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DETERMINATION OF ACYL-CoA CONCENTRATIONS USING
PANCREATIC LIPASE

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SUMMARY

A rapid and sensitive assay for acyl-CoA thiol esters has been devised using pancreatic lipase as a hydrolytic agent in the presence of dithionitrobenzoic acid to detect the liberated CoA. Acyl thiol esters containing 12–22 carbon atoms and 0–6 double bonds are measurable in this system whereas acetyl-CoA and malonyl-CoA were not cleaved.

INTRODUCTION

Enzymes catalysing the hydrolysis of a variety of acyl thiol esters have been reported¹. Several have been implicated in exergonic processes which help to drive the synthesis of other compounds^{2–4}, but the biological function of most remains obscure^{5,6}.

During the course of some experiments in which pancreatic lipase⁷ was added to acyltransferase reaction mixtures, we noted an acyl-CoA hydrolase activity associated with the lipase preparation. The enzyme, in the presence of acyl-CoA and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), seems able to liberate free CoA as evidenced by an increase in the absorbance at 413 nm (ref. 8). We thought that the hydrolytic activity could be used as the basis for an assay to routinely measure acyl-CoA concentrations in various solutions. The present communication describes the conditions and characteristics of this assay system.

METHODS

Reagents

Pancreatic lipase, 30 units/mg, was purchased from Worthington. Tris was a product of Sigma Chemical Company and DTNB was obtained from Aldrich Chemical Company. "Pancreatin" was purchased from Viobin Corporation.

Abbreviation: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid)

Enzyme solutions

Enzyme solutions (20 mg/ml) were prepared in 0.1 M Tris-HCl buffer (pH 7.4) and stored at -10° . DTNB was dissolved in deionized water, (titrated to pH 4 with NaOH) and stored at 4° . Acyl-CoA derivatives were prepared from the corresponding acid chlorides by the method of SEUBERT⁹ and stored at -10° . Santoquin, a gift of Monsanto Chem. Co., was added to unsaturated acyl-CoA solutions as an antioxidant. Phosphate analyses were performed on acid hydrolysates of acyl-CoA solutions as described by EIBL AND LANDS¹⁰.

Spectrophotometric measurements

These measurements were performed using a Gilford Model 2000 recording spectrophotometer at ambient temperature. Cuvettes of 1 cm pathlength were used throughout. The extinction coefficient for free TNB was taken to be $13\,600\text{ M}^{-1}\text{ cm}^{-1}$ at 413 nm (ref. 8).

Standard assay system

The standard assay contained 90 μ moles of Tris-HCl buffer (pH 7.4), 10 μ moles of DTNB and 0.1–1.0 mg pancreatic lipase in a total volume of 1.0 ml.

RESULTS

Properties of the assay system

Fig. 1 shows the spectrophotometric traces obtained when various amounts of acyl-CoA were added to the standard assay system. The absorbance at 413 nm increased rapidly after acyl-CoA was added (Point A) and the absorbance at the end-point was proportional to the amount of acyl-CoA added. Subsequent additions of acyl-CoA (Point B) led to further proportional increases in the absorbance, indicating that the hydrolase activity remained functional during the course of the assay. The very slow increase in absorbance seen in the case of Cuvette 1 might have been due to the reaction of exposed protein sulphhydryl groups with DTNB as the protein slowly unfolds. Addition of sodium deoxycholate (0.05% (w/v) final concentration) did not increase the rate seen in Cuvette 1. No increase in absorbance was detected when the enzyme was omitted or when enzyme which had been heated at 100° for 15 min was added. "Pancreatin", an acetone powder of whole pancreas, was also active in the assay system, although its specific activity was only about 10% that of the partially purified commercial lipase toward either stearoyl-CoA or eicosatrienoyl-CoA. Addition of free CoA, β -mercaptoethanol or dithiothreitol to the assay system gave instantaneous absorbance increases in the presence or absence of the enzyme. The rate of release of CoA from the thiol ester was a linear function of the amount of lipase present from 0 to 100 μ g when either 91 μ M lauroyl-CoA or 81 μ M arachidonoyl-CoA were used as substrate.

Fig. 2 shows the total amount of CoA released *versus* the volume of acyl-CoA solution added. It can be seen that the response was linear up to 80 nmoles. The deviation from linearity above this point was also seen when free CoA was added. Thus it cannot be related to the nature of the enzyme interaction with the acyl groups and may reflect a limiting absorbance in the instrument used. Only those experiments in which the change in absorbance was less than 1.1 were considered reliable for quan-

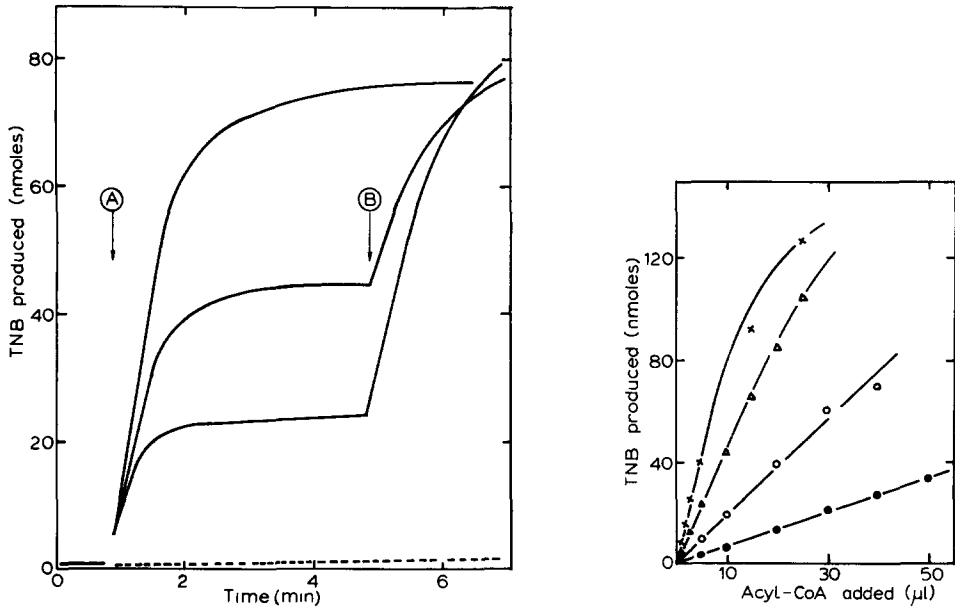


Fig 1 Spectrophotometric traces seen when 0, 5, 10 and 15 μ l of a solution of eicosanoyl-CoA are added to Cuvettes 1, 2, 3 and 4 respectively (Point A) containing the standard assay components described under METHODS and 1.0 mg of pancreatic lipase. At Point B, 15 and 10 μ l of eicosanoyl-CoA are added to Cuvettes 2 and 3, respectively. Experiments were performed on the Gilford Model 2000 recording spectrophotometer at 413 nm with full scale absorbance equal to 1.36.

Fig 2 The final amount of CoA released is plotted as a function of the volumes of various CoA solutions added. The values were obtained from experiments such as those shown in Fig 1 using lauroyl-CoA (●—●), linoleoyl-CoA (○—○), free CoA (Δ — Δ) and eicosatrienoyl-CoA (x—x).

titative assays and used in subsequent calculations. The concentration of the acyl-CoA in the solutions was then calculated from the average of several determinations at differing levels such as those shown in Figs 1 and 2.

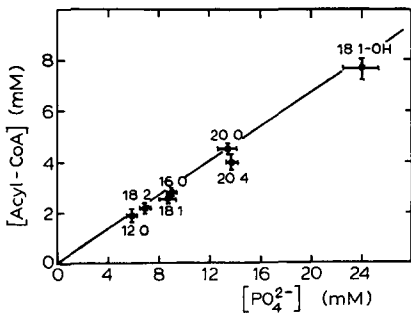


Fig 3 A comparison of the results obtained using the lipase assay method with those obtained by phosphate analysis of the same solutions of acyl-CoA. The horizontal and vertical bars indicate the observed standard deviations for both assays. Phosphate analyses¹⁰ were performed in triplicate while the lipase assays represent averages of 3-9 determinations. The line represents the expected molar ratio of phosphate to thiol ester groups. The nature of the acyl group is indicated for each point.

A comparison of the results obtained from the lipase assay method with those obtained by a series of phosphate analyses of the same solutions is presented in Fig. 3. The expected molar ratio of 3:1 for phosphate to acyl-thiol ester was obtained in each case within experimental error. Thus the lipase assay system gives valid and useful results with a variety of long chain acyl-CoA substrates.

Kinetic properties of the lipase system

All of the long chain acyl-CoA's tested (12-22 carbon atoms, 0-6 double bonds) served as substrates for the pancreatic lipase "hydrolase," activity and hence could be assayed by this method. However, it was noticed that the rate of CoA release is dependent not only on the concentration of acyl-CoA but also upon the nature of the acyl moiety. We therefore decided to investigate more closely the effect of the acyl group on the reaction rate. Accordingly, the initial velocity of CoA release was determined at 5 or 6 concentrations of each acyl-CoA. The K_m and V values were estimated from plots of reciprocal initial velocity *versus* reciprocal substrate concentration. The relationship was linear in each case and no inhibition by high concentrations of acyl-CoA was noted. Such inhibition has been seen with several acyl-CoA phospholipid acyltransferases^{11,12} and with rat-liver acyl-CoA hydrolase¹². The resulting kinetic parameters for various acyl-CoA's are given in Table I. Some variation of the V values was noticed for preparations which had been stored for various times, although the K_m values were not dependent on the length of storage. The results presented in Table I were all obtained with freshly prepared solutions of the lipase. It should be noted that acetyl-CoA and malonyl-CoA were found to be inactive with this enzyme preparation. It should also be pointed out that a plot of $\log K_m$ *versus* chain length for the series of fully saturated acyl-CoA's is linear, indicating a possible thermodynamic relationship between chain length and apparent effectiveness as a substrate.

TABLE I

KINETIC PARAMETERS FOR VARIOUS ACYL-CoA'S

These parameters were obtained as described in the text. Maximal velocities are given per mg of pancreatic lipase protein added.

Acyl-CoA	V (nmoles/min per mg)	K_m (μM)
Acetyl	1.0	—
Malonyl	1.0	—
12:0	90	600
14:0	750	80
16:0	250	30
18:0	180	17
20:0	20	8
18:1 (n-9)	250	30
18:2 (n-6)	450	15
20:1 (n-9)	400	200
20:2 (n-9)	400	30
20:3 (n-6)	500	25
20:4 (n-6)	100	200
20:5 (n-3)	80	—
22:6 (n-3)	125	100

DISCUSSION

The pancreatic lipase preparation has proved to be a convenient, and thereby valuable, tool for measuring the concentration of acyl-CoA in solutions. Although we have used the method with purified synthetic acyl-CoA substrates it could, in principle, be used in the measurement of acyl-CoA contents in reaction mixtures or perhaps in biological fluids¹⁴⁻¹⁶. We have found the method especially useful for the assay of solutions of polyunsaturated acyl-CoA's to which antioxidants have been added for protection during storage. The latter compounds preclude using the convenient assay absorption at 232 nm and 260 nm which reflect the presence of the thiol ester and adenine groups, respectively⁵. Although the sensitivity of the lipase-DTNB method is not as great as some techniques available for the measurement of free CoA (ref 14-18) the commercial availability of the enzyme and the stability of the necessary reagents can make it a very useful tool.

The acyl-CoA hydrolase activity of pancreas resembles many liver acyl-transferases and hydrolases¹⁹ in that it is not inhibited by the presence of DTNB. This result suggests that enzymic thiol groups may not be involved in the hydrolytic mechanism. Such a conclusion could not be made for the acyl-glutathione thiolesterase from mouse liver which is strongly inhibited by thiol reagents such as *p*-chloro-mercuribenzoate²⁰.

The *V* values of the hydrolase toward saturated acyl-CoA thiol esters decrease in the order 14:0, 16:0, 18:0, 12:0, 20:0. Esters of 14-18 carbons are clearly preferred over shorter and longer chains. This specificity is very similar to that reported for a palmitoyl thioesterase from *E. coli*⁶, and similar also to the specificity of rat liver acyl-CoA hydrolase reported by BARDEN AND CLELAND¹² except that in the latter case palmitoyl-CoA was hydrolysed at almost ten times the rate of other saturated acyl-CoA's.

Although it is not possible for us to identify the enzyme(s) responsible for the observed hydrolysis of acyl-CoA's, it is interesting to compare the acyl specificity of this system with that of pancreatic triglyceride lipase. This enzyme, known to be in high concentration in the preparations used in this study, cleaves triglycerides and produces monoglycerides, diglycerides and fatty acids. Lauroyl, myristoyl, palmitoyl and stearoyl as well as oleoyl and linoleoyl groups are removed at about the same rate by triglyceride lipase whereas acetyl groups are cleaved at only 20% the rate of the others⁷. Certain long-chain polyunsaturated fatty acyl groups (*e.g.* 20:5 and 22:6 but not 22:5) are resistant to hydrolysis²¹. The *V* values reported in Table I for cleavage of acyl-CoA thiol esters are only vaguely similar to the general pattern reported for acyl-glycerol (oxygen) ester cleavage by triglyceride lipase. The lack of agreement may be a function of the different micellar states of the thio and oxo derivatives tested. However, pancreas also contains other "esterases" which are separable from triglyceride lipase and the activity that we observe may be due to one of these⁷. The answer to this question must await further purification of the acyl-CoA hydrolase(s).

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