The third hemoprotein isolated in the hydroxylapatite chromatography (cytochrome *b*-562) could better be purified from the soluble fraction obtained from the sonicate of intact cells by DEAE-cellulose column chromatography followed by ammonium sulfate fractionation and hydroxylapatite column chromatography. As shown in Fig. 2, the pigment thus purified showed absorption bands at 562 mμ (α), 532 mμ (β) and 427 mμ (Soret) in the reduced form and at 420 mμ (Soret) in the oxidized form. These spectra resembled those reported for "cytochrome *b*" of *Bacterium anitratum*[11]. The prosthetic group of this cytochrome was identified as protoheme from its pyridine hemochromogen spectrum. This pigment was definitely different from cytochrome b_1 (ref. 7) not only in its spectral properties but also in its unusual non-autoxidizability. Cyanide and CO again showed no affinity to this cytochrome.

The three soluble cytochromes described in this paper seemed to be present also in *E. coli* B, *E. coli* var communior and *Serratia marcescens* grown under similar conditions.

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Citryl-CoA and the citrate condensing enzyme

Previous work from this laboratory[1,2] has shown that a preparation of synthetic citryl-CoA which was cleaved by the citrate cleavage enzyme to acetyl-CoA and oxaloacetate, inhibited the citrate condensing enzyme. It is the purpose of this paper to extend our earlier observations and present evidence which indicates that the inhibition by citryl-CoA is competitive for both acetyl-CoA and oxaloacetate. It is further shown that the slow cleavage of citryl-CoA catalyzed by condensing enzyme, which was first reported by EGGERER AND REMBERGER[3], occurs at the same site as acetyl-CoA and oxaloacetate condensation.

Abbreviation: DTNB, 5,5′-dithio-*bis*-2-nitrobenzoic acid.

ELLMAN[4] has reported that when DTNB reacts with free sulfhydryl groups 2-nitrobenzoate mercaptide is formed which has a strong absorption maximum at 412 mμ. If DTNB is added to a reaction mixture containing condensing enzyme and its substrates, the rate of appearance of CoASH can be assayed spectrophotometrically by observing the rate of change of absorption at 412 mμ[5,6].

Since the conversion of DTNB to the mercaptide has an isosbestic point at 355 mμ, it is possible to follow the utilization of oxaloacetate generated from malate, NAD^+ and malate dehydrogenase (EC 1.1.1.37) by the appearance of NADH at 355 mμ and simultaneously the appearance of CoASH by its reaction with DTNB at 412 mμ. Table I shows the change in absorbancy occurring simultaneously at

TABLE I

INHIBITION OF CONDENSING ENZYME BY CITRYL-CoA

Cuvettes contained 100 μmoles Tris–HCl (pH 8.1), 100 μmoles potassium malate, 0.4 μmole NAD^+, 0.1 unit malate dehydrogenase, 0.12 μmole acetyl-CoA, 0.03 μmole DTNB, and 0.011 unit condensing enzyme in a total volume of 1 ml. Citryl-CoA, prepared by reacting the mixed anhydride of citric acid with CoASH[7], was purified using the CoA chromatography method of MOFFATT AND KHORANA[8]. Citryl-CoA was assayed using the citrate cleavage enzyme[7,9].

Contents of cuvette	*ΔA per min*	
	412 mμ	*355 mμ*
Complete system	0.155	0.034
+ 6 mμmoles citryl-CoA	0.015	0.000

412 mμ and 355 mμ in a citrate condensing enzyme assay system. Upon the addition of citryl-CoA an inhibition of oxaloacetate utilization and CoASH liberation occurs. These results are in agreement with our early work[1,2] and with the data reported by EGGERER AND REMBERGER[3]. The data shown in Table II confirm the observations of these authors that in the presence of large quantities of condensing enzyme a hydrolysis of citryl-CoA occurs. The rate of this reaction is about one-tenth the rate of the citrate condensing reaction.

As pointed out by EGGERER AND REMBERGER[3] one would expect the v_{max} for hydrolysis of citryl-CoA to be at least as high as that for the condensing reaction. Since the chemical synthesis of citryl-CoA leads to the formation of diastereomers

TABLE II

HYDROLYSIS OF CITRYL-CoA BY CONDENSING ENZYME

Cuvettes contained 200 μmoles of Tris–HCl (pH 8.1), 0.1 μmole DTNB and water to make 1 ml.

Enzyme (units)	*Citryl-CoA (mμmoles)*	*ΔA per min (412 mμ)*
0.35	12	0.560
0.35	3.0	0.095
0.35	1.2	0.030
0.035	3.0	0.033
0.035	2.4	0.028
0.035	1.8	0.017

and since the condensing enzyme is highly stereospecific, these results could be explained if the unnatural isomer is an inhibitor for the reaction.

The results in Table III show that inhibition of condensing enzyme reaction by synthetic citryl-CoA can be overcome by increasing either the acetyl-CoA or oxaloacetate concentration. The synthetic citryl-CoA preparation contains, therefore, not only a substrate but a potent competitive inhibitor for both substrates.

TABLE III

EFFECT OF ACETYL-CoA AND OXALOACETATE ON CITRYL-CoA INHIBITION

Cuvettes contained in addition to the components listed 200 μmoles Tris–HCl (pH 8.1), 0.1 μmole DTNB and 0.007 unit condensing enzyme in a total volume of 1 ml.

Acetyl-CoA (μmoles)	*Oxaloacetate (μmoles)*	*Citryl-CoA (mμmoles)*	*Rate (ΔA at 412 mμ per min)*	*Inhibition (%)*
0.06	0.1	0.0	0.130	—
0.06	0.1	6.0	0.037	72
0.06	1.0	0.0	0.175	—
0.06	1.0	6.0	0.053	70
0.3	0.1	0.0	0.280	—
0.3	0.1	6.0	0.145	48
0.3	1.0	0.0	0.340	—
0.3	1.0	6.0	0.335	1.5

By allowing large amounts of citrate condensing enzyme to cleave consecutive aliquots of citryl-CoA the natural diastereomer is hydrolyzed and one can accumulate the "unnatural" isomer. The results of such an experiment shown in Table IV indicate the accumulation of an inhibitor, since each successive addition of citryl-CoA is hydrolyzed more slowly than the preceding one.

Further evidence which shows that the site that catalyzes the condensation reaction is probably the same as the site which catalyzes the hydrolysis of citryl-CoA. The crystalline condensing enzymes from moth muscle and pigeon breast muscle[5,6] also catalyzed the hydrolysis of citryl-CoA. The ratios of the rate of condensing enzyme reaction to the rate of citryl-CoA hydrolysis for the three enzymes are very similar. Condensing enzyme is inactivated under mild acetylation

TABLE IV

CUMULATIVE INHIBITORY EFFECT OF CITRYL-CoA ON ITS HYDROLYSIS

Cuvettes contained 0.175 unit of condensing enzyme, 200 μmoles Tris–HCl and 0.1 μmole DTNB in a total volume of 1 ml. In each case the reaction proceeded to completion before the next addition of substrate.

Citryl-CoA	*Time for 0.050 ΔA at 412 mμ (sec)*
First addition, 6.0 mμmoles	35
Second addition, 6.0 mμmoles	53
Third addition, 6.0 mμmoles	85
Fourth addition, 6.0 mμmoles	155

conditions. When 3–4 acetyl groups are introduced per mole of enzyme the condensation activity is reduced 70%. Inactivation of condensing enzyme by acetylation results in a parallel loss of condensing enzyme activity against acetyl-CoA and oxaloacetate and against citryl-CoA (Table V).

In a recent report from this laboratory we have shown that citryl-CoA is cleaved by the citrate cleavage enzyme[9]. The rate of cleavage of citryl-CoA to acetyl-CoA and oxaloacetate was slightly greater than the rate of the overall reaction from citrate, ATP, and CoA. In the cleavage of citryl-CoA by cleavage enzyme no evidence was obtained for an inhibition by the unnatural diastereomer.

The results indicate therefore that citryl-CoA can serve as a substrate for both

TABLE V

LOSS OF CONDENSING ENZYME ACTIVITY AND CITRYL-CoA HYDROLYSIS ACTIVITY BY ACETYLATION OF ENZYME

Acetic anhydride addition (µl)*	*Condensing enzyme (% activity)*	*Citryl-CoA hydrolysis (% activity)*
0	100	100
30	35	27
40	21	13
50	9	2.0
60	1.5	1.5
70	0.0	0.1

* Diluted 1:25 in tetrahydrofuran.

the citrate cleavage and citrate condensing enzymes. There is no direct evidence, however, that indicates it is an intermediate in the catalyzed reactions.

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