxyacyl-CoA derivative on the enzyme, there is no need to assume that the enzymatic hydrolysis must occur like that of the chemical model. In the enzyme catalyzed reaction, the hydrolysis of the thioester might be facilitated otherwise, *e.g.* through the formation of an acyl-imidazole enzyme from the thioester directly, rather than from the anhydride.

One must take into account, however, that the synthesis of citrate from acetyl-CoA and oxaloacetate is reversible[12]. This reversal amounts to the formation of an acyl-CoA-derivative without an external supply of energy. As discussed by CORN-

[12] J. R. STERN, in P. D. BOYER, H. LARDY and K. MYRBÄCK, The Enzymes, Vol. 5, p. 367, Academic Press, New York 1961.

FORTH[13], the energy required to form the CO-S bond is so high that such a process appears most unlikely in the absence of some special characteristic of the acid concerned. The easily occurring formation of the five-membered ring provides a special characteristic in facilitating the formation of the "energy rich" anhydride and hence that of the thioester. That citric acid in aqueous solution exists indeed in equilibrium with the anhydride has been demonstrated by HIGUCHI *et al.*[14]. Similar in principle to the mechanisms described in this paper, the neighbouring group participation of the carboxyl group facilitates an otherwise unlikely chemical reaction.

In summary it appears that the chemical evidence presented in this paper favours the formation of a citric anhydride on the enzyme citrate synthase. Proof, however, for this particular phase of the biological conversion can be obtained only by investigations with the enzyme itself. Some further attempts to answer this problem are described in a following communication[15].

Support of this work by the DEUTSCHE FORSCHUNGSGEMEINSCHAFT is gratefully acknowledged.

Experimental

General

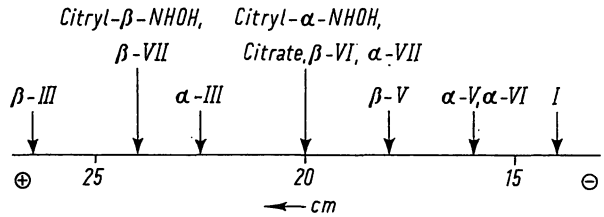
Microanalyses were performed by H. SCHULZ in the Analytical Laboratory of the Institut für Organische Chemie der Universität München. Melting points are uncorrected. Equivalent weights were determined by titration with 0.1N NaOH; salts were passed through Dowex-50 ($H^{\oplus}$, 200—400 mesh; 0.8×5 cm) and the acid in the effluent was titrated.

Paper electrophoresis

Hochspannungspherograph, Modell Frankfurt, Hormuth & Vetter, Heidelberg-Wiesloch. Paper: Whatman 1; buffer solution: pyridine-acetate, pH 6.2 (10 m*l* glacial acetic acid, 100 m*l* pyridine and 890 m*l* water); voltage: 45 V/cm; time interval: 90 min.

Detection of acids: In order to ensure complete removal of pyridinium acetate, the paper was dried at 70°C and treated with steam repeatedly. Detection was accomplished with 0.05% methylred in 0.05M borate buffer, pH 8, or with 0.1% aqueous $KMnO_4$ solution. Hydroxamates and glycine were detected with a solution of 5% $FeCl_3$ in 0.1N ethanolic HCl, and with 0.2% ninhydrin in acetone, respectively.

Like citrate[16], its α-derivatives are oxidatively decarboxylated by $KMnO_4$ rapidly yielding colorless spots on the paper. The β-derivatives, however, are less easily oxidized and can thus be differentiated from the corresponding α-derivatives. The β-derivatives of citrate migrate faster than the corresponding α-derivatives. As compared to other di- and tricarboxylic acids, citrate and the α-derivatives migrate more slowly. This may indicate intra- or intermolecular association involving the alcoholic hydroxyl and the β-carboxyl groups. A schematic representation of the separation by paper electrophoresis of several citryl derivatives is given in Scheme 4. Conditions as outlined above; size of the zones approx. ±1 cm from the centre indicated on the scheme. All preparations of citryl derivates described below were followed and checked by application of this method.



Scheme 4: Paper electrophoretic separation of citryl derivatives. For a key to the Roman numerals see Scheme 2.

^{14}C-Determinations

The electrophoretically separated compounds were detected with a strip counter (Frieseke-Höpfner Radiochromatograph FH 452) and, yielding congruent zones, with methylred. The radioactive zones were integrated by calculation from the height and width of the recorded peaks.

UV-Measurements were performed with a Zeiß Spektralphotometer PM Q4.

Hydrolysis of citryl-CoA (Fig. 1)

(*RS*)-Citryl-CoA was prepared[8] and contained 14mM total thioester (determined as hydroxamate[8]) and 4mM (*S*)-citryl-CoA (determined with citrate synthase[4]). Small amounts of CoA-SH present in the solution were oxidized with alcoholic iodine solution and excess iodine was extracted with ether. For the determination of the rate of hydrolysis, each cuvette, $d = 1$ cm, contained initially, in a total volume of 1.0 m*l*, 0.14 μmoles citryl-CoA (determined as hydroxamate), buffer solution (pH 0: 1M HCl; pH 2.3 and 3.1: 50mM glycine-HCl;

[13] J. W. CORNFORTH, J. Lipid Res. **1**, 15 [1959].

[14] T. HIGUCHI, L. EBERSON and J. D. MCRAE, J. Amer. chem. Soc. **89**,, 3001 [1967].

[15] P. WUNDERWALD and H. EGGERER, Europ. J. Biochem. [Berlin] **11**, 97 [1969].

[16] A. C. KUYPER, J. Amer. chem. Soc. **55**, 1722 [1933].

pH 4.1: 50mM citrate; pH 5: 25, 50 and 200mM acetate; pH 6.0—8.25: 50mM phosphate; pH 8.85 and 9.85: 50mM carbonate) and KCl (350—490mM) in order to ensure constant ionic strength ($\mu = 0.5$) in all samples.

The rate of hydrolysis was followed at 25°C for a time interval of 15 min by the decrease of thioester absorption at 235 mμ. The extinction difference observed in solutions above pH 7 was corrected for mercaptide ion absorption ($CoA\text{-}SH \rightleftharpoons CoA\text{-}S^{\ominus} + H^{\oplus}$; $pK = 9.6$[17]) by application of the following molar extinction coefficients ($l \cdot Mol^{-1} \cdot cm^{-1}$): ε (pH 0—7) $= 4.7 \cdot 10^3$; ε (pH 8.26) $= 4.6 \cdot 10^3$; ε (pH 8.85) $= 4.1 \cdot 10^3$; ε (pH 9.5) $= 1.5 \cdot 10^3$.

v_0 in Fig. 1 represents the rate of hydrolysis as obtained by extrapolation to time zero of the time course, v_5 represents that determined at time = 5 min. The observed lower value of v_5 as compared to v_0 at pH values above 7 is in agreement with the back reaction of CoA-SH with the anhydride II.

Glycinolysis of the thioester I and its dependence on pH (Fig. 2)

Theory

1) Intramolecular reaction: Assuming the formation of the anhydride II (Scheme 1) as the rate determining step in the reaction sequence leading to both citryl derivatives (α-III and β-III), their formation may be described as follows:

$$d[\alpha_1]/dt = -(1-p)\, d[E]/dt = -(1-p)\, k_1 [E],$$

$$d[\beta]/dt = -p\, d[E]/dt = -p k_1 [E],$$

α = α-III; β = β-III; E = I; p = frequency factor, indicating the attack of glycine anion at the β-carbonyl group of II. $(1-p)$ likewise indicates the frequency of attack at the α-carbonyl group by the glycine anion.

2) Intermolecular reaction: The thioester I is directly attacked by the glycine anion.

$$d[\alpha_2]/dt = -d[E]/dt = -k_2 [E]\, [Gly^{\ominus}].$$

$Gly^{\ominus} = H_2NCH_2CO_2^{\ominus}$.

3) Combination of intra- and intermolecular reaction sequence yields

$$-d[\alpha]/dt = -(d[\alpha_1] + d[\alpha_2])/dt = k_1(1-p)\,[E] + k_2 [E]\, [Gly^{\ominus}]$$

and $-d[\beta]/dt = k_1 p [E]$.

The ratio of the products α-III and β-III, α/β, corresponds to the ratio of their rates of formation:

$$\alpha/\beta = \frac{k_1(1-p) + k_2[Gly^{\ominus}]}{k_1 p}.$$

This ratio is independent of the thioester concentration but dependent on the concentration of the glycine anion, and thus on pH. In Fig. 2, the experimentally determined yield of α-III was plotted against pH. The unknown constants k_2/k_1 and p can be determined by fitting a curve to the experimental points from the following equation:

$$\frac{[\alpha]}{[\alpha] + [\beta]} = 1 - \frac{p}{1 + k_2/k_1 \dfrac{[Gly]_{total}}{1 + [H^{\oplus}]/K}}$$

K = dissociation constant of glycine ($1.7 \cdot 10^{-10}$M).

It is more convenient, however, to rearrange this equation into that of a straight line:

$$\frac{[\alpha] + [\beta]}{[\beta]} = \frac{1}{p} + \frac{1}{p}\frac{k_2}{k_1}[Gly^{\ominus}].$$

Intersection of the straight line with the $(\alpha + \beta)/\beta$ axis yields $1/p$, and hence k_2/k_1 from the slope. The theoretical curves (solid lines) in Fig. 2 were calculated from this equation using the values of p and k_2/k_1, experimentally determined as described below.

Results

1) Ratio of glycinolysis to hydrolysis of the thioester I and its dependence on glycine concentration:

The reaction mixtures, final volume 0.10 m*l*, contained 5mM thioester I, 40mM phosphate buffer, pH 7.0, and glycine as indicated below. The solutions were kept at 25°C for 86 h, then extracted with ether in order to remove precipitated mercaptan. Citrate present in the aqueous solution was determined with the aid of citrate lyase according to GRUBER and MOELLERING[18].

Glycine applied [mmoles/l]	Citrate found [%]
0	100
10	47
20	32
50	14
100	4
200	3
500	< 1
1000	< 1

A 0.5M solution of glycine is thus sufficient at pH 7 to prevent the hydrolytic reaction of the anhydride II and to ensure its quantitative glycinolysis. A like result was obtained at pH 10.

2) Two series of investigations were performed for the pH-dependence of glycinolysis of I (Fig. 2):

a) Reaction of the thioester I (50mM) in the presence of 2M unlabelled glycine: Each incubation mixture, vol. 0.10 m*l*, contained in addition 0.5M buffer substance (phosphate for pH 7, $KHCO_3/K_2CO_3$ for pH 9—11) and

[17] H. BEINERT, R. W. VON KORFF, D. E. GREEN, D. A. BUYSKE, R. E. HANDSCHUMACHER, H. HIGGINS and F. M. STRONG, J. biol. Chemistry **200**, 385 [1953].

[18] W. GRUBER and H. MOELLERING, Biochem. Z. **344**, 85 [1966].

was kept at 25°C for 25 h. Liberated mercaptan was extracted with 0.2 m*l* ethyl acetate in each case, and 20-µ*l* portions of each aqueous solution was applied to 3-cm strips for paper electrophoretic separation. The zones were labelled with methylred and their intensity was estimated visually.
Result: Experimental points of curve I, Fig. 2.

b) Reaction of I (45mM) in the presence of 570mM 1-^{14}C-labelled glycine (0.04 µC/µmole): Each incubation mixture in a total volume of 0.055 m*l* contained in addition 570mM Tris buffer (pH 8.1—11.0) and 570mM KCl. The Tris-glycine buffers were prepared from a stock solution containing Tris, glycine and KCl, each 0.715M. 5N KOH or 5N HCl was added to adjust to the appropriate pH. The reaction mixtures were kept at 25°C for 25 h and liberated mercaptan was extracted with 0.2 m*l* ethyl acetate. Each aqueous solution was applied to 9-cm strips for paper electrophoretic separation. These strips, after the separation, were cut into three strips of 3 cm breadth, each of which was analyzed in the strip counter. The results are summarized in the Table.

The relative yield of α- and β-citryl derivatives in glycinolysis of I as a function of pH. The ratio of $\alpha/(\alpha+\beta)$ is indicated in Fig. 2.

pH	Glycine anion [mmoles/*l*]	α-III* [mm²]	β-III* [mm²]	(α+β)/β	Average
8.1	13	331	369	1.83	
		448	597	1.75	1.77
		480	660	1.73	
8.75	50	416	341	2.18	
		920	840	2.10	2.08
		1060	1100	1.96	
9.0	83	1007	572	2.75	
		1260	720	2.75	2.85
		665	324	3.05	
9.2	120	1030	542	2.90	2.92
		1090	560	2.94	
9.4	182	1254	520	3.41	
		924	280	4.29	3.92
		729	260	4.05	
11.0	538	1850	112	17.5	14.15
		1100	120	10.8	

* Peak of radioactivity.

From a graph of $(\alpha+\beta)/\beta$ against the concentration of glycine anion, the frequency factor of glycine attack at the β-carbonyl group of the anhydride II was determined as $p = 0.63$. This result indicates that on reaction of the pure anhydride with glycine, 63% of the β- and 37% of the α-citryl derivative would be formed as products. The competition constant was determined as $k_2/k_1 = 0.63 \cdot 11 = 7$ *l*/Mol. This result indicates that the glycine anion must be present in a concentration of $^1/_7$ Mol/*l* = 0.14M in order to compete equally well with the carboxylate anion.

Hydroxylaminolysis of the thioester I and its dependence of hydroxylamine concentration

Each reaction mixture, which was kept at 25°C for 30 h, in a total volume of 0.60 m*l* contained 25mM thioester I and hydroxylamine solution of pH 7 in concentrations as indicated below. 20-µ*l* portions of each solution were analyzed by paper electrophoresis; the hydroxamates were detected with $FeCl_3$-solution and the intensity of the detected zones was estimated visually. The barium salt of α-citrylhydroxamate[8] was used as a marker.

Hydroxylamine [mmoles/*l*]	Citryl hydroxamate
1000	only α
100	α and trace amounts of β
50	α:β ca. 20:1
25	α:β ca. 10:1

PREPARATION OF CITRYL DERIVATIVES FROM THE THIOESTER I

α-Citrylglycine (α-III)

2.1 g (5 mmoles) *mono potassium salt of I*[8] was dissolved in 100 m*l* 1.33M *glycine*-KOH buffer, pH 10, and stirred for 17 h at room temperature. Precipitated disulfide (*N.N'*-bis-capryloylcystamine) was extracted with ether (3 · 30 m*l*) and the aqueous phase was acidified by adding 100 m*l* moist Dowex-50. The suspension was placed on a Dowex-50 column (4×10 cm) and the acids were eluted with water. The residue obtained after evaporation of the effluent *in vacuo* and drying over phosphorus pentoxide was dissolved in 10 m*l* acetone and neutralized with *cyclohexylamine* to pH 8. Crystallization from water-acetone was induced by scratching with a glass rod to yield 2.0 g (73%) tricyclohexylammonium salt of α-III within three days. After recrystallization this became 1.8 g (66%), m. p. 173—174°C (decomp.). The salt is soluble in water and ethanol, insoluble in other common organic solvents.

$C_8H_{11}NO_8 \cdot 3\ C_6H_{13}N$ (546.7)

Calc. C 57.12 H 9.22 N 10,25 equiv. weight 182,2
Found. C 57.42 H 9.52 N 10.31 equiv. weight 177

Citric α-cyclohexylamide (α-V)

415 mg (1 mmole) *monopotassium salt* of I[8] was dissolved in 10 m*l* aqueous 1M *cyclohexylamine* and kept at 25°C for 15 h. The solution was then extracted with ether (3 · 5 m*l*) and passed through Dowex-50 (2×6 cm). The residue obtained on evaporation of the effluent *in vacuo* was crystallized from ethyl acetate/petroleum ether to yield 200 mg (73%) needles, m. p. 140°C (decomp.). After recrystallization this gave 150 mg, m. p. 145°C (decomp.). The amide, on paper electrophoretic analysis, was identical with α-V prepared

from the dioxolanone IV and could be completely separated from β-V (c. f. Scheme 4). The mixed m. p. with α-V, 145°C (decomp.), and β-V, 152—153°C (decomp.) (prepd. from the dioxolanone IV) were 145°C (decomp.) and 135°C (decomp.), respectively.

Influence of the solvent in the aminolysis of I

Each 1 m*l* 0.1M solution of the thioester I in 1M aqueous and 1M ethanolic cyclohexylamine was kept at 25°C for 15 h. To each was then added 1 m*l* of ethanol and of water, respectively, and the reaction mixtures were extracted with ether (3 · 2 m*l*). Samples of the aqueous phases were analyzed by paper electrophoresis and the intensity of the zones, labelled with methylred, was estimated visually:

Solvent used	Ratio of products found
Water	α-V:β-V ca. 19:1
Ethanol	α-V:β-V ca. 1:2

α-*Methylcitrate* (α-VII)

The suspension of 415 mg (1 mmole) *mono potassium salt of I*[8] in 25 m*l* 0.1M methanolic *sodium methylate* was refluxed for two minutes to transiently yield a solution. 10 g of air-dried Dowex-50 was added to the cooled reaction mixture and the resin was separated and washed twice with 20-m*l* portions of methanol. The acidic effluents were combined, evaporated to dryness *in vacuo*, and the residue was extracted with a mixture of 5 m*l* water and 10 m*l* ether. The aqueous phase was brought to dryness again and the residue was purified by precipitation from hot ethyl acetate with petroleum ether. After recrystallization (ethyl acetate/petr. ether) the yield was 83 mg (40%), m. p. 119—121°C.

$C_7H_{10}O_7$ (206.2)

Calc. C 40.78 H 4.89 equiv. weight 103.1
Found C 40.87 H 4.98 equiv. weight 104

PREPARATION OF CITRYL DERIVATIVES FROM THE DIOXOLANONE IV

β-*Citrylglycine* (β-III)

10 g (36 mmoles) *dioxolanone IV*[8] and 22 m*l* (220 mmoles) *glycine ethylester*[19] were dissolved in 400 m*l* *tert.*-butanol and refluxed for 45 min with exclusion of moisture. 200 m*l* water was then added and benzaldehyde was extracted with ether (3 · 100 m*l*). The aqueous solution, after concentration *in vacuo* to approx. 50 m*l*, was passed through Dowex-50 (2.5 · 25 cm) and the acidic effluent was brought to dryness *in vacuo*. The partially crystalline material obtained on drying the oily residue over phosphorus pentoxide *in vacuo* was dissolved in approx. 10 m*l* ethyl acetate to yield crystalline β-citrylglycine ethylester (β-VI). After recrystallization from acetone the yield was 5.6 g (56%) β-VI, m. p. 146—147°C. (Soluble in water, ethanol; insoluble in ethyl acetate, ether.)

$C_{10}H_{15}NO_8$ (227.2)

Calc. C 43.32 H 5.45 N 5.05 equiv. weight 138.6
Found C 43.33 H 5.56 N 5.06 equiv. weight 138

2.8 g (10 mmoles) β-*VI* was dissolved in 40 m*l* 1N *NaOH* at room temperature and 5 min later passed through Dowex-50 (2 · 10 m*l*). The residue obtained after evaporation of the effluent *in vacuo* was dissolved in 10 m*l* acetone and neutralized with *dibenzylethylenediamine*. Crystallization was induced by the addition of water until slight turbidity appeared; the yield was 5.6 g (92%) β-III, m. p. 125—130°C. After recrystallization from acetone/water the needles melted at 132—134°C.

$C_8H_{11}NO_8 \cdot 1.5\ C_{16}H_{20}N_2$ (609.7)

Calc. C 63.04 H 6.78 N 9.19 equiv. weight 203.2
Found C 62.76 H 7.06 N 8.98 equiv. weight 204

α-*Citrylglycine* (α-III)

The *mother liquor* (ethyl acetate) of the crystallisation *of* β-*VI* was evaporated *in vacuo* and the oily residue was redissolved in approx. 2 m*l* ethyl acetate to yield a further small crop of β-VI. The mother liquor was then diluted with 30 m*l* ethyl acetate and neutralized with *cyclohexylamine* to yield an oily, slowly crystallizing precipitate. After recrystallization from water/acetone it yielded 2.5 g (14%) dicyclohexylammonium salt of α-VI, m. p. 169—170°C (decomp.).

$C_{10}H_{15}NO_8 \cdot 2\ C_6H_{13}N$ (475.6)

Calc. C 55.56 H 8.69 N 8.84 equiv. weight 237.8
Found C 55.82 H 8.55 N 8.55 equiv. weight 241

α-VI on treatment with 1N KOH was hydrolyzed as easily as β-VI. The product of the hydrolysis of α-VI, on paper electrophoretic analysis, was identical with α-III, prepared from the thioester I.

Citric β-*cyclohexylamide* (β-V)

5.6 g (20 mmoles) *dioxolanone IV*[8] was thoroughly mixed with 50 m*l* (440 mmoles) *cyclohexylamine* and kept in a pressure vessel at 110°C for 24 h. Solution of the cooled, solid reaction mixture in 100 m*l* water resulted in the formation of two phases which were extracted with ether (3 · 50 m*l*). The aqueous phase was evaporated *in vacuo* and the residue was recrystallized from ethanol to give 4.7 g (50%) dicyclohexylammonium salt of β-V, m. p. 191—193°C (decomp.). 6.8 g (40 mmoles) of this salt was dissolved in water and the base was removed by treatment with Dowex-50. The crystalline residue obtained after evaporation of the effluent *in vacuo* was recrystallized from ethanol giving 2.2 g (56%) β-V, m. p. 152—153°C (decomp.).

$C_{12}H_{19}NO_6$ (273.3)

Calc. C 52.74 H 7.01 N 5.13 equiv. weight 136.6
Found C 52.91 H 7.18 N 5.13 equiv. weight 140

[19] G. HILLMANN, Z. Naturforsch. **1**, 683 [1946].

Citric α-cyclohexylamide (α-V)

The *mother liquor* (ethanol) of the crystallization of the dicyclohexylammonium salt of β-V was evaporated to dryness *in vacuo* and the residue was crystallized from water/acetone yielding 2.1 g (22%) dicyclohexylammonium salt of α-V, m. p. 172—174°C. The free acid was obtained from the salt on treatment with Dowex-50. After recrystallization from acetone/petr. ether: 0.9 g (82%) was obtained as needles, m. p. 144—145°C (decomp.).

$C_{12}H_{19}NO_6$ (273.3)

Calc.	C 52.74	H 7.01	N 5.13	equiv. weight 136.6
Found	C 52.85	H 6.93	N 5.14	equiv. weight 136

β-*Methylcitrate* (β-VII)

1 m*l* 2.5M methanolic *sodium methoxide* was added to solution of 280 mg (1 mmole) *dioxolanone IV*[8] in 4 m*l* methanol, and the reaction mixture was kept at room temperature for 5 min. 10 g air-dried Dowex-50 was then added, the resin was removed by suction filtration and washed twice with each 20-m*l* portions of methanol. Evaporation of the acidic filtrate *in vacuo* and recrystallization (ethyl acetate/petr. ether) of the residue yielded 200 mg (95%) β-VII, m. p. 164—166°C (decomp.). After recrystallization this had m. p. 167°C (decomp.).

$C_7H_{10}O_7$ (206.2)

Calc.	C 40.78	H 4.89		equiv. weight 103.1
Found	C 41.17	H 4.98		equiv. weight 100