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Enzymatic Methods for the Preparation of Acetyl-CoA and Analogs

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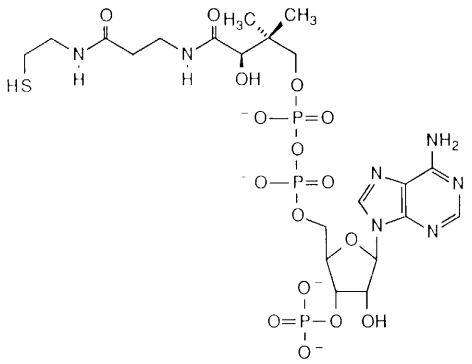
The enzymes phosphotransacetylase (EC 2.3.1.8) or carnitine acetyltransferase (EC 2.3.1.7) and their respective substrates (acetyl phosphate and L-acetylcarnitine) provide the basis for efficient recycling systems for acetyl-CoA. This paper also surveys syntheses and substrate activities of analogs of these two substances.

INTRODUCTION

Coenzyme A thioesters serve as substrates in a large variety of enzyme-catalyzed reactions (*1*), and acetyl-CoA is central to biological acetylation reactions. CoA thioesters play a pivotal role in cholesterol biosynthesis (3-hydroxymethylglutaryl-CoA) and fatty acid metabolism (malonyl-CoA). The complex structure of Coenzyme A **(1)** is still a challenge for synthesis (*2*). Synthetic analogs of CoA are more readily accessible than natural CoA and have proven useful as substitutes for it (*3*). The high cost of Coenzyme A ($80/mmol) limits its use as a stoichiometric reagent in bioorganic synthesis.



Our interest in the development of enzymology as an adjunct to organic synthesis led us to look for an *in situ* recycling system for acyl-CoA derivatives, initially focusing on acetyl-CoA, but also investigating the enzymatic synthesis of other CoA thioesters and their regeneration.

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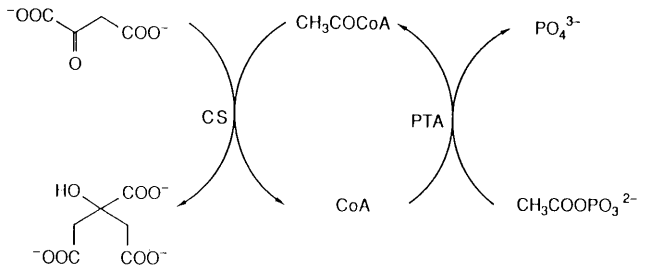
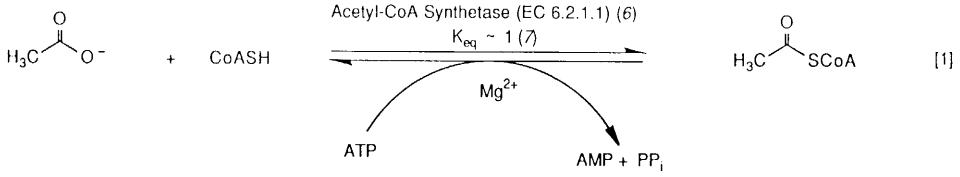


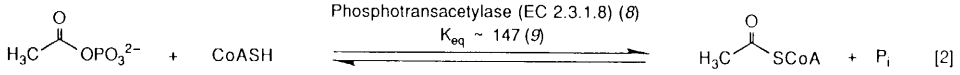
SCHEME 1. Citrate synthesis with recycling of acetyl-CoA, with acetyl transfer catalyzed by phosphotransacetylase (PTA) (CS, citrate synthase).

Two previous studies presented methods for recycling acetyl-CoA[2] for use in organic synthesis. Reactions with dextran-bound CoA using the system shown in Scheme 1 (where CoA and AcCoA were replaced with CoA-dextran and AcCoA-dextran, respectively) (*4*) produced citrate (4% in 5 h, 14 turnovers[3] of the CoA analog) but were impractical due to the poor affinity of the enzymes for the CoA analog. Walt and co-workers (*5*) showed that a system composed of acetate, ATP, and acetyl-CoA synthetase (EC 6.2.1.1) worked well for the regeneration of acetyl-CoA (Scheme 2). They reported a turnover number of 1000 for CoA. The disadvantages of this system were the high price of acetyl-CoA synthetase ($32.50/10 U) and the need for ATP (which was not recycled in their work).

RESULTS AND DISCUSSION

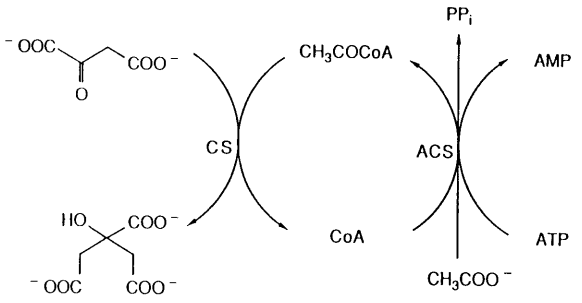
Recycling of acetyl-CoA. A number of enzyme-catalyzed reactions might, in principle, be used to transform CoA into acetyl-CoA (Eqs. [1]–[5]). Only two, however, seem to provide a practical basis for an efficient recycling system. For a practical system, the enzymes and substrates used should be commercially avail-



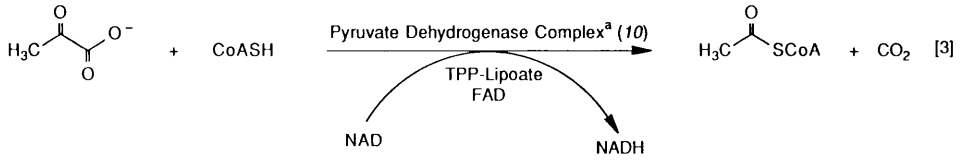


[2] N^6 of the adenosine residue of CoA was linked to BHP-activated dextran as CoA-N^6-$CH_2CONH(CH_2)_6NH$-dextran.

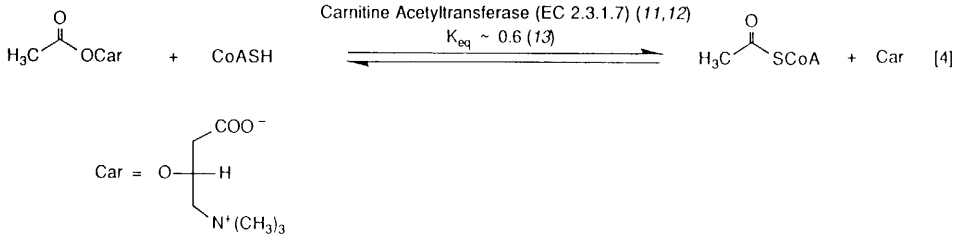
[3] Turnovers or turnover numbers reported for cofactors are defined as moles product/mole cofactor.

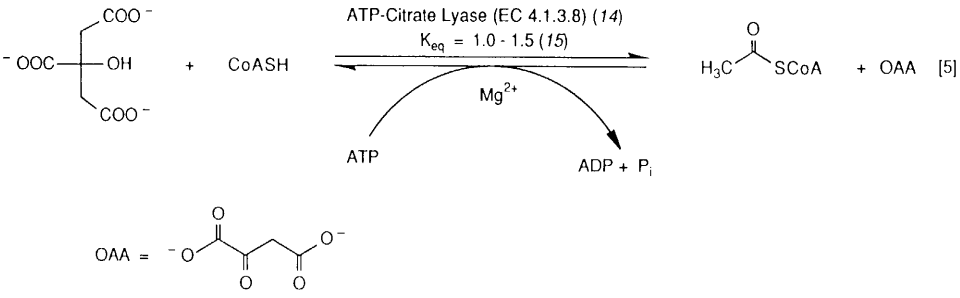


SCHEME 2. Citrate synthesis with recycling of acetyl-CoA, with acetyl transfer catalyzed by acetyl-CoA synthetase (ACS).

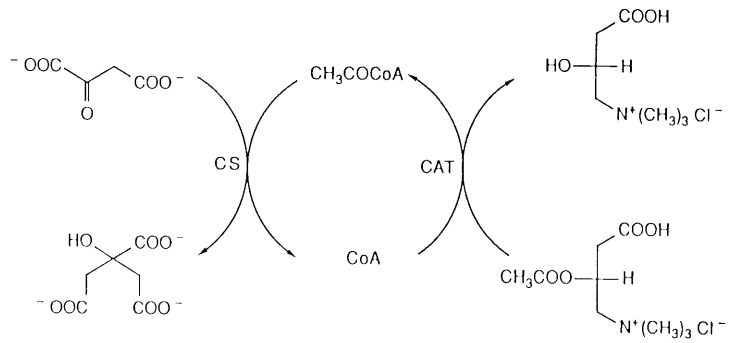


[a] consists of pyruvate dehydrogenase (EC 1.2.4.1), lipoate acetyltransferase (EC 2.3.1.12) and lipoamide dehydrogenase (EC 1.6.4.3)





able, inexpensive, and fairly stable. Reactions that themselves require a cofactor (Eqs. [1], [3], [5]) are not very attractive, because a secondary recycling system would be needed, even though acetate, pyruvate, and citrate are inexpensive



SCHEME 3. Citrate synthesis with recycling of acetyl-CoA, with acetyl transfer catalyzed by carnitine acetyltransferase (CAT).

commercially. The phosphotransacetylase-catalyzed acetyl transfer from acetyl phosphate to CoA (Eq. [2]) appeared to be the simplest method, but had the disadvantage that acetyl phosphate is intrinsically unstable in solution. The system based on carnitine acetyltransferase/L-acetylcarnitine (Eq. [4]) also seemed attractive, as L-acetylcarnitine is more stable than acetyl phosphate. Both acetyl phosphate (*16, 17*) and acetylcarnitine can be prepared easily (*18*).

The development of a method for *in situ* regeneration of acetyl-CoA required a coupled reaction for utilization of acetyl-CoA. We chose the first step of the tricarboxylic acid cycle as the acetyl-CoA consuming reaction. In this reaction, oxalacetate and acetyl-CoA form citrate in an aldol reaction catalyzed by citrate synthase (EC 4.1.3.7) (*19*). Although the synthesis of citrate is of little practical interest (except, perhaps, for isotopic labeling), citrate synthase is commercially available and has been extensively studied. The equilibrium constant for citrate formation is large ($K_{eq} = 8 \times 10^3$ M^{-1}),[4] and this reaction goes essentially to completion.

Our experiments with the systems acetyl phosphate/phosphotransacetylase (Scheme 1)[5] and acetylcarnitine[6]/carnitine acetyltransferase (Scheme 3) for the regeneration of acetyl-CoA both gave satisfactory results. The yields of citrate were excellent (>90%, determined by enzymatic assay) in preparative (1 to 15 mmol) scale reactions using phosphotransacetylase with either high (190 mM) or low (35 mM) acetyl phosphate concentrations. Measured turnover numbers for CoA in two experiments were 590 and 620. Preliminary assay-scale experiments were carried out to arrive at the reaction conditions described (see experimental section). These experiments suggested that a ratio of 2 U : 1 U of phosphotransacetylase : citrate synthase was most effective in assuring high reaction rates. In

[4] K_{eq} was defined as ([citrate][P_i])/[Acetyl-CoA][Oxalacetate][H_2O].

[5] This recycling system has been studied by Walt and co-workers (*5*).

[6] We used the racemic mixture of the acylcarnitines in our experiments. Carnitine acetyltransferase was from pigeon breast muscle. Differences in the substrate specificity of the enzyme from pigeon breast muscle and the enzyme from rat or pig liver have been noted (*11*).

addition, these experiments indicated that 50 U of PTA[7] per mmol of oxalacetate was sufficient to give complete reaction overnight (ca. 17 h). They also demonstrated that a CoA concentration beyond 0.1 mM was not effective in further increasing reaction rates and that decreasing the concentration (in order to increase the turnover number for CoA) greatly slowed the reaction. In this system, fast reaction was desirable, as acetyl phosphate is hydrolytically unstable, and decarboxylation of oxalacetate occurs during the reaction and competes with citrate formation if the reaction rate is too low. The reactions were carried out in Tris buffer solution at pH 7.5, and, thus, there were no problems with changes in pH due to formation of the product. However, the isolation of citrate from the buffered solution was difficult. Separation of citrate from the lyophilized reaction mixture by extraction with ethyl acetate gave an unsatisfactory yield of 12%. To alleviate this problem in the system utilizing acetylcarnitine/CAT for recycling, reactions in unbuffered media with pH control were used. Separation of the citrate from the unbuffered reaction mixture by ion exchange chromatography gave a yield of 87%.

The recycling of acetyl-CoA with acetylcarnitine/carnitine acetyltransferase also gave yields of citrate higher than 90% as determined by enzymatic assay and a turnover number of 690 for CoA. From a reaction in unbuffered media, we easily separated citrate from acetylcarnitine/carnitine by ion exchange chromatography on Dowex 50W. After esterification with diazomethane and chromatography on a short column, trimethyl citrate was isolated in 87% yield. Acetylcarnitine/carnitine was eluted from the ion exchange column with hydrochloric acid.

With the carnitine acetyltransferase-catalyzed regeneration of acetyl-CoA, the reaction time was slightly longer (20 h compared to 17 h) than with the phosphotransacetylase-catalyzed regeneration. A ratio of about 2:1 for carnitine acetyltransferase to citrate synthase gave better results than a 1:1 ratio. While carnitine acetyltransferase is more expensive than PTA ($10/100 U for CAT vs $3/100 U for PTA), it appeared to be more stable than phosphotransacetylase. At the end of the reaction in buffered media, we determined remaining activities of ~70% for carnitine acetyltransferase but from 0 to 60% for phosphotransacetylase. We did not establish the origin of this large variability in remaining activity.

Preparation of substrate analogs and their activities with phosphotransacetylase and carnitine acetyltransferase. We wished to know whether the enzyme systems that transform CoA into acetyl-CoA would also be useful in the preparation of acyl derivatives of CoA other than acetyl. In particular, we wanted to know whether phosphotransacetylase and carnitine acetyltransferase accept substrates other than acetyl phosphate and L-acetylcarnitine and whether these materials are readily available. Our interest in acyl analogs of acetyl-CoA was not, of course, restricted to synthesis of analogs of citric acid. A number of other enzyme-catalyzed reactions that require CoA thioesters as substrates are also attractive. For instance, α,β-unsaturated acyl-CoA derivatives are substrates for the enoyl-CoA hydratase (crotonase, EC 4.2.1.17) that catalyzes the stereospecific

[7] Abbreviations used: PTA, phosphotransacetylase; CAT, carnitine acetyltransferase.

formation of β-hydroxy acyl-CoA esters (*20*). Representative compounds of interest to us as analogs of acetyl phosphate and L-acetyl carnitine include **1–15:**

CH_3COX	**(1)**	$ClCH_2COX$	**(9)**
CH_3CH_2COX	**(2)**	$CH_2{=}CHCOX$	**(10)**
$CH_3(CH_2)_2COX$	**(3)**	$CH_2{=}C(CH_3)COX$	**(11)**
$CH_3(CH_2)_3COX$	**(4)**	$CH_3CH{=}CHCOX$	**(12)**
$NCCH_2COX$	**(5)**	$CH_3CH_2CH{=}CHCOX$	**(13)**
$PhCONHCH_2COX$	**(6)**	$AcOCH_2COX$	**(14)**
$CH_3CONHCH_2COX$	**(7)**	$MeOCH_2COX$	**(15)**
N_3CH_2COX	**(8)**		

$X = PO_4^{2-}$ **a** $X = O{-}CH(CH_2COOH)(CH_2N^+(CH_3)_3Cl^-)$ **b**

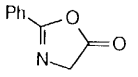
Analogs of acetyl phosphate. Acyl phosphates are usually prepared by reaction of an activated acid derivative, such as an acid anhydride, acyl ethyl carbonate, oxazolone, or acyl imidazole with orthophosphate in buffered aqueous solution at 0°C (Eqs. [6]–[9]) (*16, 17, 21*). With water-immiscible acid derivatives the application of a phase transfer catalyst was useful (*17*). Following this general procedure, we obtained propionyl (**2a,** (*17*)), butyryl **(3a)**, and valeryl phosphate **(4a)** as their dilithium salts in 79, 79, and 84% yields. The acyl phosphates were identified by

$$(CH_3(CH_2)_nCO)_2O \xrightarrow[2.\ LiOH]{1.\ K_2HPO_4,\ pyridine} CH_3(CH_2)_nCOPO_4^{2-} \quad [6]$$

n = 1 **2a** 79 % (isolated)
n = 2 **3a** 79 % (isolated)
n = 3 **4a** 84 % (isolated)

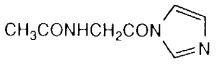
$$(CH_2{=}CHCO)_2O \xrightarrow[(CH_3)_3(C_{16}H_{33})N^+Br^-]{K_2HPO_4} CH_2{=}CHCOPO_4^{2-} \quad [7]$$

10a 43 %



$$\xrightarrow{Na_2HPO_4,\ pyridine} PhCONHCH_2COPO_4^{2-} \quad [8]$$

6a 30 %



$$\xrightarrow[(CH_3)_3(C_{16}H_{33})N^+Br^-]{K_2HPO_4,\ CH_3CN\text{-}H_2O} CH_3CONHCH_2COPO_4^{2-} \quad [9]$$

7a 41 %

$$H_2NCH_2COPO_2(OCH_2Ph)_2 \xrightarrow{H_2,\ Pd(C)} H_2NCH_2COPO_4^{2-} \quad [10]$$

16 17

NMR spectroscopy, since we did not obtain analytical samples. Acryloyl phosphate **(10a)** was generated in 43% yield as determined by ^{31}P NMR spectroscopy, but attempts to isolate this species by precipitation gave low yields. The *N*-substituted glycyl phosphates **6a** and **7a** were prepared from 5-phenyloxazolone and *N*-acetylglycyl imidazole in 30 and 41% yield. They were less stable than the simple acyl phosphates. The *N*-benzoyl glycyl phosphate **(6a)** hydrolyzed to the extent of 80% in 24 h (pH 7, r.t.). Synthesis of chloroacetyl **(9a),** methoxyacetyl **(15a),** acetoxyacetyl **(14a),** cyanoacetyl **(5a),** or azidoacetyl phosphate **(8a)** from their anhydrides or acyl ethyl carbonates failed, probably because these compounds are unstable in aqueous solution. We also explored another method that has been reported in the literature for the preparation of the very unstable glycyl phosphate (**17,** Eq. [10]) (*22*). This substance was prepared as the protected dibenzyl glycyl phosphate **(16)** and deprotected by hydrogenation. Our attempts to synthesize heteroatom-substituted acyl phosphates such as methoxyacetyl **(15a),** acetoxyacetyl **(14a),** cyanoacetyl **(5a),** or azidoacetyl phosphate **(8a)** using this method were unsuccessful. Considering that water-unstable substrates would not be of value for an enzyme reaction run in aqueous solution, no further efforts were made to prepare these phosphates. We concluded from these efforts that only simple alkyl carboxyl phosphates were easily obtained and sufficiently stable to be useful for our purposes.

The substrate activity of the acyl phosphates was investigated by incubating the enzyme with the substrate analog and measuring the appearance of acyl-CoA by uv spectrophotometry at 233 nm, an assay that has been used previously (*23*). Based on the assumption that the molar extinction coefficients of saturated acyl-CoA derivatives do not differ greatly, Michaelis–Menten constants (K_m) and relative reaction rates (V_{rel}) were determined based on the value for acetyl-CoA.

Kinetic studies by Satchell (*23, 24*) had already indicated a high specificity for the phosphotransacetylase from *Clostridium kluyveri*. His results are included with our results for comparison in Table 1. With increasing bulk of the substituents on the carboxyl phosphate, the rate of acyl transfer to CoA dropped rapidly. While propionyl phosphate **(2a)** was still accepted at a fairly high rate, butyryl **(3a)** and valeryl phosphate **(4a)** reacted with 2 and 0.05% of the rate for acetyl phosphate **(1a)**. The glycyl derivatives **6a** and **7a** and benzoyl phosphate **(20)** did not appear to react. Acryloyl phosphate **(10a)** was accepted as substrate, but at a rate much lower than that for propionyl phosphate **(2a),** the saturated analog.

Analogs of acetylcarnitine. Derivatives of acetylcarnitine promised to be better starting materials for the enzymatic synthesis of CoA thioesters than acyl phosphates. They would be more stable toward hydrolysis and possibly easier to prepare. Judging from the literature that was available concerning carnitine acetyltransferase (EC 2.3.1.7) (*11, 26, 27*), this enzyme appeared to be less specific than phosphotransacetylase. The acylcarnitines were prepared by reaction of carnitine with acid chlorides in acidic solution (*28–31*). Carnitine was heated to 45 or

TABLE I

Substrates for Phosphotransacetylase[a]

Substrate		K_m (mM)	V_{rel} (%)	Ref.
$CH_3COPO_4^{2-}$	**(1a)**	0.78	100	(*24*)
$CH_3CH_2COPO_4^{2-}$	**(2a)**	0.66	93	
		0.30	47	(*24*)
$CH_3(CH_2)_2COPO_4^{2-}$	**(3a)**	1.1–2.0	2	
$CH_3(CH_2)_3COPO_4^{2-}$	**(4a)**	3.4	0.05	
$(CH_3)_2CHCOPO_4^{2-}$	**(18)**	1.1	0.4	(*24*)
$ClCH_2COPO_4^{2-}$	**(9a)**	1.0	17	(*24*)
$FCH_2COPO_4^{2-}$	**(19)**	—	100	(*25*)
$PhCOPO_4^{2-}$	**(20)**		<0.05[b]	(*23*)
$CH_2{=}CHCOPO_4^{2-}$	**(10a)**	0.6	7[c,d]	
$PhCONHCH_2COPO_4^{2-}$	**(6a)**		<0.05	
$CH_3CONHCH_2COPO_4^{2-}$	**(7a)**		<0.05	

[a] The phosphotransacetylase from *Clostridium kluyveri* was used in all experiments unless indicated otherwise.

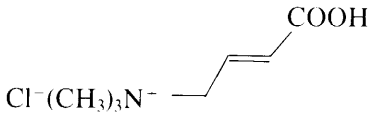
[b] Reactions slower than 0.05% would not have been detected. We were not able to detect any reaction with these compounds at the highest concentrations used (3.8 mM).

[c] Phosphotransacetylase from *Bacillus stearothermophilus*.

[d] This rate value was calculated on the basis of the molar extinction coefficient of acetyl-CoA.

80°C in a mixture of an excess of carboxylic acid and an equimolar quantity of analogous acid chloride or, with better results, of thionyl chloride. The mixture was preheated for several hours before the carnitine was added and may also contain carboxylic acid anhydride. Trifluoroacetic acid was used as solvent for those systems that did not dissolve carnitine. After completion of the reaction, the solvent was removed, and the acylcarnitine was crystallized from dry methanol/acetone/ether mixtures.

These rigorous methods are necessitated by the insolubility of carnitine in most organic solvents, and, perhaps, by deactivation of the β-hydroxyl group by hydrogen bonding. The β-acyloxy group of the products is a very good leaving group and is easily eliminated. Several of our target molecules were formed only slowly and even then were accompanied by the elimination product 4-trimethylammonium butyric acid **(21)**:



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We synthesized DL-propionyl- **(2b)** and DL-butyrylcarnitine **(3b)** in good yields (77 and 87%) as reported (*30*). The α,β-unsaturated DL-crotonyl- **(12b)** and DL-2-pentenoylcarnitine derivatives **(13b)** were isolated in 70 and 39% yield after crys-

tallization, although the crude reaction product showed 100% conversion by ^1H NMR. DL-Methacryloylcarnitine **(11b)** was isolated in 29% yield by chromatography. Methoxyacetyl and acetoxyacetyl chloride reacted only slowly and with formation of elimination product **21.** Some acid chlorides (especially cyanoacetyl chloride) seemed to be unstable under the conditions used.

To determine rates of reaction for the various substrate analogs with carnitine acetyltransferase, a reliable nonspecific assay was needed. The usual coupled assay (Assay A; Eqs. [11]–[13]) that measures the formation of NADH by uv spectrophotometry at 340 nm is specific for L-acetylcarnitine (*13*, *32*) and could not be used. The direct method, measuring the formation of acetyl-CoA at 233 nm ((*33*), Assay B; Eq. [11]), has the disadvantage of a large background absorption (from CoA), which limits the substrate concentration of CoA. Since the equilibrium constant for acetyl-CoA formation is, however, unfavorable ($K = 0.67$) and the K_m for L-acetylcarnitine is high ($K_m = 350\ \mu m$), it would be advantageous to have a large excess of CoA over acylcarnitine. One can thus only use small concentrations of acylcarnitine, which means that the time interval over which the reaction rate can be assumed to be a linear function of the acylcarnitine concentration (and the CoA concentration can be assumed to be constant) is very short.

Assay A:

L-acetylcarnitine + CoASH ⟶ L-carnitine + acetyl-CoA [11]

acetyl-CoA + oxalacetate ⟶ citrate + CoASH [12]

malate + NAD ⟶ oxalacetate + *NADH* [13]

Assay B:

L-acetylcarnitine + CoASH ⟶ L-carnitine + *acetyl-CoA* [11]

Assay C:

L-acetylcarnitine + CoASH ⟶ L-carnitine + acetyl-CoA [11]

CoASH + EII-S-S-EII ⟶ CoA-S-S-EII + $^-$*S-EII* [14]

An assay for CoA based on reaction with Ellman's reagent (Assay C) has been employed successfully for measuring reaction rates of CoA thioesters with carnitine—that is, the inverse of the reaction in which we are interested (Eqs. [11] and [14]; (*33*)). We used it successfully to assay for the amount of unreacted CoA. This method has the advantage that there are no limits to concentrations of reac-

TABLE 2

Substrates for Carnitine Acetyltransferase

Substrate		Assay[a]	K_m (mM)	V_{rel} (%)
DL-Acetylcarnitine	**(1b)**	B	0.70	108
		C	0.82	100
DL-Propionylcarnitine	**(2b)**	B	2.0	78(77)[b]
DL-Butyrylcarnitine	**(3b)**	B	0.72 (0.46)[b]	50(41)[b]
DL-Crotonylcarnitine	**(12b)**	C	6.0	5
DL-Pentenoylcarnitine	**(13b)**	C	14.0	2
DL-Methacryloylcarnitine	**(11b)**	C	—	Very slow

[a] B is a direct assay for acyl-CoA thioesters based on Eq. [11]; C is an assay based on estimation of unreacted CoASH (Eqs. [11] and [14]).

[b] The values in parentheses were determined by Chase for the L-acylcarnitines (*26*).

tants or to reaction conditions. The assay does, however, require taking aliquots (and is thus not as convenient as a continuous assay) and is subject to interference from any species capable of reducing Ellman's reagent.

Both assays (B and C) were used to determine the substrate activity of acetylcarnitine analogs. Table 2 summarizes the results. The data determined for acetylcarnitine by both assays are comparable. For L-acetyl- **(1b),** L-propionyl- **(2b),** and L-butyrylcarnitine **(3b),** literature references were available (*26*). While the relative reaction rates were similar, the K_m for the pure enantiomer was much lower. This result is not surprising, because the D-enantiomer of acylated or free carnitine is an inhibitor of carnitine acetyltransferase (*12*).

Although the substrate specificity of carnitine acetyltransferase is broader than that of phosphotransacetylase, it is also limited. The synthetic potential of this system is further limited by the difficulty in separating many acylcarnitines of possible interest, especially those having heteroatom-substituted acyl groups.[8]

CONCLUSION

This paper demonstrates an application of the enzymes phosphotransacetylase and carnitine acetyltransferase, and their substrates acetyl phosphate and acetylcarnitine, for the recycling of acetyl-CoA. The use of acetylcarnitine/carnitine acetyltransferase has the advantage that enzyme and substrate are more stable than phosphotransacetylase and acetyl phosphate.

Both phosphotransacetylase and carnitine acetyltransferase are of limited value in preparing acyl-CoA analogs. Both the narrow substrate specificity of phosphotransacetylase (Table 1) and the difficulty in synthesizing the acyl phosphates limit

[8] Chase has reported that chloro- and bromoacetylcarnitine, both of which can be prepared by the method described, are irreversible inhibitors of carnitine acetyltransferase (*34, 35*).

the number of substrate analogs that can be employed in this system. Carnitine acetyltransferase accepts a variety of substrates with low activity (Table 2, (*26, 29*)), but currently available syntheses of acylcarnitines are also of limited generality.[9]

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